SURVEYING ANTIMICROBIAL RESISTANCE: THE NEW COMPLEXITY OF THE PROBLEM

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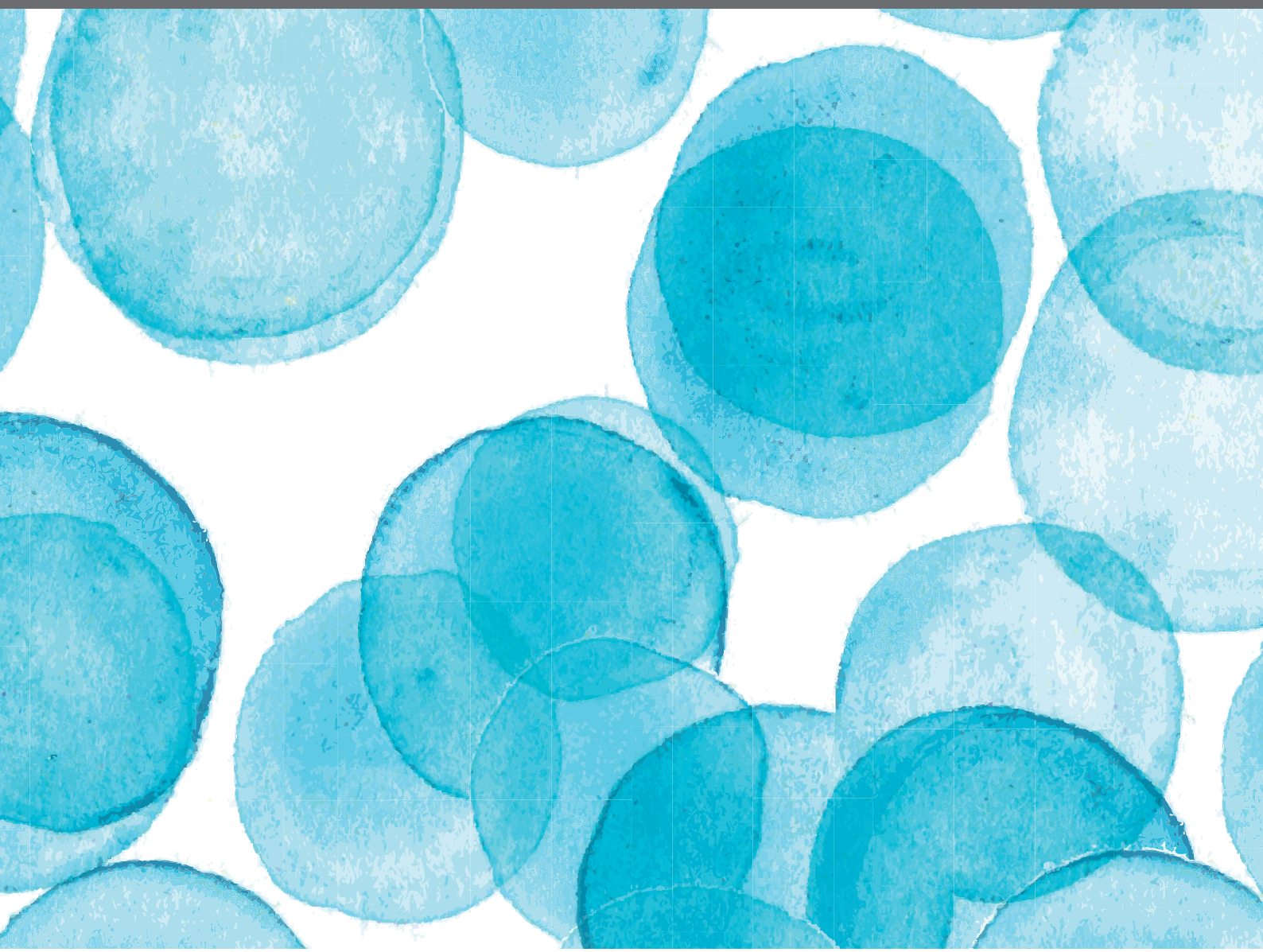

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SURVEYING ANTIMICROBIAL RESISTANCE: THE NEW COMPLEXITY OF THE PROBLEM

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In January of 2015, under the 1st International Caparica Conference in Antibiotic Resistance, a Research Topic entitled: "Surveying Antimicrobial Resistance: Approaches, Issues, and Challenges to overcome", was published (http://journal.frontiersin.org/researchtopic/3763/surveying-antimicrobial-resistance-approaches-issues-and-challenges-to-overcome). The problem of antimicrobial resistance (AMR), caused by excessive and inappropriate use of antibiotics, is a public health issue that concerns us all. The introduction of penicillin in the 1940s, the start of the antibiotics era, has been recognized as one of the greatest advances in therapeutic medicine. However, according to the World Health Organization (WHO), AMR infections are now an increasing worldwide public health threat and a post-antibiotic era is imminent, where common infections and minor injuries could be fatal. AMR is a typical 'One Health' problem, in which livestock animals and the environment constitute AMR reservoirs and transmission routes to and from the human population. Without effective antimicrobials to counter and prevent infections, other major achievements in modern medicine, such as organ transplantation, cancer chemotherapy and major surgery, risk being compromised.

AMR infections in animals have negative outcomes on animal health, welfare, biosecurity and production. In 2006, the ban of growth promoting antibiotics highlighted antibiotic use in animal production as a risk factor in the development of antibiotic resistant bacteria. Bacteria can be transferred to humans via several routes; consumption of animal products, exposure through contact with animals, and the contamination of ground and surface waters by animal waste products. Therefore, it is of utmost importance that antimicrobial use in animals is reduced to a minimum, without compromising animal health and welfare.

Mechanisms of bacterial antibiotic resistance are classified according to the types of antibiotic molecules or their targets in the cell. Environmental antibiotic-resistance genes are spread then acquired by clinically relevant microorganisms. Many resistance genes are conveyed into pathogen genomes via mobile genetic elements such as plasmids, transposons or integrons, increasing the propagation of potential resistant

pathogens. Substantial progress has already been made in elucidating the basic regulatory networks that endow bacteria with their extraordinary capacity to adapt to a diversity of lifestyles and external stress factors.

So how will we face bacteria in the future?

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EDITORIAL
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Editorial: Surveying Antimicrobial Resistance: The New Complexity of the Problem

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Keywords: antibiotics, AMR, genomics, microbiology, omics

Editorial on the Research Topic

Surveying Antimicrobial Resistance: The New Complexity of the Problem

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The 2nd International Caparica Conference in Antibiotic Resistance (IC2AR) was held in Caparica, Portugal from 11 to 15 June 2017. This very successful meeting had a clear One Health vision and attracted 216 attendees from 39 countries keen to exchange knowledge and expertise on diverse but interrelated topics. Formal contributions totaled over 131 oral presentations, 19 short presentations and 49 posters. The results and insights from this meeting are now being made accessible to the general scientific community in this special issue of the Frontiers in Microbiology Research Topic.

The introduction of penicillin in the 1940s, the start of the antibiotics era, has been recognized as one of the greatest advances in therapeutic medicine. However, according to the World Health Organization (WHO), antimicrobial resistant infections are now an increasing worldwide public health threat and a post-antibiotic era is imminent when even common infections and minor injuries could be fatal. Antimicrobial resistance (AMR) reduces the effectiveness of treatment and patients remain infected for a longer period, thereby increasing the potential to spread resistant microorganisms to others, according to WHO. Without effective antimicrobials to counter and prevent infections, other major achievements in modern medicine, such as organ transplantation, cancer chemotherapy and major surgery, risk being compromised. According to The State of the World's Antibiotics, two-thirds of the 100,000 tons of antibiotics produced globally each year are used in animal husbandry, and of the 27 antimicrobials used in animals, 18 are also used for human medicine. In terms of global sales in 2009, the top three antimicrobial classes for use in animals were macrolides, penicillins and tetracyclines, all of which are categorized as being critical for human medicine. The growth of global trade and travel allows resistant microorganisms to be spread rapidly to distant countries and continents, which threatens health security and risks damaging trade and economics.

AMR is becoming one of the most threatening public health issues worldwide. In Europe, the Mediterranean countries are most at risk, possibly due to a complex combination of antibiotic use practices, socio-economic factors and climate changes. For economies that rely heavily on tourism and export of food crops, the current situation is delicate. For the well-being and safety of the populations and for socio-economic stability, the increase in AMR must be reversed.

AMR infections in animals have negative outcomes on animal health, welfare, biosecurity and production. Growth promoting antimicrobials have been banned in the EU countries in 2006, however they are in widespread use in other countries outside the EU. Antibiotic use in animal production was highlighted as a risk factor in the development of antibiotic resistant bacteria that can be transferred to humans via several routes.

With the increasing resistance of bacterial pathogens to present-day antibiotics and the lack of a robust pipeline to generate novel antimicrobial substances, more innovative and efficient approaches are needed to develop anti-infective drugs. Proteomics and genomics technologies already offer sensitive and specific methods for identification of microbial food contaminants and their toxins. So, there is a lot to learn and discuss about these cutting-edge methods.

AMR within populations of different infectious agents is a worldwide public health threat. Already the available treatment options for common infections in some settings are becoming ineffective. There are now reports of bacterial resistance to all antibiotic classes used in either human or veterinary medicine, and in several cases, of an association between antibiotic use and the development of clinical resistance. To counter this emergent problem, the World Health Organization has appealed for urgent and concerted action by governments, health professionals, industry, civil society and patients to slow down the spread of drug resistance, limit its impact today, and so preserve medical advances for future generations.

The prevalence of AMR varies greatly between and within countries and between different pathogens. The widespread use of antimicrobial agents in human and veterinary medicine for therapeutic and prophylactic purposes has been identified as the main determinant for the emergence and spread of resistant bacteria. However, there are hardly any specific integrated studies that indicate how the risk could be limited. Progress has been made in recent years in understanding the AMR mechanisms underlying the emergence of the resistance genes and their spread, but there are still major gaps. Co-integrated research on resistance in animals and the environment together with in-depth pharmacokinetics and pharmacodynamics of antibiotics will contribute to this understanding. As One Health Initiatives get underway, a global perspective must be encouraged and maintained even for very focused investigations.

Livestock and the environment constitute AMR reservoirs and transmission routes to and from the human population. Environmental antibiotic resistance genes are spread then acquired by clinically relevant microorganisms. Many resistance genes are conveyed into pathogen genomes via mobile genetic elements such as plasmids, transposons or integrons, increasing the propagation of potentially resistant pathogens and the intricacies of these adaptive mechanisms are still the focus of investigation. This Research Topic presents original research on integrative and conjugative elements and the staphylococcal cassette chromosome, as well as new studies of resistance gene variants borne by plasmids or transposons, and characterization of the regulation of their gene expression.

Substantial progress has already been made in elucidating the basic regulatory networks that endow bacteria with their extraordinary capacity to adapt to a diversity of lifestyles and external stress factors. The articles collated here describe microbial life in a vast spectrum of natural and manmade settings. Just to illustrate this variety, micro-organism samples studied have been collected from 2 m depth of sediment on the Red Sea coast and from the International Space Station orbiting 400 km above the Earth's surface (Rehman and Leiknes; Sobisch et al.). Microbes from aquatic ecosystems of seas, rivers and wetlands have also been analyzed (Rehman and Leiknes; Tuo et al.; Sen et al.). Farming and food production contexts cover organic, conventional and intensive agriculture (Zheng et al.; Cadena et al.; Liu et al.; Miranda et al.; Armalyte et al.; McMillan et al.; Zajac et al.). The non-food animal hosts studied range from wild primates in Brazilian forests and flocks of crows over US farmland to pet cats and dogs in Spanish homes (Grassotti et al.; Roberts et al.; Gómez-Sanz et al.; Sen et al.). Clinical research comes from hospitals in a range of different healthcare systems with presentation of a range of pathologies and includes the analysis of historical specimens providing some longer-term perspective that is valuable in depicting the timescale of mutation and spread of resistance (Manageiro et al.; Bostanghadiri et al.; Ferreira et al.; Palmeiro et al.; Pinto et al.). The fundamental ecology of microbiota is still a strong focus with investigations of quorum sensing, biofilm formation, stress responses and resistance mechanisms (Knight et al.; Rehman and Leiknes; Martins et al.; Sobisch et al.; Yang et al.). Without a robust pipeline to generate novel antimicrobial substances, more innovative and efficient approaches are needed to develop anti-infective drugs and some of these will be based on specific biological functions or the dynamics and interactions of microbial populations (Grassotti et al.; Igrejas et al.; Jäger et al.; Troiano et al.; Zhang et al.; Armalyte et al.).

Improvement of food safety standards helps to strengthen the competitiveness of the food industry. To achieve this, microbial food contamination, risks and exposures must be analyzed, assessed, monitored, controlled and traced throughout the food supply chains from production and storage to processing, packaging, distribution, catering, and preparation at home. Many of the papers published here deal with some stage or aspect of this complex process (Dominguez et al.; Cui et al.; McMillan et al.; Zajac et al.). It is important to design research that contributes to ensuring the safety of food of animal origin while addressing the sustainability of food production, supply and consumption, along the whole food chain and related services from field to fork. When dealing with the issue of safe food, healthy diets and sustainable consumption, the control of foodborne outbreaks must always be a priority (Isidro et al.). Current research focuses strongly on the detection of foodborne pathogens and specific spoilage organisms from food of animal origin along different production chains (slaughterhouses, restaurants, meat product manufacturers, fisheries). Important microbiological hazards responsible for foodborne outbreaks are analyzed, such as those involving *Salmonella* sp., *Campylobacter* spp., *E. coli*, *Listeria* spp., or *Aeromonas* spp. (Bai et al.; Hormeño et al.; Peng et al.; Beshiru et al.; Cyoia et al.; Islam et al.). Researchers will continue to develop new approaches to analyze and interpret more complex and emerging microbial pathogens

using molecular, serotyping and phylogenetic methods. Expected developments will be in pinpointing and surveying prevalence, contamination sources, public health risks, and strategies to improve food safety and quality (Dandachi et al.; Igrejas et al.; Domokos et al.; Zeineldin et al.). For example, packaging, temperature treatments, and traditional methods for meat preservation (fermentation, drying, spices and herbs, wine) may be revisited with modern technologies (Sparo et al.; Igrejas et al.). With the policies to reduce the use of additives and promote environmentally sustainable production of meat products, research to develop and validate organic preservation procedures will be necessary (Beshiru et al.; Li et al.; Sen et al.; Wieczorek et al.).

On the subject of food safety, studies on the resurgence of AMR as a pandemic threat must be included. Presently and in the near future, antimicrobial peptides produced by different microorganisms will be characterized to generate novel applications in human and veterinary medicine and in food conservation. Such discoveries will also facilitate research on antibiotic resistance and molecular characterization of virulence factors in microbiota from different ecological niches. Antimicrobial peptides are indeed the subjects of original research, review, and opinion articles published here which give some indication of current strategic thinking (Pizzolato-Cezar et al.; Vasilchenko and Rogozhin).

Proteomics and genomics technologies already offer sensitive and specific methods for identification of microbial food contaminants and their toxins. A perusal of the techniques and technologies used in AMR research shows that whole-genome sequencing is now well-entrenched alongside conventional molecular and microbiological techniques, an approach that is clearly increasing the diversity, depth and pace of AMR monitoring and basic research. Impact studies that analyze and assess some of the cumulated economic, epidemiological or environmental data are also featured here (Annavajhala et al.).

To summarize, this Research Topic brings together a group of leading researchers from all over the world who have described different aspects of AMR patterns found in diverse ecosystems. The articles address the epidemiology of resistance in animal and zoonotic pathogens, mobile elements containing resistance genes, the omics of AMR, emerging AMR mechanisms, control of resistant infections, establishing antimicrobial use and resistance surveillance systems, and alternative strategies to overcome the problem of AMR worldwide. In this conference an attempt was made to present the latest research on possibilities to manage this question. The meeting carried out an integrated approach to research and presented a universal vision of the importance of antimicrobial resistance in different ecosystems and what can be done about it.

We want to thank the reviewers for their many thoughtful and insightful comments, and the authors for their high-quality contributions. In closing, we would like to encourage readers to participate in the 4th edition of the International Caparica Conference in Antibiotic Resistance to be held in 2021 (http://www.bioscopegroup.org/index.php/congresses).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
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High Prevalence of CTX-M-15-Type ESBL-Producing *E. coli* from Migratory Avian Species in Pakistan

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The increased presence of clinically relevant multidrug resistant bacteria in natural environments is an emerging challenge for global health care. Little is known regarding the occurrence of extended-spectrum beta-lactamase producing *Escherichia coli* (ESBL-*E. coli*) from environmental sentinels in Pakistan. The goal of the current study was to gain insights into the prevalence and phylogenetic relationships of ESBL-*E. coli* recovered from wild birds in Pakistan during winter migration. After initial screening of fecal samples on selective chromogenic agar, ESBL-*E.coli* were analyzed phenotypically using the Vitek-2 automated system. Genotypic characterization was performed using whole genome sequencing (WGS) followed by an in-depth in silico analysis. Of 150 birds screened, 26 (17.3%) were fecal carriers of ESBL-*E. coli.* Of these, 88.4% isolates exhibited multidrug resistance (MDR) phenotypes. Resistance to cefotaxime, ceftazidime, ampicillin, doxycycline, tetracycline and sulfamethoxazole/trimethoprim (CTX-CAZ-AM-DC-TE-SXT) represented the most common pattern of MDR (76.9%). WGS data analysis found $bla_{\text{CTX-M-15}}$ as the predominant ESBL genotype (92.3%). Other genes encoding resistance to sulfonamides (*sul1/sul2/sul3*), aminoglycosides (*strA*, *strB*, *aadA1, aadA2, aadA5, aac(3)-IId-like, aac(3)-IVa-like* and *aph(4)-Ia)*, trimethoprim *(dfrA14* or *dfrA17),* tetracyclines [*tet(A)/tet(B)*], and fluoroquinolones (*qnr*S1) were detected commonly, often encoded on IncF-type plasmids (76.9%). ESBL-*E. coli* were assigned to 17 different sequence types (STs) of which ST10 and ST7097 (4 isolates each) were the most abundant followed by ST4720, ST93, and ST1139 (2 isolates each). Core-genome phylogeny of the isolates found low numbers (0–29) of single nucleotide polymorphisms (SNPs) in isolates belonged to ST7097 originated from two different locations (Chashma barrage and Rasul barrage). Similar trends were found among isolates belong to ST1139. In addition, WGS-based plasmid typing and S1-digestion found plasmids of the same pMLST type (IncF[F-:A-:B53]) and similar sizes in different bacterial and avian hosts suggesting horizontal gene transfer as another possibility for the spread of ESBL-*E. coli* in avian wildlife in Pakistan.

Keywords: antimicrobial resistance, wild birds, ESBL-producing *E. coli*, genomic epidemiology, Pakistan

INTRODUCTION

The intensive use of antimicrobials in human and veterinary medicine has resulted in an emergence of antimicrobial resistance (AMR) in humans, animals and the environment at large (Radhouani et al., 2014; Berendonk et al., 2015). Enterobacteriaceae producing ESBLs have increasingly emerged due to the widespread use of cephalosporins and represent a major challenge in infection control (Pitout and Laupland, 2008). Currently, the most commonly encountered ESBL enzyme is the plasmid-encoded CTX-M-type. In particular, an *E. coli* clone of sequence type 131 (ST131) carrying the CTX-M-15 ESBL has been commonly found in clinical and non-clinical settings (Nicolas-Chanoine et al., 2014).

Previous studies have suggested the environment including water, soil and wildlife as the source for clinically relevant ESBL-*E. coli* (Wright, 2010; Blaak et al., 2015; Guenther et al., 2017), thereby possibly transmitting certain ESBL-*E. coli* clonal lineages or ESBL-plasmids from natural environments to humans, livestock or companion animals. Wild migratory birds have been discussed as sentinels and a potential vectors for the transboundary spread of ESBL- producing bacteria (Raza et al., 2017). Furthermore, wildlife has been considered as reservoir of potentially zoonotic extra-intestinal pathogenic *E. coli* (ExPEC) strains in earlier studies (Ewers et al., 2009; Gordon and Cowling, 2012).

Recently, it has been suggested that certain clonal lineages distinguished by very low number of single nucleotide polymorphisms (SNPs) circulate at the human-animal-environment interfaces which strongly supports the One Health perspective of AMR (Falgenhauer et al., 2016; Schaufler et al., 2016). Pakistan is among the Asian countries that harbor a large number of migratory birds during winter migration along the Indus route coming from Siberia and Central Asia. In this study, we screened wild migratory birds from four different wetland habitats along the Indus migration route in Pakistan to assess the prevalence of ESBL-*E. coli* and to subsequently characterize them in-depth via whole genome sequencing to assess AMR genes, multi locus sequence types (MLST), plasmid replicon types, and virulence-associated genes (VAGs). Additionally, the core genomes of identical STs were analyzed for SNPs.

MATERIALS AND METHODS

Sample Collection and Isolation of ESBL-*E. coli*

In a study conducted between 2013 and 2015, fecal samples of 150 wild migratory birds were collected from four wetland habitats in Pakistan (**Figure 1**; Raza et al., 2017). These birds included Eurasian coot (*Fulica atra*: $n = 60$), mallard duck (*Anas platyrhynchos*: $n = 20$), common pochard (*Aythya farina*: $n = 15$), red headed pochard (*Netta rufina*: $n = 10$), shoveler duck (*Anas clypeata*: $n = 15$), Eurasian wigeon (*Anas penelope*: $n = 15$) and rosy starling (*Pastor roseus*: $n = 15$). Fecal samples were directly streaked on CHROMagar-ESBL plates (CHROMagar Co., Paris, France) and incubated at 37°C overnight. One putative *E. coli* colony per sample was selected and confirmed by API 20E biochemical strips (bioMérieux, Marcy l'Etoile, France).

ESBL Confirmation and Antimicrobial Susceptibility Testing

Confirmation of the ESBL production was done by double disc synergy test according to the CLSI guidelines (CLSI, 2012) and approved using the Vitek-2 compact system (AST-card GN38, bioMérieux, Germany), which was also used for analyzing additional phenotypic AMRs. Multi-drug resistance (MDR) was defined as resistance to three or more different classes of antimicrobials (Magiorakos et al., 2012).

Whole Genome Sequencing

DNA extraction of confirmed ESBL-*E. coli* isolates were performed using MasterPure™ Purification Kit (Epicenter Biotechnologies, WI) according to the manufacturer's instruction. Whole genome sequencing (WGS) and assembly of reads was performed as previously described (Schaufler et al., 2016; Guenther et al., 2017). Briefly, WGS was performed on an Illumina MiSeq (Illumina, San Diego, CA) using an Illumina Nextera XT library with 300 bp paired-end sequencing. Quality control (QC) was performed using the NGS tool kit (70% of bases with a phred score >20). QC report from the assembled genomes has been provided in (Table S1). *De novo* assembly of high-quality filtered reads into contiguous sequences (contigs) and nodes was done using SPAdes. For each *E. coli* analyzed by WGS, a minimum 90-fold coverage was yielded.

In Silico Analysis

WGS data from multiple bacterial isolates were analyzed simultaneously for their multi-locus sequence types (MLSTs), antibiotic resistance genes, plasmid replicon types and pMLST using the Bacterial Analysis Pipeline Tool at the web service of Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) (Thomsen et al., 2016). In the case of quinolone resistance genes *gyrA* and *parC* detection, the Resistance Gene Identifier (RGI) tool of CARD (Comprehensive Antibiotic Resistance Database) was used (McArthur et al., 2013). Virulence associated genes (VAGs) were detected with an in-house reference sequence collection which maps Illumina reads against chromosomal and plasmid virulence genes found in the Virulence Factor Database for *E. coli* (http://www.mgc.ac.cn/VFs/). In case of strains lacking plasmids, the chromosomal location of the $bla_{\mathrm{CTX-M}}$ gene was also analyzed with Geneious v. 7.1.2 (Guenther et al., 2017).

For phylogenetic analysis, SNPs between the core genome of isolates were calculated using Harvest suite 1.0 (parsnp) (Treangen et al., 2014) and the number of SNPs in any two isolates were calculated using distance matrix generated in MEGA 7.0 Software (http://www.mega software.net/). The phylogenetic tree of the core genomes was visualized using iTOL 3 (http://itol.embl.de/) (Letunic and Bork, 2016).

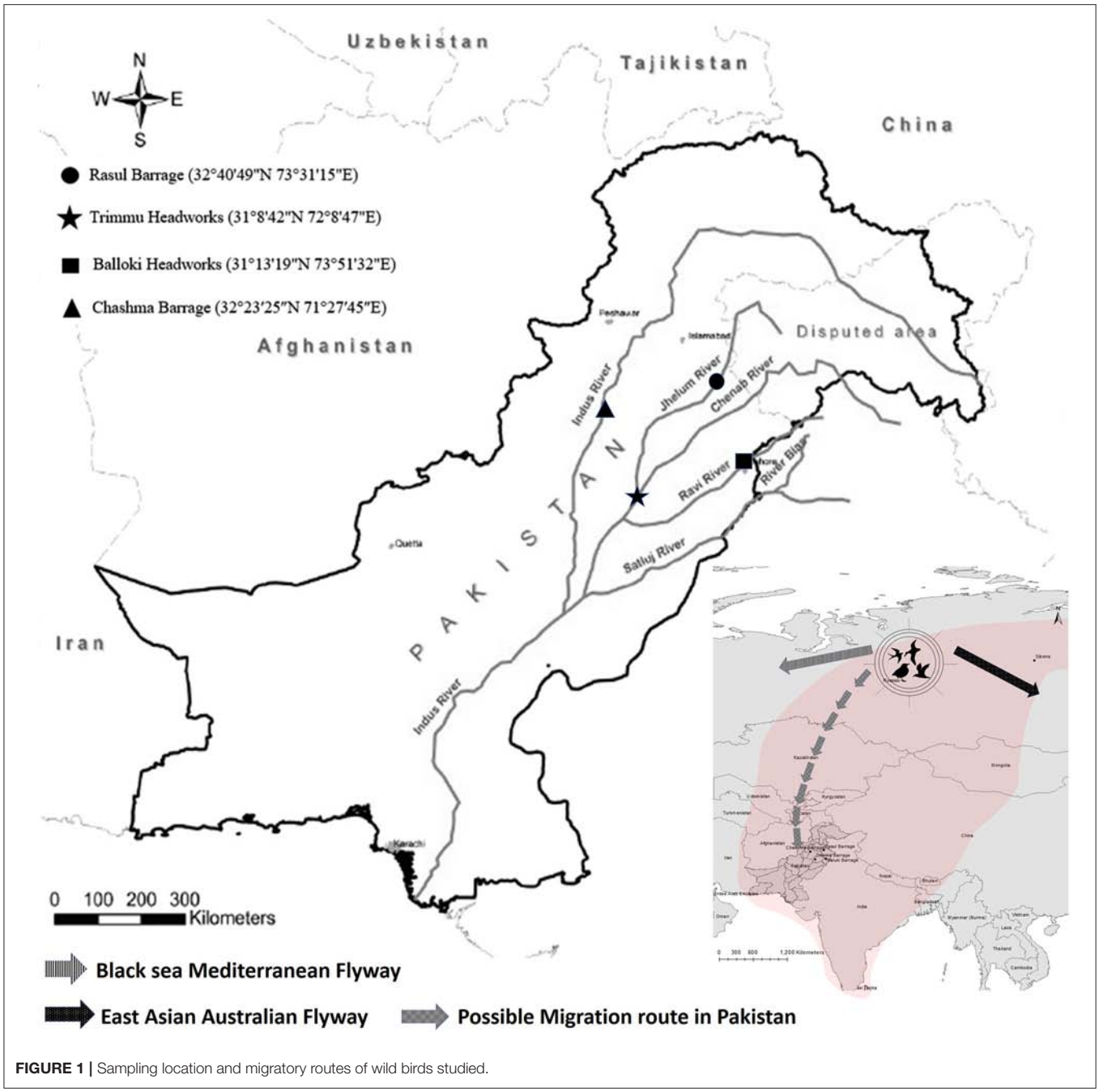

FIGURE 1 | Sampling location and migratory routes of wild birds studied.

S1 Digestion

Isolates displaying the pMLST type IncF[F-:A-:B53] were analyzed by S1-nuclease PFGE (Guerra et al., 2004) using the following running conditions: 1–25 s, 17 h, 6 V/cm, 120 V.

RESULTS

Prevalence and Phenotypic Resistance of ESBL-producing *E. coli*

Twenty-six of 150 birds were fecal carriers of ESBL-producing *E. coli* (17.3%), which correspond to six different avian species spread across all sampling areas (**Table 1**). Of 26 ESBL- producing *E. coli* isolates, 23/26 (88.4%) showed a MDR phenotype. The most common MDR phenotype was cefotaxime, ceftazidime, ampicillin, doxycycline, tetracycline and sulfamethoxazole /trimethoprim (CTX-CAZ-AM-DC-TE-SXT) found in 20/26 (76.9%) isolates (**Table 1**). In general, trimethoprim/sulfamethoxazole resistance was the most common non-beta-lactam phenotype (92.3%) followed by resistance to tetracycline (84.6%), doxycycline (80.7%), marbofloxacin and enrofloxacin (15.3%). One of these isolates (Pk-13) showed resistance to colistin and has been reported

TABLE 1 | Characteristics of the ESBL producing *E. coli* isolates from wild migratory birds in Pakistan.

Sample ID	Host Species	Date of Isolation	Sampling location	Antibiotic resistances	Beta-lactam genes	Colistin	Aminoglycoside	Sulphonamide	Quinolone	Trimethoprim	Tetracycline	Phenicol	Fosfomycin	ST	Plasmid replicon types	pMLST summary	S1-digest plasmid sizes	VAGs
Pk-1	Rosy Starling (*Pastor roseus*)	16/12/2013	Balloki Headworks	CTX, CAZ, AMP, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)-like*			ST-202	IncFIB	IncF[F-:A-:B53]	n.d.	*astA (East-1), malX, ompA*
Pk-2	Rosy Starling (*Pastor roseus*)	16/12/2013	Balloki Headworks	CTX, CAZ, AMP, C, DC, TE, ENR, MRB, SXT	*blaCTX-M-15*		*aadA1,aadA2*	*sul3*		*dfrA12*	*tet(A)-like*	*cmlA1-like*		ST-224	No replicon		n.d.	*matA (ecpR), astA (East-1), malX, ompA*
Pk-3	Rosy Starling (*Pastor roseus*)	01/01/2014	Trimmu headworks	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2-like*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-10	IncFIB	IncF[F-:A-:B53]	130kb	*fimC, matA (ecpR), astA (East-1), malX, ompA*
Pk-4	Red-headed pocahard (*Netta rufina*)	16/12/2013	Balloki headworks	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-10	IncFIB, IncI1	IncF[F-:A-:B53], IncI1[Unknown ST]	110/90kb	*fimC, sitA, astA (East-1), malX, ompA*
Pk-5	Eurasian coot (*Fulica atra*)	11/02/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-10	IncY		n.d.	*fimC, fyuA, irp2, astA (East-1), malX, ompA*
Pk-6	Eurasian coot (*Fulica atra*)	11/02/2014	Chashma barrage	CTX, CAZ, AMP,DC, TE, SXT	*blaCTX-M-1, blaTEM-1C*		*aadA5,strA,strB*	*sul2*		*dfrA17*	*tet(A)*			ST-4720	IncFIC, IncI1, IncFIB, IncFII	IncI1[ST-3], IncF[F18:A-:B1]	n.d.	*bfpm, fimC, metaA (ecpR), tsh, astA (East-1), fyuA, iroN, irp2, iucD, iutA, sitA, sitB, sitC, sitD, cvi, traT, ompA, malX*
Pk-7	Eurasian coot (*Fulica atra*)	11/02/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-1, blaTEM-1C*		*aadA5,strA,strB-like*	*sul2*		*dfrA17*	*tet(A)-like*			ST-4720	IncFIC, IncI1, IncFIB, IncFII	IncI1[ST-3], IncF[F18:A-:B1]	n.d.	*bfpm, fimC, metaA (ecpR), tsh, astA (East-1), fyuA, iroN, iucD, iutA, sitA, sitB, sitC, sitD, cvi, traT, ompA, malX*
Pk-8	Eurasian coot (*Fulica atra*)	20/01/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-7097	IncFIB	IncF[F-:A-:B53]	110/90kb	*astA (East-1), sitA, sitB, sitC, sitD, malX, ompA*
Pk-9	Eurasian coot (*Fulica atra*)	20/01/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(B)*			ST-1722	No replicon		n.d.	*fimC, matA (ecpR), sfaX, astA (East-1), chuA, malX, ompA*
Pk-10	Red-headed pocahard (*Netta rufina*)	16/12/2013	Balloki headworks	CTX, CAZ, AMP	*blaCTX-M-15*				*QnrS1*					ST-58	No replicon		n.d.	*matA (ecpR), astA (East-1), malX, ompA*
Pk-11	Eurasian coot (*Fulica atra*)	11/02/2014	Chashma barrage	CTX, CAZ, AMP	*blaCTX-M-15, blaTEM-33-like*									ST-361	IncFIC, IncFIB, IncY	IncF[F46*:A-:B16]	n.d.	*matA (ecpR), ompA*
Pk-12	Eurasian coot (*Fulica atra*)	11/02/2014	Chashma barrage	CTX, CAZ, AMP, GM, TM, C, DC, TE, SXT	*blaCTX-M-15*		*aac(3)-IVa-like, aph(4)-Ia, strA-like, strB-like*	*sul2*		*dfrA14-like*	*tet(A)*	*catA2-like*	*fosA*	ST-602	IncFIB, IncFIA, IncFIC, IncFII	IncF[F18:A5:B1]	n.d.	*fimC, metaA (ecpR), astA (East-1), iroN, iucD, iutA, sitA, sitB, sitC, sitD, cvi, traT, ompA, malX*
Pk-13	Eurasian coot (*Fulica atra*)	11/02/2014	Chashma barrage	CTX, CAZ, AMP, CO, PO, DC, TE, ENR, MRB, SXT	*blaCTX-M-15, blaTEM-1B*	*mcr-1*	*aadA1,aadA2-like,strA,strB*	*sul2,sul3*		*dfrA14-like*	*tet(B)*	*cmlA1-like*		ST-354	IncFII, IncHI2, IncFIB, IncFIA, IncI2	IncHI2[ST-3], IncF[F36:A6*:B1]	n.d.	*fimC, metaA (ecpR), astA (East-1), chuA, iroN, iucD, iutA, sitB, sitC, cvi, ompA, malX*
Pk-14	Eurasian coot (*Fulica atra*)	11/02/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15,blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-10	IncY		n.d.	*astA (East-1), fyuA, irp2, malX, ompA*
Pk-15	Mallard duck (*Anas platyrhynchos*)	16/02/2015	Rasul barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-1139	IncFIB, p0111	IncF[F-:A-:B53]	130/100kb	*astA (East-1), malX, ompA*
Pk-16	Shoveler duck (*Anas clypeata*)	16/12/2013	Balloki headworks	CTX, CAZ, AMP, GM, TM, ENR, MRB, SXT	*blaCTX-M-15, blaTEM-1B*		*aac(3)-IId-like,aadA2,strA-like,strB*	*sul1,sul2*		*dfrA12*				ST-617	ColRNAI		n.d.	*matA (ecpR), astA (East-1), malX, ompA, tia*

(Continued)

TABLE 1 | Continued

Sample ID	Host Species	Date of Isolation	Sampling location	Antibiotic resistances	Beta-lactam genes	Colistin	Aminoglycoside	Sulphonamide	Quinolone	Trimethoprim	Tetracycline	Phenicol	Fosfomycin	ST	Plasmid replicon types	pMLST summary	S1-digest plasmid sizes	VAGs
Pk-17	Shoveler duck (*Anas clypeata*)	16/12/2013	Balloki headworks	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-1303	IncFIB	IncF[F-:A-:B53]	130kb	*matA (ecpR), astA (East-1), fyuA, irp2, malX, ompA*
Pk-18	Eurasian wigeon (*Anas penelop*)	01/01/2014	Trimmu headworks	CTX, CAZ, AMP, SXT	*blaCTX-M-15, blaTEM-1B-like*			*sul2*	*QnrS1*	*dfrA1*				ST-2914	IncFII, IncQ1, IncB/O/K/Z	IncF[F55*:A-:B-]	n.d.	*matA (ecpR), astA (East-1), chuA, kpsMT_II, traT,malX, ompA*
Pk-19	Eurasian wigeon (*Anas penelop*)	01/01/2014	Trimmu headworks	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-3716	IncFIB	IncF[F-:A-:B53]	130kb/ 40kb	*astA (East-1), malX, ompA*
Pk-20	Mallard duck (*Anas platyrhynchos*)	01/01/2014	Trimmu headworks	CTX, CAZ, AMP, DC, TE, ENR, MRB, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-1421	IncFIB	IncF[F-:A-:B53]	130/30kb	*astA (East-1), malX, ompA*
Pk-21	Mallard duck (*Anas platyrhynchos*)	01/03/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-7097	IncFIB	IncF[F-:A-:B53]	130/ 40/30kb	*astA (East-1), sitA, sitB, sitC, sitD, malX, ompA*
Pk-23	Mallard duck (*Anas platyrhynchos*)	01/03/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A),tet(B)*			ST-93	IncFIB	IncF[F-:A-:B53]	60kb	*hek/hra, matA (ecpR), astA (East-1), sitA, sitB, sitC, sitD, kpsMT_II, malX, ompA*
Pk-24	Mallard duck (*Anas platyrhynchos*)	16/02/2015	Rasul barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15,blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-7097	IncFIB	IncF[F-:A-:B53]	n.d.	*astA (East-1), sitA, sitB, sitC, sitD, malX, ompA*
Pk-26	Eurasian coot (*Fulica atra*)	01/03/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-7097	IncFIB	IncF[F-:A-:B53]	130/100kb	*astA (East-1), sitA, sitB, sitC, sitD, malX, ompA*
Pk-29	Eurasian coot (*Fulica atra*)	01/03/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A)*			ST-1139	IncFIB, p0111	IncF[F-:A-:B53]	130/ 00kb	*astA (East-1), malX, ompA*
Pk-30	Eurasian coot (*Fulica atra*)	01/03/2014	Chashma barrage	CTX, CAZ, AMP, DC, TE, SXT	*blaCTX-M-15, blaTEM-1B*		*strA,strB*	*sul2*	*QnrS1*	*dfrA14-like*	*tet(A),tet(B)*			ST-93	IncFIB	IncF[F-:A-:B53]	60kb	*hek/hra, matA (ecpR), astA (East-1), sitA, sitB, sitC, sitD, kpsMT_II, malX, ompA*

AMP, ampicillin; C, chloramphenicol; CO, colistin, DC, doxycycline; ENR, enrofloxacin; GM, gentamicin; MRB, marbofloxacin; SXT, PO, polymyxin; sulfamethoxazole/trimethoprim; TE, tetracycline; TM, tobramycin astA (East-1), heat stable cytotoxin associated with enteroaggregative E. coli; malX, phosphotransferase system enzyme II; matA, ecp operon encodes EcpR; ompA, outer membrane protein A; fimC, Type 1 fimbria; sitA, sitB, sitC, sitD, Salmonella iron transport system; bfpm, bundle-forming pilus morphogenesis; fyuA, yersiniabactin receptor; irp2, iron repressible protein; tsh, temperature sensitive hemagglutinin; iroN, siderophore receptor; iucD, aerobactin; iutA, iron uptake transport; cvi, structural genes of colicin V operon; traT, transfer protein; kpsMTT_II, group II capsule antigen; hek/hrA, heat resistant hemagglutinin; chuA, E. coli haem utilization; tia, toxigenic invasion locus; sfaX, fimbriae

in our previous publication (Mohsin et al., 2016; **Table 1**). All isolates were susceptible to carbapenems.

Antibiotic Resistance and Virulence Genes

WGS revealed that all of 26 ESBL-*E. coli* isolates harbored the bla_{CTX-M} gene with bla_{CTX-M}-15 as the most dominant 24/26 (92.3%) genotype (**Table 1**). Of these, 19 isolates also harbored bla_{TEM-1B} whereas two isolates carried $bla_{CTX-M-1}$ together with *bla* TEM-1C. Among non-beta-lactam resistance, genes conferring resistance to sulfonamide and trimethoprim were predominant 24/26 (92.3%) followed by aminoglycosides 23/26 (88.4%), tetracycline 22/26 (84.6%) and quinolones 19/26 (73%). We found that most of the isolates carried the *sul2* gene, alone or in combination with *sul1* or *sul3* for sulfonamide resistance. A total of 7 different genes encoding resistance for aminoglycoside were detected. Of these, most common were *strA* and *strB*, alone or in combination with *aadA1, aadA2, aadA5, aac(3)-IId-like, aac(3)-IVa-like,* and *aph(4)-Ia.* Overall, genotypic data strongly correlated with phenotypic resistance data. Virulence gene analysis exhibited an overall low number of VAGs in wild birds studied. ExPEC were defined as suggested previously which is mainly based on the presence of at least two VAGs including P fimbrial genes *papA* and *papC*, S frimbriae genes *sfa/foc*, afimbrial adhesion genes *afa/dra*, group 2 polysaccharide capsule gene *kpsMTII* and iron acquisition gene *iutA* (Nowak et al., 2017). According to this definition, none of the isolates is regarded as ExPEC (**Table 1**). All isolates contained *E. coli* outer membrane protein A gene (*ompA*). Other common genes were *malX, astA* and *iha* coding phosphotransferase system enzyme II, enteroaggregative heat-stable toxin EAST1 and iron-regulated-gene-homologue adhesion, respectively.

MLST, Plasmid Replicon Types and Plasmid Profile Analysis

In this study, 17 different STs were observed among the 26 sequenced ESBL-*E. coli*. Among the known STs, the most common ones were ST10 and ST7097 (each n=4) followed by ST4720, ST93, and ST1139 (2 isolates each) whereas one isolate each of ST1421, ST354, ST224, ST1303, ST2914, ST202, ST602, ST58, ST617, ST361, ST3716, and ST1722 were found (**Table 1**). In silico plasmid replicon typing revealed the IncF-type plasmid as the most common (20/26; 76.9%). The other replicon types detected in this study included IncY, IncI1, IncI2, IncHI2, IncQ1, IncB/O/K/Z. Out of 20 isolates with IncF replicon type, 19 belonged to IncFIB class followed by IncFII ($n = 5$), IncFIC ($n = 4$) and IncFIA ($n= 2$). pMLST of the IncF plasmids revealed the presence of one common plasmid type F-:A-:B53 ($n = 14$). Analysis of the plasmid size with S1 digestion showed a 130 kb plasmid in most of the isolates (**Table 1**). In contrast, no replicons were detected in the Pk-2, Pk-9 and Pk-10 but those isolates harbored bla_{CTX-M}-15 encoded on large contigs whose annotation pointed toward a chromosomal integration of the resistance gene.

Whole Genome Phylogeny

Core-genome based phylogenetic analysis of 26 isolates grouped *E. coli* into four clusters. Most of the sequenced isolates clustered together in accordance with their ST (**Figure 2**). Core genome alignment showed very few SNPs ranging from 0 to 29 among isolates Pk-8, Pk-21, Pk-24, and Pk-26 (**Figure 2** and Table S2). All of these strains belonged to ST7097 and originated from two different hosts (Eurasian coot and mallard duck) and sampling locations (Chashma barrage and Rasul barrage). Likewise, only 29 SNPs were present between Pk-15 and Pk-29 isolates although recovered from different hosts (Eurasian coot and mallard duck) and locations (Chashma barrage and Rasul barrage). More strikingly, only one SNP was found between Eurasian coot isolates Pk-5 and Pk-14 originated from Chashma barrage. Fewer than 28 SNPs were observed between Pk-23 and Pk-30 (isolated from mallard duck and Eurasian coot from Chashma barrage). Two $bla_{CTX-M-1}$-producing *E. coli* Pk-6 and Pk-7 were marked by only four SNPs and were recovered from a similar geographic location and host (**Figure 2**). Numbers of SNPs for the individual isolates are displayed in Table S2.

DISCUSSION

Wild migratory birds have been suggested as a reservoir of ESBL-producing *E. coli* in a number of studies worldwide (Guenther et al., 2011, 2012; Bonnedahl et al., 2015; Atterby et al., 2016). More recently, we reported the occurrence of $bla_{CTX-M-15}$ producing *Klebsiella pneumoniae* (Raza et al., 2017) in wild migratory bird populations in Pakistan. We therefore also screened for ESBL-producing *E. coli* and their clonal relatedness using WGS, as there is lack of knowledge regarding genetic diversity of ESBL-*E. coli* isolates from environmental niches in Asia. *E. coli* is an excellent indicator species to study the spread of AMR through fecal pollution of water and waterfowl can be considered as sentinel of AMR in the environment (Guenther et al., 2011). The present study indicates high carriage rates of ESBL-producing *E. coli* (17%) in migratory birds along the Indus migration route in Pakistan. This high prevalence mirrors those reported in migratory gulls from Bangladesh (17.3%) (Hasan et al., 2014) and is comparable to another study from Bangladesh which reported 30% ESBL-*E. coli* from wild ducks (Hasan et al., 2012). This is underlining the important role of waterfowl as carrier of ESBL-producing *E. coli* in Asia and also adding the important Indus avian migration route to the environments influenced by human healthcare practices.

WGS showed $bla_{CTX-M-15}$ was the predominant ESBL genotype in this study. This is in agreement with some previous findings from wild birds in Bangladesh (Hasan et al., 2014), Germany (Guenther et al., 2010) and North America (Poirel et al., 2012). CTX-M-15 has now a worldwide distribution and although it is commonly associated with human and pet ESBL-isolates, it is also very common in avian wildlife (Wang et al., 2017).

In fact, summing up the current literature it becomes obvious that the emergence of ESBL-producing *E. coli* in wildlife is associated with the success of the bla_{CTX-M} family in hospitals (Guenther et al., 2011). The reason why bla_{CTX-M} producing *E. coli* are also very successful in the environment remain unclear

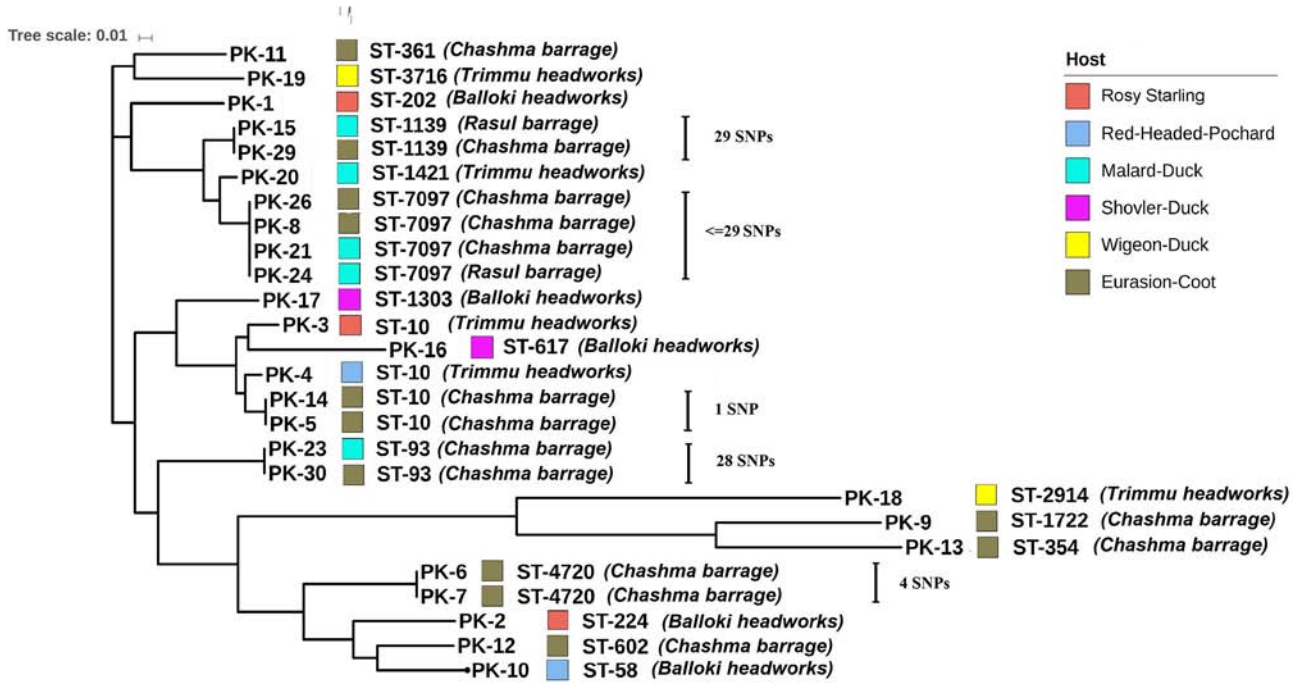

FIGURE 2 | Whole Genome phylogeny based upon core genomes of 26 avian ESBL-*E. coli*. The tree was produced using Harvest Suite and drawn by MEGA5 software.

but recent studies suggest that plasmids carrying those genes confer more advantages than mere resistance to the bacterial host strains (Schaufler et al., 2016). A previous study also indicated high rates of $bla_{CTX-M-15}$ from human clinical isolates in Pakistan (Habeeb et al., 2014), however as we did not include human isolates in this study their relatedness remains to be clarified in the future.

Besides their spread via plasmids, very recently the new trend of chromosomal integration of ESBL-encoding genes has been demonstrated in clinical *E. coli* isolates of ST38, ST410, ST131 and ST648 (Hirai et al., 2013; Rodríguez et al., 2014; Falgenhauer et al., 2016) and also in non-clinical ST38 isolates from wild birds (Guenther et al., 2017). Similarly, we detected the chromosomal insertion of $bla_{CTX-M-15}$ genes among *E. coli* of different STs (ST224, ST1722 and ST58), which have been found as plasmid carrying ESBL-producers in clinical and non-clinical samples, worldwide (Zurfluh et al., 2013; Leangapichart et al., 2016). This scenario has also been recently shown for *E. coli* strains of ST38 from Mongolian wild birds, which were very closely related to a clinical outbreak strain from the UK (Guenther et al., 2017).

As mentioned above, wildlife has been reported to carry ExPEC strains, we therefore also screened for the occurrence of VAGs to gain information on pathotype. However, we detected no ExPEC strain in our isolates. Most of the strains harbored only a few VAGs and are likely commensal strains. However, all the *E. coli* carried serum resistance *ompA* gene (**Table 1**). We also found high frequency of *astA* and *iha* genes. These are only putative virulence genes and their exact involvement in the pathogenesis is not well understood, although they have been frequently reported in enteroaggregative *E. coli* and avian pathogenic *E. coli* (Nowak et al., 2017).

We found a large diversity of sequence types within the avian isolates including typical ESBL-associated sequence types like ST10, ST224, ST617 (Guenther et al., 2011; Sherchan et al., 2015), and ST354 (Zhang et al., 2016). However, globally distributed high risk clones like ST131, ST410, and ST648 were not found in this study. Earlier studies from human clinical *E. coli* isolates from Pakistan reported those sequence types including ST131 and ST648 (Mushtaq et al., 2011; Pesesky et al., 2015), indicating that different clonal population of *E. coli* might be present in wild birds and the human population in Pakistan but this finding can also be due to the low number of birds sampled.

Interestingly we found identical STs in isolates originating from different avian host species and geographic locations (**Figure 2**). Core genome phylogenetic analysis of those isolates showed that within identical STs only a small number of SNPs ranged from 1 to 29 were found. This suggests a recent interspecies transmission and long-distance dissemination of certain clonal ESBL-lineages by wild birds as it has been reported earlier (Guenther et al., 2017). The origins of most of these birds are remote areas in Siberia and Central Asia and exposure to antimicrobials is less likely in these areas. The high rates of MDR isolates detected from the wild migratory bird are of concern and could be due to anthropogenic activities from the surrounding environment. In addition to the clonal spread of certain STs our data showed the common occurrence of a plasmid replicon type (IncFIB, F-:A-:B53) linked to a 130 kb plasmid. This plasmid was found in all four wetlands tested and in five of the seven

different avian species. Together with the large number of minor STs points toward the spread of a bla_{CTX-M} resistance plasmid of the pMLST type F-:A-:B53 among a naive *E. coli* population in the avian hosts.

The transmission dynamics of ESBL-producing *E. coli* in a natural environment are complex. Wild birds have been suggested as sentinels for the spread and transmission of multi-resistant strains in the environment. It is widely believed that the spread of ESBL-*E. coli* is driven both by plasmid transfer in commensal and pathogenic strains as well as by the clonal spread of certain lineages in local areas. In this study we were able to detect both main mechanisms in wild migratory birds in Pakistan underlining the suitability of avian sentinels. In addition our data highlights the potential for regional and intercontinental transmission of ESBL-producing *E. coli* clones and resistance plasmids via migratory birds.

AUTHOR CONTRIBUTIONS

MM, SG: conceived and designed the experiments; MM, SR, and FS: collected the data and samples; MM, KS, NR, FS, and PS: performed laboratory analysis; SG, MM, SR, and TS: analyzed the data; TS and SG: performed WGS; MM and SG: wrote the article. All authors have read and approved the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02476/full#supplementary-material

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REVIEW
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Acinetobacter spp. Infections in Malaysia: A Review of Antimicrobial Resistance Trends, Mechanisms and Epidemiology

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Acinetobacter spp. are important nosocomial pathogens, in particular the *Acinetobacter baumannii-calcoaceticus* complex, which have become a global public health threat due to increasing resistance to carbapenems and almost all other antimicrobial compounds. High rates of resistance have been reported among countries in Southeast Asia, including Malaysia. In this review, we examine the antimicrobial resistance profiles of *Acinetobacter* spp. hospital isolates from Malaysia over a period of nearly three decades (1987–2016) with data obtained from various peer-reviewed publications as well as the Malaysian National Surveillance on Antibiotic Resistance (NSAR). NSAR data indicated that for most antimicrobial compounds, including carbapenems, the peak resistance rates were reached around 2008–2009 and thereafter, rates have remained fairly constant (e.g., 50–60% for carbapenems). Individual reports from various hospitals in Peninsular Malaysia do not always reflect the nationwide resistance rates and often showed higher rates of resistance. We also reviewed the epidemiology and mechanisms of resistance that have been investigated in Malaysian *Acinetobacter* spp. isolates, particularly carbapenem resistance and found that bla_{OXA-23} is the most prevalent acquired carbapenemase-encoding gene. From the very few published reports and whole genome sequences that are available, most of the *Acinetobacter* spp. isolates from Malaysia belonged to the Global Clone 2 (GC2) CC92 group with ST195 being the predominant sequence type. The quality of data and analysis in the national surveillance reports could be improved and more molecular epidemiology and genomics studies need to be carried out for further in-depth understanding of Malaysian *Acinetobacter* spp. isolates.

Keywords: *Acinetobacter*, antimicrobial resistance, Malaysia, surveillance data, epidemiology, resistance mechanisms

Abbreviations: *Abc* complex, *Acinetobacter baumannii–calcoaceticus* complex; ADC, *Acinetobacter*-derived cephalosporinase; CC, clonal complex; HSA, Hospital Sultanah Aminah; HSNZ, Hospital Sultanah Nur Zahirah; HUSM, Hospital Universiti Sains Malaysia; IMR, Institute of Medical Research; LPS, lipopolysaccharide; MBL, metallo-β-lactamase; MDR, multidrug resistance; MLST, multilocus sequence typing; NSAR, National Surveillance of Antibiotic Resistance; UKMMC, Universiti Kebangsaan Malaysia Medical Centre; UMMC, University of Malaya Medical Centre; WGS, whole genome sequencing.

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INTRODUCTION

Acinetobacter spp. are Gram-negative opportunistic pathogens associated with severe nosocomial infections including pneumonia, bloodstream, urinary tract and wound infections, as well as meningitis. The majority of infections are due to the *A. baumannii–A. calcoaceticus* (*Abc*) complex with *A. baumannii* being the most clinically important species (Dijkshoorn et al., 2007; Clark et al., 2016; Gonzalez-Villoria and Valverde-Garduno, 2016). The genus *Acinetobacter* is taxonomically complex with unambiguous identification at the species level particularly problematic (Gundi et al., 2009). *A. baumannii, A. nosocomialis*, *A. pittii* and *A. calcoaceticus*, which is usually an environmental species, along with two novel pathogenic species, *A. seifertii* and *A. djikshoorniae* cannot be reliably differentiated by phenotypic tests, and are thus usually grouped together as the *Abc* complex (Gerner-Smidt et al., 1991; Nemec et al., 2015; Cosgaya et al., 2016; Marí-Almirall et al., 2017). Accurate identification at the species level requires sequencing of the RNA polymerase β-subunit gene, *rpoB*, and/or the DNA gyrase B gene, *gyrB* (Gundi et al., 2009), with full-length 16S rRNA gene sequencing proven unreliable (Wang et al., 2014).

Carbapenems are broad-spectrum β-lactam antibiotics that have been the treatment of choice for *Acinetobacter* infections, particularly in critically ill patients (Fishbain and Peleg, 2010). However, the increasing prevalence of carbapenem-resistant *A. baumannii*, particularly in the last two decades, has been of immense concern such that carbapenem-resistant *A. baumannii* is now listed as the top priority pathogen in urgent need of new antimicrobials by the World Health Organization in February 2017 (World Health Organization, 2017). This is due to *Acinetobacter* spp., especially *A. baumannii*, having extensive intrinsic antimicrobial resistance mechanisms coupled with the inherent ability to easily acquire new resistance determinants through mobile genetic elements such as plasmids, transposons and genomic islands (Peleg et al., 2008; Doi et al., 2015). Carbapenem-resistant *A. baumannii* is the most common pathogen associated with nosocomial infections in Southeast Asia (Mendes et al., 2013; Suwantarat and Carroll, 2016), a region which groups together 11 nations with disparate incomes and levels of development. The surveillance of antimicrobial resistance among common pathogens was one of the important recommendations issued by the World Health Organization (WHO) in 2001 to slow down the emergence and contain the spread of bacterial resistance (WHO, 2001). Only four Southeast Asian countries, namely Singapore, Thailand, Malaysia and the Philippines have established national antimicrobial surveillance programs; poorer countries such as Myanmar and East Timor (or Timor-Leste) are hampered by limited microbiology laboratory capabilities (Hsu et al., 2017). Malaysia, which is considered as an upper middle income nation and with an active national antimicrobial surveillance program, has surprisingly few publications and little comprehensive data available on *Acinetobacter* spp. infections (McNeil et al., 2016). A recent paper that estimated the mortality attributable to multidrug-resistant pathogens in nosocomial infections in Thailand clearly showed that *Acinetobacter* spp. is the leading cause of hospital-acquired infections with the highest attributable mortality at around 40% (Lim et al., 2016). It would not be surprising if similar burdens of *Acinetobacter* infection are present in neighboring Malaysia but such data have not been published.

In this review, we look at the resistance trends of several antimicrobials for *Acinetobacter* spp. isolated in Malaysia with data obtained from individual studies (which usually involves strains isolated from single institutions/healthcare centers) as well as from the Malaysian National Surveillance on Antibiotic Resistance (NSAR), and spanning a period of nearly three decades, between 1987 and 2016. We also cover the various mechanisms of resistance that have been elucidated, in particular carbapenem resistance, and finally, we review the epidemiological and genomic studies of *Acinetobacter* spp. that have been published, thereby giving us an overview of the state of *Acinetobacter* antimicrobial resistance and epidemiology in this Southeast Asian nation.

ANTIBIOTIC SUSCEPTIBILITY PROFILES

The Institute for Medical Research (IMR), Malaysia, publishes the NSAR results from 2003 onward (except year 2006) online[1] which surveys isolates from various hospitals throughout Malaysia, including Sabah and Sarawak in Borneo. The number of hospitals involved and the sample sizes differ each year but have increased from just 12 hospitals in 2007 to 41 hospitals in 2016. Prior to 2007, the NSAR data only presented the total number of isolates that were analyzed for that particular year (i.e., for 2003–2005) without indicating the source of these isolates. The names of the participating hospitals were only published from 2009 onward. Nevertheless, the data did not indicate the prevaling resistance rates for individual participating hospitals but rather was analyzed as a total cumulative pool of isolates.

The Clinical and Laboratories Standard Institute (CLSI) currently lists 24 antimicrobial agents from nine groups with breakpoints for *Acinetobacter* spp. (CLSI, 2017). A joint initiative between the European Centre for Disease Prevention and Control (ECDC) and the US Centers for Disease Prevention and Control (CDC) led to the development of standard definitions of MDR, extensive drug resistance (XDR) and pandrug resistance (PDR) in an effort to harmonize the antimicrobial resistance surveillance systems (Magiorakos et al., 2012). The ECDC-CDC recommendation for *Acinetobacter* spp. covered 22 of the 24 CLSI antimicrobial agents (omitting piperacillin from the penicillin group and gatifloxacin from the fluroquinolone group; see **Table 1**) (Magiorakos et al., 2012). In the Malaysian NSAR reports, only six groups of antimicrobials were regularly tested (no data was available for antibiotics under the folate pathway inhibitor group and limited data available for the lipopeptides polymyxin B and colistin). The NSAR data do not give any indication on the prevalence of MDR (let alone XDR or PDR) among the isolates that were tested. No mention was

[1]http://www.imr.gov.my/en/component/content/article/75-english-content/national-collabration/1469-nsar.html

made in the NSAR reports to differentiate between infection and colonization and whether the isolates were obtained from hospital-acquired or community-acquired infections. The source of the bacterial isolates (i.e., whether they were isolated from blood, pus, tracheal aspirates, or other clinical samples) were only stated in the NSAR reports of 2015 onward. We are thus unable to assess the quality assurance or the validity of the NSAR data but these are nevertheless presented here as they are the only publically available nationwide data available for Malaysia. Besides NSAR, there were also scattered reports from other researchers throughout Malaysia who obtained *Acinetobacter* spp. samples from various hospitals throughout the country, albeit only in Peninsular Malaysia and not in the states of Sabah and Sarawak in Borneo (see **Figure 1** for the geographical location of these studies). These *Acinetobacter* spp. were isolated from clinical specimens in the respective hospital laboratories and the sources of these isolates were usually presented in these reports. However, whether these were hospital-acquired or community-acquired infections are not known. The panel of antibiotics used by these researchers differs from the NSAR report, thus making meaningful comparisons difficult. Nevertheless, there are some common antimicrobials that were used throughout the few research papers that have been published and here, we summarize and review these results.

Carbapenems

Carbapenems are usually the drug of choice for serious *Acinetobacter* infections; nevertheless their utility is increasingly compromised by the rapid emergence of resistance (Peleg et al., 2008; Doi et al., 2015). *Acinetobacter* spp. isolates ($n = 21$) from the UMMC, which is located in the capital city of Kuala Lumpur, and collected in 1987 showed imipenem resistance rates of only 4.8% but a decade after that, imipenem resistance rates have increased to 36.4% for isolates collected between 1996 and 1998 ($n = 88$) (**Figure 2**) (Misbah et al., 2004). The first NSAR data in 2003 showed that the national resistance rate for meropenem was slightly below 30% and this was also reflected in a study of isolates from HUSM, located in the northeastern state of Kelantan, from 2003–2004 (Deris et al., 2009). However, by 2008, the NSAR data showed that the resistance rates for meropenem as well as imipenem have reached 50%. Nevertheless, there has not been any drastic increase in the nationwide carbapenem resistance rates from 2008–2016 which has stayed around 50–60%. Several studies on *A. baumannii* isolates from individual hospitals showed carbapenem resistance rates higher than the national average: ICU isolates from the UMMC collected from 2006–2009 showed very high resistance rates for imipenem at 96.5% and meropenem at 98.2% (Kong et al., 2011), as did isolates from several ward in Hospital Selayang (located also in Kuala Lumpur) in 2010 with a 92.5% resistance rate for meropenem whereas the imipenem resistance rate was lower at 67.5% (Nazmul et al., 2012). Likewise, *A. baumannii* isolates collected in 2010 and 2011 from various ward in HSA in the southern state of Johor, displayed resistance rates of 88% for both imipenem and meropenem (Dhanoa et al., 2015). Resistance rates of >70% were also reported for isolates from UKMMC (located south of Kuala Lumpur) in 2010–2011 (Biglari et al., 2015, 2017) and HSNZ (located in the east coast state of Terengganu) in 2011(Lean et al., 2014).

Cephalosporins

The national *A. baumannii* resistance rates for the extended-spectrum cephalosporins of the third generation, ceftazidime, and the fourth generation, cefepime, were around 30% in 2003 but increased to around 50% between 2005 and 2009 (**Figure 3**). The resistance rates for both ceftazidime and cefepime remained within the 50–60% range throughout 2010–2014. From 2015 onward NSAR only reported rates for ceftazidine, which maintained between 55 and 60%. Reports of strains that were

TABLE 1 | List of antimicrobials recommended by the European Centre for Disease Prevention and Control (ECDC) and the United States Centers for Disease Prevention and Control (CDC) for standard definitions of multidrug resistance, extensive drug resistance and pandrug resistance for *Acinetobacter* spp. (Magiorakos et al., 2012) along with the antimicrobial agents with available breakpoints as given by the Clinical and Laboratories Standard Institute (CLSI) in its 2017 edition (CLSI, 2017).

Antimicrobial agent with CLSI breakpoints	Inclusion in ECDC-CDC recommendation
Penicillins	
Piperacillin	No
β-lactam/β-lactamase inhibitor	
Ampicillin/Sulbactam	Yes
Piperacillin/Tazobactam	Yes
Ticarcillin/Clavulanante	Yes
Cephams	
Ceftazidime	Yes
Cefepime	Yes
Cefotaxime	Yes
Ceftriaxone	Yes
Carbapenems	
Doripenem	Yes
Imipenem	Yes
Meropenem	Yes
Lipopeptides	
Colistin	Yes
Polymyxin B	Yes
Aminoglycosides	
Gentamicin	Yes
Tobramyxin	Yes
Amikacin	Yes
Netilmycin	Yes
Tetracycline	
Doxycycline	Yes
Minocycline	Yes
Tetracycline	Yes
Fluoroquinolones	
Ciprofloxacin	Yes
Levofloxacin	Yes
Gatifloxacin	No
Folate pathway inhibitors	
Trimethoprim-sulfamethoxazole	Yes

Antimicrobial groups are given in bold.

FIGURE 1 | Map of Malaysia indicating the geographical location of the hospitals in which the *Acinetobacter* spp. isolates were obtained for the various individual studies that had been conducted and reviewed in this paper. The various states within Malaysia are indicated in blue whereas neighboring countries are labeled in brown. HUSM, Hospital Universiti Sains Malaysia; HSNZ, Hospital Sultanah Nur Zahirah; HSA, Hospital Sultanah Aminah; HRPB, Hospital Raja Perempuan Bainun; UKMMC, Universiti Kebangsaan Malaysia Medical Centre; UMMC, University Malaya Medical Centre.

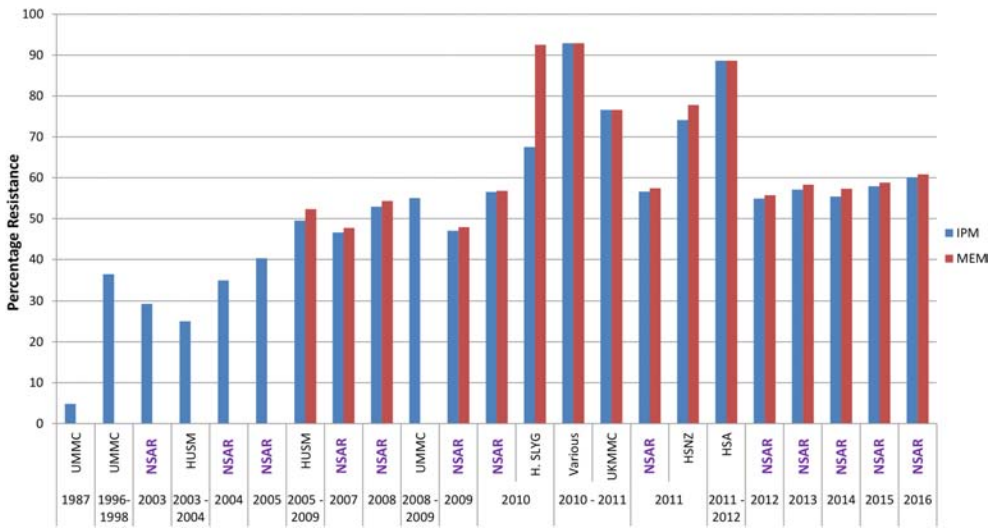

FIGURE 2 | Carbapenem resistance rates for Malaysian *Acinetobacter* spp. isolates (1987–2016). IMP, imipenem; MEM, meropenem. Data from the National Surveillance for Antibiotic Resistance (NSAR) is included and labeled as "NSAR" in purple-colored fonts. Data from the other studies are as follows: UMMC from 1987 and between 1996 and 1998, (Misbah et al., 2004); HUSM between 2003 and 2006, (Deris et al., 2009); and between 2005 and 2009, (Ariffin et al., 2012); UMMC between 2008 and 2009, (Dhabaan et al., 2012); Hospital Selayang (H. SLYG) in 2010, (Nazmul et al., 2012); UKMMC between 2010 and 2011, (Biglari et al., 2015, 2017); Various, collected from various hospitals mainly around the town of Ipoh in the state of Perak in 2010 and 2011, (Kor et al., 2014); HSNZ in 2011, (Lean et al., 2014); and Hospital Sultanah Aminah (HSA) between 2011 and 2012 (Dhanoa et al., 2015).

isolated from individual hospitals showed higher resistance rates for ceftazidime and cefepime when compared to the national average: strains from HSA in 2010 and 2011(Dhanoa et al., 2015) showed resistance rates of nearly 90% whereas strains from UKMMC from 2010 and 2011 (Biglari et al., 2015) and HSNZ in 2011 (Lean et al., 2014) showed resistance rates of around 70%. Ceftazidime resistance rates for *A. baumannii* isolates from Hospital Selayang in 2010 (Nazmul et al., 2012) were closer to the national resistance rate of 58% for that year, as was the resistance rate for cefepime of isolates from UMMC in 2008–2009 (51%) although the resistance rate for ceftazidime was about 10% higher than the national resistance rate for that period of time (Dhabaan et al., 2012). In stark contrast, all 170 isolates obtained from the ICU of UMMC in 2006–2009 were resistant to ceftazidime and cefepime (Kong et al., 2011). Very high ceftazidime resistance rates had earlier been reported for *Acinetobacter* spp. isolates from UMMC that were isolated in 1987 (81%) and between 1996 and 1998 (97.7%) (Misbah et al., 2004).

The resistance rates for another third generation extended-spectrum cephalosporin, cefotaxime, were consistently higher than ceftazidime and cefepime (**Figure 3**). NSAR first reported the national resistance rates for cefotaxime in 2007 and this was already at 75.4%. An earlier study from HUSM from 2003–2004 showed an even higher cefotaxime resistance rate at 88% (Deris et al., 2009) and this reached 94.7% in strains isolated from the same hospital between 2005 and 2009 (Ariffin et al., 2012).

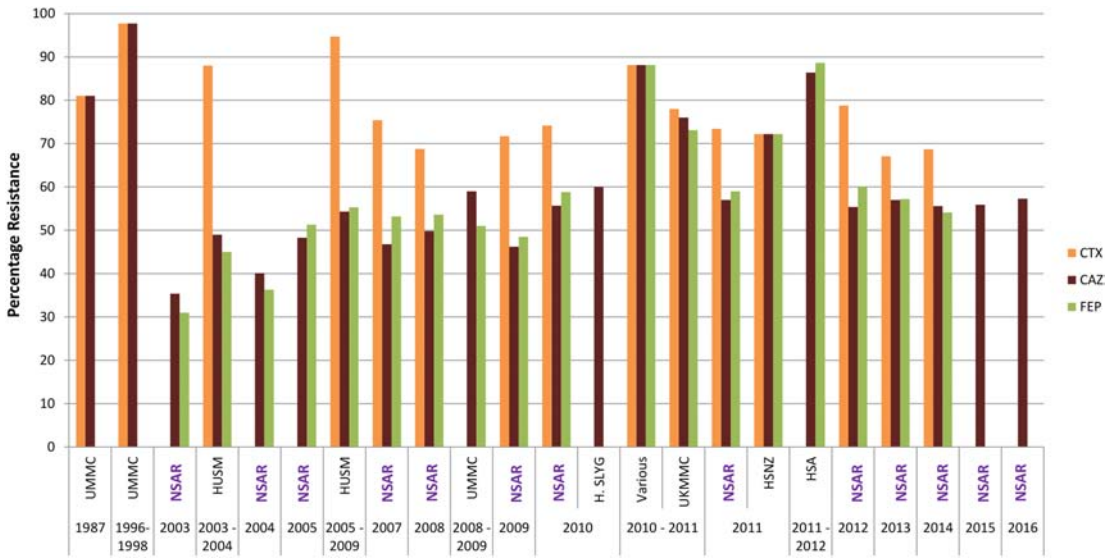

FIGURE 3 | Cepholosporin resistance rates for Malaysian *Acinetobacter* spp. isolates (1987–2016). CTX, cefotaxime; CAZ, ceftazidime; and FEP, cefepime. Data from the National Surveillance for Antibiotic Resistance (NSAR) is included and labeled as "NSAR" in purple-colored fonts. Data from the other studies are as follows: UMMC from 1987 and between 1996 and 1998, (Misbah et al., 2004); HUSM between 2003 and 2006, (Deris et al., 2009); and between 2005 and 2009, (Ariffin et al., 2012); UMMC between 2008 and 2009, (Dhabaan et al., 2012); Hospital Selayang (H. SLYG) in 2010, (Nazmul et al., 2012); UKMMC between 2010 and 2011, (Biglari et al., 2015, 2017); Various, collected from various hospitals mainly around the town of Ipoh in the state of Perak in 2010 and 2011, (Kor et al., 2014); HSNZ in 2011, (Lean et al., 2014); and Hospital Sultanah Aminah (HSA) between 2011 and 2012 (Dhanoa et al., 2015).

The national resistance rates for cefotaxime remained above 70% for 2009–2012 but dipped slightly below 70% in 2013–2014. Cefotaxime resistance rates for UKMMC in 2010–2011 (Biglari et al., 2015) and HSNZ in 2011 (Lean et al., 2014) were similar to the national resistance rate at that time frame (i.e., around 70%). Interestingly, cefotaxime resistance for *Acinetobacter* spp. isolates from UMMC from 1987 was even higher at 81% and this further increased to 97.7% in isolates obtained from 1996–1998 (Misbah et al., 2004). No data for cefotaxime were available in the NSAR reports for 2015 and 2016.

No NSAR data is also available for the fourth extended-spectrum cephalosporin that was listed in the CLSI and the ECDC-CDC guidelines, i.e., ceftriaxone. However, data from *Acinetobacter* spp. isolates obtained from UMMC in 1987 showed a high resistance rate of 90.5% and this further increased to 97.7% for isolates in 1996–1998 (Misbah et al., 2004). By the following decade, a 100% resistance rate to ceftriaxone was reported for *Acinetobacter* isolates from the UMMC ICU (collected from 2006–2009) (Kong et al., 2011).

Aminoglycosides

The NSAR report from 2003 showed a nationwide gentamicin resistance rate of 39.1% and an amikacin resistance rate that is four-fold lower at 8.8%. Resistance rates steadily increased and by 2008, the resistance rates for both aminoglycosides were similar although the rates for amikacin were around 2–5% lower than that of gentamicin (**Figure 4**). Throughout this period, gentamicin resistance rates increased from 39.1% in 2003 to about 50% in 2010 and remained around that level until the latest NSAR report for 2016. When looking at the aminoglycoside resistance data from individual hospitals as reported by other groups of researchers, the resistance rates for gentamicin were generally higher than for amikacin as shown in the NSAR data (**Figure 4**). However, isolates from three hospitals showed around 20% higher resistance rates than the NSAR data: UKMMC in 2010–2011 (70.2% for gentamicin) (Biglari et al., 2015), HSNZ in 2011 (66.7% for gentamicin, 57.4% for amikacin) (Lean et al., 2014) and HSA in 2011–2012 (79.5% for gentamicin, 72.4% for amikacin) (Dhanoa et al., 2015). A random sample of 42 *A. baumannii* isolates from various hospitals in Malaysia taken from 2008–2009 yielded a gentamicin resistance rate of 76.2% (Kim et al., 2013), which is also above the national resistance rate as reported by NSAR, although for this particular study, the isolates chosen were all carbapenem resistant.

Fluoroquinolones

Only ciprofloxacin from the fluoroquinolone group of antimicrobials has been used to assess the antimicrobial susceptibility rates for *Acinetobacter* spp. in Malaysia. The NSAR data showed that ciprofloxacin resistance rates increased from about 20% in 2003 to around 50% in 2008 with rates remaining around 50–55% until the latest report for 2016. Results from individual hospitals more or less reflected the national trend with the exception of UKMMC in 2010–2011 which showed a resistance rate of 79.6% (Biglari et al., 2017), HSNZ in 2011 with a rate of 66.1% (Lean et al., 2014) and HSA in 2011–2012 with a rate of 84.1% (Dhanoa et al., 2015). ICU isolates from UMMC (2006–2009) showed highest ciprofloxacin resistance rates at 99.4% (Kong et al., 2011).

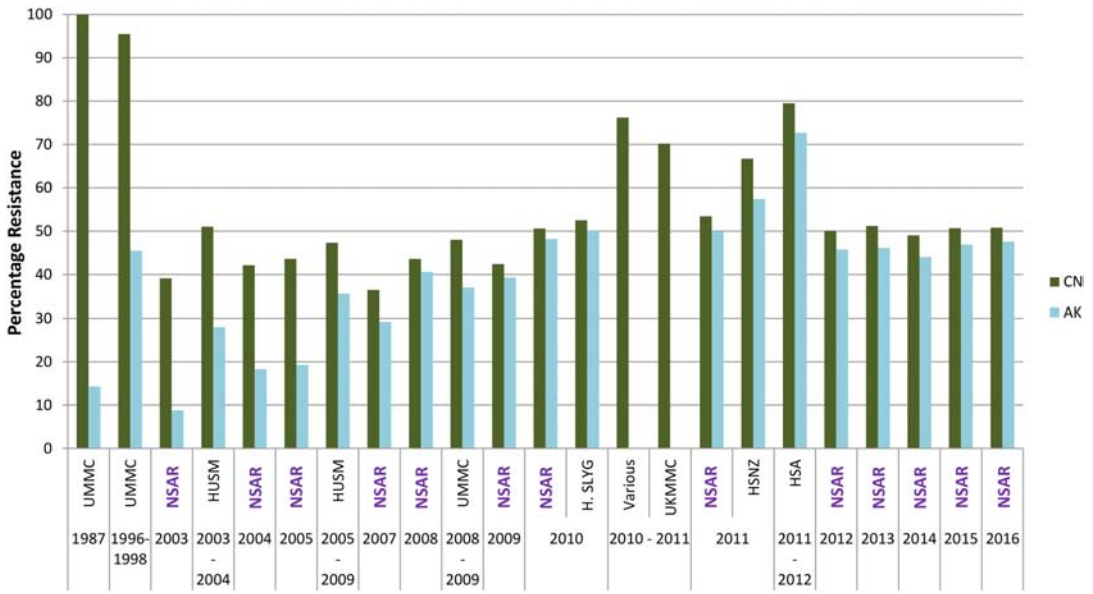

FIGURE 4 | Aminoglycoside resistance rates for Malaysian *Acinetobacter* spp. isolates (1987–2016). CN, gentamicin; AK, amikacin. Data from the National Surveillance for Antibiotic Resistance (NSAR) is included and labeled as "NSAR" in purple-colored fonts. Data from the other studies are as follows: UMMC from 1987 and between 1996 and 1998, (Misbah et al., 2004); HUSM between 2003 and 2006, (Deris et al., 2009); and between 2005 and 2009, (Ariffin et al., 2012); UMMC between 2008 and 2009, (Dhabaan et al., 2012); Hospital Selayang (H. SLYG) in 2010, (Nazmul et al., 2012); UKMMC between 2010 and 2011, (Biglari et al., 2015, 2017); Various, collected from various hospitals mainly around the town of Ipoh in the state of Perak in 2010 and 2011, (Kor et al., 2014); HSNZ in 2011, (Lean et al., 2014); and Hospital Sultanah Aminah (HSA) between 2011 and 2012 (Dhanoa et al., 2015).

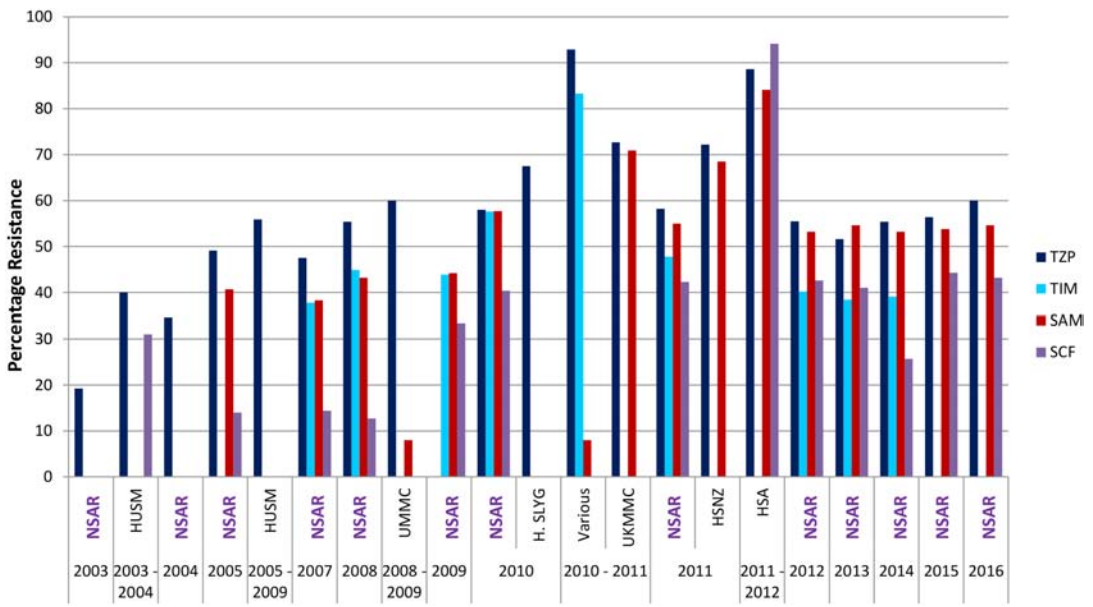

FIGURE 5 | Resistance rates for β-lactam/β-lactamase combination in Malaysian *Acinetobacter* spp. isolates (2003–2016). TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanate; SAM, ampicillin/sulbactam; SCF, cefoperazone/sulbactam. Data from the National Surveillance for Antibiotic Resistance (NSAR) is included and labeled as "NSAR" in purple-colored fonts. Data from the other studies are as follows: HUSM between 2003 and 2006, (Deris et al., 2009); and between 2005 and 2009, (Ariffin et al., 2012); UMMC between 2008 and 2009, (Dhabaan et al., 2012); Hospital Selayang (H. SLYG) in 2010, (Nazmul et al., 2012); UKMMC between 2010 and 2011, (Biglari et al., 2015, 2017); Various, collected from various hospitals mainly around the town of Ipoh in the state of Perak in 2010 and 2011, (Kor et al., 2014); HSNZ in 2011, (Lean et al., 2014); and Hospital Sultanah Aminah (HSA) between 2011 and 2012 (Dhanoa et al., 2015).

Penicillins

NSAR reported *Acinetobacter* spp. resistance rates for ampicillin and piperacillin from 2007 to 2014. The Malaysian *Acinetobacter* isolates displayed very high resistance rates for ampicillin, which averaged at 89.2% whereas piperacillin showed a lower average resistance rate of 55.6% within the 7-year surveillance period.

β-Lactam/β-Lactamase Inhibitor Combination

The national resistance rate of *Acinetobacter* spp. toward the combination of piperacillin/tazobactam was relatively low (at 19.2%) in 2003 but this gradually increased to 55.8% by 2008 (**Figure 5**). NSAR data showed that from 2008 to 2016, the national resistance rates for piperacillin/tazobactam remained within the 55–60% range. However, reports of strains isolated from individual hospitals showed markedly higher resistance rates, as had been observed for other antimicrobials. Isolates from HSA in 2011 and 2012 (Dhanoa et al., 2015) showed resistance rates of about 90% whereas the resistance rates were lower at around 70% for UKMMC in 2010 and 2011 (Biglari et al., 2015), and HSNZ in 2011 (Lean et al., 2014) (**Figure 5**).

NSAR data for the combination of ticarcillin/clavulanate was available from 2007–2014 and the national *Acinetobacter* spp. resistance rates remained around the 40% level with the exception of 2010 when it spiked to 57.6% before decreasing to 47.8% the following year (**Figure 5**). The national resistance rate for ampicillin/sulbactam was around 40% from 2005 to 2009, thereafter increasing to between 50 and 60% from 2010 to 2016 (**Figure 5**). Reported resistance rates for the ampicillin/sulbactam combination from individual hospitals were higher, at 84.1% in the HSA *A. baumannii* isolates obtained in 2011 and 2012 (Dhanoa et al., 2015), and about 70% for the UKMMC isolates between 2010 and 2011 (Biglari et al., 2015) and the HSNZ isolates in 2011 (Lean et al., 2014). Lower resistance rates were generally observed for the sulbactam/cefoperazone combination when compared to ampicillin/sulbactam. When NSAR first reported data for sulbactam/cefoperazone in 2005, the resistance rate was at 14% and remained around that level for 2007–2008. The national sulbactam/cefoperazone resistance rate increased considerably to 33.4% in 2009 and it remained between 40 and 45% from 2010 to 2016 with the notable exception of 2014 where the reported rate was at 25.7%. However, *A. baumannii* isolates from HSA (in 2011 and 2012) showed a much higher sulbactam/cefoperazone resistance rate of 94.1%, higher than the ampicillin/sulbactam resistance rate of 84.1% (Dhanoa et al., 2015).

Tetracyclines

There are very few reports on the prevalence of tetracycline resistance in Malaysian *Acinetobacter* isolates. Lean et al. (2014) reported that out of 54 *A. baumannii* isolates that were collected from various ward in HSNZ in Terengganu during 2011, 87% were resistant to tetracycline while 61.1% were resistant to doxycycline. Similar high resistance rates for tetracycline were reported (79.1%) for a collection of 43 MDR *A. baumannii* isolates that were obtained from various hospitals mainly around the town of Ipoh, Malaysia although the year of their collection and the identity of the hospitals were not stated (Kor et al., 2014).

Tigecycline is a semisynthetic antibiotic belonging to the tetracycline-derived glycylcycline family and along with the lipopeptides or polymyxins (i.e., polymyxin B and colistin, or polymyxin E), tigecycline is considered one of the 'last resort' drugs for the treatment of *Acinetobacter* infections (Lim et al., 2011; Doi et al., 2015; Li et al., 2015; Pogue et al., 2015). However, it should be noted that guidelines such as the latest Infectious Diseases Society of America (IDSA) and the American Thoracic Society (ATS) for the management of adults with hospital-acquired pneumonia and ventilator-associated pneumonia (HAP/VAP) strongly recommends against the use of tigecycline in *Acinetobacter* infections (Kalil et al., 2016). Latest systematic reviews and meta-analyses also disfavor the use of a tigecycline-based regimen for the treatment of MDR *A. baumannii* infections, despite its lower nephrotoxicity compared with colistin (Ni et al., 2016; Kengkla et al., 2017). NSAR only reported tigecycline resistance rates for *A. baumannii* blood isolates from 2013–2016 with fairly constant rates of 15–18% for the 4 year period. An earlier study from the UMMC with isolates obtained from 2008–2009 indicated a 5% intermediate susceptibility to tigecycline for their clinical isolates but a much higher percentage (60%) of intermediate susceptibility for hospital environmental isolates (Dhabaan et al., 2012), which is surprising and a cause for concern. On the other hand, Kor et al. (2014) had reported a 58.1% tigecycline resistance rate on their collection of 43 MDR *A. baumannii* from various hospitals in Ipoh but their susceptibility testing for tigecycline was performed using the Kirby-Bauer disk diffusion assay for which no standard breakpoints were available. The 2008–2009 UMMC isolates were assessed for tigecycline susceptibility using both *E*-test and broth microdilution, and the MIC breakpoints from the United States Food and Drug Administration (FDA) were used for their interpretation of tigecycline susceptibility (Dhabaan et al., 2012), a move which was recently supported (Nicolau et al., 2015) in the absence of any CLSI guidelines for tigecycline until now (CLSI, 2017). Broth microdilution is recommended for determining tigecycline MIC values as a report had shown that tigecycline MICs varied greatly according to the *in vitro* testing methods used with Etest giving significantly elevated MICs and were thus, deemed inaccurate (Marchaim et al., 2014).

Polymyxins (Lipopeptides)

NSAR only reported *A. baumannii* resistance rates for colistin from 2015 onward where rates were low at 0.8% in 2015 and all isolates were susceptible in 2016. Data for the other polymyxin, polymyxin B, was only reported for blood isolates of *A. baumannii* from 2013–2016 with a resistance rate of 1.4% in 2013 and all isolates susceptible in 2014–2016. In stark contrast, Lean et al. (2014) had reported an alarmingly high resistance rate of 25.9% for polymyxin B in HSNZ. So far, this is the only peer-reviewed, published report of polymyxin resistant *A. baumannii* from Malaysia. The UMMC study on *A. baumannii* isolates obtained from 2008 and 2009 did not detect any polymyxin resistance (Dhabaan et al., 2012), as were isolates

obtained from the UMMC ICU earlier (between 2006 and 2009) (Kong et al., 2011). Likewise, no polymyxin-resistant isolates were found in the 2011–2012 HSA study (Dhanoa et al., 2015) and the 2010– 2011 UKMMC study (Biglari et al., 2013).

RESISTANCE MECHANISMS

Carbapenem Resistance

Carbapenem resistance in *Acinetobacter* spp. is now increasingly reported worldwide and is usually mediated by enzymatic inactivation (via carbapenemases), active efflux of drugs and target site modification (i.e., altered penicillin-binding proteins) (Zarrilli et al., 2009). More than 210 β-lactamases belonging to 16 families have been identified in *Acinetobacter* spp. (Zhao and Hu, 2012) with class D β-lactamases being the most widespread carbapenemase in *A. baumannii* (Zarrilli et al., 2009; Bush, 2013). Class B metallo-β-lactamases (MBL; IMP-, VIM-, SIM- and NDM-types) have also been sporadically reported worldwide in *A. baumannii*, being able to hydrolyze carbapenems and other β-lactams, except aztreonam, and resistant to clinically available β-lactamase inhibitors (Zhao and Hu, 2012). Several insertion sequence (IS) elements such as ISAba1, ISAba2, ISAba3 and IS18, have been found to increase the expression of class D β-lactamase genes (including $bla_{OXA-23-like}$ and $bla_{OXA-58-like}$ genes) when they are inserted immediately upstream due to the presence of an outward-directing promoter at the ends of these IS elements (Zarrilli et al., 2009; Hsu et al., 2017). The *A. baumannnii* chromosome also encodes an intrinsic $bla_{\text{OXA-51-like}}$ gene that is weakly expressed but does not confer resistance to carbapenems. However, it has been demonstrated that insertion of an ISAba1 element upstream of the gene conferred carbapenem resistance (Turton et al., 2006).

There are very few papers that have investigated the possible carbapenem resistance mechanisms in *Acinetobacter* spp. isolates from Malaysia. The $bla_{\text{OXA-23}}$ gene appeared to be the predominant acquired carbapenemase in the Malaysian *A. baumannii* isolates, which is not surprising as $bla_{\text{OXA-23}}$ is the most common cause of carbapenem resistance in *A. baumannii*, and the most widely spread acquired OXA carbapenemase worldwide (Kamolvit et al., 2015). The prevalence of the $bla_{\text{OXA-23}}$ gene was 75.9% in the 2011 *A. baumannii* HSNZ isolates (Lean et al., 2014) and 82% in the 2010–2011 UKMMC isolates (Biglari et al., 2015, 2017). In an earlier study, nearly 95% of carbapenem-resistant *Acinetobacter* spp. isolated in 2003–2004 from UMMC, were positive for $bla_{\text{OXA-23}}$ (Wong et al., 2009). However, almost half of the UKMMC isolates that contained the ISAba1-$bla_{\text{OXA-51-like}}$ configuration were susceptible to carbapenems, leading the authors to conclude that ISAba1 may not upregulate the expression of the intrinsic $bla_{\text{OXA-51-like}}$ gene and mediate carbapenem resistance (Biglari et al., 2015), as had been previously proposed (Turton et al., 2006). No $bla_{OXA-24-like}$ and $bla_{OXA-58-like}$ genes were detected so far in the Malaysian *A. baumannii* isolates (Biglari et al., 2015; Lean et al., 2014) although these class D β-lactamases have been found elsewhere, particularly in European isolates (D'Andrea et al., 2009; Merino et al., 2010; Novovic et al., 2015; Chatterjee et al., 2016). Among the Class B MBLs, only bla_{IMP} has been reported albeit only in 9.9% of the UKMMC *A. baumannii* isolates (Biglari et al., 2015) and 5.1% in the carbapenem-resistant 2003–2004 UMMC *Acinetobacter* spp. isolates (Wong et al., 2009), whereas neither bla_{IMP} nor bla_{VIM} was found in the HSNZ *A. baumannii* isolates from 2011 (Lean et al., 2014). Southern hybridization localized the bla_{IMP-4} gene in an *A. calcoaceticus* isolate from UMMC to a class 1 integron on an approximately 35 kb plasmid (Wong et al., 2009). Interestingly, genome sequencing of an *A. pittii* isolated in 2014 from a hospital in the state of Perak (in Peninsular Malaysia) led to the discovery of bla_{NDM-1} and bla_{OXA-58} co-residing in the isolate (Ang et al., 2016). The bla_{NDM-1} gene was found within a 10,038 bp composite transposon which resided on a 140 kb megaplasmid whereas the bla_{OXA-58} gene was located on a 35 kb plasmid. Metallo-β-lactamase production in this *A. pittii* strain was validated by testing with the Etest MBL kit from BioMériux (Ang et al., 2016).

Cephalosporin Resistance

Acinetobacter spp. are known to encode *Acinetobacter*-specific AmpC cephalosporinases in the chromosome, designated ADCs (Hujer et al., 2005). More than 45 variants of ADCs (ADC-1 to ADC-56) have been categorized for the genus *Acinetobacter* with many more that remain uncategorized (Zhao and Hu, 2012). In cephalosporin-resistant *A. baumannii* isolates from UKMMC, the bla_{ADC} gene was present in 93.7% of the isolates and in most of these bla_{ADC}-positive isolates, ISAba1 was detected upstream of the bla_{ADC} gene (Biglari et al., 2015). ADCs are normally expressed at low levels and are not inducible (Hujer et al., 2005) but the insertion of ISAba1 upstream often leads to the overexpression of these cephalosporinases (Héritier et al., 2006). The specific ADC type was, however, not determined for the UKMMC isolates. The bla_{ADC} sequence from 3 cephalosporin-resistant *A. baumannii* from HSNZ isolated in 2011 (i.e., AC12, AC29 and AC30) were found to be a hitherto uncategorized ADC (with R80S and G246S mutations in reference to ADC-7) (Lean et al., 2015, 2016). However, these bla_{ADC} genes were characterized as belonging to the *ampC* allele 20 in a recent paper reporting on the re-curation of the *A. baumannii*-encoded *ampC* genes in a new database hosted at http://pubmlst.org/abaumannii (Karah et al., 2017). These bla_{ADC} genes from *A. baumannii* AC12, AC29 and AC30 were cloned into a pET30a expression vector and expressed in *Escherichia coli* BL21, leading to the recombinant *E. coli* strains displaying resistance to ceftazidime, cefepime, aztreonam and even imipenem (Lean et al., 2016). This suggests that the ADC from these isolates were indeed extended-spectrum *Acinetobacter*-derived AmpC (ESAC) as ADCs typically hydrolyze penicillins, narrow- and extended-spectrum cephalosporins but not carbapenems and zwitterionic cephalosporins such as cefepime (Rodríguez-Martínez et al., 2010; Lean et al., 2016).

Other Resistance Mechanisms

The main mechanisms of fluroquinolone resistance are mutations that alter the target sites DNA gyrase (encoded by *gyrA* and *gyrB*) and DNA topoisomerase IV (encoded by *parC* and *parE*) (Jacoby, 2005). Ciprofloxacin-resistant *A. baumannii*

isolates from UKMMC and *A. baumannii* AC12, AC29 and AC30 from HSNZ all displayed the characteristic serine-to-leucine substitution at position 83 for GyrA and position 80 for ParC (Lean et al., 2015, 2016; Biglari et al., 2017), mutations which have been implicated in fluoroquinolone resistance in *Acinetobacter* (Wisplinghoff et al., 2000; Fournier et al., 2006).

Resistance to polymyxins (polymxin B and colistin) in *A. baumannii* is mediated by multiple factors but is mainly due to modification of the LPS moieties that form the outer membrane layer of the cell (Olaitan et al., 2014; Jeannot et al., 2017; Poirel et al., 2017). In some polymyxin-resistant *A. baumannii*, phosphoethanolamine is enzymatically added to the lipid A of LPS (Arroyo et al., 2011) whereas in other resistant isolates, the LPS part of the outer membrane is completely absent due to mutations in the genes involved in LPS biosynthesis (Moffatt et al., 2010, 2011; Henry et al., 2012). These LPS alterations decrease the net negative charge, preventing the binding of the cationic polymyxin molecules to the bacterial surface (Jeannot et al., 2017; Poirel et al., 2017). PmrAB is a two-component regulatory system that regulates the expression of the genes involved in LPS modification; some mutations in *pmrAB* resulted in polymyxin resistance due to constitutive upregulation of the LPS modification pathway (Arroyo et al., 2011; Park et al., 2011; Lim et al., 2015; Dahdouh et al., 2017). Investigations into the polymyxin-resistant *A. baumannii* isolates from HSNZ in 2011 indicated a P102H mutation in the *pmrA* gene in all resistant isolates and several point mutations in the *lpxC*, *lpxD* and *lpsB* genes involved in LPS biosynthesis (Lean et al., 2014). Further experimental studies on two of these polymyxin-resistant isolates, *A. baumannii* AC12 and AC30, indicated upregulation of the *pmrB* gene as well as possible impairment (but not total loss) of the LPS (Lean et al., 2016). These mutations are intrinsic, and not transmissible, and are likely the result of selective pressure (Jeannot et al., 2017; Poirel et al., 2017). However, the recent discovery of the transmissible polymyxin-resistant genes, *mcr-1*, *mcr-1.2,* and *mcr-2* (which encode phosphoethanolamine transferases) in Enterobacteriaceae (Liu et al., 2015; Giamarellou, 2016) raised the alarming possibility of its spread to *Acinetobacter* spp. and other bacteria. Although no reports of *mcr*-positive *Acinetobacter* spp. have emerged until now, it is likely just a matter of time as the *mcr* genes are carried on transmissible plasmids (Malhotra-Kumar et al., 2016; Jeannot et al., 2017). A recent report highlighted this when it was shown that laboratory transformation of an *mcr-1*-encoded recombinant plasmid into several strains of *A. baumannii* led to the development of colistin resistance in these strains (Liu et al., 2017).

EPIDEMIOLOGY AND GENOMICS

Prior to the current accessibilty of WGS, various molecular methods were available for investigating the epidemiology of *A. baumannii.* Pulsed-field gel electrophoresis (PFGE) was the gold standard for epidemiological investigations of pathogenic bacteria including *A. baumannii* but suffers from limitations such as its labor- and time-intensiveness (2–4 days) and the lack of reliable inter-laboratory reproducibility despite the availability of guidelines for comparison of band positions (Rafei et al., 2014). Other electrophoretic band-based typing methods such as random amplified polymorphic DNA (RAPD) and repetitive sequence-based PCR (Rep-PCR) have been used for *A. baumannii*, but both suffer from lack of intra- and inter-laboratory reproducibility (van Belkum et al., 2007; Rafei et al., 2014). MLST remains the most widely accepted typing technique to study clonality and population structure of *A. baumannii* even in the era of WGS (Zarrilli et al., 2013; Rafei et al., 2014). MLST accesses the genetic variation that occurs in housekeeping genes by considering each unique sequence of the housekeeping gene as an allele type with a sequence type (ST) defined by combination of allele types for each gene in the MLST scheme. There are currently two MLST schemes for *A. baumannii*: (1) the Bartual or the Oxford scheme, which is based on seven genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*) (Bartual et al., 2005; Wisplinghoff et al., 2008), and (2) the Institut Pasteur scheme which is also based on seven genes (*cnp60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB*) (Diancourt et al., 2010), three of which (i.e., *cpn60*, *recA* and *gltA*) is common with the Oxford scheme.

Despite the availability of various molecular typing methods for *A. baumannii*, papers reporting on the molecular epidemiology of *A. baumannii* in Malaysia are few and far between. *Acinetobacter* isolates from UMMC obtained from 1987 and from 1996–1998 were subjected to Rep-PCR fingerprinting (Misbah et al., 2004) whereas those obtained from the same medical centre in 2006–2009 were analyzed by PFGE (Kong et al., 2011). PFGE profiles revealed the likelihood of a persistent *A. baumannii* clone endemic to the ICU with several environmental isolates and an isolate from the hands of a healthcare worker showing closely related PFGE profiles with isolates from patients (Kong et al., 2011). Similarly, Rep-PCR fingerprints indicated the presence of two distinct *Acinetobacter* lineages at UMMC that could have persisted from 1987 to 1996–1998 (Misbah et al., 2004). However, any meaningful comparisons between these two studies could not be made due to the different fingerprint methods that were used. Hence, an opportunity has been lost to assess the evolution of *Acinetobacter* spp. in the same medical center over a span of two decades. PFGE has also been used to investigate the *A. baumannii* isolates from HSNZ in 2011 (Lean et al., 2014) and *Acinetobacter* spp. isolates from HSA in 2010–2011 (Dhanoa et al., 2015). In both cases, endemicity of a prevalent clone in the respective hospitals as determined by their closely related pulsed-field *Apa*I profiles, was inferred and all isolates belonging to these prevalent clones were carbapenem resistant (Lean et al., 2014; Dhanoa et al., 2015). Clonal relatedness of *A. baumannii* isolates from UKMMC (2010–2011) was assessed by Rep-PCR which indicated 31 clones among the 162 *A. baumannii* isolates at a cutoff value of 90% similarity (Biglari et al., 2017). Unlike the HSNZ and HSA studies, the UKMMC study did not have any strong inference of a prevalent clone within the hospital during the time period of the investigation, based on the Rep-PCR profiles which showed considerable diversity between the isolates (Biglari et al., 2017).

TABLE 2 | Available whole genome sequences of *A. baumannii* isolated from Malaysia in the NCBI GenBank database.

A. baumannii strain	Source of isolate	ST (Oxford scheme)	ST (Pasteur scheme)	Accession number	Reference*
AC12	Blood	ST195	ST2	CP007549.3	Lean et al., 2015
AC29	Endotracheal secretion	ST195	ST2	CP007535.2	Lean et al., 2016
AC30	Endotracheal secretion	ST195	ST2	CP007577.1	Lean et al., 2016
PR07	Blood	ST734	ST239	CP012035.1	Izwan et al., 2015
269	Mucoid sputum	Unknown	ST119	JQNV00000000	NA
863	Mucoid sputum	ST938	ST2	LZTF00000000	NA
461	Wound swab	ST195	ST2	LCTE00000000	NA
341	Mucopurulent sputum	ST938	ST2	JQSD00000000	NA

*NA, not available .

Kim et al. (2013) gave an indication of the Oxford scheme STs that were prevalent in Malaysian *A. baumannii* isolates when they characterized 38 isolates obtained from Malaysia as part of the Asian Network for Surveillance of Resistance Pathogens (ANSORP) study on hospital-acquired pneumonia from 2008–2009. The majority of the Malaysian isolates (30 isolates; 78.9%) belonged to clonal complex 92 (CC92), out of which ST92 (12 isolates; 31.6%), ST195 (7 isolates; 18.4%) and ST426 (7 isolates; 18.4%) were the most frequently identified STs (Kim et al., 2013). Three *A. baumannii* isolates from HSNZ (2011) that were subjected to WGS (namely AC12, AC29 and AC30) were all found to be ST195 (Lean et al., 2015, 2016). Similarly, when MLST was performed on seven selected *A. baumannii* UKMMC isolates (based on their major Rep-PCR profiles), six were found to be ST195 whereas the other isolate was found to be ST208 (Biglari et al., 2017). We mined the GenBank database for *A. baumannii* genome sequences from Malaysia (**Table 2**) and found that only one of the other five available genomes were ST195 (*A. baumannii* strain 461). *A. baumannii* 269 had an unknown ST based on the Oxford scheme but was typed as ST119 using the Pasteur scheme (**Table 1**). Hence, based on the small number of isolates and limited studies that are available, it would appear that the *A. baumannii* isolates from Malaysia mainly belonged to the Global Clone 2 (GC2) CC92, with ST195 being the predominant ST.

CONCLUSION

In this review, we have comprehensively examined the trends of antimicrobial resistance in *Acinetobacter* spp. isolated from various hospitals in Malaysia covering a period of nearly three decades from 1987 to 2016. The national *Acinetobacter* spp. carbapenem resistance rate currently stands at around 60%, which is similar to the levels reported for 2015 in two of Malaysia's neighboring countries which have national surveillance programs, i.e., Singapore (50%), and the Philippines (54.1%), whereas Thailand reported a higher rate of 73.7% (Hsu et al., 2017). The major acquired carbapenemase gene in *Acinetobacter* spp. isolated from Malaysia is $bla_{\mathrm{OXA-23}}$, as had been reported in these three neighboring countries although it should be noted that these data were obtained from individual studies and not through their respective national surveillance programs (Hsu et al., 2017). Although results from the Malaysian national surveillance program, NSAR, are publically available online from 2003 onward, the data and analysis could be vastly improved, as we had pointed out here and in a recent commentary (McNeil et al., 2016). Good quality surveillance data is an important component in the global fight against the spread of antimicrobial resistance and the paucity of such essential epidemiological data often leads to delayed or suboptimal revisions in policies and guidelines, which in turn, strengthens the vicious cycle of the careless use of antibiotics by medical practitioners (Laxminarayan et al., 2013). Ideally, a comprehensive surveillance programme should also include molecular epidemiological testing which would enable us to have an in-depth understanding of the origins and extent of the antimicrobial resistance problem (Hsu et al., 2017) but this will likely not be implemented in the near future due to the limited resources of these countries with the exception of perhaps Singapore. Closer collaborations between institutes that handle the national surveillance programs with other academic or research institutions with the relevant resources and skills for molecular epidemiology and WGS should be fostered to better expedite and improve the quality of the surveillance data. This is particularly pressing for priority pathogens such as *Acinetobacter* spp. for which containing and preventing the spread of antimicrobial resistance is of paramount importance to prevent a possible "antibiotic apocalypse" whereby such bacterial infections would no longer be treatable with antibiotics.

AUTHOR CONTRIBUTIONS

Conception and design of study: CCY, NIAR, SI, and SCC; acquisition of data: FMR and AGA; analysis and interpretation of data: FMR, CCY, AGA, DWC, and SCC; drafting of the manuscript: FMR and CCY; critical revisions of the manuscript: NIAR, SI, AGA, DWC, and SCC. All authors have approved the final article.

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Occurrence and Genomic Characterization of ESBL-Producing, MCR-1-Harboring *Escherichia coli* in Farming Soil

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The emergence and spread of the mobile colistin resistance gene (*mcr-1*) has become a major global public health concern. So far, this gene has been widely detected in food animals, pets, food, and humans. However, there is little information on the contamination of *mcr-1*-containing bacteria in farming soils. In August 2016, a survey of ESBL-producing *Escherichia coli* isolated from farming soils was conducted in Shandong Province, China. We observed colistin resistance in 12 of 53 (22.6%) ESBL-producing Enterobacteriaceae isolates from farming soil. Six *mcr-1*-positive *E. coli* strains originating from a livestock-intensive area were found. The isolates belonged to four different STs (ST2060, ST3014, ST6756, and ST1560) and harbored extensive additional resistance genes. An *E. coli* with bla_{NDM-1} was also detected in a soil sample from the same area. Comparative whole genome sequencing and S1-PFGE analysis indicated that *mcr-1* was chromosomally encoded in four isolates and located on IncHI2 plasmids in two isolates. To our knowledge, we report the first isolation of *mcr-1* in ESBL-producing *E. coli* from farming soils. This work highlights the importance of active surveillance of colistin-resistant organisms in soil. Moreover, investigations addressing the influence of animal manure application on the transmission of *mcr-1*-producing bacteria are also warranted.

Keywords: *mcr-1*, ESBLs, *Escherichia coli*, farming soil, animal manure

INTRODUCTION

Antimicrobial resistance determinants, the dissemination of which are facilitated by human activities, are increasingly being recognized as emerging environmental contaminants with the potential to pose a threat to human health (Sanderson et al., 2016). It is well-recognized that large amounts of antibiotics are released from humans and animals into agricultural fields by manure fertilization (Jechalke et al., 2014). Subsequently, these substances may affect the structure

and function of *in situ* bacterial communities and further lead to an increased abundance and transferability of antibiotic resistance genes (ARGs) (Jechalke et al., 2014). Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae is an important group of multidrug-resistant (MDR) bacteria which constitutes a major public health concern (Bush and Fisher, 2011). Antimicrobial therapy with colistin alone, or in combination with other antibiotics, is regarded as a "last-line" treatment option against bacterial infections caused by MDR Gram-negative pathogens (Paterson and Harris, 2016). Globally, there are increasing reports of colistin-resistant Enterobacteriaceae. Bacteria that produce ESBLs or carbapenemases in particular, are associated with colistin resistance; these colistin-resistant bacteria pose a severe health threat due to the limited therapeutic options available (van Duin and Doi, 2015).

Recently, concerns were raised regarding the increasing prevalence of colistin-resistant Enterobacteriaceae due to the discovery of the first plasmid-mediated colistin resistance gene, *mcr-1*, which was identified in China (Liu et al., 2016). Since the first report of *mcr-1*, *mcr* genes, including *mcr-1/2/3/4/5* have been detected in animals, food, human microbiota, and clinical samples in over 30 countries (Gao et al., 2016; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017). Notably, our and other research groups have already found Enterobacteriaceae isolates containing MCR-1 and carbapenemases, raising serious concerns about the possible global dissemination and spread of pan-resistant pathogens (Zheng et al., 2016).

To date, the *mcr* gene has been detected worldwide in human and animal samples; however, its occurrence in environmental samples has rarely been described. Several previous studies have documented the emergence of *mcr*-harboring, ESBL-producing Enterobacteriaceae in river and waste water (Zurfuh et al., 2016; Ovejero et al., 2017; Sun P. et al., 2017), suggesting that the *mcr* gene has spread from veterinary to aquatic environments. Colistin resistance is a threat to human and animal health worldwide, and soil ecosystems are one of the major environmental contamination sectors of antibiotic-resistant bacteria. However, the extent and significance of emergence of MCR-producing isolates in soil has not been elucidated.

The aim of this study was to describe the occurrence of *Escherichia coli* isolates harboring both the $bla_{\text{CTX-M}}$ and *mcr* genes that were originally isolated from farming soils in China. We also sought to reveal the genomic structure of *mcr*-positive *E. coli* isolates and to decipher the colistin resistance mechanisms among these environmental isolates.

MATERIALS AND METHODS

Study Site and Soil Sampling

In August 2016, we collected farming soil samples from 32 distinct rural sites in Shandong Province, China (Supplementary Figure S1). The families at the study sites most commonly lived in a four-room house with an outdoor toilet located in the yard. Most families kept chicken and pigs in the yard. Toilet waste was disposed by the family itself and manure from animals were often applied to agricultural fields. Three non-repeated samples were obtained from each site, which is geo-positioned with a precision <0.5 m. All samples were collected from deeper layers (depth 3–10 cm) within a 20 cm × 20 cm area and kept on ice during transport.

Isolation of ESBL-Producers

Each sample (2.0 g) was homogenized with a fivefold volume of sterile Luria-Bertani (LB) liquid medium (∼10 ml) and cultured at 37°C overnight. The enriched solutions were plated on MacConkey agar plates with 2 mg/L cefotaxime for 18–24 h at 37°C to isolate potential ESBL-producing strains. ESBL production was confirmed via the double-disk synergy test (DDST) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). ESBL-producing isolates were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Antimicrobial Susceptibility Testing and Detection of Resistance Genes

Broth microdilution was performed for antimicrobial susceptibility testing of ESBL producers, and the results were interpreted using CLSI breakpoints. EUCAST breakpoints were used for colistin and tigecycline[1]. The ESBL-producing isolates were further subjected to PCR for the detection of *mcr* genes (*mcr-1*, *mcr-2*, *mcr-3*, and *mcr-4*), carbapenemase genes and ESBL genes, as previously described (Branas et al., 2015; Liu et al., 2016; Xavier et al., 2016; Carattoli et al., 2017; Yin et al., 2017).

Multilocus Sequence Typing and Pulsed-Field Gel Electrophoresis

Multilocus sequence typing (MLST) was undertaken in accordance with protocols described in the *E. coli* database (Wirth et al., 2006) and the *Klebsiella pneumoniae* database (Brisse et al., 2009). The clonality of *mcr-1*-positive isolates was assessed by XbaI-pulsed-field gel electrophoresis (PFGE) and cutoff lines at 85% were used to analyze genetic relatedness (Zheng et al., 2015). S1-PFGE, hybridization, and conjugation experiments were performed as previously described (Zheng et al., 2016).

Whole Genome Sequencing (WGS) and *in Silico* Analyses

To characterize the genetic features of the *mcr*-bearing isolates, whole-genome sequencing (WGS) was performed on six isolates using the Illumina HiSeq platform (Illumina, San Diego, CA, United States). WGS data quality control was performed as previously described (Zhang et al., 2014). Sequencing data were assembled using SOAPdenovo (Luo et al., 2012) and queries were then generated by utilizing the ResFinder 2.1 (Zankari et al., 2012) database to identify acquired ARGs. PlasmidFinder

[1]http://www.eucast.org

1.3 was employed to identify plasmid replicon types (Carattoli et al., 2014). Plasmid profiling using plasmidSPAdes to assemble plasmids from WGS data was also performed (Antipov et al., 2016).

Conjugation Experiments and Plasmid Analysis

The transferability of *mcr*-bearing plasmids from isolates was determined using filter mating with *E. coli* J53 as the recipient strain, mixed at a ratio of 1:1 in broth culture, as previously described (Zheng et al., 2015). The resulting transconjugants were selected on BHI agar plates amended with colistin (2 mg/L). The colonies were identified as *E. coli* J53 via MALDI-TOF MS and such colonies were screened and sequenced for the presence of *mcr-1* gene. Plasmid sizes were determined using the S1-nuclease PFGE (S1-PFGE) method (Zheng et al., 2015). Additionally, Southern blotting analysis was performed to determine genetic location using specific probes for the *mcr* gene. Identification of replicon types of the plasmid incompatibility (Inc) groups was performed by multiplex PCR, as described previously (Carattoli et al., 2005).

Accession Numbers

The whole genome sequences of *mcr-1*-positive *E. coli* strains were deposited in GenBank under the following accession numbers: accession no. MVOR00000000 (E4), MVOS00000000 (E11), MVOT00000000 (E24), MVOU00000000 (E38), MVOV00000000 (E43), and MVOW00000000 (E47).

RESULTS AND DISCUSSION

Identification of ESBL-Producing Enterobacteriaceae

Analysis of 96 soil samples led to the isolation of 53 ESBL-producing Enterobacteriaceae, including 42 *E. coli* isolates and 11 *K. pneumoniae* isolates. MIC results demonstrated that 50 (96.2%) isolates exhibited multidrug resistance, which was defined as resistance to at least three different classes of antimicrobial agents (Supplementary Table S1). The highest susceptibility rate was observed for imipenem (100%), followed by meropenem (96.2%), tigecycline (94.3%), colistin (79.2%), and polymyxin B (75.5%). $bla_{\text{CTX-M}}$ genes were detected in 50 (96.2%) isolates. The most prevalent $bla_{\text{CTX-M}}$ gene was $bla_{\text{CTX-M-14}}$ ($n = 21$), followed by $bla_{\text{CTX-M-27}}$ ($n = 13$), $bla_{\text{CTX-M-65}}$ ($n = 10$), $bla_{\text{CTX-M-55}}$ ($n = 9$), $bla_{\text{CTX-M-11}}$ ($n = 2$), and $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-17}}$ ($n = 1$ for each) (Supplementary Table S2). For *E. coli* in a clinical context, ST10, ST38, ST131, and ST405 are responsible for the dissemination of CTX-M worldwide (Hernandez and Gonzalez-Acuna, 2016). The STs among the ESBL-producing *E. coli* observed in this study were quite different and only ST10 ($n = 2$) was detected among the aforementioned STs. Notably, although NDM-1-producing strains are rarely recovered from soil (Wang and Sun, 2015), the $bla_{\text{NDM-1}}$ gene was identified in strain E28 (Supplementary Table S1). In addition, 10 (23.8%) *E. coli* and 2 (18.2%) *K. pneumoniae* were resistant to colistin and polymyxin B. The currently known resistance mechanisms to colistin involve modifications of the lipopolysaccharide and can either be encoded chromosomally or by the plasmid-borne *mcr-1/2/3/4* (Poirel et al., 2017). In our study, six isolates were positive for *mcr-1* and none of the isolates carried *mcr-2/3/4* determinants. DNA sequencing of the full-length *mcr* gene revealed 100% matching nucleotide identity with the *mcr-1* sequence described in the original publication. Interestingly, *mcr-1*-producing isolates were recovered from five sampling sites, all of which were located in an area with intensive livestock farming (Supplementary Figure S1). In addition, except for isolates E31 and E7, isolates E91, E95, K63, and K64 were highly resistant to colistin (>16 mg/l). The resistance mechanism responsible for the high MICs observed could be due to mutations in the two-component system *pmrAB,* which can lead to increases in the extent of LPS modifications which in turn lowers the affinity to colistin (Poirel et al., 2017).

Occurrence of MCR-1-Harboring *E. coli* in Farming Soil

The six *mcr-1*-producers belonged to ST2060 ($n = 3$), ST3014, ST6756, and ST1560 (**Figure 1**). These STs have not been previously reported to be associated with *mcr-1*. The diverse STs exhibited genetic heterogeneity, which has also been observed in other reports on MCR-1-producing *E. coli* (Veldman et al., 2016; Wang et al., 2016). These findings imply the complex genetic diversity of both the *mcr-1* gene and its *E. coli* hosts in soils in China. As a consequence, there is an urgent need to formulate a comprehensive strategy to prevent further dissemination of *mcr-1* in multidrug-resistant isolates. The isolates E38, E43, and E47 presented highly similar profiles, indicating the clonality of these MCR-1-producing strains (**Figure 1**). S1-PFGE and hybridization showed that the MCR-1-producing isolates had multiple plasmids that ranged from 30 to 250 kb (**Figure 2A**). Moreover, the *mcr-1* gene was located on a 220 kb plasmid in isolates E11 and E24. Interestingly, southern blot and conjugation experiments produced negative results for E4, E38, E43 and E47, indicating that the *mcr-1* gene was chromosomally encoded in these isolates (**Figure 2B** and Supplementary Table S3). Chromosome-based *mcr-1* genes have also been found in previous studies (Falgenhauer et al., 2016; Li et al., 2016). Our study revealed unexpected diversity in the *mcr-1*-harboring strains present in the examined soil samples.

China produces an estimated 2.1 trillion kg of swine and chicken annually (Zhou et al., 2016). Prior to the Chinese government's ban of colistin as a feed additive for animals in Nov 1, 2016, the consumption of colistin was more than 8,000 tons (Walsh and Wu, 2016). The long-term usage of huge amounts of colistin may have established a selection pressure facilitating the generation and dissemination of colistin-resistant isolates in feces, especially in chicken, as antimicrobial agents were often administered orally to these animals (Nguyen et al., 2016). Predictably, colistin-resistant strains have been widely

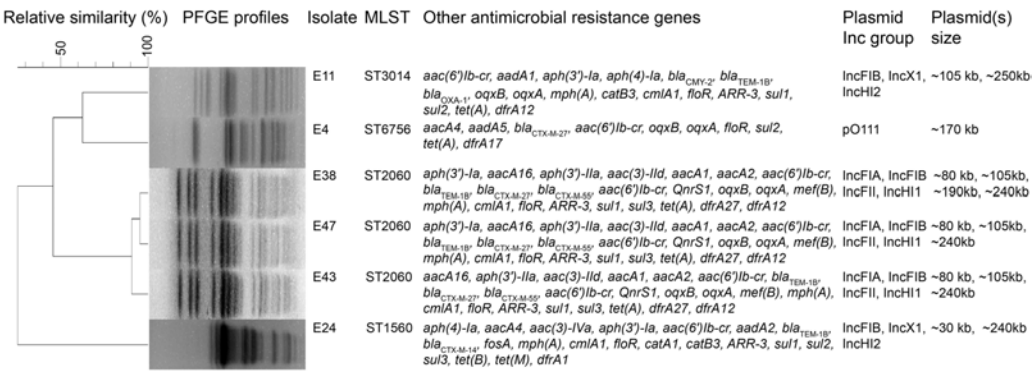

FIGURE 1 | Molecular and genotypic profiles of six *mcr-1*-producing *Escherichia coli* isolates from farming soil. Summary of molecular epidemiological characteristics of the six *mcr-1*-producing *E. coli* isolates. The dendrogram of PFGE patterns was constructed using BioNumerics v6.6 with UPGMA clustering. The scale bar indicates percentage of genetic relatedness.

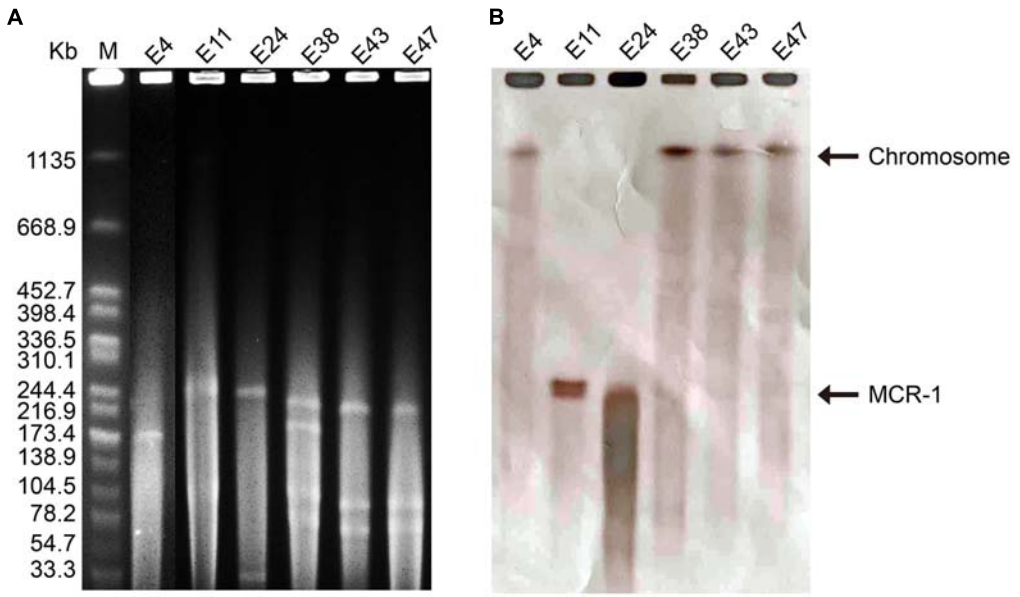

FIGURE 2 | (A) Plasmid profiles of six *mcr-1*-positive isolates generated using the restriction enzyme S1, with *Salmonella enterica* serovar Branderup as the molecular mass marker. **(B)** Southern blot hybridization with a *mcr-1*-specific probe. The MCR-F1 (5′-TGCAGCATACTTCTGTGTGGT-3′) and MCR-R1 (5′-CACCGAGTAGATTGGCATGA-3′) primers were used.

detected in fecal samples from food animals in China (Bai et al., 2016). To the best of our knowledge, no report to date have described *mcr*-positive Enterobacteriaceae isolated from soil samples. However, *mcr*-positive *E. coli* have been identified in river water, vegetable samples (Zurfuh et al., 2016) and sewage water (Ovejero et al., 2017). Interestingly, one study investigated the transmission of *mcr-1*-containing bacteria into the environment around farm areas in Germany and found seven *mcr-1*-positive *E. coli* strains originating from environmental boot swabs, dog feces, stable flies, and manure (Guenther et al., 2017). More pertinently, a recent report revealed that *mcr-1* producers have been identified in drinking water from Shandong Province (Sun P. et al., 2017). Notably, in rural areas of China, especially areas with intensive livestock farming, animal manure is widely used as organic fertilizer (Zhu et al., 2013). These findings were consistent with our results, although the contribution of soil-contaminant routes to the spread of *mcr-1*-harboring bacteria requires additional investigation. Our data suggest potential contamination of soil with bacteria harboring the *mcr-1* gene from animal manure, since in our study, all of the isolated *mcr-1*-producers were recovered from a livestock-intensive area.

Genomics Features of MCR-1-Producing Isolates

Whole-genome sequencing produced 4,717,954, 5,886,228, 4,302,436, 5,043,375, 4,164,486, and 5,989,082 pairs of 150-bp reads for E4, E11, E24, E38, E43, and E47, respectively. Assembly

of these isolates' genomes resulted in 109, 179, 124, 116, 119 and 113 contigs larger than 500 bp, comprising 4.9 megabases of sequence and representing a median 309-fold coverage (Supplementary Table S4).

The wide-spread use of antibiotics in animal production leads to a contamination of animal feces and urine with the parent antimicrobial compound and MDR bacteria, resulting in contamination of the farming soils with ARGs (Xu et al., 2015). All of the sequenced *mcr-1*-positive isolates found in this study harbored multiple resistance genes, inducing multidrug resistance, and multiple plasmid Inc types, suggesting that multiple plasmids were present, a finding consistent with our plasmid profiling results (**Figure 2**). The bla_{TEM-1B}, *floR*, and *sul1* genes and aminoglycoside resistance genes [*aac(6′)Ib-cr*, *aph(3′)-Ia* or *aadA*] were detected in all *mcr-1*-positive *E. coli* strains; these findings explain the extensively drug-resistant phenotype of these *E. coli* isolates (**Figure 1** and Supplementary Table S1). The E38, E43, and E47 strains were genetically closely related; this finding was consistent with our observations for PFGE profiles, indicating the isolate-driven spread of the *mcr-1* gene. Interestingly, isolates E11 and E24 shared the same plasmid Inc types although PFGE results showed their relative heterogeneity, indicating the prevalence of *mcr-1*-bearing plasmids in this livestock-intensive area and their broad-host-range characteristics which facilitates the dissemination of the *mcr-1* gene (**Figures 1**, **2**). A recent study also revealed that the worldwide dissemination of *mcr-1* was mainly mediated by highly promiscuous plasmids rather than several populations of *mcr-1*-carrying clones (Matamoros et al., 2017). The clones may have the intrinsic ability of acquiring antimicrobial resistance genes, including *mcr-1*, enabling them to play a potential role as a reservoir for this gene and facilitate the prevalence of *mcr-1* gene in local regions.

We identified plasmid replicons in all six isolates, including one type of plasmid in E4, three types of plasmids in E11, and four types of plasmids in E24, E38, E43, and E47. Via BLAST analysis of the plasmid sequences assembled by plasmidSPAdes, we also found seven different types of plasmids in these strains, a result consistent with the S1-PFGE findings (**Figure 2A**). In isolate E11, *mcr-1* was carried on an IncHI2 plasmid. A search of the nr/nt database revealed sequence homology between the assembled large plasmid contig (60.4 kb) and the annotated *mcr-1*-positive IncHI2 plasmid pHNSHP45-2 (GenBank: KU341381) (Supplementary Figure S2A). For isolate E24, a *mcr-1*-harboring contig (37.5 kb) was found to be 99% identical to the *mcr-1*-positive IncHI2 plasmid pMR0516mcr (GenBank: KX276657) (Supplementary Figure S2B). Notably, the sequence of *pap2-mcr-1*-IS*Apl1* region was identified in both plasmids, which is usually found in *mcr-1*-carrying plasmids (Wang et al., 2017). In addition, the genetic context of the chromosomally encoded *mcr-1* genes was similar to that reported in a previous study, i.e., *mcr-1* was observed in a structure consisting of IS*Apl1*-IRR-*mcr-1-hp* (Supplementary Figure S3) (Sun J. et al., 2017). IS*Apl1* is a member of the IS*30* family, and contributes to the mobilization of the *mcr-1* cassette into the chromosome through recognition of different related IRRs, which could perfectly match with 3′-end of *mcr-1-hp* to form a circular intermediate (Dona et al., 2017; Sun J. et al., 2017).

CONCLUSION

To the best of our knowledge, this investigation involved the first survey of MCR-1 in ESBL-producing *E. coli* isolates from farming soils. It is well-known that the *mcr-1* gene can spread through food chains. This study further highlights the possibility that *mcr-1* may enter humans via soil contamination and thereby threaten public health. Rates of *mcr-1* carriage are likely to rise rapidly in the examined region due to the environmental contamination with *mcr-1* described in this work and a previous study (Sun P. et al., 2017). Therefore, investigations addressing the influence of animal manure application on the transmission of *mcr-1* producers are of great significance, and improved multisectoral surveillance for colistin-resistant *E. coli* in Zhucheng City and nearby regions is warranted.

AUTHOR CONTRIBUTIONS

BZ, YX, and LL conceived and designed the experiments. BZ, CH, HX, JZ, LJ, and XW performed the experiments. LG, XJ, and XY analyzed the data. BZ, XL, YF, and YX wrote the paper.

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SUPPLEMENTARY MATERIAL

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Characterization of Resistance Patterns and Detection of Apramycin Resistance Genes in *Escherichia coli* Isolated from Chicken Feces and Houseflies after Apramycin Administration

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The aim of this study was to evaluate the influence of apramycin administration on the development of antibiotic resistance in *Escherichia coli* (*E. coli*) strains isolated from chicken feces and houseflies under field conditions. Chickens in the medicated group (n = 25,000) were given successive prophylactic doses (0.5 mg/l) of apramycin in their drinking water from Days 1 to 5, while no antibiotics were added to the un-medicated groups drinking water (n = 25,000). Over 40 days, a total of 1170 *E. coli* strains were isolated from fecal samples obtained from medicated and un-medicated chickens and houseflies from the same chicken farm. Apramycin MIC90 values for *E. coli* strains obtained from the medicated group increased 32–128 times from Days 2 to 6 (256–1024 μg/ml) when compared to those on Day 0 (8 μg/ml). Strains isolated from un-medicated chickens and houseflies had consistently low MIC90 values (8–16 μg/ml) during the first week, but showed a dramatic increase from Days 8 to 10 (128–1024 μg/ml). The apramycin resistance gene *aac(3)-IV* was detected in *E. coli* strains from medicated (n = 71), un-medicated (n = 32), and housefly groups (n = 42). All strains positive for *aac(3)-IV* were classified into 12 pulsed-field gel electrophoresis (PFGE) types. PFGE types A, E, and G were the predominant types in both the medicated and housefly groups, suggesting houseflies play an important role in spreading *E. coli*-resistant strains. Taken together, our study revealed that apramycin administration could facilitate the occurrence of apramycin-resistant *E. coli* and the apramycin resistance gene *acc(3)-IV*. In turn, these strains could be transmitted by houseflies, thus increasing the potential risk of spreading multi-drug-resistant *E. coli* to the public.

Keywords: apramycin resistance genes, *Escherichia coli*, PFGE, chicken feces, housefly

INTRODUCTION

Antimicrobial resistance emerges from the use of antimicrobials in animals and the subsequent transfer of resistance bacteria from those animals to the broader environment (Berendonk et al., 2015). The influence of antimicrobial usage on the prevalence of resistant strains in animals is of great concern for wider public health (da Costa et al., 2008; Martins da Costa et al., 2011; Sato et al., 2014).

Apramycin is an aminoglycoside antibiotic that has been used in animal husbandry since the early 1980s. It is still used in several European countries and it was approved for use in China in 1999 (Zhang et al., 2009). It is used to treat or prevent infections caused by Gram-negative bacteria such as colibacillosis, salmonellosis, and bacterial enteritis in poultry, swine, and calves (Antunes et al., 2011). Epidemiological investigations of apramycin-resistant bacteria from food producing animals showed differential prevalence of apramycin resistance in different animals (Choi et al., 2011). To date, there are two known resistance genes that confer resistance to apramycin in *E. coli*. One is the most prevalent apramycin resistance gene, *aac(3)-IV*, which codes for an aminoglycoside 3-*N*-acetyltransferase type-IV enzyme (Davies and Oconnor, 1978). The other is *npmA*, which was identified in a clinical *E. coli* strain in 2007 and subsequently found to encode for a 16S rRNA m^1A1408 methyltransferase (Wachino et al., 2007).

According to a previous study in China, apramycin-resistant *E. coli* are not only resistant to apramycin itself, such strains have also been found to be multi-resistant to several other antimicrobial agents (Zhang et al., 2009). This could complicate therapeutic options for bacteriosis treatment in both farm animals and humans (Zhang et al., 2009). A few studies have shown that apramycin treatment caused significant selective pressure in prevalence of resistance *E. coli* in swine (Mathew et al., 2003; Jensen et al., 2006). However, its influence on *E. coli* found in chicken has not yet been investigated.

The risk of flies disseminating resistant bacteria from livestock and poultry farms to the public has been a subject of increasing concern. Flies captured from different animal rearing facilities had been shown to be vectors for different microorganisms, some of which may be foodborne pathogens that are potentially threatening to human health (Forster et al., 2007). Moreover, flies also function as transmission vehicles for ESBL-producing *E. coli* from cattle (Usui et al., 2013) as well as laying hens and broilers (Blaak et al., 2014). However, the influence of apramycin administration on the development of antibiotic resistance in *E. coli* from chicken feces and houseflies has not been fully investigated.

Given this, our study was designed to evaluate three questions: (i) the influence on the development and persistence of apramycin resistance in *E. coli* isolated from fecal and houseflies in a chicken farm after preventive use of apramycin; (ii) the relationships between apramycin-resistant *E. coil* isolated from chicken feces and houseflies; and (iii) the characterization of apramycin-resistant *E. coli* found in houseflies.

MATERIALS AND METHODS

Study Setting

This study was conducted in a chicken farm with two different poultry houses (1000 m^2 each). The two houses were separated about 50 m to each other. After hatching, 50,000 chickens were equally and randomly allocated into two poultry houses (Day 0). Chickens in the medicated group (n = 25,000) were given successive prophylactic doses (0.5 mg/l) of apramycinsulfate (Shandong Qilu King-phar Pharmaceutical Co., Ltd., Shandong, China) in their drinking water from Days 1 to 5. In comparison, the un-medicated group (n = 25,000) was given drinking water without apramycin. No other antibiotics were used during the study period. Add antibiotic to drinking water for 5 days is the normal production behavior of the laying hens company. This study was carried out without any additional interference with the growth of the chickens. The protocol was approved by the Animal Ethics Committee of Sichuan University. We confirm that the best practice veterinary care and informed consent has been granted by the owners.

Samples were taken from each group as described in **Table 1**. Specifically, 15 cloacal swabs were collected from both the medicated and un-medicated groups at Day 0 and placed separately into sterile plastic bags. Fifteen sterilized plates were randomly placed under selected cages along two main diagonals of the poultry house containing both the medicated and un-medicated groups. Plates were placed at 12:00 am and withdrawn at 3:00 pm to allow for the collection of fresh fecal samples. Collections occurred on Days 1, 2, 3, 4, 5, 6, 8, 10, 15, 20, 30, and 40. Flies were captured using a sweep net on each sampling day from both of the two houses and approximately 30 flies were individually placed into sterile tubes for later morphological classification. All samples were placed into cool boxes containing ice packs and transported to the lab within 4 h for immediate bacterial isolation.

Bacterial Isolation

The cloacal swabs (n = 30) were separately put into 10 ml phosphate-buffered saline (PBS) and thoroughly vortexed. The resulting suspension was then 10-fold serial diluted with PBS and 100 μl of the dilution was plated onto eosin methylene blue (EMB) agar (Hangzhou Microbial Reagent Co., Ltd., Hangzhou, China) and incubated at 37°C overnight.

Fecal samples were collected from medicated (n = 15) and un-medicated groups (n = 15) at each sampling time. From these fresh fecal samples, 0.1 g was put into 10 ml PBS and thoroughly vortexed. The resulting suspension was 10-fold serial diluted with PBS and 100 μl was plated onto EMB agar and incubated at 37°C overnight.

Houseflies were collected at each sampling time, as previously described. Collected houseflies were morphologically identified using a stereomicroscope and 15 houseflies were randomly chosen for subsequent *E. coli* isolation. Each housefly was put into 10 ml PBS and thoroughly vortexed. The resulting suspension was 10 times gradient diluted with PBS, 100 μl was plated onto EMB agar, then incubated at 37°C overnight.

TABLE 1 | Sample collection and *E. coli* isolation.

Groups	Sample types	Number of samples/number of *E. coli* isolated			Total number of *E. coli*
		Pre-medication[a]	On-medication[b]	Off-medication[c]	
Un-medicated group	Cloacal swab	15/30	–	–	390
	Fresh feces	–	15/30	15/30	
Medicated group	Cloacal swab	15/30	–	–	390
	Fresh feces	–	15/30	15/30	
Houseflies group	Housefly	15/30	15/30	15/30	390
Total number of *E. coli*		90	450	630	1170

[a]*Sampling time at day 0 when chicken was hatched and transferred to the farm.* [b]*Sampling time at Days 1–5 when apramycin was administrated.* [c]*Sampling time at Days 6, 8, 10, 15, 20, 30, and 40 after apramycin was administrated.*

After overnight incubation, two colonies from each plate were selected for each sample. All isolates were then confirmed as being *E. coli* using a biochemical identification kit for *Enterobacteriaceae* (Hangzhou Microbial Reagent Co. Ltd., Hangzhou, China). All the confirmed *E. coli* isolates were kept frozen (−70°C) with 25% glycerol pending further analysis.

Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of apramycinsulfate (China Institute of Veterinary Drugs Control, Beijing, China) for all *E. coli* isolates was determined using the agar dilution method following the guidelines of the Clinical and Laboratory Standards Institute [CLSI] (2012a). In short, *E. coli* strains were subcultured on Luria Bertani (LB) agar at 37°C for 12 h. A clearly separate colony of the *E. coli* isolate was picked and a suspension of each strain in saline solution was adjusted to match the 0.5 McFarland standard. Mueller–Hinton (MH) plates that contain different apramycinsulfate concentration (0.125–1024 μg/ml) were seeded with a multipoint inoculum replicator and incubated at 35°C for 16–18 h. *E. coli* ATCC 25922 was used as the quality control strain. MIC data were only accepted if MICs of the control strains were within the required reference ranges. MIC90 (the MIC that ≥90% tested bacteria were inhibited for each sampling group) was used to evaluate the changes trend of apramycin resistance.

Apramycin Resistance Gene Detection

For detection of apramycin resistance genes, genomic DNA was prepared using a QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA, United States). Apramycin resistance genes *aac(3)-IV* and *npmA* were screened for all *E. coli* isolates as previously described (Yates et al., 2004; Zhou et al., 2010).

Pulsed-Field Gel Electrophoresis (PFGE) Typing of *aac(3)-IV*-Positive Strains

The clonal relatedness of *aac(3)-IV*-positive isolates were typed by PFGE as previously described (Gautom, 1997). Briefly, 145 *aac(3)-IV*-positive isolates were subcultured on LB agar at 37°C for 12 h. A single colony of each isolate was suspended with cotton swab in about 2 ml of TE buffer. The cell suspensions were adjusted to 20% transmittance by using a bioMérieux Vitek (Hazelwood, MO, United States). Proteinase K and lysozyme were added into 100 ml cell suspensions at final concentration of 1 mg/ml each and then incubated at 37°C for 10–15 min. Following the lysozyme–proteinase K incubation, 7 ml of 20% sodium dodecyl sulfate (50°C) and 140 ml of 1.2% InCert Agarose (50°C) were mixed with each bacterial suspension. Then the mixture was immediately added to plug molds (Bio-Rad Laboratories). After that, each solid plug was transferred to 2-ml round-bottom tubes with 1.5 ml of ESP buffer and incubated at 55°C for 2 h in a water bath. Then five times washes with 8–10 ml TE buffer (50°C) each in a shaker water bath for 15 min were carried out. For restriction endonuclease digestion, two 1-mm-thick slices of each plug were incubated at 37°C for 3 h with 50 U of *Xba*I enzyme. The plugs were then soaked in standard 0.5 Tris–borate–EDTA (TBE) prior to electrophoresis. The electrophoretic conditions used were as follows: initial switch time, 2.16 s; final switch time, 54.17 s; run time, 22 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14°C; ramping factor, linear. PFGE profiles were analyzed using the BioNumerics Program (Applied Maths, Sint-Martens-Latem, Belgium) as previously described (Yates et al., 2004). The clonal clusters with a similarity cutoff value of 80% were used in this study.

Antimicrobial Resistance Phenotype and Genotype of *aac(3)-IV*-Positive Strains

To investigate the antimicrobial resistance patterns and resistance genes of *aac(3)-IV*-positive isolates belonging to different PFGE types, we tested one isolate of each PFGE type for susceptibility to 22 antimicrobial agents. This process was conducted using the disk diffusion method according to CLSI guidelines (Clinical and Laboratory Standards Institute [CLSI], 2012b). Briefly, MH agar plate was inoculated with suspensions of bacteria, equivalent to standard 0.5 McFarland. Subsequently, the disks of different antimicrobial agents were placed on media and then incubated at 35°C for 16–18 h. The tested antimicrobial agents were as follows: ampicillin (10 μg), piperacillin (100 μg), cefazolin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), amoxicillin/clavulanic acid (20/10 μg), ampicillin/sulbactam (10/10 μg), piperacillin/tazobactam (100/10 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), tetracycline (30 μg), doxycycline (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), gentamicin (10 μg), amikacin

(30 μg), sulfamethoxazole/trimethoprim (1.25/23.75 μg), chloramphenicol (30 μg), and florfenicol (30 μg). All tested antimicrobial agents were obtained from Oxoid (Basingstoke, United Kingdom). *E. coli* ATCC 25922 was used as the control strain. The obtained data were interpreted according to CLSI recommendations (Clinical and Laboratory Standards Institute [CLSI], 2016).

Finally, we screened for the presence of 25 additional types of resistance genes and integron integrates genes in the 12 *aac(3)-IV*-positive isolates were screened using primers and PCR conditions as previously described: bla_{TEM}, bla_{SHV}, $bla_{\mathrm{OXA-1-like}}$, $bla_{\mathrm{CTX-M-group\,1}}$, $bla_{\mathrm{CTX-M-group\,2}}$, $bla_{\mathrm{CTX-M-group\,9}}$, $bla_{\mathrm{CTX-M-group\,8/25}}$ (Dallenne et al., 2010), *tetA*, *tetB*, *tetM* (Ng et al., 2001), *qnrA*, *qnrB*, *qnrC*, *qnrD* (Schink et al., 2012), *aac(3)-IIa*, *aac(6′)-Ib*, *ant(3″)-Ia*, *aph(3′)-IIa* (Zhang et al., 2012), *sulI*, *sulII* (Kerrn et al., 2002), *cfr*, *cmlA*, *floR* (Keyes et al., 2000; Kehrenberg and Schwarz, 2006), *IntI*, and *IntII* (Ishikawa, 2011).

Statistical Analysis

Statistical analysis was performed using SPSS software for Windows, version 18.0 (SPSS Inc., Chicago, IL, United States). Data were analyzed using descriptive statistics and χ^2 tests. A P-value < 0.05 was considered statistically significant.

RESULTS

Bacterial Isolation

Over the course of the 40-day testing period, a total of 585 samples were collected. Two *E. coli* strains were selected from each sample. As shown in **Table 1**, a total of 1170 *E. coli* isolates from the medicated group ($n = 390$), un-medicated group ($n = 390$), and housefly group ($n = 390$) were obtained. Prior to apramycin administration (Day 0), 90 *E. coli* strains were collected from the included samples, 450 *E. coli* strains were collected during apramycin administration (Days 1–5), and 630 *E. coli* strains were collected after apramycin administration.

The Changes of MIC90 for Apramycin

Minimum inhibitory concentration for apramycin was tested for all 1170 *E. coli* isolates. MIC90 was used to evaluate the changes trend of apramycin resistance (**Figure 1**).

For *E. coli* isolates obtained from the medicated group, apramycin MIC90 was at a low level (8 μg/ml) prior to apramycin administration (Day 0). After the addition of apramycin, MIC90 increased significantly from Days 2 to 6 and was maintained above 512 μg/ml compared to that in Day 0 and Day 1 ($P < 0.05$). This was with the exception of Day 5, which sustained a level of 256 μg/ml. However, ending apramycin administration resulted in a substantial decrease in MIC90 (8–16 μg/ml) from Days 8 to 15. To our surprise, MIC90 increased again (above 512 μg/ml) from Days 20 to 40.

For *E. coli* isolates obtained from the un-medicated group, apramycin MIC90 was remained at low level (8–16 μg/ml) from Days 0 to 8. This was with the exception of Day 3, which sustained a level of 64 μg/ml. Days 10–20 saw a dramatic increase (128–1024 μg/ml), but a subsequent decrease to 8 μg/ml from Days 30 to 40. Significant difference was found for the MIC90 values between *E. coli* isolates from the un-medicated group and medicated group ($P < 0.05$).

For *E. coli* isolated from houseflies, apramycin MIC90 remained at a low level (8–16 μg/ml) from Days 0 to 6, then increased and fluctuated between 256 and 1024 μg/ml from Days 8 to 40. MIC90 values for apramycin were significantly different between 1–6 days and 8–40 days for *E. coli* isolated from houseflies ($P < 0.05$).

Detection Rates of Apramycin Resistance Gene

Apramycin resistance genes *aac(3)-IV* and *npmA* were screened for all 1170 *E. coli* isolates. *Aac(3)-IV* was detected in 32, 71, and 42 *E. coli* isolates from the un-medicated, medicated, and housefly groups, respectively. *npmA* gene was not detected in any samples from this study. The change of *aac(3)-IV* frequency is shown in **Figure 2**.

For the medicated group, *aac(3)-IV* detection rate was 6.67% before treatment (Day 0) and showed a steady increase from Day 1 (3.33%) to Day 4 (63.33%). Rates then decreased and fluctuated between 0 and 23.33% from Days 5 to 40. Noticeably, *aac(3)-IV* detection rates were still higher than Day 0. This rate held even 35 days after treatment (Day 40).

For the un-medicated group, *aac(3)-IV* detection rate showed no drastic change when compared to Day 0. Rates fluctuated between 3.33 and 16.67% for the entirety of the experiment.

For the housefly group, *aac(3)-IV* detection rate was low from Days 0 to 6 (0–3.33%), then increased and fluctuated between 13.33 and 36.67% from Days 8 to 40.

The *aac(3)-IV* detection rate was significantly different between medicated group and un-medicated group from days 3 to 4 ($P < 0.05$). No significant difference was found between un-medicated group and housefly group ($P > 0.5$).

PFGE Typing of *aac(3)-IV*-Positive Strains

A total of 145 *aac(3)-IV*-positive *E. coli* isolates from the un-medicated ($n = 32$), medicated ($n = 71$), and housefly groups ($n = 42$) were analyzed using PFGE and 12 PFGE types were characterized (**Figure 3**). Among these, the three predominant PFGE types that emerged in the un-medicated group were types A ($n = 12$), B ($n = 4$), and D ($n = 5$). In the medicated group, the three major types were types A ($n = 8$), E ($n = 39$), and G ($n = 9$) and the housefly group were types A ($n = 7$), E ($n = 11$), and G ($n = 19$). PFGE types A, E, and G were the predominant types in both the medicated and housefly groups, suggesting houseflies play an important role in the spread of antibiotic-resistant *E. coli*.

Characterization of Antimicrobial Resistance Phenotype and Genotype of *aac(3)-IV*-Positive Strains

Antimicrobial resistance profiles of the 12 *E. coli* isolates from each PFGE type are shown in **Table 2**. All tested isolates were multi-resistant, showing an antimicrobial-resistant phenotype to 10–18 antibiotics. Furthermore, all 12 isolates

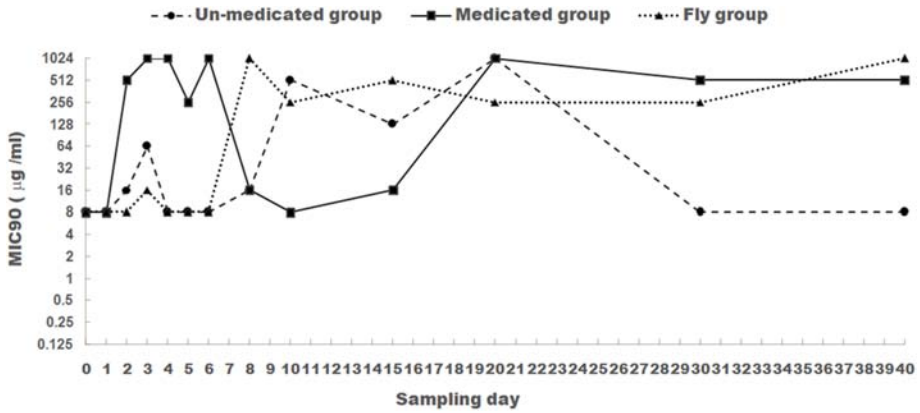


FIGURE 1 | The changes of MIC90 for apramycin of *E. coli* isolated from chicken feces (medicated and un-medicated groups) and houseflies. Apramycin was administrated from Days 1 to 5 in their drinking water (0.5 mg/l) for the medicated group.

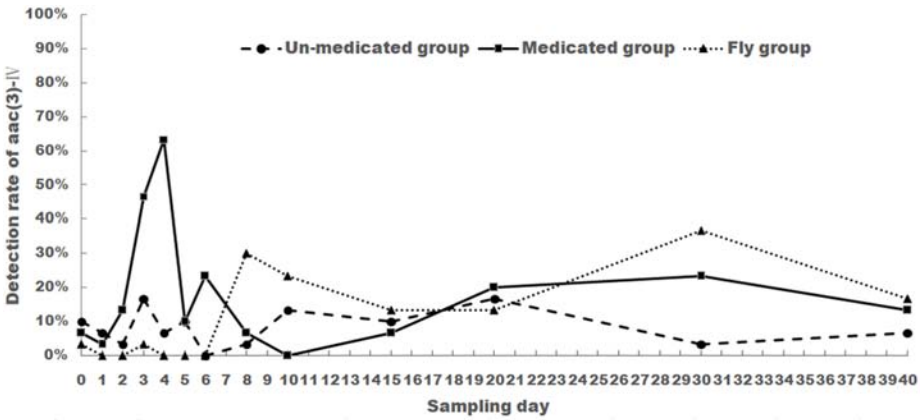


FIGURE 2 | The changes of *aac(3)-IV* detection rate of *E. coli* isolated from chicken feces (medicated and un-medicated groups) and houseflies. Apramycin was administrated from Days 1 to 5 in their drinking water (0.5 mg/l) for the medicated group.

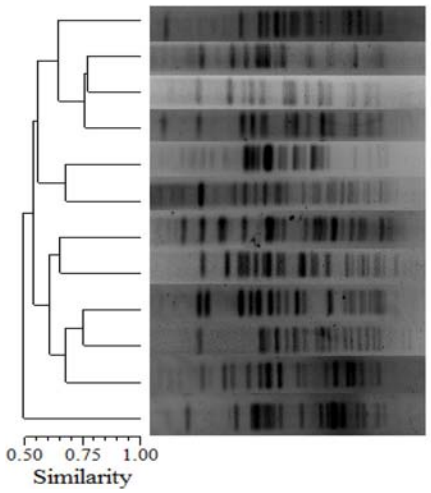
were co-resistant to the following antibiotics: ampicillin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, gentamicin, and sulfamethoxazole/trimethoprim. They showed sensitivity to piperacillin/tazobactam, imipenem, meropenem, and amikacin. The number of isolates resistant to other antimicrobials ranged from 4 to 11 (**Table 2**).

Resistance gene screening results showed multiple resistance genes co-existed in all 12 different *E. coli* isolates from each PFGE type (**Table 2**). The isolates among the 12 different PFGE types harboring resistance genes other than *aac(3)-IV* are shown in **Table 2**. Remarkably, 10 isolates harbored at least one ESBL genes ($bla_{\mathrm{CTX-M-group\,1or9}}$). Moreover, among the 12 isolates, 11 were positive for the type I integrase gene *intI*.

DISCUSSION

Increasing attention has been paid to verify whether the extensive uses of antibiotics in food animals poses a risk to human health. Studies regarding the association between antibiotic administration and the development and persistence of resistant bacteria may provide guidance for more accurate antibiotic usage in animal husbandry.

Previous studies have suggested that apramycin administration can promote resistance *E. coli* isolated from swine (Mathew et al., 2003; Jensen et al., 2006). However, the influence of apramycin administration on *E. coli* resistance in chicken has not yet been reported. In this study, we demonstrated that the use of apramycin could facilitate *E. col I* resistance from the first day after administration to 1 day after cessation. Apramycin MIC90 dropped to a relatively low level 3 days after cessation, but increased again from Days 20 to 40 after cessation. Some studies have investigated the influence of other antibiotics on resistance changes of *E. coli* isolated from different farm animals (Smith et al., 2007; Martins da Costa et al., 2011; Sato et al., 2014). These previous studies have also demonstrated that antimicrobials caused selective pressure and resulted in increased resistance to bacteria originating from animals.



PFGE types	No. of isolates		
	Un-medicated group	Medicated group	Fly group
A	12	8	7
B	4	1	3
C	0	4	0
D	5	4	0
E	1	39	11
F	3	2	1
G	1	9	19
H	2	0	0
I	2	0	0
J	0	3	0
K	0	0	1
L	2	1	0

FIGURE 3 | PFGE analysis of *aac(3)-IV*-positive *E. coli* isolates. A total of 145 *aac(3)-IV*-positive *E. coli* isolates from the un-medicated (n = 32), medicated (n = 71), and housefly groups (n = 42) were characterized into 12 PFGE types.

TABLE 2 | Antimicrobial resistance profile of *aac(3)-IV*-positive *E. coli* isolates of different PFGE types.

PFGE type	Resistance phenotype[a]	Resistance genotype
A	AMP, PRL, KZ, CTX, CRO, AMC, SAM, ATM, TE, DO, CIP, LEV, CN, SXT, C	bla_{OXA}, $bla_{CTX-M-group\,9}$, *tetA, SulI*, *SulII*, *aac(6′)-Ib*, *ant(3′′)-Ia*, *aac(3)-IV*, *cmlA*, *intI*
B	AMP, PRL, KZ, CTX, CRO, SAM, TE, DO, CIP, LEV, CN, SXT, C, FFC	bla_{TEM}, $bla_{CTX-M-group\,9}$, *tetA, SulII*, *ant(3′′)-Ia*, *aac(3)-IV*, *floR*, *cmlA*, *intI*
C	AMP, PRL, KZ, CAZ, CTX, CRO, FEP, SAM, ATM, TE, DO, CIP, LEV, CN, SXT, C, FFC	bla_{TEM}, $bla_{CTX-M-group\,1}$, $bla_{CTX-M-group\,9}$, *tetA*, *SulI*, *SulII*, *aac(3)-IV*, *floR*, *intI*
D	AMP, SAM, TE, DO, CIP, LEV, CN, SXT, C, FFC	bla_{OXA}, *tetA*, *SulI*, *SulII*, *aac(6′)-Ib*, *ant(3′′)-Ia*, *aac(3)-IV*, *floR*, *cmlA*, *intI*
E	AMP, PRL, KZ, CAZ, CTX, CRO, FEP, AMC, SAM, ATM, TE, DO, CIP, LEV, CN, SXT, C, FFC	bla_{TEM}, bla_{OXA}, $bla_{CTX-M-group\,1}$, *SulI*, *SulII*, *aac(3)-IIa*, *aac(6′)-Ib*, *ant(3′′)-Ia*, *aac(3)-IV*, *floR*, *cmlA*, *intI*
F	AMP, PRL, AMC, SAM, TE, DO, CIP, LEV, CN, SXT, C, FFC	bla_{TEM}, bla_{OXA}, *tetA*, *SulI*, *SulII*, *aac(3)-IIa*, *aac(6′)-Ib*, *ant(3′′)-Ia*, *aac(3)-IV*, *floR*, *cmlA*, *intI*
G	AMP, PRL, KZ, CAZ, CTX, CRO, FEP, AMC, SAM, ATM, TE, DO, CIP, LEV, CN, SXT	bla_{TEM}, bla_{OXA}, $bla_{CTX-M-group\,1}$, *tetA*, *SulI*, *SulII*, *aac(6′)-Ib*, *aac(3)-IV*, *intI*
H	AMP, PRL, KZ, CTX, CRO, FEP, SAM, ATM, TE, DO, CIP, LEV, CN, SXT, C, FFC	$bla_{CTX-M-group\,9}$, *SulII*, *aac(3)-IV*, *floR*
I	AMP, PRL, KZ, CTX, CRO, ATM, TE, DO, CIP, LEV, CN, SXT, C, FFC	$bla_{CTX-M-group\,9}$, *tetA*, *SulI*, *SulII*, *ant(3′′)-Ia*, *aac(3)-IV*, *floR*, *intI*
J	AMP, PRL, KZ, CTX, CRO, FEP, ATM, TE, DO, CIP, LEV, CN, SXT, C, FFC	$bla_{CTX-M-group\,9}$, *tetA*, *SulI*, *SulII*, *ant(3′′)-Ia*, *aac(3)-IV*, *floR*, *intI*
K	AMP, PRL, KZ, CTX, CRO, SAM, TE, DO, CIP, LEV, CN, SXT, C, FFC	bla_{OXA}, $bla_{CTX-M-group\,9}$, *tetA*, *SulI*, *SulII*, *aac(6′)-Ib*, *ant(3′′)-Ia*, *aac(3)-IV*, *floR*, *cmlA*, *intI*
L	AMP, PRL, KZ, CAZ, CTX, CRO, FEP, SAM, ATM, TE, DO, CIP, LEV, CN, SXT, C, FFC	$bla_{CTX-M-group\,1}$, $bla_{CTX-M-group\,9}$, *tetA*, *SulII*, *aac(3)-IV*, *floR*, *intI*

[a]*AMP, ampicillin; PRL, piperacillin; KZ, cefazolin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; AMC, amoxicillin/clavulanic acid; SAM, ampicillin/sulbactam; ATM, aztreonam; TE, tetracycline; DO, doxycycline; CIP, ciprofloxacin; LEV, levofloxacin; CN, gentamicin; SXT, sulfamethoxazole/trimethoprim; C, chloramphenicol; FFC, florfenicol.*

Noticeably, a high MIC90 was persistent even after stopping antibiotic treatment in the medicated group (Days 20–40). This value was higher than prior to antibiotic treatment, results that have also been found in a separate study (Smith et al., 2007). These findings could be due to the clonal dissemination of resistant strains and the capacity of *E. coli* to exchange resistance genes (da Costa et al., 2009). One of the potential reasons could be due to the dissemination of resistant strains by flies. Because according to the results of MIC90 of the flies group (**Figure 1**), the MIC90 values remained at a high level (256–1024 mg/ml) from days 20 to 40 in the housefly group.

MIC90 in the un-medicated group also increased at Day 3 and again from Days 10 to 20. This change in antibiotic resistance has also been observed in other studies featuring no antimicrobial treatment (Diarra et al., 2007; da Costa et al., 2009). These findings might be due to the influence of resistant

strains in the farm environment and animal feed on microbial composition in the chicken gut (Apajalahti et al., 2004; Martins da Costa et al., 2011). We also hypothesized that the change of resistant phenotype of the un-medicated group was due to the spread of the resistant strains from the medicated group to un-medicated group through environmental factors (e.g., air, dust, mice, and flies). There are two reasons for this: first, compared with medicated group, the increase of MIC90 values of the un-medicated group was relatively delayed. Second, the trend of drug-resistant phenotype of the un-medicated group and housefly group was very similar, which suggested the resistant strains might be spread from the medicated group to un-medicated group by houseflies.

Furthermore, the influence of antimicrobial administration on resistance phenotype and genotype of *E. coli* isolated from houseflies captured from a poultry farm was investigated for the first time. Our study found that apramycin administration also promoted resistance of *E. coli* isolated from houseflies. However, the change of apramycin resistance in *E. coli* isolated from houseflies group was not as synchronous as that seen in the medicated group. To this end, MIC90 values rose from Days 2 to 6 (except for Day 5) in the medicated group, but remained at a low level (8–16 μg/ml) in the housefly group. Furthermore, while MIC90 values dropped from Days 8 to 15 in the medicated group, values rose above 256 μg/ml in the housefly group.

Pulsed-field gel electrophoresis analysis of *aac(3)-IV*-positive *E. coli* isolates indicated that the same strains were present in both fecal samples and houseflies. Furthermore, the predominant three PFGE types in the medicated group (A, E, and G) were also the predominant three PFGE types in the housefly group. This suggests that houseflies are transmission vehicles from chicken feces for resistant bacterial strains. Therefore, as the use of antimicrobials increases the presence of resistant strains in food producing animals, it will also likely increase the potential for further dissemination by houseflies to the public. Similar results have been found in pig farms, as *E. coli* isolates from flies and pigs showed the same resistance phenotype, genes, and PFGE profiles (Literak et al., 2009).

Resistance profiles of the *aac(3)-IV*-positive isolates of different PFGE types indicated multi-drug resistance was very common, which is consistent with other studies (da Costa et al., 2009; Zhang et al., 2009). Therefore, apramycin administration does not only cause selective effects on resistance itself, but also to other antimicrobials. Noticeably, among these apramycin-resistant isolates, the ESBL-producing strains were very common (10/12). More critically, some of these ESBL-producing strains also existed in houseflies. This would only increase their disseminating opportunity, posing a great potential risk to public health. Other studies have also shown that flies were capable of spreading ESBL-producing *E. coli* from poultry and cattle (Usui et al., 2013; Blaak et al., 2014).

CONCLUSION

Our study found that apramycin administration increased the occurrence of *aac(3)-IV*-resistant isolates from chicken feces and houseflies. Moreover, houseflies transmitted resistant bacteria from chicken feces, thus increasing the potential risk of spreading these multi-resistant isolates to the public. Critical management strategies of antimicrobial usage in animal husbandry and pest control should be undertaken to better control and reduce this risk.

AUTHOR CONTRIBUTIONS

AZ and HW designed the study. ZG, YY, and YL carried out the sampling work. ZG, HT, and DL performed the experiments. AZ, CX, and CL analyzed the data. AZ and ZG drafted the manuscript. All authors have read and approved the final manuscript.

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frontiers in Microbiology

REVIEW
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Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms

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The Chilean salmon industry has undergone a rapid development making the country the world's second largest producer of farmed salmon, but this growth has been accompanied by an intensive use of antibiotics. This overuse has become so significant that Chilean salmon aquaculture currently has one of the highest rates of antibiotic consumption per ton of harvested fish in the world. This review has focused on discussing use of antibiotics and current status of scientific knowledge regarding to incidence of antimicrobial resistance and associated genes in the Chilean salmonid farms. Over recent years there has been a consistent increase in the amount of antimicrobials used by Chilean salmonid farms, from 143.2 tons in 2010 to 382.5 tons in 2016. During 2016, Chilean companies utilized approximately 0.53 kg of antibiotics per ton of harvested salmon, 363.4 tons (95%) were used in marine farms, and 19.1 tons (5%) in freshwater farms dedicated to smolt production. Florfenicol and oxytetracycline were by far the most frequently used antibiotics during 2016 (82.5 and 16.8%, respectively), mainly being used to treat *Piscirickettsia salmonis*, currently considered the main bacterial threat to this industry. However, the increasing development of this industry in Chile, as well as the intensive use of antimicrobials, has not been accompanied by the necessary scientific research needed to understand the impact of the intensive use of antibiotics in this industry. Over the last two decades several studies assessing antimicrobial resistance and the resistome in the freshwater and marine environment impacted by salmon farming have been conducted, but information on the ecological and environmental consequences of antibiotic use in fish farming is still scarce. In addition, studies reporting the antimicrobial susceptibility of bacterial pathogens, mainly *P. salmonis*, have been developed, but a high number of these studies were aimed at setting their epidemiological cut-off values. In conclusion, further studies are urgently required, mainly focused on understanding the evolution and epidemiology of resistance genes in Chilean salmonid farming, and to investigate the feasibility of a link between these genes among bacteria from salmonid farms and human and fish pathogens.

Keywords: antibiotics, salmon farming, antimicrobial resistance, *Piscirickettsia salmonis*, Chile

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INTRODUCTION

It is well known that many fisheries resources have been overexploited, and that many are currently depleted, and unable to support the global demand for seafood. In this context, world aquaculture is seen as a key industry in satisfying the growing demand for food for human consumption. Currently, aquaculture supplies more than 50% of all the seafood produced for human consumption, having increased production 20-fold between 1970 and 2010 (up from 2.6 to 60.4 million of tons per year) with a mean annual growth rate of 7.8% (Troell et al., 2014), resulting in the fastest growing food-production industry in the world (FAO, 2014).

Chile is the eighth largest producer of aquaculture products in the world, with the salmonids (Atlantic salmon *Salmo salar*, rainbow trout *Oncorhynchus mykiss*, and Coho salmon *Oncorhynchus kisutch* – in order of relevance) and blue mussels (*Mytilus chilensis*) as the principal products (FAO, 2014). Chilean salmon aquaculture has developed rapidly over the last three decades, making Chile the world's second largest producer of salmon after Norway, producing more than 900 thousand tons in 2014 (SERNAPESCA, 2017a). However, this high productivity has been achieved by intensive farming, i.e., huge biomass grown at high densities of fish per unit of water volume, which has resulted in an increased susceptibility of fish to diseases caused by viruses, bacteria, fungi, and parasites (Quesada et al., 2013). Common intensive husbandry practices as well as management procedures on salmon farms, such as stripping of broodstock, handling, vaccination, crowding, grading, starvation, antimicrobial treatments as well as loading and transport can lead to an increased susceptibility to a wide range of diseases. These stressors can also lead to injury and the impaired performance of reared salmon, which are usually kept in crowded conditions which facilitate the transmission of infectious pathologies (Poppe et al., 2002; Håstein, 2004). Thus, over recent decades, this increase in productivity has been accompanied by an increased use of chemicals, mainly antibiotics, which are commonly used for prevention and treatment of bacterial disease in salmon farming (Miranda, 2012). Antimicrobials used in salmonid farming are mainly administered to the fish through medicated feed, thus there is significant potential for a large proportion of the drug to enter the environment via uneaten medicated feed in addition to through urinary and fecal excretion (Cravedi et al., 1987; Kemper, 2008). It has been demonstrated that a significant amount of oxytetracycline is released through leaching from uneaten feed (Capone et al., 1996) and losses from uneaten feed may increase during a disease outbreak, especially if the disease or the lower palatability of medicated feed results in a loss of appetite (Hustvedt et al., 1991). This leads to the accumulation of antibiotic residues in the aquatic environment especially in marine sediments, where they can persist for months, favoring the selection of resistant microorganisms and consequently affecting the natural microbial activity and biogeochemical processes (Hollis and Ahmed, 2014).

Traditionally, antibiotics have been widely used in aquaculture to prevent and treat bacterial diseases (Romero et al., 2012). Excessive use of antibiotic in aquaculture in many countries has caused problems and concerns due to the development and dissemination of bacterial resistance, food safety hazards and environmental issues (World Health Organization, 2016). However, despite the negative impact of the use of antibiotics, the role of antibiotic usage in aquaculture in the development of resistance and dissemination of antimicrobial resistance genes (ARG) is still poorly understood (Done et al., 2015). Evidence suggests that antibiotics also promote the selection and spread of a broad and diverse set of ARG that form the resistome, facilitating the horizontal transfer of these genes among different bacteria and posing a health risk when they are transferred to human pathogens.

In this context, antibiotic use by the Chilean aquaculture is a particular case worth studying, because as far as it is known and based on the data available, production in Chile has one of the highest rates of antibiotic consumption per ton harvested worldwide. This is even more relevant, considering that high amounts of antibiotics are discharged annually into the waters of Chilean Patagonia, a pristine area of high conservation value, which contains a mosaic of unique ecosystems and three World Biosphere Reserves.

Various reviews have addressed at least partially the issue of antibiotic use in Chilean salmon farming (Cabello, 2004, 2006; Burridge et al., 2010; Millanao et al., 2011; Miranda, 2012; Romero et al., 2012; Cabello et al., 2013, 2016), mainly focusing on the potential impacts on human health, but studies providing information on the environmental consequences of the use of antibiotics in Chilean salmonid farming are still scarce. This review is focused on the available knowledge, encompassing information on antibiotic utilization over the last decade in Chilean salmonid aquaculture and the available published studies concerning antibiotic resistance in the farm associated microbiota and fish bacterial pathogens.

USE OF ANTIBIOTICS IN CHILEAN SALMON AQUACULTURE

Antibiotics are not only utilized in human medicine, but also worldwide in livestock to treat bacterial infections and/or to promote animal growth (Du and Liu, 2012). Despite the lack of information on antibiotic use in many countries, worldwide antibiotic usage has been estimated to be in the range of 100–200 thousand tons per year (Wise, 2002; Kümmerer, 2003), with about half of this amount being used for veterinary purposes (Sarmah et al., 2006). For example, in 2009 13,000 tons were used in animal production within the United States of America alone (FDA, 2009), whereas 382.5 tons were used by the Chilean salmon industry during 2016. These levels must be of concern if it is taken into account that most of them are poorly absorbed at the tissue level and then excreted, at levels of between 40 and 90%, into the environment via animal urine or feces (Kemper, 2008).

The amount of antibiotics used in aquaculture worldwide is very difficult to estimate as the different countries involved vary

widely with respect to their registration systems, and for this reason in many cases information is unavailable or impossible to compare due to gaps in the data (Heuer et al., 2009; Romero et al., 2012). However, within countries that have a registration system, a large variation in antibiotic use has been reported. For example, while Norway uses 1 g per ton of salmon produced, Vietnam requires 700 g per ton of shrimp (Smith, 2008). In fact, shrimp cultured in Vietnam along with Chilean salmon farming, are examples of industries exhibiting the highest rates of aquaculture antibiotic consumption in the world (Van Boeckel et al., 2015).

Chile is the second largest producer of salmon, accounting for approximately one third of the global salmonid production, behind only by Norway, and ahead of Scotland and Canada (Ibieta et al., 2011; Asche et al., 2013). However, Chile has significantly higher rates of antibiotic consumption than the other three countries. The amount used to produce 1 ton of salmon in Chile between 2011 and 2015 was on average more than 1,500 times higher than in Norway (NORM/NORM-VET, 2016; SERNAPESCA, 2017b).

This is of significant concern considering that the geographic area used by Chile for salmon farming is 4 times smaller than that used by Norway (Buschmann et al., 2006). Despite the fact that Norwegian production of farmed salmonids has more than doubled between 2003 and 2014, the use of antibacterials in aquaculture there has decreased by half over the same period (Directorate of Fisheries, 2015). This low antibiotic consumption is mainly a consequence of the availability of highly effective vaccines against furunculosis and vibriosis pathologies, as well as the rapid implementation of efficient zoo-sanitary measures and a significant improvement in biosecurity policies such as zoning and the spatial re-arrangement of marine production sites to minimize the horizontal spread of infections (Midtlyng et al., 2011). Unlike Norway, the higher mortality in Chile is attributed to bacterial infections as opposed to viruses, particularly the intracellular pathogen *Piscirickettsia salmonis* which causes the highest mortality in the marine phase of the culture and for which there are currently no effective vaccines nor an efficient and reliable antibiotic therapy (Rozas and Enríquez, 2014).

Looking at the antibiotic per ton of harvested salmon, during the last four years (2013–2016), Chilean companies used annually on average 580 g of antibiotic per ton of harvested salmon, surpassing the average levels used during the period 2005–2012 (438 g of antibiotic per ton of harvested salmon). Over recent years a consistent increase in the amount of antimicrobials used by Chilean salmonid farms, from 143.2 tons in 2010 to 382.5 tons in 2016, has been observed (SERNAPESCA, 2017b). During 2016, Chilean companies utilized approximately 0.53 kg of antibiotic per ton of harvested salmon, surpassing the levels used during 2005 and 2006 (0.39 and 0.53 kg per ton of harvested salmon, respectively), just prior to the infectious salmon anemia virus outbreak and the subsequent collapse of Chilean farmed fish production (**Table 1**). This indicates that beyond the fluctuations in the use of antibiotic during the last decade, the levels of antibiotic use by the Chilean farming salmon are far from decreasing. Of the 382.5 tons of antibiotics used on Chilean salmon farms during 2016, 363.4 tons (95%) were used in marine farms, whereas only 19.1 tons (5%) were used in freshwater centers dedicated to smolt production. These large differences in the quantities used are explained by the amount of antibiotic used to treat the *P. salmonis* bacterium in marine environments (SERNAPESCA, 2017b).

TABLE 1 | Antibiotic use in Chilean salmon industry (SERNAPESCA, 2011, 2017b).

Year	Antimicrobial use (tons)	Harvested fish (thousands of tons)	Ratio (kg per harvested ton)
2005	239.2	614.0	0.39
2006	343.8	647.6	0.53
2007	385.6	600.6	0.64
2008	325.6	630.6	0.52
2009	184.5	474.2	0.39
2010	143.2	466.9	0.31
2011	206.8	649.5	0.32
2012	337.9	826.9	0.41
2013	450.7	786.1	0.57
2014	563.2	955.2	0.59
2015	557.2	883.1	0.63
2016	382.5	727.8	0.53

Among the six antibiotics currently approved for use in Chilean salmon aquaculture, florfenicol and oxytetracycline were by far the most frequently used during 2016 (82.5 and 16.8%, respectively) (SERNAPESCA, 2017b). It must be noted that the use of antibiotics has changed since 2005 (**Figure 1**), with an observable progressive increase in the use of florfenicol and oxytetracycline compared to the decrease in the use of the quinolones, oxolinic acid, and flumequine (SERNAPESCA, 2011, 2017b). The dominance of florfenicol in marine-based salmonid faming in Chile is mainly because it is the first choice for the treatment of *P. salmonis*, currently considered the main bacterial threat to the salmonid farm industry. The quinolones are a class of highly effective antibiotics extensively used in human medicine and consequently their use in animal production has been severely restricted by the World Health Organization, however, their use in animal production is not prohibited in many countries (Collignon et al., 2016). Despite the fact that during 2016 Chilean salmon farms did not report any use of oxolinic acid and that only 0.3% of the antimicrobials used was flumequine (**Figure 1**), it is clearly a priority to implement new regulations in the Chilean salmon industry, prohibiting the use of quinolones.

Despite the regulations and control of antibiotic usage in aquaculture imposed by the Chilean government, it must be concluded that until 2015 the use of antibiotics in this industry was higher than the amount reported. As an example only 22 out of 25 Chilean salmon farming companies agreed to release individualized information on their antimicrobial use in the marine phase of culture during 2015 (SERNAPESCA, 2016). To solve this issue, from 2016 it has been mandatory for all salmon companies in Chile to

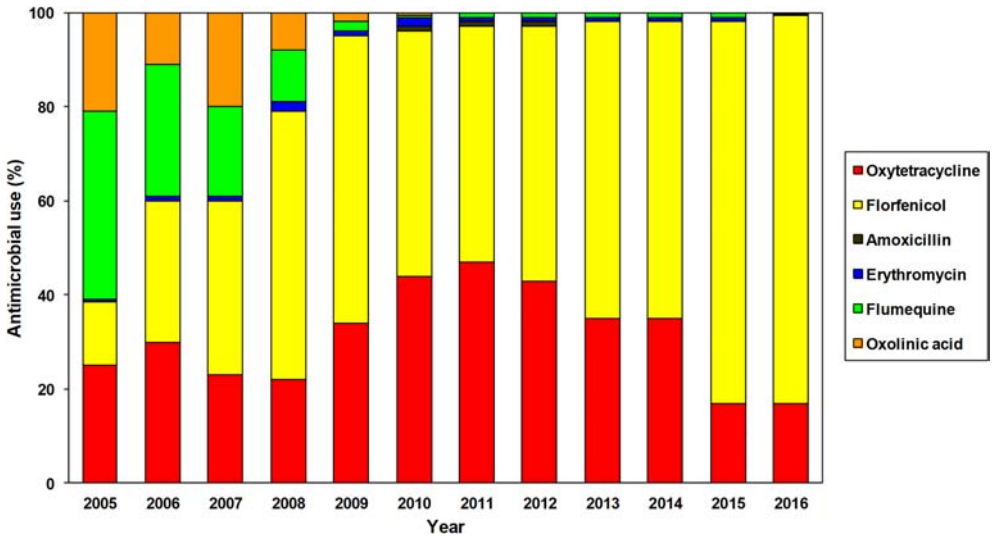

FIGURE 1 | Annual use of antimicrobials authorized for use in Chilean salmon farming between 2005 and 2016 (SERNAPESCA, 2011, 2017b).

provide the information of their use of antibiotics during fish culture.

ANTIBIOTIC RESISTANCE IN THE AQUATIC AND SALMON FARM ENVIRONMENTS

For many decades, the general opinion of scientists and physicians was that resistance to antibiotics and the presence of genetic determinants was a problem confined to the hospital environment. Only recently has it been recognized that antibiotic resistant microorganisms and associated resistance determinants are ubiquitous in nature, and that they are even present in pristine environments which have never been exposed to antimicrobial contamination (Allen et al., 2010; Knapp et al., 2011; Miranda, 2012). Several studies have indicated the occurrence of a great diversity of resistance genes, leading to the suggestion that the environment is a reservoir and an important source of new and emerging antibiotic resistance genes (ARGs) (Riesenfeld et al., 2004; D'Costa et al., 2006, 2007; Dantas et al., 2008; Allen et al., 2010; Donato et al., 2010; Wright, 2010). This discovery has led to a rethink on the origin of bacterial antibiotic resistance in pathogenic bacteria, accepting the assumption that the emergence of ARGs in pathogenic bacteria is likely to have arisen in natural environments (Nesme and Simonet, 2015). The term "resistome" was proposed in order to aid in our understanding of the origin, evolution and emergence of antibiotic resistance and was defined as the collection of all genes that might contribute to antimicrobial resistance (Wright, 2007). The resistome encompasses not only the genes encoding for antimicrobial resistance associated with bacterial pathogens, but also includes all the genes present in non-pathogenic species that dominate the natural environment (D'Costa et al., 2006). Thus, the resistome of a particular environment could include: precursor genes that express low resistance to antimicrobial molecules or affinity; cryptic resistance genes with no or low phenotypic expression in their host; and clinical resistance genes such as broad spectrum beta-lactamases, which confer resistance to high concentrations of antibiotics (Wright, 2007). It has been noted that ARGs present in pathogens can undertake different roles when they are found in an environmental host, as it is the host and the genomic context in which the gene is found that determines its phenotypic expression (Nesme and Simonet, 2015).

Traditionally, most of studies concerning antibiotic resistant bacteria and their resistance-encoding genes are based on techniques developed for cultivable bacteria, or molecular procedures using polymerase chain reaction primers only able to detect specific known antibiotic resistance-encoding genes (Miranda and Zemelman, 2002b; Buschmann et al., 2012; Di Cesare et al., 2013), but these techniques are unable to detect unknown ARGs (Petersen et al., 2002; Dang et al., 2008; Taviani et al., 2008). Furthermore, even when the use of these techniques has produced important findings, it has been concluded that they have the limitation of covering only a small fraction (< 0.1% in the marine environment) of the ARGs in the environment (Vaz-Moreira et al., 2014). The exponential increase in databases including sequences from genomes and metagenomes has allowed *in silico* sequence analysis of ARGs on the basis of comparisons with sequences described from pathogenic bacteria (Gibson et al., 2014; Nesme and Simonet, 2015). Functional metagenomics is a methodology that covers all components of a bacterial community (culturable and non-culturable) and does not depend on databases of previously known sequences which are generally isolated from bacteria from clinical settings (Mullany, 2014). Indeed, when genes with resistance phenotypes from metagenomic libraries are compared with known genes, frequently less than 65% of similarity at the amino-acid level is observed (Pehrsson et al., 2013). In a recent

study using functional metagenomics on soil samples, nearly 3,000 genes encoding for antibiotic resistance were described, and most of them were new undescribed genes (Forsberg et al., 2014). Thus, different studies using functional metagenomics have found that ARGs are highly diverse and widely distributed, exhibiting little or no similarity to sequences of known genes (Lang et al., 2010; Schmieder and Edwards, 2012; Su et al., 2014).

The ARGs in natural ecosystems evolved over millions of years, long before the therapeutic use of antibiotics (Baquero et al., 2009). Currently, environmental resistomes are a vast and diverse collection of resistance genes, and also constitute a potential source of resistance genes for human pathogens (Martínez, 2008). There is significant evidence that various resistance genes present in human pathogenic bacteria have an environmental origin, strongly supporting the hypothesis that the transfer of genes encoding for antimicrobial resistance from the aquatic to the human clinic compartment is of importance.

However, until now it has been difficult to demonstrate the transfer of ARGs from the environment to clinically relevant bacteria or identify the mechanisms involved in this transfer (Finley et al., 2013; Perry and Wright, 2013; Vaz-Moreira et al., 2014). This may be due to the existence of restrictions or "bottlenecks" that modulate the transfer of resistance determinants from the original host to human pathogens, such as ecological connectivity, founder effects, and fitness costs as was noted by Martínez (2011).

The enhancement of selection and the environmental distribution of antibiotic resistant bacteria by the intensive use of antibiotics in aquaculture have been well-established (Smith, 2008; Miranda, 2012). Antibiotics used in fish aquaculture are typically administered via medicated feed, thus the first contact the antibiotic has with microorganisms occurs in the intestine of the fish. Considering the high densities of the bacterial populations present, the intestinal environment provides optimal conditions for the selection of antibiotic resistant bacteria (Le Bris et al., 2007). In fact, the increase in the levels of antibiotic resistant bacteria in the digestive system of fish under antimicrobial therapy is well documented (Austin and Al-Zahrani, 1988; DePaola et al., 1995). The next step is the dispersal of commensal or pathogenic antibiotic resistant bacteria from the intestinal environment to the water column or sediments through fish feces (Herwig et al., 1997; Samuelsen et al., 2000; Navarrete et al., 2008). It should also be considered that the medicated feed can also be ingested by wild fish living around the salmon cages, increasing the levels of antibiotic resistant bacteria in the intestine of these fishes also (Björklund et al., 1990; Ervik et al., 1994). Furthermore, the presence of antibiotic residues inside fish muscle has also been demonstrated, and obviously these residues can enter the human intestine if the fish is consumed without cooking (Fortt et al., 2007). The detection of tetracycline and quinolones in wild fish living near fish farms suggests that the environmental effects of antibiotic use in aquaculture have spread beyond the salmon farming cages (Fortt et al., 2007).

Marine sediments beneath fish cages are also an important compartment where selection of antibiotic resistant bacteria and the dissemination of the ARGs can be strongly enhanced. Many studies have demonstrated a strong correlation between the antibiotic use and the increase in antibiotic resistant bacteria in the sediments beneath the fish farm cages (Björklund et al., 1991; Herwig et al., 1997; Schmidt et al., 2000). In fact, bacteria resistant to antibiotics frequently administered in fish farms have been detected at high frequencies in fish farms and the surrounding aquatic environments (Nygaard et al., 1992; Samuelsen et al., 1992; Schmidt et al., 2000; Petersen et al., 2002; Cabello et al., 2016). Furthermore, the prophylactic and therapeutic utilization of antibiotics in aquaculture not only favors the selection of antibiotic resistant bacteria, but also the selection and dissemination of their respective antibiotic resistance-encoding genes (Yang et al., 2013). Consequently, genes codifying different resistances have been detected and quantified in fish farm environments (Tamminen et al., 2010; Muziasari et al., 2014). Similar results have been described for several tetracycline resistance genes [*tet(A)*, *tet(C)*, *tet(H)*, and *tet(M)*] (Tamminen et al., 2010). In another study, using one plasmid metagenomic library and high throughput sequencing, 58 genes codifying for resistance against 11 antibiotics were detected in marine sediments impacted by a fish farm (Yang et al., 2013). Many of these genes shared more than 90% similarity with transposons and plasmids described for human pathogens, suggesting the occurrence of an important frequency of mobility of these ARGs to human pathogenic bacteria (Yang et al., 2013). Another recent study performed on sediment samples from fish farms located in the Northern Baltic Sea, indicated that the resistome associated with fish farms can be from native ARGs enriched by antibiotic use, modifying the diversity and distribution of ARGs in the sediment (Muziasari et al., 2017). At the same time the enrichment of mobile genetic elements by antibiotic use was also detected, which indicates the potential risk of the ARGs spreading to other environments (Muziasari et al., 2017).

STUDIES ON ANTIBIOTIC RESISTANCE ASSOCIATED WITH CHILEAN SALMON FARMING

Farm-Associated Microbiota

Antibiotic use in aquaculture, as well as in other anthropogenic activities, has been widely associated with the selection and prevalence of resistant bacteria, and also the spread of their resistance genes (Cabello et al., 2013, 2016). This is something which must be of concern to the Chilean salmon industry, considering the large amounts of antibiotics used and the resulting high concentrations released into the surrounding aquatic environment (Kemper, 2008). Despite this concern, only a few studies concerning antimicrobial resistance in Chilean salmonid farming have been conducted in Chile (**Table 2**), and of these, only a few were related to the impact of this activity on the surrounding environment (**Table 3**). Among these, Buschmann et al. (2012) found barely measurable antibiotic concentrations, with the exception of flumequine, that was detected at trace levels in 8 of 36 collected sediment samples, with no significant differences between the control and impacted sites.

TABLE 2 | Studies of antibacterial resistance in Chilean salmonid farming.

Issue	Number	Year
Resistant microbiota		
Freshwater	6	2002–2015
Marine	5	2012–2018
Fish pathogens		
Piscirickettsia salmonis	9	1996–2017
Flavobacterium psychrophilum	2	2012, 2016
Aeromonas salmonicida	1	2015
Vibrio ordalii	1	2013
Streptococcus phocae	1	2011

The authors argued that presence of residues of flumequine in the sediment from an apparently pristine control site was probably the result of transport by water currents of both unchanged antimicrobials and their antimicrobially active metabolites, concluding that excessive use of antimicrobials in Chilean salmon aquaculture may also have an effect on marine sediments far from where these activities take place (Buschmann et al., 2012). Additionally, Contreras and Miranda (2011) detected no residues of oxytetracycline, florfenicol, flumequine, or oxolinic acid in sediments from eight salmon farms located in Southern Chile. Apparently, the persistence of antimicrobial residues in salmon farm impacted-sediments is higher at freshwater-based farms than in those below marine farms.

Based on the previous descriptions of the fate of antimicrobials in the aquatic environment, the lack of detection of highly persistent antimicrobials such as oxytetracycline, flumequine, and oxolinic acid in aquaculture impacted sediments, strongly suggests that these antimicrobials are mainly diluted and carried off by currents. In under-cage sediments, adsorption or attachment of antibiotics to particulate matter will usually result in their inactivation, but considering that these processes are dynamic and reversible, adsorbed antibiotics are expected to leach from these sites with their antibacterial activity intact and able to select for antimicrobial resistant bacteria, exerting a continuous low level selective pressure on the sedimentary microbiota. This could explain the recovery of high levels of antibiotic-resistant bacteria in under-cage sediments from farms with no history of antimicrobial usage, as was demonstrated by Miranda and Rojas (2007).

In Chile the detection and reporting of antimicrobial residues associated with the salmon farming industry is currently not mandatory. However, many salmon farming companies in Chile commonly carry out monitoring of various parameters, including assessments of sedimentary antibacterial residues from beneath salmon cages. Unfortunately this data is not made public nor is it made available to the Chilean regulatory agency. It is essential that the concentrations of antimicrobial residues in freshwater and marine sediments impacted by the Chilean salmonid industry are known in order that efficient guidelines for their regulation can be implemented. Currently only a veterinarian prescription is required to approve their use, and their progressive impact on the surrounding environment is not considered. It is strongly believed that the accumulation of antibacterial residues in sediments beneath salmon pens must preclude their use and that a rotation of the administered drugs is required.

It must be noted that even in the absence of detectable amounts of antimicrobials in water or sediments impacted by Chilean salmon farming, these environments are commonly associated with a high incidence of antibiotic multi-resistant bacteria and their respective resistance genes against a high diversity of antimicrobials, including oxytetracycline, florfenicol, and oxolinic acid (Miranda and Zemelman, 2002b; Miranda and Rojas, 2007; Buschmann et al., 2012). These results suggest that these environments enhance the persistence of resistant bacteria and associated genes even in absence of a selective pressure.

The most intensively used antibacterial in Chilean freshwater salmonid farms is oxytetracycline, comprising 86.8% of the total drugs used in freshwater-based farms for the treatment of flavobacteriosis during 2016 (SERNAPESCA, 2017b) and consequently various studies assessing the levels of oxytetracycline-resistant bacteria as well as characterizing their associated *tet* genes have been performed (Miranda and Zemelman, 2002a,b; Miranda et al., 2003; Roberts et al., 2015). Miranda and Zemelman (2002b) found a high proportion of bacterial resistance to high levels of oxytetracycline (100 $\mu g\ mL^{-1}$) mainly from fingerling and effluent samples of a land-based farm (19.2 and 39.8%, respectively), as well as from the pelletized feed used in other salmon farms (34.3%). They found that resistant strains recovered from sampled farms showed high levels of resistance to oxytetracycline, exhibiting minimum inhibitory concentrations (MICs) ranging from 64 to 2,048 $\mu g\ mL^{-1}$. Furthermore, Miranda and Zemelman (2002a) studied 103 oxytetracycline-resistant strains recovered from various sources at four Chilean freshwater salmonid farms, finding high taxonomic variability within the resistant microbiota, with a predominance of multi-drug resistant *Pseudomonas* strains. In addition, a high simultaneous resistance to various antimicrobials was detected in the studied strains, with 74 strains exhibiting resistance to 6–10 antimicrobials. Most of these strains showed resistance to amoxicillin, erythromycin and furazolidone, as well as a high frequency of resistance to florfenicol, cefotaxime, and trimethoprim–sulfamethoxazole, but a low incidence of resistance to quinolones.

In another study by Miranda and Rojas (2007) florfenicol resistance among microbiota associated with two Chilean freshwater-based salmon farms with different histories of antimicrobial usage and located in two different lakes was investigated providing evidence of high levels of resistance to florfenicol in under-cage sediments (26.4%) at the salmon farm with a recent history of florfenicol usage, whereas under-cage sediments at the salmon farm with no recent history of antimicrobial usage exhibited low levels of resistance (0.69%). However, it must be noted that non-impacted control sediments from one of the studied lakes also exhibited high levels of resistance (18.6%) with a high predominance of *Pseudomonas* species. The authors also observed the important occurrence of intrinsic resistance among resistant bacteria, as was observed by Kerry et al. (1994) for marine sediments free from anthropogenic impact, where a high incidence of pseudomonads, a group

TABLE 3 | Studies of antibiotic resistance of bacteria associated to Chilean salmonid farming.

Source		No. of isolates	Main result	Reference
Freshwater	Water, Pellet Sediment, Fish	103[A]	High proportions of low- and high-level OTC-resistant bacteria mainly from pellet and effluent samples. Resistant bacteria were mostly non-fermenting bacteria (77.7%), exhibiting MICs ranging from 64 to 2,048 $\mu g\ mL^{-1}$.	Miranda and Zemelman, 2002b
	Water, Pellet Sediment, Fish	103[A]	A high number of bacteria resistant to AML, ERY, and FR, and an important frequency of resistance to FFC, CTX, and SXT was found, whereas resistance to G, K, FLU, and ENR was rather low. A high frequency (74 strains) of resistance to 6–10 antibacterial agents was detected.	Miranda and Zemelman, 2002a
	Water, Pellet Sediment, Fish	25[A]	Fifteen of the isolates carried one of seven different tetracycline (*tet*) genes [*tet(A)*, *tet(B)*, *tet(E)*, *tet(H)*, *tet(I)*, *tet(34)*, and *tet(35)*] and 10 had unknown *tet* genes	Miranda et al., 2003
	Water, Pellet Sediment, Fish	70[A]	Proportions of florfenicol resistance in under-cage sediments from salmon farm under florfenicol therapy (26.40%) were significantly higher than those from a farm with no recent history of antibacterial therapy (0.69%), detecting high levels of resistance to AML, ERY, FR, and SXT and susceptibility to G, K, and ENR	Miranda and Rojas, 2007
	Water, Pellet Sediment, Fish	119[A]	The *floR* gene was detected in 26 strains (21.8%) and most of the *floR*-carrying strains were glucose fermenters resistant to S and OTC. FFC resistance in most of non-fermenters (82 strains), was partially mediated by non-specific efflux pumps	Fernández-Alarcón et al., 2010
	Water, Pellet Sediment, Fish	10[A]	Six of the isolates carried the *tet(39)* gene, encoding for an efflux protein, such as the *Corynebacterium*, *Pseudomonas*, and *Psychrobacter* species.	Roberts et al., 2015
Seawater	Sediment	$24^A + 24^C$	Increase of resistance to FFC, OT, and OA in aquaculture site. Detection of genes *Tet(A)*, *tet(B)*, *tet(S)*, *tet(K)*, *tet(M)*, *aac(6')-Ib-cr*, and *intI1* among resistant isolates.	Buschmann et al., 2012
	Sediment	$124^A + 76^C$	32, 16, and 53% of resistance to FFC, OT, and OA at aquaculture site. Detection of genes *tet(A)*, *tet(G)*, *dfrA1*, *dfrA5*, *dfrA13*, *sul1*, *sul2*, and bla_{TEM} in resistant isolates.	Shah et al., 2014
	Sediment	4[A]	Isolates carried the *aac(6')-Ib-cr* gene, conferring reduced susceptibility to quinolones and kanamycin.	Aedo et al., 2014
	Sediment Water	$24^A + 24^C$	Genes *tet(A)*, *tet(B)*, *tet(K)*, *tet(M)*, *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr* were detected in marine bacteria from aquaculture and control sites.	Tomova et al., 2015
	Sediment Water	$23^A + 23^C$	*intI1* gene detected in isolates from aquaculture (11) and control (11) sites. *qnrA*, *qnrB*, and *qnrS* genes in four marine isolates were chromosomally located.	Tomova et al., 2018

[A], aquaculture impacted site; [C], control site; FFC, florfenicol; OT, oxytetracycline; OA, oxolinic acid; AML, amoxicillin; ERY, erythromycin; FR, furazolidone; CTX, cefotaxime; SXT, trimethoprim–sulfamethoxazole; G, gentamicin; K, kanamycin; FLU, flumequine; ENR, enrofloxacin; S, streptomycin.

that exhibits innate resistance to various antimicrobials, was detected (Sengeløv et al., 2003). Finally, the use of unmedicated pelletized feed in a lake-based salmon farm was high (34.8%), suggesting that in certain cases this could be an important source of resistant bacteria for Chilean aquaculture impacted environments.

From these studies, an important number of resistant strains were demonstrated to carry several specific genes encoding for antimicrobial resistance such as *floR* and *tet* genes (Miranda et al., 2003; Fernández-Alarcón et al., 2010). In addition, a high number of other resistant strains were probably carrying new and previously uncharacterized antimicrobial-resistance encoding genes. This was recently demonstrated by Roberts et al. (2015), who studied 10 tetracycline-resistant strains isolated in 1999 from Chilean freshwater salmon farms, which tested negative for 22 *tet* genes, but six strains were later found to be carrying the *tet*(39) gene, while the other four strains most probably carried other unknown *tet* genes. To date, only two studies assessing the mobility of resistance encoding genes carried on bacteria recovered from various Chilean salmon farms have been conducted. Miranda et al. (2003) and Roberts et al. (2015) demonstrated the ability of a diverse group of *tet* genes to be transferred to an *Escherichia coli* recipient. This suggests that salmon farming is highly relevant to the enrichment of the environmental resistome, exhibiting the characteristics required to spread enteric bacterial species, which could play an important role in waterborne human disease. Despite the intensive use of large amounts of antimicrobials in the Chilean salmon farming industry and its role as an important reservoir of resistant bacteria carrying antibiotic-encoding genes, no studies of the transfer of genes encoding for antimicrobial resistance from salmon farming associated bacteria to pathogens have been conducted.

More recently, additional studies assessing antimicrobial resistance in the marine environment impacted by Chilean salmon farming have been conducted. In a study by Buschmann et al. (2012) strains recovered near to salmon culture cages in Chile exhibited high incidences of *tet*, *qnr*, and *floR* genes encoding for resistance to tetracyclines, quinolones and

florfenicol, respectively, but in a later study the authors confirmed the absence of *qnr* and *floR* genes among these strains (Shah et al., 2014). In the most recent study, the authors found an important incidence of genes encoding for sulfonamide and trimethoprim resistance (*sul* and *dfrA*, respectively) as well as the presence of mobile genetic elements such as class 1 and 2 integrons (Shah et al., 2014). In addition, the same group identified the *aac(6)-Ib-cr* gene, encoding for an aminoglycoside acetyltransferase that confers reduced susceptibility to quinolone and kanamycin in marine bacteria associated with sediments impacted by a Chilean salmon farm, identical to the gene carried by urinary tract isolates of *E. coli*, suggesting the occurrence of a flow of this gene between these bacteria isolated from different environments (Aedo et al., 2014). In a more recent study, Tomova et al. (2015) studied a number of marine strains recovered from a Chilean aquaculture site at the same location, detecting in some of them the presence of *tet*, *qnr*, and *floR* genes, but concluding that undescribed tetracycline, quinolone and florfenicol resistance genes were probably carried by the majority of these strains. It must be noted that *qnr* genes encode for a low-level resistance to quinolones and are frequently associated with plasmids, suggesting a high feasibility of their mobility by horizontal transfer. Tomova et al. (2015) reported a high incidence of the *qnrB* gene among quinolone-selected bacteria and demonstrated that quinolone-resistant urinary *E. coli* isolated from patients living close to the sampled site were significantly enriched with *qnrB*, *qnrS*, and *qnrA* genes, compared to isolates from other regions not associated with aquaculture. The authors found that sequences of some of these genes were identical to those detected in the antimicrobial-resistant marine bacteria, and suggested the occurrence of horizontal gene transfer between antimicrobial-resistant marine bacteria and human pathogens (Tomova et al., 2015). Using the same isolates the authors detected the integrase encoding gene *intI1* in an important number of isolates recovered from non-impacted (11 isolates) and aquaculture impacted (11 isolates) sites (Tomova et al., 2018). Otherwise, the authors detected the chromosomally located *qnrA*, *qnrB*, and *qnrS* genes in four marine isolates, but these genes were no associated to integron gene cassettes (Tomova et al., 2018). In conclusion, these studies demonstrated a high concordance between the used antibiotics and the occurrence of associated resistance genes in Chilean salmonid farming providing evidence of an important occurrence of genes encoding for resistance to florfenicol (*floR*), tetracyclines (*tet*), and sulfonamides (*sul*), which suggest that this industry plays an important role as a reservoir of these genes.

Finally, it must be noted that all previous studies dealing with the issue of antimicrobial resistance in Chilean salmonid aquaculture have only considered the antibiotic resistant bacteria and some of the ARGs belonging to the culturable bacterial pool, which is known to be less than 1% of all environmental bacteria. Despite having proven that aquaculture supporting environments are an important source of new ARGs, the occurrence of important biases and limitations in our understanding of the real consequences of the release of these antibiotics into the aquatic environments must be recognized, and that an increased focus is required to demonstrate a direct relationship between environmental- and human-pathogenic antibiotic resistomes.

Bacterial Pathogens

It should be noted that various studies reporting the antimicrobial resistance of several bacterial pathogens associated with Chilean salmon farms have been published (**Table 4**). In the absence of stated clinical breakpoints most of the studies of bacterial pathogens in Chilean aquaculture aim to generate standard protocols and establish epidemiological cut-off values to differentiate between wild-type (WT) and non-wild-type (NWT) populations. It must be noted that variations in cut-off values are indicative of changes in the antibiotic susceptibility of populations of the pathogenic species, but epidemiological cut-off (CO_{WT}) values are protocol specific and need to be developed for all salmonid pathogens in Chile. Avendaño-Herrera et al. (2011) calculated the epidemiological cut-off values of florfenicol, erythromycin and oxytetracycline for *Streptococcus phocae* strains mostly recovered from diseased Atlantic salmon (*Salmo salar*), indicating that of the 19 strains isolated from 2004 onward, 18 strains were classified as NWT (non-fully susceptible). The authors suggested the importance of reducing oxytetracycline use for the streptococcal treatment. In another study, Henríquez-Núñez et al. (2012) studied a total of 40 *Flavobacterium psychrophilum* isolates obtained from Chilean salmon farms to determine their antimicrobial susceptibility to oxytetracycline, florfenicol, and oxolinic acid, finding that 90, 92.5, and 85%, respectively of strains were resistant to the three antimicrobials. Furthermore, 39 of the 40 isolates carried a single plasmid or combinations of two plasmids, but a relationship between plasmid and resistance could not be established. In a recent study, Miranda et al. (2016) determined the susceptibility of 125 *F. psychrophilum* Chilean isolates to antimicrobials used in fish farming and calculated their CO_{WT} values by using an agar dilution MIC method and a disk diffusion method. The data generated by the disk diffusion protocol used in this work were shown to have low precision, in agreement with Henríquez-Núñez et al. (2012), confirming that MIC determination would be the preferred method for susceptibility testing for this species. The NWT frequencies obtained using MIC data, were 24% for amoxicillin, 8% for florfenicol, and 70% for oxytetracycline, whereas for the quinolones oxolinic acid, flumequine, and enrofloxacin the frequencies of NWT isolates were 45, 39, and 38%, respectively using MIC data. The significant frequencies of isolates exhibiting reduced susceptibility to oxytetracycline and quinolones may result from treatment failures when these agents were used (Miranda et al., 2016). The occurrence of resistance to oxytetracycline, florfenicol, and oxolinic acid among some Chilean strains of *Vibrio ordalii* isolated from diseased salmonids has also been reported (Poblete-Morales et al., 2013). In a further study, Valdés et al. (2015) studied the draft genome sequence of an antibiotic-resistant strain of *Aeromonas salmonicida* isolated from infected rainbow trout, finding various efflux pumps and putative genes that confer resistance to macrolides, β-lactamics, florfenicol, and quinolones, concluding that efflux pumps are the main mechanisms of resistance to non-β-lactamic antibiotics.

TABLE 4 | Studies of antibiotic resistance of pathogenic bacteria associated to Chilean salmonid farming.

Species	No. of isolates	Main result	Reference
Piscirickettsia salmonis	4	MIC and MBC values of CM, G, OTC, OA, and FLU using cytopathic effect on cell cultures	Smith et al., 1996
	2	A formulated medium is proposed to be used in antimicrobial susceptibility assays for *P. salmonis*	Yáñez et al., 2014
	20	Single point mutation in *gyrA* gene is responsible for the quinolone resistant phenotype	Henríquez et al., 2015
	292	ECO_{WT} values of FFC, OTC, OA, and FLU	Henríquez et al., 2016
	2	Florfenicol can modulate RND gene expression and increase efflux pump activity	Sandoval et al., 2016
	3 (genome)	Six specific genes, encoding for specific transporter proteins eventually relevant in conferring resistance to FFC and OTC	Cartes et al., 2017
	58	ECO_{WT} values of FFC and OTC using MIC data	Contreras-Lynch et al., 2017
	1 (genome)	The genome of an oxytetracycline-resistant strain bearing a multidrug-resistance plasmid is described	Bohle et al., 2017
	247	Resistance to quinolones (71.3%) and oxytetracycline (8.1%)	Saavedra et al., 2017
Flavobacterium psychrophilum	40	ECO_{WT} values of FFC, OTC, and OA for MIC data	Henríquez-Núñez et al., 2012
	125	ECO_{WT} values of AML, FFC, OTC, OA, FLU, and ENR using MIC and antibiogram data	Miranda et al., 2016
Aeromonas salmonicida	1 (genome)	Strain isolated from infected rainbow trout contained several efflux pumps and putative genes that confer resistance to macrolides, β-lactamics, florfenicol, and quinolones	Valdés et al., 2015
Vibrio ordalii	24	ECO_{WT} values of FFC, OTC and OA using MIC and antibiogram data	Poblete-Morales et al., 2013
Streptococcus phocae	31	ECO_{WT} values of ERY, FFC, and OTC	Avendaño-Herrera et al., 2011

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; CM, chloramphenicol; G, gentamicin; AML, amoxicillin; FFC, florfenicol; OTC, oxytetracycline; OA, oxolinic acid; FLU, flumequine; ENR, enrofloxacin; ERY, erythromycin; RND, resistance nodulation division; ECO_{WT}, epidemiological cut-off value for the fully susceptible to the antibacterial agent population (wild-type).

Piscirickettsiosis, the disease caused by the intracellular pathogenic bacteria *P. salmonis*, is currently the most important bacterial pathology of seawater salmonid farming in Chile, accounting during 2016 for the 74.6 and 86.8% of the mortality in the Chilean salmon industry for Atlantic salmon and rainbow trout, respectively (SERNAPESCA, 2017a), and consequently it is the main target of antimicrobial therapies administered in the Chilean salmon industry (Rozas and Enríquez, 2014). With this in mind, based on a systematic review of available scientific literature, Mardones et al. (2018) concluded that the emergence and frequency of *P. salmonis* antibiotic resistant strains are topics which require further research, but the authors claimed that there is no published work that developed harmonized schemes for monitoring antimicrobial resistance and effectiveness against *P. salmonis*, neither the ecological impact nor costs associated with treatment strategies. However, various studies addressing the susceptibility to antimicrobial agents among Chilean *P. salmonis* strains have been conducted (**Table 4**). Smith et al. (1996) studied the antimicrobial susceptibility of four Chilean strains of *P. salmonis* by using cell monolayer-based MIC assays which detected significant variation in antimicrobial susceptibility patterns, whereas Yáñez et al. (2014) found a high susceptibility to florfenicol and oxytetracycline, but only three *P. salmonis* strains were studied. In another study, Henríquez et al. (2015) reported an important incidence of resistance to quinolones mediated by a single point mutation in the *gyrA* gene among *P. salmonis* strains isolated from diseased salmon in Chile. More recently, Henríquez et al. (2016) studied the susceptibility to quinolones, florfenicol, and oxytetracycline of 292 *P. salmonis* strains collected over 5 years, providing evidence of a high incidence of strains exhibiting resistance to quinolones, but suggesting that resistance to florfenicol and oxytetracycline is still developing. In further study, Sandoval et al. (2016) detected different florfenicol susceptibilities in two Chilean *P. salmonis* strains, observing that in the less susceptible strain florfenicol could modulate the gene expression of the multi-drug resistance-related efflux pump belonging to the resistance nodulation division (RND) family and increasing efflux pump activity. The authors concluded that the *acrAB* efflux pump is essential for *P. salmonis* survival at critical florfenicol concentrations and for the generation of antibiotic-resistant bacterial strains. More recently, Cartes et al. (2017) analyzed whole genomes of 3 *P. salmonis* isolates exhibiting different degrees of susceptibility to florfenicol and oxytetracycline, detecting genes encoding for specific transporter proteins. The authors suggested that these strains possess a greater number of membrane carriers, such as MDR (multidrug resistance) type (Cartes et al., 2017). On the other hand, Saavedra et al. (2017) studied a high number of isolates of this species, finding a high incidence of non-susceptible isolates to quinolones, but only a low percentage of non-susceptible to oxytetracycline, whereas all studied isolates were susceptible to florfenicol. In another recent study, Bohle et al. (2017) described the genome of an oxytetracycline-resistant *P. salmonis* isolate bearing a multidrug-resistance plasmid unique to this isolate and harboring a *tet* determinant, but no other resistance-encoding genes were described. Finally, in an attempt

to standardize protocols and criteria for studying antibacterial susceptibility of this pathogen, Contreras-Lynch et al. (2017) proposed a standard protocol and stated the epidemiological cut-off values for florfenicol and oxytetracycline for this species.

CONCLUSION

The growth of salmon aquaculture in Southern Chile is an example of industrial development that over only a few decades has gained a prominent place in global seafood markets. Along with this explosive development, this salmon farming industry has excessively utilized antibiotics to treat or prevent salmon diseases. Currently, 0.53 kg of antibiotics per ton of harvested salmon are used in the treatment and prevention of salmon diseases (data for 2016), 95% of which is used in the marine culturing phase to treat *P. salmonis* infections and 99.6% is comprised of just two antibiotics, florfenicol and oxytetracycline (SERNAPESCA, 2017b). Under this scenario, hundreds of tons of antibiotics enter the marine environment causing possibly negative environmental consequences and potential risks for human health. If we take account of the pharmacokinetic properties of both antibiotics, and assume that all administered antibiotic (by feed) was consumed, we can estimate that 40 tons of oxytetracycline and 3 tons of florfenicol were released into the marine environment in 2016. This is highly significant considering that in the last 10 years these antibiotics have been the most frequently used by industry.

Antibiotics entering marine environments favor the selection of antibiotic resistance among environmental bacteria and fish pathogens, and may also affect the activity of bacteria driving biogeochemical cycles in marine sediments. Furthermore, these chemicals can modify resistomes by selecting antibiotic resistance genes (ARGs) and increasing the rates of horizontal gene transfer, thereby increasing the probability of antibiotic resistance gene transfer from environmental to human pathogenic bacteria. These effects are of significant importance for Southern Chile, where antibiotics are used excessively in salmonid farming when compared to the other salmon producing countries. Therefore, antibiotic use by Chilean salmon farms has become a controversial issue due to the possible effects of high concentrations of antibiotics being released into nominally pristine environments, such as Chilean Patagonia. The Pacific coast of Patagonia is comprised of a vast area of fjords and canals, much of which is protected either within National Parks or close to them. Yet despite this protection the areas being used for aquaculture are constantly expanding into ever more remote and previously unimpacted areas.

Despite that over the last two decades only few studies assessing antimicrobial resistance and the resistome in the freshwater and marine environment impacted by salmon farming have been conducted, most of them demonstrated that Chilean salmonid farm industry plays an important role as a reservoir of antibiotic resistant microbiota and associated resistance genes. Furthermore, previous studies have shown that even in the absence of detectable amounts of antimicrobials in several sediments impacted by Chilean salmon farming, these environments are commonly associated with a high incidence of antibiotic multi-resistant bacteria and their respective resistance genes against a high diversity of antimicrobials, including oxytetracycline, florfenicol, and oxolinic acid. This might suggest that these environments enhance the selection and persistence of resistant bacteria and associated genes even in the absence of a selective pressure.

Considering that the Chilean salmon farming industry is one of the worldwide leaders in the use of antibiotics, studies on antibiotic resistant microbiota and related resistome are still very scarce and much data is required to understand the role of these environments in the maintenance and dissemination of antibacterial resistance. Thus, studies aimed at increasing knowledge of environmental resistomes associated with Chilean salmon farming and the possibility of their mobilization to the human clinical compartment are crucial for managing the potential threat to human public health. In this trend, surveillance studies of antibacterial resistance in under-cage sediments must be mandatory for all Chilean salmonid farms to avoid spread of selected resistance/genes to the human clinical compartment.

Furthermore, the growing incidence of antimicrobial-resistance among bacterial pathogens causing outbreaks in the Chilean salmon industry is probably a consequence of the intensive use of antibiotics in this industry, suggesting the urgent requirement for the application of a strict controls in order to avoid the overuse of antimicrobials, and the implementation of a regular surveillance program in order to detect the emergence and prevalence of ARGs in the environment. The observed irregular effects of antimicrobial therapies in controlling *P. salmonis* in Chilean salmonid farms suggest that the bacterium has developed some level of resistance. Thus, it is important that the rational and well-controlled use of antimicrobials is implemented soon in order to decrease the selective pressure imposed on this pathogenic species and consequently avoid the selection of multi-drug resistant strains.

In conclusion, further studies are urgently required, mainly focused on understanding the evolution and epidemiology of resistance genes in Chilean salmonid farming, particularly those encoding for resistance to antibiotics used in humans and to determine the feasibility of a link between these genes among bacteria from salmonid farms and human and fish pathogens. Furthermore, a harmonization of protocols and epidemiological cut-off values used to categorize pathogen isolates in all diagnostic labs is urgently required to avoid therapy failures. Considering that *P. salmonis* is a particularly important pathogen in Chilean salmon farming, causing the highest mortalities from infectious diseases (SERNAPESCA, 2017a), the development of efficient strategies for its control as well as understanding its antimicrobial susceptibility status, should be an urgent priority for the industry. Because of this trend, is understandable that most of published studies are related to this pathogenic species. Various Chilean researchers are currently elucidating the antibacterial resistance mechanisms involved in detected non-susceptible isolates, in accordance with the conclusions stated in a recent study (Mardones et al., 2018). Finally, having demonstrated the high prevalence of antibiotic

resistant bacteria carrying transferable resistance genes in the Chilean salmonid farm industry it is an urgent necessity to implement antibiotic resistance surveillance programs and a high number of complementary initiatives to reduce the rate of increase of resistance in this industry. It is important to note that dissemination of surveillance data should not be restricted to the scientific community but must include all major stakeholders including the Chilean government regulatory agencies.

AUTHOR CONTRIBUTIONS

CM and FG conceived the review outline, researched and drafted the manuscript, and are the corresponding authors and primary contacts during the manuscript submission, review, and publication process. ML contributed significantly to the drafting and revisions of the manuscript. All authors have made intellectual contribution to the work, and approved it for publication.

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Identification of a New Antimicrobial Resistance Gene Provides Fresh Insights Into Pleuromutilin Resistance in *Brachyspira hyodysenteriae*, Aetiological Agent of Swine Dysentery

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Brachyspira hyodysenteriae is the aetiological agent of swine dysentery, a globally distributed disease that causes profound economic loss, impedes the free trade and movement of animals, and has significant impact on pig health. Infection is generally treated with antibiotics of which pleuromutilins, such as tiamulin, are widely used for this purpose, but reports of resistance worldwide threaten continued effective control. In *Brachyspira hyodysenteriae* pleuromutilin resistance has been associated with mutations in chromosomal genes encoding ribosome-associated functions, however the dynamics of resistance acquisition are poorly understood, compromising stewardship efforts to preserve pleuromutilin effectiveness. In this study we undertook whole genome sequencing (WGS) and phenotypic susceptibility testing of 34 UK field isolates and 3 control strains to investigate pleuromutilin resistance in *Brachyspira hyodysenteriae*. Genome-wide association studies identified a new pleuromutilin resistance gene, *tva*(A) (*t*iamulin *v*alnemulin *a*ntibiotic resistance), encoding a predicted ABC-F transporter. *In vitro* culture of isolates in the presence of inhibitory or sub-inhibitory concentrations of tiamulin showed that *tva*(A) confers reduced pleuromutilin susceptibility that does not lead to clinical resistance but facilitates the development of higher-level resistance via mutations in genes encoding ribosome-associated functions. Genome sequencing of antibiotic-exposed isolates identified both new and previously described mutations in chromosomal genes associated with reduced pleuromutilin susceptibility, including the 23S rRNA gene and *rplC*, which encodes the L3 ribosomal protein. Interesting three antibiotic-exposed isolates harboured mutations in *fusA*, encoding Elongation Factor G, a gene not previously associated with pleuromutilin resistance. A longitudinal molecular epidemiological examination of two episodes of swine dysentery at the same farm indicated that *tva*(A) contributed to development of tiamulin resistance *in vivo* in a manner consistent with that seen experimentally *in vitro*. The *in vitro* studies further showed that *tva*(A) broadened the mutant selection window and raised the mutant prevention

concentration above reported *in vivo* antibiotic concentrations obtained when administered at certain doses. We show how the identification and characterisation of *tva*(A), a new marker for pleuromutilin resistance, provides evidence to inform treatment regimes and reduce the development of resistance to this class of highly important antimicrobial agents.

Keywords: *Brachyspira hyodysenteriae*, swine dysentery, antimicrobial resistance, tiamulin, pleuromutilin, antimicrobial resistance gene

INTRODUCTION

Pigs are an important source of meat and provide the second highest share of meat consumed worldwide (OECD)[1]. Swine dysentery (SD) is a severe mucohaemorrhagic colitis affecting pigs and is of significant economic, and pig health and welfare importance (Hampson, 2012; Alvarez-Ordóñez et al., 2013). Economic costs of SD can be large, estimated at $115 million to the US swine industry in 1994 and $8.30 to medicate each SD-positive animal in 1990 (Burrough et al., 2013). In the United Kingdom (UK) SD was estimated to cost £4–10 per infected pig in 2012 (Alderton, 2012). These losses arise from reduced feed conversion and weight gain, the high morbidity of disease (up to 90%), costs associated with treatment of clinical disease and metaphylaxis, hygiene measures, and disruption to the trade of pigs (Hampson, 2012; Alvarez-Ordóñez et al., 2013). The disease is distributed worldwide and the classical etiological agent is *Brachyspira hyodysenteriae*, an anaerobic spirochaete which resides in the large intestine of infected pigs. Antibiotic treatment is critical for control of disease on infected units, and is also part of treatment and elimination programmes for SD, especially as no commercial vaccines against SD are available. In most jurisdictions however the number of efficacious antibiotics available to treat SD is severely limited (Hampson, 2012; Kulathunga and Rubin, 2017). For example, in the UK antibiotics authorised for the treatment of SD are limited to the pleuromutilins tiamulin and valnemulin, the macrolides tylosin and tylvalosin, and lincomycin (a lincosamide); although off-label use of other antibiotics (e.g., doxycycline) is permitted under the cascade system, a risk-based decision tree that allows veterinarians to employ clinical judgement to treat an animal with an alternative product when there is no appropriate authorised veterinary medicine available. Tiamulin is the most widely used antibiotic for treatment of SD, due to efficacy towards *B. hyodysenteriae* and relatively short withdrawal periods (van Duijkeren et al., 2014). The World Organisation for Animal Health has classed tiamulin and valnemulin as Veterinary Highly Important Antimicrobial Agents, given their critical importance for the treatment of SD and the lack of alternatives (Anonymous, 2007). In the USA the proposed withdrawal of carbadox (Anonymous, 2014), a compound used to control SD which is already withdrawn from use in the European Union and Canada, and recent recommendations in the European Union to withdraw the indication for oral formulations of tylosin (European Medicines Agency, 2014b) and certain oral lincomycin (European Medicines Agency, 2017) and lincomycin-spectinomycin combinations (European Medicines Agency, 2014a) for treatment of SD caused by *B. hyodysenteriae* would further restrict antimicrobial therapy options available to veterinarians.

A major threat to the effective control of SD is resistance of *B. hyodysenteriae* to pleuromutilins and/or other antibiotics, indeed isolates with reduced susceptibility have been reported in North America, Europe, Japan, and Australia, and the prevalence of resistance appears to be increasing (Karlsson et al., 2002; Lobová et al., 2004; Hidalgo et al., 2009; Pringle et al., 2012; Swedres-Svarm, 2015; Kajiwara et al., 2016; Mirajkar et al., 2016; Mahu et al., 2017; De Luca et al., 2018). Reduced antibiotic susceptibility can lead to suboptimal or ineffective antibiotic treatment, resulting in increased economic impact to producers, adverse effects on pig health and welfare, and development of antibiotic resistance. Furthermore, multidrug resistance has been reported and in some herds *B. hyodysenteriae* has become resistant to all authorised antimicrobials, leaving depopulation and elimination of infection through thorough cleansing and disinfection, and then restocking as the only effective course of action (Hampson, 2012; Strugnell et al., 2013), which has significant cost. Reduced antibiotic susceptibility in *B. hyodysenteriae* has been associated with the presence of *lnu*(C) (lincosamides) (De Luca et al., 2018) and point mutations at specific positions in the 16S rRNA gene (doxycycline), 23S rRNA gene (macrolides, lincosamides, and pleuromutilins) and *rplC*, the gene encoding the L3 ribosomal protein (pleuromutilins) (Karlsson et al., 1999; Pringle et al., 2004, 2007; Hidalgo et al., 2011; Hillen et al., 2014; De Luca et al., 2018). The development of resistance to pleuromutilins in *B. hyodysenteriae* is thought to occur in a stepwise manner both *in vitro* and *in vivo*, suggesting that multiple mutations are required for the emergence of high level resistance (Karlsson et al., 2001; Hidalgo et al., 2011; van Duijkeren et al., 2014), however the dynamics and mechanisms of emergence of resistance to pleuromutilins remain poorly defined. Furthermore *B. hyodysenteriae* isolates with reduced susceptibility to pleuromutilins but without relevant point mutations have been described, while for other mutations there is debate on their role in conferring resistance (Pringle et al., 2004; Hidalgo et al., 2011; Hillen et al., 2014; Mahu et al., 2017). This debate indicates that our understanding is incomplete and suggests that other unidentified mutations and/or genes may be involved in pleuromutilin resistance in *B. hyodysenteriae*.

[1]OECD. Meat comsumption (indicator).

In this study we have examined the molecular basis for antimicrobial resistance in *B. hyodysenteriae* isolates recovered from pigs in the UK ($n = 34$) and ATCC control strains ($n = 3$) by whole genome sequencing (WGS) and antimicrobial susceptibility testing. Genome-wide association studies were employed to screen for new genes associated with reduced pleuromutilin susceptibility. We additionally investigated mechanisms underlying the emergence of pleuromutilin resistance *in vitro* by sequencing mutant isolates obtained after single exposure of isolates to inhibitory tiamulin concentrations or following repeated culture in sub-inhibitory concentrations. Antibiotic exposure can select for mutational changes conferring resistance to the antimicrobial which has been used, with cross-resistance occurring where those mutations confer resistance to several antimicrobial compounds (Karlsson et al., 1999, 2001; Pringle et al., 2004). We applied the principles of the mutation prevention concentration (MPC) hypothesis, which defines the antibiotic concentration at which mutations giving rise to resistance do not occur (Drlica and Zhao, 2007), when exposing isolates to inhibitory tiamulin concentrations. The MPC has been applied to assess the development of resistance to various antibiotics including quinolones, macrolides, tetracyclines, and pleuromutilins in many bacterial species including *Escherichia coli*, *Salmonella enterica*, *Mycoplasma gallisepticum*, and *Staphylococcus aureus* (Randall et al., 2004; Drlica and Zhao, 2007; Ozawa and Asai, 2013; Zhang et al., 2017). Maintaining antibiotic concentrations above the MPC during therapy is thought to help reduce the development of resistance (Drlica and Zhao, 2007) and we related our findings to published tiamulin pharmacokinetic and pharmacodynamic parameters in pigs to help inform veterinary options for the treatment of SD.

MATERIALS AND METHODS

Isolates and Culture Methods

Thirty three UK field isolates of *B. hyodysenteriae* recovered from submissions to the Animal and Plant Health Agency between 2005 and 2013 from 22 pig holdings were used in this study (**Table 1**). Isolates were derived from diagnostic samples ($n = 32$) or samples collected to assess infection status as part of disease control ($n = 1$). Samples were of three types: excreted faecal samples not collected directly from live pigs ($n = 20$); faeces or intestinal contents collected from dead pigs ($n = 12$, no animals were euthanased specifically for this publication); or rectal swabs ($n = 1$) collected from individual live pigs by veterinary surgeons, which did not require anaesthesia, and was not harmful to the pigs. This sampling strategy is part of the normal veterinary diagnostic investigation of this type of disease on a farm and as such is not for scientific purpose and therefore not covered by the Animal (Scientific Procedures) Act 1986. Sampling which is for the immediate or long term benefit of the individual animal, its immediate cohort or the wider epidemiological group, is covered as an act of veterinary clinical practice within the Veterinary Surgeon's Act 1966. The UK field strain P18A was also included in the panel, which was isolated from a pig with swine dysentery in the late 1970s (Lemcke and Burrows, 1981) and is used as a control for susceptibility testing at APHA (Griffiths et al., 2008). All isolates were recovered from cases of swine dysentery, except BH23 which was isolated from an apparently healthy animal that showed no clinical signs of swine dysentery. At Holdings A and B isolates were recovered on different sampling dates, allowing on-farm disease episodes to be followed; information on tiamulin use was also available for these farms. Additionally, three reference strains were included in this work: $B78^T$ (ATCC 27164), B204 (ATCC 31212), and WA1 (ATCC 49526).

Isolates were cultured on fastidious anaerobe blood agar (FABA) in an anaerobic cabinet (Don Whitley Scientific) in anaerobic gas (10% H_2, 10% CO_2, and 80% N_2) at 38°C for 3–5 days. Broth cultures of *B. hyodysenteriae* were prepared by aseptically picking from the agar surface with a sterile inoculation loop and inoculating into pre-reduced Brain Heart Infusion Broth (BHIB) with 10% Horse Serum (Oxoid or E and O Laboratories Ltd.).

Susceptibility Testing

Minimum Inhibitory Concentrations (MICs) for tiamulin, valnemulin, tylosin, tylvalosin, doxycycline, and lincomycin were determined by broth dilution using VetMIC Brachy plates (National Veterinary Institute, Uppsala, Sweden) (Karlsson et al., 2003). Isolates were plated from stock culture onto FABA plates and sub-cultured twice before testing according to the manufacturer's instructions. Plates were incubated for 4 days at 38°C with shaking at 80 rpm and the MIC was recorded as the lowest concentration of the antimicrobial agent that prevented visible growth. For all samples purity was demonstrated and viable counts (CFU ml^{-1}) estimated by creating a 10-fold dilution series in pre-reduced BHIB + 10% FCS and plating on FABA plates. Strain $B78^T$ was used as control in each batch of tests (Pringle et al., 2006).

Selection for Resistant Mutants at Inhibitory Concentrations

The isolates selected for these experiments comprised the reference strains $B78^T$ and WA1 and 16 field isolates, with different tiamulin MICs spanning ≤0.063 to 4 mg/L and different genotypes (STs) (Table S4). Isolates were plated from stock culture onto FABA and sub-cultured twice. For each isolate the growth from four plates was harvested into 10 ml broth culture and incubated overnight at 38°C with shaking at 100 rpm. The McFarland of the broth was determined using a densitometer (Grant Instruments) and 100 μl when then plated onto each of four FABA plates supplemented with dilutions of tiamulin hydrogen fumarate (Sigma-Aldrich, UK) at the MIC as determined by broth dilution and three doubling concentrations above this (Table S4). The purity and CFUml^{-1} of the broth culture was determined by creating a 10-fold dilution series in broth and plating on FABA plates. Plates were incubated for up to 5 days at 38°C. Zones of haemolysis on the antibiotic containing plates indicative of resistant colonies were counted, picked and streaked onto FABA containing tiamulin at the same concentration as the plate picked from. A single CFU was then picked and sub-cultured on FABA with tiamulin until there was sufficient growth to create a stock culture and a cell pellet for DNA extraction. Subsequently, stock cultures of mutant isolates were tested for antibiotic susceptibility as described above. The mutation frequency was calculated as the number of mutants

TABLE 1 | Summary of the 34 field isolates and three reference strains examined in this project, sorted by tiamulin Minimum Inhibitory Concentration (MIC).

Isolate ID	ST	Holding	Year and month	Tiamulin MIC (ECOFF >0.25 mg/L)	Valnemulin MIC (ECOFF >0.125 mg/L)	23S rRNA			L2 protein	L3 protein	*tva*(A)
						G2032A	G2201T	G2535A	T50N	N148S	
WA1	26	Reference strain	2000s-n/a	<=0.063	<=0.031						
B204	54	Reference strain	1970s-n/a	<=0.063	<=0.031						
B78T	56	Reference strain	1970s-n/a	<=0.063	<=0.031						
P18A	4	Not known	1970s-n/a	<=0.063	<=0.031						
BH13	88	A	2009-8	<=0.063	<=0.031						
BH26	88	B	2012-1	<=0.063	<=0.031						
BH8	88	C	2008-10	<=0.063	<=0.031						
BH35	91	AR	2012-1	<=0.063	<=0.031						
BH7	239	R1	2008-9	<=0.063	<=0.031						
BH9	88	C	2008-11	0.125	<=0.031						
BH15	8	X	2010-5	0.25	**0.5**			A			*tva*(A)
BH2	8	Z	2005-11	0.25	**0.5**						*tva*(A)
BH3	8	Z	2005-11	0.25	**0.5**						*tva*(A)
BH20	52	CB	2010-12	0.25	**0.25**						*tva*(A)
BH28	88	B	2012-2	0.25	<=0.031					Ser	
BH34	8	CQ	2012-9	**0.5**	**0.5**			A			*tva*(A)
BH16	87	CN	2010-6	**0.5**	0.125		C	A		Ser	
BH14	88	A	2009-10	**0.5**	**1**						*tva*(A)
BH29	88	B	2012-3	**0.5**	**0.5**						*tva*(A)
BH37	240	G	2013-5	**0.5**	**1**			A			*tva*(A)
BH24	52	CM	2011-1	**1**	**0.5**						*tva*(A)
BH6	240	II	2008-7	**1**	**1**			A			*tva*(A)
BH23	167	BF	2010-12	**2**	**1**			A	Asn		*tva*(A)
BH38	52	CP	2013-9	**4**	**4**						*tva*(A)
BH25	8	AB	2011-6	**8**	**4**			A			*tva*(A)
BH27	8	CO	2012-1	**8**	**2**						*tva*(A)
BH17	87	J	2010-7	**8**	**>4**		C	A		Ser	*tva*(A)
BH30	240	H	2012-3	**8**	**2**					Ser	*tva*(A)
BH32	240	H	2012-3	**8**	**4**					Ser	*tva*(A)
BH12	87	J	2009-7	**>8**	**>4**		C	A		Ser	*tva*(A)
BH33	87	K	2012-6	**>8**	**>4**		C	A		Ser	*tva*(A)
BH36	87	O	2013-1	**>8**	**>4**		C	A		Ser	*tva*(A)
BH18	88	A	2010-10	**>8**	**>4**	A					*tva*(A)
BH19	88	A	2010-10	**>8**	**>4**	A					*tva*(A)
BH21	88	A	2010-11	**>8**	**>4**	A					*tva*(A)
BH22	88	A	2010-11	**>8**	**>4**	A					*tva*(A)
BH31	240	H	2012-3	**>8**	**>8**					Ser	*tva*(A)

Holding of origin is given as an anonymized letter code, together with year and month of sampling. The MICs for tiamulin and valnemulin are shown; bold text indicates MICs above the ECOFF values as given in column headers. The presence of SNPs identified in the 23S rRNA gene and amino acid substitutions in L2 and L3 proteins associated with reduced pleuromutilin susceptibility are indicated; blank, wild-type; ST, Sequence Type. Presence of tva(A) indicated.

recovered per CFUml^{-1} and the selection index was calculated as the MPC:MIC ratio by dividing the MPC values by the MIC values.

Selection for Resistant Mutants at Sub-inhibitory Concentrations

Ten isolates (Table S6) were plated from stock culture onto FABA and sub-cultured twice. Isolates were then plated onto FABA and a tiamulin MIC Test Strip (Launch Diagnostics, UK) aseptically applied. Subsequently, isolates were sub-cultured twice a week, by harvesting growth along the line of inhibition and re-plating in the presence of a tiamulin MIC Test Strip. As the growth became rich the concentration of tiamulin was increased, using doubling concentrations prepared in sterile discs (Oxoid, UK and Sigma-Aldrich, UK). At points during the experiment a portion of growth was plated onto FABA in the absence of antibiotic, cultured for 3–4 days and used to prepare a stock culture for storage at −80°C and a cell pellet for DNA extraction. Five of the isolates were additionally sub-cultured twice weekly in the absence of tiamulin and stock cultures prepared during the

experiment. Selected stock cultures were tested for antibiotic susceptibility as described above.

Whole Genome Sequencing and Analysis

DNA extracts were prepared from cell pellets using Prepman Ultra (Life Technologies, UK) according the manufacturer's protocol. Nextera XT libraries were prepared for WGS (Illumina, Lesser Chesterford, UK) and sequenced on an Illumina MiSeq platform v2 2x 150 bp paired-end protocol. The raw sequences for each isolate were analysed with the Nullarbour pipeline (version 1.20; Seemann et al.[2]) using the closed genome of WA1 (Bellgard et al., 2009) as reference, and SPAdes version 3.9.0 (Bankevich et al., 2012) and Prokka version 1.11 (Seemann, 2014) for genome assembly and annotation respectively. The published genomes of 41 *B. hyodysenteriae* isolates from swine (Black et al., 2015; La et al., 2016b) were included in this analysis. A maximum likelihood phylogenetic tree using the SNPs located within chromosomal regions present for all the strains was constructed using FastTree (Price et al., 2009). Species were assigned by Kraken (Wood and Salzberg, 2014) (version 0.10.5-beta) and Roary (Page et al., 2015) used to generate gene presence/absence lists. Genome-wide association studies to identify genes having significant association ($p < 0.05$; after Bonferroni correction for multiple tests) with reduced susceptibility to tiamulin and valnemulin were performed using Scoary (Brynildsrud et al., 2016).

Additionally each isolate was analysed using SeqFinder (Anjum et al., 2016), in which the raw sequences were filtered and trimmed using Trimmomatic (Bolger et al., 2014), with the parameters for the minimum quality threshold equal to 20, sliding window equal to 10, and minimum sequence length equal to 36. The raw trimmed and filtered data was mapped onto the genome of the WA1 chromosome (Accession number NC_012225) and plasmid (Accession number NC_012226) (Bellgard et al., 2009) using SMALT (Sanger Institute). The published genomes of 41 *B. hyodysenteriae* isolates from swine (Bellgard et al., 2009; Black et al., 2015) were also mapped to WA1. Single nucleotide polymorphisms (SNPs) with respect to WA1 were calculated using SAMTOOLS software (Li and Durbin, 2009; Li et al., 2009). SNPs were filtered using the quality thresholds of minimum coverage equal to 4, minimum proportion of raw sequences agreeing with the SNP call equal to 80%, and SAMTOOLS SNP quality score >150. Isolate sequence type (ST) was determined by extracting the seven house-keeping genes of the *B. hyodysenteriae* MLST scheme (*adh*, *alp*, *est*, *gdh*, *glpK*, *pgm*, and *thi*) (La et al., 2009) and interrogation of the PubMLST database (https://pubmlst.org/brachyspira/). Differences between the genomes of closely related isolates (e.g., parent and mutant isolates) were examined by comparison of the SNPs determined by SeqFinder using custom scripts and by extracting mapped genes of interest for alignment using the Clustal V method in MegAlign (version 11; DNAstar Inc.).

The whole genome sequences and sequence of *tva*(A) from isolate BH14 were deposited in the European Nucleotide Archive under study accession number PRJEB24023.

The presence of SNP mutants associated with reduced susceptibility to antibiotics in the VetMIC Brachy panel was assessed as follows: doxycycline and mutation at G1058 in the 16S rRNA gene (Pringle et al., 2007); tylosin and lincomycin and mutation at A2058 in the 23S rRNA gene (Karlsson et al., 1999; Hidalgo et al., 2011); tylvalosin and a mutation at A2058 and/or A2059 in the 23S rRNA gene (Hidalgo et al., 2011). Reduced susceptibility to tiamulin and valnemulin was assessed using mutations at positions G2032, C2055, G2201, G2447, C2499, C2504, and G2535 in the 23S rRNA gene and with SNPs causing non-synonymous substitutions at amino acids N148 and S149 in the 50S ribosomal protein L3 (Pringle et al., 2004; Hidalgo et al., 2011; Hillen et al., 2014). *E. coli* numbering was used for the 16S and 23S rRNA genes and polypeptide sequences were numbered according to sequence in strain WA1. The correlation of the presence of a SNP with reduced susceptibility was evaluated by two-by-two table analysis (Mackinnon, 2000), where test specificity, sensitivity, and the predictive value of a positive and negative test were calculated using the following criteria: mutant SNP and MIC > ECOFF value were true positive (TP), wild type SNP and MIC ≤ ECOFF value were true negative (TN), mutant SNP but MIC ≤ ECOFF value were false positive (FP), and wild type SNP but MIC > ECOFF value were false negative (FN). The correlation was also evaluated for each antibiotic in this manner for the presence/absence of *tva*(A).

RESULTS AND DISCUSSION

Genome Sequencing Revealed Considerable Diversity in UK *B. hyodysenteriae*

The 34 UK *B. hyodysenteriae* isolates sequenced for this study were obtained from submissions to APHA between 2005 and 2013, except P18A which was a historical UK strain isolated from a pig with swine dysentery in the late 1970s (Lemcke and Burrows, 1981; **Table 1**). The genome properties of these isolates, including genome size, GC%, and number of predicted coding sequences were similar to the reference strain WA1 (Bellgard et al., 2009) and other published *B. hyodysenteriae* genomes (Black et al., 2015; La et al., 2016b; De Luca et al., 2018; Table S1). As only 43 *B. hyodysenteriae* genomes have been published to date, the *B. hyodysenteriae* MLST scheme (La et al., 2009) was used to place these UK isolates into a global context. Each UK isolate was assigned to one of eight sequence types (ST) of which two were new variants not represented in the MLST database (https://pubmlst.org/brachyspira/), five had previously been identified in the UK and/or other European countries (**Figure 1**) and the historical strain P18A (1970s) was ST4 which has been previously described in the UK (NX; 2010s) and Canada (FMV89.3323; 1990s). The Australian isolate WA100 (2010s) was also ST4 by genome analysis, but in the MLST database

[2]Seemann, T., Goncalves da Silva, A., Bulach, D. M., Schultz, M. B., Kwong, J. C., Howden, B. P. (San Francisco: Github). Available online at: https://github.com/tseemann/nullarbor [Accessed: August 03 Aug 2016].

(La et al., 2009) is classed as ST130 due to a difference in one allele. Importantly, none of the 33 contemporary UK isolates (2005–2013) had STs associated with regions outside Europe, such as North America, Asia and Australia, possibly reflecting pig trading relationships.

A maximum likelihood phylogenetic tree was constructed using core genome SNPs from the WGS of the 34 UK isolates and 43 published *B. hyodysenteriae* genomes (Bellgard et al., 2009; Black et al., 2015; La et al., 2016b; De Luca et al., 2018; **Figure 1**). Most UK isolates from this study ($n = 32$) formed a sub-cluster that also contained isolates from Germany and the Canadian ST4 isolate. The two UK isolates that did not fall into this group (BH7 and BH35) formed a separate sub-cluster containing a German, a Canadian and a previously sequenced UK isolate. A number of UK and German isolates had considerable diversity in their core genome and formed a distinct sub-cluster (**Figure 1**). The most distantly related isolates were BH23 from the UK and the weakly haemolytic German isolates JR11-13 (La et al., 2016b). Interestingly, BH23 also had a weak haemolysis phenotype in culture and was isolated from an apparently healthy animal that showed no clinical signs of swine dysentery.

The phylogenetic tree further showed that, while there is considerable diversity in the *B. hyodysenteriae* core genome, the core genome of individual clones remained very stable over prolonged periods of times as demonstrated by the relatively low numbers of SNPs between isolates collected at different times from the same holdings, such as the isolates from Holding A (≤69 SNPs). The diversity and stability of the *B. hyodysenteriae* genome has been noted previously (Black et al., 2015) but these data provide new insight in the farm environment. The phylogenetic tree also gave greater resolution than MLST into the molecular epidemiological investigation of disease episodes at different holdings and identified, for example, three distinct sub-clades from three different holdings within the ST88 branch (**Figure 1**), including holdings A and B which had known epidemiological links (Strugnell et al., 2013). It is also interesting to note the high degree of core genome conservation in ST4 isolates from three continents collected in different decades, particularly as Australia banned imports of live pigs in the mid-1980s (La et al., 2016a).

Reduced Antibiotic Susceptibility Can Be Predicted From Genotype

The susceptibility of the 34 field strains and 3 ATCC strains was determined by broth dilution (Karlsson et al., 2003) for tiamulin and valnemulin (**Table 1**) and for tylsoin, tylvalosin, lincomycin and doxycycline (Table S2). For each antibiotic, an isolate was defined as having reduced susceptibility if the MIC exceeded the environmental cut-off (ECOFF) value (Pringle et al., 2012). The WGS of each isolate was examined for mutations in the 16S rRNA, 23S rRNA, and *rplC* genes associated with resistance to these antibiotics (**Table 1**). Reduced susceptibility to tylosin, lincomycin, tylvalosin, and doxycycline (i.e., antimicrobial phenotype) correlated well with the presence of relevant mutant SNPs (i.e., genotype), giving good (≥80%) sensitivity, specificity, positive predictive values, and negative predictive values, as calculated using two-by-two table analysis (Table S3) (Mackinnon, 2000), in accordance with previous studies (Karlsson et al., 1999, 2003; Pringle et al., 2007; Hidalgo et al., 2011; Alvarez-Ordóñez et al., 2013; Mahu et al., 2017; De Luca et al., 2018). A new polymorphism (G1058T) associated with reduced doxycycline susceptibility was identified in the 16S rRNA gene of isolates BH6 and BH37.

TABLE 2 | Reduced tiamulin and valnemulin susceptibility predicted by genome sequence based on the presence of mutations in chromosomal genes or the presence of *tva*(A).

	Tiamulin		Valnemulin	
	Chromosomal SNPs	*tva*(A)	Chromosomal SNPs	*tva*(A)
Sensitivity	77%	**95%**	68%	**100%**
Specificity	**87%**	73%	**83%**	**100%**
Positive predictive value	**89%**	**84%**	**89%**	**100%**
Negative predictive value	72%	**92%**	56%	**100%**
True positive	17	21	17	25
True negative	13	11	10	12
False positive	2	4	2	0
False negative	5	1	8	0

*Isolate genotypes and susceptibilities described in **Table 1**. Specificity, sensitivity, and the predictive value of a positive and negative test were calculated using two-by-two table analysis (Mackinnon, 2000) with the following criteria: SNP/tva(A) presence and MIC > ECOFF value were true positive (TP), wild type SNP and MIC ≤ ECOFF value were true negative (TN), SNP/tva(A) presence but MIC ≤ ECOFF value were false positive (FP), and wild type SNP but MIC > ECOFF value were false negative (FN). Values ≥ 80% are indicated in bold font.*

Correspondence between SNPs and reduced susceptibility to tiamulin and valnemulin was poorer, largely due to the greater number of isolates with reduced susceptibility but no mutation (**Table 2**), a phenomenon previously noted by others (Pringle et al., 2004; Hidalgo et al., 2011; Hillen et al., 2014; Mahu et al., 2017). To identify new mutations potentially associated with reduced pleuromutilin susceptibility we examined genes encoding the 50S ribosomal proteins L2, L4, and L22 for amino acid substitutions, as they have a possible role in pleuromutilin resistance (Hillen et al., 2014). There was no variation in the L4 amino acid sequence and L22 was also highly conserved. The predicted amino acid sequence of the L2 protein was identical in all but one isolate: BH23 which had a T50N substitution at a conserved threonine residue and a tiamulin MIC of 2 mg/L (**Table 1**).

Identification of a New Pleuromutilin Resistance Gene

We next employed a genome-wide association study to search for genes associated with reduced pleuromutilin susceptibility and identified one gene significantly associated with isolates having reduced valnemulin susceptibility ($p < 0.000003$ after Bonferroni correction for multiple tests). Two-by-two table analysis using this gene as a predictor of reduced valnemulin susceptibility gave 100% sensitivity and specificity (**Table 2**). This

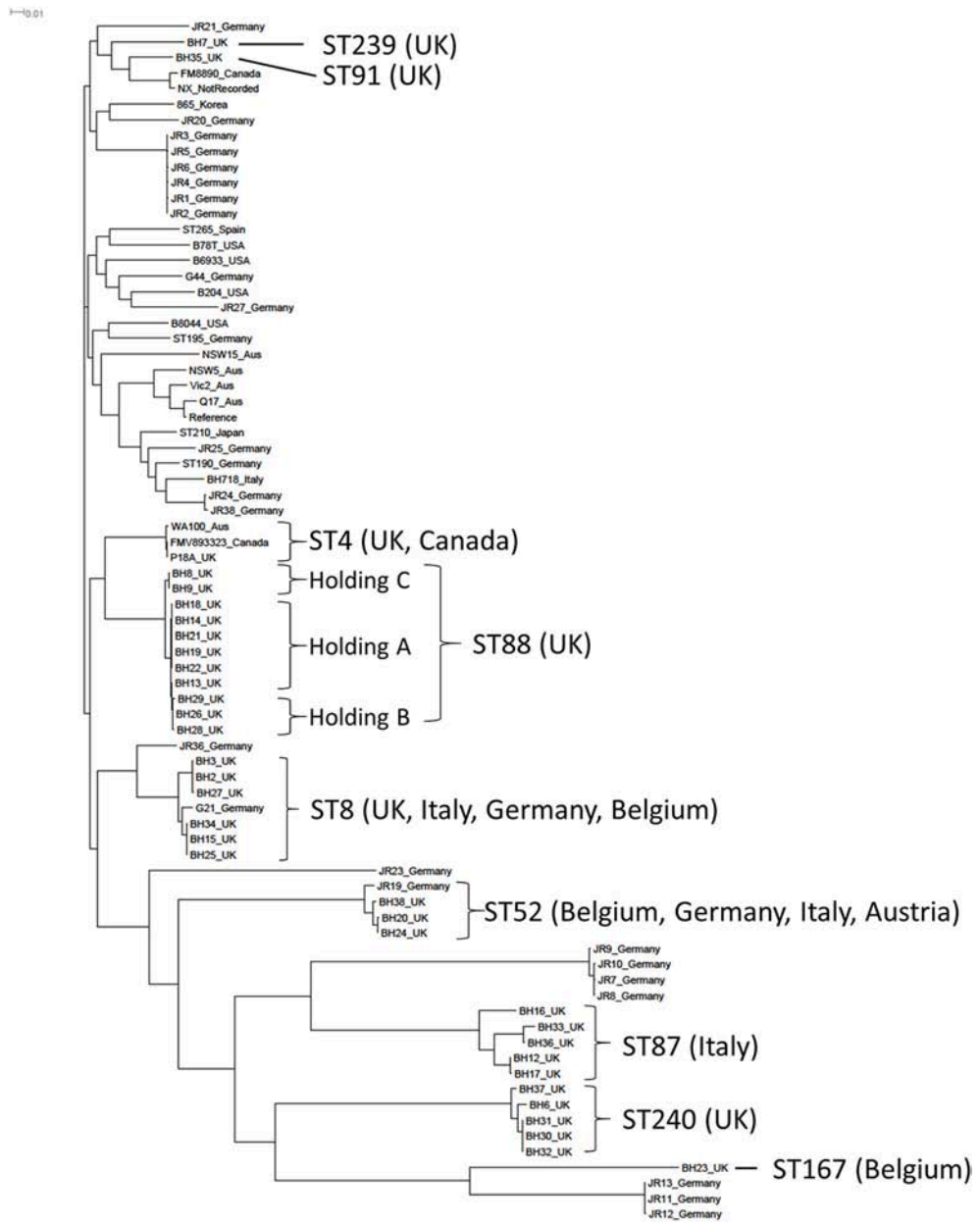

FIGURE 1 | A phylogenetic construction of *Brachyspira hyodysenteriae* isolates using a maximum-likelihood tree. The 34 field isolates sequenced in this project have been included together with 43 published *B. hyodysenteriae* genomes. The Sequence Type (ST) of the 34 isolates sequenced in this study together with countries in which these STs have been described previously is shown.

gene was also identified when examining reduced susceptibility to tiamulin but the association was not significant ($p < 0.0606$ after Bonferroni correction). However using the gene as a predictor of reduced tiamulin susceptibility gave an improved sensitivity and negative predictive value compared to SNPs only (**Table 2**); the lower specificity arose because four isolates with a tiamulin MIC at the ECOFF value carried this gene (**Table 1**). One isolate with reduced tiamulin susceptibility (BH16) did not possess this gene although it did carry three mutations in ribosome-associated genes associated with reduced pleuromutilin susceptibility (**Table 1**). Of the 14 isolates which had a tiamulin MIC > 2 mg/L, and thus meeting criteria proposed for clinical resistance (Duinhof et al., 2008; Swedres-Svarm, 2015), 12 (86%) carried both the newly identified gene and one or more SNPs associated with reduced pleuromutilin susceptibility (**Table 1**).

The newly identified 1,518 bp gene encoded a highly conserved 505 amino acid polypeptide in which Pfam analysis

(Finn et al., 2016) identified two regions with strong similarity to ABC transporter domains (*E*-values ≤ 3e-10), each containing a Walker A, Walker B, and ABC signature motif, but no transmembrane domain (Figure S1). This structure is found in ATP-binding cassette (ABC) proteins of the ABC-F subfamily (Kerr et al., 2005; Wilson, 2016). Antibiotic resistance ABC-F proteins act as ribosome protection proteins (Sharkey et al., 2016) and have been described in Gram positive bacteria, falling into three main groups according to the resistance phenotypes they confer (Kerr et al., 2005; Sharkey et al., 2016). The newly identified ABC-F gene had an overall amino acid identity of <23% to proteins from these groups and was distantly related in a phylogenetic tree (Figure S2). The new gene was also present in 10 published *B. hyodysenteriae* genomes, but only the Italian isolate BH718 has published pleuromutilin susceptibilities, having tiamulin and valnemulin MICs above the ECOFF (De Luca et al., 2018). Furthermore, a closely related gene (86% amino acid identity) was identified in the *Brachyspira pilosicoli* isolates WesB and B2904 (Figure S2). We have named the *B. hyodysenteriae* ABC-F gene *tva*(A) (*t*iamulin *v*alnemulin *a*ntibiotic resistance) and the *B. pilosicoli* variant *tva*(B). Pleuromutilins target the ribosomal peptidyl transferase centre (Long et al., 2006) and we therefore propose that *tva*(A) may reduce susceptibility to these antibiotics by acting as an ABC-F ribosome protection protein. In future the cloning and overexpression of *tva*(A) in a heterologous system, such as *Escherichia coli*, can be undertaken to examine this further.

Resistance mediated by ABC-F proteins in Gram-positive bacteria is often transferable as the genes can reside on mobilisable plasmids. Analysis of the nucleotide region surrounding *tva*(A) for all isolates indicated that it was located on the chromosome and not on the only plasmid present in *B. hyodysenteriae*. Furthermore, the synteny of *tva*(A) was identical in every isolate, being invariably placed between a cell division protein (WA1 locus ID RS04455) and an operon containing an oxidoreductase (RS04460) and an efflux pump of the multi-drug and toxic compound extrusion (MATE) family (RS04465), as shown for two isolates in **Figure 2**. Although the synteny of *tva*(B) within the two *B. pilosicoli* genomes was identical they had no similarity to *B. hyodysenteriae* synteny (**Figure 2**). In contrast to the lincomycin resistance gene *lnu*(C) recently reported in *B. hyodysenteriae* (De Luca et al., 2018), no transposon and/or insertion element sequences were identified in the vicinity of *tva*(A). However sequence alignment identified highly conserved motifs upstream and downstream of *tva*(A), absent in isolates without *tva*(A). For example, an AC dinucleotide motif flanked *tva*(A) (Figure S3), which may have been duplicated following insertion and with the subsequent loss of the transposon or insertion sequence, as has recently been described for *mcr-1* in *Moraxella* spp. (AbuOun et al., 2017). Furthermore an inverted repeat flanked *tva*(A) and may indicate a site of recombination (Figure S3). However at present there is insufficient evidence to unambiguously conclude that *tva*(A) is mobilisable but it is interesting to note that the *tva*(A) GC content was not greatly different to the *B. hyodysenteriae* average (27.5% vs. 29.5%).

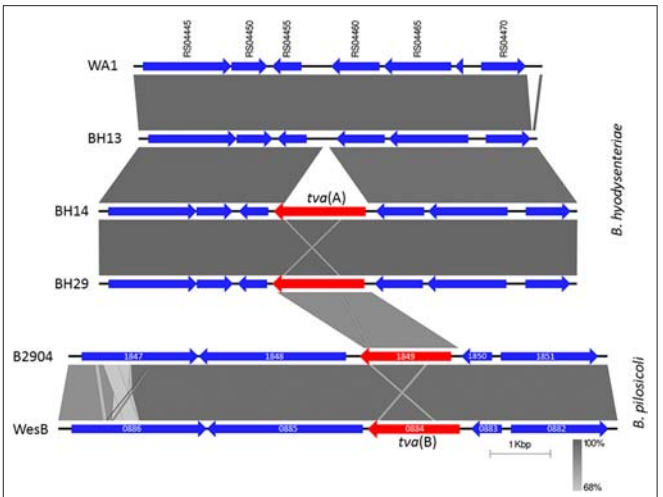

FIGURE 2 | Chromosomal arrangement of genes in *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* surrounding *tva*(A) and *tva*(B) respectively. *B. hyodysenteriae* genes labelled according to the locus tag in the reference strain WA1 (Accession number NC_012225); *tva*(A) is not present in WA1 and therefore has no locus tag. Genes have been coloured to indicate *tva*(A) and *tva*(B) in red and other genes in blue. WA1 and BH13 were tiamulin susceptible (MIC ≤ 0.063 mg/L). Field isolates BH14 and BH29 were recovered from different holdings, harboured *tva*(A) and had intermediate tiamulin MICs (0.5 mg/L). Also shown is the region surrounding *tva*(B) from *B. pilosicoli* isolates B2904 (Accession number CP003490; locus tag B2904_orf1849) and WesB (Accession number HE793032; locus tag WESB_0884); other genes labelled according to their locus tags. Regions of homology between isolates are shown by grey shading. Image generated using EasyFig (Sullivan et al., 2011).

The Dynamics of Tiamulin Resistance Development *in Vitro*

We next examined the development of tiamulin resistance and the significance of *tva*(A) using *in vitro* studies. In one set of experiments 18 isolates were cultured at inhibitory tiamulin concentrations: the MIC as determined by broth dilution and three doubling concentrations above this (Table S4). This approach allowed us to investigate the tiamulin MPC and define the mutant selection window (MSW), which lies between the MIC and the MPC and is the concentration range at which resistant mutants may arise (Drlica and Zhao, 2007). No mutants were observed with the six isolates which did not carry *tva*(A) (Table S4). Fifteen mutants were recovered from five of the six isolates tested which carried *tva*(A) and had a tiamulin MIC between 0.25 and 1 mg/L (Table S4). Of the five tiamulin resistant isolates tested, all harbouring *tva*(A), one mutant was recovered from BH38 (Table S4), the only resistant isolate tested in which no resistance mutations in ribosome-associated genes were identified (**Table 1**). For 17/18 isolates tested, the MPC was within the tiamulin concentration range used and less than three doublings above the MIC; two mutants were recovered from BH29 at the highest tiamulin concentration used for this isolate (Table S4). All isolates that did not harbour *tva*(A) had MPCs which did not exceed 0.5 mg/L and the selection index (MPC:MIC ratio) was 1, whereas isolates harbouring *tva*(A), and with a tiamulin MIC ≤ 2 mg/L, had MPCs from 0.5 to

at least 8 mg/L and a selection index of 1–8 (Table S4). These results indicate that *tva*(A) raises the MPC and widens the MSW. Mutants were only recovered from isolates harbouring *tva(A)* and the geometric mean of mutation frequency was 1.88×10^{-8}, similar to mutation rates reported for *E. coli* and *S. enterica* exposed to quinolones (Randall et al., 2004; Ozawa and Asai, 2013).

Ten of the 16 mutants were recovered from one isolate (BH20) which we termed as "hypermutable" due to the large numbers of mutants it generated in comparison to other isolates. All purified mutants showed an increase in tiamulin and valnemulin MICs compared to their parent isolate (Table S5) but no increased MICs for tylosin, lincomycin, tylvalosin, and doxycycline. Analysis of the WGS of mutants showed that each had between one and nine new SNPs, absent in the parent isolate, with 10 isolates having only one new SNP (Table S5). Twelve mutants had a SNP in the 23S rRNA gene, of which nine were at positions previously associated with tiamulin resistance (G2032A, C2055T, and C2499T) and three were at new positions (G577A, G1846T, and C1902T), as detailed in Table S5. The polymorphisms at C2055 and C2499 are new mutations associated with tiamulin resistance, as only adenine substitutions at these positions have been reported previously (Pringle et al., 2004). Two mutants had a SNP in *rplC*, one resulting in a S149I amino acid change in the L3 ribosomal protein (described previously Pringle et al., 2004) and the other gave a new amino acid change (S149R). Another mutant had a SNP in the *fusA* gene, encoding Elongation Factor-G (EF-G), which resulted in an A261V substitution at the conserved alanine residue in the G5 box. One mutant isolate had no SNPs in ribosome-associated genes but had a single SNP in a gene encoding ribose-phosphate pyrophosphokinase that resulted in an amino acid substitution (Table S5), but the role of this enzyme in tiamulin resistance is unknown. SNPs not associated with the ribosome were also identified in six other mutants, with most (16/21) located in non-coding regions (Table S5).

In a separate *in vitro* screen for tiamulin resistant mutants, 10 isolates were repeatedly sub-cultured in the presence of sub-inhibitory concentrations of tiamulin for up to 70 sub-cultures, with concentrations increased during this period as growth became rich, similar to earlier studies (Karlsson et al., 1999; Pringle et al., 2004). Five of these isolates were also repeatedly sub-cultured in the absence of tiamulin for the same time period to determine any baseline changes that may occur. Isolates were collected after 30, 45, 60, and/or 70 sub-cultures and tested by broth dilution to determine changes that may have occurred in antibiotic susceptibilities (Table S6). The tiamulin resistant isolate BH30 showed no significant alteration in tiamulin susceptibility after 60 sub-cultures in either the presence or absence of the antibiotic and, following WGS, SNPs were detected in the tiamulin exposed and non-exposed BH30 sub-cultures but none were present in ribosome-associated genes (Table S6). The remaining nine isolates exposed to tiamulin showed from 2 to 5 two-fold increases in tiamulin MIC (Table S6) and a concomitant increase in valnemulin MIC but no alteration in susceptibilities to tylosin, tylvalosin, doxycycline, and lincomycin. Interestingly, for isolates without *tva*(A) (initial MIC $\leq$ 0.25 mg/L) the MIC post-tiamulin exposure did not exceed 2 mg/L, whereas for isolates harbouring *tva*(A) (initial MIC 0.25–1 mg/L) MICs post-exposure were >2 mg/L, indicating resistance to tiamulin. Genome sequencing showed that 8/9 of these tiamulin-exposed isolates had SNPs in ribosome-associated genes, which were absent in the parent isolate (Table S6). Six isolates had mutations in the 23S rRNA, four at previously described positions (G2032A, C2055T, and G2447T), one at a position also identified in the MPC experiment (G577T) and another at a new position (C2179T). Two isolates (BH15 and BH37) acquired non-synonymous SNPs in the *fusA* gene, which together with the *fusA* mutant from the MPC experiment, provide the first evidence for an association of EF-G with reduced pleuromutilin resistance. EF-G acts during the translocation step of the elongation cycle of bacterial protein synthesis, the step immediately following the peptide-bond formation step which is inhibited by pleuromutilins (Wilson, 2014). Mutations in *fusA* of *Staphylococcus* spp. confer resistance to fusidic acid, an antibiotic that inhibits translocation (Farrell et al., 2011). BH37 additionally acquired a SNP in *rplC* causing as S149I substitution in the L3 protein. The newly identified mutations were not present in any of the UK field isolates analysed or published *B. hyodysenteriae* genomes.

Importantly, sub-culture of the susceptible isolates B78T, BH13, and BH20 in the absence of tiamulin did not alter tiamulin or valnemulin MICs or give rise to mutations in ribosome-associated genes (Table S6). However, for BH14 there was an increase in pleuromutilin MICs both with and without exposure to tiamulin, although no SNPs were identified in ribosome-associated genes in sub-cultured strains from either group, which requires further investigation, as it may represent a subset of strains that can become "naturally" resistant to tiamulin without any exposure possibly through de-repression or up-regulation of some key regulatory genes.

The Role of *tva*(A) in the Development of Pleuromutilin Resistance Development

The results provided by the two *in vitro* experiments provide the basis of a hypothesis describing how resistance to pleuromutilin antibiotics develops in *B. hyodysenteriae*, which is summarised in **Table 3**. The hypothesis derives from the observation that isolates which did not carry *tva*(A) were generally susceptible to tiamulin, and no mutants were recovered from these isolates when exposed to inhibitory tiamulin concentrations. Furthermore, although isolates without *tva*(A) could acquire resistance mutations and consequently reduced pleuromutilin susceptibility following repeated exposure to sub-inhibitory tiamulin concentrations, they did not develop clinical resistance despite prolonged exposure to high tiamulin concentrations (e.g., discs containing 1,000 μg tiamulin). In contrast, generally all isolates with MICs between the ECOFF value and the clinical breakpoint carried *tva*(A); as did four isolates with a tiamulin MIC at the ECOFF (**Table 1**). Clinically resistant mutants were recovered from 5/6 *tva*(A) isolates following exposure to inhibitory tiamulin

TABLE 3 | Hypothesis for pleuromutilin resistance development in *B. hyodysenteriae*.

Tiamulin resistance phenotype		Resistance genotype		Tiamulin inhibitory concentration (Single exposure)	Tiamulin sub-inhibitory concentration (Repeated exposure)	No antibiotic (Repeated exposure)
Category	**MIC (mg/L)**	**Chromosomal SNPs**	***tva*(A)**			
Susceptible	≤0.25	No	No[a]	No change *(remains susceptible)*	**Becomes intermediate *(not resistant)***	No change *(remains susceptible)*
Intermediate	>0.25 to ≤2	No	Yes[b]	**Can become resistant**	**Becomes resistant**	No change *(remains intermediate)*
Resistant	>2	Yes[c]	Yes	No change *(remains resistant)*	No change *(remains resistant)*	No change *(remains resistant)*

Susceptible isolates have tiamulin MICs equal to or less than the ECOFF value (Pringle et al., 2012), resistant isolates exceed the proposed clinical breakpoint (Duinhof et al., 2008; Swedres-Svarm, 2015), and intermediate isolates reside in between.

[a] *Four susceptible isolates had an MIC of 0.25 mg/L and harboured tva(A).*

[b] *One intermediate isolate had an MIC of 0.5 mg/L, did not carry tva(A) but did harbour chromosomal SNPs associated with resistance.*

[c] *Two resistant isolates did not harbour known chromosomal SNPs associated with resistance.*

concentrations and from the six isolates repeatedly exposed to sub-inhibitory tiamulin concentrations. Therefore our results indicate that *tva*(A) is critical for the development of clinical pleuromutilin resistance and most highly resistant isolates harboured both *tva(A)* and mutations in ribosome-associated genes. Further support is provided by the fact that the same mutation in the 23S rRNA gene increased the tiamulin MIC of an isolate without *tva*(A) to an intermediate level (>0.25 to ≤2 mg/L) whereas an isolate with *tva*(A) harbouring these mutations became highly resistant (>2 mg/L); e.g., compare isolates BH13 and BH20 in Table S6.

The development of resistance observed *in vitro* during sustained exposure to tiamulin was mirrored *in vivo*, as shown in a longitudinal molecular epidemiological examination of two episodes of swine dysentery at the same farm (Holding A). The same clone was found to be responsible for the two disease episodes, which were separated by 1 year (**Figure 1**; **Table 1**). The first episode was treated with tiamulin and the initial isolate (BH13) was susceptible to tiamulin and did not harbour *tva*(A). Isolate BH14, obtained 2 months later during the first episode, had a raised tiamulin MIC of 0.5 mg/L, had only 69 SNPs difference to BH13 in the core genome but now carried *tva*(A). Four isolates recovered a year later (BH18, BH19, BH21, and BH22), during the second disease episode, were clinically resistant and retained *tva*(A) but now carried 1–4 new SNPs not present in BH14, including a G2032A mutation in the 23S rRNA gene (**Table 1**).

We therefore propose that *tva*(A) confers reduced pleuromutilin susceptibility in *B. hyodysenteriae* that does not lead to clinical resistance but facilitates the development of higher-level resistance via mutations in ribosome-associated genes. This proposed mechanism of resistance development to pleuromutilins aligns with and refines the stepwise manner proposed previously (Karlsson et al., 2001; Hidalgo et al., 2011; van Duijkeren et al., 2014). It is similar to that reported for plasmid-mediated quinolone resistance genes in several species (Jacoby et al., 2014), and likely explains reported contradictions regarding the capability of particular mutations to confer tiamulin resistance.

Evidence to Inform Swine Dysentery Control On-Farm

The data we present on the tiamulin MSW and MPC for *B. hyodysenteriae* can also help inform measures designed to prevent the development of resistance on-farm. In the UK, the authorised dosage for tiamulin products for pigs provides for two treatment regimens: high doses at an inclusion level of 100–200 ppm (5–10 mg/kg bodyweight) in feed for 7–10 days to treat clinical swine dysentery caused by *B. hyodysenteriae* and a lower dosage at an inclusion level of 40 ppm (2 mg/kg bodyweight) in feed for 2–4 weeks for the metaphylaxis of swine dysentery (https://www.vmd.defra.gov.uk/ProductInformationDatabase/). Similar regimes are employed in other jurisdictions. There are limited data available for the pharmacokinetics and pharmacodynamics of tiamulin in pigs, but one report presents estimated colon contents concentrations (CCC) for tiamulin following treatment at doses of 38.5 ppm (CCC <1.98 mg/L), 110 ppm (CCC 2.84 mg/L), and 220 ppm (CCC 8.05 mg/L) in feed for 14 days (Burch and Hammer, 2013). Comparison of CCC to the MSW and MPC defined in this work shows that for isolates without *tva*(A), the MPC was exceeded by the CCC obtained at all three doses. Thus either treatment regimen could be expected to deliver sufficient antibiotic to treat infection and prevent emergence of resistance. However, in *tva*(A) positive isolates, the MSW was expanded and the MPC was higher than the CCC obtained with doses at 38.5 ppm and also at 110 ppm for some isolates tested. Thus isolates harbouring *tva*(A) have the potential to acquire high level resistance under these treatment regimens, whereas the higher therapeutic dose should limit development of clinical resistance. Therefore it would be valuable

to establish whether the *tva*(A) gene is present or absent in the *B. hyodysenteriae* infecting the pigs. This is particularly important when tiamulin is used at the metaphylactic dose as this would provide an extended opportunity for *B. hyodysenteriae* harbouring *tva*(A) to remain within the MSW, increasing the potential for clinical resistance to develop. Regular determination of isolate susceptibility on farms using tiamulin for metaphylaxis is recommended, particularly if *tva*(A) was present. Use of different licensed antimicrobials presents another option if the *tva*(A) gene is detected, however resistance to these can be common. Increasing the metaphylactic dose to the treatment dose level might limit development of resistance but would require a change in authorisation and further research on issues such as animal safety, environmental impact and other aspects relating to tiamulin use.

The existence and potential mobilisation of *tva*(A) may also prove relevant to human clinical medicine due to the sustained interest in the use of pleuromutilins to treat human bacterial infections; retapamulin was approved for topical use in the USA in 2007 and lefamulin, highly active against multidrug resistant *S. pneumoniae* and *S. aureus*, was recently reported as being in phase III development for systemic use (Eyal et al., 2016).

In conclusion this work has provided new insights into the diversity of *B. hyodysenteriae* genomes, an important aetiological agent for swine dysentery, and demonstrated the utility of WGS approaches for the molecular epidemiological investigation of disease episodes. Reduced antibiotic susceptibility can be confidently predicted from genome sequences and we have described an expanded repertoire of genes and SNPs associated with pleuromutilin resistance. Indeed, the identification of *tva*(A) gives a deeper understanding of the development of resistance to pleuromutilins and provides evidence-based science that can be practically applied on-farm to assist efforts to reduce the development of resistance to this class of highly important veterinary antimicrobial agents.

AUTHOR CONTRIBUTIONS

MFA, CT, SW: Funding acquisition. MFA, CT, SW, BS, RC: Project conceptualization. RC, JR, ES, RE, MA, JN-G: Investigation, methodology, and data curation. RC, JN, MA, ES: Data analysis and software. RC: Preparation of draft manuscript. All authors reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01183/full#supplementary-material

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Tetracycline and Sulfonamide Antibiotic Resistance Genes in Soils From Nebraska Organic Farming Operations

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There is widespread agreement that agricultural antibiotic resistance should be reduced, however, it is unclear from the available literature what an appropriate target for reduction would be. Organic farms provide a unique opportunity to disentangle questions of agricultural antibiotic drug use from questions of antibiotic resistance in the soil. In this study, soil was collected from 12 certified organic farms in Nebraska, evaluated for the presence of tetracycline and sulfonamide resistance genes (n = 15 targets), and correlated to soil physical, chemical, and biological parameters. Tetracycline and sulfonamide antibiotic resistance genes (ARGs) were found in soils from all 12 farms, and 182 of the 196 soil samples (93%). The most frequently detected gene was tetG (55% of samples), followed by *tet*(Q) (49%), *tet*(S) (46%), *tet*(X) (30%), and *tetA*(P) (29%). Soil was collected from two depths. No differences in ARGs were observed based on soil depth. Positive correlations were noted between ARG presence and soil electrical conductivity, and concentrations of Ca, Na, and Mehlich-3 phosphorus. Data from this study point to possible relationships between selected soil properties and individual tetracycline resistance genes, including *tet*(O) which is a common target for environmental samples. We compared organic farm results to previously published data from prairie soils and found significant differences in detection frequency for 12 genes, eight of which were more commonly detected in prairie soils. Of interest, when tetracycline ARG results were sorted by gene mechanism, the efflux genes were generally present in higher frequency in the prairie soils, while the ribosomal protection and enzymatic genes were more frequently detected in organic farm soils, suggesting a possible ecological role for specific tetracycline resistance mechanisms. By comparing soil from organic farms with prairie soils, we can start to determine baseline effects of low-chemical input agricultural production practices on multiple measures of resistance.

Keywords: soil, antibiotic resistance, antibiotic resistance gene, ARG, organic, farm, agriculture, environment

INTRODUCTION

The global emergence of antibiotic resistance has led to the immediate need to find ways to mitigate resistance in the environment. Agricultural antibiotic resistance is an issue that has gained national and international attention (Topp et al., 2017), and there is concern that resistance from cropland and livestock will be transferred through the environment and cause untreatable infectious disease in people and animals (Durso and Cook, 2014). In conventional food animal production, livestock, and poultry are commonly given antibiotics to treat and prevent illnesses (Du and Liu, 2012). However, research has indicated that only 10 to 20% of antibiotics administered are absorbed into animal tissue: the majority is excreted in manure (Ok et al., 2011). The presence of antibiotic residues in manure may lead to selection and proliferation of strains of antibiotic resistant bacteria (ARB); thus, manure from livestock facilities is a source of antibiotic drugs, ARB, and antibiotic resistance genes (ARGs) excreted into the environment (Binh et al., 2008; Heuer et al., 2011). There is widespread agreement and support for the idea that agricultural antibiotic resistance should be reduced, with an emphasis on reducing transfer of resistance from practices such as land application of animal manures (Heuer et al., 2011; Pruden et al., 2013; Marti et al., 2014), and spraying of antibiotics to control bacterial disease in fruit crops (Stockwell and Duffy, 2012). However, the details of a realistic reduction target are elusive. In order to develop effective methods to reduce resistance, it is important to first obtain baseline information on how basic agricultural practices are involved in resistance transfer. There remain many knowledge gaps surrounding the basic ecology of antibiotic resistance on farms and in fields, such as how variable is any particular measure of resistance within or between farms? And from a human and animal health standpoint, which types of resistance should be measured or tracked?

Organic farms provide a unique and valuable opportunity to disentangle questions of agricultural drug use from questions of antibiotic resistance. Since antibiotic drugs use is severely restricted in organic operations, these farms provide a natural starting place for assessing background and baseline levels of ARB and ARG in agricultural production settings (Rothrock et al., 2016b).

In Nebraska, over 90% of the land mass is devoted to agriculture, with cattle, corn, soybeans, hogs, and eggs being the top agricultural commodities, in order of value (Nebraska Department of Agriculture [NDA], 2017). In 2016, the USDA National Agricultural Statistics Service reported a total of 48,400 farm operations in Nebraska (National Agriculture Statistics Service [NASS], 2016). Of these, 267 were certified organic according to the USDA Agricultural Marketing Service's Organic Integrity Database (United States Department of Agriculture [USDA], 2017). A previous study characterized ARB/ARG in native Nebraskan prairie soils, providing a reference point for resistance in soils with minimal anthropogenic inputs (Durso et al., 2016); however, data on resistance in organic farm soils from this region are lacking. Here we assess prevalence and distribution of selected tetracycline and sulfonamide resistance genes in soil from 12 USDA certified organic farming operations in Nebraska. Resistance gene distributions were compared within and among different organic operations and at different soil sampling depths. In addition, this study explored relationships between ARGs and soil physical, chemical, and biological characteristics. There is some indication that soil nutrient levels may impact the prevalence of ARB/ARG (Udikovic-Kolic et al., 2014; Zhou et al., 2017); therefore, we hypothesize that relationships will be observed between ARG frequency and selected soil characteristics.

MATERIALS AND METHODS

Soil Collection and Analyses

Soil samples were collected from 12 certified organic farms in Nebraska. The crops grown are listed in **Table 1**. There were no animals on pasture at the time of collection. Information on whether or not manure had been used as a soil amendment within the last three years is provided in **Table 1**. A total of 98 soil cores (15.24 cm) were collected between May 22 and June 6, 2013. Aboveground residue and large roots were removed. Soil for microbiological analysis was collected using a gardener's trough which was cleaned following each sample, placed in polyethylene bags and immediately stored on ice for transport to the laboratory. Soil for chemical analysis and aggregate stability were collected using a spade. In total, 98 cores were collected from 12 farms. Samples were collected at two depths (0.0–7.6 cm and 7.6–15.2 cm), homogenized by hand-mixing of the bag, and stored at −80°C, resulting in a total of 196 soil samples that were evaluated for ARG targets. Soil analyses, including determination of coarse particular organic matter (CPOM), fine particulate organic matter (FPOM), microaggregates (MicAg), large and small macroaggregates (Lmac, Smac), pH, electrical conductivity (EC), and fatty acid profiles were performed as part of a separate study, using methods that have previously been described (Cambardella and Elliott, 1994; Drijber et al., 2000; Cambardella et al., 2001; Grigera et al., 2006). Chemical analyses were performed at Ward Laboratories, Kearney, NE, United States. Briefly, Nitrate-nitrogen was extracted using a Ca solution to flocculate soil clays, and analyzed using a cadmium reduction procedure, with a flow injection analyzer; phosphorus was extracted by the Mehlich P-3 test, using an extracting solution of 0.013 N HNO_3 and 0.015 N NH_4F; potassium was extracted using 1 N ammonium acetate, and analyzed with a flame emission mode of an atomic absorption spectrophotometer; sulfur was extracted using calcium phosphate, followed by barium sulfate turbidity determined by flow injection analysis; micronutrients were extracted with a chelated DTPA solution and Ca and Mg were extracted using an ammonium acetate solution an measured with an atomic absorption spectrophotometer.

Molecular Analyses

Isolation and purification of DNA from bulk soil samples (n = 196) was conducted with the DNeasy PowerSoil Kit (Qiagen Sciences Inc., Germantown, MD, United States) according to the manufacturer's protocol. A Bead Ruptor 24 homogenizer

TABLE 1 | Description of sample collection sites.

Farm	No. of cores	Crop at time of collection	Previous crop(s)	Recent manure
1	6	W. wheat	Soybeans, corn	Yes
2	4	Warm and cool perennial grasses	Warm and cool perennial grasses	Yes
3	6	Wheat, fallow, millet	Wheat, fallow, millet	Yes
4	2	Mix vegetables	Mix vegetables	No
5	11	Oats, corn, alfalfa, pasture mix*	Oats, corn, alfalfa, pasture mix*	Yes
6	7	Pasture, oats, w. wheat	Soy, pasture, oats, corn, w. wheat	Yes
7	10	Corn	Soybeans, corn	Yes
8	10	Soy, oats, alfalfa, corn, pasture	Soy, oats, alfalfa, corn, pasture	Yes
9	7	Pasture, oats, corn, sorghum, millet	Pasture, oats, corn, sorghum, millet	Yes
10	8	Soybeans	Corn	Yes
11	16	Popcorn, hay, pasture, soy, barley	Popcorn, hay, pasture, soy, barley	Yes
12	11	Wheat, soy, corn, alfalfa, pasture, oats	Wheat, soy, corn, alfalfa, pasture, oats	Yes

*Samples for each depth were formed in the field at the time of sampling, resulting in two soil samples location. A "yes" in the "recent manure" column means that some or all of the samples from that farm were collected from areas which had received manure within the last three years. Not all fields within a farm necessarily had "recent manure." *Pasture mix contains (pasture, oats, buck wheat, turnip, radish). "W. wheat" indicates winter wheat. "Soy" indicates soybean.*

(OMNI International, Kennesaw, GA, United States) was used for sample mixing during DNA isolation. Purified DNA was quantified using a NanoDrop3300 (ThermoFisher, Waltham, MA, United States), and used directly in the polymerase chain reactions (PCRs). All samples were subjected to the PCR for detection of 15 tetracycline and sulfonamide resistance genes (**Supplementary Table S1**), resulting in 2,940 total PCR assays performed. There are 29 genes known to code for resistance to tetracyclines (Roberts, 2005), and four genes known to code for resistance to sulfonamide (Razavi et al., 2017). We chose a subset of the tetracycline resistance genes for which multiplex PCR reactions had previously been described (Ng et al., 2001). Since *sul*1 is one of the most frequently detected sulfonamide resistance genes (Phuong Hoa et al., 2008), and since it has been closely associated with class 1 integrons responsible for transfer of ARGs between bacteria, we chose *sul*1 for this study. The PCR reactions were performed as previously described for ARG in soils (Ng et al., 2001; Pei et al., 2006; Durso et al., 2016). In brief, thermocycling conditions were one cycle of 94°C for 2 min, followed by 30 cycles of denaturation at 94°C (60 s), annealing at primer-specific temperatures (see **Supplementary Table S1**) for 60 s, and extension at 72°C (90 s), with a 5-min final extension at 72°C for 5 min. Bands were visualized using Invitrogen SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA, United States) added directly to tris-acetate-EDTA 2% agarose gels, and documented using a UVP Gel Doc-It^{TS3} imaging system (UVP, LLC, Upland, CA, United States). Note that standard PCR assays can only report the presence or absence of the selected target, and do not provide information on the amount of the targets in the sample.

Data Analysis

The SAS GLM procedure was used to determine differences for each of the soil physical, chemical, and biological parameters between samples positive and negative for each ARG target (SAS Institute, 2008). Results are reported for both $P \leq 0.05$ and $P \leq 0.1$ probability levels. Significant correlations between number of positive ARG targets per sample and various soil parameters were identified at the ($P \leq 0.05$) level using Pearson correlation coefficients. The MEANS procedure was used to examine farm-level depth-based differences in soil parameters. Differences in the proportions of ARG between surface and deeper cores or between organic farms and prairies were determined using the TABLES statement in PROC FREQ and designating the CHISQ option (equivalent to a Z test for the equality of proportions). Antibiotic fingerprinting was performed as previously described by concatenating individual ARG target results (Durso et al., 2011). Individual ARG assay results were coded as 1 if the target was detected in the sample and 0 if the target was not detected in the sample. Then, these results were combined into a 14-digit binary "fingerprint" for each sample, and used for comparison purposes.

RESULTS

Tetracycline and/or sulfonamide resistance genes were found in soils collected from all 12 organic farms (100%) (**Supplementary Figure S1**), in 94 of 98 cores (96%) and in 178 of the 196 soil samples (91%). This study examined 15 ARG targets, and all but one [*tet*(C)] were found in at least one of the 196 soil samples (**Figure 1**). The most frequently detected genes at the farm level (n = 12 farms) were *tet*(G), *tetA*(P), and *tet*(Q) with 83%, 92%, and 100% of the farms positive for each of these targets, respectively (**Figure 1**). At the individual soil sample level (n = 196 samples), the most frequently detected genes were *tet*(G) (55% of samples), followed by *tet*(Q) (49%), *tet*(S) (46%), *tet*(X) (30%), and *tetA*(P) (29%) (**Figure 1**). Most of the samples (91%) were positive for at least one of the 15 targets, and 82% were positive for two or more of the tested ARGs. The number of positive ARG targets (n = 15 total) ranged from 3 to 11 at any single farm, and from 0 to 8 in any single soil sample. The distribution of multi-gene detection at the farm, core, and sample level is displayed in (**Supplementary Figure S2**).

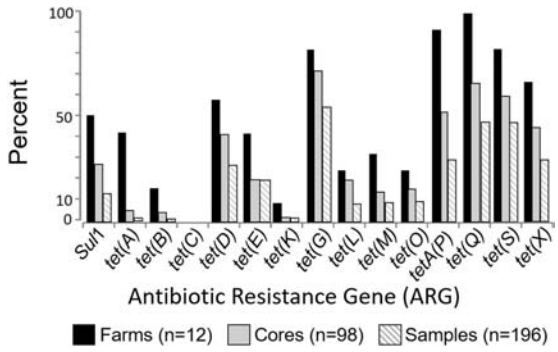

FIGURE 1 | Farm, core, and sample level prevalence of selected antibiotic resistance genes.

Each of the 98 cores was split into 0.0–7.6 cm and 7.6–15.2 cm depths. Although some of the soil physical, chemical, and biological properties differed with depth (**Table 2**), significant depth-based differences were generally not observed for individual resistance genes. The only ARG for which a difference was observed was *tet*(L). When the depth-based data was analyzed at the core level by farm, *tet*(L) was detected more frequently ($P = 0.025$) in the surface soils (0–7.1 cm) compared to soils from the lower depth (7.1–15.2 cm). Since major depth-based differences were not observed, further analysis of ARGs and soil parameters were performed only on the surface samples.

For each tetracycline resistance gene, mean values for soil parameters in the upper surface cores (0–7.1 cm) were compared for the samples that were positive vs negative for each ARG (**Table 3**). **Table 3** reports that 86 out of 476 gene-by-soil-parameter analyses were statistically significant. This analysis examined mean values for each soil parameter in the ARG positive samples, compared to the mean values in the ARG negative samples. Examining this set of data for those results likely to be biologically significant, four stand out because they were significant for four or more genes which trend positively for that soil parameter. EC, Ca, Na, and Mehlich-3 phosphorus (MehP) values were all consistently higher in the ARG positive soils. These four parameters are related to each other and together influence EC. In addition to having higher mean values for the positive ARG soils, these four measures (EC, Ca, Na, and MehP) were also positively correlated with the total number of ARG-positive targets (**Supplementary Table S2**). The relationships between number of detected resistance genes and soil physical and chemical parameters were examined using Pearson Correlation Coefficients (**Supplementary Table S2**). Significant differences ($P < 0.05$) or tendencies to differ ($P < 0.1$) were observed. The proportion of positive samples that were and were not exposed to manure within three years of collection are described in **Supplementary Table S3**, with statistically significant increases of *sulI*, *tet*(G), and *tet*(O) in the manured plots, and *tet*(D) in the non-manured plots.

Examining which gene targets had similar results for individual soil parameters (**Table 3**), EC and Ca had significantly higher mean values in samples where *tet*(B) and *tetA*(P) were detected. The Na and MehP values were higher in soil samples where *tet*(B), *tet*(L), *tet*(M), *tet*(O), and *tet*(S) were detected. Organic carbon, soil organic matter, and organic nitrogen measurements tended to have lower mean values in soils positive for *tet*(G) but were greater in soils positive for *tet*(B), *tet*(O), and *tet*(Q). The *tet*(B) gene appears at first glance to be most frequently associated with non-random changes in soil properties in positive compared to negative soils, however, note that there are only three positive samples in this group, so it is unlikely that there is any biological significance to these numbers (**Table 3**).

For each sample, each ARG is coded as detected = 1 or not detected = 0. These values are concatenated (i.e., linked together in a series) to create an ARG profile or fingerprint (Durso et al., 2011), serving as a molecular antibiogram. The ARG diversity profiles of the 12 farms sampled is presented in **Figure 2**. On average, 72% of the profiles were unique for each farm, with a range between 43% and 100% of profiles from each farm found exclusively in that farm With the exception of Farm 11, the majority of the samples within each farm had a unique ARG profile (range 0.43–1.00, mean 0.72, median 0.74), where a value of 1.0 indicates that every sample had a unique profile.

Using the SAS two sample test of equality of proportions (SAS Institute, 2008), we compared frequency of detection of targets from the current set of certified organic farm soils with results from a previously published set of native Nebraskan prairie soils (Durso et al., 2016). Significant differences were seen in the frequency of detection from certified organic farms compared to native prairie soils for 12 of 15 targets at the farm level (**Table 4**).

DISCUSSION

Organic farms present a unique opportunity to determine impacts of agriculture on antibiotic resistance in soil, without the routine antibiotic drug inputs associated with conventional production practices. Soils from 12 USDA certified organic farms in Nebraska were probed for the presence of tetracycline and sulfonamide resistance genes. All farms were positive for at least three, and up to 12 of the 15 assayed genes, demonstrating that ARGs are common in agricultural soils, even in the absence of routine antibiotic drug or pesticide use. These data support other work done in organic farming operations examining ARGs in organic cattle, swine, and poultry production (Stanton et al., 2011; Rothrock et al., 2016a; Sancheza et al., 2016), where ARGs were also detected even when antibiotic drugs were not administered to animals. It was not surprising to detect sulfonamide and/or tetracycline ARGs at every farm sampled, as they occur naturally in soils, and have been detected in soils and water from around the globe, including ungrazed native prairie soils from the same region of Nebraska in which this study was conducted (D'Costa et al., 2006, 2007; Allen et al., 2010; Durso et al., 2012, 2016; Cytryn, 2013).

There is broad consensus that agricultural antibiotic resistance needs to be reduced, but little information is available to inform what a target level should be, and no consensus on which targets to measure. As part of identifying which targets

TABLE 2 | Mean soil measurements by depth.

Soil factor	Mean 0.0–7.6 cm	Mean 7.6–15.2 cm	*P*-value
Coarse particulate organic matter (g/kg soil)	0.44	0.26	<0.0001
Fine particulate organic matter (g/kg soil)	0.60	0.61	–
Organic nitrogen (g/kg soil)	0.21	0.17	<0.0001
Organic carbon (g/kg soil)	2.02	1.67	–
Carbon (g/kg soil)	9.78	9.60	–
Large macroaggregates (% soil wt)	15.28	16.53	–
Small macroaggregates (% soil wt)	38.01	38.47	–
Micro aggregates (% soil wt)	23.90	24.92	–
Total water saturation (C/kg soil)	77.18	79.92	–
pH (unitless)	7.02	6.68	–
Buffer pH* (unitless)	7.09	7.02	–
Electrical conductivity (dS/m)	0.39	0.35	<0.05
Soil organic matter (%)	3.30	2.83	<0.0001
Nitrate [NO_3] (mg/kg soil)	19.22	11.75	<0.05
Potassium [K] (mg/kg soil)	672.26	544.10	<0.05
Sulfur [S] (mg/kg)	14.18	12.87	<0.05
Zinc [Zn] (mg/kg soil)	3.14	2.16	<0.05
Calcium [Ca] (mg/kg soil)	3129.54	3117.73	–
Magnesium [Mg] (mg/kg soil)	384.36	387.46	–
Sodium [Na] (mg/kg soil)	18.48	24.54	–
Cation exchange capacity (cmol/kg)	21.33	21.50	–
Mehlich-3 phosphorus (mg/kg)	105.68	82.53	–
Total fatty acid (nmol/g soil)	112.15	70.85	<0.0001
Fatty acids fungi:bacteria (ratio)	0.30	0.22	<0.0001
Fatty acids bacteria (nmol/g soil)	56.59	37.29	<0.0001
Fatty acids actinomycetes (nmol/g soil)	7.35	4.34	<0.0001
Fatty acids cyclopropyl (nmol/g soil)	10.28	7.62	<0.0001
Fatty acids bacteria:cyclopropyl (ratio)	5.59	4.97	<0.0001
Fatty acids eukaryotes (nmol/g soil)	3.62	2.33	<0.0001
Fatty acids arbuscular mycorrhizal fungi [AMF] (nmol/g soil)	7.60	6.16	<0.05
Fatty acids saprophtes:fungi (ratio)	14.62	7.04	<0.0001
Sand (%)	19.70	20.01	–
Clay (%)	28.11	30.40	–
Silt (%)	50.37	48.10	–

**Buffer pH is used to determine the lime rate. A buffering solution of lime that is 0.6 effective Ca carbonate equivalent is added to each sample. To determine lime recommendation, pH is compared to buffer pH. If the difference is large, it suggests that the soil pH is easily changed.*

to measure in agricultural and environmental settings, it is informative to examine the frequency of detection for the tetracycline and sulfonamide gene targets in the 12 Nebraskan certified organic farms. In this instance, *tet*(G), *tet*(Q), *tet*(S), *tet*(X), and *tetA*(P) were most frequently detected (**Figure 1**), and are recommend as the most informative for future studies in these soils. The *sul*(I) gene has been proposed as a marker of human impacts (Pruden et al., 2006). In the current study *sul*(I) was detected at 50% of the farms, but in only 14% of the individual soil samples. This suggests that the utility of this gene as a general marker of anthropogenic agricultural activity might vary depending on the frequency and depth of sampling.

No statistically significant differences were observed for the incidence of various resistance genes from soil collected between 0 and 7.6 cm and that from 7.6 to 15.2 cm samples, with exception of *tet*(L). It is unclear from the data if the *tet*(L) result is biologically significant, as there were only three farms positive for *tet*(L) in this study, and the differences between the depths can be attributed to values from a single farm. Because the two depths compared in this study are both found within in the upper soil horizon, we conclude that these soils can be sampled within the top 15.2 cm without affecting ARG prevalence data. We know that bacterial phylogeny is correlated with ARG profiles (Fosberg et al., 2016), so it is expected that changes in a bacterial community structure will impact overall ARG carriage. However, for this set of soil and ARG targets, no changes in ARG profiles were observed at the two depths. This is an interesting disconnect with our current understanding that soil bacterial communities change with depth (Zhang et al., 2016), a finding that is reflected in the summary FAME data for these organic farms (**Table 2**). Although no qualitative differences in ARGs were observed for

TABLE 3 | Mean soil values for ARG positive samples.

Soil factor	Mean All[§]	Tetracycline resistance gene														
	(*n* = 98)	A	B	C	D	E	K	G	L	M	O	A(P)	Q	S	X	*Sul*1
Number of samples positive >		6	3	0	53	26	1	108	18	17	19	58	98	92	60	
Coarse particulate organic matter (g/kg soil)	0.44	–	–	–	–	–	–	–	–	–	–	0.33*	–	–	–	–
Fine particulate organic matter (g/kg soil)	0.60	–	–	–	–	–	–	0.53	0.42	0.45	–	0.51	–	–	–	–
Organic nitrogen (g/kg soil)	0.21	–	–	–	–	–	–	0.20	0.24*	–	0.26	–	0.22	–	–	–
Organic carbon (g/kg soil)	2.0	–	0.4	–	–	–	–	1.9*	–	–	2.5*	–	2.1	–	–	–
Carbon (g/kg soil)	9.7	–	4.15	–	–	–	–	–	9.4	9.3	9.4	10.1	9.9	–	–	9.4
Large macroaggregates (% soil wt)	15	–	–	–	–	–	–	–	–	–	–	–	–	12*	–	–
Small macroaggregates (% soil wt)	38	–	–	–	–	49	–	–	–	–	–	42	41	–	–	–
Micro aggregates (% soil wt)	24	–	–	–	–	–	–	–	–	–	–	–	–	–	27*	–
Total water saturation (C/kg soil)	77	–	49	–	83	85	–	–	–	70	–	–	80	–	–	–
pH (unitless)	7.0	–	8.3*	–	6.7	7.5	–	7.1	–	–	–	7.2*	–	–	–	–
Buffer pH (unitless)	7.0	–	–	–	–	–	–	7.1	–	–	6.9	7.2*	–	–	–	–
Electrical conductivity (dS/m)	0.39	–	0.61*	–	0.43	0.46	–	–	–	–	–	0.42*	–	–	–	–
Soil organic matter (%)	3.3	–	5.90	–	–	–	–	3.2*	–	–	3.9	–	3.5	–	–	–
Nitrate [NO_3](mg/kg soil)	19	–	–	–	24*	30	–	–	–	–	–	–	–	–	–	–
Potassium [K] (mg/kg soil)	672	–	–	–	–	382	–	–	–	–	958	–	–	–	–	–
Sulfur [S] (mg/kg)	14	–	–	–	–	–	–	–	–	–	16	–	–	–	–	–
Zinc [Zn] (mg/kg soil)	3.1	–	–	–	–	–	–	2.5*	6.1	5.7	7.5	–	–	4.3	–	–
Calcium [Ca] (mg/kg soil)	3129	–	4694	–	–	4028	–	–	–	–	–	159	3309	–	–	–
Magnesium [Mg] (mg/kg soil)	384	657	998	–	–	300	–	–	521	481	530	–	–	–	–	–
Sodium [Na] (mg/kg soil)	18	–	302	–	–	–	–	–	38	45	55	–	–	24*	–	–
Cation exchange capacity (cmol/kg)	21	–	36	–	–	24	–	–	24	–	25	23	–	–	19	–
Mehlich-3 phosphorus (mg/kg)	105	–	484	–	–	–	–	–	229	228	263	–	–	140	–	153*
Total fatty acid (nmol/g soil)	112	–	239	–	–	–	–	–	–	133	146	–	–	–	–	–
Fatty acids fungi:bacteria (ratio)	0.30	–	–	–	–	–	–	–	–	–	–	–	–	–	0.35	–
Fatty acids bacteria (nmol/g soil)	56	–	109	–	–	–	–	–	65*	–	76	–	–	–	–	–
Fatty acids actinomycetes (nmol/g soil)	7.35	–	–	–	–	6.3*	–	6.2*	–	–	10	–	–	–	–	–
Fatty acids cyclopropyl (nmol/g soil)	10	–	27	–	–	–	–	–	5.4	–	15	–	–	–	–	–
Fatty acids bacteria:cyclopropyl (ratio)	5.6	–	4.0	–	5.4	–	–	–	–	–	5.1	–	–	–	5.8	5.8*
Fatty acids eukaryotes (nmol/g soil)	3.6	–	7.8	–	–	–	–	–	–	4.4	–	–	–	–	–	–
Fatty acids arbuscular mycorrhizal fungi (nmol/g soil)	7.6	–	45*	–	–	–	–	–	–	11.2	11*	–	–	–	–	–
Fatty acids saprophtes:fungi (ratio)	15	–	–	–	–	–	–	–	–	–	–	–	–	–	16	–
Sand (%)	20	–	–	–	15*	27*	–	–	12*	–	–	–	–	14	–	–
Clay (%)	28	–	–	–	–	–	–	31*	38*	–	–	–	–	31*	24*	–
Silt (%)	50	–	–	–	–	40	–	–	–	–	–	–	–	–	–	–

Listed values have a $P \leq 0.05$, except where noted by an asterisk, denoting $P \leq 0.01$. "–" indicates no significant difference from the mean for all (combined ARG positive and negative) samples. [§] The number of samples in the positive group represents all 98 samples, measured at both depths.

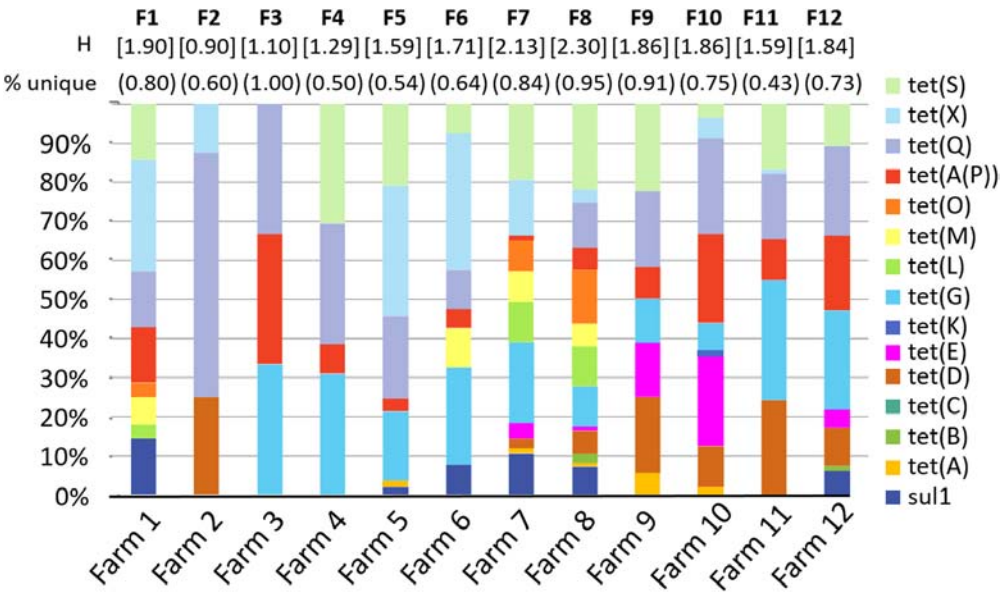

FIGURE 2 | Diversity of selected antibiotic resistance genes by farm. Graph shows the percent of samples from each farm that were positive for each gene. "Percent unique" indicates the number of sample ARG profiles that are unique for each farm. For example, in farm 1, 8 of the 10 profiles were unique = 0.80. *H* is the Shannon diversity index for each farm. "F1–F12" indicates farms 1–12.

TABLE 4 | Comparison of tetracycline and sulfonamide resistance gene prevalence in organic farms and native prairies in Nebraska.

Gene	Mechanism	Conventional manure prevalence (%)	Organic farm soil (*n* = 98)	Prairie soil (*n* = 100)	*P*-value
Sul1	Enzyme	100[A,B]	16	91	<0.0001
tet(A)	Efflux	8[C]	2	52	<0.0001
tet(B)	Efflux	0–4[B,C]	2	27	<0.0001
tet(C)	Efflux	0–100%[B,C,D]	0	14	0.0001
tet(D)	Efflux	No data	29	55	0.0004
tet(E)	Efflux	28[B]	14	15	0.887
tet(G)	Efflux	No data	56	15	<0.0001
tet(K)	Efflux	No data	0	9	0.0024
tet(L)	Efflux	No data	13	34	0.0006
tet(M)	Ribosomal	80–100%[B,D]	11	15	0.4316
tet(O)	Ribosomal	85–100%[B,D]	8	37	<0.0001
tetA(P)	Ribosomal	No data	30	17	0.359
tet(Q)	Ribosomal	80–100%[B,D]	48	0	<0.0001
tet(S)	Ribosomal	49[B]	45	12	<0.0001
tet(X)	Enzymatic	No data	33	2	<0.0001
Mean # ARGs**			3.07	3.94	

*P-value is for comparison of gene % positive in organic vs prairie soils. Manure prevalence % values are calculated from data from peer-reviewed publications that measured gene prevalence from various manure-impacted substrates. [A]Data from Marti et al. (2014). [B]Data from Storteboom et al. (2010). [C]Data from Sengeløv et al. (2003). [D]Data from Jindal et al. (2006). **Based on n = 15 assayed for this study.*

these soils, it may be that quantitative depth-based differences exist for the ARG targets in organic farm soils within the upper 15.2 cm, but they were not revealed with presence/absence data we collected. The ARG antibiogram results reported here are a strong indicator that additional sampling would likely yield additional unique profiles. It is possible, therefore, that the data reported here are an underestimation of the prevalence and distribution of the assayed genes.

ARGs and Soil Properties

Antibiotic resistance genes are ubiquitous in soil (D'Costa et al., 2006; Durso et al., 2012), and the soil is thought to be a direct source for resistance genes that are associated with untreatable infectious disease in hospitals and clinics (Fosberg et al., 2012). As such, there is value in exploring the impact of soil properties on survival and persistence of ARGs in the soil matrix. It has been shown that the presence of metals in soil can provide a selective

pressure for antibiotic resistance (Knapp et al., 2011), but little is known about the impacts of other physical and chemical parameters as they relate to antibiotic resistance. In this study, we identified relationships between multiple physical and chemical properties of the soil, and frequency of detection of sulfonamide and tetracycline resistance genes.

We observed higher EC values in ARG positive vs negative soils. EC is considered an indicator of soil health, influencing crop yield, nutrient availability and activity of soil microorganisms. EC values are also used to identify areas of manure deposition in feedlots and fields (Woodbury et al., 2009). Manure is a common amendment in organic systems, whether deposited via grazing or applied directly as a soil amendment, and it is known to enrich for ARGs in the soil (Udikovic-Kolic et al., 2014; Kyselkova et al., 2015), but this study was specifically not structured to discern the specific role of manures on ARGs in organic production systems. However, statistically significant greater numbers of ARGs were detected at sites having some history of manure application (**Supplementary Table S3**). If the patterns observed in this study apply more broadly, then EC measurements might be helpful in identifying soil regions that are more or less likely to be enriched for tetracycline or sulfonamide resistance genes. Soil Ca, Na, and MehP values were also consistently higher in the ARG positive soils, and may also be useful indicators either individually or as they related to and influence EC.

Sand, clay, TotWSA, pH, C, CEC, Bac:Cyclo, and FPOM did not seem to cluster with the other three groups or with each other, and they had varying relationships with tetracycline resistance genes. Interestingly FPOM had a consistently lower mean value with selected ARG targets [*tet*(G), *tet*(L), *tet*(M), *tet*(O), *tet*A(P)]. FPOM is an easily decomposable part of non-living soil organic matter. It provides resources for microorganisms and nutrients for plant growth. It is possible that the patterns we observed were related to complex interactions involved in active rhizosphere growth. Fatty acid data support the idea that there were active rhizosphere interactions in these soils. The cyclopropane fatty acids are found in a subset of Gram-negative bacteria, including a number of enteric and gut-associated bacteria like *Escherichia* and *Salmonella*, as well as soil dwelling bacteria such as *Rhizobium* (Grogan and Cronan, 1997). Because of the large number of enterics in this group, this fatty acid profile is of particular interest when exploring antibiotic resistance. We observed two ARG targets [*tet*(B), *tet*(O)] associated with significantly higher cyclopropane values as measured by fatty acid methyl ester analysis.

Comparison With Pristine and Conventional Agriculture Sites

The Nebraskan certified organic farm data can be compared to previous data collected from 20 ungrazed native prairie sites, also in Nebraska (Durso et al., 2016). Identical methods were used for gene detection in both studies. Surprisingly, of the 12 targets that were significantly different between certified organic farm and prairie sites, 8 of 12 were less frequently detected in the farm soils than the prairie soils. We initially assumed that anthropogenic practices, such as farming, were likely to increase any measure of AR. However, in this instance we observed that the native prairies had "more resistance" than the farm soils, as measured by frequency of detection of selected ARG targets. Additionally, the mean number of different ARGs (n = 15 total) in the native prairie soils was 3.94, compared to only 3.07 for the organic farms. Again, numerically, the native prairie soils have "more resistance" than the farm soils. Since ARGs are, for the most part, carried inside of bacteria, and since bacterial phylogeny has a strong influence on the types of ARGs present in a sample (Fosberg et al., 2016), the fact that ARGs were more frequently detected in native prairie soils, and that there was a greater diversity of tetracycline resistance genes in native prairie soils, could potentially be explained by the expected greater microbial diversity in native prairie compared to farmed soils (Convention on Biological Diversity, 2010). Importantly, these data compare the number of different gene types, and do not take into account the absolute amount of each gene present. Our conclusions do not exclude the possibility that agricultural systems might have a greater total number of the target genes (absolute number or per 16S), as that was not measured as part of the current study. It is also important to note that there is currently no direct evidence that links soil ARG numbers or diversity with human health outcomes: the data collected in this study was not intended to address risk to human populations from agriculture. Finally, gene-based studies, such as the one reported here, can provide a valuable insight into the ecology of ARGs in agroecosystems, but PCR methods only reveal if a target is present in a sample. We have no information on whether or not the gene is expressed, or whether the gene is contained within a viable cell.

There are three main mechanisms of action for tetracycline resistance (**Table 4**). When the tetracycline resistance gene results were sorted by gene mechanism of action, the tetracycline efflux genes were generally present in higher frequency in the prairie soils, while the genes with ribosomal protection and enzymatic mechanisms of action were generally present in higher frequency in the organic farm soils. Individual ARGs each have their own ecologies (Durso et al., 2016). And although the current study design prevents us from drawing conclusions beyond the specific sites studied, the interpretation of our current results raises the possibility that there might be functional ecological significance that correlates with tetracycline resistance gene mechanism of action.

The long-term applied goal of studies of these types is to identify which ARG targets are the most relevant for agricultural production settings, and provide a starting point for identifying realistic targets for ARGs on farms and in fields. To that end, despite limited data, we can also compare our organic farm soil results to data collected from manures at conventional animal operations, where antibiotics would be used more frequently (**Table 4**). The *tet*(M) gene occurred at 15% or less of samples in both the organic farm and prairie soils. However, this same target was measured in 80–100% of conventionally raised animal manures in studies by Jindal et al. (2006) and Storteboom et al. (2010). This suggests that *tet*(M) prevalence could serve

as a useful indicator of recent manure-borne resistance in the environment, and that there is potential utility in monitoring this gene over time when manures are land applied. Our conclusion on *tet*(M) supports European efforts that have identified *tet*(M) detection as a possible tool to track and monitor ARG transport from and within agricultural systems (Berendonk et al., 2015).

Organic farm soils can serve as a baseline for determining realistic target levels of ARGs in agricultural production settings. They also provide valuable information for studies probing the ecology of antibiotic resistance on farms and in fields. By comparing organic farms with less disturbed soils, such as native prairies, we can start to determine what kinds of impacts agricultural production practices may have on multiple measures of resistance. It is unclear if the relationships we observed are due to management, underlying macroecological (i.e., weather), or geophysical (i.e., soil type) factors. Additional studies are needed to determine if these relationships are broadly applicable across different spatial and temporal scales.

AUTHOR CONTRIBUTIONS

This work was conceived and planned by LD and MC, with input from DM, HW, and CW. Samples were collected and processed by RD and CW, and tested in the laboratory by MC, BC, and RD. Analysis was performed by MC, LD, DM, and BC, with help from HW, RD, and CW. The manuscript was drafted by MC and LD, with substantial input from DM, HW, BC, RD, and CW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01283/full#supplementary-material

FIGURE S1 | Percent of samples per farm with at least one of the targeted antibiotic resistance genes.

FIGURE S2 | Number of target antibiotic resistance genes per sample **(A)** and farm **(B)**.

TABLE S1 | PCR primers and annealing temperatures.

TABLE S2 | Pearson correlation coefficients, based on number of positive ARG targets (n = 15 total ARG targets) per sample. $P < 0.05$ is considered significant. $P < 0.1$ is reported as a possible trend.

TABLE S3 | Proportion of positive samples coming from sites that had received recent manure.

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Quorum-Quenching Bacteria Isolated From Red Sea Sediments Reduce Biofilm Formation by *Pseudomonas aeruginosa*

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Quorum sensing (QS) is the process by which bacteria communicate with each other through small signaling molecules such as *N*-acylhomoserine lactones (AHLs). Certain bacteria can degrade AHL molecules by a process called quorum quenching (QQ); therefore, QQ can be used to control bacterial infections and biofilm formation. In this study, we aimed to identify new species of bacteria with QQ activity. Red Sea sediments were collected either from the close vicinity of seagrass or from areas with no vegetation. We isolated 72 bacterial strains, which were tested for their ability to degrade/inactivate AHL molecules. *Chromobacterium violaceum* CV026-based bioassay was used for the initial screening of isolates with QQ activity. QQ activity was further quantified using high-performance liquid chromatography-tandem mass spectrometry. We found that these isolates could degrade AHL molecules of different acyl chain lengths as well as modifications. 16S-rRNA sequencing of positive QQ isolates showed that they belonged to three different genera. Specifically, two isolates belonged to the genus *Erythrobacter*; four, *Labrenzia*; and one, *Bacterioplanes*. The genome of one representative isolate from each genus was sequenced, and potential QQ enzymes, namely, lactonases and acylases, were identified. The ability of these isolates to degrade the 3OXOC12-AHLs produced by *Pseudomonas aeruginosa* PAO1 and hence inhibit biofilm formation was investigated. Our results showed that the isolate VG12 (genus *Labrenzia*) is better than other isolates at controlling biofilm formation by PAO1 and degradation of different AHL molecules. Time-course experiments to study AHL degradation showed that VG1 (genus *Erythrobacter*) could degrade AHLs faster than other isolates. Thus, QQ bacteria or enzymes can be used in combination with an antibacterial to overcome antibiotic resistance.

Keywords: quorum quenching, marine bacteria, *N*-acylhomoserine lactone degradation, Red Sea sediments, biofilm inhibition

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INTRODUCTION

Quorum sensing (QS) is the molecular mechanism by which bacteria monitor their population density in the local environment and regulate their behavior in a collective manner (Fuqua et al., 1994). QS is achieved by bacteria through the production of small chemical signaling molecules, collectively known as auto-inducers. Bacteria produce various kinds of auto-inducers that differ

in chemical structure and mechanism of action. Broadly, auto-inducers are categorized into three types: (i) acylhomoserine lactones (AHLs), (ii) auto-inducing peptides (AIPs), and (iii) auto-inducer 2 (AI-2) (Huang et al., 2016). QS is used by bacteria to regulate biofilm formation, conjugal DNA transfer, pathogenesis, production of extracellular polysaccharides, and other processes (Galloway et al., 2011). QS blockade is hypothesized to be of use to control infections and biofilm formation by bacteria.

Quorum quenching (QQ) refers to the mechanism by which bacterial communication can be interrupted. QQ can be achieved by inhibiting the production of auto-inducers, their detection by receptors, or their degradation (Natrah et al., 2011). Interference of QS by blocking signal production is not very common and few reports discuss this approach (Hentzer and Givskov, 2003). Many organisms such as algae (Givskov et al., 1996), plant (Gao et al., 2003), and bacteria (Teasdale et al., 2009) produce molecules that are structurally similar to AHLs, and therefore, competitively inhibit their binding to receptors. Certain mammalian cells (Yang et al., 2005) and bacteria (Dong and Zhang, 2005; Romero et al., 2011; Torres et al., 2016) produce enzymes that can degrade or modify AHLs. Bacteria from both terrestrial and marine environments are known to produce AHL-degrading/modifying enzymes (Dong et al., 2002; Romero et al., 2008). The widespread prevalence of QQ enzymes in bacterial communities suggest that it provides competitive advantage to the producer in terms of food and space.

In the wake of rising antimicrobial resistance and toxic impact of antimicrobials on the environment, it is necessary to explore alternative methods to control bacterial infections. QQ is one such alternative, which has been successfully tested in diverse industries (Bzdrenga et al., 2017). For example, QQ has been successfully employed to reduce the pathogenicity of common plant pathogens (Zhang et al., 2007). Similarly, QQ can reduce membrane biofouling in wastewater treatment plants (Oh et al., 2012; Kim et al., 2015; Huang et al., 2016). Successful utilization of QQ in lab-scale wastewater treatment plants has allowed its application in large pilot-scale wastewater treatment plants (Lee et al., 2016). In aquaculture industry, QQ has shown positive results in the disruption of bacterial infections (Cao et al., 2012; Romero et al., 2014; Vinoj et al., 2014; Torres et al., 2016). Recently, QQ was tested for its ability to mitigate the biofouling of reverse osmosis membranes used in seawater desalination (Oh et al., 2017). QQ also has other potential applications such as control of biofouling on the hulls of shipping vessels and fishnets and bio-corrosion of oil production wells. Therefore, there is a need to identify new/novel bacterial species that can produce robust enzymes for use in non-conventional environments; our study is an attempt toward this.

Bacteria can produce three different types of enzymes that can degrade or modify AHLs (Dong and Zhang, 2005): AHL-lactonases hydrolyze the lactone moiety of AHLs (Dong et al., 2001), AHL-acylases hydrolyze the amide bond between lactone ring and acyl chain (Lin et al., 2003), and AHL-oxidoreductase oxidize or reduce the third carbon of the acyl chain of AHL molecules. Generally, hydrolysis of AHL molecules results in complete loss of activity, while oxidation/reduction reduces their activity (Chowdhary et al., 2007). This suggests that lactonases and acylases are more potent and useful in inhibiting bacterial communication.

Quorum quenching is gaining importance as a new way to control bacterial biofilms in medical and industrial domains, aquaculture, and water treatment plants (Torres et al., 2016; Bzdrenga et al., 2017). In this study, we attempted to isolate bacteria from sea sediments that can degrade AHLs and interfere with bacterial communication. We focused on QQ based on AHL inhibition because AHL-based QS is predominantly used by gram-negative bacteria, which are the dominant bacteria found in marine environments and are regarded as early colonizers during biofilm formation (Dang and Lovell, 2000; Zhang et al., 2006). For bacterial isolation, we used sediments from the Red Sea because this particular niche has not been explored from the point of view of QQ. Furthermore, this niche might help us identify new/novel species of bacteria that can be used for biofilm control for applications wherein terrestrial bacteria cannot be used. Screening of these isolates helped identify bacteria with QQ activity. Sequencing the genomes of these isolates allowed us to identify open reading frames (ORFs) encoding QQ enzymes. We further showed that these isolates can be used to degrade a wide range of AHL molecules as well as inhibit biofilm formation by *Pseudomonas aeruginosa* PAO1.

MATERIALS AND METHODS

Sample Collection and Isolation of Bacteria

Red Sea sediment cores were collected at a depth of 1–2 m from the coastal area (22.389778 and 39.135556) 12 km north of Thuwal, Saudi Arabia, in February 2016. Samples were collected from two different areas: one with vegetation (seagrass) and one without vegetation. Sediments were sampled using 30-cm-long acrylic cylindrical tube with a diameter of 5 cm. An ∼20-cm sediment core was collected, and the remaining headspace was filled with indigenous seawater. After sediment collection, rubber stoppers were inserted to seal the two ends of the cylinder. Sampled sediments were stored at 30°C and used to isolate QQ bacteria at the earliest to avoid any negative effect of storage. About 1 g of sea sediments collected from a depth of 2 cm from the surface of the sampling cylinder was suspended in 1 mL of 0.2-μm filtered autoclaved seawater and vortexed. Samples were allowed to stand for 1–2 min to allow the particles to settle down. The supernatant was then subjected to 10-fold serial dilution. Each dilution was plated on Marine Agar (MA) (HIMEDIA, India), R2A agar (HIMEDIA, India), and Casamino acids (CAS) agar (VWR, United States). Both R2A and CAS agar were prepared in 75% of 0.2-μm-membrane-filtered autoclaved seawater. The plates were incubated at 30°C for 1 week. Colony-forming units observed on plates (with 30–300 colonies) were enumerated, and the colonies were further subcultured onto sterile agar plates based on macroscopic characteristics. Single colonies were further streaked twice to obtain pure cultures.

QQ Assay

The isolated strains were tested for QQ activity by using the AHL biosensor strain *Chromobacterium violaceum* CV026. This sensor strain has been used to detect C6-AHLs in various studies (McClean et al., 1997; Romero et al., 2011; Torres et al., 2016). The isolates were grown in 0.5 mL of the isolation medium and incubated at 30°C with shaking at 150 rpm. C6-AHLs were added to this bacterial culture to reach a final concentration of 10 μM (2 μg/mL) and further incubated for 24 h at 30°C with shaking. The pH of this mixture was measured to confirm that the observed degradation of AHLs was not caused by alkaline pH (Yates et al., 2002). C6-AHLs mixed with cell-free medium were used as the negative control. The bacterial cultures were centrifuged to pellet the cells, and the remaining C6-AHLs in the culture supernatant were detected by the following method. Luria-Bertani (LB) agar plates were overlaid with 5 mL of 1/100th-dilution of an overnight culture of the biosensor strain CV026 mixed with LB soft agar (0.7%). After the biosensor layer was solidified, 6-mm wells were created in the medium by using sterile pipette tips. These wells were filled with the culture supernatant and incubated at 30°C for 24 h. Solvent without C6-AHLs was used as the blank. The appearance of a purple halo around the well-indicated the absence of QQ activity. On the other hand, strains with QQ activity degraded C6-AHLs, and therefore, the biosensor strain was not activated. Thus, halo formation was not observed. Furthermore, the culture supernatant of QQ isolates was tested for the production of C6-AHLs.

No purple halos were observed in the CAS and R2A cell-free media (negative controls), which showed that these media cannot be used for QQ assay. Therefore, for these isolates, we slightly modified the QQ assay, as described previously (Uroz et al., 2005; Shepherd and Lindow, 2009). Briefly, 24-h-old bacterial cultures were centrifuged to obtain cell pellets. These pellets were suspended in 0.5 mL of 1X phosphate-buffered saline (PBS) containing 10 μM C6-AHLs and incubated overnight at 30°C with shaking. The remaining procedure was as described above.

QQ Assay With Heat-Inactivated Bacteria

To ensure that the loss of C6-AHL activity observed in QQ-positive strains was not due to the adsorption of these molecules onto the cell surface, the bacterial cells were heat killed. Bacterial cells were heated at 100°C for 15 min. Heat-killed bacterial cells were allowed to cool down for 10 min at room temperature. QQ assay was performed as described above. Bacterial cell death was confirmed by plating 150 μL of the heat-treated cell suspension on respective culture medium.

Detection and Localization of AHL-Degradation Activity

This assay was performed as described previously with slight modifications (Romero et al., 2008; Torres et al., 2016). About 200 mL of the overnight culture suspension of QQ-positive isolates was centrifuged at 7000 × *g* for 10 min. Cell pellets were washed with an equal volume of 1X PBS and re-suspended in 50 mL of PBS. Cells were lysed by intermittent ultra-sonication (Qsonica, United States) for 5 min in a cold water bath at a frequency of 15 kHz. Lysed cells were centrifuged at 16000 × *g* for 30 min at 4°C. Cell lysates were filtered through a 0.2-μm-pore-sized-membrane filter. The protein concentration of the cell lysates was determined with Qubit (Invitrogen, United States). To determine AHL-degradation activity, 500 μL of the cell lysates was incubated with 10 μM C6-AHLs for 24 h at 30°C, with shaking at 140 rpm. The remaining C6-AHLs were detected by a well-diffusion agar plate assay, as described above. Cell lysate without C6-AHLs was used as the control. To understand the chemical nature of QQ activity, the cell lysates were heated at 95 and 105°C for 10 min. Furthermore, the cell lysates were fractionated using 10-kDa centrifugal filters (Amicon, United States) and QQ activity was analyzed for both the retentate and filtrate of cell lysates.

HPLC-MS-Based Analysis of AHLs

The ability of isolates to degrade different types of AHLs was studied by using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS) as described previously (Romero et al., 2011). Briefly, overnight bacterial cultures were centrifuged and re-suspended in PBS containing 10 μM AHL. This mixture was incubated overnight at 30°C with shaking. For the time-course experiment, the samples were withdrawn every hour for 5 h. To extract AHLs, the cells were separated by centrifugation at 7000 × *g* for 5 min, and the PBS was extracted twice with an equal volume of ethyl acetate (Fisher Scientific, United States). Ethyl acetate was evaporated under a flux of nitrogen at 40°C, and the final extract was suspended in 400 μL of acetonitrile (Fisher Scientific, United States) for HPLC-MS. PBS containing equal amount of AHLs was used as the negative control. To determine whether the QQ activity was caused by the hydrolysis of lactone ring (lactonolysis), the bacterial cells were incubated overnight with PBS containing 50 μM 3OHC10-AHLs. The cell-free supernatant was acidified to a pH of 2 by adding 10 mM hydrochloric acid (HCl). The acidified supernatant was incubated overnight at room temperature to allow re-cyclization of lactone ring. AHLs were extracted from this solution as described above.

High-performance liquid chromatography 1100 series equipped with ZORBAX Eclipse XDB-C18 (4.6 mm × 250 mm column; 5-μm particle size; Agilent Technologies, United States) kept at 45°C was used for analysis. About 10 μL of the extract was injected at a flow rate of 0.45 mL/min. For elution, a mobile phase consisting of solvent B (methanol with 0.1% formic acid) and solvent A (25 mM ammonium formate with 0.1% formic acid) was used. The gradient profile used was 1 min of 10% solvent B, followed by a linear gradient gradually increasing to 95% of solvent B over 15 min. Solvent B (95%) was then stabilized for 4 min. The column was re-equilibrated for a total of 5 min. MS data were obtained on TSQ Vantage triple-quadruple mass spectrometer (Thermo Fisher Scientific, United States) by using positive-ion electrospray and multiple-reaction-monitoring (MRM) mode.

Bacterial Identification Based on 16S-rRNA Gene Sequencing

About 500 μL of the overnight bacterial suspension was centrifuged and the cell pellets were re-suspended in 500 μL of nuclease-free water. Bacterial cells were lysed by heating at 95°C for 10 min, followed by cooling for 15 min at room temperature. The lysed bacterial cells were centrifuged at 12000 × *g* for 3 min, and 1 μL of the supernatant was used as template DNA for polymerase chain reaction (PCR). A set of three primer pairs, namely, (27F-785R), (341F-907R), and (785F-1492R) was used to amplify the I6S-rRNA gene. Primer sequences are available in Supplementary Table 4. Following PCR conditions were used: initial denaturation at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s; primer annealing at 52°C (27F-785R), 62°C (341F-907R), and 53°C (785F-1492R) for 30 s; and extension at 72°C for 1 min. Final extension was performed at 72°C for 5 min. The PCR product was analyzed by gel electrophoresis and purified using the ExoSap-IT PCR product cleanup kit (Affymetrix, United States), according to manufacturer's instructions. The purified DNA was submitted for Sanger sequencing. The three overlapping sequences were aligned to obtain a single rRNA molecule for use in BLAST search (Altschul et al., 1997) against the 16S-rRNA gene sequences available in the GenBank database. The 16S-rRNA gene sequences of close relatives, as determined by BLAST and the QQ bacteria described in literature, were used for phylogenetic analysis.

For phylogenetic analysis, the SINA software package available in SILVA rRNA database (Quast et al., 2013) was used to align 16S-rRNA gene sequences. The aligned sequences were subjected to phylogenetic tree construction by using MEGA7 (Kumar et al., 2016) software at default parameters.

Acylhomoserine Lactones (AHLs)

Following AHLs were used in this study; *N*-butyryl-DL-homoserine lactone (C4-AHLs), *N*-hexanoyl-DL-homoserine lactone (C6-AHLs), *N*-decanoyl-DL-homoserine lactone (C10-AHLs), *N*-tetradecanoyl-DL-homoserine lactone (C14-AHLs), *N*-(3-oxodecanoyl)- DL-homoserine lactone (3OXOC10-AHLs), *N*-(3-hydroxydecanoyl)- DL-homoserine lactone (3OHC10-AHLs), and *N*-(3-oxododecanoyl)- L-homoserine lactone (3OXOC12-AHLs). All AHLs used in this study were purchased from Sigma, United States.

Biofilm Formation and Quantification

The impact of QQ bacteria on biofilm formation by *P. aeruginosa* PAO1 was studied using a recently described segregated culture bioassay (Oh et al., 2017). In this assay, QQ bacteria are physically separated from PAO1 by using a semipermeable membrane (Transwell polycarbonate membrane cell inserts; Corning, NY, United States). PAO1 (OD_{600} = 0.01) was directly inoculated into the wells of a 24- or 6-well microtiter plates. QQ bacteria (live or dead) were added into the membrane inserts and installed into the wells. As a control, QQ bacteria were killed by incubating the cells in 4% paraformaldehyde for 30 min at room temperature. Cell death was confirmed by spreading the cell suspension on R2A or MA plates. The inoculated microtiter plates were incubated for 24 h at 30°C with shaking at 60 rpm. The membrane inserts were removed, and the OD_{600} of the PAO1 cell suspension was measured to determine the effects of QQ bacteria on growth, if any. Biofilm formation by PAO1 on the wells was measured using the crystal violet assay (Coffey and Anderson, 2014). PAO1 culture was also used for the extraction and quantification of 3OXOC12-AHLs, as described above. Furthermore, 3OXOC12-AHLs were quantified in the control sample (LB) as well as VG1, VG12, and NV9.

Genome Sequencing and Annotation

Genomic DNA of the strains to be sequenced was extracted using QIAGEN genomic-tip 100/G columns (QIAGEN, Germany). A genome-sequencing library was prepared using the Pacific Biosciences (PacBio) 20-kb template preparation kit by employing the BluePippin size selection system and sequenced on PacBio RS platform. The PacBio reads were assembled using the CANU WGS assembler version 1.4 (Koren et al., 2017). The assembled genome was annotated using the Automatic Annotation of Microbial Genomes (AAMG) pipeline (Alam et al., 2013). For functional annotation, the predicted ORFs were compared to the latest version of UniProt (The Uniprot Consortium, 2017) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2014).

Statistical Analysis

Mean and standard deviation were calculated for AHL degradation and biofilm inhibition assays. Further statistical analyses such as analysis of variance (ANOVA) with Bonferroni corrected *post hoc t*-test and also Student's *t*-test were preformed where statistical significance was (p-value < 0.0063) or (p-value < 0.05) respectively. All the statistical analyses were performed in Microsoft® Excel version 16.9.

RESULTS

Bacterial Isolates

Different number of CFUs were obtained, from both types of sea sediment samples by using three different culture media. The CFU/gram of sea sediment is listed in Supplementary Figure 1. For all culture media, the number of CFUs obtained from the samples from non-vegetative areas was higher compared to that from the samples collected from the vicinity of vegetation (Supplementary Figure 1).

Higher CFUs were observed on MA medium, compared to R2A and CAS media. The highest number of CFUs, that is, 6.6×10^4, was obtained from the samples obtained from non-vegetative areas that were plated on MA. For the samples collected from the vicinity of vegetation, 4.4×10^4 CFUs were obtained on MA (Supplementary Figure 1), which was four-times higher than that obtained on R2A and CAS media. Isolates exhibiting different colony morphology were selected for QQ assay.

Biosensor-Based Detection of QQ Activity

About 71 bacterial isolates were screened for QQ activity. A solid plate assay was performed using the biosensor strain *C. violaceum* CV026, which produces a purple pigment violacein in response to C6-AHLs (McClean et al., 1997). The QQ strains can degrade AHLs, which, in turn, did not allow the development of any color. The number of isolates tested and those testing positive for QQ activity is listed in **Table 1**. Of the 14 QQ-positive isolates, 64.3% were isolated on the R2A medium, followed by MA (21.5%) and CAS (14.3%). However, the QQ-positive CAS and MA isolates showed only partial degradation of C6-AHLs, as indicated by the small/faint purple halos (Supplementary Figures 2, 3). Most QQ-positive isolates obtained on R2A showed complete degradation of AHLs. Overall, 22.4% isolates from the samples obtained from areas near vegetation and 13.6% isolates from the samples obtained from areas without vegetation were positive for QQ activity (**Table 1**). C6-AHL production by the QQ isolates was not detected.

Some previous studies investigating the QQ potential of marine bacteria used marine broth for QQ assay (Romero et al., 2011; Torres et al., 2016). Therefore, we tried to cultivate the isolates obtained on R2A and CAS media in marine broth. However, except VG12, none of them grew in marine broth, although they did grow on MA. VG12 cultivated in marine broth continued to remain positive for QQ activity.

Quorum quenching assay performed using heat-killed QQ isolates ruled out the possibility that the observed loss of AHLs was due to adsorption onto bacterial cells (data not shown). Bacterial isolates that retained QQ activity after heat treatment were not included in further analyses. Of the 14 isolates, eight were selected for further analyses.

QQ Analysis Based on HPLC–MS

The QQ activity of the positive strains, as determined by the biosensor assay, was further confirmed by HPLC-MS. The ability of QQ bacteria to degrade different types of AHLs was also investigated. For this, QQ-positive bacterial cultures were mixed with AHLs of different acyl chain lengths and modifications (C4-AHLs, C6-AHLs, C10-AHLs, 3OXOC10-AHLs, 3OHC10-AHLs, and C14-AHLs). After 24 h of incubation, the final pH was < 7.5, which excluded the possibility of the hydrolysis of the lactone ring of AHL molecules due to alkalinity. The remaining AHLs were extracted and quantified (**Figures 1A–E**, **2A**). All the strains showed significant reduction in the amount of AHLs, compared to the negative control (**Figure 1**). Analysis of variance (ANOVA) along with Bonferroni's corrected *post hoc t*-test was applied, which showed significant reduction of AHLs by the QQ strains (p-value < 0.0063), compared to the blank sample. The degradation capacity of all isolates was higher for C10-AHLs and C14-AHLs, compared to C6-AHLs (**Figures 1A–C**). All QQ bacteria caused > 90% reduction in the quantities of C10 and C14-AHLs (**Figures 1A,B**). These results are in agreement with previous reports wherein the reduction in the amount of long-acyl-chain AHLs was higher compared to that in case of short-acyl-chain AHLs (Romero et al., 2008; Romero et al., 2011; Torres et al., 2016).

The ability of these QQ bacteria to degrade differently modified C10-AHLs (3OXO-AHLs and 3OH-AHLs) was also investigated (**Figures 1D,E**). The strain VG16 displayed inconsistent cultivability; therefore, it was not included in further analyses. In a recent study, most QQ bacteria were able to degrade a wide range of AHLs, but they could not effectively degrade 3OHC10-AHLs (Torres et al., 2016). Similar to this, all QQ-positive isolates in this study could degrade 3OXOC10-AHLs more effectively, compared to 3OHC10-AHLs (**Figures 1D,E**).

We also studied the ability of bacteria to degrade C4-AHLs. For this analysis, three QQ-positive bacteria (VG1, VG12, and NV9) belonging to different genera were selected. Of these, VG12 showed maximum degradation (>80 ± 8.9%) of C4-AHLs (**Figure 2A**), while NV9 showed only 26 ± 13% reduction and VG1 did not show significant degradation (**Figure 2A**).

To identify the nature of QQ activity, i.e., lactonase or acylase, 3OXOC10-AHLs degraded by VG1, VG12, and NV9 were treated with HCl. Acidification resulted in the reformation of lactone ring that suggested lactonase activity (Yates et al., 2002; Romero et al., 2008). In NV9, ∼63.5 ± 4% of 3OXOC10-AHLs was recovered after HCl treatment. In VG12 and VG1, only 2 ± 0.003 and 0.004 ± 0.009% of AHLs, respectively, were recovered after acidification (**Figure 2B**).

TABLE 1 | Number and QQ activity of the strains isolated from different samples (vegetative and non-vegetative) by using different media.

Sample/medium	Isolates tested for QQ	QQ based on CV026
Vegetative		
MA	21	2
R2A	18	7
CAS	10	2
Non-vegetative		
MA	6	1
R2A	10	2
CAS	6	0
Total	71	14

Number of isolates with positive QQ activity (based on C6-AHL degradation in the Chromobacterium violaceum CV026 assay) is shown.

QQ Activity and Its Localization

The location of QQ activity (extracellular or intracellular) was studied for VG1, VG12, and NV9. Cell-free supernatants and lysates were incubated with C6-AHLs. Cell lysates and culture supernatant of VG1 were able to degrade C6-AHLs (Supplementary Figure 2B). No QQ activity was detected in the culture supernatant and cell lysates of VG12 and NV9 (Supplementary Figure 2B). Heat treatment of the cell lysates of VG1 at 95 and 105°C did not result in loss of QQ activity. After fractionation of the cell lysates of VG1 by using 10-kDa filters, QQ activity was detected only in the retentate but not in the filtrate (data not shown). This suggested that the molecules responsible for QQ activity are larger than 10-kDa.

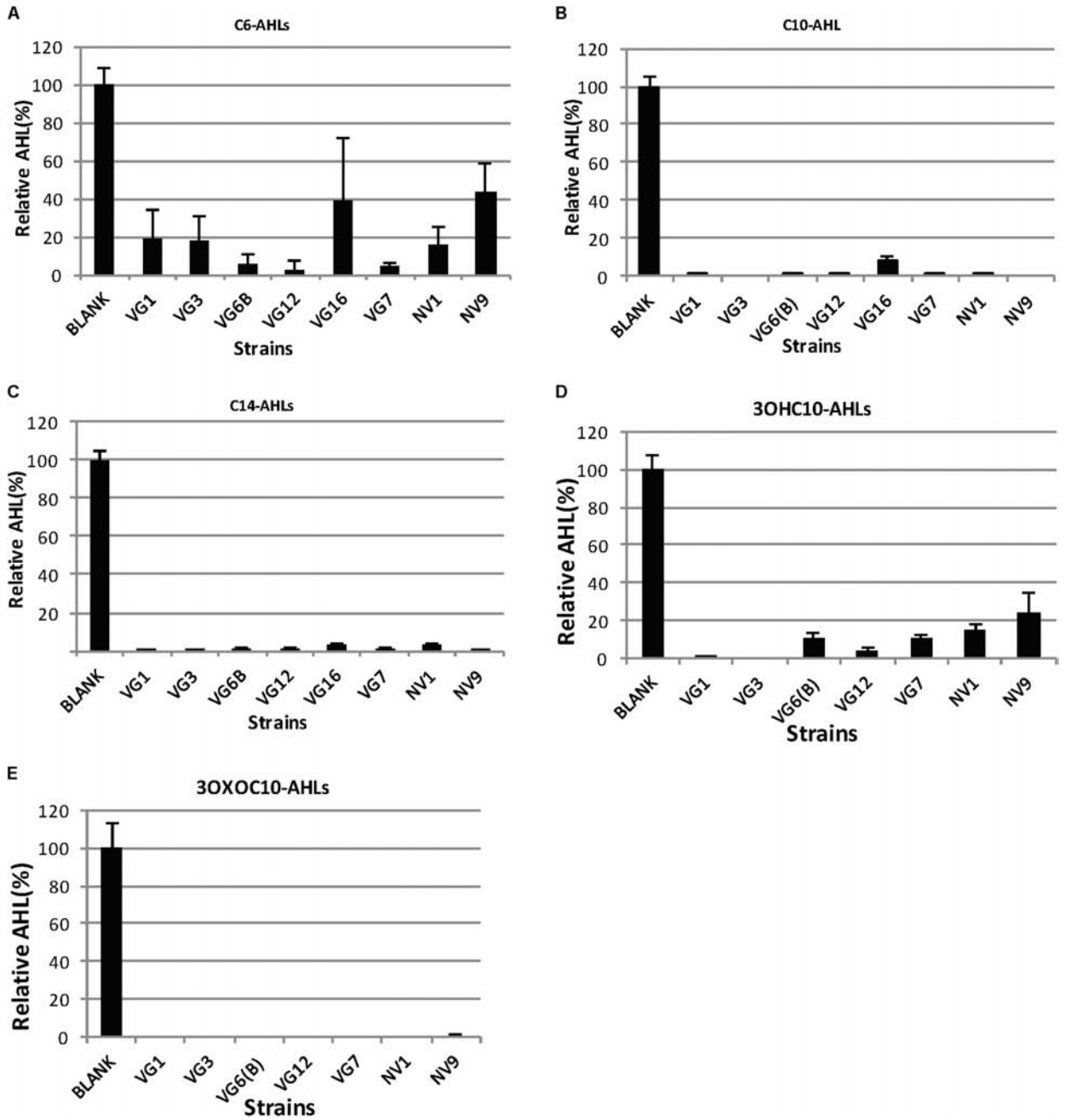

FIGURE 1 | Degradation of AHLs by the isolated bacteria. The amount of AHLs degraded by different isolates is listed, relative to the negative control. Quantification of AHLs was performed as described in Section "Materials and Methods." Briefly, AHLs were extracted with ethyl acetate, which was evaporated under a flux of nitrogen gas. The extracted AHLs were re-suspended in acetonitrile and quantified by HPLC-MS. Cell-free PBS was used as the negative control (100%). Values are the mean of three replicates; error bars represent standard deviation. Charts **(A–E)** illustrates the degradation of C6, C10, C14, 3OXOC10-AHLs, and 3OHC10-AHLs, respectively.

Time-Course Experiment of AHL Degradation

The kinetics of the degradation of 3OXOC10-AHLs by VG1, VG12, and NV9 was also investigated. The isolate VG1 caused 98.7 ± 0.11% reduction in the first hour, while VG12 caused 58 ± 1.4% reduction and NV9 caused only 26.9 ± 8.2% reduction in the amount of AHLs (**Figure 3**). However, after 2 h, the amount of AHLs degraded by VG1 and VG12 was almost equal, i.e., 99.9 ± 0.01 and 98 ± 0.7%, respectively, while only 50 ± 3.4% of the AHLs was degraded by NV9. Maximum reduction of 3OXOC10-AHLs by NV9 occurred after 4 h (**Figure 3**).

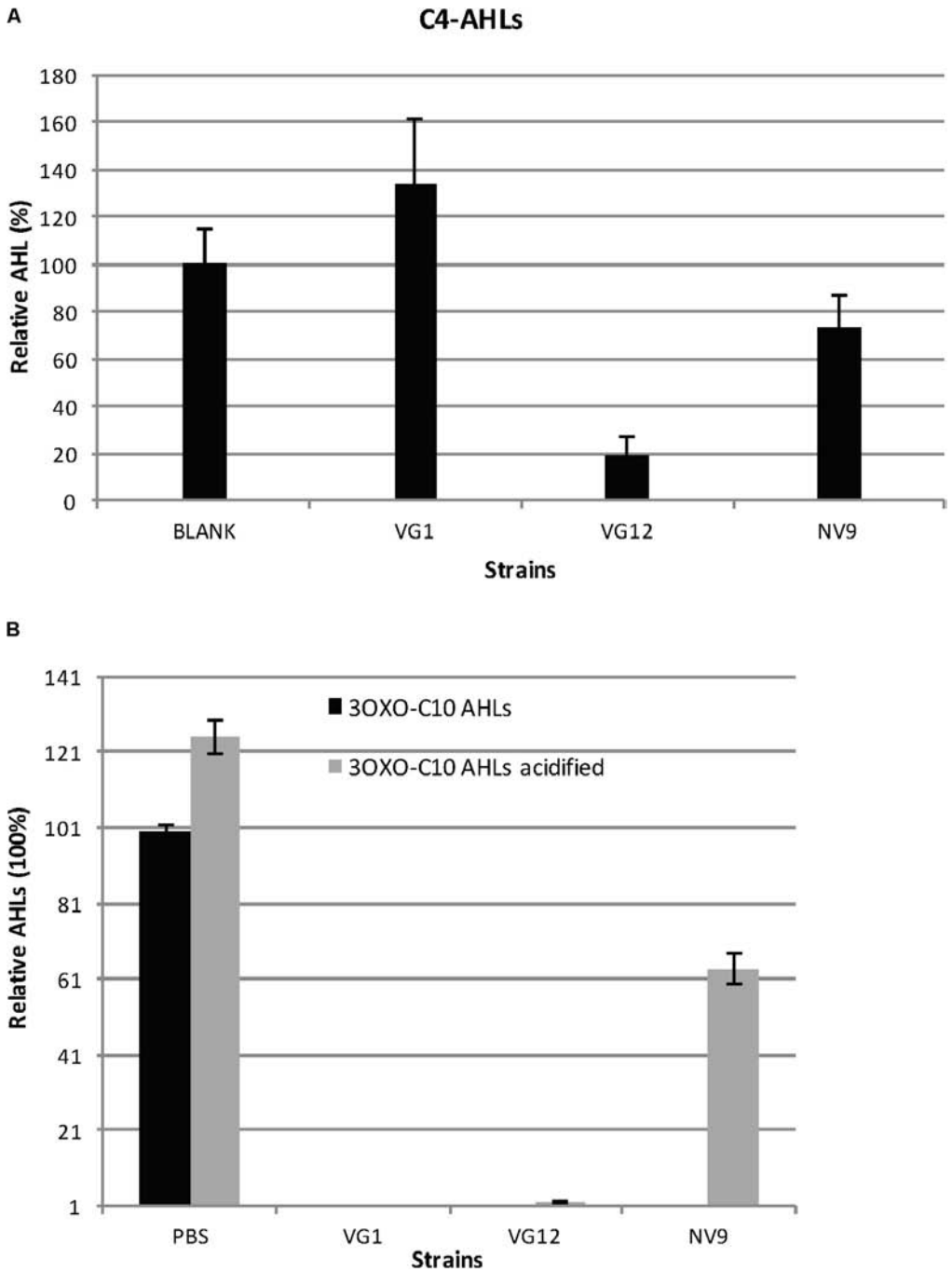

FIGURE 2 | Degradation and acidification of AHLs. **(A)** Relative amount of C4-AHLs degraded by the three isolates is given. For quantification, the C4-AHLs were extracted with ethyl acetate and subsequently dried and re-suspended in acetonitrile for injection in HPLC-MS. Cell-free PBS served as the negative control (100%). Experiments were performed in triplicate; error bars represent the standard deviation of the mean value. Student's *t*-test showed significant reduction in the amount of C4-AHLs by VG12 (p-value = 0.003) and NV9 (p-value = 0.03). No significant degradation of C4-AHLs by VG1 was observed (p-value = 0.11). **(B)** Acidification of 3OXOC10-AHLs after incubation with QQ bacteria. Relative amount of AHLs before and after acidification is given. Black bars represent the amount of AHLs after incubation with PBS (negative control is 100%) or QQ bacteria. Gray bars represent the amount of AHLs recovered after acidification. Error bars represent the standard deviation for the three independent replicates.

Identification of QQ Isolates

Phylogenetic analyses showed that all the seven QQ isolates belonged to the phylum Proteobacteria (Supplementary Table 1). Except NV9, all other isolates [VG1, VG3, VG6(B), VG12, VG7, and NV1] belonged to the class Alphaproteobacteria and two different genera *Erythrobacter* and *Labrenzia*.

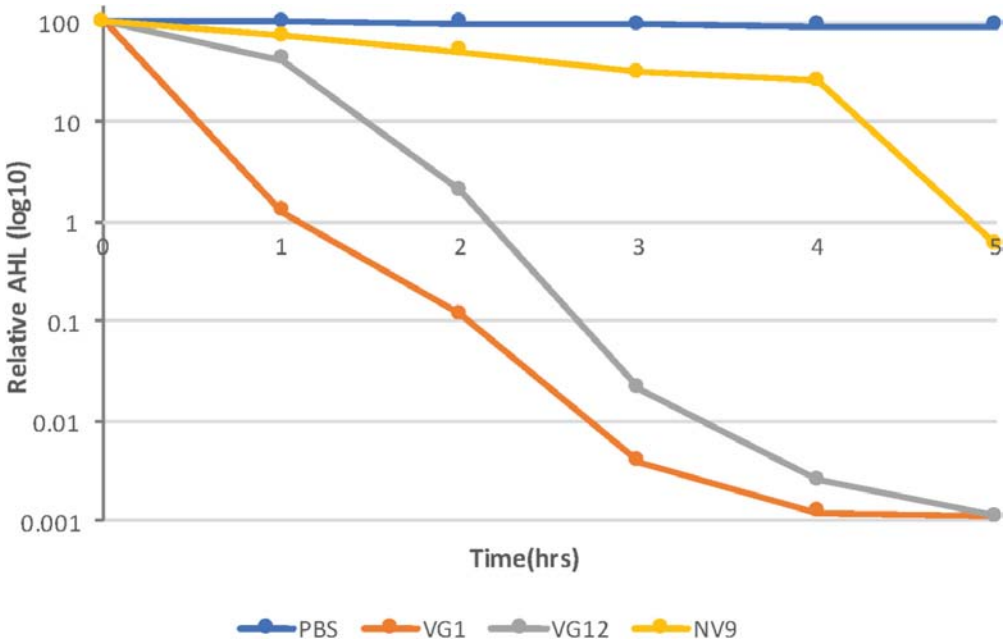

FIGURE 3 | Time-course experiment to study AHL degradation. Log_{10} of the relative amount of AHLs quantified at each time point is given along the y-axis. AHLs were quantified using HPLC–MS, as described in Section "Materials and Methods." The amount of AHLs at 0 h is considered 100%. Control (PBS) sample and different strains are represented by different colors, as indicated in the legend. Standard deviation at each time point was <10%.

Isolate NV9 belonged to the class Gammaproteobacteria and genus *Bacterioplanes* (Supplementary Figure 4 and **Table 1**).

Isolates VG1 displayed 100% identity to *Erythrobacter flavus* SW-52, which was also isolated from the marine environment (Yoon et al., 2003). As described for *E. flavus*, VG1 formed yellow colonies on agar plates. Isolate VG3 showed 99% identity to *Erythrobacter* sp. JL-378 and also formed yellow colonies on R2A agar.

Four isolates, namely, VG6(B), VG12, VG7, and NV1, belonged to the genus *Labrenzia*. Different species of *Labrenzia* that were identified based on 16S-rRNA gene sequence homology are listed in Supplementary Table 1. VG12 showed 99% identity to *Alphaproteobacterium* JL001 that was isolated from marine sponges. Phylogenetic analysis showed that VG12 is closely related to the other *Labrenzia* species identified in this study and previously (Supplementary Figure 4). Isolate VG7 showed 99% identity to *Labrenzia* sp. A-3-20, which was recently isolated from the soft corals found in Baltic sea (Pham et al., 2016). NV1 displayed 99% identity to *Labrenzia* sp. R-666638. All species of genus *Labrenzia* that have been identified so far, have been isolated from marine environments (Biebl et al., 2007; Camacho et al., 2016).

The 16S-rRNA sequence of the QQ isolate NV9 (obtained from areas without vegetation) showed 99% identity to that of a recently proposed bacterial species *Bacterioplanes sanyensis* (Wang et al., 2014), also isolated from marine environment.

The phylogenetic relationship of the QQ isolates discussed in this study and other marine bacteria is illustrated in Supplementary Figure 4.

Effect of QQ Bacteria on Biofilm Formation

VG12 was able to significantly reduce biofilm formation by PAO1. Live VG12 cells could reduce biofilm formation by $25 \pm 0.018\%$ compared to dead VG12 cells (**Figure 4A**). However, no significant reduction was induced by VG1 and NV9 in the biofilm formation of PAO1.

Pseudomonas aeruginosa PAO1 produces 3OXO-C12AHLs, which directly or directly control the expression of virulence factors and biofilm formation (Williams and Camara, 2009). Therefore, the amount of 3OXO-C12AHLs in the supernatant of PAO1 incubated with live/dead QQ bacteria was also quantified. However, no significant degradation of 3OXO-C12AHLs was detected (**Figure 4B**). No 3OXO-C12AHLs were detected in case of LB, VG1, VG12, and NV9.

Identification of Lactonases and Acylases in the Genome Sequences

For each strain, the genomic features and their counts are listed in Supplementary Table 3. The genome sequences were submitted to GenBank; the accession numbers for VG1 is CP022528, VG12 is CP022529, and NV9 is CP022530. Annotations for VG1 are available[1], VG12[2], and NV9[3]. Based on average nucleotide identity (ANI), a new quality control test implemented by GenBank, VG12 was designated as *Labrenzia* sp. VG12

[1]https://bit.ly/2uoXhX1
[2]https://bit.ly/2mr70HU
[3]https://bit.ly/2NpfrPA

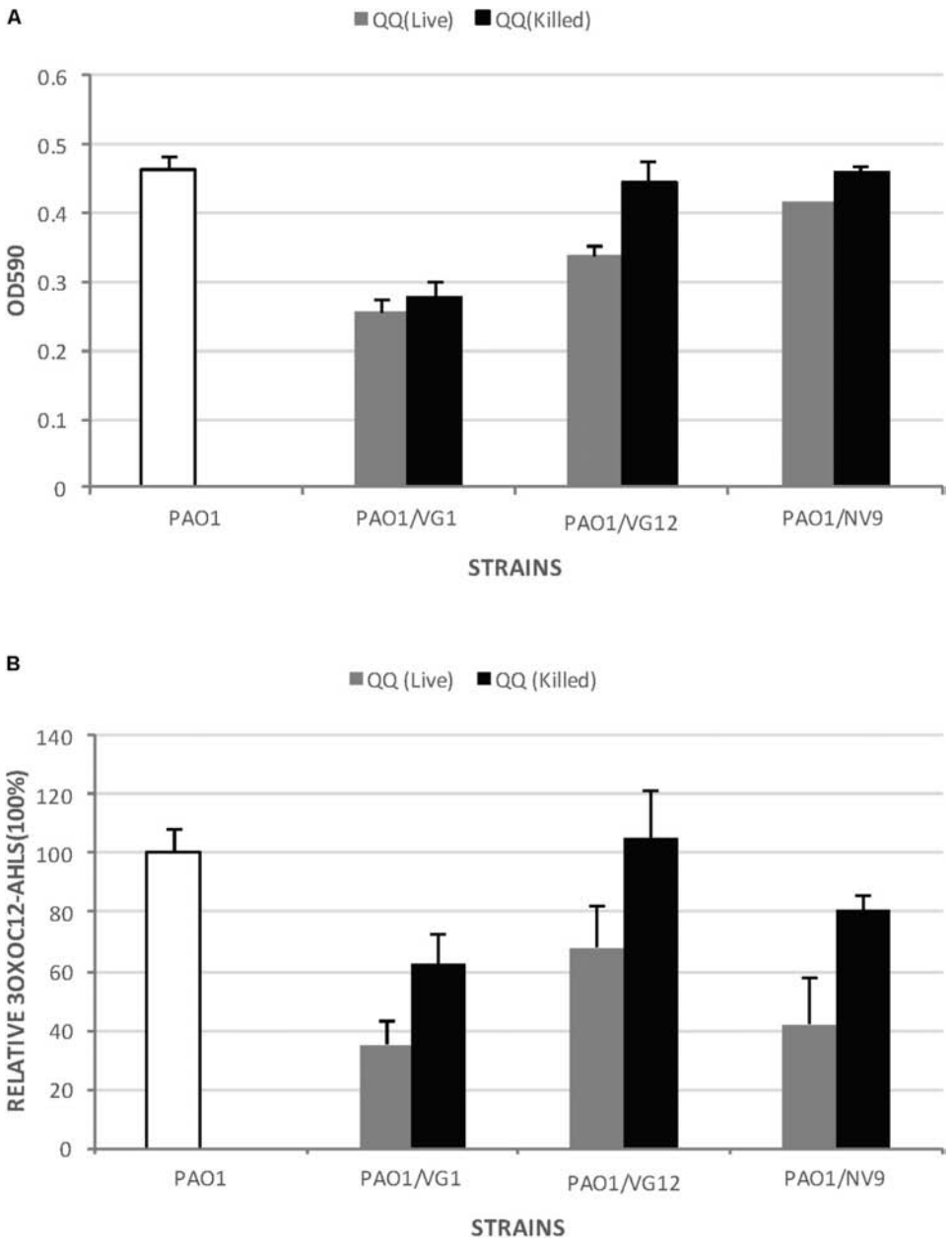

FIGURE 4 | Biofilm formation by *Pseudomonas aeruginosa* PAO1 incubated with live and dead QQ strains. **(A)** This experiment was performed in microtiter plates with membrane inserts for wells, as described in Section "Materials and Methods." The y-axis indicates the OD_{590} of the crystal violet bound to the wells. White bars represent biofilm formation by PAO1 without any live or dead QQ bacteria. Gray bars represent biofilm formation by PAO1 incubated with live QQ cells, while black bars represent biofilm formation by PAO1 incubated with dead QQ bacteria. LB broth was used as the negative control. Error bars represent the standard deviation for the three replicates. Student's *t*-test was applied to determine significance; *p*-values: VG1 (0.29), VG12 (0.04), and NV9 (0.09). **(B)** Relative amount of 3OXOC12-AHLs in the supernatant of PAO1 incubated with live or dead QQ bacteria. The amount of AHLs in the supernatant of PAO1 incubated with live QQ bacteria is shown as gray bars, while that detected in the presence of dead QQ bacteria is shown as black bars. The amount of AHLs produced by PAO1 (without live/dead QQ bacteria) is shown by white bars (100%). Error bars represent the standard deviation. Student's *t*-test showed no significant difference in the amount of 3OXOC12-AHLs in the PAO1 supernatant incubated with live/dead VG1 (*p*-value = 0.16), VG12 (*p*-value = 0.219), and NV9 (*p*-value = 0.22).

because of its low similarity with the type strain *Labrenzia alba*.

Annotated genomes were searched for the homologs of AHL lactonases and acylases, which are members of the metallo-beta-lactamase (MBL) and N-terminal nucleophile hydrolases (Ntn-hydrolases) superfamilies, respectively (Utari et al., 2017).

VG1 genomic annotations showed that the two ORFs (VG1_000001122 and VG1_000002328) are KEGG orthologs of AHL-lactonases (K13075). UniProt annotations

TABLE 2 | Genomic IDs of the ORFs of the sequenced strains, showing homology to AHL-lactonases or AHL-acylases.

Strains	ORFs (ID)	
	Lactonases	**Acylases**
VG1	VG1_000001122	VG1_000002924
	VG1_000002328	
VG12	VG12_000006578	
	VG12_000000021	
	VG12_000000913	
	VG12_000003727	
	VG12_000004165	
NV9		NV9_000000564

Open reading frames predicted by both KEGG and UniProt are listed; the amino acid sequences of these ORFs can be accessed using the annotation links given in Section "Results."

further confirmed that these proteins are beta-lactamases. Similarly, both KEGG (K07116) and UniProt annotations suggest that ORF VG1_000002924 is an AHL-acylase (**Table 2**).

For VG12, both KEGG Orthology and UniProt predicted that VG12_000000021, VG12_000006578, VG12_000000913, and VG12_000004165 belong to the lactonase group and/or are MBL members. The protein product (VG12_000003727) was predicted as AHL-lactonase by KEGG, but UniProt showed it to be Ribonuclease Z. BLAST analysis of this ORF showed that it is 90% identical to the MBL superfamily of proteins (**Table 2**). No homolog of AHL-acylases was identified for VG12, neither by KEGG nor UniProt.

For NV9, both KEGG and UniProt annotations indicated that NV9_000000564 is an AHL-acylase (**Table 2**).

Apart from these ORFs, the genomes of VG1, VG12, and NV9 carry other proteins that are homologous to MBL and amidases. The locus IDs of these ORFs are given in Supplementary Table 2.

DISCUSSION

The emergence of antimicrobial resistance has underscored the need to develop new strategies to control bacterial infections and biofilms. Furthermore, the environmentally toxic biocides used in water treatment, agriculture, and oil and shipping industry warrant the search of sustainable and non-toxic alternatives. QS is a potential target for use as a new therapeutic approach because of its role in bacterial infection and biofilm formation. One such opportunity can be identified by exploring QQ because of its potential benefits.

In this study, cultivable bacteria were isolated from Red Sea sediments collected from two different niches, i.e., areas with and without vegetation. Unexpectedly, a higher number of bacteria was isolated from the samples collected from areas without vegetation, which can be attributed to the fact that vegetative bacteria require the compounds produced by plants for their growth (Supplementary Figure 1). This can also be attributed to the inherent bias observed in the plate count method. By screening all isolates, we identified that ∼20% of the isolates exhibit QQ activity (**Table 1**). These results are similar to those of previous studies, which reported higher prevalence of QQ-positive bacteria in the marine environment, compared to the terrestrial environment (Romero et al., 2011; Saurav et al., 2016). It is important to note that we only used *C. violaceum* CV026-based assay for the initial screening of QQ bacteria, and thus, the number of positive isolates might be underestimated.

Quorum quenching bacteria have been detected and isolated from dense microbial communities in various systems (Tan et al., 2015; Saurav et al., 2016). Similarly, in this study, a higher percentage of QQ bacteria was detected from samples collected from areas with vegetation compared to those from areas without vegetation (**Table 1**). However, the vegetative bacteria identified in this study might not be permanently associated with seagrass because their close relatives have been isolated from different marine niches. It can also be that the microbial community associated with seagrass is dynamic, and that the QS and QQ activities play a role in the assembly of functional communities, as reported in case of tobacco rhizosphere and granular sludge community (d'Angelo-Picard et al., 2005; Tan et al., 2015). However, the biotechnological significance of QQ bacteria renders the association of these isolates with seagrass less important.

Not all QQ-positive isolates completely degraded the C6-AHLs involved in CV026 bioassay (Supplementary Figures 2A, 3). Moreover, some isolates did not show reproducible QQ activity, and thus, this inconsistency (Saurav et al., 2016) warrants further exploration of the regulatory mechanisms of expression of QQ activity. Isolates that displayed QQ activity even after heat killing (data not shown) indicate that either QQ activity is non-enzymatic and/or the loss of AHLs was due to adsorption onto cellular debris. However, it could also be attributed to the fact that the QQ is enzymatic and that these enzymes are heat resistant. A recent study has shown that Aii20J, an AHL-lactonase from *Tenacibaculum* sp. 20J, can retain its activity even after heating up to 100°C for 10 min (Mayer et al., 2015). We will investigate the possibility of heat-resistant enzymes in our future studies.

Based on our findings (**Figures 1**, **2A**) and those of others (Romero et al., 2011; Torres et al., 2013; Tan et al., 2015), there appears to be a general feature: QQ bacteria capable of degrading small-chain AHLs can almost always degrade medium- and long-chain AHLs. A recent study, wherein 12 QQ bacteria were identified, showed that these bacteria could degrade a variety of different AHLs, but none of them could degrade C4-AHLs (Torres et al., 2016). This suggests that future studies in search of QQ bacteria should primarily focus on identifying bacteria capable of degrading small-acyl-chain AHLs.

Although QQ activity has been observed in either cell lysate or cell-free supernatant (Uroz et al., 2005; Shepherd and Lindow, 2009), to the best of our knowledge, it has not been detected in both fractions. The QQ activity found in both the cell lysate and supernatant of VG1 might represent a new class of QQ enzymes (Supplementary Figure 2B). However, it is possible that

QQ molecules were released into the supernatant during sample preparation. Our results showing that the cell lysates of VG1 retain QQ activity even after heating at 105°C (data not shown) appear to contradict the heat killing of whole cells that can result in the loss of QQ activity. The exact reason for this observation is unknown, but it is possible that in case of cell lysates, the QQ enzyme can reform its 3D structure when cooled after heating. Fractionation of VG1 cell lysates with the 10-kDa-filter rule out the possibility that QQ is caused by small-molecular-weight metabolites that could be heat resistant. Unexpectedly, for VG12 and NV9, QQ activity was lost upon cell lysis. It is possible that the QQ enzymes of these strains are sensitive to our methods of cell disruption (sonication) or that these enzymes need certain factors/conditions for their activity, which are lost on cell lysis.

All QQ-positive isolates identified in this study belong to Proteobacteria (Supplementary Figure 4 and **Table 1**). These results are consistent with those of previous reports, where majority of the QQ bacteria identified were also Proteobacteria (Romero et al., 2011; Tan et al., 2015; Saurav et al., 2016; Torres et al., 2016). This is not surprising because Proteobacteria is predominant in various marine environments (Gonzalez and Moran, 1997).

Although disputed, it has been suggested that AHL-acylases are not active against small-acyl-chain AHLs such as C4-AHLs (Shepherd and Lindow, 2009; Czajkowski et al., 2011). If this is correct, then degradation of C4-AHLs and restoration of the degraded 3OXOC10-AHLs after acidification suggest that the QQ activity observed in NV9 is primarily caused by lactonase (**Figures 2A,B**). Genomic annotation of NV9 identified one ORFs (NV9_000000104) (Supplementary Table 2) that belongs to the MBL superfamily, which could be responsible for the observed QQ activity. In VG12, although the acidification of degraded AHLs restored only 2% of AHLs (**Figure 2B**), the ability of VG12 to effectively degrade C4-AHLs suggests lactonase activity (**Figure 2A**). It is possible that, in case of VG12, the hydrolyzed lactone ring of 3OXOC10-AHLs was further modified and was unable to reform the lactone ring. Furthermore, the prediction of only AHL-lactonases in the genome sequence of VG12 (**Table 2** and Supplementary Table 2) suggests that lactonases are responsible for QQ activity. Similarly, the genome sequence of a close relative of VG12, namely, *Labrenzia alba* CECT 755, carries only AHL-lactonases (CTQ52848.1, CTQ54016.1, CTQ52453.1, CTQ55013.1, and CTQ55918.1); no AHL-acylase was detected. For VG1, although both AHL-lactonases and acylases are predicted in the genome sequence (**Table 2** and Supplementary Table 2), its inability to degrade C4-AHLs (**Figure 2A**) and inability to relactonize 3OXOC10-AHLs after acidification (**Figure 2B**) suggest that AHL-acylases are responsible for QQ activity in this case. Interestingly, unlike VG1, the genome annotation of *Erythrobacter* species such as *E. longus* strain DSM 6997 (GenBank: JMIW0000000.1), *Erythrobacter* sp. HL-111 (GenBank: LT629743.1), and *E. citreus* strain LAMA915 (GenBank: JYNE00000000.1) show only AHL-acylases (KEO91396.1, SDS44800.1, SDT09981.1, and KNH01491.1) while no AHL-lactonase was detected in these bacteria. However, this difference might be caused by the different annotation methods/pipelines used. It is important to note that some recently discovered QQ enzymes did not show any sequence homology to the typical AHL-lactonases and acylases (Torres et al., 2017). Hence, it remains possible that the observed QQ activity is caused by a new class of enzymes.

The time-course experiment showed that VG1 can quickly degrade AHLs, closely followed by VG12 (**Figure 3**). We used 3OXOC10-AHLs for this assay, and it is possible that slow degradation by NV9 reflects its specificity for AHLs with a certain kind of acyl chains.

None of the QQ isolates was able to completely inhibit biofilm formation (**Figure 4A**), may be because biofilm formation is a complex process involving many factors (Flemming et al., 2016).We also tested QQ isolates for their ability to degrade the 3OXOC12-AHLs produced by PAO1 (**Figure 4B**), because 3OXOC12-AHLs lie higher in the hierarchy of the QS signaling cascade and regulate the expression of other QS molecules (C4-AHLs), production of virulence factors, and formation of biofilms (Whitehead et al., 2001; Williams and Camara, 2009). The observed ineffective degradation of 3OXOC12-AHLs and biofilm inhibition might be caused by certain PAO1 metabolites that inhibited the QQ activity of our isolates. It is also possible that 3OXOC12-AHLs are not a preferred substrate for our QQ isolates. A significant reduction in biofilm formation by VG12 might be the result of effective degradation of C4-AHLs caused by this isolate (**Figures 2A**, **4A**). Based on our results, VG12 appears to be best isolate among others for the inhibition of biofilm formation and kinetics and diversity of AHL degradation (**Figures 1–3**, **4A**). It also appears to be the best candidate for future studies employing bacteria as an anti-biofouling agent.

Quorum quenching alone might not completely abolish bacterial infections and biofilms, but it can be used in combination with other antimicrobial agents to achieve desired results. Combinatorial therapies are gaining importance because no single therapy or drug can effectively control bacteria for longer time periods, given that the bacteria will eventually develop resistance (Fischbach, 2011). Furthermore, QQ enzymes can confer resistance against antibiotics (Kusada et al., 2017). Therefore, improved understanding of these enzymes will provide opportunities to overcome such resistance.

In this study, we found bacteria belonging to three different genera, namely, *Erythrobacter, Labrenzia*, and *Bacterioplanes,* that can degrade AHLs. Although extracted metabolite-based QQ activity has been described for *Erythrobacter* and *Labrenzia* (Saurav et al., 2016), the bacteria identified in this study represent a new species whose QQ activity has not been described before. We have identified potential QQ genes and our future studies will focus on cloning these genes and investigating their mechanism of action.

AUTHOR CONTRIBUTIONS

ZR and TL designed the experiments and wrote and revised the manuscript. ZR performed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01354/full#supplementary-material

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Simultaneous Carriage of *mcr-1* and Other Antimicrobial Resistance Determinants in *Escherichia coli* From Poultry

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The use of antimicrobial growth promoters (AGPs) in sub-therapeutic doses for long periods promotes the selection of resistant microorganisms and the subsequent risk of spreading this resistance to the human population and the environment. Global concern about antimicrobial resistance development and transference of resistance genes from animal to human has been rising. The goal of our research was to evaluate the susceptibility pattern to different classes of antimicrobials of colistin-resistant *Escherichia coli* from poultry production systems that use AGPs, and characterize the resistance determinants associated to transferable platforms. *E. coli* strains ($n = 41$) were obtained from fecal samples collected from typical Argentine commercial broiler farms and susceptibility for 23 antimicrobials, relevant for human or veterinary medicine, was determined. Isolates were tested by PCR for the presence of *mcr-1*, extended spectrum β-lactamase encoding genes and plasmid-mediated quinolone resistance (PMQR) coding genes. Conjugation and susceptibility patterns of the transconjugant studies were performed. ERIC-PCR and REP-PCR analysis showed a high diversity of the isolates. Resistance to several antimicrobials was determined and all colistin-resistant isolates harbored the *mcr-1* gene. CTX-M-2 cefotaximase was the main mechanism responsible for third generation cephalosporins resistance, and PMQR determinants were also identified. In addition, co-transference of the *qnrB* determinant on the *mcr-1*-positive transconjugants was corroborated, which suggests that these resistance genes are likely to be located in the same plasmid. In this work a wide range of antimicrobial resistance mechanisms were identified in *E. coli* strains isolated from the environment of healthy chickens highlighting the risk of antimicrobial abuse/misuse in animals under intensive production systems and its consequences for public health.

Keywords: Colistin, *mcr-1*, food-borne bacteria, *Escherichia coli*, CTX-M-2, *qnrB*, multi drug resistance

INTRODUCTION

Antimicrobial agents have been used extensively for prevention and treatment of infectious diseases in food animals (Dibner and Richards, 2005; Niewold, 2007). The concomitant risk of spreading antibiotic resistance to human population through the food supply chain and the environment is important since many classes of these antimicrobial agents are also used in human medicine. Therefore, increased global concern regarding development of antimicrobial resistance and transference of resistance genes from animals to humans has been rising (Ljungquist et al., 2016; Madec et al., 2017; Wang et al., 2017).

Various antimicrobials have been widely used by the poultry industry as antibiotic growth promoters (AGPs) since the 1950s. To reduce costs of production, AGPs have been added into feed to promote weight gain by optimizing feed conversion ratios (Moore and Evenson, 1946; Jukes et al., 1950). In contrast to therapeutic usages of antimicrobials that are administered at high doses for a limited period of time, AGPs are used in sub-therapeutic doses during longer periods. This situation is particularly favorable for the selection of resistant microorganisms (Diarra et al., 2007).

Any use of antimicrobial agents may contribute to clinical relevant antimicrobial resistance. One of the first findings that led to strong recommendations (and even banning) for the use of AGP in the European Union (EU) was the finding that administration of avoparcin, a glycopeptide AGP, was involved in emerging glycopeptide-resistant bacteria (Howarth and Poulter, 1996). In the same way, use of colistin as an AGP in livestock led to the emergence and silent dissemination of plasmid-mediated mechanisms involved with polymyxin resistance (Rhouma et al., 2016). International organizations responsible for human, animal health, and food production (World Health Organization-WHO/World Organization for Animal Health-OIE/ Food and Agriculture Organization-FAO) carried out systematic evaluations on the impact of veterinary antimicrobial resistance on public health, and they stated that the misuse and overuse of antimicrobials is accelerating the processes of antimicrobial resistance. As a result, this topic is now considered as one of the critical issues in developed and developing countries as indicated by the United Nations General Assembly in 2016.

As part of a technical support program to national poultry producers, our team conducted studies to understand the antimicrobial resistance evolution in food-borne bacteria under commercial production systems in Argentina. Our studies included the selection of *Escherichia coli* as an indicator microorganism and concluded that almost 50% of the strains were found to be resistant to colistin used as AGP (Dominguez et al., 2017) which was much higher than reported in studies published previously. Therefore, the aim of this work was to evaluate the susceptibility pattern to different classes of antimicrobials of colistin-resistant *E. coli* isolated from poultry production systems that use AGP, and to characterize the resistance determinants associated to transmissible elements.

MATERIALS AND METHODS

Sampling and *E. coli* Isolation

Fresh fecal samples were collected from 129 commercial broiler farms located in the most relevant production areas of Argentina (Entre Rios and Buenos Aires Provinces). At the moment of the sampling, healthy 4–6 week-old broiler chickens were at the end of the rearing cycle in the farms (Dominguez et al., 2017). Each *E. coli* strain was isolated from a pool of 10 feces samples collected in different sections of each barn. All samples were placed into boxes containing ice packs and immediately transported to the laboratory to isolate the microorganism by culture on non-antibiotic-supplemented MacConkey agar plates at 37°C for 18–24 h. Isolates were initially selected by the morphology of the colonies and further identified by standard biochemical tests (Brenner and Farmer, 2015). According to the size of the farms, a fixed number of isolates were arbitrarily selected: 2 isolates from small (less than 50,000 birds), 3 from medium (between 50,000 and 150,000 birds) and 6 from large (more than 150,000 birds) farms. Overall 304 *E. coli* isolates were obtained (Dominguez et al., 2017). In the present study a subset of 31 strains resistant to colistin and 10 susceptible -according to EUCAST criteria- were analyzed (EUCAST 2017)[1]. These strains were isolated from 11 farms belonging to 3 different integrated companies located at Entre Rios and Buenos Aires Provinces.

Phenotypic Antimicrobial Susceptibility Testing

Antibiotic susceptibility was determined by agar disk diffusion test against 23 antibiotics representing seven antimicrobial classes, commonly used in human and veterinary medicine. Antimicrobial susceptibility was determined for the following agents:

- ß-lactams including:
  - Penicillins: Ampicillin (AMP), Amoxicillin-Clavulanic Acid (AMC)
  - Second generation cephalosporins: Cefuroxime (CXM)
  - Third generation cephalosporins (TGC): Ceftiofur (CFT), Cefotaxime (CTX), Ceftriaxone (CRO), Ceftazidime (CAZ)
  - Cephamycins: Cefoxitin (FOX)
  - Fourth generation cephalosporins: Cefepime (FEP)
  - Monobactams: Aztreonam (ATM)
  - Carbapenems: Imipenem (IMI), Meropenem (MEM)
- Aminoglycosides: Kanamycin (KAN), Gentamicin (GEN), Amikacin (AMI), Streptomycin (STR)
- Tetracyclines: Tetracycline (TET)
- Quinolones: Nalidixic Acid (NAL), Ciprofloxacin (CIP), Enrofloxacin (ENR)
- Sulfonamides: Trimethoprim-Sulfamethoxazole (SXT)
- Phenicols: Chloramphenicol (CLR)
- Polymyxins: Colistin (COL)

The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria, (CLSI, 2017) and

[1]http://www.eucast.org

TABLE 1 | Targets, primers, sequence, and product size used for PCR and sequencing of *mcr-1*, BLEE, ESBL, AmpC, and PMQR genes.

Targets	Primers	Nucleotide Secuence (5′-3′)	Size (bp)	References
mcr-1	CLR5-F	CGGTCAGTCCGTTTGTTC	344	Liu et al., 2016
	CLR5-R	CTTGGTCGGTCTGTA GGG		
$bla_{CTX-M-like}$	CTX-M GRAL F	ATGTGCAGYACCAGTAARGTKATGGC	500	Ghiglione, 2015
	CTX-M GRAL R	CCGCTGCCGCTYTTATCVCCBAC		
$bla_{CTX-M-group1}$	CTX-M-1 CF	ATGGTTAAAAAATCACTGC	864	Saba Villarroel et al., 2017
	CTX-M-1 CR	GGTGACGATTTTAGCCGC		
	CTX-M-1 FpK	AAATGGTTAAAAAATCACTGC	876	Ghiglione, 2015
	CTX-M-1 RpK	CTACAAACCGTCGGTGACGAT		
$bla_{CTX-M-group2}$	CTX-M-2 FpK	TAATGATGACTCAGAGCATTCGC	900	Ghiglione, 2015
	CTX-M-2 RpK	GCATCAGAAACCGTGGGTTACG		
	CTX-M-2 CF	TTAATGATGACTCAGAGCATTC	910	Bertona et al., 2005
	CTX-M-2 CR	GATACCTCGCTCCATTTATTGC		
$bla_{CTX-M-group8}$	CTX-M-8 CF	TGAATACTTCAGCCACACG	923	Saba Villarroel et al., 2017
	CTX-M-8 CR	TAGAATTAATAACCGTCGGT		
	CTX-M-8 FpK	AGATGATGAGACATCGCGTTAAGC	1184	Ghiglione, 2015
	CTX-M-8 RpK	TTAATAACCGTCGGTGACG		
$bla_{CTX-M-group9}$	CTX-M-9 CF	ATGGTGACAAAGAGAGTGC	876	Saba Villarroel et al., 2017
	CTX-M-9C R	TCACAGCCCTTCGGCGATG		
	CTX-M-9 FpK	AGATGGTGACAAAGAGAGTGC	876	Ghiglione, 2015
	CTX-M-9 RpK	TTACAGCCCTTCGGCGATG		
$bla_{CTX-M-group25}$	CTX-M-25 CF	ATGAGAMAWMGCGTWARGC	878	Saba Villarroel et al., 2017
	CTX-M-25 CR	TAGAATTAATAACCGTCGGTGAC		
bla_{pAmpC}	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520	Cejas et al., 2012
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C		
	CITMF	TGG CCA GAA CTG ACA GGC AAA	462	
	CITMR	TTT CTC CTG AAC GTC GCT GGC		
	DHAMF	AAC TTT CAC AGC TGT GCT GGG T	405	
	DHAMR	CCG TAC GCA TAC TGG CTT TGC		
	ACCMF	AAC AGC CTC AGC AGC CGG TTA	346	
	ACCMR	TTC GCC GCA ATC ATC CCT AGC		
	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302	
	EBCMR	CTT CCA CTG CGG CTG CCA GTT		
	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190	
	FOXMR	CAA AGC GCG TAA CCG GAT TGG		
bla_{CMY-2}	CMY -F	ATGATGAAAAAATCGTTATGCT	1146	
	CMY-R	TTATTGCAGCTTTTCAAGAATGCG		
qnrA	qnrA-F	AGAGGATTTCTCACGCCAGG	580	Cruz et al., 2013
	qnrA-R	TGCCAGGCACAGATCTTGAC		
qnrS	qnrS-F	GCAAGTTCATTGAACAGGGT	428	
	qnrS-R	TCTAAACCGTCGAGTTCGGCG		
qnrC	qnrC-F	GGGTTGTACATTTATTGAATCG	330	
	qnrC-R	CACCTACCCATTTATTTTCA		
qnrD	qnrD-F	CGAGATCAATTTACGGGGAATA	582	
	qnrD-R	AACAAGCTGAAGCGCCTG		
qnrB	qnrB-F	GGMATHGAAAATCGCCACTG	264	
	qnrB-R	TTTGCYGYYCGCCAGTCGAA		

(Continued)

TABLE 1 | Continued

Targets	Primers	Nucleotide Secuence (5′-3′)	Size (bp)	References
	qnrBIF-F	ATGWYGYCATTACTGTATA	676	
	qnrBIF-R	CCMATHAYMGCGATRCCAAG		
	qnrBcf-F	GTTRGCGAAAAAATTRACAG	626	
	qnrBIF-R	CCMATHAYMGCGATRCCAAG		
qepA	qepA-F	ACATCTACGGCTTCTTCGTCG	501	
	qepA-R	AACTGCTTGAGCCCGTAGATC		
acc(6′)-Ib	aac(6′)Ib-F	CGATCTCATATCGTCGAGTGTT	447	
	aac(6′)Ib-R	TTAGGCATCACTGCGTGTTC		
oqxA	oqxA-F	CTCGGCGCGATGATGCT	393	
	oqxA-R	CCACTCTTCACGGGAGACGA		
oqxB	oqxB-F	TTCTCCCCCGGCGGGAAGTAC	513	
	oqxB-R	CTCGGCCATTTTGGCGCGTA		

(CLSI, 2013). Susceptibility to colistin was evaluated by broth microdilution and results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST).

E. coli strains resistant to three or more antimicrobial classes were categorized as multidrug resistant (MDR). Phenotypic screening for extended spectrum β-lactamase (ESBL) and plasmid mediated AmpC (pAmpC) was conducted performing synergy test using cefotaxime/clavulanic acid (CTX/CLA, 30/10 μg), ceftazidime/clavulanic acid (CAZ/CLA, 30/10 μg) and phenyl-boronic acid (PBA, 300 μg) containing disks, respectively (Yagi et al., 2005; CLSI, 2017). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were included as control.

Molecular Analysis of Resistance

All strains were tested by PCR for the presence of transferable resistance markers (*mcr-1*, ESBL, *pAmpC*, and plasmid mediated quinolone resistance—PMQR- coding- genes) using primers listed in **Table 1**. In the case of *mcr-1* detection, the full *mcr-1* gene was amplified and sequenced by using CLR5-F in combination with MCR1-R (5′-TGCGGTCTTTGACTTTGTC) (this study). Total DNA was obtained by boiling bacterial suspensions and plasmid DNA was purified according to Kado and Liu method (Kado and Liu, 1981).

Plasmid Conjugation Studies

To assess *mcr-1* plasmid transferability, conjugation studies by liquid mating were performed. *Salmonella* M1744 and *E. coli* J53 strains were used as recipient and randomly chosen *mcr-1*-positive strains from each farm were used as donors. After the conjugation, the transconjugants obtained from *Salmonella* M1744 were selected in TSA media supplemented with colistin (2 μg/mL), whereas those obtained from *E. coli* J53 were selected with sodium azide (200 μg/mL) and colistin (1 μg/mL). To confirm successful conjugation, colonies obtained in the selective media were screened for *mcr-1* gene by PCR and then colistin MIC was determined for both transconjugant and parental *E. coli* strains by the broth microdilution as described before. In addition, co-resistance to other antimicrobials was assessed by agar disk diffusion method as previously described.

Molecular Typing by PCR-Based Techniques

Clonality of the isolates was determined by the homology relationships among fragments amplified by ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus) and REP-PCR (Repetitive Extragenic Palindromic) according to Versalovic et al. (1991). Dendrograms were constructed by GelJ 1.0 program, using UPGMA algorithm and applying the DICE correlation coefficient.

Statistical Analysis

Significant differences ($p < 0.05$) in the association among strains according to the presence of genes were determined by Pearson's Chi-squared test with Yates continuity correction using Epidat software (version 4.1).

RESULTS AND DISCUSSION

Resistance to Colistin and *mcr*−1 Gene Detection

The presence of *mcr-1* in Argentina was already detected in *E. coli* isolates recovered from invasive infections in humans (Rapoport et al., 2016) and has also been found in bacteria isolated from domestic animals (Dominguez et al., 2017). The *E. coli* strains included in the present report were classified in the base of their susceptibility to colistin following the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017). All strains considered resistant to colistin harbored the *mcr-1* gene as demonstrated by PCR, and the sequenced gene was identical to the previously published sequence, accession number KP347127.1 (Liu et al., 2016). Additionally, from the 10 strains classified as colistin-susceptible, 3 of them were positive for the *mcr-1* gene (**Table 2**).

From the 31 colistin resistant/*mcr-1*-positive *E. coli* strains, 28 showed MICs ranging from 4 to ≥ 32 μg/mL. Although the disk

TABLE 2 | Characteristics of *Escherichia coli* recovered from different farms in Buenos Aires and Entre Ríos, Argentina, 2014.

Provinces	Farms	Strain	MIC to colistin (μg/mL)	Resistance determinant*							
				mcr-1	*AmpC*	*bla*$_{CTX-M}$	*qnrA*	*qnrB*	*qnrD*	*qnrS*	*oqxAB*	*qepA*
Buenos Aires	1	*E. coli* 190-02	2 (S)									
		E. coli 241-S1	8 (R)			CTX-M-14						
		E. coli 241-S3	1 (S)									
	2	*E. coli* 190-06	0.5 (S)									
		E. coli 241-S2	1 (S)									
		E. coli 241-S4	1 (S)			CTX-M-2						
	3	*E. coli* 241-L1	8 (R)									
		E. coli 241-L2	2 (S)			CTX-M-2						
		E. coli 190-08	8 (R)		CMY-2	CTX-M-2						
		E. coli 190-10	8 (R)			CTX-M-2+ CTX-M14						
		E. coli 241-L3	0,5 (S)			CTX-M-2						
	4	*E. coli* 190-13	8 (R)			CTX-M-2+ CTX-M14						
		E. coli 190-14	8 (R)			CTX-M-2						
		E. coli 241-P2	4 (R)			CTX-M-2						
		E. coli 241-P3	8 (R)		CMY-2							
		E. coli 241-P4	2 (S)			CTX-M-2+ CTX-M14						
		E. coli 241-P1	8(R)			CTX-M-2						
	5	*E. coli* 190-15	8(R)									
		E. coli 190-16	4 (R)									
		E. coli 241-Z1	8 (R)			CTX-M-2						
		E. coli 241-Z2	16 (R)			CTX-M-2						
		E. coli 241-Z3	8(R)			CTX-M-2						
	6	*E. coli* 190-17	8 (R)		CMY-2	CTX-M-2						
		E. coli 190-18	4 (R)			CTX-M-2						
		E. coli 241-K1	8 (R)			CTX-M-14						
		E. coli 241-K2	8 (R)			CTX-M-2						
		E. coli 241-K3	2 (S)			CTX-M-2						
		E. coli 241-K4	8 (R)			CTX-M-2+ CTX-M14						
	7	*E. coli* 241-B2	2 (S)									
		E. coli 241-B3	8 (R)			CTX-M-2						
		E. coli 241-B1	4(R)			CTX-M-2						
Entre Ríos	8	*E. coli* 191-08	8 (R)			CTX-M-2						
		E. coli 191-07	8 (R)		CMY-2	CTX-M-2						
	9	*E. coli* 191-11	8 (R)									
		E. coli 191-12	32 (R)									
		E. coli 191-13	16 (R)									
	10	*E. coli* 191-16	4 (R)									
		E. coli 191-17	8 (R)			CTX-M-2						
	11	*E. coli* 191-21	8 (R)			CTX-M-2						
		E. coli 191-23	32 (R)			CTX-M-2+ CTX-M14						
		E. coli 191-22	8 (R)			CTX-M-2						

**The squares in gray indicate presence of the gene; while the squares in white indicate absence of the studied gene. (R) resistant and (S) susceptible by MIC determinations with colistin.*

diffusion method (10 μg colistin disk) is not yet standardized for polymyxins, all 28 strains displayed colistin inhibition zone ≤11 mm. The remaining strains (3/31) showed MICs between 4 and 8 μg/mL but nevertheless displayed inhibition zones ≥ 11 mm with colistin. Although molecular detection is the most appropriate technique for *mcr-1* identification, a strong association with the phenotypic methodologies to detect *mcr-1*-mediated colistin resistance was observed.

Previous works describe the transferable nature of the *mcr-1* gene (Liu et al., 2016). In the present work, the *mcr-1*-mediated colistin resistance was successfully transferred by conjugation to both recipient laboratory strains (*E. coli* J53

and *Salmonella* M1744). Two out of ten *mcr-1*-positive strains from each farm (**Table 3**) were obtained from liquid mating experiments performed using poultry *E. coli* strains as plasmid donors. Plasmids carrying the *mcr-1* gene conjugated at a transfer frequency of $\sim 1.5 \times 10^{-3}$ transconjugants per donor cell. Accordant to results obtained by Liu et al. (2016), we found that MIC for colistin of the transconjugants increased four- and eight-fold compared to the original recipient strains.

Molecular typing analysis by the ERIC-PCR and REP-PCR showed that all *E. coli* carrying *mcr-1* gene from this study had high clonal diversity and thus considered as genetically unrelated **Figure S1**. These results are in concordance with previous reports that describe the wide distribution of the *mcr-1* gene among *E. coli* isolates independently of bacterial source or host species, suggesting a non-clonal spread of colistin resistance (Fernandes et al., 2016; Rapoport et al., 2016). In addition, this study reports a successful plasmid–gene combination of these *E. coli* strains in healthy broiler chickens, which may play a role in the emergence and spread of this gene.

Resistance to Other Antimicrobials

Resistance to Fluoroquinolones and Detection of Plasmid-Mediated Quinolone Resistant (PMQR) Genes

Further determinations of antimicrobial susceptibility of *mcr-1*-positive strains demonstrated high rates of multidrug resistance, since 85% (29/34) of the tested strains were resistant to at least three different classes of antimicrobial agents (**Figure 1**).

Simultaneous resistance to colistin and quinolones or fluoroquinolones was relatively high (**Figure 1A**), since 94% (32/34) of the *mcr-1*-positive strains were resistant to nalidixic acid (NAL), 67.6% (23/34) to ciprofloxacin (CIP), and 76.5% (26/34) to enrofloxacin (ENR). Almost three from every four strains (76.5%) harbored a PMQR marker and the most prevalent determinants were *qnrS* (20/34) and *qnrB* (18/34). Almost three from every four strains (76.5%) harbored a PMQR marker and the most prevalent determinants were *qnrS* (20/34) and *qnrB* (18/34). Other PMQRs such as *qnrA* (2/34), *qnrD* (1/34) and the efflux pumps *oqxAB* (5/34) and *qepA* (5/34) were also identified. These results are consistent with the analysis made by Huang et al. (2009) in isolates from China, who also found a high ratio of *E. coli* strains harboring PMQR determinants and the authors suggest that this fact may be related to the extended use or misuse of antimicrobials in poultry.

Although no significant genotypic relation ($p > 0.05$) was found between *mcr-1*-positive strains and plasmid mediated quinolone resistance genes (PMQR), results obtained in conjugation experiments suggest that fluoroquinolone and colistin resistance can be simultaneously co-transferred, since both transconjugants (EC 190-14 TC and EC 191-07 TC or EC 191-07 TCS) displayed decreased susceptibility to NAL and were positive for *qnrB* gene detection (**Table 3**). However, the large number of strains carrying genetic determinants for fluoroquinolones in healthy broilers was relatively high; this scenario suggests that other selective forces such as colistin used as AGP (Morales et al., 2012) or therapeutic antimicrobial misuse are driving the selection of fluoroquinolone-resistant bacteria.

Resistance to β-Lactams and Detection of Extended Spectrum β-Lactamase (ESBL) and Plasmidic AmpC β-Lactamase

The antimicrobial susceptibility analysis showed a relatively high percentage of AMP resistance 82.4% (28/34) among the *mcr-1*-positive strains and a strong relation between susceptibility to both antimicrobials as determined by disk diffusion tests (R: 0.33, $p < 0.05$). Considering the susceptibility showed to AMP, a high percentage of resistance (between 76.5 and 79.4%) was also observed in oxyimino-cephalosporins (CTX, CRO and CFT, a cephalosporin used in veterinary medicine) and FEP (70.6%). However, very little resistance to CAZ and FOX was detected, while all isolates remained susceptible to carbapenems (IMI and MEM) (**Figure 1B**). In contrast, most clinical *E. coli* strains were found to be susceptible to a wide range of antimicrobials, including carbapenems (Lai et al., 2017).

TABLE 3 | Plasmid conjugation studies.

Strains	MIC to colistin (μg/mL)	Disk diffusion test*		PCR
		NAL	CIP	*mcr-1*/PMQR/CTX-M
E. coli 190-14	8	R	R	*mcr-1*+ *qnrB*+*qnrS*+CTX-M-2
EC190-14 TC**	4	I	S	*mcr-1*+ *qnrB*
E. coli 191-07	8	I	S	*mcr-1*+ *qnrB*+ CTX-M-2+ CMY-2
EC191-07 TC**	4	I	S	*mcr-1*+ *qnrB*
E. coli J53	0,5	S	S	–
EC191-07 TCS***	8	I	S	*mcr-1*+ *qnrB*
Salmonella M1744	1	S	–	–

*(R) resistant, (I) intermediate and (S) susceptible by disk diffusion test: Nalidixic Acid (NAL), Ciprofloxacin (CIP).

**TC: transconjugants obtained using E. coli J53 as the recipient strain.

***TCS: transconjugants obtained using Salmonella M1744 as the recipient strain.

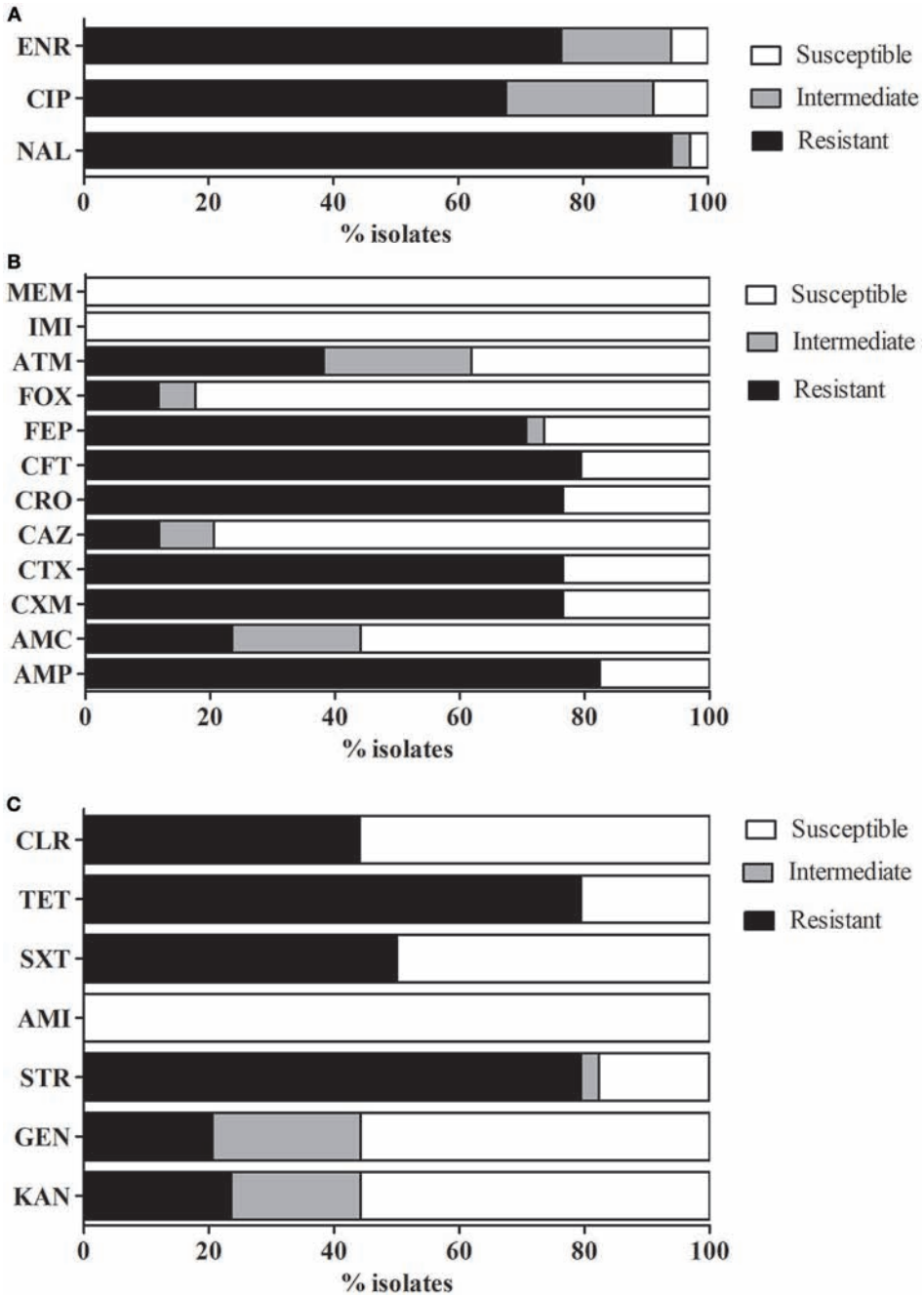

FIGURE 1 | Antimicrobial susceptibility profiles. Percentage of antimicrobial susceptibility in the isolates analyzed. **(A)** Fluoroquinolones, **(B)** β-lactams, and **(C)** Other antimicrobials. AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CFT, ceftiofur; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; IMI, imipenem; MEM, meropenem; NAL, nalidixic acid; CIP, ciprofloxacin; ENR, Enrofloxacin; KAN, kanamycin; GEN, gentamicin; STR, streptomycin; AMI, amikacin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; and CLR, chloramphenicol.

CTX-M-producing enterobacteria are widespread among human population and an increasing number of reports describes their presence in livestock environments as well as in food from animal origin (Lazarus et al., 2015). Our findings demonstrate that also healthy birds may act as a reservoir of $bla_{\mathrm{CTX-M-2}}$ and $bla_{\mathrm{CTX-M-14}}$ genes. In the recent past, CTX-M-2 was the dominant ESBL group among human clinical *Enterobacteriaceae* isolates in South America (Quinteros et al., 2003; Minarini et al., 2007; Saba Villarroel et al., 2017). From 26 extended- spectrum cephalosporins (ESC)-resistant and *mcr-1*-positive strains, 18 strains (18/34, 56%) were CTX-M-2 producers and two produce CTX-M-14. Five strains harbored both CTX-M genes. CMY-2

was identified in 4 strains (3 were also CTX-M-2 producers) (**Table 2**). According to these results, the main mechanism responsible for TGC resistance was the production of CTX-M cefotaximases which explained the low resistance rates to FOX and CAZ. ESBLs from groups CTX-M-2 and CTX-M-14 were previously identified in *mcr-1*-carrying *E. coli* recovered from human samples (Rapoport et al., 2016) and from wild birds (kelp gulls) in the south of Argentina (Liakopoulos et al., 2016).

The cosmopolitan CTX-M-15 variant belonging to the CTX-M-1 subfamily, which is also widespread in human clinical isolates from Argentina, (Sennati et al., 2012), could not be found in this study. This finding was unexpected since reports from Brazil, where poultry productive systems are similar to Argentina (Botelho et al., 2015), described the presence of the CTX-M-15 ESBL and the coexistence of CTX-M-8 and CMY-2 in *E. coli* isolates recovered from chicken meat.

Many studies in *E. coli* strains, most of them involving isolates from animals, have demonstrated the presence of *mcr-1* gene together with ESBL (Rhouma and Letellier, 2017). In the present work, despite the presence of the CTX-M-2 gene in the parental strain, no co-selection of ESC-resistant was observed in the transconjugants (**Table 3**).

Although 50% of the *E. coli* strains analyzed carried both sets of ESBL and PMQR genes, no association between the presence of ESBL and a specific PMQR mechanism ($p > 0.05$) was observed. Additionally, *aac (6′)-Ib-cr* gene was not detected. It is remarkable the absence of *aac (6′)-Ib-cr* gene, and the lack of association between ESBL and PMQR which is usually found in some *Enterobacteriaceae* isolated from human (Andres et al., 2013; Cruz et al., 2013) in Argentina.

A large variability of PMQR determinants was also observed in TGC-sensitive (without ESBL) and *mcr-1*-positive strains with a similar proportion of *qnrB* and *qnrS* genes. To a lesser extent, some of these strains also showed *oqxAB* gene. According to the results of this study, we suggest that *E. coli* strains from broiler chickens could be the reservoir not only of the *mcr-1* gene, but also of PMQR and ESBL genes.

Resistance to Other Antimicrobials-Multiple Drug Resistance (MDR)

Most of the *mcr-1*-positive strains were determined to carry ESBL or PMQR-genes and also most of them were resistant to other classes of antimicrobial agents. This is probably due to the fact that the aforementioned genes are commonly found in mobile elements such as conjugative plasmids that also harbor resistant determinants to different groups of antimicrobials and confer the MDR phenotype. It is of particular concern that 39/41 (95.1%) strains considered in this study (including *mcr-1*-negative strains) expressed a multi-resistance phenotype.

The percentage of strains resistant to aminoglycosides and *mcr-1*-positive strains was variable and drug dependent. Resistance rates to this family was STR>KAN (79.4%, 23.6%) and GEN (20.6%). All strains remained susceptible to AMI (**Figure 1C**). Resistance to TET (79.4%) was relatively high, as expected considering the extensive use in animal medicine, which is in concordance with previous studies were TET resistance markers are frequently found in *E. coli* strains (Argudín et al., 2017). To a lesser extent, also resistance to SXT (50%) and CLR (44%) was also detected.

CONCLUSIONS

The results highlight that commercial broiler farms can be an important reservoir of *mcr*-1-carrying *E. coli* strains. In fact, the high occurrence of *E. coli* isolates (76%) carrying the *mcr-1* gene is alarming and has not been reported in any other part of the world (Delgado-Blas et al., 2016; Fernandes et al., 2016; Kawanishi et al., 2017; Meinersmann et al., 2017; Monte et al., 2017; Whang et al., 2018). These differences could be associated with the method of screening used in the present report since a higher number of unrelated farms separated from a relatively high distance were considered. Although this may be related to particularities of the productive system, the local practices are quite similar to the ones from other countries in South America that were administering colistin without any restriction. A potential combination of antibiotics used in the productive system, climatic variations and other variables, may influence the spread of *mcr-1* and these scenarios could also contribute to the selection of multi-resistant bacteria.

In this study, we determined the presence of resistance determinants in colistin-resistant *E. coli* strains from the environment of an intensive production system such as broiler chickens destined to consumption. A wide range of phenotypic resistance to both antibiotics in veterinary and human medicine was identified and resistance to colistin, quinolones and β-lactams was observed in the analyzed strains. The proportion of resistance to other antimicrobial families (SXT, TET, CLR, and aminoglycoside) was relatively high, underlining the presence of a large number of isolates with a MDR profile. The ability the co-transference of the *qnrB* determinant on the *mcr-1*-positive transconjugants was corroborated, which suggests that these resistance genes are likely to be located in the same plasmid thus transforming it into a more successful clone.

AUTHOR CONTRIBUTIONS

JD, LR, GG, JC, and MF participated in the design of the study. JD, RF, and DC performed the experiments. JD, LR, JC, and MF analyzed the data. JD, LR, PC, and MF collected *E. coli* strains. JD, LR, PC, JC, and MF wrote the paper. All authors contributed to the critical revision of the manuscript and have seen and approved the final draft. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01679/full#supplementary-material

Figure S1 | Phylogenetic tree and patterns of ERIC-PCR on agarose gel electrophoresis.

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Acinetobacter nosocomialis: Defining the Role of Efflux Pumps in Resistance to Antimicrobial Therapy, Surface Motility, and Biofilm Formation

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Acinetobacter nosocomialis is a member of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex. Increasingly, reports are emerging of the pathogenic profile and multidrug resistance (MDR) phenotype of this species. To define novel therapies to overcome resistance, we queried the role of the major efflux pumps in *A. nosocomialis* strain M2 on antimicrobial susceptibility profiles. *A. nosocomialis* strains with the following mutations were engineered by allelic replacement; Δ*adeB*, Δ*adeJ,* and Δ*adeB/adeJ*. In these isogenic strains, we show that the Δ*adeJ* mutation increased susceptibility to beta-lactams, beta-lactam/beta-lactamase inhibitors, chloramphenicol, monobactam, tigecycline, and trimethoprim. The Δ*adeB* mutation had a minor effect on resistance to certain beta-lactams, rifampicin and tigecycline. In addition, the Δ*adeJ* mutation resulted in a significant decrease in surface motility and a minor decrease in biofilm formation. Our results indicate that the efflux pump, AdeIJK, has additional roles outside of antibiotic resistance in *A. nosocomialis.*

Keywords: *Acinetobacter*, RND-efflux, motility, biofilm, antimicrobial resistance

INTRODUCTION

Acinetobacter nosocomialis is a Gram-negative opportunistic pathogen that is grouped into the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex (Nemec et al., 2011; Visca et al., 2011). The ability of *A. nosocomialis* to cause disease in humans is well-recognized (Wisplinghoff et al., 2012; Chusri et al., 2014; Huang et al., 2014), although studies suggest the virulence of this bacterium may be lower than the closely related bacterium *Acinetobacter baumannii* (Peleg et al., 2012; Lee et al., 2013; Yang et al., 2013; Fitzpatrick et al., 2015). Many potential virulence factors have been identified in *A. nosocomialis* and include a CTFR inhibitory factor (Cif), a protein O-glycosylation system, a type-I secretion system, a type-II secretion system, secretion of outer membrane vesicles, the OmpA protein, the CpaA protease, and quorum sensing (Niu et al., 2008; Bahl et al., 2014; Harding et al., 2015, 2016, 2017; Nho et al., 2015; Weber et al., 2015; Kim et al., 2016; Kinsella et al., 2017).

A. nosocomialis strain M2 was isolated in 1996 from a hip infection and has been extensively studied, particularly with respect to the virulence factors described above. M2 was formerly classified as *A. baumannii,* but whole genome sequencing resulted in its reclassification (Carruthers et al., 2013). While *A. nosocomialis* can be highly resistant to antibiotics, the role of RND-type efflux pumps in this process has not been investigated in this bacterium. Two primary efflux systems in the closely related *A. baumannii* are the AdeABC and AdeIJK efflux systems (Magnet et al., 2001; Damier-Piolle et al., 2008). Each efflux system is composed of an outer membrane channel (AdeC, AdeK), a membrane fusion protein (AdeA, AdeI) and an inner membrane transporter (AdeB, AdeJ). In addition to the efflux of antimicrobials, these systems can impact additional phenotypes in the cell, such as surface motility, biofilm formation, and virulence (Yoon et al., 2015; Richmond et al., 2016).

In this study, we investigated the role of AdeABC and AdeIJK orthologs in *A. nosocomialis*. Similar to what is observed in *A. baumannii,* loss of AdeIJK had a major impact on antibiotic susceptibility profiles. In contrast, the loss of AdeABC had a minimal impact on susceptibility. Interestingly, the loss of AdeIJK reduced surface motility, indicating additional roles for this RND-type efflux system in *A. nosocomialis*.

MATERIALS AND METHODS

Bacterial Growth Conditions, Strains, and Plasmids

A. nosocomialis strain M2 was used for all studies and has been described previously (Carruthers et al., 2013). *E. coli* strains EC100D and CC118 were used for general cloning. *E. coli* strain SM10 was used for bacterial conjugations. Growth media consisted of 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter. Agar was added at 15 g per liter. For sucrose counter-selections, media was prepared as described above, but without NaCl and containing 10% sucrose. Cloning vectors were pBC.SK- (Agilent) and pKNG101 (Kaniga et al., 1991).

Construction of *adeB* and *adeJ* Mutations

Internal fragments of the *adeB* and *adeJ* genes were obtained by PCR amplification of M2 genomic DNA using the following primers. peg93.for 5′- TTGCTAAGTATTCCTAAATTAC-3′ and peg93.rev 5′- TTAGGAAGAGATTTTTTTC−3′ for *adeB,* and peg1681.for 5′- ATGGCACAATTTTTTATTCATC−3′ and peg1681.rev 5′- TCACGATTTATGCTCCTGAG-3′ for *adeJ*. The resulting PCR generated fragments were cloned into the pBC.SK digested plasmid with SmaI, creating padeB and padeJ. The padeB plasmid was then digested with NarI, which digests once in the middle of the *adeB* gene and treated with T4 DNA polymerase to create blunt ends. This was then re-ligated to create a frameshift mutation in *adeB*. The plasmid padeJ was digested with SphI, which cuts once in the middle of *adeJ*, treated with T4 DNA polymerase to create blunt ends and re-ligated to create an *adeJ* frameshift mutation. The mutated *adeB* and *adeJ* genes were then excised as an XbaI-SalI fragment and cloned into the suicide vector pKNG101 digested with XbaI and SalI. Each plasmid was transformed into *E. coli* SM10 and then introduced into the *A. nosocomialis* M2 chromosome by conjugation. Exconjugants were grown for 10 generations in LB broth without antibiotic and dilutions were plated on lysogeny broth (LB) plates without sodium chloride and containing 10% sucrose. Colonies containing the *adeB* or *adeJ* frameshift mutations were identified by PCR amplifying each gene and the digesting the resulting PCR products with either NarI for *adeB* or SphI for *adeJ*. The presence of each chromosomal mutation was indicated by the failure of each enzyme to digest the fragment and each mutation was verified by DNA sequence analysis. To create an *adeB, adeJ* double mutant, the *adeB* mutant was used as the parent and the *adeJ* mutation was crossed into the chromosome as described above. To create an *adeB::Km* mutation, an EZ-Tn5<Kan-2> insertion centrally located in the *adeB* gene present in pKNG101 was recombined into the chromosomal copy of *adeB* as described above.

Antimicrobial Susceptibility Testing

A. nosocomialis strain M2 and its isogenic derivatives were subject to antimicrobial susceptibility testing using E-Test Strips, Trek, and MicroScan platforms. Additionally, disk diffusion assays were performed using Mueller Hinton agar for several antibiotics alone and in combination with boronic acid transition state inhibitor (BATSI) compounds SM23 and S02030 (Powers et al., 2014; Nguyen et al., 2016). For TREK, strains were tested once. For the disc diffusion and Etest assays, strains were tested in duplicate.

Motility Assays

The base media for motility assays consisted of 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter. Media was solidified using 0.35% Eiken agar (Eiken Chemical Ltd. Tokyo, Japan). Plates were used the same day they were prepared. For testing the motility of the M2 strain and various mutants, cultures were grown up to early log phase, adjusted to the same optical density of $A_{600} = 0.15$ by the addition of sterile LB broth and a 1 μl drop was placed on the center of the plate. Plates were incubated at 30°C and motility was measured after 14 h. Statistical analysis was done using the Student's *T*-test.

Biofilm Analysis

Cells for biofilm analysis were taken directly from freezer stocks and grown in 2 ml 0.5X LB without shaking at room temperature to an optical density A_{600} of 0.1. Each tube was then used to inoculate wells of a 96 well microtiter plate with 150 μl of culture. Plates were incubated stationary at 30 or 37°C for 24 h. The optical density of each well was read at A_{600} for cell growth and the planktonic cells were removed. To stain biofilms, 250 μl of 10% crystal violet was added to each well for 30 min. The crystal violet was gently decanted and each well was gently washed three times with distilled water. Three hundred microliters of 33% acetic acid was added to each well to solubilize the crystal violet and the absorbance of a 1/10 dilution was read at A_{585}. Statistical analysis was done using the Student's *T*-test.

TABLE 1 | Antimicrobial susceptibility profiles.

	M2 wild-type	M2 Δ*adeB*	M2 Δ*adeJ*	M2 Δ*adeB*, Δ*adeJ*
E-TEST (mg/L)				
Ampicillin	24	16	8	8
Cefotaxime	>32	24	1.5	1.5
Ceftriaxone	>32	>32	3	3
Chloramphenicol	64	64	6	6
Amikacin	4	3	3	3
Rifampin	12	8	3	3
Tigecycline	0.25	0.19	0.032	0.023
Trimethoprim	>32	>32	1.5	1.5
TREK (mg/L)				
Piperacillin/tazobactam	16/4	≤8/4	≤8/4	<8/4
Ceftazidime	4	4	≤1	≤1
Cefuroxime	16	16	≤4	≤4
Amikacin	≤4	≤4	≤4	≤4
Aztreonam	>16	>16	4	4
Meropenem	<1	<1	<1	<1

TABLE 2 | Disk diffusion results (zone size in mm).

Antibiotic (mg/L) + inhibitor (mg/L)	M2	M2, Δ*adeB*	M2, Δ*adeJ*	M2 Δ*adeB*Δ*adeJ*
Ampicillin 10	12	12	18	18
Ampicillin 10 + SM23 10	15	15	21	21
Ampicillin 10 + S02030 10	15	14	19	19
Ceftazidime 10	18	18	23	23
Ceftazidime 10 + SM23 10	18	18	23	24
Ceftazidime 10 + SM02030 10	18	19	24	23
Cefotaxime 10	16	16	24	24
Cefotaxime 10 + SM23 10	16	17	27	26
Cefotaxime 10 + S02030 10	16	16	26	25
Meropenem 10	24	24	30	30
Meropenem 10 + SM23 10	25	25	30	30
Meropenem 10 + S02030 10	24	24	30	29

RESULTS

Analysis of AdeABC and AdeIJK RND-Efflux Systems in *A. nosocomialis*

A. nosocomialis strain M2 contains orthologs of AdeA, AdeB and AdeC that share 94, 98, and 92 percent amino acid identify, respectively, to the corresponding proteins in *A. baumannii* strain AB5075.UW. In addition, orthologs of the AdeIJK proteins were found with 97, 99, and 98 percent identity to the corresponding proteins in *A. baumannii* AB5075.UW. To investigate the function of each RND-type efflux system, null alleles in the *adeB* and *adeJ* genes, encoding the inner membrane transporter for each system were constructed by introducing frameshift mutations in each gene into the chromosome by allelic replacement (section Materials and Methods).

The antibiotic susceptibility profile of each mutant was then determined for a panel of antibiotics representing different classes (**Table 1**). The loss of *adeB* had a minimal effect on the overall levels of resistance and a slight increase in susceptibility was observed for ampicillin, cefotaxime, amikacin, rifampin, and tigecyline (**Table 1**). This result was surprising as the AdeABC system has a prominent role in antibiotic resistance in *A. baumannii* (Magnet et al., 2001). To determine if this *adeB* frameshift mutation was somehow being suppressed or was not a null allele, we constructed an *adeB::Km* mutation, where the *adeB* gene was disrupted in the middle of the coding region. However, this *adeB::km* mutant displayed the same level of resistance to ampicillin (128 μg/ml), tetracycline (2 μg/ml), and ciprofloxacin (0.38 μg/ml) as wild-type, indicating that the previously isolated frameshift mutation in *adeB* was non-functional.

The effect of a mutation in *adeJ* on antibiotic susceptibility was far more pronounced, where cells became more susceptible to the following antibiotics; ampicillin (3-fold), cefotaxime (>15-fold), ceftriaxone (>10-fold), chloramphenicol (>10-fold), rifampin (4-fold), tigecycline (8-fold), and trimethoprim (>20-fold) (**Table 1**). The antibiotic susceptibility profiles were also examined for an *adeB/adeJ* double mutant to determine if the loss of both efflux systems had additional effects. However, the *adeB/adeJ* double mutant essentially phenocopied the *adeJ* single mutant (**Table 1**).

We next assayed the role of AdeB and AdeJ efflux pumps in the handling of the boronic acid transition state inhibitors (BATSIs) SM23 and S02030 (Powers et al., 2014; Nguyen et al., 2016). These BATSIs either mimic the acylation or deacylation transition state. Paired with a penicillin (ampicillin), carbapenem (meropenem), or cephalosporin (ceftazidimne or cefepime) as performed herein, the BATSI can act to inhibit serine based beta-lactamases *in-vitro*. As a result of this mechanism of action, class C cephalosporinases possess the greatest affinity for these compounds (e.g., ADC cephalosporinase in *A. nosocomialis*). Our results indicate that the BATSI studied are substrates for the AdeIJK efflux pump in *A. nosocomialis* (**Table 2**). In particular, the susceptibility of wild-type M2 to cefotaxime is unaffected by these inhibitors, but in the presence of the *adeJ* mutation, these inhibitors now increase susceptibility to cefotaxime (**Table 2**).

Role of AdeABC and AdeIJK in Motility

A. nosocomialis strain M2 is capable of rapidly translocating across soft agar surfaces (Clemmer et al., 2011). Although the mechanism responsible for this motility is unclear, a number of genes have been identified that reduce motility including mutations in the *abaI* autoinducer synthase responsible for quorum sensing signal production (Clemmer et al., 2011). We tested the wild-type M2 parent and the isogenic *adeB* and *adeJ* mutants for their motility phenotypes at 30 degrees. The *adeB* mutation did not significantly alter surface motility (**Figures 1A,B**). In contrast, the *adeJ* mutation had a pronounced effect on surface motility, with a greater than 50% reduction relative to the wild-type M2 parent (**Figure 1**). Interestingly, this motility defect was temperature dependent, at 37 degrees the

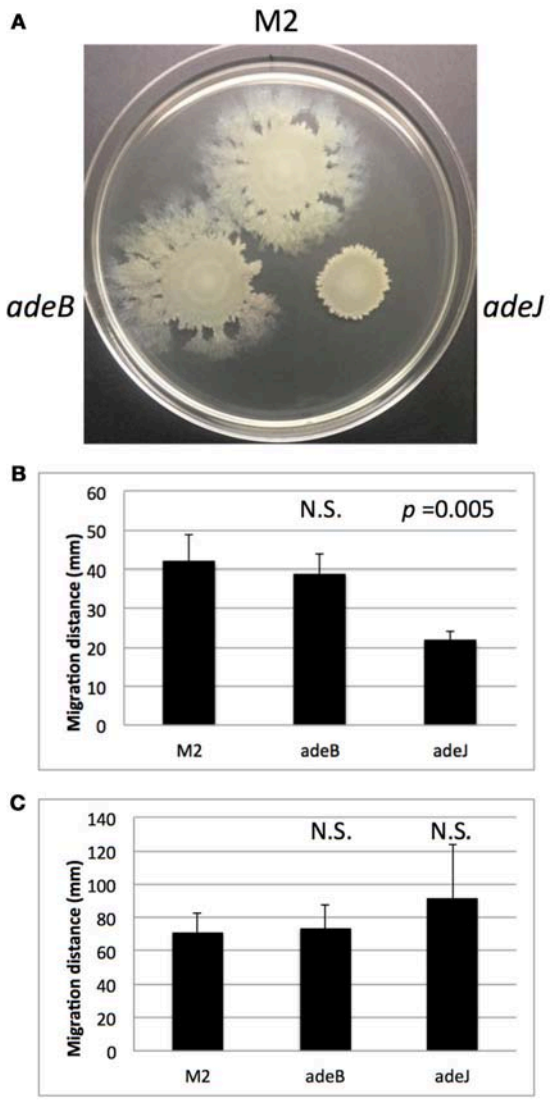

FIGURE 1 | Surface motility of wild-type M2 and efflux mutants. Wild-type M2 and the isogenic *adeB* and *adeJ* mutants were assayed for surface motility as described in the Materials and Methods. In **(A)**, motility of the indicated strains is shown after 14 h at 30°C. Motility was quantitated from 4 separate experiments at 30°C **(B)** and 37°C **(C)**. Error bars represent standard deviation of the mean. N.S. indicates a *p*-value > 0.05.

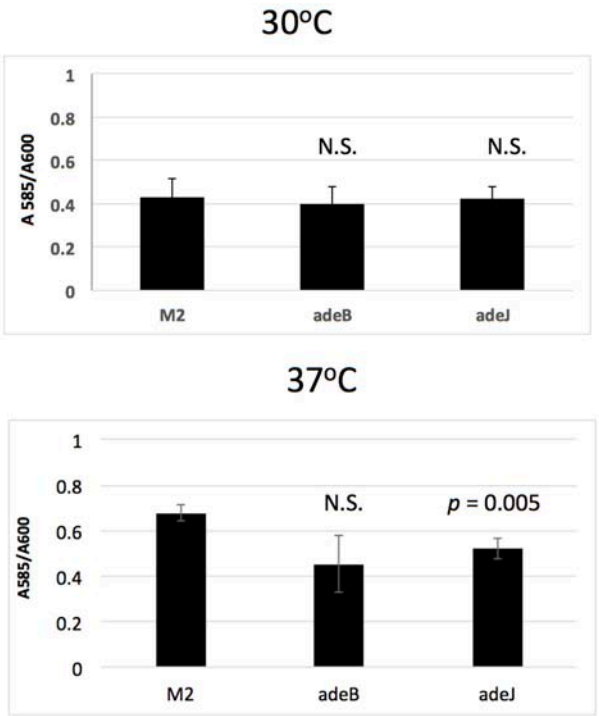

FIGURE 2 | Biofilm formation. Wild-type M2 and the isogenic *adeB* and *adeJ* mutants were assayed for biofilm formation in microtiter wells grown at 30 or 37°C for 24 h. Values represent crystal violet staining/cell density (A_{585} / A_{600}) ratio and error bars represent standard deviation of the mean. N.S. indicates a *p*-value > 0.05.

adeJ mutant exhibited a similar level of motility as wild-type (**Figure 1C**).

In a previous study, the motility of *A. nosocomialis* M2 was shown to be dependent on production of the quorum sensing signal 3-OH C_{12}-HSL (Clemmer et al., 2011). To investigate the possibility that the motility defect in the *adeJ* mutant was due to the failure to export 3-OH-C_{12}-HSL, an *Agrobacterium tumefaciens traG-lacZ* biosensor strain was used to assay signal production in the *adeJ* mutant and wild-type M2 (Niu et al., 2008). However, no significant differences in signal production were observed between these strains (**Supplementary Figure 1**).

Role of AdeABC and AdeIJK in Biofilm Formation

The role of AdeABC and AdeIJK in biofilm formation was also examined. When biofilms were formed on the surface of polystyrene microtiter wells, biofilm formation by the *adeB* and *adeJ* mutants were similar to wild-type M2 after 24 h of growth at 30°C (**Figure 2**). At 37°C, only the *adeJ* mutant showed a statistically significant reduction in biofilm formation, with a 24% decrease relative to wild-type (**Figure 2**).

DISCUSSION

In this study, the roles of AdeABC and AdeIJK orthologs in *A. nosocomialis* were addressed. Both a frameshift allele in the *adeB* gene and an *adeB::Km* disruption did not result in a major change in antibiotic resistance profiles, which is in contrast to that observed in *A. baumannii* (Magnet et al., 2001). Several possibilities can account for these differences. First, the *adeABC* genes may be expressed at very low levels in *A. nosocomialis* M2, therefore, the loss of this efflux system would have a minimal impact. In *A. baumanii,* the AdeABC system is typically expressed at low levels and inactivation of these genes in some strains does not produce a phenotype (Yoon et al., 2015; Leus et al., 2018). Increased expression can

result from mutations in the AdeRS two-component system. In *A. nosocomialis* M2, the AdeR and AdeS proteins did not contain amino acid substitutions previously associated with increased AdeABC expression (Marchand et al., 2004; Yoon et al., 2013; Gerson et al., 2018). Based on this information, we propose that the AdeABC genes are tightly regulated by AdeRS and the levels of expression in the M2 strain do not contribute to intrinsic resistance. We also tested the role of AdeABC in both surface motility and biofilm formation and no significant changes were observed in the *adeB* mutant relative to wild-type (**Figures 1**, **2**).

In contrast, the AdeIJK efflux system was shown to play a significant role in antibiotic efflux, where a mutation inactivating this system had a pronounced effect on antibiotic susceptibility (**Table 1**). This observation is consistent with previous studies in *A. baumannii* demonstrating that efflux mediated by AdeIJK contributes substantially to antibiotic resistance. In addition, the loss of AdeIJK strongly reduced surface motility with a greater than 50% reduction compared to wild-type (**Figure 1**). The loss of AdeIJK resulted in a modest (24%) reduction in biofilm formation, which is also consistent with previous studies in *A. baumannii,* where the loss of AdeIJK resulted in a 20% reduction in biofilm formation (Yoon et al., 2015). The decreased surface motility and biofilm formation in the *adeJ* mutant were not the result of decreased production of the quorum sensing signal 3-OH C_{12}-HSL, which has been shown to be important for both surface motility and biofilm formation in *A. nosocomialis* (Niu et al., 2008; Clemmer et al., 2011).

The mechanism that results in loss of motility when the AdeIJK system in inactivated is unknown, but may indicate a role for AdeIJK in secretion of a lipopeptide surfactant that is required for motilty (Clemmer et al., 2011; Rumbo-Feal et al., 2017) or in the secretion of 1,3-diaminopropane, also required for motility (Skiebe et al., 2012). This also indicates that in addition to antibiotic efflux, there are cellular functions mediated by AdeIJK, indicating a general role for this RND-type efflux system in general physiology of *A. nosocomialis*.

AUTHOR CONTRIBUTIONS

DK, SR, and PR conducted experiments. PR and RB wrote the manuscript. SR, RB, and PR edited the manuscript.

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SUPPLEMENTARY MATERIAL

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Surveillance for Azole-Resistant *Aspergillus fumigatus* in a Centralized Diagnostic Mycology Service, London, United Kingdom, 1998–2017

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Background/Objectives: *Aspergillus fumigatus* is the leading cause of invasive aspergillosis. Treatment is hindered by the emergence of resistance to triazole antimycotic agents. Here, we present the prevalence of triazole resistance among clinical isolates at a major centralized medical mycology laboratory in London, United Kingdom, in the period 1998–2017.

Methods: A large number (n = 1469) of clinical *A. fumigatus* isolates from unselected clinical specimens were identified and their susceptibility against three triazoles, amphotericin B and three echinocandin agents was carried out. All isolates were identified phenotypically and antifungal susceptibility testing was carried out by using a standard broth microdilution method.

Results: Retrospective surveillance (1998–2011) shows 5/1151 (0.43%) isolates were resistant to at least one of the clinically used triazole antifungal agents. Prospective surveillance (2015–2017) shows 7/356 (2.2%) isolates were resistant to at least one triazole antifungals demonstrating an increase in incidence of triazole-resistant *A. fumigatus* in our laboratory. Among five isolates collected from 2015 to 2017 and available for molecular testing, three harbored TR_{34}/L98H alteration in the *cyp51A* gene that are associated with the acquisition of resistance in the non-patient environment.

Conclusion: These data show that historically low prevalence of azole resistance may be increasing, warranting further surveillance of susceptible patients.

Keywords: *Aspergillus fumigatus*, azole resistance, *cyp51A*, antifungal agents, surveillance

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INTRODUCTION

Aspergillus fumigatus is a ubiquitous ascomycete mold and the primary etiologic agent of aspergillosis which varies in severity and clinical presentation. These manifestations include a spectrum of conditions including colonization, allergic response in allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis, aspergilloma, and to the most severe form, invasive aspergillosis (Kosmidis and Denning, 2015). Sensitization to *Aspergillus* in patients with severe asthma is another form of disease. *Aspergillus* bronchitis is a recently described condition, especially in patients with cystic fibrosis (CF) or bronchiectasis, lung transplant recipients, and those receiving mechanical ventilation in intensive care units (Kosmidis and Denning, 2015). Moreover, aspergillosis may occur in immunocompetent hosts with influenza infection (Crum-Cianflone, 2016).

Triazoles have been the most widely used antifungal agents in prophylaxis and treatment of *Aspergillus*-related infections (Verweij et al., 2015). The Infectious Diseases Society of America (IDSA) guideline recommends the triazole antifungal voriconazole as the first line agent for the primary treatment of IA (Patterson et al., 2016). Since the late 2000s there has been a steady increase in the number of reported resistance to azole antifungals in *A. fumigatus*, causing a major clinical concern with subsequent treatment failure among some patients (Verweij et al., 2007; Chowdhary et al., 2013). The emergence and global spread of azole-resistant isolates led to a fundamental question as to whether first line clinical use of mold-active triazoles can be retained (Verweij et al., 2015).

The molecular basis of resistance to triazoles in *A. fumigatus* mainly involves the environmentally driven polymorphism TR_{34}/L98H, which consists of a tandem repeat (TR) of 34 bases in the promoter of the *cyp51A* gene and a leucine-to-histidine change at codon 98 (Mellado et al., 2007); this polymorphism is globally widespread in environmental and clinical isolates (Chowdhary et al., 2015, 2017). Another *cyp51A*-mediated resistance alteration that leads to high-level voriconazole resistance, TR_{46}/Y121F/T289A, has also been described in *A. fumigatus* (van der Linden et al., 2013). In contrast, non-synonymous mutations in the *cyp51A* gene cause structural alterations due to amino acid substitutions and are sufficient to induce resistance to some or all triazole drugs. Numerous mutations in *cyp51A* have been reported that confer resistance to triazoles *in vitro*. These resistant mutations often evolve during prolonged azole treatment in patients with chronic forms of aspergillosis (Hagiwara et al., 2016). In *A. fumigatus*, *cyp51A* gene encodes lanosterol 14-α-sterol demethylase which is required for the biosynthesis of ergosterol, an essential component of the fungal cell membrane (Chowdhary et al., 2014).

The true prevalence of azole resistance in *A. fumigatus* is largely unknown and multiple factors including sample size, method of detection and geographical differences in the studied samples might affect the prevalence rate of azole-resistant isolates (Verweij et al., 2016a). Overall azole resistance rates of 0–27.8% have been determined from different surveys (Vermeulen et al., 2013; Hagiwara et al., 2016; Chowdhary et al., 2017). Despite the global emergence of triazole resistance, the prevalence data on azole-resistant *A. fumigatus* in the United Kingdom is limited to reports (Howard et al., 2009; Bueid et al., 2010; Denning et al., 2011) generated by the National Aspergillosis Centre in Manchester. Howard and co-workers (Howard et al., 2009) have reported an increase in azole resistance (5%) in clinical *A. fumigatus* isolates since 2004. Later, another alarming increase in azole resistance frequency to 14% in 2008 and 20% in 2009 was reported by Bueid et al. (2010). Recent published data from the Public Health England, showed 8, 7, and 4.5% of clinical isolates referred to the National Mycology Reference Laboratory in, 2016 were resistant to itraconazole, posaconazole, and voriconazole, respectively (Public Health England, 2017), though the mechanism of resistance among these azole-resistant isolates remained unknown.

We hypothesized that prevalence of azole-resistant *A. fumigatus* reported by specialist or referral centers may not represent the true prevalence of azole resistance occurring in other institutions with different patient populations, thus surveillance studies at regional level are warranted. Our centralized mycology laboratory provides diagnostic service to eight major hospitals with mixed specialties in North West London, plus over 100 primary care providers, all serving a population of 2.5 million. The main patient population at risk of invasive fungal infections is diverse with high adult and pediatric hematology, oncology, renal transplant and intensive care caseloads. To investigate the prevalence of azole-resistance in clinical *A. fumigatus* isolates, antifungal susceptibility profiles of a large collection of unique clinical isolates of *A. fumigatus* collected over 17 years were retrospectively reviewed.

MATERIALS AND METHODS

Data Collection

Our objective was to investigate the prevalence of azole-resistance in *A. fumigatus* isolates in a major centralized diagnostic mycology service based at the Imperial College Healthcare National Health Service trust, which provides diagnostic mycology service to the North West London area. Retrospective data on antifungal susceptibility profiles of a large collection of clinical *A. fumigatus* isolates tested between January 1998 – December 2017 was extracted from the laboratory database. This database is populated with antifungal minimum inhibitory concentration (MIC) data against fungal isolates cultured from diverse clinical samples from a mixed and unselected patient population. No data were available for period of January 2012–December 2014 as no susceptibility testing was carried out for molds during this period.

Fungal Isolates

All isolates were identified as *A. fumigatus* species complex based on culture colonial morphology and microscopic characteristics. Adhesive tape technique was used for microscopic examination of fungal cultures. In addition, growth at 45°C was used to exclude most cryptic species. The identification of isolates with elevated azole MICs was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry

(MALDI–TOF MS). Identification by MALDI–TOF MS was performed with a Microflex LT system (Bruker Daltonics, Bremen, Germany) using Biotyper 3.0 software with the additional fungi library (Bruker Daltonics, Bremen, Germany) according to the manufacturer's recommendations. Exact identification of azole-resistant *A. fumigatus* isolates was confirmed by sequencing the partial calmodulin gene (*CaM* locus) as previously described (Samson et al., 2014).

Antifungal Susceptibility Testing (AFST)

Antifungal susceptibility testing was carried out according to the CLSI M38-A2 standard broth microdilution method (Clinical and Laboratory Standards Institute, 2008) for filamentous fungi (isolates tested 1998–2011) and EUCAST EDEF 9.1 (Arendrup et al., 2012) (isolates tested 2015–2017). MICs were read at 48 h as the concentration of drug that elicited 100% inhibition of growth (amphotericin B, itraconazole, posaconazole, voriconazole) or as the minimum effective concentration (MEC) for caspofungin, anidulafungin, micafungin, in which the end-point is read as the lowest concentration at which the fungal hyphae can be seen to be stunted with swollen tips using an inverted microscope. For interpretation of MIC values, the EUCAST clinical breakpoints for *A. fumigatus* were used (Arendrup et al., 2012). For itraconazole, voriconazole, and amphotericin B MICs of $\leq$1 mg/L (susceptible) and >2 mg/L (resistant) and posaconazole MICs of $\leq$0.125 mg/L (susceptible) and >0.25 mg/L (resistant). No clinical breakpoints for the echinocandins have yet been established for *Aspergillus*. Quality control for AFST was ensured by testing the following type strains: *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *A. fumigatus* NCPF 7097 and *A. fumigatus* NCPF 7100.

Fungal DNA Extraction

Fungal genomic DNA was extracted as previously described (Abdolrasouli et al., 2015). Briefly, gDNA was extracted with an optimized MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Cambridge, United Kingdom) with an additional bead-beating step included. Harvested conidia were homogenized using 1.0-mm-diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK, United States) in a FastPrep-24 system (MP Biomedicals, Solon, OH, United States) at 4.5 m/s for 45 s. DNA samples were stored at −80°C for molecular testing.

Genotype Testing

Five isolates collected from 2015 to 2017 with raised MICs to at least one triazole agent were available for molecular analysis. A commercially available real-time PCR and high-resolution melt-curve analysis, AsperGenius® (PathoNostics, Maastricht, Netherlands) was utilized to detect alterations in *cyp51A* conferring resistance to triazole antifungal agents. The AsperGenius® resistance multiplex assay targets the single-copy *cyp51A* gene of *A. fumigatus* and detects the TR_{34}, L98H, Y121F, and T289A mutations to differentiate wild-type from mutant *A. fumigatus* isolates *via* melting curve analysis. This real-time PCR was performed according to the manufacturer's instructions. The detection of four different fluorescent labels (emission spectra, 495 nm, 530 nm, 598 nm, and 645 nm) was enabled by using the Rotor-Gene Q (Qiagen, Heidelberg, Germany) for all experiments.

Statistical Analysis

Categorical variables were reported as counts and percentages and were compared using Fisher's exact tests. Statistical analyses were two sided, and $P < 0.05$ was considered to have statistical significance. Analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, La Jolla, CA, United States).

RESULTS

Fungal Isolates

A total of 1,469 fungal isolates identified as *A. fumigatus* at the diagnostic mycology service based in North West London between 1998 and 2017 included 12 isolates with (minimum inhibitory concentrations) MICs above the breakpoints for itraconazole, posaconazole and/or voriconazole. Due to difference in antifungal susceptibility testing methodology (methods), results were presented in two time periods (retrospective 1998–2011; prospective 2015–2017).

Retrospective Surveillance

From 1998 to 2011, a total of 1,151 isolates were identified as *A. fumigatus*. Respiratory samples were the most common (966/1151, 84%) source of isolation. Overall 0.43% (5/1151) of *A. fumigatus* isolates from five patients displayed elevated MICs to triazole antifungal agents. **Table 1** summarizes the characteristics and MIC results of resistant isolates in this study. For itraconazole, 3/1151 isolates had MIC values above the sensitive breakpoint (MIC > 2 mg/L). For voriconazole, 3/1151 were classified as resistant (MIC > 2 mg/L). Among 720 isolates tested against posaconazole, three isolates displayed reduced susceptibility (MIC $\geq$ 0.25 mg/L). No isolate in this collection displayed high level of resistance (MIC > 16 mg/L) to three tested triazole antifungal agents.

All azole-resistant *A. fumigatus* isolates were cultured from sputum samples. Two patients had hematological underlying diseases, two had chronic respiratory disease and one had human immunodeficiency virus (HIV) infection. With the exception of one case (case 1) with unknown outcome, four cases died (case 2–5). However, we were not able to determine if the death was attributed to azole-resistant aspergillosis or the underlying clinical conditions in these patients. One azole-resistant isolate displayed a concomitant raised MEC (8 mg/L) to caspofungin. However, all azole-resistant isolates remained susceptible to amphotericin B. No clinical information on prior azole therapy was available on any of five patients. The first azole-resistant *A. fumigatus* from these five patients was isolated in 2001.

Prospective Surveillance

From 2015 to 2017, a total number of 356 clinical isolates were identified as *A. fumigatus* over a 3-year period. Antifungal susceptibility testing (AFST) was conducted on 318 out of 356

TABLE 1 | Characteristics of *A. fumigatus* isolates of this study.

Case	Year	Gender/Age/ Underlying disease	Sample type	Azole therapy	MIC or MEC (mg/L)					AFST method	*cyp51A* mutation
					ITC	VRC	PCZ	AMB	CAS		
1	2001	Gender/age unknown HIV	Sputum	Unknown	0.12	4	ND	0.25	ND	CLSI M38-A2	ND
2	2007	M/63, respiratory	Sputum	Unknown	4	0.5	0.25	0.06	0.007	CLSI M38-A2	ND
3	2008	M/34, hematology	Sputum	Unknown	8	4	2	0.25	8	CLSI M38-A2	ND
4	2008	M/49, respiratory	Sputum	Unknown	4	1	0.25	0.06	0.007	CLSI M38-A2	ND
5	2009	M/35, hematology	Sputum	Unknown	0.5	4	0.12	0.06	0.007	CLSI M38-A2	ND
6	2015	M/55, unknown	Sputum	Unknown	>16	1	>16	0.25	0.06	EUCAST	WT
					>16	2	1	0.5	0.06		WT
					2	2	1	0.5	0.06		ND
7	2016	M/73, necrotizing aspergillosis of the lung	Sputum	Yes	16	>16	4	0.5	0.03	EUCAST	TR_{34}/L98H
8	2016	F/68, asthma, and bronchiectasis	Sputum	Yes	>16	2	0.5	0.5	0.06	EUCAST	TR_{34}/L98H
9	2017	M/39, trauma, and Intensive care	BAL	No	4	0.5	0.12	0.25	0.03	EUCAST	TR_{34}/L98H
10	2017	M/66, HIV, aspergilloma, and hemoptysis	Sputum	Yes	8	0.5	1	0.5	0.06	EUCAST	ND

AFST, antifungal susceptibility testing; AMB, amphotericin B; CAS, caspofungin; CLSI, Clinical Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; F, female; HIV, human immunodeficiency virus; ITC, itraconazole; M, male; MEC, minimum effective concentration; MIC, minimum inhibitory concentration; ND, not done; PCZ, posaconazole, VRC, voriconazole; WT, wild-type.

(89.3%) isolates. Among isolates with AFST data, the majority (289/318, 90.9%) were cultured from either sputum (n = 179) or bronchoalveolar lavage (BAL) fluid (n = 110) samples.

Seven isolates showed MIC of ≥2 mg/L to itraconazole (2.2%). This ranged from one isolate being intermediate (MIC = 2 mg/L), two isolates had MIC of 4 and 8 mg/L and four isolates displayed high level itraconazole resistance (MIC ≥ 16 mg/L) (**Table 1**). Only one isolate demonstrated high level resistance to voriconazole (MIC > 16 mg/L) while the remaining isolates showed intermediate (n = 3) or susceptible phenotype to voriconazole (n = 3). While six isolates displayed resistance to posaconazole (MIC > 0.25 mg/L), high level of resistance against posaconazole was only detected in one isolate. Notably, one isolate from case 7, displayed a pan-azole resistant phenotype.

The seven azole-resistant isolates were recovered from five patients. All of the resistant strains remained sensitive to amphotericin B and caspofungin. All triazole-resistant isolates were cultured from respiratory samples. All clinical isolates with azole-resistant phenotype were confirmed as *A. fumigatus* using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS).

Azole Resistance Prevalence

Overall, reduced susceptibility to triazoles antifungal agents remained low in a large collection of unselected clinical *A. fumigatus* isolates tested in our centralized mycology laboratory in London. In total, only 0.81% (12/1469) isolates with available AFST data displayed reduced susceptibility to at least one triazole antifungal agent over a period of 17 years. However, prevalence of azole-resistant *A. fumigatus* was increased from 0.43% in 1998–2011 to 2.2% in 2015–2017 ($P < 0.05$, Fisher Exact test). Within the study period, pan-azole resistance has been recorded amongst tested isolates, however its occurrence remains rare (n = 1).

Mechanism of Resistance

From 12 clinical *A. fumigatus* isolates with azole-resistant phenotype, five isolates collected from 2015 to 2017 were available to investigate their molecular mechanism of resistance. When tested for mechanism of resistance using AsperGenius® high resolution melt-curve analysis, TR_{34}/L98H was present in three isolates (60%). Two isolates with azole-resistant phenotype did not show any alteration in TR_{34}/L98H when tested by AsperGenius® assay.

DISCUSSION

Although the prevalence of azole resistance in *A. fumigatus* has been investigated in diverse populations and in different countries (Hagiwara et al., 2016; Verweij et al., 2016b; Chowdhary et al., 2017), the true frequency of resistance in the United Kingdom remains largely unknown. This is predominantly because previous reports from United Kingdom were all based on studies (Howard et al., 2006, 2009; Bueid et al., 2010; Denning et al., 2011) carried out in the National Aspergillosis Centre in Manchester which represented a very specific patient sub-population predominantly with chronic forms of aspergillosis. In essence therefore this population did not represent other general and mixed patient cohorts in other centers with

different underlying diseases such as hemato-oncology or solid organ transplantation. Furthermore there is no national surveillance program to actively screen clinical or environmental isolates for resistance to triazole antifungal agents in the United Kingdom. The prevalence of azole-resistant *A. fumigatus* may differ considerably from center to center depending on the geographical location of studied hospitals. To compound this, most clinical microbiology laboratories in United Kingdom either do not routinely test *Aspergillus* isolates for antifungal susceptibility testing or refer those isolates deemed clinically significant to reference laboratories. Expert recommendation by the recently instituted International Society for Human and Animal Mycology (ISHAM) *Aspergillus* Resistance Surveillance Working Group has highlighted the importance of performing local surveillance in order to determine the presence of azole resistance and adjust treatment guidelines if necessary (Resendiz Sharpe et al., 2018).

In the present study the prevalence of azole-resistant *A. fumigatus* recovered from clinical samples collected from unselected patient populations remains low. Among all clinical isolates tested from 1998 to 2011, only 0.43% (5/1151) demonstrated reduced susceptibility to at least one triazole antifungal agent. This figure increased to 2.2% (7/318) between 2015 and 2017, when a prospective passive surveillance program was carried out over a 3-year period in the same laboratory. This increase in the incidence of triazole resistance among mixed patient population, was in agreement with the general increase described in the recently published ESPAUR report (Public Health England, 2017). Arguably, the 8.5% itraconazole resistance among *A. fumigatus* isolates tested at the national reference laboratory may represent a bias due to testing clinical isolates that were predominantly originated from patients with refractory disease or in whom more exposure to medical triazoles was expected. Internationally, resistance prevalence in populations comprising of mixed patient groups is consistent with published data from countries such as Denmark (Jensen et al., 2016), France (Alanio et al., 2011), India (Chowdhary et al., 2015), Iran (Mohammadi et al., 2016), Pakistan (Perveen et al., 2016), Kuwait (Ahmad et al., 2014), Australia (Kidd et al., 2015), and United States (Berkow et al., 2018) where, overall, prevalence of antifungal resistant *A. fumigatus* remained low.

This study showed a clear difference in the prevalence of azole-resistant *A. fumigatus* in London when compared to the previously published data from NAC in Manchester (Howard et al., 2009; Bueid et al., 2010; Denning et al., 2011). This significant variation in the proportion of resistance, suggested that patients with chronic airway diseases might be at higher risk of colonization and/or infection with azole-resistant *A. fumigatus* when compared to general or mixed patient cohorts. Similarly, prevalence of azole resistance in clinical *A. fumigatus* in French patient populations was dependent on underlying clinical conditions; 1.1% in hematological patients, 1.8% in unselected patients (Alanio et al., 2011), and 8% to 12.2% in patients with cystic fibrosis (Morio et al., 2012; Guegan et al., 2018). This finding supports the recommendations by van der Linden et al. (2016) about the need to determine azole resistance frequency at the hospital level and within different patient groups. Additionally, recording clinical data to include triazole duration and dose administered for prophylaxis and/or treatment in conjunction with therapeutic drug monitoring will elucidate the potential relationship between previous azole exposure and development of antifungal resistance.

To investigate the common *cyp51A*-dependent mechanisms of resistance, DNA extracts from five available fungal isolates with azole-resistant phenotype were tested with AsperGenius® multiplex RT-PCR assay followed with high resolution melt-curve analysis. TR_{34}/L98H detected in 3/5 (60%). This is the first time that presence of this polymorphism has been shown in London. Two *A. fumigatus* with azole-resistant phenotype (both from case 6 isolated in 2015) did not demonstrate any TR_{34}/L98H or TR_{46}/Y121F/T289A alterations. Unfortunately none of the five azole-resistant isolates found between 2001 and 2009 were available for further analysis so we cannot, at this stage, determine whether the incidence of these alleles has increased across the period of study. Furthermore, recovering azole-resistant *A. fumigatus* from patients with retroviral and hematological underlying conditions in this study indicates that isolation of azole-resistance *A. fumigatus* is not limited patients with respiratory disorders.

Limitations of our study include the absence of data for a period of 3 years (2012–2015) when antifungal susceptibility testing was excluded from the routine diagnostic service. Molecular basis of azole-resistant in two isolates with no alteration in *cyp51A* gene remains unknown. Mutations in hot spots in *cyp51A* gene or other non-*cyp51A*-related mechanisms like efflux pumps might be responsible for elevated MIC values to triazoles in these two isolates. Furthermore, six azole-resistant *A. fumigatus* isolates were not available for molecular testing.

The current study identifies an overall low proportion of azole resistance (0.81%) in clinical *A. fumigatus* isolates obtained from a mixed and diverse patient population in London, United Kingdom. However, there are signs that this may be changing as there has been an increase in recent years showing that further cross-sectional studies are necessary to establish whether this trend is mirroring that seen in other European countries. It is also necessary to conduct similar surveillance studies in specific patient populations such as those with chronic respiratory diseases at regional level to investigate whether the prevalence of triazole resistance varies between different patient cohorts. The discovery of environmentally driven TR_{34}/L98H among azole-resistant *A. fumigatus* isolates is of clinical significance suggesting a spillover of environmentally acquired antifungal resistance into susceptible patients. Systematic, continual and multi-center surveillance programs at a nation-wide scale are warranted to provide comprehensive epidemiological data on triazole-resistant *A. fumigatus* in United Kingdom.

AUTHOR CONTRIBUTIONS

AA, MCF, and DA-J contributed to the conception and design of the research. AA performed all experiments and wrote the manuscript. MAP carried out AFST on retrospective fungal isolates. All authors contributed to the analysis and interpretation of data and the drafting and revising of this manuscript.

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REVIEW
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Prevalence and Emergence of Extended-Spectrum Cephalosporin-, Carbapenem- and Colistin-Resistant Gram Negative Bacteria of Animal Origin in the Mediterranean Basin

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In recent years, extended ESBL and carbapenemase producing Gram negative bacteria have become widespread in hospitals, community settings and the environment. This has been triggered by the few therapeutic options left when infections with these multi-drug resistant organisms occur. The emergence of resistance to colistin, the last therapeutic option against carbapenem-resistant bacteria, worsened the situation. Recently, animals were regarded as potent antimicrobial reservoir and a possible source of infection to humans. Enteric Gram negative bacteria in animals can be easily transmitted to humans by direct contact or indirectly through the handling and consumption of undercooked/uncooked animal products. In the Mediterranean basin, little is known about the current overall epidemiology of multi-drug resistant bacteria in livestock, companion, and domestic animals. This review describes the current epidemiology of ESBL, carbapenemase producers and colistin resistant bacteria of animal origin in this region of the world. The CTX-M group 1 seems to prevail in animals in this area, followed by SHV-12 and CTX-M group 9. The dissemination of carbapenemase producers and colistin resistance remains low. Isolated multi-drug resistant bacteria were often co-resistant to non-beta-lactam antibiotics, frequently used in veterinary medicine as treatment, growth promoters, prophylaxis and in human medicine for therapeutic purposes. Antibiotics used in veterinary medicine in this area include mainly tetracycline, aminoglycosides, fluoroquinolones, and polymyxins. Indeed, it appears that the emergence of ESBL and carbapenemase producers in animals is not related to the use of beta-lactam antibiotics but is, rather, due to the co-selective pressure applied by the over usage of non-beta-lactams. The level of antibiotic consumption in animals should be, therefore, re-considered in the Mediterranean area especially in North Africa and western Asia where no accurate data are available about the level of antibiotic consumption in animals.

Keywords: ESBL, carbapenemase, *mcr-1*, Mediterranean, livestock

BACKGROUND

Antimicrobial resistance is an emerging and rapidly evolving phenomenon. This phenomenon is currently observed in all bacterial species including clinically important Gram negative bacilli (GNB) (Rubin and Pitout, 2014). Gram negative bacilli, "enterobacteriaceae and non-fermenters" are normal inhabitants of the human intestinal microflora (Vaishnavi, 2013); they are responsible for the most common hospital and community acquired infections. Antibiotic resistance in GNB is mediated by target drug modification (Lambert, 2005), changes in bacterial cell permeability (Delcour, 2009) and, most importantly, the production of hydrolyzing enzymes, namely beta-lactamases. The most common beta-lactamases which are now widespread include the extended spectrum beta-lactamases (ESBL) (SHV, TEM, OXA, and CTX-M types), AmpC beta-lactamases, and carbapenemases (MBL, KPC, and class D oxacillinases) (Giedraitiene et al., 2011; Poirel et al., 2011). These enzymes provide the bacterium with resistance toward the majority of therapeutic options available in the clinical market. Furthermore, resistance determinants of these enzymes are often located on plasmids carrying resistance genes to other non-beta-lactam antibiotics, thus further limiting treatment options (Guerra et al., 2014).

The emergence of colistin resistance in GNB is another concern. Colistin belongs to the polymyxin group of polypeptide antibiotics (Olaitan et al., 2014a). Previously abandoned due to its nephrotoxicity and neurotoxicity, it is now in use once again and is considered to be the last resort antimicrobial agent against carbapenem resistant GNB (Kempf et al., 2013). Colistin resistance can be mediated either by the acquisition of the plasmid mediated "*mcr*" gene or by chromosomal mutations that lead to modification of the lipid A moiety of lipopolysaccharide (LPS), which is considered the primary target of colistin in Gram negative bacilli (Baron et al., 2016).

It is currently known that, in addition to the human intestinal microflora, resistant GNB can be found in water, soil, and fecal animal matter (Verraes et al., 2013). In fact, there is increasing evidence that animals constitute a potent reservoir of resistant GNB (Ewers et al., 2012). This is mainly due to the over- and misuse of antibiotics in veterinary medicine (Guerra et al., 2014): antibiotics are not only prescribed for treatment but are also administered for disease prevention and growth promotion (Economou and Gousia, 2015). Although studies have shown that the direct threat of resistant GNB to human health is still controversial (Olsen et al., 2014), the wide dissemination of these resistant organisms is worrying due to their ease of transmission (Rolain, 2013) and their high potential contribution to the spread of bacterial resistance across all ecosystems (Pomba et al., 2017). In this review, we attempt to describe the epidemiology of ESBL, AmpC and carbapenemase producing GNB of animal origin in the Mediterranean region. Colistin resistance in GNB in the same area is also described. The Mediterranean basin is a region of the world that compromises a wide diversity of populations. It includes five Asian countries (Cyprus, Israel, Lebanon, Syria, and Turkey), eleven European countries (Albania, Bosnia, Croatia, France, Greece, Herzegovina, Italy, Monaco, Montenegro, Slovenia, and Spain) and five African countries (Algeria, Egypt, Libya, Morocco, and Tunisia).

DISTRIBUTION OF ESBLS AND AMPC PRODUCERS IN ANIMALS

Chicken and Food of Poultry Origin

Poultry production is a complex system in the food and agricultural industry. It includes breeding chickens for meat and eggs. Chickens are kept either as a "breeding flock" or as a "broiler flock" for human consumption. Along with eggs, broilers are traded and transported across different countries around the world (Dierikx et al., 2013). This trade results in a vulnerable system that can be hacked by multi-drug resistant organisms (MDRO), i.e., once a MDRO is introduced into the production chain, it can be transferred internationally. This is why the dissemination of ESBL and AmpC-producing GNB, recently extensively reported in chicken farms (Blaak et al., 2015) is worrying, as these can contribute to not only local but global dissemination of antimicrobial resistance (Dierikx et al., 2013). Studies have shown that the carriage of ESBL and AmpC producers in chicken is persistent (Huijbers et al., 2016). ESBL and AmpC producers are isolated from grandparent breeding stock (Nilsson et al., 2014), broiler chickens (Reich et al., 2013), retail meat (Choi et al., 2015), and at the slaughterhouses (Maciuca et al., 2015).

In the Mediterranean basin, the first detection of ESBL in chicken dates back to 2000 in Greece, when a CTX-M-32 harboring *Salmonella enterica* was isolated from poultry end products (Politi et al., 2005). Since then, many studies have reported the emergence of ESBL in poultry in the Mediterranean area. In Italy for instance, the first ESBL reported was a case of SHV-12 detected in *Salmonella* spp (Chiaretto et al., 2008). *Salmonella infantis* species harboring CTX-M-1 were later isolated in 2011 from broiler chicken flocks. These strains led to human infection in Italy in 2013–2014 (Franco et al., 2015). In both studies, isolated strains were co-resistant to non-beta-lactam antibiotics, notably nalidixic acid, sulfonamide, trimethoprim, and tetracyclines. According to the European Food Safety Authority and the European Center for Disease Prevention and Control recent report, *S. infantis* is the fourth most common serovar detected in humans in the European Union and that is mostly being observed in the turkey and broiler chain. In this report, it has been stated that this serovar has been able to extensively disseminate along the broiler production chain (EFSA, 2017). Indeed it has been suggested that the consumption of contaminated chicken meat is among the most common sources of salmonellosis in humans (Antunes et al., 2016). Furthermore, in Italy, opportunistic pathogen such as *Escherichia coli* isolates producing CTX-M-32, CTX-M-1, and SHV-12 type beta-lactamases were also reported (Giufrè et al., 2012). These strains were retrieved from flocks which had no prior treatment with cephalosporins. It is proposed that the prescription of other antimicrobials such as enrofloxacin and tylosin is responsible for the co-selection of the aforementioned resistant organisms (Bortolaia et al., 2010). Reports on chicken feces (Giufrè

et al., 2012), broiler chicken samples, and retail chicken meat (Ghodousi et al., 2016) showed that these latter carried *E. coli* producing CTX-M-grp-1, CTX-M-grp-2, and CTX-M-grp-9 enzymes in Italy. The co-existence of these enzymes with AmpC beta-lactamases was also reported, including CTX-M-1/CMY-2 (Accogli et al., 2013) and CIT-like/CTX-M (Ghodousi et al., 2015) in *E. coli* of poultry origin. CTX-M and AmpC beta-lactamase producers in the Italian poultry belong mostly to the A and B phylogroups with the genes being carried mainly on IncI1 plasmids. In France, the only report from poultry was the detection of two CTX-M-1-producing *E. coli* isolates (Meunier et al., 2006). CTX-M-1 was linked to the insertion sequence IS*Ecp1* (Meunier et al., 2006). This insertion sequence has been previously described as being a potent contributor to the mobilization and insertion of *bla*CTX-M genes (El Salabi et al., 2013). Although no studies described the emergence of ESBL in the Slovenian animal sector, one study reported the presence of CTX-M-1 and SHV-12-producing in Slovenian raw chicken meat samples sold on the Swiss market (Zogg et al., 2016).

In Spain, the Spanish Veterinary Antimicrobial Resistance Surveillance Network (VAV) monitored antimicrobial resistance of *Salmonella enterica* in healthy broilers in 2003–2004: two CTX-M-9 producers were isolated (Riaño et al., 2006). During the same period, ESBL-producing *E. coli* were also detected (Mesa et al., 2006; Moreno et al., 2007). Indeed, it seems that early monitoring systems often targeted resistance in *Salmonella* species, as these are common causative agents of human infections of food of animal origin (Antunes et al., 2016). Thereafter, as bacterial resistance became widely disseminated in all environments (Stoll et al., 2012), researchers began to think of poultry as a reservoir of resistance in enteric organisms. For instance, Egea et al. found that the prevalence of retail poultry meat colonized by CTX-M and/or SHV producing *E. coli* increased from 62.5% in 2007 to 93.3% in 2010 (Egea et al., 2012). During these three years, a significant increase was observed at the level of A0 and D1 phylogroups. Egea et al. suggested that the rise of meat colonization is muli-clonal since only 2 strains from the main phylogroup detected in this study showed genetic relatedness by PFGE typing. Thus, it appears that the diffusion of ESBL producers in retail chicken meat is related rather to successful spread of one or several plasmids carrying the *bla*CTX-M and *bla*SHV genes (Egea et al., 2012). Apart from *E. coli*, ESBL production in the poultry production system in Spain was also detected in *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Proteus mirabilis,* and *Serratia fonticola* (Ojer-Usoz et al., 2013). In parallel, CMY-2 is the only AmpC beta-lactamase type reported in *E. coli* originating from chicken in this country (Blanc et al., 2006; Cortés et al., 2010; Solà-Ginés et al., 2015b). Apart from chicken, one study in Spain reported the detection of CTX-M-1, CTX-M-9, CTX-M-14 harboring *E. coli* strains in flies surrounding chicken farms (Solà-Ginés et al., 2015a). For instance, the detection of ESBL producers in flies reflects on one side the contamination status of the farm housing environment; and on the other side, it contributes to the colonization of other broilers with ESBL producing *E. coli* strains (Solà-Ginés et al., 2015a).

In Turkey, the first ESBL production in animals was detected in *K. pneumoniae* and *Klebsiella oxytoca* in 2007–2008 (Gundogan et al., 2011). In 2012–2014, *E. coli* producing CTX-M-1, CTX-M-3, CTX-M-15, CTX-M-8 as well as SHV-5 and SHV-12 were identified in raw chicken meat samples in different areas across the country (Perrin-Guyomard et al., 2016)-(Tekiner and Ozpinar, 2016). The A, D1 and D2 were the most common phylogroups detected. In the same aforementioned study, ESBL was also detected in *E. cloacae*, *Citrobacter werkmanii,* and *K. pneumoniae* (CTX-M-1) (Tekiner and Ozpinar, 2016). Similarly, CMY-2 type beta-lactamase was detected in *E. coli* (Pehlivanlar Onen et al., 2015) as well as in *E. cloacae* (Tekiner and Ozpinar, 2016). In Lebanon, CTX-M type beta-lactamase followed by CMY AmpC beta-lactamase appear to dominate the Lebanese chicken farms (Dandachi et al., 2018b). MLST typing of CTX-M positive *E. coli* strains revealed the presence of different sequence types across the territory. Furthermore, a significant resistance of ESBL producers toward gentamicin was observed. The spread of ESBL producers in Lebanon could be attributed in part to the co-selective pressure applied by the heavy usage of gentamicin in the veterinary sector as previously reported (Dandachi et al., 2018b). In Israel, only one study showed the presence of CTX-M-producing *E. coli* of A, B, and D phylogroups in liver samples of dead broiler chickens and ready-to-market chicken meat (Qabajah et al., 2014).

Concerning Africa, ESBL was first detected in *E. coli* strains isolated from foods of poultry origin in Tunisia in 2006. These harbored SHV-5, CTX-M-8, CTX-M-14, and CTX-M-1 type beta-lactamases (Jouini et al., 2007). It appears that in this country, *bla*CTX-M-1 and *bla*CMY-2 are the dominant genes responsible for ESBL and AmpC production in *E. coli* isolated from chicken samples (Ben Slama et al., 2010; Ben Sallem et al., 2012). This is in addition to *bla*CTX-M-15, *bla*CTX-M-14 (Maamar et al., 2016), and *bla*CTX-M-9 that were detected in *E. coli* isolated from the fecal samples of dead/diseased chickens (Grami et al., 2014). ESBL genes in Tunisia appear to be located on various plasmids carried by different *E. coli* phylogroups. These include mainly IncI1 followed by IncF and IncFIB (**Table 2**). *bla*CTX-M as well as CMYgenes in Tunisia were found to be also associated to the IS*Ecp1* insertion sequence. Furthermore, apart from the CMY gene, AmpC production in *E. coli* strains in this country was found to be also mediated via mutations in the promoter region of the chromosomal AmpC gene (Ben Slama et al., 2010). In Algeria, CTX-M-like enzymes were detected in *E. coli* (Mezhoud et al., 2015; Chabou et al., 2017) as well as in other species such as ST15 *Salmonella* Heidelberg (Djeffal et al., 2017). In their study, Djeffal et al. reported the detection of the same sequence type "ST15" of *Salmonella* spp isolated from both chicken and human. This emphasizes on the hypothesis that the poultry production system could constitute a potent contributor to the diffusion of multi-drug resistant *Salmonella* in the human population (Djeffal et al., 2017). In parallel, *bla*SHV-12 and CMY-2 genes were detected in *E. coli* strains recovered from slaughtered broilers' intestinal swabs (Belmahdi et al., 2016).

In Egypt, *E. coli* producing CTX-M-15 and CMY-2 were initially reported from blood samples from the hearts of

septicemic broilers in 2011 (Ahmed and Shimamoto, 2013). CTX-M-15 and CTX-M-14 were further detected in *E. coli*, *K. pneumoniae*, *K. oxytoca,* and *Enterobacter spp* isolated from chicken carcasses in the north of Egypt (Abdallah et al., 2015; Ahmed and Shimamoto, 2015). *E. coli* isolates harboring SHV-12 have also been reported in Egypt; although they originated from liver and heart samples of chickens affected with colibacillosis (El-Shazly et al., 2017; **Figure 1**). Similarly to other countries in the Mediterranean basin, ESBL producers in the Egyptian poultry sector belong mainly to the A and B1 phylogroups with the *bla*CTX-M genes being associated with IS*Ecp1* (**Table 2**).

Cattle and Sheep

Cattle and sheep are essential members of the human food and agricultural system. For humans, cattle and sheep serve as a source of meat and milk. In agriculture, their feces are commonly used as manure for artificial fertilization (Nyberg et al., 2014). As it is now widely recognized that animals' intestines are a normal habitat for wild type and resistant micro-organisms (Nelson et al., 2013), it has been suggested that if resistant bacteria contaminated animal manures are used without prior treatment, there is a potential risk of transmitting this resistance to the surrounding environment and to the human population (Hruby et al., 2016). This transmission may occur through irrigation and drinking water without treatment (Hruby et al., 2016) or through animals grazing on contaminated lands (Bagge et al., 2009).

In France, the first identification of an ESBL producer in cattle dates back to 2004 when *E. coli* strains harboring CTX-M-1 and CTX-M-15 were isolated from cows (Meunier et al., 2006). *E. coli* producing the CTX-M-15 type ESBL were later isolated from the fecal sample of a dead calf (Valat et al., 2012) and from the feces of cattle located in 10 different geographical areas in France (Madec et al., 2012). In the aforementioned study, CTX-M-15 was carried on IncI1 plasmids but also on F31:A4:B1/IncFII and F2:A–:B–/IncFII plasmids which has been extensively reported in humans (Madec et al., 2012). Although CTX-M-15 appears to be dominant in French cattle, other ESBL types were also reported in *E. coli* (Hartmann et al., 2012) and *Klebsiella* species (Dahmen et al., 2013b; Haenni et al., 2014a) such as CTX-M-1, CTX-M-14, CTX-M-9, CTX-M-2, CTX-M-32, CTX-M-57, CTX-M-3 (Dahmen et al., 2013b; Haenni et al., 2014a), and TEM-71(Hartmann et al., 2012). These latter were carried by *E. coli* strains of different sequence types such as ST23, ST58, ST10, ST45, ST88, ST2210, ST2212-ST2215, ST2497, and ST2498 (**Table 1**); no epidemic clones such as ST101 were detected. Moreover, two studies in France detected AmpC-producing *E. coli* in calves. In both, AmpC beta-lactamase production was suggested as being due to highly conserved mutations in the promotor/attenuator region and to an over-expression of the

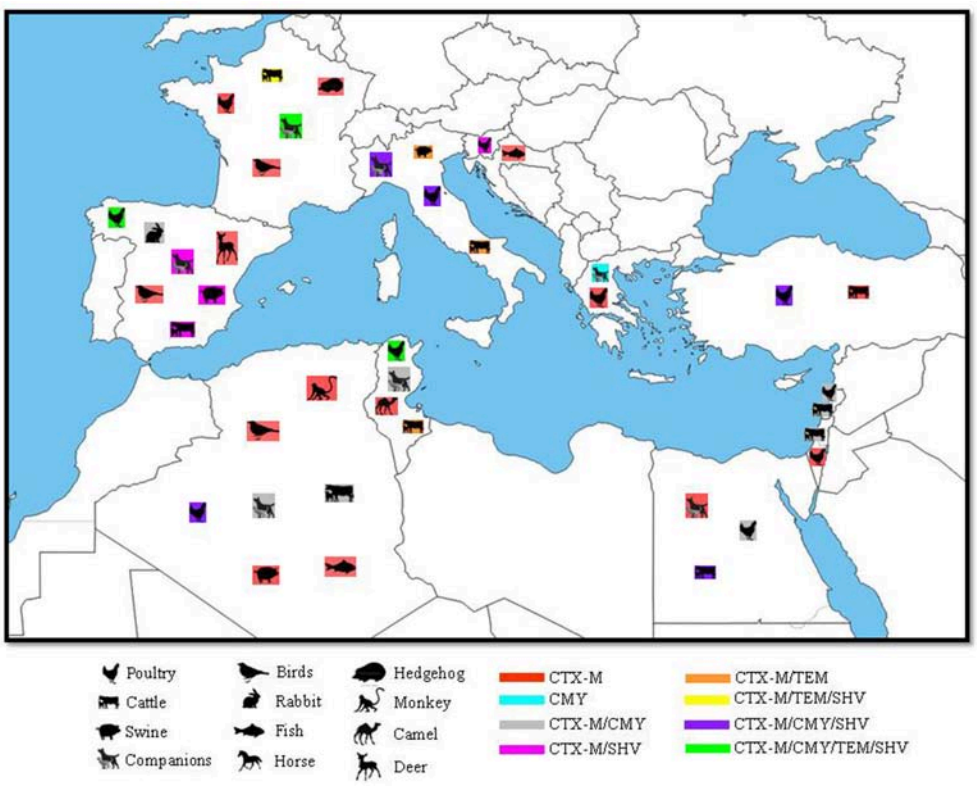

FIGURE 1 | Geographical distribution of ESBLs and their correspondent animal hosts in the Mediterranean Basin. N.B: only SHV and TEM genes confirmed by sequencing as ESBL were included.

TABLE 1 | Non Beta-lactam resistance in MDR of animal origin vs. antibiotic consumption in the Mediterranean Basin.

Country	Animal host	Species (number)	*bla*gene Type (number)	Non beta-lactam Resistance	Antibiotic usage	References
Algeria	Poultry	*E. coli* (17)	CTX-M (17)	CMX,NAL,SXT	Unknown	Mezhoud et al., 2015
	Poultry	*E. coli* (16)	CTX-M (2), SHV (14), CMY (4)	AMK, CIP, KAN, NAL, STR, TOB		Belmahdi et al., 2016
	Poultry	*Salmonella spp* (11)	CTX-M (11)	CIP		Djeffal et al., 2017
	Cattle	*A. baumannii* (1)	NDM (1)	CIP		Chaalal et al., 2016; Yaici et al., 2016
	Cattle	*E. coli* (4)	NDM (4), CTX-M (4), CMY (4),			Yaici et al., 2016
	Birds	*E. coli* (11)	CTX-M (11)	CIP, NAL, NEO SXT, TET,		Meguenni et al., 2015
	Birds	*A. baumannii* (4)	OXA (4)			Morakchi et al., 2017
	Dogs	*E. coli* (1)	NDM (1)	FLU, TET		Yousfi et al., 2015
	Dogs	*E. coli* (15)	CTX-M (13), SHV (3)	CIP, GEN, NAL, SUL, SXT, TET, TMP, TOB		Yousfi et al., 2016b
	Dogs	*E. coli* (3)	CTX-M (1), CMY (1), NDM (1), OXA-48 (2)	GEN, CIP, NAL, SXT, TEM, TOB,		Yousfi et al., 2016a
	Cats	*E. coli* (2)	CMY (1), OXA-48 (2)	CIP, GEN, NAL, SXT, TEM, TOB		Yousfi et al., 2016a
	Cats	*E. coli* (5)	CTX-M (5)	CIP, NAL, SUL, SXT, TET, TMP, TOB		Yousfi et al., 2016b
	Fish	*E. coli* (22)	CTX-M (16), TEM (6)	AMK, CIP, CMX, GEN, KAN, NAL, NET, OFX		Brahmi et al., 2016
	Fish	*A. baumannii* (2)	OXA-23 (2)	CIP, GEN, KAN, SXT		Brahmi et al., 2016
	Macaques	*K. pneumoniae* (7)	CTX-M (7)	CIP, FOS, GEN, SXT		Bachiri et al., 2017
	Wild Boars	*E. coli* (30)	CTX-M (30)	AMK, CIP, FOS, GEN, SXT, TET		Bachiri et al., 2017
		K. pneumoniae (10)	CTX-M (10)			
Tunisia	Poultry	*E. coli* (13)	CTX-M (12), CMY (1)	CIP, CHL, GEN, NAL, SXT, SUL, STR, TET	Streptomycin, Tetracycline, Sulphonamides, Trimethoprim	Ben Slama et al., 2010; Ben Sallem et al., 2012
	Poultry	*E. coli* (67)	CTX-M (42), CMY (24)	AMK, GEN, NAL, NOR, SXT, TET		Mnif et al., 2012
	Poultry	*E. coli* (16)	CTX-M (16)	NAL, SXT, STR, SUL, TET		Kilani et al., 2015
	Poultry	*E. coli* (7)	CTX-M (7)	NAL, STR, TET, SUL, TMP		Grami et al., 2013
	Poultry	*E. coli* (10)	CTX-M (8), TEM (1), CMY (2)	NAL, SXT, SUL, TET, STR		Ben Sallem et al., 2012
	Poultry	*E. coli* (48)	CTX-M (35), CMY (13)	AMK, CIP, GEN, MIN, NAL, SXT, TET		Maamar et al., 2016
	Poultry	*E. coli* (5)	CTX-M (4), SHV (1)			Jouini et al., 2013
	Cattle	*E. coli* (1)	CTX-M (1)	GEN, TOB, TET		Grami et al., 2014
	Beef	*E. coli* (1)	CTX-M (1)	CIP, NAL, SXT, SUL, TET		Ben Slama et al., 2010
	Beef	*E. coli* (5)	CTX-M (5)	CHL, GEN, STR, SUL, SXT, TET, TOB		Jouini et al., 2013
	Sheep	*E. coli* (3)	CTX-M (5), TEM (1)	CIP, GEN, NAL, SXT, SUL, STR, TET		Ben Slama et al., 2010
	Dogs	*E. coli* (6)	CTX-M (6)	CHL, ENR. GEN, KAN, NAL, NET, SUL, STR, TET, TMP, TOB		Grami et al., 2013
	Dogs	*E. coli* (6)	CTX-M (5), CMY (1)	CIP, NAL, SXT, STR, SUL, TET		Sallem et al., 2013
	Cats	*E. coli* (1)	CTX-M (1)	NAL, STR, SUL, TET, TMP,		Grami et al., 2013
	Cats	*E. coli* (8)	CTX-M (8)	CIP, KAN, NAL, STR, SXT, SUL, TET		Sallem et al., 2013
	Dromedaries	*E. coli* (1)	CTX-M (1)	SUL, TET		Ben Sallem et al., 2012

(Continued)

TABLE 1 | Continued

Country	Animal host	Species (number)	*bla*gene Type (number)	Non beta-lactam Resistance	Antibiotic usage	References
Egypt	Poultry	*E. coli* (18)	CTX-M (7), CMY (11)	CHL, CIP, KAN, NAL, SPX, STR, SXT, TET	Fluoroquinolones, Tetracyclines, Aminoglycosides, Cefotaxime	Ahmed and Shimamoto, 2013; Dahshan et al., 2015
	Poultry	*E. coli* (9)	CTX-M (2), SHV (1), TEM (1), CMY (1)	CIP, CMX, DOX, GEN, STR		El-Shazly et al., 2017
	Poultry	*K. pneumoniae* (15)	NDM (15), KPC (14), OXA (12)	-		Hamza et al., 2016
	Poultry	*K. pneumoniae* (11) , *K. oxytoca* (1)	NDM (12)			Abdallah et al., 2015
		E. coli (8)	CTX-M (8)			
		K. pneumoniae (40)	CTX-M (40)			
		K. oxytoca (2)	CTX-M (2)			
		Enterobacter spp (9)	CTX-M (9)			
	Cattle	*E. coli* (112)	CTX-M (106), OXA (6)	FOS, FLU, CMX, CHL, MLS, TET,	Tetracycline, quinolones	Braun et al., 2016
	Cattle	*E. coli* (8)	CTX-M (2), SHV (5), CMY (1)	NAL, SXT, STR, TET		Ahmed et al., 2009
	Beef	*E. coli* (4)	CTX-M (1), SHV (1), CMY (2)	CHL, CIP, GEN, KAN, NAL, SPX, STR, SXT, TET	Fluoroquinolones	Ahmed and Shimamoto, 2015
	Cats	*E. coli* (5)	CTX-M (5)			Abdel-Moein and Samir, 2014
	Dogs	*E. coli* (11)	CTX-M (11)			Abdel-Moein and Samir, 2014
		K. pneumoniae (3)	CTX-M (3)			
		P. mirabilis (1)	CTX-M (1)			
Palestine	Cattle	*E. coli* (287)	CTX-M (287)	SXT, STR, TET	Chlortetracycline, doxycycline, Norfloxacin, Cephalexin, Ceftiofur, Sulfa agents, Gentamicin, Monensin	Adler et al., 2015
		K. pneumoniae (4)	SHV (4)	CHL, CIP, GEN		
	Poultry	*E. coli* (9)	CTX-M (9)			Qabajah et al., 2014
Lebanon	Poultry	*E. coli* (217), *K. pneumoniae* (8), *P. mirabilis* (3), *E. albertii* (2), *E. fergusonii* (1), *E. cloacae* (3),	CTX-M, CMY	CIP, GEN, SXT	Gentamicin, Tetracyclines	Dandachi et al., 2018a
	Cattle	*E. coli* (27)	CTX-M (27)	CHL, ENR, GEN, KAN, NAL, STR, SUL, TET, TMP	Penicillin G - Streptomycin, Ampicillin, Amoxicillin Oxytetracycline, Gentamicin,	Gundogan et al., 2011; Diab et al., 2016
	Fowl	*A. baumannii* (1)	OXA-48 (1)	AMK, GEN, TOB	Unknown	Al Bayssari et al., 2015b
	Horse	*A. baumannii* (1)	OXA-143 (1)			Rafei et al., 2015
	Rabbit	*A. pitii* (1)	OXA-24 (1)			
Turkey	Poultry		CTX-M (60), SHV (4), CMY (18)	CHL, KAN, NAL, STR, SUL, TET, TMP	Tetracycline, Quinolones	Politi et al., 2005; Pehlivanlar Onen et al., 2015
	Cattle	*E. coli* (3)	CTX-M (2), CMY (1)	NAL, SXT, STR, TET		

(Continued)

TABLE 1 | Continued

Country	Animal host	Species (number)	*bla*gene Type (number)	Non beta-lactam Resistance	Antibiotic usage	References
	Poultry	*E. coli* (15)	CTX-M (15)			Tekiner and Ozpinar, 2016
	Cattle	*E. coli* (19)	CTX-M (19)			
Croatia	Mussel	*Aeromonas. Caviae* (25)	CTX-M (11), SHV (11), FOX (3)		Tetracycline, Amphenicol, Penicillins, Sulfonamides, Trimethoprim, Fluoroquinolones, Aminoglycosides, Polymixins	Maravić et al., 2013; EMA/ESVAC, 2014
		A. Hydrophila (8)	CTX-M (8), SHV (2)			
Greece	Poultry	*Salmonella enteric* (2)	CTX-M (2)	CHL, KAN, STR, SUL, TMP, TET	Unknown	Politi et al., 2005
	Dogs	*E. coli* (8)	CMY (8)	FLU		Vingopoulou et al., 2014
Slovenia	Poultry	*E. coli* (6)	CTX-M (2), SHV (4)	GEN, NAL, STR, SUL	Ceftiofur	Chiaretto et al., 2008
Italy	Poultry, Cattle, Swine				Tetracyclines, Amphenicol, Penicillins, 3rd/4th Cephalosporins, Sulfonamides, Trimethoprim, Macrolides, Lincosamides, Fluoroquinolones, Aminoglycosides, Polymixins, Pleuromutilins, Tylosin, Flumequine,	
	Poultry	*E. coli* (8)	CTX-M (7), SHV (1),	CIP		Giufrè et al., 2012
	Poultry	*E. coli* (60)	CTX-M (45), CIT-like (15)	CIP, GEN, SXT, TET		Ghodousi et al., 2015
	Poultry	*E. coli* (67)	CTX-M (24), SHV (43)	CIP, NAL, SUL, TMP, TET		Bortolaia et al., 2010
	Poultry	*Salmonella spp* (12)	SHV (12)	GENT, NAL, SUL, STR, TET		Chiaretto et al., 2008
	Poultry	*Salmonella infantis* (30)	CTX-M (30)	CIP, NAL, SUL, TMP, TET		Franco et al., 2015
	Swine	*Salmonella infantis* (2)	CTX-M (2)			
	Cattle	*K. ozaenae* (5)	CTX-M (5), TEM (1)			Stefani et al., 2014
	Swine	*E. coli* (15)	CTX-M (10), TEM (7)			
	Dogs	*K. oxytoca* (2)	SHV (2), DHA (2)	CIP, GEN, KAN, STR, SUL, TET, TMP		Donati et al., 2014
		K. pneumoniae (11)	CTX-M (11), SHV (5), DHA (1)	CIP, GEN, KAN, NAL, TET, TMP		
	Dogs	*K. pneumoniae* (1)	CTX-M (1), SHV (1)	CIP, LEV		Bogaerts et al., 2015
		E. coli (1)	CMY (1)	CIP, LEV		
	Cats	*K. oxytoca* (2)	CTX-M (2)	CIP, SUL, TMP, TET		Donati et al., 2014
		K. pneumoniae (4)	CTX-M (2), SHV (2), DHA (1), CMY (1)	CIP, KAN, NAL, SUL, TET, TMP		
	Cats	*E. coli* (7)	CTX-M (7), CMY (2)	CHL, ENR, GEN, NAL, NIT, SPX, STR, SUL, TET, TMP.		Nebbia et al., 2014
France	Poultry, Cattle, Swine				Tetracycline, Amphenicol, Penicillins, 1st/2nd/3rd/4th Cephalosporins, Sulfonamides, Trimethoprim, Macrolides, Lincosamides, Fluoroquinolones, Aminoglycosides, Polymixins, Pleuromutilins	EMA/ESVAC, 2014

(Continued)

TABLE 1 | Continued

Country	Animal host	Species (number)	*bla*gene Type (number)	Non beta-lactam Resistance	Antibiotic usage	References
	Cattle	*E. coli* (26)	CTX-M (21), TEM (5)	CHL, GENT, SXT		Hartmann et al., 2012
	Cattle	*E. coli* (3)	CTX-M (3)	CHL, ENR, FFC, GEN, KAN, NAL, STR, SUL, TET, TMP		Meunier et al., 2006
	Cattle	*A. baumannii* (9)	OXA-23 (9)	FOS, KAN, TET		Poirel et al., 2012
	Cattle	*E. coli* (9)	CTX-M (9)	CHL, ENR, GEN, KAN, NAL, NET, OFX, STR, SUL, TET, TOB, TMP		Madec et al., 2012
	Cattle	*E. coli* (5)	CTX-M (5)	APR, CHL, ENR, GEN, KAN, NAL, NET, OFX, STR, SUL, TET, TOB, TMP		Dahmen et al., 2013b
		K. pneumoniae (1)	CTX-M (1)			
	Sheep	*K. pneumoniae* (3)	CTX-M (3), DHA (3)	NAL, SUL, SXT, TET		Poirel et al., 2013
		E. fergusonii	CTX-M (1)			
	Veal calves	*E. coli* (147)	CTX-M (147)	APR, CHL, ENR, FFC, GEN, KAN, NAL, NET, SUL, STR, TET, TOB, TMP		Haenni et al., 2014a
		K. pneumoniae (3)	CTX-M (2), SHV (1)	FLU, SUL, STR, TET, TMP		
	Swine	*E. coli* (3)	CTX-M (3)	CHL, NAL, STR, SUL, TET, TMP		Meunier et al., 2006
	Dog	*E. cloacae* (11)	CTX-M (10), SHV (1)	FLU, GEN, KAN, QUI, TET, SUL, STR, TMP		Haenni et al., 2016c
	Dog	*E. coli* (47)	CTX-M (47), CMY (24)	CHL, GEN, KAN, STR, TOB ENR, FFC, NAL, NET, OFX, SUL, TET, TMP		Haenni et al., 2014a
	Dog	*E. coli* (9)	CTX-M (8), TEM (1)	GEN, SUL, TET		Poirel et al., 2013
		K. pneumoniae (8)	CTX-M (8), DHA (1)	GEN, NAL, SUL, SXT, TET		
		K. oxytoca (2)	CTX-M (2)			
	Dog	*P. mirabilis* (14)	CTX-M (1), CMY (7), DHA (2), VEB (6)	APR, CHL, ENR, GEN, KAN, NAL, NET, STR, SUL, TOB, TMP		Schultz et al., 2017
	Dog	*A. baumannii* (2)	OXA-23 (2)	CIP, SXT		Hérivaux et al., 2016
	Dog	*E. coli* (3)	CMY (2), OXA-48 (1)	GEN, NAL		Melo et al., 2017
	Cat	*A. baumannii* (1)	OXA-23 (1)	GEN, NAL, SUL, STR, TET		Ewers et al., 2016
	Cat	*K. pneumoniae* (3)	CTX-M (3), DHA (3)	NAL, SUL, SXT, TET	Unknown	Poirel et al., 2013
		E. coli (3)	CTX-M (3)	GEN, SUL, TET	Unknown	
	Cat	*P. mirabilis* (1)	CMY (1)	ENR, NAL, SUL, TMP		Schultz et al., 2017
		P. rettgeri (1)	CTX-M (1)	ENR, NAL, SUL, TMP		
	Cat	*E. coli* (2)	CTX-M (2)	STR, TMP		Melo et al., 2017
	Cat	*E. cloacae* (11)	CTX-M (10), SHV (1)	FLU, GEN, KAN, QUI, SUL, STR, TET, TMP		Haenni et al., 2016c
	Companions	*E. coli* (19)	CTX-M (19)	CIP, NAL, SUL, STR, TET		Dahmen et al., 2013a
	Hedgehog	*E. coli* (1)	CTX-M (1), DHA (1)	NAL, SUL, SXT, TET	Unknown	Poirel et al., 2013
	Tawny Owl	*E. coli* (1)	CTX-M (1)			
	Domestic goose	*E. coli* (1)	CTX-M (1)			
	Rock Pigeon	*E. coli* (1)	CTX-M (1)			
	Horse	*E. cloacae* (14)	CTX-M (8), SHV (6)	FLU, GEN, KAN, QUI, SUL, STR, TET, TMP		Haenni et al., 2016c
	Horse	*P. mirabilis* (14)	VEB (2)	ENR, CHL, KAN, NAL, NET, SUL, STR, TOB, TMP	Unknown	Schultz et al., 2017

(Continued)

TABLE 1 | Continued

Country	Animal host	Species (number)	*bla*gene Type (number)	Non beta-lactam Resistance	Antibiotic usage	References
Spain	Poultry, Cattle, Swine				Tetracycline, Amphenicol, Penicillins, 3rd/4th Cephalosporins, Sulfonamides, Trimethoprim, Macrolides, Lincosamides, Fluoroquinolones, Quinolones, Aminoglycosides, Polymixins, Pleuromutilins	Abreu et al., 2014; EMA/ESVAC, 2014
	Poultry	*E. coli* (64)	CTX-M (44), SHV (6), TEM (2), CMY (13)	CHL, CIP, FUR, GEN, KAN, NAL, SUL, SXT, TET, TOB, TMP		Blanc et al., 2006
	Poultry	*S. enterica* (2)	CTX-M (1), SHV (1)	NAL, SXT, STR, SUL, TET,		Riaño et al., 2006
	Poultry	*E. coli* (116)	CTX-M (116)	CIP, NAL, SXT		Abreu et al., 2014
	Poultry	*E. coli* (11)	CTX-M (6), SHV (2), CMY (2)	CHL, CIP, FFC, GEN, KAN, NAL, STR, SUL, TET, TMP		Solà-Ginés et al., 2015b
	Poultry	*E. coli* (50)	CTX-M (40), CMY (10)	NAL		Cortés et al., 2010
	Poultry	*E. coli* (62)	CTX-M (20), SHV (42)	CIP, NAL		Egea et al., 2012
	Swine	*E. coli* (20)	CTX-M (20)			Solà-Ginés et al., 2015b
	Swine	*S. enteric* (1)	SHV (1)	SUL, STR, TET		Riaño et al., 2006
	Swine	*E. coli* (39)	CTX-M (27), SHV(12)	CIP, CHL, FUR, GEN, KAN, NAL, SUL, SXT, TET, TMP, TOB		Blanc et al., 2006
	Swine	*E. coli* (20)	CTX-M (8), SHV (12)	APR, CIP, GEN, NAL, STR, SUL, TET, TMP		Escudero et al., 2010
	Dog	*E. coli* (1)	SHV (1)	CHL, CIP, NAL, SUL, TET, TMP		Teshager et al., 2000
	Dog	*E. coli* (1)	CMY (1)			Bogaerts et al., 2015
		P. mirabilis (2)	CMY (2)	DOX, MIN		
	Dog	*K. pneumoniae* (2)	CTX-M (1), VIM (1), DHA (1)			González-Torralba et al., 2016
		E. cloacae (1)	SHV (1)			
	Deer	*E. coli* (1)	CTX-M (1)	CIP, CHL, NAL, SXT, TET	Unknown	Alonso et al., 2016
	Rabbit	*E. coli* (1)	CMY (1)		Unknown	Blanc et al., 2006
		E. cloacae (3)	CTX-M (3)			

**APR, refers to apramycin; AMK, amikacin; CIP, ciprofloxacin; CHL, chloramphenicol; CMX, co-trimoxazole; DOX, doxycycline; ENR, enrofloxacin; FFC, florfenicole; FLU, fluoroquinolones; FOS, fosfomycin; FUR, furazolidone; GEN, gentamicin; KAN, kanamycin; LEV, levofloxacin; MIN, minocycline; MLS, Macrolides; NAL, nalidixic acid; NET, netilmicin; NIT, nitrofurantoin; NOR, norfloxacin; OFX, oxofloxacin; QUI, quinolones; SPX, spectinomycin; SXT, trimethoprim-sulfamethoxazole; TEM, temocillin; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin.*

chromosomal AmpC gene, respectively (Haenni et al., 2014a,c). In sheep, only one study was conducted in France in which one CTX-M-1 *E. fergusonii* and three *K. pneumonia* harboring both *bla*CTX-M-15 and DHA genes were detected (Poirel et al., 2013). The three *K. pneumoniae* were co-resistant to nalidixic acid, sulfonamides, trimethoprim-sulfamethoxazole and tetracycline and belonged to the same sequence type ST274. In Spain, ESBL-producing Gram-negative bacilli were isolated from beef samples collected from different geographical locations (Doi et al., 2010; Ojer-Usoz et al., 2013). In Italy, Stefani et al. reported the isolation of five *Klebsiella ozaenae* harboring CTX-M-1, CTX-M-1/TEM-24 and CTX-M-15 ESBL types from cattle (Stefani et al., 2014).

In Turkey, a study conducted in 2007–2008, showed the presence of ESBL-producing *K. pneumoniae* and *K. oxytoca* in raw calf meat (Gundogan et al., 2011). Later on, CTX-M-3 and CTX-M-15 harboring *E. coli* were isolated from beef samples sold in a market in the south of Turkey (Conen et al., 2015). Recently, a study conducted by Tekiner et al. reported the isolation of ESBL-producing *E. coli*, *E. cloacae,* and *Citrobacter brakii* from raw cows' milk collected from different cities of Turkey. In these areas, CTX-M-1 was dominant (Tekiner and Ozpinar, 2016). In Lebanon the situation differs, in that unlike Turkey but similarly to other Mediterranean countries, *bla*CTX-M-15, *bla*SHV-12, and *bla*CTX-M-14 are the dominant ESBL genes prevailing in *E. coli* in the Lebanese cattle (Diab et al., 2016). In this latter study, various sequence types were detected. Of special interest is the detection of ST10. ST10 was heavily reported in the literature as being shared between animal and human isolates all over

the world: Chile (Hernandez et al., 2013), Denmark (Huijbers et al., 2014), Vietnam (Nguyen et al., 2015), Germany (Belmar Campos et al., 2014). Indeed, it has been suggested that ST10 became associated with the production and dissemination not only of CTX-M-type ESBLs but also of *mcr-1* in animals, humans and environment (Monte et al., 2017). In Israel, Adler et al. reported the identification of CTX-M-1/CTX-M-9 and SHV-12 beta-lactamase producing *E. coli* and *K. pneumoniae* strains respectively, which were isolated from cattle farms situated in the main farming locations across the country (Adler et al., 2015).

In Egypt, SHV-12 (Ahmed et al., 2009) in addition to CTX-M-1/15 and CTX-M-9 were detected in *E. coli* strains isolated from cattle (Braun et al., 2016). On study targeting raw milk samples reported the detection of SHV-12 /CTX-M-3, in addition to CMY-2-producing *E. coli* strains (Ahmed and Shimamoto, 2015). In Tunisia, *E. coli* strains producing CTX-M-1 and TEM-20 were isolated from beef and sheep situated in different areas across the country (Jouini et al., 2007; Ben Slama et al., 2010). Furthermore, *bla*CTX-M-15 was detected in an ST10 *E. coli* isolate recovered from the milk sample of cattle affected with mastitis (Grami et al., 2014). Similarly, In Algeria, Yaici et al. reported the detection of four ST1284 *E. coli* strains carrying CTX-M-15, CMY-42, and NDM-5 in raw milk samples (Yaici et al., 2016).

Swine

Meat from pigs is used by humans for consumption and their feces are used as manure for land fertilization. Studies have shown that antibiotics are usually detected in higher concentrations in pig manures compared to that of other farm animals (Hou et al., 2015). This finding reflects high and uncontrolled antimicrobial usage in swine farms (Woolhouse et al., 2015). Heavy antibiotic usage creates a selective pressure that contributes to the emergence and spread of bacterial resistance; in this regard, pigs are suggested as a potential source of resistant bacteria.

Reports concerning the prevalence of ESBL of swine origin in the Mediterranean area are very scarce with the majority being reported from Spain where a *bla*SHV-12 positive *Salmonella enterica* was isolated in the early 2000s (Riaño et al., 2006). Furthermore, CTX-M-grp-9 (Doi et al., 2010; Ojer-Usoz et al., 2013), SHV-5 and CTX-M-grp-1 carried by A phylogroup *E. coli* strains and SHV-12 carried by B1 *E. coli* and *bla*SHV-5 were detected (Blanc et al., 2006; Cortés et al., 2010). One study conducted in 13 different Spanish provinces found seven AmpC-producing *E. coli*. In these cases, AmpC production was due to a mutation in the promoter region of the chromosomal AmpC gene (Escudero et al., 2010). In Italy, TEM-52, CTX-M-1, CTX-M-15, and CTX-M-1/TEM-201 carrying *E. coli* were reported in pigs (Stefani et al., 2014). Franco et al. reported also the presence of *Salmonella* infantis carrying CTX-M-1 in swine (Franco et al., 2015). In France, only one study conducted at the beginning of the Twenty-first century reported the detection of CTX-M-1-producing *E. coli* strains in pigs (Meunier et al., 2006). Similarly to what is widely observed in the Mediterranean basin, the CTX-M-1 was associated with the insertion sequence IS*Ecp1*(Meunier et al., 2006). In Algeria, CTX-M-15 harboring *E. coli* and *K. pneumoniae* strains were isolated in 2014 from wild boars (Bachiri et al., 2017). MLST typing showed the *K. pneumoniae* belongs to the ST584 while on the other hand several sequence types (ST617, ST131, ST648, ST405, ST1431, ST1421, ST69, ST226) were observed among *E. coli* strains (Bachiri et al., 2017). The aforementioned study was the only one to investigate the epidemiology of ESBL-producing Gram-negative bacilli in the African and Asian countries lining the Mediterranean Sea.

Companion Animals

Unlike food producing animals, companion animals are not used as consumption source of human food, nor are their feces used as manure for land fertilization. Instead, these animals are kept for the individual's protection, entertainment and company. The number of companion animals has significantly increased in modern society in recent decades (Pomba et al., 2017). Despite regular close contact with people, little attention has been given to the prevalence of antimicrobial resistance in these animals (Scott Weese, 2008). The close contact between companion animals such as dogs, cats, and horses and their owners makes the transmission of resistant organisms more likely to occur (Dierikx et al., 2012). As such, it is essential to investigate the prevalence of resistant bacteria in companion animals as well as to identify the possible risk factors for the transmission of resistant organisms to humans (Rubin and Pitout, 2014).

In the Mediterranean basin, the first detection of ESBL in companion animals was in Spain where an *E. coli* harboring SHV-12 was isolated from a dog with a urinary tract infection (Teshager et al., 2000). Subsequently, between 2008 and 2010, three strains carrying CMY-2 (one ST2171 *E. coli* and two *P. mirabilis*) were recovered from dogs infected with respiratory, urinary tract and skin and soft tissue infections, respectively (Bogaerts et al., 2015). In all three strains, the CMY-2 genes were associated with the IS*Ecp1*. More recently, one *K. pneumoniae* and one *E. cloacae* producing CTX-M-15/DHA and SHV-12, respectively, were isolated from the fecal swabs of healthy dogs in this same country (González-Torralba et al., 2016).

In Italy, a study conducted by Donati et al. on 1,555 dog samples of clinical cases and necropsy specimens with suspicious bacterial infections, between the center and the north of Italy found two *K. oxytoca* harboring SHV-12/DHA-1 and 11 *K. pneumoniae* carrying the following genes: *bla*CTX-M-15 (six strains), *bla*CTX-M-15/DHA-1, *bla*CTX-M-15/SHV-28, *bla*CTX-M-1/SHV-28, and *bla*CTX-M-1 (Donati et al., 2014). In this same study, 429 cats' samples were also investigated revealing the presence two *K. oxytoca* producing CTX-M-9 and four *K. pneumoniae* producing CTX-M-15 (two isolates), CTX-M-15/ DHA-1 and SHV-28/CMY-2 beta-lactamases (Donati et al., 2014). The beta-lactamase and AmpC genes in *K. oxytoca* strains isolated from dogs and cats were located on different plasmid types: IncL/M versus IncHI2 respectively. This is unlike the *K. pneumoniae* strains where the *bla*CTX-M-15 was localized on the same plasmid IncR and both strains in dogs and cats shared the same ST340. ST15 and ST101 were also common between dogs and cats in this study. ST15 and ST101 are among the most international clones carrying ESBL as well as carbapenemase genes which became highly detected recently worldwide (Donati et al., 2014). Another study conducted reported the detection of CTX-M-1-producing *K. pneumoniae* was further reported from a

dog with urinary tract infection and an *E. coli* carrying the CMY-2 type beta-lactamase associated to IS*Ecp1* also in a diseased cat with a urinary tract infection (Bogaerts et al., 2015). Infections in pets with *E. coli* strains carrying CTX-M-14 (three isolates), CTX-M-15, CTX-M-1, and CTX-M-14/CMY-2 (two isolates) were also reported in Italy (Nebbia et al., 2014). The strains also showed different sequence types and phylogroups (A "ST3848, ST3847," B2 "ST131, ST155, ST555, ST4181," B1 "ST602") emphasizing that apparently the dissemination of ESBL and AmpC beta-lactamase producers is most likely due to the successful spread of various plasmids carrying these resistance genes (Nebbia et al., 2014).

In France, the highest number of studies addressing the prevalence of extended-spectrum-cephalosporin resistance in companion animals in the Mediterranean was conducted. In dogs, CTX-M-grp 1 (CTX-M-1, CTX-M-15, CTX-M-3, CTX-M-32) and CTX-M-grp 9 in addition to CMY-2 and TEM-52 prevail in *E. coli* (Dahmen et al., 2013a; Poirel et al., 2013; Haenni et al., 2014b; Bogaerts et al., 2015; Melo et al., 2017). These genes were mostly carried on IncI1, IncFII, and IncHI2 plasmid types and were harbored by strains of different sequence types and phylogroups. Furthermore, *K. pneumoniae* isolated from dogs showed to produce the CTX-M-15, CTX-M-32, SHV-12, and DHA-1 have been reported (Poirel et al., 2013; Haenni et al., 2014b). In parallel, *P. mirabilis* showed to produce CMY-2, DHA-16, VEB-6, and CTX-M-15 have been described (Schultz et al., 2017) and *E. cloacae* the CTX-M-15, CTX-M-14, CTX-M-3, and SHV-12 have been identified (Haenni et al., 2016c). In addition, CTX-M-15 and CMY-2 were also decribed in *K. oxytoca* and *Salmonella enterica*, respectively isolated from dogs in this same country (Poirel et al., 2013; Haenni et al., 2014b). On the other hand, in cats, the following distribution was observed: in *E. coli* (CTX-M-1, CTX-M-15, CTX-M-32, CTX-M-3, CTX-M-14) (Poirel et al., 2013; Melo et al., 2017), in *K. pneumoniae* (CTX-M-15/DHA) (Poirel et al., 2013), in *E. cloacae* (CTX-M-15, SHV-12) (Haenni et al., 2016c), in *P. mirabilis* (CMY-2) and in *Proteus rettgeri* (CTX-M-1) (Schultz et al., 2017). The dissemination of extended-spectrum-cephalosporin resistance in companion animals in France necessitates studies addressing the risk factors responsible for the acquisition of these strains in pets as well as novel approaches to control the spread of resistance in these animals. Furthermore, the contribution of the pet animals to the spread of resistance in the common population in France should be also investigated. Moreover, France is the only Mediterranean country in which studies reporting ESBL and/or AmpC-producing bacteria in horses are available. Between 2010 and 2013, *E. cloacae* harboring CTX-M-15, CTX-M-1, and SHV-12 were isolated from clinical samples of horses. These genes were located on IncHI2 and IncP plasmids and were harbored by strains of various sequence types such as ST127, ST372, ST145, ST114, ST135, ST118, ST268, ST107 (Haenni et al., 2016c). Later on, VEB-6 carrying *P. mirabilis* were isolated from healthy horses (Schultz et al., 2017). In Greece, CMY-2 carried on IncI1 plasmid and harbored by ST212 *E. coli* strains were isolated from diseased canines in 2011 (Vingopoulou et al., 2014). More recently, a study conducted in Greek households revealed the detection of extended-spectrum-cephalosporin-resistant *E. coli* isolates. The strains presented with different sequence types including the human pandemic ST131 clone which suggests a possible from humans to animals and vice-versa (Liakopoulos et al., 2018).

In Egypt, CTX-M beta-lactamases have been detected in *E. coli* recovered from cats' rectal swabs. In this same study, CTX-M-producing *E. coli, K. pneumonia,* and *P. mirabilis* were isolated from dogs (Abdel-Moein and Samir, 2014). In Algeria, only one study reported the detection of *E. coli* strains carrying *bla*CTX-M-1, *bla*CTX-M-15 in cats and *bla*CTX-M-1, *bla*CTX-M-15, *bla*SHV-12 in dogs (Yousfi et al., 2016b). In Tunisia, CTX-M-1 carrying *E. coli* were isolated from cats; while from dogs CTX-M-1, CTX-M-15, and CMY-2-producing *E. coli* were detected (Grami et al., 2013; Sallem et al., 2013). CTX-M-1 was mostly carried on IncI1 plasmid whereas CTX-M-15 on IncFII (Grami et al., 2013). The *bla*CTX-M-1 and CMY-2 genes were also found associated with the IS*Ecp1*. Indeed it appears that the insertion sequence IS*Ecp1* might be also responsible for the dissemination of CMY-2 AmpC genes apart from the *bla*CTX-M ones.

Wild Birds and Domestic Animals

Besides companion and food producing animals, scattered reports exist on the isolation of ESBL from domestic animals such as wild birds and dromedaries in the Mediterranean. For instance, CTX-M-producing *E. coli* was isolated from wild birds in Algeria (Meguenni et al., 2015), Turkey (Yilmaz and Guvensen, 2016), *bla*CTX-M-1 in addition to *bla*CTX-M-15 carrying *E. cloacae* in France (Bonnedahl et al., 2009). Furthermore, in France, CTX-M-1 and CTX-M-15 were detected in ST93, ST124, and ST10 *E. coli* strains recovered from tawny owls/rock pigeons and domestic geese, respectively. In addition, a CTX-M-15/DHA-producing ST274 *K. pneumoniae* was isolated from a hedgehog living in the same city (Poirel et al., 2013). Rooks carrying CTX-M-14 type ESBL in *E. coli* have been described in Italy and Spain (Jamborova et al., 2015). Furthermore, in Spain, *E. coli* and *K. pneumoniae* harboring CTX-M-14, CTX-M-1, CTX-M-32, CTX-M-9, CTX-M-15, CTX-M-14b, CTX-M-3, and CTX-M-8 were recovered from the fecal samples of gulls (Stedt et al., 2015). In rabbits, CMY-2-producing *E. coli* and CTX-M-14, CTX-M-9-producing *E. cloacae* were isolated (Blanc et al., 2006; Mesa et al., 2006). More recently, *bla*CTX-M-1 was identified in *E. coli* isolated from the fecal sample of a deer living in the Los Alcornocales natural park in southern Spain (Alonso et al., 2016). In Algeria, *bla*CTX-M-15 and *bla*CTX-M-9 genes were detected in *E. coli* isolated from the gut and gills of fish caught in the Mediterranean across Bejaia city (Brahmi et al., 2016). In this study, it has been suggested that the presence of beta-lactamase producers is due to contamination of the fish from river water and the rising amount of untreated waste that is released into the Mediterranean Sea from the agricultural as well as the industrial operations (Brahmi et al., 2016). These findings emphasizes on the importance of the natural environment in the dissemination of resistance from humans to animals and vice versa. Furthermore, Bachiri et al. also reported the detection of CTX-M-15-producing ST584 *K. pneumoniae* in Barbary macaques situated in national parks in the north of Algeria (Bachiri et al., 2017). In both Tunisia and Egypt, CTX-M beta-lactamases were detected in *E. coli*

and *Pseudomonas aeruginosa* recovered from dromedaries and camels, respectively (Ben Sallem et al., 2012; Elhariri et al., 2017). In Croatia, the only study investigating the prevalence of ESBL in animals was conducted in 2009–2010 in mussels caught in the Adriatic Sea. In this study, 18 *Aeromonas* species carrying SHV-12, CTX-M-15, FOX-2, and PER-1 were identified (Maravić et al., 2013).

Prevalence of Carbapenemase Producers in Livestock and Domestic Animals

Carbapenems are beta-lactam antibiotics often considered as the last resort antimicrobial agent against multi-drug resistant organisms (Temkin et al., 2014). Carbapenems are active against ESBL and AmpC-producing Gram negative bacilli. Due to the wide dissemination of multi-drug resistant organisms, these antimicrobials recently became heavily used in human medicine. As a result, the emergence of carbapenem resistance has accelerated and it is now a normal phenomenon encountered in hospital settings and, to a lesser extent, community settings. The production of hydrolyzing enzymes called "carbapenemases" is one of the mechanisms by which carbapenem resistance is mediated in Gram negative bacilli. These include (a) class A carbapenemases (KPC, GES, SME, IMI, NMC-A), (b) class B metallo beta-lactamases "MBL" (NDM, VIM, IMP and TMB), and (c) class D oxacillinases (Martínez-Martínez and Gonzalez-Lopez, 2014).

In the Mediterranean basin, in Egypt, OXA-48 and OXA-181 carbapenemases were detected in *E. coli* strains recovered from dairy cattle farms (Braun et al., 2016). In the poultry production system, one study reported the isolation of *K. pneumonia* and *K. oxytoca* harboring NDM metallo beta-lactamases (Abdallah et al., 2015). Another study described the identification of *K. pneumoniae* carrying OXA-48, NDM and KPC type carbapenemases. Isolated strains were recovered from the liver, lungs, and trachea of broiler chicken (Hamza et al., 2016). In Algeria, NDM-1 and NDM-5 were observed, respectively, in ST85 *Acinetobacter baumannii* and ST1284 *E. coli* originating from raw milk in the west and north of the country (Chaalal et al., 2016; Yaici et al., 2016). In *E. coli*, NDM-5 was located on an IncX3 plasmid (Yaici et al., 2016). In broilers, OXA-58 was identified (Chabou et al., 2017) while in pigeons, in addition to OXA-58 and OXA-23 were detected (Morakchi et al., 2017). In terms of companion animals, NDM-5 and OXA-48-producing *E. coli* were reported from healthy dogs Algeria (Yousfi et al., 2015, 2016a). The NDM-5 was harbored by an *E. coli* strain having the same sequence type ST1284 previously described in cattle (Yousfi et al., 2015; Yaici et al., 2016). OXA-48 was further detected in healthy and diseased cats in the same city (Yousfi et al., 2016a). Furthermore, in this same country, two *A. baumannii* producing OXA-23 were isolated from fish (Brahmi et al., 2016). In Lebanon, *A. baumannii* with different sequence types (ST294, ST491, ST492, ST493) were detected in a horse's mouth carrying OXA-143 (Rafei et al., 2015), and in pigs and cattle carrying OXA-23(Al Bayssari et al., 2015a). Furthermore, in cattle, a VIM-2-producing *P. aeruginosa* was isolated (Al Bayssari et al., 2015a). In fowl, Bayssari et al. reported the detection of OXA-23 and OXA-58 harboring *A. baumannii* and OXA-48-producing *E. coli* as well as VIM-2 producing *P. aeruginosa* (Al Bayssari et al., 2015b). VIM-2 producers in fowl and cattle were of different sequence types suggesting the presence of plasmid that is mediating the spread of this resistance gene. In France, OXA-23-producing *Acinetobacter* species were described in cows and dogs (Poirel et al., 2012; Hérivaux et al., 2016). Melo et al. reported the detection of OXA-48 located on an IncL plasmid and carried by an ST372 *E. coli* strain from dogs in France (Melo et al., 2017). In contrast, in Spain, only one study reported the isolation of a VIM-1-producing ST2090 *K. pneumoniae* from a dog's rectal swab (González-Torralba et al., 2016; **Figure 2**).

Clonal Relationship of Beta-Lactamase Producers and Plasmid Types of Beta-Lactamase Genes Isolated From All Animal Sources

The different phylogroups and sequence types of beta-lactamase and *mcr-1* positive strains as well as the type of plasmids carrying ESBL, AmpC, carbapenemase, and *mcr-1* genes detected in all animal sources in the Mediterranean region are summarized in **Table 2**. In this area of the world, it appears that multi-drug resistance in the veterinary sector is mediated by the spread of different phylogroups and sequence types with the main ones being A, B, and D phylogroups (**Table 2**). The detection of ST10 in CTX-M producers in poultry, cattle, pets, and domestic animals in Algeria, Tunisia, Lebanon, and France is of special interest. ST10 was often described in the literature as being common to ESBL *E. coli* strains of human and avian origin worldwide such as in Germany (Belmar Campos et al., 2014), Denmark (Huijbers et al., 2014), Vietnam (Nguyen et al., 2015), and Chile (Hernandez et al., 2013). ST10 was suggested as being associated with the spread of CTX-M ESBL types and *mcr-1* genes in humans, animals and environments (Monte et al., 2017). Another distinct finding is the detection of ST101 in dogs and cats in Italy. ST101 is an international sequence types frequently detected in pigs (El Garch et al., 2017), broilers (Solà-Ginés et al., 2015b) as well as in the clinical settings. In several countries, ST101 was associated to NDM-1 *E. coli* strains isolated from the clinical settings of Germany, Canada, Australia, UK, and Pakistan (Yoo et al., 2013) implying thus that ST101 is a candidate for the zoonotic transmission to the human population.

More deeply speaking, ESBL and AmpC encoding genes were mostly carried on conjugative IncI1, IncFIB, IncN, and IncK plasmids (**Table 1**). IS*Ecp1* was the most common insertion sequence associated with the CTX-M ESBL types with the main ones being *bla*CTX-M-1 and *bla*CTX-M-15 genes. IS*Ecp1* has been previously described as a potent contributor to the mobilization and insertion of *bla*CTX-M genes worldwide (El Salabi et al., 2013). As for the carbapenemase encoding genes, these latter were found to be carried by IncX3 and IncL plasmids detected in *E. coli* strains isolated from cattle, swine and dogs in Algeria, Italy, and France, respectively. Overall, the detection of a variety of sequence types and phylogroups in ESBL and AmpC producers isolated from animals of all origins within and among countries's animals suggests that the dissemination of multi-drug

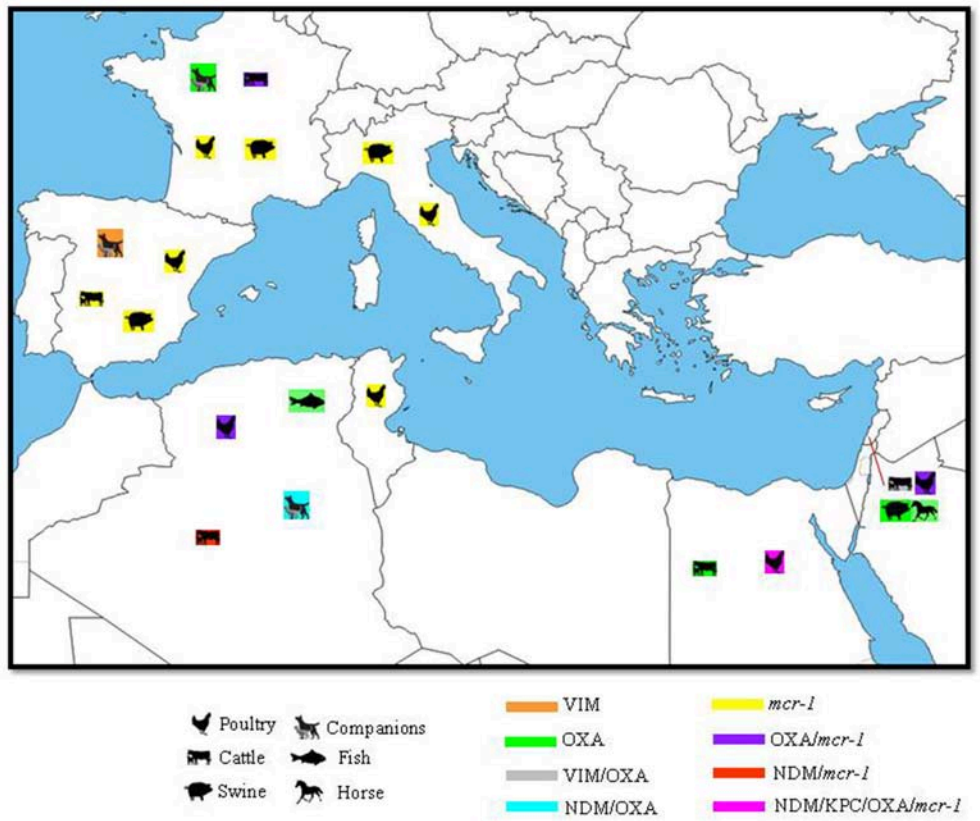

FIGURE 2 | Geographical distribution of carbapenemases and *mcr* colistin resistance gene with their hosts in the Mediterranean. N.B: only OXA genes confirmed by sequencing as carbapenemases were included.

resistance in the Mediterranean is multi-clonal and related rather to the diffusion of conjugative plasmids carrying beta-lactamase genes.

Prevalence of Colistin Resistance in Livestock and Domestic Animals

Polymyxin E (colistin) and polymyxin B are polycationic antimicrobial peptides that are considered as the last-line antibiotic treatment for multi-drug resistant (MDR) Gram-negative bacterial infections (Olaitan and Li, 2016). From the 1960s until the 1990s, colistin was considered as an effective treatment for MDR-GNB (Olaitan et al., 2014b). However, due its nephrotoxicity within the human body, the clinical use of this antimicrobial was abandoned (Olaitan and Li, 2016). Recently, the emergence of carbapenem resistance in clinically important bacteria such as *P. aeruginosa*, *A. baumannii*, *K. pneumonia,* and *Escherichia coli*, necessitated the re-introduction of colistin into clinical practice as a last-resort treatment option (Olaitan and Li, 2016).

Colistin is not only administered in humans, its use has been also described in veterinary medicine. Indeed, it has been suggested that the uncontrolled use of colistin in animals has played an important role in the global emergence of colistin-resistant bacteria (Collignon et al., 2016). The World Health Organization recently added polymyxins to the list of critically important antibiotics used in food producing animals worldwide (Collignon et al., 2016). The main use for colistin in animals includes the treatment of gastrointestinal infections caused by *E. coli* in rabbits, pigs, broilers, veal, beef, cattle, sheep, and goats; and, in particular, gastrointestinal infections caused by *E. coli* (Poirel et al., 2017). Colistin is mainly administered orally using different formulations such as premix, powder and oral solutions (Catry et al., 2015). In European countries, several epidemiological studies reported the use of colistin in veterinary medicine. In fact, Kempf et al. reported that colistin is mainly used to inhibit infections caused by *E. coli*, a Gram-negative bacillus known as a common causative agent of diarrhea, septicemia, and colibacillosis in animals (Kempf et al., 2013). In Spain, Casal et al. revealed that colistin is among the most frequent administered drug for the treatment of digestive diseases in pigs (Casal et al., 2007).

Epidemiologically speaking, the worldwide prevalence of resistance to polymyxins accounts for 10% of Gram-negative bacteria with the highest rates being observed in Mediterranean countries and Southeast Asia (Al-Tawfiq et al., 2017). For many

TABLE 2 | ST/phylogroups, IS and plasmid types associated with beta-lactamase and *mcr* genes in the Mediterranean.

Country	Animal Host	Species	*Bla* and/or *mcr* genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
Algeria	Poultry	*E. coli*	CTX-M 1	ST38, ST2179			Belmahdi et al., 2016
			SHV-12	ST1011, ST5086			
			CMY-2	ST744			
	Poultry	*S. Heidelberg*	CTX-M-1	ST15			Djeffal et al., 2017
	Cattle	*A. baumanii*	NDM-1	ST85			Chaalal et al., 2016
	Cattle	*E. coli*	NDM-5/ CMY-42/ CTX-M-15	ST1284	IncX3 (NDM-5)		Yaici et al., 2016
	Swine	*K. pneumoniae*	CTX-M-15	ST584			Bachiri et al., 2017
		E. coli	CTX-M 15	ST617, ST131, ST648, ST405, ST1431, ST1421, ST69, ST226			
	Dog	*E. coli*	CTX-M-15	A, B1, E			Yousfi et al., 2016b
			CTX-M-1/SHV-12	E			
			SHV-12	A, B1			
	Dog	*E. coli*	NDM-5	ST1284			Yousfi et al., 2015
	Dog	*E. coli*	OXA-48	A, D			Yousfi et al., 2016a
			NDM-5/ CTX-M-15/ CMY-42	A			
	Cat	*E. coli*	CTX-M-1	B1			Yousfi et al., 2016b
			CTX-M-15	A, U, E			
	Cat		OXA-48 / CMY-1	U			Yousfi et al., 2016a
			OXA-48	D			
	Barbary Macaques	*K. pneumoniae*	CTX-M-15	ST584			Bachiri et al., 2017
	Fish	*A. baumanii*	OXA-23	ST2			Brahmi et al., 2016
	Fish	*E. coli*	CTX-M-15	ST471, ST132, ST398, ST37,ST477, ST131, ST31			Brahmi et al., 2015
			CTX-M-9	ST8			
			TEM-24	ST31, ST471, ST66, ST21, ST74			
Tunisia	Poultry	*E. coli*	CTX-M-1	A, B1, D		IS*Ecp1*	Ben Sallem et al., 2012
			CMY-2	B2		IS*Ecp1*	
				D		IS*Ecp1D*-IS*10*	
	Poultry		CTX-M-1			IS*Ecp1*/IS*26*	Jouini et al., 2007
	Poultry	*E. coli*	CTX-M-1	B1, A			Ben Slama et al., 2010
			CMY-2	B1			
	Poultry	*E. coli*	CTX-M-1	A, B1, D, B2	IncI1		Mnif et al., 2012
			CTX-M-15	A, B1			
			CTX-M-1/CMY-2	B2	IncI1		
			CMY-2	A, D, B1	IncI1		
	Poultry	*E. coli*	CTX-M-1		IncI1		Grami et al., 2013
			CTX-M-9		IncI1		
	Poultry	*E. coli*	CTX-M-1	A0, A1, D2, B2			Kilani et al., 2015
	Poultry	*E. coli*	CMY-2	A, B1, D	IncI1, IncF, IncFIB, IncFIA		Maamar et al., 2016
			CTX-M-14	B1	IncF	IS*Ecp1*-IS*903*	
			CTX-M-1	B1, D, A	IncI1, IncF, IncFIB, IncK, IncY, IncP, IncN		
			CTX-M-15	D		IS*Ecp1*and IS*Ecp1*-IS*5*	

(Continued)

TABLE 2 | Continued

Country	Animal Host	Species	*Bla* and/or *mcr* genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
	Poultry	*E. coli*	CTX-M-1/*mcr-1*	D, H, K	IncHI2/ST4		Grami et al., 2016
	Poultry	*E. coli*	CMY-2/*mcr-1*	A (ST2197)	IncP (*mcr-1*) IncI1 (CMY-2)	IS*Apl1*	Maamar et al., 2018
	Cattle	*E. coli*	CTX-M-1	A, B1			Ben Slama et al., 2010
			CTX-M-1/ TEM-20	B1			
	Cattle	*E. coli*	CTX-M-1			IS*Ecp1*/IS*26*	Jouini et al., 2007
			CTX-M-14			IS*Ecp1* and IS*903*	
	Cattle	*E. coli*	CTX-M-15	ST10		IS*Ecp1*	Grami et al., 2014
	Dog	*E. coli*	CTX-M-1		IncI1		Grami et al., 2013
			CTX-M-15		IncFII		
	Dog	*E. coli*	CMY-2	B1		IS*Ecp1*	Sallem et al., 2013
			CTX-M-1	D, B1, A		IS*Ecp1*	
	Cat	*E. coli*	CTX-M-1	B1, A, D		IS*Ecp1*	Sallem et al., 2013
			CTX-M-1/ TEM-135	A		IS*Ecp1* (CTX-M-1)	
	Cat	*E. coli*	CTX-M-1		IncI1		Grami et al., 2013
	Dromedaries	*E. coli*	CTX-M-1	B1		IS*Ecp1*	Ben Sallem et al., 2012
Egypt	Poultry	*E. coli*	CTX-M-15	clonal group O25b-ST131		IS*Ecp1*	Ahmed and Shimamoto, 2013
	Poultry	*E. coli*	CTX-M	A, B1, B2, D			Abdallah et al., 2015
	Poultry	*E. coli*	CTX-M-14	D			El-Shazly et al., 2017
			SHV-12	D			
			CMY-2	A, B1, D			
	Poultry	*E. coli*	*mcr-1*	phylotype A, F, B1	IncFIB; IncI1; IncI2		Lima Barbieri et al., 2017
	Cattle	*E. coli*	*mcr-1*	ST10			Khalifa et al., 2016
Lebanon	Poultry	*E. coli*	CTX-M	ST156, ST5470, ST354, ST155, ST3224			Dandachi et al., 2018a
	Poultry	*E. coli*	*mcr-1*	ST515			Dandachi et al., 2018b
	Cattle	*E. coli*	CTX-M-15	A (ST1294, ST2325, ST1303, ST4623, ST5204)			Diab et al., 2016
				B1 (ST58, ST162, ST4252, ST155, ST196, ST540)			
				D (ST69)			
			CTX-M-14	D (ST457)			
			CTX-M-15/SHV-12	A (ST10, ST2450, ST5442)			
			CTX-M-14/SHV-12	D (ST457)			
			SHV-12	A (ST218, ST617, ST5204, ST1303,ST5728,ST1140, ST746)			
	Cattle	*A. baumanii*	OXA-23	ST2			Al Bayssari et al., 2015a
		P. aeroginosa	VIM-2	ST1762, ST1759			
	Swine	*A. baumanii*	OXA-23	ST491			Al Bayssari et al., 2015a
	Fowl	*A. baumanii*	OXA-23	ST492, ST493			Al Bayssari et al., 2015b

(Continued)

TABLE 2 | Continued

Country	Animal Host	Species	*Bla* and/or *mcr* genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
			OXA-58/OXA-23	ST20			
		P. aeroginosa	VIM-2	ST1760, ST1761			
	Fowl	*E. coli*	OXA-48	ST38			Al Bayssari et al., 2015b
	Horse	*A. baumanii*	OXA-143	ST294			Rafei et al., 2015
	Rabbit	*A. pitii*	OXA-24	ST221			Rafei et al., 2015
Palestine	Poultry	*E. coli*	CTX-M	A, B, D			Qabajah et al., 2014
Turkey	Poultry	*E. coli*	CMY-2	A0, B2 D1, D2			Pehlivanlar Onen et al., 2015
			CTX-M-1/CMY-2	A0			
			CTX-M-1	A1, A0, D1, D2			
			CTX-M-1/SHV-5	D1			
			CTX-M-3	A0, D1			
			CTX-M-15	B1, D1, D2			
			SHV-12	D1			
			CTX-M-15/SHV-12	D2			
Italy	Poultry	*E. coli*	SHV-12		IncI1, IncFIB		Bortolaia et al., 2010
			CTX-M-1		IncI1, IncFIB, IncN		
			CTX-M-32		IncN		
	Poultry	*E. coli*	CTX-M-1		IncI1		Accogli et al., 2013
			CMY-2		IncI1		
	Poultry	*E. coli*	CTX-M	A, B1, B2, D			Ghodousi et al., 2015
			CIT like	B1, B2, D			
	Poultry	*E. coli*	CTX-M	B2, ST131			Ghodousi et al., 2016
	Swine	*E. coli*	OXA-181	B1 (ST359), A (ST641)	IncX3		Pulss et al., 2017
			mcr-1	A (ST641)	IncX4		
			CMY-2	A (ST641)	IncI1		
	Cat	*E. coli*	CMY	A		IS*Ecp1*/IS*26*	Bogaerts et al., 2015
	Dog	*K. oxytoca*	SHV-12, DHA-1	N.I	IncL/M		Donati et al., 2014
		K. pneumoniae	CTX-M-15,DHA-1	ST340	IncR (CTX-M-15)		
			CTX-M-15	ST101			
			SHV-28,	ST15			
			CTX-M-15,SHV-28,	ST15			
			CTX-M-1,SHV-28	ST15	CTX-M-1 in IncN and IncR		
			CTX-M-1	ST11			
	Cat	*K. oxytoca*	CTX-M-9	N.I	IncHI2		Donati et al., 2014
		K. pneumoniae	CTX-M-15, DHA-1	ST340	CTX-M-15/DHA-1 on IncR		
			SHV-28, CMY-2	ST15	CMY-2 on InCI1		
			CTX-M-15	ST101			
	Cat	*E. coli*	CTX-M-14/CMY-2	A (ST3848, ST3847)			Nebbia et al., 2014
			CTX-M-14	B2 (ST555, ST4181), B1 (ST602)			
			CTX-M-1	B2 (ST155)			
			CTX-M-15	B2 (ST131)			

(Continued)

TABLE 2 | Continued

Country	Animal Host	Species	*Bla* and/or *mcr* genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
Slovenia	Poultry	*E. coli*	CTX-M-1	D			Zogg et al., 2016
			SHV-12	B1 and D			
Spain	Poultry	*E. coli*	CTX-M-14	ST101, ST156,ST165, ST350, ST889, ST1137	IncK		Solà-Ginés et al., 2015b
			SHV-12	ST350, ST533	IncI1		
			CMY-2	ST429, ST131	IncK		
	Poultry	*E. coli*	CMY-2	A, D			Cortés et al., 2010
			CTX-M-14	A, B1, B2			
			CTX-M-32	A			
			CTX-M-9	B1			
			SHV-12				
			TEM-52	B1			
	Poultry	*E. coli*	CTX-M-9	O25b:H4-B2-ST131.			Mora et al., 2010
	Poultry	*E. coli*	CTX-M, SHV	A, B1, D1			Egea et al., 2012
	Poultry, Swine, Cattle	*E. coli*	CTX-M, SHV	B2, D			Doi et al., 2010
	cattle	*E. coli*	*mcr-1* /mcr-3/ CTX-M-55	ST533	non mobilizable IncHI2		Hernández et al., 2017
	Swine	*E. coli*	CTX-M-1	A			Cortés et al., 2010
			SHV-5	A			
			SHV-12	B1			
	Dog	*E. coli* (1)	CMY (1)	ST2171	IncK	IS*Ecp1*	Bogaerts et al., 2015
		P. mirabilis (2)	CMY (2)				
	Dog	*K. pneumoniae*	VIM-1	ST2090			González-Torralba et al., 2016
	Deer	*E. coli*	CTX-M-1	ST224	IncN	IS*26*	Alonso et al., 2016
Croatia	Mussel	*Aeromonas spp*	CTX-M-15		IncFIB		Maravić et al., 2013
France	Poultry	*E. coli*	CTX-M-1			IS*Ecp1*	Meunier et al., 2006
	Cattle	*E. coli*	CTX-M-1			IS*Ecp1*	Meunier et al., 2006
			CTX-M-15			IS*Ecp1*	
	Cattle	*E. coli*	CTX-M-15	B1		IS*Ecp1*	Valat et al., 2012
	Cattle	*E. coli*	CTX-M-1	ST2497, ST2498			Hartmann et al., 2012
			TEM-71	ST178			
	Cattle	*E. coli*	CTX-M-15,	ST2212, ST2213, ST2210, ST2214,ST2215, ST88	F31:A4:B1/IncFII F2:A–:B–/IncFII and IncI1		Madec et al., 2012
	Cattle	*K. pneumoniae*	CTX-M-14	ST45	F2:A-:B-IncFII		Dahmen et al., 2013b
		E. coli	CTX-M-14	ST23, ST58, ST10, ST45	F2:A-:B-IncFII		
			CTX-M-1	ST23, ST58	IncI1/ST3		
	Sheep	*K. pneumoniae*	CTX-M-15, DHA	all ST274			Poirel et al., 2013
	Swine	*E. coli*	CTX-M-1			IS*Ecp1*	Meunier et al., 2006
	Dogs	*E. coli*	CTX-M-15	A (ST410, ST617)	IncFII		Dahmen et al., 2013a
			CTX-M-1	A (ST10), B1 (ST1303, ST1249)	IncFII		
					IncFII		
	Dog	*A. baumanii*	OXA-23	ST25			Hérivaux et al., 2016
	Dogs	*E. coli*	CTX-M-1	ST345, ST1001, ST124	IncI1		Poirel et al., 2013
			CTX-M-15	NEW ST	N.T		

(Continued)

TABLE 2 | Continued

Country	Animal Host	Species	*Bla* and/or *mcr* genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
			TEM-52	ST359			
		K. pneumoniae	CTX-M-15, DHA-1	ST274			
			CTX-M-15,	ST15			
	Dogs	*E. coli*	CTX-M-1	A, B1,D	*bla*CTX-M-1/IncI1/ST3		Haenni et al., 2014b
			CTX-M-grp9	B2			
			CMY-2	A, B1, B2, D	CMY-2/IncI1/ST2		
	Dog	*E. cloacae*	CTX-M-15	ST114,ST136,ST270,ST100	IncHI2		Haenni et al., 2016c
			CTX-M-14	ST102	N.T		
			CTX-M-3	ST408	N.T		
			SHV-12	ST268	IncHI2		
	Dog	*E. coli*	CMY	ST55	N.T		Melo et al., 2017
			CMY	ST963	N.T		
			OXA-48	ST372	IncL		
	Cat	*K. pneumoniae*	CTX-M-15, DHA	ST274			Poirel et al., 2013
		E. coli	CTX-M-1	ST124, ST641			
			CTX-M-14	ST141			
	Cats	*E. coli*	CTX-M-15	A (ST617, ST410)			Dahmen et al., 2013a
			CTX-M-32	B1 (ST224)			
			CTX-M-3	B2 (ST493)			
			CTX-M-14	B1, (ST359), B2 (ST131)			
	Cat	*E. cloacae*	CTX-M-15	1 ST136, others ST114	IncHI2		Haenni et al., 2016c
			SHV-12	N.T	IncA/C		
	Cat	*E. coli*	CTX-M-14	ST68	IncF		Melo et al., 2017
			CTX-M-1	ST673	IncFIB		
	Cat	*A. baumanii*	OXA-23	ST1/ST231			Ewers et al., 2016
	Hedgehog	*K. pneumoniae*	CTX-M-15, DHA	ST274			Poirel et al., 2013
	Tawny Owl	*E. coli*	CTX-M-1	ST93			Poirel et al., 2013
	Domestic goose	*E. coli*	CTX-M-15	ST10			Poirel et al., 2013
	Rock pigeon	*E. coli*	CTX-M-1	ST124			Poirel et al., 2013
	Horse	*E. cloacae*	CTX-M-15	ST127, ST372, ST145, ST114	IncHI2		Haenni et al., 2016c
			SHV-12	ST135,ST145,ST118	IncHI2		
			CTX-M-1	ST268	N.T		
				ST107	IncP		
Greece	Dog	*E. coli*	CMY-2	ST212	IncI1/ST65		Vingopoulou et al., 2014

Bla, beta-lactamase; ST, sequence type; IS, insertion sequence; N.T, non typeable.

years, colistin resistance was thought to be mainly mediated by chromosomic mutations, with no possibility of horizontal gene transfer. However, the emergence of the *mcr-1* plasmid mediated colistin resistance gene (Liu et al., 2016) has thoroughly altered the view of colistin resistance as a worldwide problem (Baron et al., 2016). The current epidemiology of colistin resistance is poorly understood.

In the Mediterranean area (**Figure 2**), the first detection of *mcr-1* was in an *E. coli* strain isolated from chickens in Algeria (Olaitan et al., 2016). This same isolate was further detected in sheep in another region of this country in 2016 (Chabou et al., 2017). In Tunisia, Grami et al. reported a high prevalence of multi-clonal *E. coli* carrying the *mcr-1* gene in three chicken farms imported from France (Grami et al., 2016). Isolated strains were found to co-harbor the *bla*CTX-M-1 ESBL gene along with *mcr-1* on an IncHI2/ST4 plasmid (**Table 1**; Grami et al., 2016). Apart from colistin resistance, these strains were also co-resistant to tetracyclines, quinolones, fluoroquinolones, trimethoprim, and sulfonamides (Grami et al., 2016). The co-existence of ESBL and *mcr-1* genes on the same plasmid facilitates the dissemination

of colistin resistant strains by the co-selective pressure applied via the use of colistin as well as possibly the utilization of non-beta-lactam antibiotics. Molecular analysis targeting the co-localization of ESBL and *mcr* genes along with the ones mediating resistance toward non-beta-lactams is however warranted in order to validate this hypothesis. Also in Tunisia, two colistin resistant *E. coli* strains positive for *mcr-1* and harboring the CMY-2 gene were recently detected in chicken. Both strains shared the same sequence type "ST2197" in addition to their PFGE patterns. The *mcr-1* gene in these latter was associated with the IS*Apl1* and was carried by IncP plasmid while the CMY-2 gene was located on an IncI1 plasmid type (Maamar et al., 2018). Furthermore, in this same country, a recent study revealed the absence of *mcr-1* and *mcr-2* positive Gram-negative bacilli in camel calves in southern Tunisia (Rhouma et al., 2018). Likewise, in Egypt, *mcr-1* was detected in *E. coli* isolated from diseased chickens as well as from cows displaying subclinical mastitis (Khalifa et al., 2016; Lima Barbieri et al., 2017). The emergence of *mcr-1* in Egypt can be related to the use of colistin in animal agriculture, and its ready application as a therapeutic agent for colibacillosis as well as other infections, in rabbits and calves (Lima Barbieri et al., 2017). In Southeast Asia, Dandachi et al. reported the detection of the *mcr-1* plasmid mediated colistin resistance gene in *E. coli* in poultry in the south of Lebanon (Dandachi et al., 2018a). This strain had a sequence type of ST515 that was not reported before in *mcr-1 E. coli* strains of poultry origin (Dandachi et al., 2018a).

Of the European countries bordering the Mediterranean, Spain was the first to report the detection of *mcr-1* in *E. coli* and *Salmonella enterica* isolated from farm animals (Quesada et al., 2016). This could be related to the fact that Spain is one of the countries were colistin is extensively used in veterinary medicine (de Jong et al., 2013). More recently, *mcr-1* co-existing with *mcr-3* on the same non mobilizable IncHI2 plasmid was detected in an *E. coli* strain recovered from cattle feces in a slaughterhouse (Hernández et al., 2017). In France, as part of routine surveillance by the French agricultural food sector, *mcr-1* was identified in four *Salmonella* spp isolated from sausage, food of poultry origin, and boot swabs taken from broiler farms (Perrin-Guyomard et al., 2016; Webb et al., 2016). *E. coli* harboring *mcr-1* was also isolated in France from pig, broiler and turkey samples (Haenni et al., 2016a). Haenni et al. reported the identification of unique IncHI2/ST4 plasmid co-localizing *mcr-1* and ESBL genes in an *E. coli* strain isolated from French veal calves (Haenni et al., 2016b). In Italy, Carnevali et al. reported the detection of *mcr-1* in *Salmonella* spp strains isolated from poultry and pigs (Carnevali et al., 2016). Subsequently, *mcr-1* was further detected in *E. coli* of swine origin. In the aforementioned report, *mcr-1* was co-existent with the carbapenemase OXA-181 in the same bacterium and was carried on an IncX4 plasmid type (Pulss et al., 2017). In the Mediterranean basin, likewise ESBL producers, *mcr* positive strains belong to different phylogroups and appear to be not clonally related; however, they were not associated to a common plasmid or an insertion sequence type. This questions the molecular mechanism by which the *mcr* genes are being disseminating in this region of the world. More molecular work is warranted in this area especially that *mcr* genes are often located on plasmids carrying ESBL and/or carbapenemase genes.

Antibiotic Use in Animals and Potential Impact on Public Health

For many years, the use of antibiotics in the veterinary medicine has increased animal health via lowering mortality and the incidence of infectious diseases (Hao et al., 2014). However, in view of the heavy dissemination of resistant organisms namely ESBL, AmpC, and carbapenemase producers in addition to the emergence of colistin resistance in livestock and animals with frequent contacts with human; the efficiency of antibiotic administration to animals has been reconsidered. Indeed, antibiotic use in animals is not controlled, in that these latter are not only prescribed for treatment, but are also given for prophylaxis and as growth promoters (Economou and Gousia, 2015). In its recent publication, the world health organization recommended a reduction but an overall restriction of the use of medically important antibiotics for prophylaxis and growth promotion in farm animals (WHO, 2017). According to the world health organization list of Critically Important Antimicrobials for Human Medicine (WHO CIA list), these include mainly extended spectrum cephalosporins, macrolide, ketolides, glycopeptides and polymixins (WHO CIA, 2017). The control of antibiotic use in the veterinary sector aims to reduce the emergence of resistance in addition to preserving the efficacy of important classes for treatment in the human medicine.

In the Mediterranean region, tetracyclines, aminoglycosides, sulfonamides, fluoroquinolones, and polymixins are the most common antimicrobial classes prescribed in the veterinary sector (**Table 1**). The usage level of each antibiotic class in addition to its real purpose of administration apart from treatment is limited and not well understood in this area of the world. In fact, it is nowadays accepted that the over-use of antibiotics in animals is the main driven for the dissemination of multi-drug resistance (Barton, 2014). As shown in **Table 1**, ESBL, AmpC, and carbapenemase producers are often co-resistant to non-beta-lactam antibiotics with the most common being gentamicin, streptomycin, tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, and ciprofloxacin. One study conducted in healthy chicken in Tunisia showed the presence of *tetA, tetB, sul1,* and *sul2* on the same plasmids carrying the *bla*CTX-M genes (Maamar et al., 2016). Another study in Egypt, reported the detection of *tetB, qnrB2, qnrA1, aadA1* on the same gene cassette along with the *bla*CMY-2 AmpC beta-lactamase gene (Ahmed and Shimamoto, 2013). In Italy, *strA/B, tetD, qnrB, aadA1, sulI* genes were associated with the *bla*CTX-M and *bla*SHV ESBL genes types in companion animals (Donati et al., 2014). Furthermore, in this same country, aminoglycoside modifying enzymes (*aadA1, aadA2*), quinolone resistance genes (*qnrS1*), florfenicol/chloramphenicol resistance gene (*floR*), in addition to tetracycline and sulfonamide resistance genes (*tetA, sul1, sul2, sul3*) were found associated with OXA-48/181 and OXA-48/181/ CMY-2 /*mcr-1* positive *E. coli* strains isolated from pigs (Pulss et al., 2017). In *Salmonella enterica*, Franco et al. reported the detection of a megaplasmid harboring

the *bla*CTX-M-1 ESBL gene along with *tetA, sulI, dfrA1,* and *dfrA14* conferring thus additional resistance toward tetracycline, sulfonamide, and trimethoprim (Franco et al., 2015). Beta-lactamase producing Gram-negative bacilli appear thus to be selected by the co-selective pressure applied by the use of non-beta-lactam antibiotics in livestock and companion animals. Surveillance studies addressing the types, purpose and level of antibiotic classes' administration in animals of the Mediterranean region are warranted in order to develop approaches that control the use of antibiotics while preserving animal's health. This is especially in Syria, Cyprus, Albania, Montenegro, Bosnia, Herzogovina, Monacco, Morocco, and Libya where even no data exists on the prevalence and epidemiology of multi-drug resistant organisms in animals.

The spread of multi-drug resistant organisms of animal origin is sparked by the concern of being transmitted to humans; these latter can then be causative agents for infections with limited therapeutic options (Bettiol and Harbarth, 2015). The transfer of resistant organisms from animals to humans can occur either via direct contact or indirectly via the consumption of under/uncooked animals products (Dahms et al., 2014). Recent studies have also highlighted the importance of the farms surrounding environment in the transmission chain. Air (von Salviati et al., 2015), dust (Blaak et al., 2015), contaminated waste waters (Guenther et al., 2011), and soil fertilized with animal manures (Laube et al., 2014) are all potential sources from which resistant organisms can be transferred to the general population. In their study, Olaitan et al. demonstrated the transfer of a colistin resistant *E. coli* strain from a pigs to its owner (Olaitan et al., 2015). This was documented by both strains (in the pig and its owner) having the same sequence types and sharing the same virulence as well as same PFGE patterns (Olaitan et al., 2015). The increased risk of ESBL fecal carriage in humans with frequent contact with broilers has been further taken as an evidence of transmission (Huijbers et al., 2014). Furthermore, sharing the same sequence types, virulence and PFGE patterns in addition to common plasmids/ESBL genes are all proofs for the possible transfer of resistant organisms and/or genes from the veterinary sector to the human population (Leverstein-van Hall et al., 2011). In Algeria, Djeffal et al. reported the detection of a common sequence type (ST15) in *Salmonella spp* producing ESBL isolated from both humans and avian isolates (Djeffal et al., 2017). In Egypt, Hamza et al. showed an abundance of carbapenemase genes namely *bla*OXA-48, *bla*KPC and *bla*NDM in chicken, drinking water, and farm workers suggesting a possible transmission of carbapenemase encoding genes from broilers to farmers and the surrounding environment (Hamza et al., 2016). Another study conducted in Italy reported the spread of a multi-drug resistant clone of "*Salmonella enterica* subsp. enterica serovar Infantis" that was first detected in 2011 in broiler farms and few years later led to human infections most likely via transmission from the broiler industry (Franco et al., 2015). In Spain, common *bla*CTX-M-grp1 and *bla*CTX-M-grp9 ESBL genes were detected in retail meat as well as in *E. coli* strains isolated from infected and colonized patients in the same region (Doi et al., 2010). In France, Hartmann et al. showed a clonal relationship among CTX-M carrying *E. coli* strains in cattle and farm cultivated soils (Hartmann et al., 2012). Another study in cattle, demonstrated that CTX-M-15 harboring plasmids in non-ST131 *E. coli* strains are highly similar to those detected in humans suggesting thus a multi-clonal plasmidic transmission of multi-drug resistant organisms from livestock to the humans (Madec et al., 2012). The detection of common genes and sequence types among animals and humans and the surrounding environment emphasizes the need to have a global intervention measures to avoid the dissemination of multi-drug resistance in the one health concept.

CONCLUSION

Antimicrobials have been used in veterinary medicine for more than 50 years. The use of antibiotics proved to be crucial for animal health by lowering mortality and incidence of diseases, in addition to controlling the transmission of infectious agents to the human population. Recently, the dissemination of ESBL, carbapenemase, and colistin resistant Gram negative bacteria in food producing animals brought into question the real efficacy of antibiotic administration in animals in terms of treatment, prophylaxis and growth promotion. Indeed, the emergence of MDR in food producing animals has been suggested to be largely linked to the over and misusage of antibiotics in veterinary medicine. The level of antibiotic consumption in animals varies between countries. Although, cephalosporins are not often prescribed in veterinary medicine, the use of other non-beta-lactams could account for the co-selection of multi-drug resistant bacteria. As shown in **Table 1**, ESBL and carbapenemase producers were frequently co-resistant to aminoglycosides, tetracyclines and fluoroquinolones, with these latter being mostly used in the veterinary field. Furthermore, the aforementioned antibiotics are classified by the World Health Organization as critically important antibiotics for human medicine that should be restricted in the animal field (Collignon et al., 2016). That said, the direct public health effect of the transmission of MDR bacteria from animals to humans is still controversial. Several studies have demonstrated a direct link of transmission between these two ecosystems. Resistant bacteria once transmitted to humans can be further selected by the over-use of antimicrobial agents in the clinical and community settings. This spread will promote the global dissemination of bacterial resistance across all ecosystems. The level of antibiotic consumption in animals in the European countries lining the Mediterranean is available in the European Surveillance of Veterinary Antimicrobial Consumption report (EMA/ESVAC, 2014), however this is not the case for the countries in North Africa and western Asia, where no accurate data are available. Therefore, surveillance studies investigating the levels of antibiotic prescription should be conducted in these areas. Antimicrobial prescriptions in animals should be re-considered and controlled to limit the spread of bacteria which are cross resistant to the antibiotics used in human medicine. In addition, a risk assessment of other factors contributing to the emergence

of antimicrobial resistance in animals should be conducted in future studies. Poor sanitary conditions, overcrowding and poor infection control practices in animals are all possible contributors to the robust emergence of MDR in food-producing animals.

AUTHOR CONTRIBUTIONS

ID and SC wrote the review paper. ZD and J-MR corrected the manuscript. All authors approved and revised the final version of the manuscript.

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Molecular Analysis of Two Different MRSA Clones ST188 and ST3268 From Primates (*Macaca* spp.) in a United States Primate Center

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Methicillin-resistant *Staphylococcus aureus* (MRSA) were identified in macaques, their environmental facility, and nasal cultures of personnel from the Washington National Primate Research Center [WaNPRC] and included MRSA ST188 SCC*mec* IV and MRSA ST3268 SCC*mec* V. The aim of the current study was to determine the carriage of virulence genes, antibiotic resistance genes, and other characteristics of the primate MRSA isolates to determine if there were any obvious differences that would account for differences in transmission within the WaNPRC facility. In total, 1,199 samples from primates were tested for the presence of MRSA resulting in 158 MRSA-positive samples. Fifteen ST188 isolates (all from *Macaca nemestrina*) and nine ST3268 (four from *Macaca mulatta*, two from *Macaca fascicularis,* three from *M. nemestrina*), were selected for further characterization. All but one of the 15 ST188 isolates had *spa* type t189 and the remaining one had *spa* type t3887. These isolates were resistant to β-lactams [*blaZ*, *mecA*], macrolides/lincosamides [*erm*(B)], aminoglycosides [*aacA-aphD*], and fluoroquinolones. Five isolates were additionally resistant to tetracyclines [*tet*(K)] and had elevated MICs for benzalkonium chloride [*qacC*]. In comparison, the nine ST3268 isolates had the related *spa* types t15469 (n = 5) and t13638 (n = 4). All nine ST3268 isolates were resistant to β-lactams [*blaZ*, *mecA*], and tetracyclines [*tet*(K)]. Some isolates were additionally resistant to aminoglycosides [*aacA-aphD*], fluoroquinolones and/or showed elevated MICs for benzalkonium chloride [*qacC*]. In contrast to the ST188 isolates, the ST3268 isolates had the enterotoxin gene cluster *egc* [*seg, sei, selm, seln, selo, selu*] and enterotoxin genes *sec* and *sel*. The two clones have differences regarding their *spa* types, virulence and antibiotic resistance genes as well as ST and SCC*mec* types. However, the data presented does not provide insight into why ST188 spreads easily while ST3268 did not spread within the WaNPRC in-house primates.

Keywords: MRSA, *Macaca mulatta*, *Macaca fascicularis*, *Macaca nemestrina*, novel *spa* type, multi-drug resistance, colonization, infection

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important opportunistic pathogen in human and veterinary medicine and can be a harmless colonizers but may also cause severe and live-threatening infections (Foster, 2017). MRSA consists of numerous pandemic, epidemic and sporadic clones (Monecke et al., 2011). There is very limited data on the carriage of *S. aureus* (including MRSA) in captive primates with even more limited data on MRSA carriage in wild primates in their natural habitats (Taylor and Grady, 1998; Weese, 2010; Hanley et al., 2012; Schaumburg et al., 2013; Soge et al., 2016; Roberts et al., 2018). Prior to 2014, neither *S. aureus* nor MRSA were identified in macaques from the Washington National Primate Research Center [WaNPRC], Seattle WA, United States. However, in 2014, there were nine cases of MRSA. This led to the 2015 carriage study, which determined that 17.6% of the macaques, 3.6% of the primate environmental facility samples and 2.5% of the primate personnel carried MRSA (Soge et al., 2016). Initially, all the isolates from macaques, environment and one of the personnel isolates were MRSA ST188 SCC*mec* IV [MLST profile 3, 1, 1, 8, 1, 1, 1, 1]. MRSA ST188 are not commonly found in North America[1] (Soge et al., 2016). Our previous work showed that the ST188 SCC*mec* IV represented a clone and was easily transferred between macaques in the same cage, the same room or between playmates and contaminated the primate environment. One primate researcher carried MRSA ST188 SCC*mec* IV in the nose, while another carried a normally human isolated ST8 SCC*mec* IV (Soge et al., 2016).

In May 2015, a large shipment of macaques [> 90 *Macaca nemestrina*] from out-of-state, from other United States Primate Research Centers and arrived at WaNPRC. Most of these animals were colonized with MRSA ST3268 SCC*mec* V [MLST profile 1, 14, 430, 214, 10, 303, 329] (Soge et al., 2016). This was a novel sequence type (ST) and did not seem to readily spread within the WaNPRC until later in 2015 when four MRSA ST3268-positive animals were identified. These appeared to have been exposed and acquired ST3268 from a contaminated common procedure room within in the WaNPRC. These animals were also positive for the simian immunodeficiency virus (SIV) (Soge et al., 2016). Since the first introduction of MRSA ST3268, the WaNPRC has continued to receive MRSA ST3268-positive animals with new shipments of primates but no spread of this clone was observed. More recently, MRSA ST3268 isolates and a single locus variant MRSA ST2817 isolates have been detected in Singaporean long-tailed macaques (*Macaca fascicularis*) used in experimental surgery in 2014 and one person who worked in animal husbandry at the facility. These animals originated from Vietnam (Hsu et al., 2017). ST3268 differs by one housekeeping gene [*glp*] from ST2817, which has been identified in Asia.

The hypothesis of the current study was that there were some differences in the carriage of virulence factors, antibiotic resistance genes, and other characteristics between the two MRSA clones ST188 and ST3268 that might suggest why there is a different transmission frequency among the WaNPRC macaques.

[1]https://pubmlst.org/saureus/

MATERIALS AND METHODS

Primate Sampling, MRSA Isolation and Verification

A total of 1,199 primate samples from the WaNPRC facility was tested for the presence of MRSA between May and August 2015. The animals [*M. fascicularis, Macaca mulatta*, and *M. nemestrina*] were in-house animals, as well as, out-of-state macaques shipped to the facility. The isolates were previously collected as part of the general care of the animals approved by the Institutional Animal Care and Use Committee at the University of Washington, United States, and the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Nonhuman Primates (Soge et al., 2016). In addition, other animals were obtained from different commercial vendors and different sources outside the United States and were investigated shortly after their arrival at the WaNPRC during the quarantine period. MRSA-positive animals were given baths with chlorhexidine scrub for five consecutive days. The chlorhexidine was applied to the entire body and scrubbed with a surgical scrub brush with extra time spent cleaning axillary, perianal and preputial areas. In addition, animals received nasal application of mupirocin ointment 2% given twice daily for 5 days at the same time. Animals were sampled again at two and four weeks after initial MRSA positive culture and chlorhexidine and mupirocin treatment and retreated if still MRSA positive. All animals in the colony had initial nasals cultures done, while wound and/or skin infections were also sampled when present. All samples were taken from ketamine-sedated animals using standard microbiological swabs; BD BBL CultureSwab Plus Amies Medium (Becton Dickinson, Franklin Lakes, NJ, United States) and/or Starplex Starswab II (Starplex Scientific, Etobicoke, ON, Canada) as previously described (Roberts et al., 2011; Soge et al., 2016). For the current study, colonies were identified as *S. aureus* by production of β-hemolysis on blood agar plates and a positive Staphaurex® test following manufacturer's instructions (Remel, Lenexa, KS, United States; Soge et al., 2016). No isolate was selected unless they met these criteria (Soge et al., 2016). The presence of the alternative PBP2' was determined with the Thermo Scientific PBP2' latex agglutination test kit® using instruction from the manufacturer (Thermo Fisher Scientific Remel Products, Lenexa, KS, United States). MRSA isolates were stored at −80°C. Isolates were selected without knowledge of the host primate species. This included 15 of 56 MRSA ST188 SCC*mec* IV isolates obtained from 36 animals and selected from various sample sites including animals that appeared refractory to mupirocin topical treatment. The 15 ST188 isolates came from ten *M. nemestrina* hosts and included three skin samples, and 12 nasal samples (**Table 1**). From *M. nemestrina* Z1242, three different nasal isolates Z1242N1, Z1242N2, Z1242N3, were selected taken on Feb 2, April 24, and June 5, 2015 to determine if the same strain was present over the 5 month time period. This animal was treated with chlorhexidine scrub and nasal application of mupirocin ointment between samplings. One *M. nemestrina* [Z121] had paired nasal Z121N and skin Z121S isolates taken May 29, 2015, while *M. nemestrina* Z123 had two isolates

TABLE 1 | Characterizations of the MRSA ST188 SCC*mec* V and ST3268 SCC*mec* V.

Animal ID	MLST	Host	Date collected	Source	*spa* type	PFGE type (SmaI)	Resistance patterns	Resistance genes	Enterotoxins, leukocidins, other genes
A112	ST188	*M. nemestrina*	2/27/2015	Nasal	t189	A2	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
K062	ST188	*M. nemestrina*	8/7/2015	Nasal	t189	A1	PEN, OXA, ERY, CLI, GEN, KAN, TET, CIP, BAC	*blaZ, mecA, erm*(B), *aacA-aphD, tet*(K), *qacC*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
L091	ST188	*M. nemestrina*	4/3/2015	Nasal	t189	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z121N	ST188	*M. nemestrina*	5/29/2015	Nasal	t189	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z121S	ST188	*M. nemestrina*	5/29/2015	Skin	t189	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z123N1	ST188	*M. nemestrina*	5/15/2015	Nasal	t189	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z123N2	ST188	*M. nemestrina*	5/29/2015	Nasal	t189	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z123S	ST188	*M. nemestrina*	5/29/2015	Skin	t189	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z1242N1	ST188	*M. nemestrina*	2/24/2015	Nasal	t189	A1	PEN, OXA, ERY, CLI, GEN, KAN, TET, CIP, BAC	*blaZ, mecA, erm*(B), *aacA-aphD, tet*(K), *qacC*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z1242N2	ST188	*M. nemestrina*	4/24/2015	Nasal	t189	A1	PEN, OXA, ERY, CLI, GEN, KAN, TET, CIP, BAC	*blaZ, mecA, erm*(B), *aacA-aphD, tet*(K), *qacC*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z1242N3	ST188	*M. nemestrina*	6/5/2015	Nasal	t189	A1	PEN, OXA, ERY, CLI, GEN, KAN, TET, CIP, BAC	*blaZ, mecA, erm*(B), *aacA-aphD, tet*(K), *qacC*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z130	ST188	*M. nemestrina*	2/25/2015	Nasal	t189	A1	PEN, OXA, ERY, CLI, GEN, KAN, TET, CIP, BAC	*blaZ, mecA, erm*(B), *aacA-aphD, tet*(K), *qacC*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z1304	ST188	*M. nemestrina*	7/2/2015	Nasal	t189	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z131S	ST188	*M. nemestrina*	7/2/2015	Skin	t189	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
Z143	ST188	*M. nemestrina*	2/18/2015	Nasal	t3887	A	PEN, OXA, ERY, CLI, GEN, KAN, CIP	*blaZ, mecA, erm*(B), *aacA-aphD*	*hlgA/lukF/lukS, lukD/lukE, lukX/lukY*
A140	ST 3268	*M. mulatta* WaNPRC SIV+	7/31/2015	Nasal	t15469	B	PEN, OXA, TET, CIP	*blaZ, mecA, tet*(K), *fosB*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*
A1404N	ST 3268	*M. mulatta* WaNPRC SIV+	7/20/2015	Nasal	t15469	B	PEN, OXA, TET, CIP	*blaZ, mecA, tet*(K), *fosB*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*
A1404W	ST 3268	*M. mulatta* WaNPRC SIV+	7/20/2015	Wound	t15469	B	PEN, OXA, TET, CIP	*blaZ, mecA, tet*(K), *fosB*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*
A1408	ST 3268	*M. mulatta* Vendor #1	7/17/2015	Nasal	t15469	B	PEN, OXA, TET, CIP	*blaZ, mecA, tet*(K), *fosB*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*

(Continued)

TABLE 1 | Continued

Animal ID	MLST	Host	Date collected	Source	*spa* type	PFGE type (SmaI)	Resistance patterns	Resistance genes	Enterotoxins, leukocidins, other genes
A1524	ST 3268	*M. fascicularis* China 6 mo quarantine in CA	4/8/2015	Nasal	t13638	B	PEN, OXA, (GEN), KAN, TET, CIP, BAC	*blaZ, mecA, aacA-aphD, tet(K), fosB, qacC*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*
A1525	ST 3268	*M. fascicularis* Vendor #2 China	4/8/2015	Nasal	t13638	B	PEN, OXA, GEN, KAN, TET, CIP, BAC	*blaZ, mecA, aacA-aphD, tet(K), fosB, qacC*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*
A109	ST 3268	*M. nemestrina* Indonesia 2010, arrived Seattle 2015	5/20/2015	Nasal	t13638	B1	PEN, OXA, GEN, KAN, TET, CIP	*blaZ, mecA, aacA-aphD, tet(K), fosB*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*
K990W	ST 3268	*M. nemestrina* LA NPRC	8/5/2015	wound	t13638	B	PEN, OXA, (GEN), KAN, TET, CIP, BAC	*blaZ, mecA, aacA-aphD, tet(K), fosB, qacC*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*
Z1403	ST 3268	*M. nemestrina* Born in TX arrived 2015	5/19/2015	Nasal	t13638	B2	PEN, OXA, (GEN), KAN, TET, CIP	*blaZ, mecA, aacA-aphD, tet(K), fosB*	*egc, hlgA/lukF/lukS, lukD/lukE, lukX/lukY sec, sel*

All isolates carried the mecA gene. Pen, penicillin; Oxa, oxacillin; Cip, ciprofloxacin; Gen, gentamicin; Kan, kanamycin; Tet, tetracycline; BAC, benzalkonium chloride; brackets indicate an intermediate phenotype. All isolates were mupirocin susceptible and lacked the mupA gene. The enterotoxin H gene [entH], ORF CM14, and splE were absent in all isolates. The collagen-binding adhesin [cna] and the protease genes splA, splB were present in the ST188 isolates but were not detected among the ST3268 isolates. None of the 21 isolates carried PVL genes, the toxic shock syndrome toxin 1 gene [tst1], or the exfoliative toxin genes [etA, etB, etD], or genes associated with β-haemolysin converting phages (sea, see, scn, chp).

from two nasal samples [Z123N1 and Z123N2] isolated May 15 and 29, 2015 and a skin sample [Z123S] isolated May 29, 2015 (**Table 1**). This animal was treated with chlorhexidine scrub and mupirocin ointment when first identified as MRSA positive in May 2015. All these animals were from the WaNPRC facility.

Nine of the 21 ST3268 SCC*mec* V isolates were selected from animals representing different commercial vendors and out-state-location sources for the macaques. There were seven nasal samples and two wound samples. The isolates were selected without knowledge of the host primate species and included two isolates [A1404N nasal, A1404S skin sample both taken on July 20, 2015] from a SIV-positive *M. mulatta* [A1404] from WaNPRC. *M. mulatta* A1404 had close contact with the SIV-positive animal A140 [A140 nasal] and was also from the WaNPRC (**Table 1**). Both animals had a compromised immune system and bite wounds. The other six MRSA ST3268 isolates originated from macaques shipped from other United States primate sites, macaques shipped from two different commercial vendors [A1408, A1535] or directly shipped from China and having been in quarantine for 6 months in California before shipping to the WaNPRC [A1524] (**Table 1**). These nine isolates came from two *M. mulatta* [nasal isolates A140, A1404N, and one wound isolate A1404W], two *M. fascicularis* [nasal isolates A1524, A1525] and three *M. nemestrina* [two nasal A109, Z1403, one wound site isolate K990W] (**Table 1**).

All isolates were grown on Brucella agar (Difco Laboratories, Division BD Sparks, MD, United States) slants and shipped by courier to Germany for further molecular testing.

DNA Microarray Analysis, MLST, SCC*mec* Typing and *spa* Typing

The Alere StaphyType® DNA microarray was used for all isolates as previously described (Monecke et al., 2011, 2016). The microarray includes 334 target sequences and ∼170 separate genes and allelic variants including species markers, SCC*mec*, capsule, *agr* group typing markers, common antibiotic resistance genes, toxins and microbial surface components recognizing adhesive matrix molecules [MSCRAMM] genes. The latter genes comprise among others *clfA* and *clfB* (encoding clumping factors A and B), *fnbA* and *fnbB* (encoding fibronectin binding proteins A and B), *fib* (encoding fibrinogen binding protein), *eno* (encoding laminin binding protein), and *cna* (encoding collagen binding protein), the gene products of which play a role in the initial attachment of bacteria to host tissue. The detailed protocol as well as the sequences of primers and probes have previously been published (Monecke et al., 2011).

The clonal complexes (CCs) were determined by automated comparison of the microarray hybridization profiles to a database of previously characterized isolates (Monecke et al., 2011, 2016). The *spa* typing was performed according to Harmsen et al. (2003). The *spa* types were determined using the Ridom website.

The MLST typing was done using PCR and sequencing and the SCC*mec* typing was performed as previously described prior to being sent to Germany (Soge et al., 2016).

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing was performed for 30 antimicrobial agents by broth microdilution according to the Clinical and Laboratory Standards Institute (Clinical Laboratory Standard Institute [CLSI], 2018). The microtiter plates (MCS Swalmen, Netherlands) included penicillins (penicillin, ampicillin, amoxicillin/clavulanic acid, oxacillin), carbapenems (imipenem), a macrolide (erythromycin), a lincosamide (clindamycin), tetracyclines (tetracycline, doxycycline), aminoglycosides (gentamicin, streptomycin), a quinolone (ciprofloxacin), an oxazolidinone (linezolid), a glycopeptide (vancomycin), a streptogramin combination (quinupristin/dalfopristin), a phenicol (florfenicol), a pleuromutilin (tiamulin), and the combination trimethoprim/sulfamethoxazole. The aminoglycoside kanamycin was tested by broth macrodilution (Clinical Laboratory Standard Institute [CLSI], 2018, **Supplementary Table S1**). As there are no CLSI-approved clinical breakpoints applicable to primates other than humans, we used the human clinical breakpoints as listed in the CLSI document M100, 28th edition (Clinical Laboratory Standard Institute [CLSI], 2018). The breakpoints for the categories susceptible (S), intermediate (I) and resistant (R), are as follows: penicillin ($S \leq 0.12$ μg/mL, $R \geq 0.25$ μg/mL), oxacillin $S \leq 2$ μg/mL, $R \geq 4$ μg/mL, ciprofloxacin and quinupristin/dalfopristin ($S \leq 1$ μg/mL, I = 2 mg/mL, $R \geq 4$ μg/mL), gentamicin, doxycycline and tetracycline ($S \leq 4$ μg/mL, I = 8 μg/mL, $R \geq 16$ μg/mL), erythromycin ($S \leq 0{,}5$ μg/mL, I = 1–4 μg/mL, $R \geq 8$ μg/mL), clindamycin ($S \leq 0.5$ μg/mL, I = 1–2 μg/mL, $R \geq 4$ μg/mL), linezolid ($S \leq 4$ μg/mL, $R \geq 8$ μg/mL), trimethoprim/sulfamethoxazole ($S \leq 2/38$ μg/mL, $R \geq 4/76$ μg/mL), and vancomycin ($S \leq 2$ μg/mL, I = 4–8 μg/mL, $R \geq 16$ μg/mL) (Clinical Laboratory Standard Institute [CLSI], 2018, **Supplementary Table S1**). There are no clinical breakpoints for *S. aureus* for ampicillin, amoxicillin-clavulanic acid and imipenem, but if *S. aureus* strains are classified as resistant to oxacillin they are also considered as resistant to other β-lactams. Since there are no CLSI approved kanamycin breakpoints available, isolates with MICs of ≥ 64 μg/mL were tentatively considered as resistant (Feßler et al., 2010). Florfenicol and tiamulin are not used in human medicine and thus no breakpoints are available.

Susceptibility testing of the biocides benzalkonium chloride, chlorhexidine, glutardialdehyde, and isopropanol was also performed by broth macrodilution. For this, a bacterial suspension was prepared in a tryptone-saline-diluent (TSD; 1 g tryptone-peptone, 8.5 g sodium chloride in 1 L purified water) in a concentration of in 1×10^8–1×10^9 cfu/mL from 16 to 24 h old cultures on tryptic soy agar (TSA) (Roth, Karlsruhe, Germany). This suspension was diluted 1:10. From this dilution, 20 μl were added per each ml double concentrated tryptic soy broth (2× TSB) (Roth, Karlsruhe, Germany). One ml of this inoculum was added to a 2-fold benzalkonium chloride dilution series prepared in 1 mL volumes. The test ranges were as follows: benzalkonium chloride 0.00005–0.0008%, chlorhexidine 0.000025–0.0008%, glutardialdehyde 0.03–1%, and isopropanol 4 to 12%. The results were read after 24 h incubation at 37°C (Feßler et al., 2018).

Macrorestriction Analysis With Subsequent Pulsed-Field Gel Electrophoresis (PFGE)

SmaI macrorestriction analysis with subsequent pulsed-field gel electrophoresis was performed as previously described (Murchan et al., 2003) and the gels were analyzed according to the criteria Tenover et al. (1995) and (Deng et al., 2017).

RESULTS

Basic Characteristics of the ST188 SCC*mec* IV and ST3268 SCC*mec* V Isolates

Previously, nasal cultures were performed on 596 primates and 105 (17.6%) were MRSA positives. With the exception of four animals all in-house primates carried the MRSA ST188, while the MRSA ST3258 was associated with animals that were shipped into WaNPRC from other primate facilities and commercial breeders (Soge et al., 2016). *M. nemestrina* represent 75% of the primates in the WaNPRC. All ST188 and ST3268 isolates were positive for the species markers (*rrnD1, gapA, katA, coA, nuc1, spa, sbi*), capsule and *agr* alleles and consistent with an identification as *S. aureus*. All fifteen ST188 isolates selected for the study came from *M. nemestrina* hosts and were verified to have the ST188 MLST profile (3-1-1-8-1-1-1). All but one had *spa* type t189 (07-23-12-21-17-34), while the remaining isolate [Z143] had *spa* type t3887 (07-23-12-12-34). The nine ST3268 isolates had a MLST profile of 1-14-430-214-10-303-329. Two different *spa* types were identified, t13638 ($n = 5$) and t15469 ($n = 4$). The two *spa* types differed by the presence of an additional repeat 17 in *spa* type t15469 (210-23-02-34-17-34-34-17-17-23-34) compared to *spa* type t13638 (210-23-02-34-17-34-34-17-23-34) (**Table 1**). The *spa* type t13638 isolates were cultured from *M. fascicularis* and *M. nemestrina*. This *spa* type was first described in a methicillin-susceptible *S. aureus* from the United Kingdom[2]. The *spa* type t15469, cultured from *M. mulatta*, is a novel *spa* type, first described in these primate isolates[2] (**Table 1**). In the ST3268 isolates, the *spa* types correlated with the host macaque species. The four isolates from *M. mulatta* hosts were *spa* type t15469, while the two *M. fascicularis* and *M. nemestrina* isolates were *spa* type t13638 (**Table 1**).

PFGE Profiles

Nine of 15 ST188 [L091 (nasal), Z121N (nasal), Z121S (skin), Z123N1 (nasal) and Z123N2 (nasal), Z123S (skin), Z1304 (nasal), Z131S (skin), and Z143 (nasal)], from six *M. nemestrina* had indistinguishable PFGE patterns [A]. Five ST188 isolates originating from three animals [K062 (nasal), Z1242N1, Z1242N2 and Z1242N3 (nasal), and Z130 (nasal) shared PFGE sub-pattern [A1], while the ST188 isolate A112 (nasal) had a second PFGE sub-pattern [A2] (**Table 1** and **Supplementary Figure S1**).

[2]http://spa.ridom.de/

Of the nine MRSA ST3268 isolates, seven [A140 (nasal), A1404N (nasal), A1404W (wound), A1408 (nasal), A1524 (nasal), A1525 (nasal), and K990W (wound)] had the same PFGE pattern [B]. The isolates A1404N (nasal) and A1404W (wound) were cultured from the same animal eleven days apart and were indistinguishable in their PFGE patterns, their resistance pheno- and genotypes, as well as, their virulence genes (**Table 1**). The two sub-patterns B1 and B2 were found in found in single isolates A109 (nasal) and Z1403 (nasal), respectively (**Table 1** and **Supplementary Figure S1**).

Resistance Pheno- and Genotypes of the ST188 SCC*mec* IV and ST3268 SCC*mec* V Isolates

All 21 MRSA isolates were resistant to penicillin and oxacillin. They carried the *mecA* gene and the β-lactamase gene *blaZ*. All isolates were also resistant to ciprofloxacin. In addition, all ST188 isolates were resistant to macrolides and lincosamides via the *erm*(B) gene and carried the aminoglycoside resistance gene *aacA-aphD* mediating gentamicin and kanamycin resistance. The *aacA-aphD* gene was only present in five of the ST3268 isolates, which exhibited high kanamycin MICs ($\geq$ 256 mg/L) and were classified as resistant or intermediate to gentamicin. The nine MRSA ST3268 isolates were all tetracycline resistant and carried the *tet*(K) gene, while only five MRSA ST188 isolates (K062, Z1242N1, Z1242N2, Z1242N3, and Z130), from 3 *M. nemestrina*, were resistant to tetracycline and carried the *tet*(K) gene (**Table 1**).

From some of the animals, several isolates taken at different time points [Z1242N2, Z1242N3, Z123N1, and Z123N2] were included. However, even after one or more rounds of mupirocin topical treatment and chlorhexidine baths, the MRSA isolates either persisted in the noses of these juvenile animals or the animals were re-infected or re-colonized. Treatment success was measured by MRSA-negative cultures at two and four weeks after treatment. If the animal was still MRSA-positive, it was considered as treatment failure. If this happened, the animal was retreated with mupirocin and chlorhexidine baths. This primarily happened in juvenile animals. Because this "treatment failure" was limited to juvenile animals the veterinarian staff felt that it suggested that the animals were refractory to clearance of the isolate, the isolate may have become resistant to mupirocin due to acquisition of the mupirocin gene *mupA* or an alternative resistance mechanism, or other characteristic of being a juvenile *M. nemestrina* rather than clearance and reinfection since there was no sign of clearance in two and four week samples (**Table 1**). However, none of these isolates or any of the other isolates in the study were resistant to mupirocin nor did they carry the *mupA* gene (**Table 1**).

All the isolates were tested for reduced susceptibility to benzalkonium chloride, while no change was seen with chlorhexidine, glutardialdehyde, or isopropanol. Some isolates including ST188 isolates K062, Z1242N1, Z1242N2, Z1242N3, Z130, and ST3268 isolates A1524, A1525, and K990W, had a benzalkonium chloride MIC of 0.0004% and carried the *qacC* gene. All other isolates, that did not harbor the *qacC* gene, had benzalkonium chloride MICs of 0.0001% (**Table 1**). No other change in the MIC of disinfectants were observed.

Characterization of Accessory and Virulence Genes

The nine ST3268 isolates had the enterotoxin gene cluster *egc* [*seg, sei, selm, seln, selo, selu*] and the additional enterotoxin genes *sec* and *sel*. In contrast, none of the ST188 harbored the enterotoxin gene cluster *egc*, *sec* or *sel* genes (**Table 1**). The fifteen ST188 and nine ST3268 isolates carried the *hlgA* locus [comprising of *hlgA/lukF/lukS*], leukocidin genes [*lukD/E* and *lukX/Y*], the aureolysin gene [*aur*], and the protease genes *sspA*, *sspB*, and *sspP*. The gene for the *S. aureus* surface protein G [*sasG*] was present among the ST3268 isolates but absent in the ST188 isolates. Two isolates were additionally tested with a new array and both A1403 and Z140 were positive for the carotinoid pigment gene cluster [*crtM/N/O/P*]. Other isolates were not tested.

In contrast, the enterotoxin H gene [*entH*], ORF CM14, and *splE* were absent in all isolates (**Table 1**). The collagen-binding adhesin [*cna*] and the protease genes *splA*, *splB* were present in the ST188 isolates but were not detected among the ST3268 isolates. None of the 21 isolates carried PVL genes, the toxic shock syndrome toxin 1 gene [*tst1*], exfoliative toxin genes [*etA*, *etB*, *etD*], or genes associated with β-haemolysin converting phages (*sea, see, scn, chp*) (**Table 1**).

Two ST3268 SCC*mec* V isolates, A140 from a *M. mulatta* and Z1403 from a *M. nemestrina*, were further tested for SCC*mec* accessory genes. The following genes were identified in both *mvaS, cstB-SCC2, ydhK, D1GU38, Q4LAG7, czrC, "ccrAA"* (a recombinase homologue associated with *ccrC*), and a SCC*mec* terminus type 2 (Monecke et al., 2016). This is consistent with the presence of SCC*mec* VT+*czrC* composite elements as described for the CC398 strain SO385 (GenBank accession number AM990992.1), a livestock-associated MRSA strain from Western Europe (Schijffelen et al., 2011).

All isolates from the same animal shared indistinguishable PFGE patterns, regardless of whether nasal samples were taken at different times, or nasal and skin samples taken at the same time from the same *M. nemestrina*. As shown below, isolates from the same animal were also indistinguishable with respect to their resistance pheno- and genotypes, and other genes including enterotoxin, hemolysin, leukocidin, or PVL genes (**Table 1**), suggesting the presence of the same or a closely related strain in different locations of the animal and/or the persistence of that strain over time.

DISCUSSION

There have been two different clones present in macaques from the WaNPRC facility. The in-house clone ST188 was primarily found in *M. nemestrina*, the predominant primate species [75% of the primates] in the WaNPRC facility. At this time, we believe it was introduced into the facility from primates shipped from other United States National Primate Research Facility and/or

commercial vendors around 2014. Then this clone was spread across the facility mainly via in-house transmission. ST188 has continued to be isolated from primates in 2018 and from the primate environment in 2018.

The second MRSA clone ST3268 came from primates that were originally shipped from two different commercial breeders in two different states and other primate colonies in the United States. ST3268 was identified for the first time after a United States facility shipped ∼90 animals in May 2015 to the WaNPRC. In 2016, MRSA ST3268 SCC*mec* V-positive animals were also shipped from a third commercial vendor in a third state to WaNPRC suggesting that this is the primary way ST3268 has continued to be introduced into the WaNPRC. The vendor animals primarily originated from China or Indonesia. The four MRSA ST3268-positive WaNPRC animals were those that had contact with MRSA ST3268-positive animals by following them into a treatment room. Hence, the assumption was that the treatment room was contaminated with MRSA ST3268 and the SIV-positive animals picked up the strain in the treatment room. Similarly, we have found ST188-positive macaques from both commercial vendors and other United States primate facilities. The original source of the MRSA ST188 is not as clear though it can be found in low prevalence among humans in Asia (Soge et al., 2016).

As previously reported (Soge et al., 2016) MRSA ST188 isolates have been isolated almost exclusively from Asian humans but these strains often carry other SCC*mec* types then found in the WaNPRC primates. This MLST type is very rarely reported in North America. One report has identified MSSA ST188 from sanctuary chimpanzees isolated in Uganda and ten MSSA ST188 isolated from wild Madagascar lemurs. The major differences between the two clones other than MLST and *spa* type is that ST188 has primarily been associated with *M. nemestrina*, the predominate primate in WaNPRC. In contrast, ST3268 has been identified in all three species of macaques in the WaNPRC. The two clones also differ in the carriage of antimicrobial resistance genes. For example, the *erm*(B) gene is present in all ST188 isolates studied; but none of the ST3268 isolates in the current study harbored this gene. The *tet*(K) gene is present in all ST3268 in the current study, but only in some of the ST188 isolates (**Table 1**). Only ST3268 isolates carried the *fosB* gene. All isolates from both clones were ciprofloxacin resistant. The mechanism of resistance to ciprofloxacin was not determined, however, in our previous study with related isolates from macaques in the WaNPRC center both ST188 and ST3268 isolates carried a *gyrA* mutation that resulted in the Ser84Leu amino acid substitution, suggesting that the isolates in the current study may also have this mutation (Soge et al., 2016). A few isolates of both clones had elevated benzalkonium chloride MICs.

For other genes, there were differences in the carriage of the *egc* gene cluster, *sec* and *sel* genes with all ST3268 isolates and none of the ST188 isolates carrying these genes. However, none of the differences in genes identified could readily explain the different ability to transfer between the primates within the WaNPRC or the lack of finding ST3268 in environmental sites both in 2015 and more recently in 2018 (data not shown). Recently, Hsu et al. (2017) identified six ST3268 SCC*mec* V and two ST2817 SCC*mec* isolates taken from *M. fascicularis* used in experimental surgery in 2014 in Singapore. An additional isolate was cultured from a person who worked in animal husbandry in the facility. These animals primarily came from Vietnam and were imported between 2009 and 2014. Both MLST types can be regarded as belonging to the same clonal complex (Hsu et al., 2017). The Singaporean ST3268 SCC*mec* V isolates were resistant to ciprofloxacin, gentamicin and tetracycline. MICs were determined but specific antibiotic resistance genes were not identified in the Hsu et al., 2017. One Singapore isolate, DN260, differed from ST3268 WaNPRC United States, TXA, and TXB isolates by 36 SNPs (Soge et al., 2016; Hsu et al., 2017). It was unclear in the Hsu study whether the ST3268 was able to transfer between animals within their facility or if they came into the facility carrying the MRSA. However, it is possible that the facility worker acquired his nasal MRSA ST3268 from the MRSA-positive primates or contaminated work environment.

ST3268 is genetically related to ST2817 which is found in low prevalence in Asia, previously isolated from a human surgical wound in Singapore in 2014[3]. However, except for the one worker all MRSA ST3268 SCC*mec* V isolates have been isolated from macaques and thus may very well be a primate-associated strain that is common in parts of Asia (Soge et al., 2016; Hsu et al., 2017).

The ST188 clone continues to be the dominant MRSA clone in the WaNPRC. We examined the two MRSA isolates recovered in Aug 2017 and both were ST188. As previously shown, we also found a few methicillin susceptible *S. aureus* [MSSA] strains that were ST188 which clustered with the MRSA ST188 from the WaNPRC primates (Soge et al., 2016). No MSSA that were ST3268 have been identified though the number of MSSA examined has been small (Soge et al., 2016). The MRSA ST3268 isolates characterized in the current publication were recovered over a seven month time period, and could be subdivided into two *spa* types, which were found in different species of macaques (**Table 1**).

The data from the current study as well as previous studies (Soge et al., 2016; Hsu et al., 2017) suggest that all primates should be screened and treated for MRSA carriage prior to being shipped to other facilities within a country or between countries to reduce the continual spread of primate-related MRSA.

CONCLUSION

The primate isolates belonged to two different clones, ST188 and ST3268. ST188 was the in-house clone that easily spread among primates in the colony. It was primarily identified in *M. nemestrina*, though this could be due to the predominance [75%] of this species of macaques in the WaNPRC. Fourteen of the 15 ST188 isolates exhibited the same *spa* type t189. Five isolates carried the *tet*(K) gene coding for tetracycline

[3]http://saureus.mlst.net/

resistance and all had PFGE pattern A1 with all five of these isolates harboring the *qacC* gene and showing reduced susceptibility to benzalkonium chloride. The nine ST188 isolates with PFGE pattern A were susceptible to tetracyclines and did not carry tetracycline resistance genes. The other clone, ST3268, was introduced from external macaques shipped from other United States primate facilities and United States commercial companies. ST3268 did not spread easily among the primates even though each isolate carried the *egc* enterotoxin gene cluster, *sec* and *sel* genes. One unexpected observation with the ST3268 isolates was finding that the *spa* type varied by macaque host species as did the mobile antibiotic resistance genes and reduced susceptibility to benzalkonium chloride. However, seven out of nine isolates had the same PFGE pattern B and the two variants PFGE patterns B1 and B2 did not correlate with either host macaque species or antibiotic resistance genes carried suggesting that they are members of a closely related clone. The data presented does not provide insight into why ST188 could spread easily while ST3268 did not spread within the WaNPRC facility.

ETHICS STATEMENT

Primate samples were taken as part of the general care of the animals.

AUTHOR CONTRIBUTIONS

MR designed the experiments. DN did the laboratory work in Seattle. AF and SS did the laboratory work in Germany. SM and RE helped us to understand the results. All authors worked on writing the manuscript up for publications.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02199/full#supplementary-material

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Antimicrobial Resistance Profiles in *Enterococcus* spp. Isolates From Fecal Samples of Wild and Captive Black Capuchin Monkeys (*Sapajus nigritus*) in South Brazil

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The environment, human, and animals play an important role in the spread of antibiotic-resistant bacteria. Enterococci are members of the gastrointestinal tracts of humans and animals and represent important reservoirs of antibiotic resistance genes. Until today, few studies have examined antibiotic susceptibility in enterococci isolated from primates. Therefore, the present study investigated species distribution, antibiotic susceptibility, and resistance genes in enterococci isolated from wild and captive black capuchins monkeys (*Sapajus nigritus*) in Rio Grande do Sul, South Brazil. A total of 24 swabs/fecal samples were collected, including 19 from wild monkeys living in two forest fragments [São Sebastião do Caí (SSC) and Santa Cruz do Sul (SCS)], and five in captive [Parque Zoológico da Fundação Zoobotânica (ZOO)], between August 2016 and November 2017. Fifteen colonies were randomly selected from each sample. Enterococci were identified by MALDI-TOF, tested for susceptibility to 12 antibiotics; and screened for *tet*(S), *tet*(M), *tet*(L), *msrC*, and *erm*(B) genes by PCR. Two-hundred ninety-six enterococci were isolated (SSC n = 137; SCS n = 86; ZOO n = 73) and differences in *Enterococcus* species distribution were detected on three monkey groups, with low abundance in SCS (1 − D = 0.2), followed by ZOO (1 − D = 0.68), and SSC (1 − D = 0.73). The enterococci frequently recovered include the following: *Enterococcus faecalis* (42.6%), *E. hirae* (29.1%), and *E. faecium* (15.9%). Antibiotic-nonsusceptible was observed in 202 (67.9%) strains. The rate of non-susceptibility to rifampicin, tetracycline, erythromycin, nitrofurantoin, chloramphenicol, and ampicillin was 46%, 26%, 22% and 19%, 13%, 0.3%, and 0.3%, respectively. All strains were susceptible to vancomycin, streptomycin, gentamycin, and linezolid. Forty-three (14.52%) isolates were identified as multidrug resistant (MDR), and the highest number of MDR enterococci were *E. faecium* recovered from wild monkeys living close to a hospital and water treatment plant. Elevated rates of antibiotic resistance genes

*msr*C and *tet*(L) were isolates from ZOO. In conclusion, differences in the frequency of enterococci species, antibiotic-nonsusceptible and antibiotic resistance genes in all groups of monkeys were identified. These data suggest that anthropogenic activities could have an impact in the resistome of primate gut enterococci communities.

Keywords: Enterococcus, primates, wild and captive capuchin monkeys, *Sapajus nigritus*, antimicrobial resistance

INTRODUCTION

Brazil has the greatest biodiversity on the planet, comprising approximately 103,870 different animal species and the highest diversity of Primates, around 77 species, including the howler monkey, the capuchin monkey, the marmoset, and the tamarin (Brazilian Society of Primatology [SBP], 2016). *Sapajus nigritus* (black-horned capuchin or black capuchin monkeys) are part of the Cebidae family, characterized as robust capuchin monkeys with adornments or tufts on the head (Rylands et al., 2012). They are considered the largest omnivorous Neotropical primate, which is able to adapt its diet according to food availability, thus bringing them into contact with a wide diversity of microorganisms. Their diet is composed of approximately 55% fruits, 33% insects, 8% seeds, 8% leaves (mainly young), and 2% flowers (National Research Council of the National Academies [NRC], 2003). Currently, this species occurs in Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul states, extending to the Argentinean province of Misiones (International Union for Conservation of Nature [IUCN], 2017).

The black capuchin monkeys (*S. nigritus*) live in different habitats, from large remnants or continuous to small forests fragments. Outside of their natural environment, they can be found in zoological, rehabilitation, or research centers, and even in urban and rural environments. Additionally, these animals exhibit a niche overlap with humans in the case of semi-wild areas (Muehlenbein, 2017). Since the natural habitats of primates are forests, most interactions between humans and primates occur in this high-risk interface. In many regions of the world, omnivorous primate species are adapting to human activities. Furthermore, the frequency of such interactions has increased due to ecotourism and/or increasing forest invasion, and these interactions could lead bacteria exchanges by multiple routes, namely through the offering of food (Mikich and Liebsch, 2014). Glover (2014) compared the enteric bacteria of monkeys with three levels of human contact and determined that the closer the animals were to humans, the more resistant was the enteric bacteria to antibiotics. Importantly, Rolland et al. (1985) observed that wild baboons (*Papio cynocephalus*) that fed on human debris, maintained a high proportion of antibiotic-resistant enteric bacteria than those without human contact.

The environment, humans, and animals play an important role in the emergence and spread of antibiotic-resistant bacteria. Singer et al. (2016) described three well-characterized classes of chemicals – antimicrobials, heavy metals, and biocides – related to the selection of antibiotic resistance genes. Biological fluids (e.g., urine and feces) contaminated with antimicrobials or resistant bacteria from human and animal origins are released into the environment – especially in soil, sewage, water, and wastewater – thereby contributing to the spread of resistance (Baquero et al., 2008; Gothwal and Shashidhar, 2014). The proximity to human activity has showed to increase the number of resistant bacteria in wild animals, with animals living near waste or agricultural water harboring more antibiotic-resistant bacteria than animals living close by unpolluted water (Allen et al., 2010). Recently, it was demonstrated that exposure to human antibiotics was associated with changes in the microbiota composition of baboons (Tsukayama et al., 2018).

Enterococci are a large genus of bacteria widely distributed on plants, soil, water, humans, and animals. In humans and other species, inhabit various sites including the oral cavity, genitourinary and gastrointestinal tracts (Lebreton et al., 2014). The genus *Enterococcus* consists of over 50 diverse species, and *Enterococcus faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *E. casseliflavus*, *E. gallinarum*, and *E. mundtii* are the most frequently encountered in the gastrointestinal tracts of animals (Poeta et al., 2005; Cassenego et al., 2011; Lozano et al., 2016; Medeiros et al., 2017). However, the species evaluation in the gastrointestinal tract of primates remains little known (Xavier et al., 2010; Glover, 2014). The species distribution, as well as their proportions in the different niche can change according to the host and its age, diet, underlying diseases, and prior antimicrobial therapy (Lebreton et al., 2014).

Otherwise, enterococci are considered an opportunistic pathogen, associated with serious infection, such as endocarditis, urinary, and bloodstream infections, intra-abdominal end intra-pelvic abscesses, which has been attributed, in part; to the increasing resistance to a wide range of antimicrobial agents. The presence of resistant and multidrug-resistant enterococci in patients has a clinical relevance because of limited therapeutic options (Higuita and Huycke, 2014). Antimicrobial resistance to several classes of agents is a remarkable characteristic of enterococcal isolates. These microorganisms are intrinsically resistant to some antimicrobial agents commonly prescribed for Gram-positive cocci, and exhibit resistance to a wide variety of other antimicrobials by mutation and/or acquisition of genes through the plasmids and transposons. In fact, many species are recognized for their ability to acquire and transfer resistance and virulence genes, which give a selective advantage to *Enterococcus* spp. survival and dispersion in the environment (Lebreton et al., 2014; Miller et al., 2014). The occurrence of antimicrobial resistance among enterococci is not restricted to the nosocomial setting, and therefore, resistant strains has been investigated and monitored in different habitats, providing important information

regarding about environmental disturbances (Poeta et al., 2005; Frazzon et al., 2010; Barros et al., 2011; Cassenego et al., 2011; Santos et al., 2013; Santestevan et al., 2015; Prichula et al., 2016).

To date, few studies have examined the presence of enterococci in monkeys, and these studies have focused primarily on captive animals, perhaps due to the inherent difficulty in obtaining samples from free-living wild animals (Xavier et al., 2010; Glover, 2014; Woods et al., 2017). The investigation of the persistence of enterococci in these animals highlights the impact of human activities on the environment. Moreover, antibiotic-resistant enterococci in monkeys are an important point that must be addressed in the host–microorganism–environment interactions. Therefore, the objective of the present study was to evaluate the distribution of enterococci in fecal samples of free-living and captive black capuchin monkeys from South Brazil. In addition, the prevalence of antibiotic susceptibility and antibiotic resistance genes in enterococci isolated from these primate populations were determinates.

MATERIALS AND METHODS

Sample Collection

Twenty-four samples collected from black capuchin monkeys between August 2016 and November 2017 were used in the present study, including samples from animals with free lifestyle (n = 19) and animals living in captivity (n = 5). Samples were obtained in Rio Grande do Sul, South Brazil (**Supplementary Data 1**).

Samples were taken from three groups of black capuchin monkeys. Two groups include wild animals from two forest fragments in Rio Grande do Sul State (**Figure 1**). In the first forest fragment located in São Sebastião do Caí (SSC) (29° 35′ 13″ S; 51° 22′ 17″ W), samples were obtained from 11 animals, corresponding to 30% of overall group composition. This forest fragment is located near to a hospital and water treatment plant. The area comprises 2% of vegetation, totalizing 9611 hectares of forest (SOS Mata Atlântica, 2016). In the second forest fragment, located in *Parque Municipal da Gruta dos Índios* (Indian Grotto Municipal Park) in Santa Cruz do Sul (SCS) (29° 43′ 03″ S; 52° 25′ 33″ W), samples were obtained from eight animals, corresponding to 27% of the overall group. This forest fragment is located inside of the park, and the animals come without indirect contact with any park visitor, but maintain contact with garbage and other food sources. The area comprises 13% of vegetation, totalizing 539.8 hectares of forest (SOS Mata Atlântica, 2016). The third group was in captive condition at the Zoological Park of the Zoobotânica Foundation of Rio Grande do Sul (ZOO) in Sapucaia do Sul (29° 49′ 29″ S; 51° 08′ 54″ W), and five samples were collected. The animals were isolated in quarantine at ZOO since they were rescued from illegal or abusive situations by the Wild Animals Triage Center (CETAS – IBAMA). The diet of captive monkeys was composed of extruded ration for primates (Nuvital Primatas Neotropicais, Nuvital Nutrientes S/A, Colombo, Brazil) complemented with fruits and vegetables.

Wild capuchin monkeys were captured and manipulated using conventional methods according to the protocol for sample collection described by Instituto Chico Mendes de Conservação da Biodiversidade [ICMBio] (2012) using Tomahawk-type cages. The ketamine (100 mg/mL) and xylazine (20 mg/mL) were used intramuscularly for wildlife immobilization (Miranda et al., 2011).

Rectal swabs and fecal samples were collected by veterinarians, all animals were clinically healthy and were classified according to gender and age group. Rectal swabs were collected from the perirectal area, stored in Stuart transport medium (Kasvi, Paraná, Brazil), and transported to our laboratory for microbiological analyses. Fecal samples were collected, individually or in groups, directly from cages using sterilized wooden sticks. Fecal samples were placed in sterile tubes, kept on ice, and sent to our laboratory for storage at −80°C.

This study was carried out in accordance with the recommendations of Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) and Chico Mendes Institute for Biodiversity Conservation (ICMBio). The protocol was approved by Information Authorization System in Biodiversity (SISBIO) number 56640.

Isolation and Identification of Enterococci

Isolation, enumeration, and characterization of enterococci in fecal/rectal swabs were performed as previously described by Prichula et al. (2016) and Santestevan et al. (2015). Swabs or fecal samples (0.1 g) were inoculated in 9 mL of azide dextrose broth (Himedia, Mumbai, India) and incubated for 24 h at 37°C. Aliquots of 1 mL were placed in 9 mL of saline water, and initial samples were further diluted 10-fold to obtain a final dilution factor of 1/1000. From each dilution, 100 μL was inoculated in brain heart infusion (BHI) agar plates (Himedia, Mumbai, India) supplemented with 6.5% NaCl, before being incubated as previously described (Santestevan et al., 2015; Prichula et al., 2016). Fifteen colonies were randomly selected from each sample. Phenotypic criteria, such as size/volume, shape, color, gram staining, catalase production, growth capacity at 45°C, and bile aesculin reaction were used to separate the enterococci group and the non-enterococcal strains. Selected pure colonies were stored at −20°C in a 10% (w/v) solution of skim milk (Difco, Sparks, MD, United States) and 10% (v/v) glycerol (Neon Comercial Ltda, São Paulo, SP, BR).

The isolates collected were identified using matrix-assisted laser desorption and ionization time-of-flight technique (MALDI-TOF) applied to *Enterococcus* spp. according to the protocol previously described by Sauget et al. (2017).

Antimicrobial Susceptibility Testing

Susceptibility to antimicrobial agents was performed using the Kirby–Bauer disk diffusion method recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2016). Twelve antibiotics commonly used in clinical and veterinary medicine were evaluated: ampicillin 10 μg (AMP), ciprofloxacin 5 μg (CIP),

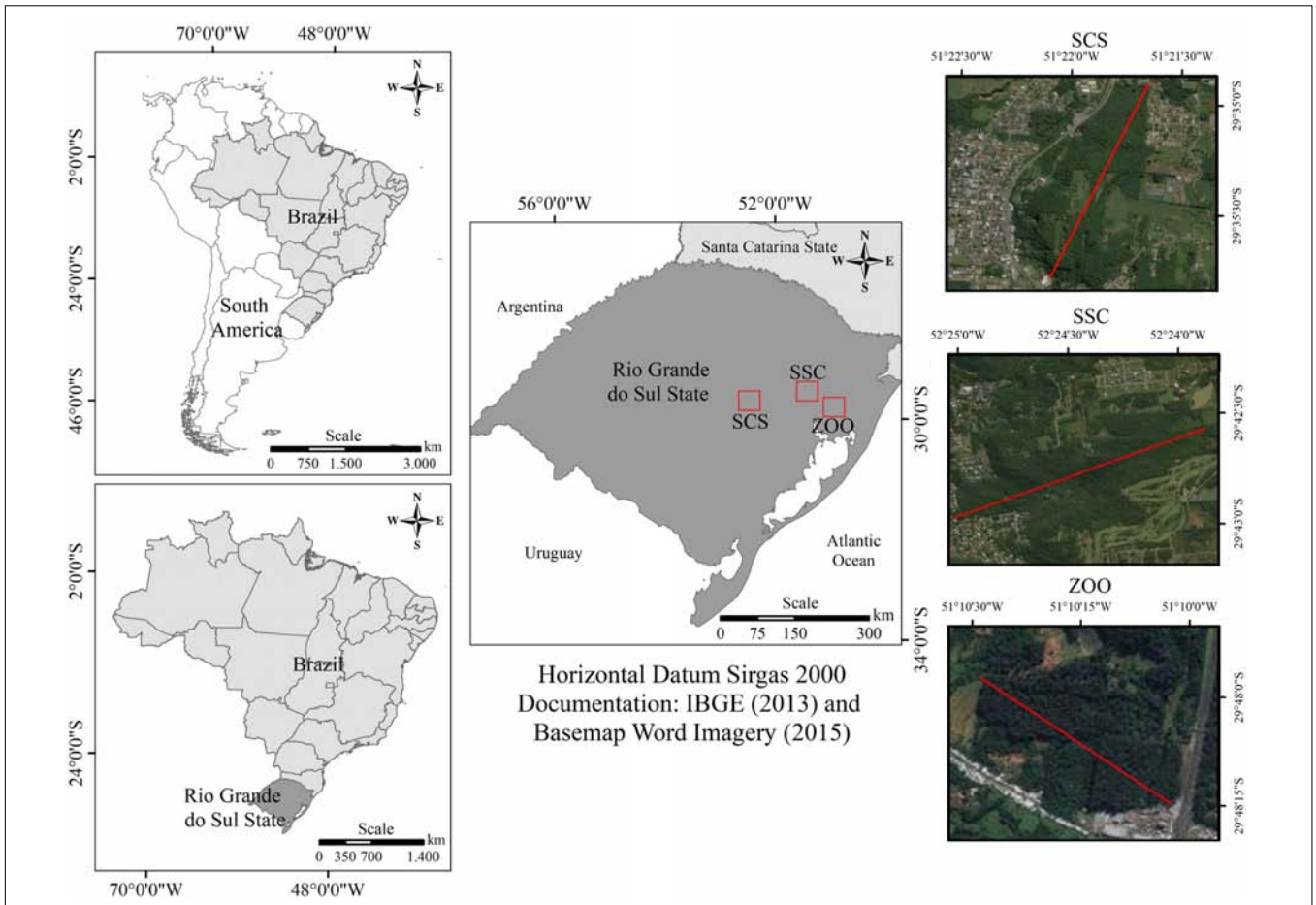

FIGURE 1 | On the left Southern American (top) and Brazil (bottom) maps. On the center, Rio Grande do Sul map showing locations of collected samples site. On the right-detailed map of the Rio Grande do Sul showing collected samples locations: Top; SSC, São Sebastião do Caí – a fragmented forest coverage of 2% vegetation of Atlantic Forest, totaling 9611 hectares of forest: Center; SCS, Santa Cruz do Sul – a fragmented forest comprised of 13% vegetation, totaling 539.8 hectares of forest; Botton; ZOO, Zoological (ZOO) at Zoobotanical Foundation of Rio Grande do Sul, in Sapucaia do Sul city.

chloramphenicol 30 μg (CHL), erythromycin 15 μg (ERY), streptomycin 300 μg (STR), gentamicin 120 μg (GEN), linezolid 30 μg (LNZ), nitrofurantoin 300 μg (NIT), norfloxacin 10 μg (NOR), rifampicin 5 μg (RIF), tetracycline 30 μg (TET), and vancomycin 30 μg (VAN). Minimum inhibitory concentration (MIC) of linezolid was determined by broth microdilution and interpretation of the results was performed following CLSI guidelines.

E. faecalis ATCC 51299 and *E. faecium* ATCC 53519 were included as control strains.

Strains resistant to three or more unrelated antibiotics were considered as multidrug-resistant (MDR). Intermediate and resistant strains were considered in a single category and classified as antibiotic-nonsusceptible.

Detection of Resistance-Related Genes in *Enterococcus* sp.

DNA extraction was performed as described by Depardieu et al. (2004). PCR was carried out for the detection of six different resistance-related genes commonly observed in clinical and environmental enterococci, namely, *erm*(B), *msr*C, *tet*(M), *tet*(S), and *tet*(L) (Sutcliffe et al., 1996; Aarestrup et al., 2000; Werner et al., 2001; Frazzon et al., 2010; Rathnayake et al., 2011). *erm*(B) encodes a ribosomal methylase that mediates macrolides, lincosamides, and type B streptogramins resistance; *msr*C encodes for a macrolide and streptogramin B efflux pump; *tet*(M) and *tet*(S) encodes for tetracycline resistance via a ribosomal protection protein mechanism; and *tet*(L) encodes for tetracycline resistance via efflux pumps proteins.

Statistical Analysis

The correlation between antimicrobial susceptibility presented by *Enterococcus* spp. and monkey collection origins were analyzed using a cross-table with Pearson's chi-square test (χ^2) ($p \leq 0.05$) and Fisher's exact test for small samples ($\leq$5). Simpson's index of diversity (D) was calculated to assess the differentiation of enterococci species among the monkeys from the different locations (Hunter and Gaston, 1988).

RESULTS

Enterococcus spp. Isolation and Identification in Fecal Samples

The distribution of *Enterococcus* species recovered from fecal/rectal samples of wild and captive black capuchins monkeys is provided in **Table 1**. A total of 296 enterococci were isolated, of those 223 (75%) were recovered from wild (SSC $n = 137$; SCS $n = 86$), and 73 (25%) from captive monkeys (ZOO). Among enterococci isolated, *E. faecalis* (42.6%; $n = 126$), *E. hirae* (29.1%; $n = 86$), and *E. faecium* (15.9%; $n = 47$) were detected in all groups of monkeys; and *E. durans* (6.8%; $n = 20$), *E. casseliflavus* (4.4%; $n = 13$), *E. raffinosus* (0.3%; $n = 1$), *E. avium* (0.3%; $n = 1$), *E. gallinarum* (0.3%; $n = 1$), and *Enterococcus* sp. (0.3%; $n = 1$) were occasionally detected in the animals.

Differences in the distribution of *Enterococcus* spp. was detected amongst the three groups of black capuchin monkeys, as shown in **Table 1**. Samples from SSC presented the higher difference and relative abundance of enterococci, when compared to SCS and ZOO. The Simpsons diversity indexes showed differences between the three groups, with low abundance to SCS ($1 - D = 0.2$), followed by ZOO ($1 - D = 0.68$) and SSC ($1 - D = 0.73$). *E. faecalis* was the predominant species recovered from wild monkeys from SCS (89.5%; $n = 77$). On the other hand, in fecal samples of wild monkeys from SSC, the more commonly observed species were *E. faecalis* (32.1%; $n = 44$), *E. hirae* (35.8%; $n = 49$), and *E. faecium* (19.0%; $n = 26$). Whereas *E. hirae* (47.9%; $n = 35$), *E. faecium* (26.0%; $n = 19$), and *E. durans* (17.8%; $n = 13$) were the most abundant species isolated in fecal samples of captive monkeys.

Antimicrobial Susceptibility Profile

Among the 296 *Enterococcus* spp. obtained from fecal samples of black capuchin monkeys, 201 (67.90%) were nonsusceptible to at least one antibiotic evaluated (**Figure 2**). Nonsusceptible to rifampicin (46%), tetracycline (26%), erythromycin (22%), and quinolones (ciprofloxacin/norfloxacin) (19%) was commonly observed, whereas nonsusceptible to nitrofurantoin, chloramphenicol, and ampicillin was observed only in 13%, 0.3%, and 0.3% of the strains, respectively. Further, all isolates were susceptible to vancomycin, streptomycin, gentamycin, and linezolid (**Table 2**). Chi-squared testing showed significant differences ($p \leq 0.05$) in tetracycline-nonsusceptible strains isolated from wild black capuchin monkeys from SSC when compared to the other groups.

In relation to species isolated from black capuchin monkeys, *E. durans* (90%) and *E. faecium* (85%), showed elevated frequency of antibiotic non-susceptibility, followed by *E. faecalis* (69%), *E. hirae* (56%), and *E. casseliflavus* (54%). *Enterococcus gallinarum* strain was only nonsusceptible to quinolones. Unlike the other species, *E. raffinosus*, *E. avium*, and *Enterococcus spp.* were susceptible to all antimicrobials tested. Regarding the source of samples, the occurrence of antibiotic non-susceptible strains was observed more frequently in isolates from SSC (**Figure 2**).

Single, double, and MDR profiles were observed in 32% ($n = 94$), 22% ($n = 64$), and 14.52% ($n = 43$) of strains, respectively. The percentages of double and MDR strains isolated from wild monkeys from SCS (10%; $n = 9$ and 7%; $n = 6$) and the captive (16%; $n = 12$ and 11%; $n = 8$) were lower compared to wild monkeys from SSC (39%; $n = 54$ and 21%; $n = 29$). Among the 29 MDR strains from SSC, *E. faecium* was the species with higher prevalence (54%; $n = 14$) (**Supplementary Data 2**).

TABLE 1 | Species distribution of enterococci in fecal samples of wild and captive black capuchin monkeys (*Sapajus nigritus*).

Species	Number (%) of Enterococci Isolated From			
	SSC	SCS	ZOO	Total (%)
E. faecalis	44 (32.1)	77 (89.5)	5 (6.8)	126 (42.6)
E. hirae	49 (35.8)	2 (2.3)	35 (47.9)	86 (29.1)
E. faecium	26 (19.0)	2 (2.3)	19 (26.0)	47 (15.9)
E. durans	7 (5.1)	–	13 (17.8)	20 (6.8)
E. casseliflavus	9 (6.6)	4 (4.7)	–	13 (4.4)
E. raffinosus	1 (0.7)	–	–	1 (0.3)
E. avium	1 (0.7)	–	–	1 (0.3)
E. gallinarum	–	–	1 (1.4)	1 (0.3)
Enterococcus sp.	–	1 (1.2)	–	1 (0.3)
Total	137 (100)	86 (100)	73 (100)	296 (100)

SSC, São Sebastião do Caí; SCS, Santa Cruz do Sul; ZOO, Sapucaia do Sul.

Frequency of Antibiotic Resistance Genes

Among the 66 erythromycin-nonsusceptible strains (11 were resistance and 56 were intermediate resistance), 24 (36%) contained the *msrC*, and none the *erm*(B) gene. Of the 77 tetracycline-nonsusceptible strains, 43 (56%) harbored only the *tet*(M), and 24 (31%) have both *tet*(M) and *tet*(L) genes. The *tet*(S) gene was not found in this study (**Table 3**).

In relation to species, the results showed that 92.5% *E. faecium*, 64% *E. hirae*, and 4% *E. faecalis* strains harbored *msrC* gene. The *tet*(M) was present in all *E. faecalis*, *E. faecium*, and *E. hirae* tetracycline-nonsusceptible strains, and *tet*(L) was detected in 14% *E. faecalis*, 57.5% *E. hirae*, and in 11% *E. faecium* tetracycline-nonsusceptible strains.

We investigated the association between resistance-related genes and the sample sources where enterococci species isolated from captive monkeys presented a higher frequency of *msr*C (95%) and *tet*(L) (57%) genes when compared to wild monkeys (**Table 3**). In addition, seven (21%) erythromycin and tetracycline-nonsusceptible strains from the ZOO harbored both *msr*C, *tet*(M), and *tet*(L) genes.

DISCUSSION

In this study using fecal samples collected of wild and captive black capuchin monkeys (*S. nigritus*) from South Brazil, we were able to detected different *Enterococcus* species. To date, only a few studies have investigated the distribution of enterococci species in the fecal samples/rectal swabs of wild and captive black capuchin monkeys. The genus *Enterococcus* was first reported in fecal samples from captive capuchin monkeys (*Cebus apella*) and common marmoset (*Callithrix penicillata*) in the Primate

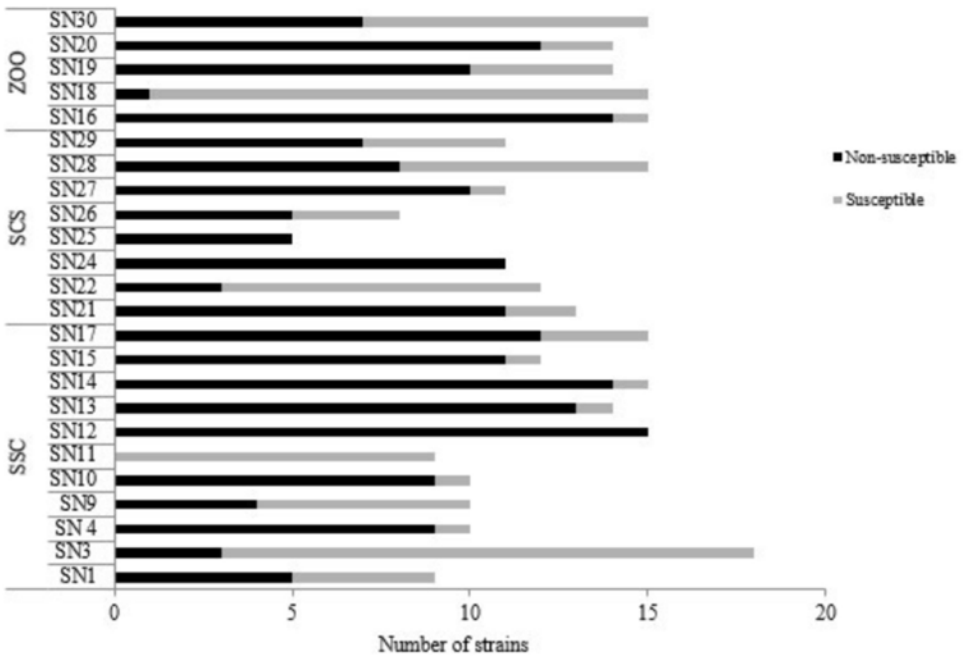

FIGURE 2 | Numbers of susceptible and nonsusceptible enterococci species isolated from fecal samples of wild (SSC and SCS) and captive (ZOO) black capuchin monkeys (*Sapajus nigritus).* SN, identification sample of black capuchin monkeys. SSC, São Sebastião do Caí; SCS, Santa Cruz do Sul; ZOO, Sapucaia do Sul.

TABLE 2 | Antibiotic resistance patterns among enterococci recovered from fecal samples and rectal of wild and captive black capuchin monkeys (*Sapajus nigritus*).

		Number (Percentage) of Nonsusceptible[1] Strains to:							Profiles		
	Species (n)	**AMP***	**QUI*,[1]**	**CHL***	**ERY***	**NIT***	**RIF***	**TET***	**SR****	**DR****	**MDR****
SSC	*E. faecalis* (44)	0	10 (23)	0	9 (20)	0	26 (59)	3 (7)	12 (27)	8 (18)	6 (14)
	E. hirae (49)	0	0	0	4 (8)	4 (8)	22 (45)	27 (55)	12 (24)	12 (24)	7 (14)
	E. faecium (26)	0	18 (69)	0	16 (61)	7 (27)	11 (42)	23 (88)	5 (19)	7 (27)	14 (54)
	E. durans (7)	1 (14)	0	0	1 (14)	4 (57)	7 (100)	1 (14)	3 (43)	2 (29)	2 (29)
	E. casseliflavus (9)	0	1 (11)	0	2 (22)	0	4 (44)	0	3 (33)	2 (22)	0
	E. raffinosus (1)	0	0	0	0	0	0	0	0	0	0
	E. avium (1)	0	0	0	0	0	0	0	0	0	0
	Subtotal (137)	1 (0.7)	29 (21)	0	32 (23)	15 (11)	70 (51)	54 (39)	35 (26)	31 (23)	29 (21)
SCS	*E. faecalis* (77)	0	18 (23)	0	14 (18)	0	41 (53)	7 (9)	36 (47)	16 (21)	4 (5)
	E. hirae (2)	0	0	0	0	0	0	0	0	0	0
	E. faecium (2)	0	2 (100)	0	1 (50)	0	1 (50)	2 (100)	0	0	2 (100)
	E. casseliflavus (4)	0	0	0	0	0	2 (50)	0	2 (50)	0	0
	Enterococcus sp. (1)	0	0	0	0	0	0	0	0	0	0
	Subtotal (86)	0	20 (23)	0	15 (17)	0	44 (51)	9 (10)	38 (44)	16 (19)	6 (7)
ZOO	*E. faecalis* (5)	0	2 (40)	0	1 (20)	0	5 (100)	4 (80)	0	3 (60)	2 (40)
	E. hirae (35)	0	2 (6)	0	7 (20)	15 (43)	8 (23)	6 (17)	7 (20)	6 (17)	4 (11)
	E. faecium (19)	0	2 (10)	1 (5)	10 (53)	2 (11)	3 (16)	3 (16)	6 (32)	4 (21)	2 (10)
	E. durans (13)	0	0	0	1 (8)	8 (61)	7 (54)	1 (8)	7 (54)	4 (31)	0
	E. gallinarum (1)	0	1 (100)	0	0	0	0	0	1 (100)	0	0
	Subtotal (73)	0	7 (10)	1 (1)	19 (26)	25 (34)	23 (31)	14 (19)	21 (29)	17 (25)	8 (11)
	Total (296)	1 (0.3)	56 (19)	1 (0.3)	66 (22)	40 (13)	137 (46)	77 (26)	94 (32)	64 (22)	43 (14)

*[1]Intermediate and resistant strains were considered in a single category and classified as antibiotic-nonsusceptible. *Antibiotics: AMP, ampicillin; QUI, quinolones (ciprofloxacin and norfloxacin); CHL, chloramphenicol; ERY, erythromycin; NIT, nitrofurantoin; RIF, rifampicin; TET, tetracycline; **Profiles: SR, single resistant; DR, double resistant; MDR, multidrug-resistant.*

SSC, São Sebastião do Caí; SCS, Santa Cruz do Sul; ZOO, Sapucaia do Sul.

TABLE 3 | Resistance-related genes among antibiotic-nonsusceptible enterococci isolated from fecal samples of wild and captive black capuchin monkeys (*Sapajus nigritus*).

		Antibiotic-Nonsusceptible Strains Tested to:								
		Erythromycin				Tetracycline				
	Strains	R^1	I^1	n^2 (%) of *msr*C	n^2 (%) of *erm*(B)	R^1	I^1	n^2 (%) of *tet*(L)	n^2 (%) of tet(M)	n^2 (%) of *tet*(S)
SSC	*E. faecalis*	1	8	0	0	3	0	1 (33)	3 (100)	0
	E. hirae	2	2	0	0	20	7	14 (52)	26 (96)	0
	E. faecium	0	16	14 (87.5)	0	21	2	0	16 (70)	0
	E. durans	1	0	0	0	1	0	0	0	0
	E. casseliflavus	0	2	0	0	0	0	ND	ND	0
	Subtotal	4	28	14 (44)	0	45	9	15 (28)	45 (83)	0
SCS	*E. faecalis*	0	14	1 (7)	0	7	0	1 (14)	7 (100)	0
	E. faecium	0	1	1 (100)	0	2	0	0	2 (100)	0
	Subtotal	0	15	2 (13)	0	9	0	1 (11)	9 (100)	0
ZOO	*E. faecalis*	0	1	0	0	4	0	0	4 (100)	0
	E. hirae	4	3	7 (100)	0	6	0	5 (83)	6 (100)	0
	E. faecium	4	6	10 (100)	0	3	0	3 (100)	3 (100)	0
	E. durans	1	0	1 (100)	0	1	0	0	0	0
	Subtotal	9	10	18 (95)	0	14	0	8 (57)	13 (93)	0
	Total	11	53	34 (51.5)	0	68	9	24 (31)	67 (87)	0

[1]*Number of resistant (R) or I, Intermediate resistant (I) strains.*
[2]*Number of positive strains; ND, not determined; SSC, São Sebastião do Caí; SCS, Santa Cruz do Sul; ZOO, Sapucaia do Sul.*

Center of the University of Brasília, Brazil (Xavier et al., 2010). Thereafter, Glover (2014) identified the genus *Enterococcus* in the fecal samples from the baboons (*Papio*) and vervet monkeys (*Chlorocebus pygerythrus*) in two rehabilitation centers in South Africa.

The enterococci species identified here from both wild and captive black capuchin monkeys have been reported to be predominant in fecal samples of different animals. Studies evaluating enterococci species in fecal samples of domestic and wild animals revealed presence of similar species (Layton et al., 2010; Cassenego et al., 2011; Franz et al., 2011; Silva et al., 2012; Nowakiewicz et al., 2014; Santestevan et al., 2015; Prichula et al., 2016; Medeiros et al., 2017). Among the species identified in the present study, *E. faecalis* was predominant. This species was also the most prevalent species in fecal samples of captive capuchin monkeys, common marmoset, domesticated mammals, birds, and wildlife feces, described in previous studies (Lanthier et al., 2010; Xavier et al., 2010). Nevertheless, it is important to highlight that some species could be underestimated in the present study due to the limitation of the method on used for enterococci isolation based on culturable methods. Although this method is widely used to isolate enterococci from different samples; we know that methods evaluating bacterial species in biological samples based on cultivation could limit the ability to recover some species occurring in small proportion.

Differences in the frequency of enterococci species in fecal samples among the three groups of monkeys were observed. Confinement, diet, and human contact are factors that may be responsible for this difference (Lebreton et al., 2014). In fecal samples of wild monkeys from SCS, the *E. faecalis* was the dominant species. In contrast, the species distribution of enterococci in samples of wild monkeys from SSC was more heterogeneous. These differences in the frequency of enterococci could be explained by the environmental conditions. In spite of the fact that both monkeys live in a free-living condition, monkeys from SCS are in a forest fragment surrounded by an urban area. Urban forest fragments are considered the most fragile area, which suffers directly the negative impacts of the anthropic action (Pereira et al., 2018). The urbanization also affects the insect species composition, as recently demonstrated by Melliger et al. (2018), whereas changes in the composition of ants and spiders were associated with increasing degree of urbanization. The anthropic action on the forest fragment in SCS may have reduced the contact of monkeys with diverse routes transmitting variable enterococci, including insect that comprised approximately 33% of the diet of these animals. Besides, the monkeys from SCS are feeding by human and have access to the garbage left by visitors on the park.

Contrasting with wild monkeys from SCS, the fecal samples from wild monkeys of SSC showed more dissimilar *Enterococcus* species, including *E. faecalis*, *E. hirae*, *E. faecium*, *E. durans*, *E. casseliflavus*, *E. raffinosus*, *E. avium*, and *E. gallinarum*. These monkeys live in a less urbanized forest fragment with a general diet, composed by insects, fruits, stems, flowers, and leaves, and consequently exposed to several *Enterococcus* species. In captive monkeys from ZOO, which are feeding with nonhuman dry food – composed by proteins, crude fiber and fat – supplemented with fruits and vegetables, the *E. hirae*, *E. faecium*, and *E. durans* were the most prevalent species. The presence of these *Enterococcus* species might be associated with the food source since enterococci were detected in the feed and feed ingredients samples as described by da Costa et al. (2007) and Ge et al. (2013).

Antibiotic-nonsusceptible enterococci species were found in captive and black capuchin monkeys. Similar studies, detected resistant bacteria in captive and wild animal from different environments (Xavier et al., 2010; Santos et al., 2013; Glover, 2014; Smith et al., 2014; Santestevan et al., 2015; Bondarczuk et al., 2016; Prichula et al., 2016; Furness et al., 2017; Bengtsson-Palme et al., 2018). In addition, samples from wild black capuchin monkeys from SSC presented a high number of antibiotic-nonsusceptible strains. The antibiotic-nonsusceptible strains isolated from wild monkeys are a matter of concern since these animals did not have a history of therapeutic antibiotic exposure. The analysis of resistant enterococci in these animals emphasizes the role of human activities on the environment. However, we cannot forget to mention that wild black capuchin monkeys from SSC live in a forest fragment near a public hospital and water treatment plant, and this proximity with these environments should represent a source of antibiotic-nonsusceptible strains in these animals. The presence of bacteria antibiotic-nonsusceptible and antibiotic resistance genes in hospital effluents has been observed and related to dissemination of resistance in the environment (Brown et al., 2006; Rodríguez-Mozaz et al., 2014; Xu et al., 2014). For example, tetracycline and erythromycin prescribed in human and animal medicine are excreted as active metabolites and remain stable in the environment (Rahardjo et al., 2011; Rudra et al., 2018; Schafhauser et al., 2018) to be considered modern pollutants in soils and aquatic environment (Gothwal and Shashidhar, 2014; Dizavandi et al., 2016). Another aspect to be considered is the antibiotic resistome (Wright, 2007; Stewart et al., 2014). Previous reports have noted the occurrence of resistant bacteria in soil independent of human activity (Allen et al., 2010). As such, we cannot exclude the possibility that the resistance found in monkeys is derived from the gut microbial communities. Tsukayama et al. (2018) showed that antibiotic resistance is an ancient feature of gut microbial communities of primate and that sharing habitats with humans may have an important impact on the structure and function of this microbiota.

In our study, 14% of the isolated strains were resistant at least to three or more drugs. The MDR enterococci species have been isolated from wild and captive animals (Nowakiewicz et al., 2014; Prichula et al., 2016). It is important to note that an elevate number of MDR *E. faecium* isolated from wild monkey that lives near to the hospital was detected. In the last years, the emergence of MDR bacteria has become a hospital-acquired infection problem and, a high number of MDR enterococcal infections are caused by *E. faecium* (Kristich et al., 2014).

The resistance-related genes commonly observed in this environment, *msr*C, *tet*(M), and *tet*(L) was detected in our samples. Those resistance genes were found in higher frequency in samples from captive monkeys when compared to wild monkeys. Perhaps, the captive condition of animals might be contributing to the acquisition/dispersion and persistence of these genes in this environmental. Up until now, only two studies have evaluated resistant genes in enterococci-resistant isolated from monkeys (Xavier et al., 2010; Woods et al., 2017). Our data demonstrated the *tet*(M) gene is widely distributed among our isolates followed by *tet*(L). Furthermore, when studying enterococci from wild marine animals, Prichula et al. (2016) identified a high prevalence (73.07%) of the *tet*(M) gene and a low prevalence (23.07%) of *tet*(L), which corroborates with the findings in the present study. Notably, Poeta et al. (2005) determined that the *tet*(M) gene is the more prevalent in enterococci from wild animals in Portugal, other than monkeys. Moreover, 50% of samples from Santestevan et al. (2015), isolated from wild sea lions presented *tet*(M) gene. Despite *erm*(B) gene is frequently observed in macrolide-resistant strains isolated from animals (Poeta et al., 2005; Cassenego et al., 2011), this gene was not detected in our samples. In addition, the *msr*C gene was detected at low frequency in wild monkeys. Additionally, Prichula et al. (2016) tested the *erm*(B) and *msr*C in enterococci strains isolated from wild marine animals and reported only the presence of the *msr*C. It is possible that other genes could be associated with erythromycin-nonsusceptible strains isolated from monkeys, like *erm*(A), *erm*(C), *erm*(D), *erm*(E), *erm*(F), *erm*(G), *erm*(Q), and the macrolide efflux pump (*msrA*).

In conclusion, the enterococci isolated in this study from monkeys living in three distinct areas, showed differences in the species, in the frequency of antibiotic-nonsusceptible and antibiotic resistance genes. These differences could be related to food web interactions, environmental pollutants, and/or antibiotic resistome. High frequency of MDR strains was observed in fecal samples of wild monkeys, which live in a forest fragment near a public hospital. The data presented in this study suggest that anthropogenic action might be affecting primate-gut enterococci community.

Finally, further research is necessary to better understand the evolution of resistance mechanisms presented by enterococci. Therefore, this study contributes in part to the comprehension of black capuchin monkey's microbiota, and to the elucidation of resistant bacterial strains and spread in wild and captive environments.

AUTHOR CONTRIBUTIONS

TG, JF, PW, and AF designed the study. RP and RS performed the MIC. TG, DZ, PW, and AA carried out the sampling work. TG, LC, JF, and AF analyzed the data and drafted the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02366/full#supplementary-material

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Pharmacokinetic/Pharmacodynamic Integration to Evaluate the Changes in Susceptibility of *Actinobacillus pleuropneumoniae* After Repeated Administration of Danofloxacin

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To evaluate the relationship between pharmacokinetic/pharmacodynamic (PK/PD) parameters and changes in susceptibility and resistance frequency of *Actinobacillus pleuropneumoniae* CVCC 259, a piglet tissue cage (TC) infection model was established. After *A. pleuropneumoniae* populations maintained at 10^8 CFU/mL in TCs, piglets were treated with various doses of danofloxacin once daily for 5 consecutive days by intramuscular injection. Both the concentrations of danofloxacin and the population of vial cells were determined. Changes in susceptibility and resistance frequency were monitored. Polymerase chain reaction (PCR) amplification of quinolone resistance-determining regions (QRDRs) and DNA sequencing were performed to identify point mutations in *gyrA*, *gyrB*, *parC*, and *parE* genes. Furthermore, the susceptibility of mutants to danofloxacin and enrofloxacin was determined in the presence or absence of reserpine to assess whether the mutants were caused by efflux pumps. The MICs and resistant frequency of *A. pleuropneumoniae* both increased when danofloxacin concentrations fluctuated between MIC_{99} (0.05 μg/mL) and MPC (mutant prevention concentration, 0.4 μg/mL). As for PK/PD parameters, the resistant mutants were selected and enriched when AUC_{24h}/MIC_{99} ranged from 34.68 to 148.65 h or AUC_{24h}/MPC ranged from 4.33 to 18.58 h. Substitutions of Ser-83→Tyr or Ser-83→Phe in *gyrA* and Lys-53→Glu in *parC* were observed. The susceptibility of mutants obtained via danofloxacin treatment at 1.25 and 2.5 mg/kg were less affected by reserpine. These results demonstrate that maintaining the value of AUC_{24h}/MPC above 18.58 h may produce a desirable antibacterial effect and protect against *A. pleuropneumoniae* resistance to danofloxacin.

Keywords: PK/PD, mutant frequency, danofloxacin, *Actinobacillus pleuropneumoniae*, tissue cage infection model

INTRODUCTION

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a severe respiratory disease that is a global problem in pig production. The acute form of this disease is highly contagious and often fatal, resulting in considerable economic losses to pig producers (Gutiérrez-Martín et al., 2006; Matter et al., 2007; Bossé et al., 2015). Historically, antibacterial therapy was a highly effective and common measure in controlling this disease. However, resistant mutants increased gradually due to the misuse of antibacterials. According to a recent report, the MIC frequency distribution of danofloxacin against *A. pleuropneumoniae* gradually increased during 2011–2015 in both the United States and Canada (Sweeney et al., 2017). Therefore, a rational antibiotic dosing regimen should be optimized, not only to eradicate bacterial infections but also to inhibit the emergence and proliferation of antibiotic-resistant strains (Toutain et al., 2002).

To design more rational dosage schedules, the antibacterial effect and pharmacokinetics of antibiotic should be considered integratedly (Aliabadi and Lees, 2000; Lees and Aliabadi, 2002). Therefore, the pharmacokinetic/pharmacodynamic (PK/PD) integration model has been commonly used as an alternative and preferred approach to dose titration studies for selection of rational dosage regimens (Toutain and Lees, 2004). To restrict selection of antibiotic-resistant mutants, various methods have been proposed. For PK/PD integration, the MIC- and MPC-related PK/PD parameters (MPC:MIC for the least susceptible single-step mutant subpopulation) have an important role in understanding the development of resistance (Firsov et al., 2003). Indeed, the relationship between PK/PD parameters and resistant mutants has been studied in several *in vitro* experiments (Firsov et al., 2003; Zinner et al., 2003, 2008; Liang et al., 2011). For *in vivo* experiments, a tissue cage (TC) infection model has been used as a feasible system in exploring the relationship between PK/PD parameters and antibacterial effects (Cui et al., 2006; Zhu et al., 2012; Zhang et al., 2014a; Xiong et al., 2016).

Danofloxacin is a third-generation quinolone with a broad-spectrum bactericidal activity and used solely in veterinary. The pharmacokinetics of danofloxacin has been investigated in several animals, such as sheep (Aliabadi et al., 2003b), goats (Aliabadi and Lees, 2001), calf (Sarasola et al., 2002), camel (Aliabadi et al., 2003a), and pigs (Richez et al., 1997). To design rational dosage regimen, the PK/PD integration model of danofloxacin against pathogenic microorgnism has been studied. A TC model was well applied to explore the antibacterial activity of danoflocaxin against bacteria, especially in ruminant. For example, one group (Aliabadi et al., 2003b) has studied the antibacterial activity of danoflocaxin against *Mannheimia haemolytica* in sheep biological fluids. After integrating the antibacterial effect and PK/PD parameters, the mean values of AUC/MIC to produce bacteriostasis, bactericidal activity, and elimination of bacteria were 17.8, 20.2, and 28.7 h for serum and 20.6, 25.5, and 41.6 h for exudate, respectively. Another study (Shojaee and Lees, 2003) focused on the PK/PD integration of danofloxacin against *M. haemolytica* 3575 in calf and the mean values of AUC/MIC to produce a bacteriostatic effect, inhibition of bacterial count by 50%, bactericidal effect, and elimination of bacteria were 15.9, 16.7, 18.15, and 33.5 h for serum and 15.0, 16.34, 17.8, and 30.7 h for transudate, respectively. In camel (Aliabadi et al., 2003a), the PK/PD modeling of danofloxacin against *Escherichia coli* 0157-H7 was developed in serum and TC fluids and the mean values of AUC_/MIC to produce a bacteriostatic activity, inhibition of bacterial count by 50%, bactericidal activity, and elimination of bacteria for serum were 17.20, 20.07, 21.24, and 68.37 h, respectively. A goat TC model (Aliabadi and Lees, 2001) has been used to estimate the antibacterial activity of danofloxacin against *M. haemolytica* and the mean values of AUC_{24}/MIC in serum to produce bacteriostasis, bactericidal effect, and elimination of bacteria were 22.6, 29.6, and 52.2 h, respectively. These studies provided abundant and original PK/PD data, which are of great significance for guiding the clinical medication of danofloxacin in animals. However, there is no paper about PK/PD integration of danofloxacin in pigs and there is also no report about correlation analysis between PK/PD parameters of danofloxacin and bacterial sensitivity changes. Therefore, PK/PD integration was developed to evaluate the changes in susceptibility of *A. pleuropneumoniae* after repeated administration of danofloxacin in pigs in this manuscript.

In the present study, a standard *A. pleuropneumoniae* CVCC 259 strain was exposed to various doses of danofloxacin in a piglet TC infection model at a population of 10^8 CFU/mL. The pharmacokinetics of danofloxacin and the changes in susceptibility and resistance frequency of *A. pleuropneumoniae* were examined. We then identified the mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* genes. Finally, the relationship between PK/PD parameters and changes in susceptibility and resistance frequency of *A. pleuropneumoniae* was analyzed. We aimed to demonstrate that this model could elucidate the relationship between emergence of resistant *A. pleuropneumoniae* and PK/PD parameters associated with danofloxacin.

MATERIALS AND METHODS

Bacterial Strain, Antibacterial Agents, and Chemicals

The *A. pleuropneumoniae* standard strain, CVCC259, was purchased from the Chinese Veterinary Culture Collection Center. Danofloxacin mesylate standard (>99%) and enrofloxacin standard (98%) were kindly supplied by Guangdong Dahuanong Animal Health Products. Pentobarbital sodium was purchased from Jian Yang Biotechnology Co., Ltd. Procainamide hydrochloride was supplied by Xin Zheng Co., Ltd., Tianjin Pharmaceutical Group. Tryptic Soy Broth (TSB) and Mueller–Hinton agar (MHA) were purchased from Guangdong Huankai Microbial Technology. Nicotinamide adenine dinucleotide (NAD, lot: 20160810) was purchased from MYM biological technology company limited (Beijing). Newborn bovine serum was provided by Guangzhou Ruite Biotechnology Ltd. Compound aminopyrine injection was purchased from Shandong Zhengmu Biological Pharmaceutical Co., Ltd.

Determination of MIC, MIC_{99}, and MPC

Actinobacillus pleuropneumoniae was grown in TSB or on MHA supplemented with 4% newborn bovine serum and 1% NAD at 1 mg/mL. The MIC was tested by an agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) reference methods (Watts, 2013). MIC_{99} and MPC were determined as previously described (Lu et al., 2003) with minor revision. Briefly, for MIC_{99}, bacterial cultures were grown for 8 h at a constant temperature of 37°C, at 180–200 rpm/min. Cultures were serially diluted and a 100 μL inoculum with a concentration of bacteria at approximately 10^6 CFU/mL was applied to agar plates containing various concentrations of danofloxacin. After incubation at 37°C, 5% CO_2 for 18–20 h, bacterial colonies were counted, and the fraction relative to the initial bacterial inoculum was calculated. The MIC is recorded as the lowest drug concentration preventing visible growth. The MIC_{99} is defined as the drug concentrations that inhibited growth of bacteria by 99%.

For MPC, approximately 10^{10} CFU *A. pleuropneumoniae* were inoculated on to multiple danofloxacin-containing agar plates. After incubation at 37°C for 72 h, plates were screened every 24 h. The lowest antibiotic concentration at which no colonies grew on an agar plate was defined as the preliminary MPC (MPC_{pr}). For exact MPC, the concentrations of danofloxacin in the agar decreased at a linear trajectory by 10%, which was based on MPC_{pr} approaching 1/2 MPC_{pr}. Then, we repeated the method for the MPC_{pr} test. The lowest antibiotic concentration at which no colonies grew on an agar plate was defined as the MPC.

Tissue Cage Infection Model

Healthy castrated crossbred piglets (Duroc × Landrace× Yorkshire), weighing 20–25 kg, were obtained from Guangzhou Fine Breed Swine Farm. They were housed in individual cages and fed antibiotic-free fodder (guangchubao premix feed for pig from the Guangzhou Zhongwang Feed Company) twice a day. Water was available *ad libitum*. All the experimental protocols were approved by the South China Agricultural University Committee on Animal Ethics (Approval number: 2017A008).

Tissue cages were made using food grade silicone tubes and the size of the TCs were the same as those described previously (Zhang et al., 2014b). Implantation surgery was performed under deep general anesthesia induced by pentobarbital sodium and local anesthesia by the injection of procainamide hydrochloride. Two TCs, sterilized with 75% ethyl alcohol and ultraviolet radiation, were implanted subcutaneously in each piglet. The TC position was perpendicular to the horizontal plane and one TC was placed on each side of the neck equidistant from the jugular vein and spinal cord. After surgery, the piglets received intramuscular (IM) injection of penicillin (1,000,000 IU/kg) to prevent infection. Animals were also treated with tetracycline ointment over the wound twice a day for 3 days. The non-steroidal anti-inflammatory drug (NSAID), aminopyrine, was simultaneously administrated by injection for post-operative analgesia. The animals were allowed to recover from surgery for 4–5 weeks to permit wound recovery and for the TC to fill with tissue cage fluid (TCF). After extraction of the TCF with disposable sterile syringes and bacteriological examination, sterile TCs were used for the study.

One milliliter of logarithmic growth phase bacterial suspension (approximately 10^{10} CFU/mL) was added to each TC. Two days after infection, 0.5 mL of TCF was extracted from each TC for bacterial enumeration. The TCFs containing a bacterial concentration exceeding 10^8 CFU/mL were used for the experiment.

Dosing Regimens and Pharmacokinetic Measurements

Sixteen piglets (eight females and eight males) were randomly allocated to one control group and seven study groups. The control group (two piglets and four TCs) was treated with 1 mL sterile physiological saline. The study groups were treated with danofloxacin at 0.4, 0.6, 0.8, 1.25, 2.5, 3.5, and 5 mg/kg (four TCs for each group) of body weight for 5 days, once daily by IM injection. TCFs (0.3 mL) were collected from the TC at 2, 4, 6, 8, 10, and 24 h after each administration. Samples were clarified by centrifugation at 3000 × *g* for 10 min and stored at −20°C avoiding light until analyzed within 2 weeks.

Danofloxacin concentrations in TCF were determined by high-performance liquid chromatography with fluorescence detection (HPLC-FD; Agilent Technologies, United States; Zhang et al., 2017). Briefly, after thawing, each sample (200 μL) including the blank sample was added to the same volume of acetonitrile for deproteinization, and was then clarified by centrifugation at 12,000 × *g* for 10 min. Two-hundred microliters of clear supernatant and 800 μL water were mixed and then transferred to an HPLC vial. The HPLC was applied with an Agilent TC-C18 column (250 mm × 4.6 mm, 5 μm) and the mobile phase was triethylamine phosphate (pH 2.4): acetonitrile (19:81, v/v) with a flow rate of 0.8 mL/min. The injection volume was 20 μL. A calibration curve was determined using nine danofloxacin concentrations (0.001–0.5 μg/mL). The mean relative recovery (RR) of danofloxacin in TCF samples was 96.9 ± 9.83% (mean ± SD).

Pharmacokinetic parameters, including C_{max} (maximum concentration of drug in samples) and AUC_{24h} (the area under the concentration–time curve over 24 h), were calculated by the non-compartmental model using WinNonlin software (version 5.2, Pharsight Corporation, Mountain View, CA, United States).

Quantification of the Time-Kill Curves and Recovery Curves of Resistant Mutants

Multiple TCFs (0.5 mL) were collected from the TCs before, during, and after the treatment (after every administration) at 24 and 48 h after the termination of treatment. To quantify the numbers of surviving bacteria and resistant mutants, each sample was serially diluted with sterile saline and 20 μL was inoculated in triplicate on to drug-free MHA or MHA containing 1 × MIC of danofloxacin. After incubation 18–20 h, the resultant bacterial colonies were counted. The detection limit was 50 CFU/mL. The time-kill curves were depicted as the number of bacteria on drug-free MHA, while the recovery curves of resistant mutants were

drawn as the populations grown on MHA containing 1 × MIC of danofloxacin.

Quantification of Changes in Susceptibility and Resistant Frequency

Loss of bacterial susceptibility in TCF was examined at before danofloxacin administration, during the treatment (after every administration), 24 and 48 h after the termination of treatment. The stability of mutants was determined by consecutive passage of *A. pleuropneumoniae* on to drug-free MHA every 24 h for 5 days. MICs were tested as described above. To evaluate the contribution of efflux, the susceptibility to both danofloxacin and enrofloxacin was then determined in the presence or absence of reserpine at 20 μg/mL.

To detect the resistant frequency of mutants, each sample was plated on to MHA containing 1 × MIC of danofloxacin (detection limit 50 CFU/mL). The definition of resistant frequency was expressed by the ratio of bacterial numbers counted in the presence of antibiotics to that in the absence of antibiotics.

Analysis of the Relationship Between PK/PD Parameters and Resistant Mutants

Pharmacokinetic/pharmacodynamic parameters such as AUC_{24h}/MIC_{99}, AUC_{24h}/MPC, %T > MIC_{99} (the percentage of the time that drug concentration remains above the MIC_{99}), %T > MPC (the percentage of time that drug concentration remains above the MPC), C_{max}/MIC_{99}, and C_{max}/MPC were calculated using WinNonlin program (version 5.2, Pharsight Corporation, Mountain View, CA, United States). Fisher's exact test was used for statistical analysis of the relationship between PK/PD indices and the changes in susceptible. Control group (two piglets and four TCs) were used as a control. $P < 0.05$ was considered to be statistically significant.

PCR Amplification of Quinolone Resistance-Determining Regions (QRDRs)

After passage for five generations, mutants with stable MIC were used for polymerase chain reaction (PCR) amplification. The nucleotide sequence of the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes were determined as previously described (Wang et al., 2010). The reagents used for PCR were purchased from Takara Bio, (Kusatsu, Japan). After amplification, the sequencing reaction was analyzed by Beijing Genomics Institute using Sanger sequencing.

RESULTS

MIC, MIC_{99}, and MPC of Danofloxacin Against *A. pleuropneumoniae*

The values of MIC, MIC_{99}, and MPC were 0.06, 0.05, and 0.4 μg/mL, respectively. All experiments were performed in triplicate on different occasions.

Antibacterial Effect and Recovery of Resistant Mutants

The time-kill curves are depicted in **Figure 1** and exhibit the antibacterial effect of danofloxcin against *A. pleuropneumoniae* CVCC259 in TCF after different doses were administered. For

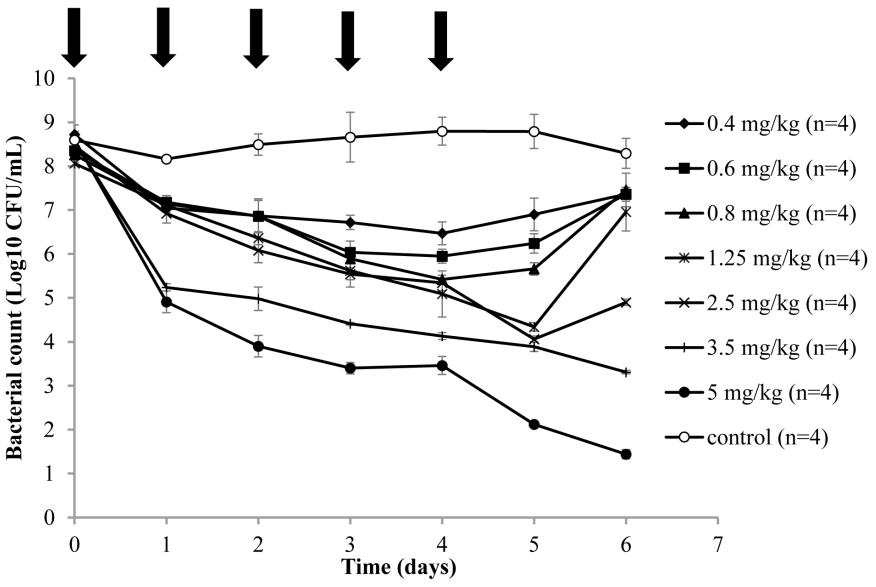

FIGURE 1 | Time-killing curves in the tissue cage fluid of danofloxacin treatment of *A. pleuropneumoniae* CVCC259. After an infection model was established, various doses (0.4, 0.6, 0.8, 1.25, 2.5, 3.5, and 5 mg/kg of body weight by IM) of danofloxacin and sterile physiological saline (control group) were administered intramuscularly once daily for 5 days (indicated by the arrow). *n* is the number of tissue cages per group.

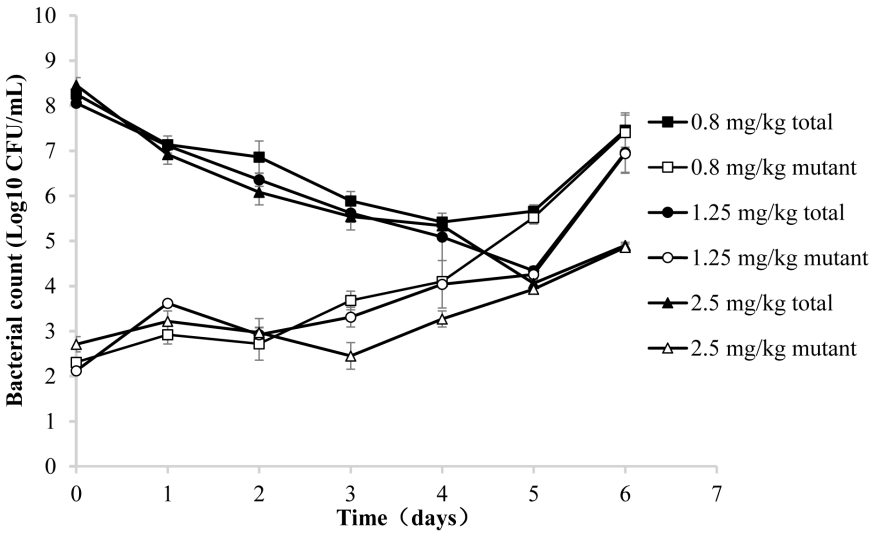

FIGURE 2 | Recovery of total and resistant bacteria after administration of danofloxacin at 0.8, 1.25, and 2.5 mg/kg. Concentrations of total bacteria and resistant mutants were determined in aliquots of tissue cage fluid obtained at the indicated time points after the initiation of treatment. Three representative examples are shown for piglets in which the danofloxacin concentrations were located between the MIC_{99} and MPC.

the control group, bacterial populations remained constant (approximately 10^8 CFU/mL). Compared to the control group, administration of danofloxacin at 0.4 mg/kg slightly decreased bacterial numbers. Bacterial numbers were reduced in response to the first 4 administrations of danofloxacin at 0.4, 0.6, and 0.8 mg/kg, although there was re-growth of bacteria after the last treatment. For the danofloxacin dosages at 1.25 and 2.5 mg/kg, bacterial numbers were obviously reduced after the five administrations, although there was re-growth at 48 h after the last administration. Administration of danofloxacin at 3.5 and 5 mg/kg caused bacterial numbers to reduce throughout treatment and they remained low during the growth recovery phase.

Three representative recovery curves are shown in **Figure 2** when the piglets were administrated danofloxacin at 0.8, 1.25, and 2.5 mg/mL. As a result, the danofloxacin concentrations were located between the MIC_{99} and MPC. Both the numbers of total and resistant bacteria are listed in **Figure 2**. The total bacterial populations reduced during treatment and then gradually increased. However, resistant bacteria numbers were initially constant or slightly reduced before amplification after several administrations. At last, the number of mutant and total bacteria were almost equal.

Pharmacokinetics of Danofloxacin

Danofloxacin concentrations collected at various time points during the treatment are depicted in **Figures 3A1–A7**. Determined by trapezoidal rules, the average values of AUC_{24h} ranged from 0.96 ± 0.34 to 18.94 ± 3.34 μg·h/mL. The average maximum concentration (C_{max}) ranged from 0.05 ± 0.01 to 1.13 ± 0.15 μg/mL. The detailed values for AUC_{24h} and C_{max} are listed in **Table 1**. The AUC_{24h} and C_{max} values in the TCF increased in a non-linear fashion with increasing doses and the correlation coefficients (R^2) were 0.95 and 0.91, respectively. After various dosages of danofloxacin were administered, the mean concentrations in the TCFs were ranged from MIC_{99} to MPC: almost completely below MIC_{99} (A1), across the MIC_{99} (A2), completely between MIC_{99} and MPC (A3–A5), across the MPC (A6), and above the MPC (A7).

Changes in Susceptibility and Resistant Frequency

Susceptibility of *A. pleuropneumoniae* in the TCFs was examined after administration with different doses of danofloxacin (**Figures 3B1–B7**). The MICs gradually increased (**Figures 3B2–B6**) when the drug concentrations were partially or completely located between MIC_{99} and MPC (**Figures 3A2–A6**). The significant increase in MICs (**Figures 3B4,B5**) were observed when the concentration of danofloxacin fluctuated between the MIC_{99} and MPC (**Figures 3A4,A5**). When danofloxacin concentrations were maintained either below the MIC_{99} (**Figure 3A1**) or above the MPC (**Figure 3A7**), MIC did not increase, either during or after treatment (**Figures 3B1,B7**).

The resistant frequencies are depicted in **Figures 3C1–C7**. Dramatic increases (>1000 fold; **Figures 3C2–C6**) were observed when the drug concentration located between MIC_{99} and MPC (**Figures 3A2–A6**). When drug concentrations were mostly below the MIC_{99} (**Figure 3A1**) or exceeded the MPC (**Figure 3A7**), the resistant frequencies slightly increased and then gradually decreased (**Figures 3C1,C7**).

Relationships Between PK/PD Parameters and Resistant Mutants

Pharmacokinetic/pharmacodynamic parameters provide an empirical way to relate antimicrobial dose to favorable treatment

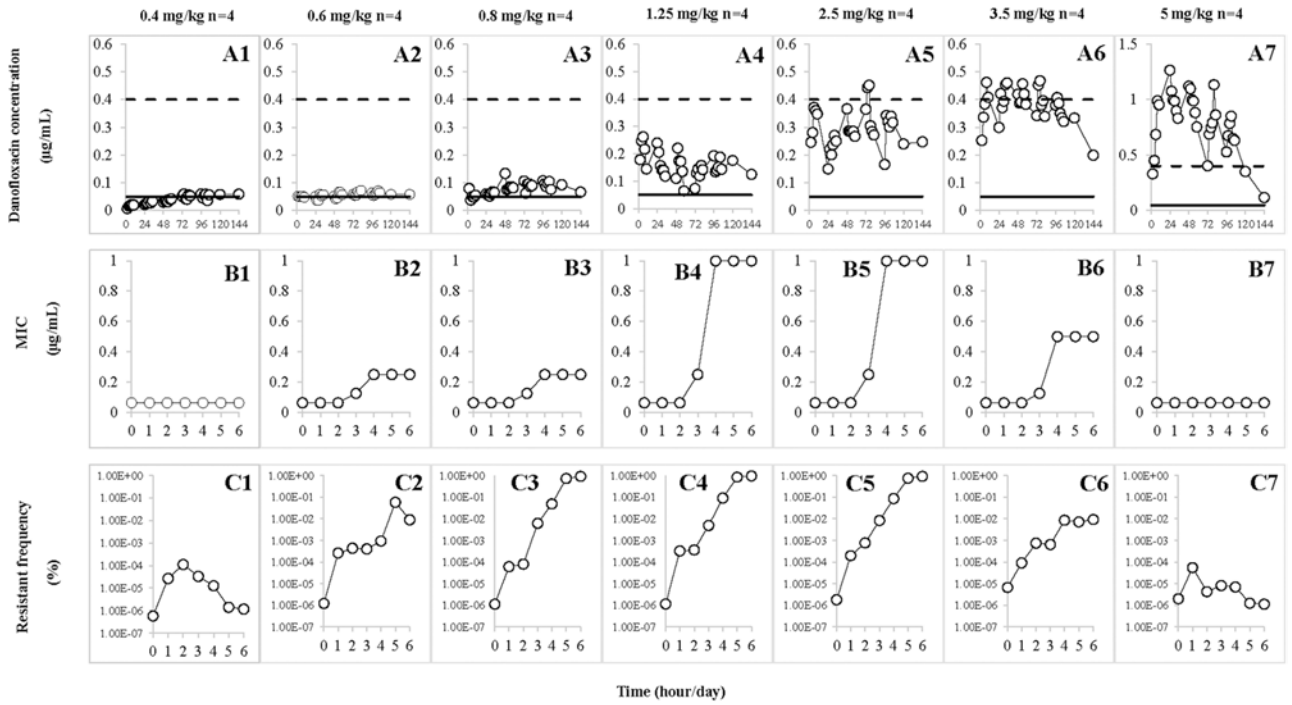

FIGURE 3 | Effect of danofloxacin concentration on loss of susceptibility and mutant enrichment. Concentrations of danofloxacin (0.4, 0.6, 0.8, 1.25, 2.5, 3.5, and 5 mg/kg) correspond to the different treatment groups once daily for 5 days. *n* is the number of tissue cages per group. **(A1–A7)** The concentration–time curves of different dosages of danofloxacin. **(B1–B7)** The values of MIC after each administration of danofloxacin, which corresponded to **A1–A7**. **(C1–C7)** The resistant frequency after each administration of danofloxacin, which corresponded to **A1–A7**. The dotted line represents MPC and the solid line represents MIC_{99} in **A1–A7**.

TABLE 1 | The pharmacokinetic parameters of danofloxacin following multiple doses in a piglet tissue-cage infection model.

Dosages (mg/kg)	AUC_{24h} (μg·h/mL)	C_{max} (μg/mL)
0.4	0.96 ± 0.34	0.05 ± 0.01
0.6	1.35 ± 0.15	0.06 ± 0.01
0.8	1.98 ± 0.35	0.11 ± 0.02
1.25	3.59 ± 0.69	0.22 ± 0.03
2.5	6.79 ± 0.35	0.38 ± 0.04
3.5	9.00 ± 0.73	0.45 ± 0.02
5	18.94 ± 3.34	1.13 ± 0.15

AUC_{24h}, 24 h area under concentration–time curve; C_{max}, maximum concentration. The AUC_{24h} and C_{max} were the mean values of five injections of danofloxacin at various dosages. Values are listed as mean ± SD.

effects associated with bactericidal agents (Mouton et al., 2005). The MIC_{99}- and MPC-related PK/PD parameters are listed in **Table 2**. Relationships between PK/PD indices, determined as steady-state values after the fifth dose, and changes in susceptibility are shown in **Table 3**. For fluoroquinolones, the AUC_{24h}/MIC_{99} index is most commonly associated with restriction of susceptible bacterial growth (Craig, 2001). Only two of eight TCs lost susceptibility when AUC_{24h}/MIC_{99} < 34.68 h (**Table 3** and **Figures 3A1,A2**). Loss of bacterial susceptibility occurred in 10 of 12 TCs when AUC_{24h}/MIC_{99} was between 34.68 and 148.65 h (**Table 3** and **Figures 3A3–A5**). Only one of eight TCs lost susceptibility when the AUC_{24h}/MIC_{99} exceeded 148.65 h (**Table 3** and **Figures 3A6,A7**). As for AUC_{24h}/MPC, mutant enrichment was observed, where 10 of 12 TCs lost susceptibility, when the AUC_{24h}/MPC was between 4.33 and 18.58 h (**Table 3** and **Figures 3A3–A5**). Only one of eight TCs occurred loss of susceptibility (**Table 3** and **Figures 3A6,A7**) when AUC_{24h}/MPC > 18.58 h.

Statistically significant correlations with selection of resistance for other PK/PD indices are also listed in **Table 3**. Mutants were selected by enrichment when the C_{max}/MIC_{99} values were between 1.09 and 8.42 or C_{max}/MPC values were between 0.14 and 1.05. Resistant bacteria were recovered from 12 of 20 TCs when the administration time of danofloxacin concentration was above the MPC for <29.63% of the dosing interval.

Characterization of the Contribution of Efflux and Gene Mutations in QRDRs

Mutants selected from danofloxacin dosages of 0.6, 0.8, and 3.5 mg/kg tended to be non-topoisomerase mutants that exhibited increased efflux. This was confirmed by adding an efflux inhibitor (reserpine), which could decrease the MIC for danofloxacin and enrofloxacin (**Table 4**). Mutants obtained from 1.25 and 2.5 mg/kg dosages were less affected by reserpine (**Table 4**). Mutations in the QRDR target genes are listed in **Table 4**. No mutant genes were observed in *gyrB* and *parE*. All mutants had a (Lys-53→Glu) substitution in *parC*. When

TABLE 2 | The PK/PD parameters of danofloxacin following multiple doses in a piglet tissue-cage infection model.

Dosages(mg/kg)	AUC_{24h}/MIC_{99}(h)	AUC_{24h}/MPC(h)	C_{max}/MIC_{99}	C_{max}/MPC	%T > MIC_{99}	%T > MPC
0.4	19.18 ± 6.73	0.91 ± 0.30	2.40 ± 0.84	0.11 ± 0.04	1.74 ± 3.77	0
0.6	26.95 ± 3.09	1.28 ± 0.15	3.37 ± 0.39	0.16 ± 0.02	72.23 ± 8.32	0
0.8	39.52 ± 7.02	2.25 ± 0.41	4.94 ± 0.88	0.28 ± 0.05	94.42 ± 11.16	0
1.25	71.79 ± 13.72	4.44 ± 0.53	8.97 ± 1.72	0.55 ± 0.07	100	0
2.5	135.87 ± 6.91	7.60 ± 0.75	16.98 ± 0.86	0.95 ± 0.09	100	15.96 ± 6.38
3.5	179.95 ± 14.53	9.02 ± 0.44	22.49 ± 1.82	1.13 ± 0.05	100	29.63 ± 25.61
5	378.81 ± 66.86	22.55 ± 3.01	47.35 ± 8.36	2.82 ± 0.38	100	98.01 ± 3.98

AUC_{24h}, 24-h area under concentration–time curve; C_{max}, maximum concentration; MIC_{99}, the minimum concentration that inhibits colony formation by 99%; MPC, antibacterial concentration that inhibits growth of the least susceptible single-step mutant subpopulation; %T > MIC_{99}, the percentage of time that drug concentration remained above MIC_{99}; %T > MPC, the percentage of time that drug concentration remained above MPC. All PK/PD parameters were calculated as the mean values of multiple doses. Values are listed as mean ± SD.

TABLE 3 | Correlation of PK/PD parameters with selection of resistance.

PK/PD index, value	Fraction of tissue cages with resistant bacteria (mutant/total)	*P*
AUC_{24h}/MIC_{99}(h)		
<34.678	2/8	0.424
34.68–148.65	10/12	0.008
>148.65	1/8	0.667
AUC_{24h}/MPC(h)		
<4.33	2/8	0.424
4.33–18.58	10/12	0.008
>18.58	1/8	0.667
C_{max}/MIC_{99}		
<1.09	0/4	NA
1.09–8.42	12/16	0.014
>8.42	1/8	0.667
C_{max}/MPC		
<0.14	0/4	NA
0.14–1.05	12/16	0.014
>1.05	1/8	0.67
T > MIC_{99}		
<17.15	2/8	0.424
>17.15	11/20	0.067
T > MPC		
<29.63	12/20	0.047
>29.63	1/8	0.667

All PK/PD parameters were determined using total drug concentrations from the tissue cage fluid. A total of 28 tissue cages were analyzed. P-values were calculated using Fisher's exact test, with a two infected but untreated piglets (four tissue cages) used as a control. NA, not applicable.

TABLE 4 | Quinolone susceptibility and identification of resistant mutants associated with different dosages of danofloxacin.

	MICs (μg/mL)				Mutations	
Dosages (mg/kg)	**Danofloxacin**	**Danofloxacin + reserpine**	**Enrofloxacin**	**Enrofloxacin + reserpine**	***gyrA***	***parC***
0	0.06	0.03	0.125	0.03	–	–
0.6 (*n* = 2)	0.25	0.125	0.5	0.125	–	K53E
0.8 (*n* = 3)	0.25	0.125	0.5	0.125	–	K53E
1.25 (*n* = 4)	1	1	2	1	S83Y	K53E
2.5 (*n* = 3)	1	1	2	1	S83Y	K53E
3.5 (*n* = 1)	0.5	0.5	1	0.25	S83F	K53E

–, No mutant was found; n, the number of tissue cages with mutant strains; gyrA, parC, the target genes of mutations in QRDRs. Reserpine concentration was at 20 μg/mL.

the dose was 0.6 and 0.8 mg/kg, no substitution was founded in *gyrA*. When the dose was 1.25 and 2.5 mg/kg, the mutants had a (Ser-83→Tyr) substitution in *gyrA*. When the dose was 3.5 mg/kg, the mutants had a (Ser-83→Phe) substitution in *gyrA*.

DISCUSSION

Danofloxacin is a synthetic fluoroquinolone that was developed solely for veterinary therapeutic purposes and shows a wide spectrum of bactericidal activity that includes Gram-negative and some Gram-positive bacteria, mycoplasma, and intracellular pathogens such as Brucella and Chlamydia species (Sappal et al., 1996; Sunderland et al., 2003; Rowan et al., 2004). However, with the abundant application of antibiotics, antibacterial resistance has emerged as a serious public health problem in both humans and animals. One of the main reasons for this phenomenon is the inappropriate dosage regimens (dose, dosage interval, duration of treatment, routes, and conditions of administration; Toutain et al., 2002). Even the commonly accepted treatment strategy of killing susceptible pathogens contributes to the problem by stimulating selective amplification of resistant mutants during treatment (Stratton, 2003). Therefore, rational antibiotic dosing regimens should be optimized, not only to eradicate the culpable pathogens but they also have an important role in inhibiting the emergence and proliferation of antibiotic-resistant strains (Toutain et al., 2002). Therefore, we considered an exploration of the relationship between the MIC- and MPC-related PK/PD parameters and emergence of resistant mutants as being important in elucidating this phenomenon.

In the present study, both the susceptibility and resistant frequency of *A. pleuropneumoniae* increased when the concentration of danofloxacin exceeded MIC_{99} and below MPC. Compared with the changes in susceptibility, the resistant frequency of mutants increased dramatically when the concentration partially or completely decreased between the MIC_{99} and MPC (**Figures 3A2–A6**) in the present study. This phenomenon was also observed by other researchers (Cui et al., 2006; Zhu et al., 2012; Zhang et al., 2014a; Xiong et al., 2016). We postulate two reasons to explain this phenomenon. One reason may be the amplification of pre-existing resistant bacteria (Blondeau, 2009). When drug concentrations were located between MIC_{99} and MPC, the total population size reduced and then gradually re-constituted after several administrations of danofloxacin. The resistant frequency of mutants significantly increased but the number of mutants changed only slightly. These data indicated that a frequency increase may result from preferential killing of susceptible bacteria. Amplification of mutants was observed after several treatments (**Figure 2**). Another reason that could explain the increase in resistance frequency may due to gene mutations that arise in bacteria (Zhang et al., 2014a). After several applications of treatment, the sequence of nucleotides may change and a new mutant can be generated (Cui et al., 2006; Zhu et al., 2012). Consequently, the total population of bacteria was almost equal to the mutant population.

To assess the clinical effects and their potential in the prevention of antibiotic resistance development, antimicrobial PK/PD parameters have been used (Leroy et al., 2012). For fluoroquinolones, AUC_{24h}/MIC can be applied commonly to predict favorable outcomes when susceptible populations are considered (Preston et al., 1998). And for MPC-related PK/PD indices, AUC_{24h}/MPC is an appropriate parameter because MPC is the MIC of the least susceptible single-step mutant (Zhao and Drlica, 2001). In the present study, we considered keeping the value of $AUC_{24h}/MPC > 18.58$ h as being a straightforward way to restrict the acquisition of resistance. The results fitted well with the conclusions of other researchers (Cui et al., 2006; Xiong et al., 2016). In an *in vivo* study, *Staphylococcus aureus* was treated with levofloxacin and AUC_{24h}/MPC was also proposed. In their study, $AUC_{24h}/MPC > 25$ h correlated with restricted growth of resistant mutant subpopulations (Cui et al., 2006). Another researcher studied the relationship between vancomycin and methicillin-resistant *S. aureus* (MRSA) *in vivo*. This group considered that resistant mutants were not enriched at a value of $AUC_{24h}/MPC > 15$ h (Zhu et al., 2012).

Other PK/PD parameters such as C_{max}/MIC_{99}, C_{max}/MPC, and T > MPC also exhibited a statistically significant correlation with resistance frequency. However, it is still not possible to accurately confirm the concentrations required to generate resistance in previously susceptible strains. For example, in an *in vitro* model, concentrations of antibiotics at the center between MIC_{99} and MPC were favorable in selecting a double mutant (Preston et al., 1998). In the present TC infection model, the concentration of danofloxacin required below MPC for 70.38% of the time to enrich mutants when those concentrations fluctuated above and below the MPC. However, the enrichment of mutants was observed when the concentration fluctuated above and below the MIC_{99} for only 17.15% of the interval time. One reason, which may explain this difference derives from more abundant pre-existing resistant mutant subpopulations being able to survive and expand near MIC99 (Zhou et al., 2000), while the mutants were killed when the drug concentration was near the MPC.

In Gram-negative bacteria, fluoroquinolone resistance occurs mainly by interplay of three mechanisms. This is realized by stepwise accumulation of mutations in the QRDRs of DNA gyrase and topoisomerase IV, active efflux of fluoroquinolones, and the presence of plasmid-borne resistance genes (qnr) protecting the target topoisomerase (Chu et al., 2005). In our experiment, the mutants had a (Ser-83→Tyr) or (Ser-83→Phe) substitution in *gyrA* and a (Lys-53→Glu) in *parC*. In a previous study (Wang et al., 2010), more mutant genes were found. They characterized the enrofloxacin-resistant *A. pleuropneumoniae* isolates and found seven different substitutions in GyrA (G75S, S83Y, S83F, S83V, D87Y, D87N, and D87H), four different substitutions in ParC (G83C, S85R, S85Y, and E89K), and five different substitutions in ParE (P440S, S459F, E461D, E461K, and D479E).

Although we successfully established a piglet TC infection model to evaluate the relationship between MIC- and MPC-based PK/PD parameters and the emergence of resistant mutants, there are some limitations to our study. First, because of the

limited number of piglets, the sample size of resistant mutants is not enough to generalize. Larger datasets should be considered in future research. Second, although a TC infection model was suitable for exploring the relationship between PK/PD indices and antibacterial effects, there are still obvious differences between TCF and clinically infected organs in animals. Therefore, for *A. pleuropneumoniae*, a lung infection model may be preferable for the study of PD and PK information in future studies.

CONCLUSION

We successfully established a piglet TC infection model and investigated the changes in susceptibility and mutant frequencies of *A. pleuropneumoniae* after different dosages of danofloxacin. After analyzing the relationship between MIC- and MPC-based PK/PD parameters and the emergence of resistant mutants, we suggest that danofloxacin concentrations should be maintained above the MPC or AUC_{24h}/MPC > 18.58 h, which could maintain effective antibacterial activity and minimize the emergence of resistant *A. pleuropneumoniae*.

AUTHOR CONTRIBUTIONS

LZ and HD contributed to the methodology, software, validation, formal analysis, data curation, writing (original draft preparation), writing (review and editing), visualization, and the project administration. LZ, ZH, ZK, and LY contributed to the investigation. LZ, QC, XS, and HD contributed to the resources. XG and HD contributed to the supervision. HD contributed to the funding acquisition.

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ant(6)-I Genes Encoding Aminoglycoside O-Nucleotidyltransferases Are Widely Spread Among Streptomycin Resistant Strains of *Campylobacter jejuni* and *Campylobacter coli*

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Thermotolerant *Campylobacter* species *C. jejuni* and *C. coli* are actually recognized as the major bacterial agent responsible for food-transmitted gastroenteritis. The most effective antimicrobials against *Campylobacter* are macrolides and some, but not all aminoglycosides. Among these, susceptibility to streptomycin is reduced by mutations in the ribosomal RPSL protein or by expression of ANT(6)-I aminoglycoside O-nucleotidyltransferases. The presence of streptomycin resistance genes was evaluated among streptomycin-resistant *Campylobacter* isolated from humans and animals by using PCR with degenerated primers devised to distinguish *ant(6)-Ia*, *ant(6)-Ib* and other *ant*-like genes. Genes encoding ANT(6)-I enzymes were found in all possible combinations with a major fraction of the isolates carrying a previously described *ant*-like gene, distantly related and belonging to the new *ant(6)-I* sub-family *ant(6)-Ie*. Among *Campylobacter* isolates, *ant(6)-Ie* was uniquely found functional in *C. coli*, as shown by gene transfer and phenotype expression in *Escherichia coli*, unlike detected coding sequences in *C. jejuni* that were truncated by an internal frame shift associated to RPSL mutations in streptomycin resistant strains. The genetic relationships of *C. coli* isolates with ANT(6)-Ie revealed one cluster of strains presented in bovine and humans, suggesting a circulation pathway of *Campylobacter* strains by consuming contaminated calf meat by bacteria expressing this streptomycin resistance element.

Keywords: *Campylobacter coli*, *Campylobacter jejuni*, streptomycin-resistance, aminoglycoside adenylyl transferases, ANT(6)-I

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INTRODUCTION

Campylobacteriosis is the main cause of foodborne diseases in the UE and in the United States [Collective Eurosurveillance Editorial Team, 2015; (Accessed March 2018)[1]]. The drugs of choice for the treatment of campylobacteriosis were, mainly erythromycin (ERY) and ciprofloxacin (CIP), although quinolones are no longer effective after a fast rise in resistance mechanisms among *Campylobacter* species (Carreira et al., 2012; Hormeño et al., 2016). Aminoglycosides, the third class of antimicrobials used worldwide after sulfonamides and beta-lactams, are a recommended alternative for the treatment of difficult infections caused by thermotolerant *Campylobacter* spp. (Wieczorek and Osek, 2013). The advantages of using aminoglycosides compared to other antimicrobials are their concentration-dependent bactericidal activity and relatively predictable pharmacokinetics, and synergism with other antibiotics (Vakulenko and Mobashery, 2003). Among aminoglycosides, the first active molecule used was streptomycin (STR), produced by *Streptomyces griseus*. STR binds to the aminoacyl-tRNA site (A site) of the 16S rRNA in the 30S ribosomal subunit, inducing codon misreading and inhibiting of translocation (Moazed and Noller, 1987; Woodcock et al., 1991) which leads to inadequate protein production. When antibiotic resistance appears it is due to target modification of ribosomal components, antimicrobial modification, or lowering drug accumulation in the cell (Vakulenko and Mobashery, 2003). Like in other bacteria, mutation K43R of S12 protein, a component of the 30S ribosomal subunit encoded by the *rpsL* gene, confers high-level of STR resistance in *Campylobacter* (Olkkola et al., 2010). Besides that, two out of four ANT(6)-I subfamily members of aminoglycoside nucleotidyltransferases (also known as aminoglycosides adenyltransferases of the AADE family), ANT(6)-Ia and ANT(6)-Ib, are frequently involved in STR resistance in *Campylobacter* strains and probably evolved from Gram-positive bacteria (Pinto-Alphandary et al., 1990; Shaw et al., 1993; Gibreel et al., 2004; Nirdnoy et al., 2005; Abril et al., 2010; Qin et al., 2012; Zhao et al., 2016). An additional role in STR resistance of ANT-like protein has been suggested in *C. coli* (Olkkola et al., 2016).

The aim of this work was to characterize the STR resistance presented in *Campylobacter* isolates of human and animal origin, establishing the role of a new enzyme of the ANT(6)-I family, ANT(6)-Ie, detected in a significant fraction of STR resistant isolates which molecular typing evidenced spread between animal and human hosts.

MATERIALS AND METHODS

Bacteria and Antimicrobial Resistance

Campylobacter spp. strains isolated from humans were previously described (Hormeño et al., 2016) and resulted from systematical screenings performed during 2010–2012 in fecal samples from gastroenteritis patients by the Microbiology services of three hospitals located in West-Center Spain: San Pedro de Alcántara, Cáceres; Campo Arañuelo, Cáceres; and Universitario de Salamanca, Salamanca. *Campylobacter* spp. isolated from bovine, fattening pigs and poultry were randomly selected in 2010–2012 from slaughterhouses located all around Spain by the Spanish Surveillance Network of Antimicrobial Resistance in Bacteria of Veterinary Origin (VAV Network; Moreno et al., 2000) and were partially described elsewhere (Florez-Cuadrado et al., 2016). From each farm, a single *Campylobacter* isolate was obtained by culturing pooled feces from animals (bovine and porcine) and cloacal or meat samples (poultry). Isolates were grown on blood agar, in a microaerophilic atmosphere (CampyGenTM, Thermo Scientific) at 42°C for 24–48 h and were identified by a Vitek-MS MALDI-TOF system (bioMérieux, Marcy-l'Etoile, France) to species level. The minimal inhibitory concentrations (MICs) for STR, ERY, gentamicin (GEN), CIP, and tetracycline (TET) were determined by agar dilution methods according to the guidelines of CLSI (Clinical and Laboratory Standards Institute [CLSI], 2010), including *Campylobacter jejuni* ATCC 33560 as the reference strain. Resistance was determined according to the EUCAST[2] (last accessed September of 2018), by using cut-off values [ecological cut-off value (ECOFF)] of 4 mg/L for STR, 4 mg/L (*C. jejuni*) or 8 mg/L (*C. coli*) for ERY, 2 mg/L for GEN, 0.5 mg/L for CIP, and 1 mg/L (*C. jejuni*) or 2 mg/L (*C. coli*) for TET. To test the presence of efflux pumps, MIC to STR were determined in the presence of the efflux pump inhibitor phenylalanine-arginine beta-naphthylamide (PaβN, Sigma) at a concentration of 20 mg/L.

Detection of Resistance Determinants

PCR was performed on DNA obtained by boiling, for 5 min, a suspension of one or two colonies from pure culture in 250 μL of milli-Q water, and recovering the supernatant after centrifugation at 10,000 × *g* for 10 min. PCR was carried out with 1 μl of DNA, 0.2 mM of each dNTP (Biotools, Madrid, Spain), 0.5 μM of each primer [Stab Service (University of Extremadura, Badajoz, Spain)], 0.025 U/μl of Taq Polymerase (Biotools, Madrid, Spain) and 1X PCR buffer containing 1.5 mM $MgCl_2$ (Biotools, Madrid, Spain), during 30 cycles of 94°C, 30 s; annealing temperature indicated in **Table 1**, 30 s; 72°C, 1 min. Amplicon purification was done with Speedtools PCR clean-up kit (Biotools, Madrid, Spain), following the manufacturer's instructions. DNA sequencing were performed by STAB Service (DNA Sequencing facilities of the Universidad de Extremadura, Spain). *In silico* data analysis was carried out with bioinformatics tools available in NCBI[3], SMS[4], and EBI[5].

Mutations in the STR resistance region of the *rpsL* gene were screened by sequencing of the PCR amplicon produced by primers and conditions previously described (**Table 1**; Olkkola et al., 2010). Similarly, the possible presence of *ant(3")-Ia* genes carried by Class-I integrons was evaluated by PCR with primers

[1] www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm

[2] www.eucast.org

[3] http://www.ncbi.nlm.nih.gov

[4] http://bioinformatics.org/sms

[5] http://www.ebi.ac.uk

TABLE 1 | Primers used in this work.

Name	Sequence (5′-3′)	T[1]	Bp[2]	Reference
RPSLF	CCAGCGCTTAAAAAT TGTCC	55	247	Olkkola et al., 2010
RPSLR	TATCAAGAGCACCA CGAACG			
INT1F	GGCTCTCGGGTAAC ATCAAGG	54	242	Leverstein-van Hall et al., 2002
INT1R	TCAGGAGATCGGAA GACCTC			
CSF	GGCATCCAAGCAGCAAG	56	VAR[3]	Lévesque et al., 1995
CSR	AAAAGCAGACTTGA CCTGA			
SAF	TGCAAAA(G/A)CC(G/C) GA(A/G)GATATGG	56	305	This work
SAR	TTCCTT(G/T)CG(G/A) CATA(G/T)CC(C/T)TT			
SBF	GATTGT(T/C)CG(T/C)CAT GAGCTGCT	57	327	This work
SBR	GTGCTATCCAGGCAGC CGGTT			
SCF	TGCCT(A/C)AAATTGG(G/A) T(G/A)AGTT	52	368	This work
SCR	ACCTAGCCA(A/G)ATTTCA AA(A/G)CCAAA			
STREJF	TGCAAAGCGAAAA AAGAAT	49	878	This work
STREJR	TTATAATTTTCTTAAAAT TTTGCAAT			
STRECF	TGCAAAATCAAGATAAAT TTTTAAAAC	51	899	This work
STRECR	TTACAATTTTCCTAAAAT TTTACAAT			
STREFF	GTATGCGCAAAAATGAT TAAAG	50	1110	This work
STREFR	AAGGAAAAATTTAAATAT TGGTTTCA			

[1]Annealing temperatures for PCR. [2]PCR-Product size in bp. [3]Variable size depending on gene-cassette structure (Lévesque et al., 1995).

specific to *intI* and *intI*-associated gene cassettes (**Table 1**). Three sets of degenerated primers were designed to amplify internal fragments of genes *ant(6)-I* (**Table 1**): *ant(6)-Ia* (primers SAF and SAR), *ant(6)-Ib* (primers SBF and SBR), and *ant(6)-Ie* (primers SEF and SER). Further analysis was performed to amplify the (almost) full coding sequences of *ant(6)-Ie* genes (**Table 1**) from *C. jejuni* (primers STREJF and STREJR) and *C. coli* (primers STRECF and STRECR). Oligonucleotide design was performed with Oligo v.6 software.

Functional Expression in *E. coli*

The expression of *ant(6)-Ie* from *C. coli* was tested through cloning the complete gene in the vector pGem-T Easy (Promega®), according to the manufacturer's instructions. The full length of the gene including its promoter sequence was amplified by using primers STREFF and STREFR (**Table 1**), designed from the genome sequence of *C. coli* Z163 (ZP_14079546.1) and assuming that σ^{70} *Campylobacter* promoters have a well-conserved −10 box and lack the −35 box presented in other bacteria (Petersen et al., 2003). The ligation mixture was electroporated in *Escherichia coli* XL1-Blue MRF' and transformants were selected in Luria-Bertani medium supplemented with 100 mg/L ampicillin.

Multilocus Sequence Typing of *Campylobacter* Isolates From Human and Animal Origin

A group of *Campylobacter* isolates was genotyped for *fla*A-SVR (short variable region of *fla*A gene) and multilocus sequence typing (MLST). PCR fragments of the housekeeping genes *asp*A (aspartase A), *gln*A (glutamine synthetase), *glt*A (citrate synthase), *gly*A (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase), and *unc*A (ATP synthase a subunit), as well as *fla*A gene (flagellin), were amplified and sequenced as described elsewhere (Ugarte-Ruiz et al., 2013). Allele numbers were assigned by sequence comparisons against the existing sequences deposited in the *Campylobacter* MLST database[6].

RESULTS

Streptomycin Resistance Phenotypes in Isolates From Human Origin

Based on the ECOFF defined by EUCAST for STR resistance of *Campylobacter* (MIC > 4 mg/L), 16 out of 141 human isolates are above the threshold (**Figure 1**). Among these it was possible to identify three different phenotypes: high-level resistance, shown by two *C. jejuni* strains (MIC > 512 mg/L), medium-level resistance, in two *C. jejuni* and five *C. coli* isolates (32 ≤ MIC ≤ 256 mg/L), and low-level resistance, with inhibition of growth immediately above ECOFF, detected in six *C. jejuni* and one *C. coli* (MIC = 8 mg/L). Treatment with the efflux pump inhibitor PAβN reduced MICs in all the isolates, with the exception of the highly resistant HSA40, with maximal susceptibility attained in two isolates from the medium-level resistance group plus in the seven isolates with the lowest resistance level (**Figure 1**). Among analyzed isolates, low susceptibility against clinically relevant antimicrobials was generally found to CIP and/or TET but not to ERY or GEN, although three low-level resistant strains to STR were also found near the cut-off for CIP and TET (HCC26, HCC27, and HCC34; **Figure 1**).

[6]http://pubmlst.org/campylobacter

Isolate	Species	MIC (mg/L)[1] STR	STR+[2]	ERY[3]	GEN[3]	CIP[3]	TET	RPSL[4]	*ant(6)-I*[5] *a*	*b*	*e*
HSA16	*C. coli*	8	0.03	1	1	16	32	WT			
HSA28	*C. coli*	128	16	8	2	32	256	WT			
HSA32	*C. jejuni*	>512	nd	2	2	32	128	K43R			
HSA40	*C. jejuni*	>512	>512	1	2	32	128	K43R			
HSA46	*C. coli*	64	16	2	2	0.25	256	WT			
HCC2	*C. coli*	128	16	1	2	64	128	WT			
HCC4	*C. jejuni*	32	0.03	1	16	64	>512	WT			
HCC26	*C. jejuni*	8	0.03	2	2	1	4	ND			
HCC27	*C. jejuni*	8	0.03	1	4	1	2	WT			
HCC30	*C. jejuni*	8	0.03	1	2	32	512	WT			
HCC32	*C. jejuni*	8	0.03	2	2	128	8	WT			
HCC34	*C. jejuni*	8	0.03	1	2	2	8	WT			
HCC40	*C. jejuni*	8	0.03	2	1	64	128	WT			
HCC56	*C. jejuni*	256	0.03	0.5	2	64	>512	WT			
HCC86	*C. coli*	128	32	2	2	128	128	WT			
HNA4	*C. coli*	32	8	2	2	2	256	WT			

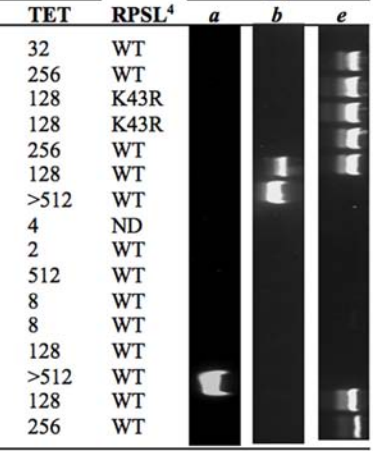

FIGURE 1 | Phenotypic and genotypic analysis of streptomycin (STR) resistant isolates. [1]Minimal inhibitory concentrations for STR, erythromycin (ERY), gentamicin (GEN), ciprofloxacin (CIP), and tetracycline (TET). [2]MIC were determined in the presence of PaβN (mg/L). [3]Data previously reported (Hormeño et al., 2016). [4]Mutations in the RPSL coding sequence were detected by sequencing (WT, no mutation). [5]Genes *ant(6)-I* were amplified with PCR with specific primers. ND, not determined.

rpsL Polymorphism Among Streptomycin Resistant Isolates

The *rpsL* gene region determining resistance to aminoglycosides (Olkkola et al., 2010) was amplified and sequenced in 15 *Campylobacter* isolates with MICs above STR ECOFF value (Accession Nos. LT605180, LT605181, LT605182, LT605184, LT605185, LT605186, LT605187, LT605190, LT605191, LT605192, LT605193, LT605194, LT605195, LT605196, and LT605197). Among 11 polymorphic positions detected, only one was expressed at protein level corresponding to mutation K43R (not shown). This occurred in two *C. jejuni* isolates, HSA32 and HSA40 (Accession Nos. LT605194 and LT605195), having both the high-level resistant phenotype (**Figure 1**).

The ANT(6)-I Family in *Campylobacter*

The NCBI database includes sequences for three members of the ANT(6) protein family previously described in *Campylobacter*: ANT(6)-Ia, ANT(6)-Ib, and ANT-like sequence cluster (Abril et al., 2010; Olkkola et al., 2016). The phylogenetic relationships previously defined within the ANT(6)-I family (Abril et al., 2010) were re-analyzed (**Figure 2**), including *C. jejuni* and *C. coli* for clusters ANT(6)-Ia and ANT(6)-Ib, plus the new and distantly related family member previously identified as ANT-like (Olkkola et al., 2016). Supported by bootstrapping with a threshold near 70%, ANT-like sequences cluster is a new member of the protein family that will be named hereafter ANT(6)-Ie (**Figure 2**), the fifth described ANT(6) (aminoglycoside 6-adenyltransferase) enzyme.

ANT(6)-I Detection in Streptomycin Resistant Isolates

The role of ANT(6)-I enzymes on STR resistance of *Campylobacter* was addressed by using specific primers designed to detect the coding sequences for ANT(6)-Ia, ANT(6)-Ib, and ANT(6)-Ie, including degenerated positions for efficient amplification of homologs of either *C. jejuni* or *C. coli* for every subfamily (**Table 1**). Among the 16 *Campylobacter* isolates resistant to STR detected in this work from human infections, nine were positive for the presence of *ant(6)-I* genes with two isolates positive for the subfamilies *ant(6)-Ia*, one for *ant(6)-Ib* and seven for *ant(6)-Ie* (**Figure 1**). The unique two *C. jejuni* isolates presenting *ant(6)-Ie* also have the RSPL polymorphism K43R and the high-resistance phenotype, whereas the six isolates with low-level of resistance did not carry any of the screened genes.

The nucleotide sequences of the seven *ant(6)-Ie* genes detected among human isolates, including the six *Campylobacter* strains presenting this gene as the unique aminoglycoside 6-adenyltransferase enzyme, revealed different functional roles on STR resistance depending on *Campylobacter* species. The *ant(6)-Ie* genes from the two *C. jejuni* isolates were found non-functional when compared with the reference used to define the protein subfamily (ZP_01070142, **Figure 2**), sharing both the unique polymorphism C-394-Δ (Accession No. LT605198, isolate HSA32), an out of frame deletion that produces the premature arrest of translation and the loss of 55% of protein sequence from its C-terminal end. In contrast, the four *ant(6)-Ie* genes from *C. coli* strains HCC2, HSA28, HSA86, and HCC46 presented identical sequences to ZP_14079546.1, whereas the polymorphism C466T originating variant P156S in the encoded protein was detected in the gene from HNA4 isolate (Accession No. LT605200).

Functional Expression in *E. coli* of ANT(6)-Ie

The coding sequence for ANT(6)-Ie from HNA4 was amplified and cloned in pGEM-T vector and *E. coli* XL1 Blue (MRF')

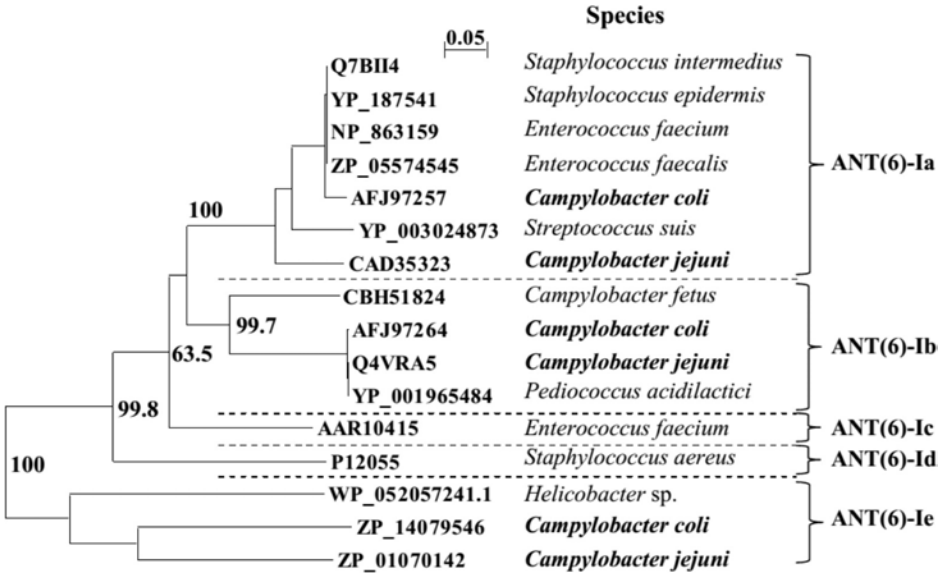

FIGURE 2 | The ANT(6)-I phylogenetic tree. Multiple sequence alignment was performed by Clustal X 2.1. The phylogenetic tree was deduced by neighbor joining algorithm, excluding positions with gaps and emulated by NJPlot 2.3. Bootstrap values (N, 100; seeds, 111) are indicated for branches supporting sequence clustering and assuming previous data (Abril et al., 2010).

cells. Cells carrying the recombinant vector expressed resistance to STR with a MIC of 64 mg/L, significantly higher than the control cells transformed with a non-recombinant vector (MIC = 8 mg/L). Besides, both recipient and transformants cells remained sensitive to other antimicrobials tested showing aminoglycoside specificity of the *ant(6)-Ie* gene: spectinomycin (MIC $\leq$ 32 mg/L), GEN (MIC $\leq$ 1 mg/L), apramycin (MIC $\leq$ 4 mg/L), and neomycin (MIC $\leq$ 4 mg/L).

Genetic and Phenotype Relationships Among Human and Animal Streptomycin Resistant Isolates Carrying ANT(6)-Ie

We screened for the three ANT(6)-I encoding genes in *Campylobacter* among 65 STR resistant isolates from the three most common food-producing livestock: poultry, pigs, and cattle (**Table 2**). All *ant(6)-I* genotypes were detected, with *C. coli* being largely the most prevalent species among streptomycin resistant isolates. Interestingly, the presence of the single-gene *ant(6)-Ie* genotype represents a major fraction of STR resistant *C. coli*, with one fourth of isolates.

Multilocus sequence typing plus *flaA* typing was performed in 14 *C. coli* isolates carrying *ant(6)-Ie* as the only determinant expressing STR resistance (**Table 3**). The multilocus analysis allowed the detection of a cluster of strains (ST-827, clonal complex 828) including two isolates from human origin plus one from bovine. Moreover, one of the human and the bovine origin isolates shared the same *flaA* allele 236 and the same resistance profile against the five clinically relevant antimicrobials tested, which is considered an indication of a probable common clonal origin.

DISCUSSION

This work shows the main role of adenylyl transferases belonging to the ANT(6)-I family on STR resistance in *Campylobacter*.

TABLE 2 | *ant(6)-I* genotypes of streptomycin resistant *Campylobacter* isolates.

Host	*ant(6)-I* profile[1]								
	a	b	e	a/b	a/e	b/e	a/b/e	Ø	Σ
Human[2]	1	1	6(4)	-	-	1(1)	-	7(1)	16 (6)
Poultry	13(8)	1(1)	-	10(9)	1(1)	-	2(1)	5(1)	32 (21)
Porcine	4(4)	-	10(10)	2(2)	7(7)	4(4)	1(1)	1(1)	29 (29)
Bovine	2(1)	-	1(1)	-	1(1)	-	-	-	4 (3)
Σ	20(13)	2(1)	17(15)	12(11)	9(9)	5(5)	3(2)	6(3)	81 (59)

*[1]Genotypes were deduced by PCR with primers SAF/R, SBF/R and SCF/R (**Table 1**). [2]Genotypes of human isolates are shown in **Figure 1**. Data in parentheses refer to number of isolates belonging to C. coli species. Ø, zero genes detected.*

TABLE 3 | Molecular and antimicrobial resistance typing of *Campylobacter* isolates carrying[1] *ant(6)-Ie*.

			MIC (mg/L)[2]							
Strain	**Year**	**Origin**	**STR**	**ERY**	**GEN**	**CIP**	**TET**	**CC[3]**	**ST[4]**	***flaA***
ZTA10/00526CPD	2010	Porcine	≥32	1	4	≥8	≥32	ST-828	7337	ND
ZTA10/00602CPD	2010	Porcine	≥32	≥64	4	≥8	≥32	ND	7340	ND
ZTA10/00794CPD	2010	Porcine	≥32	1	4	≥8	≥32	ST-828	829	ND
ZTA10/01257CPD	2010	Bovine	≥32	1	2	≥8	≥32	ST-828	827	0236
ZTA10/01418CPD	2010	Porcine	≥32	≥64	2	≥8	≥32	ST-828	1413	ND
ZTA10/02049CPD	2010	Porcine	≥32	2	2	≥8	≥32	ST-828	4950	ND
ZTA11/00514CP	2011	Porcine	≥32	≥64	2	≥8	≥32	ND	7341	0662
ZTA11/00726CP	2011	Porcine	≥32	1	4	0.13	≥32	ST-828	7338	ND
ZTA11/01342CP	2011	Porcine	≥32	≥64	4	0.25	≥32	ST-828	1413	ND
ZTA11/03282CP	2011	Porcine	≥32	0.5	1	≥8	≥32	ST-828	1096	0319
ZTA11/03389CP	2011	Porcine	≥32	≥64	2	≥8	≥32	ST-828	2733	ND
HSA028	2010	Human	128	8	2	32	256	ST-828	827	0236
HSA046	2010	Human	64	2	2	0.25	256	ST-828	827	0255
HNA4	2010	Human	32	2	2	2	256	ND	7339	0633

*[1]The fourteen C. coli isolates presenting ant(6)-Ie as the unique streptomycin (STR) resistance determinant (**Table 2**). [2]Minimal inhibitory concentrations for STR, erythromycin (ERY), gentamicin (GEN), ciprofloxacin (CIP), and tetracycline (TET). [3]Clonal Complex. [4]Sequence Types and flaA alleles were assigned by MLST database (see footnote 6). ND, not determined.*

Previous reports had described the phenotypic expression of ANT(6)-I enzymes (Nirdnoy et al., 2005; Abril et al., 2010; Qin et al., 2012; Olkkola et al., 2016), and now strong evidence is provided supporting the role of ANT(6)-Ie on STR resistance. Although ANT(6)-Ie coding sequences were detected in the two most frequent *Campylobacter* species, *C. jejuni* and *C. coli*, the association with STR resistance was only proved in *C. coli* since no *C. jejuni* isolate carried this coding sequence as the unique candidate to express the phenotype (**Figure 1** and **Table 2**).

Besides ANT(6)-I, an additional STR resistance determinant is ANT(3")-Ia or AADA which also confers resistance to spectinomycin. This enzyme is highly prevalent among enterobacteria (Shaw et al., 1993) and has been detected associated to class I integrons and their gene cassettes in *Campylobacter*, although only anecdotally (Ouellette et al., 1987; O'Halloran et al., 2004). Indeed, several reports have described the unsuccessful search of *ant(3")* in *Campylobacter* (van Essen-Zandbergen et al., 2007; Piccirillo et al., 2013). Similarly, all STR resistant isolates from humans analyzed in the present work have been screened for *int1* or associated gene cassettes, unsuccessfully (data not shown). Thus, ANT(6)-I enzymes might be the unique adenylyl transferases with significant relevance in STR resistance in *Campylobacter*.

To the best of our knowledge, this is the first report showing a RPSL mutation in *C. jejuni* isolates conferring STR resistance. In a previous study, with *C. coli*, it was found that isolates presenting high-level resistance to STR shared the mutation K43R in RPSL (Olkkola et al., 2010), similarly to the two *C. jejuni* isolates from humans, detected in this work, with MIC > 512 mg/L (**Figure 1**). Although both isolates also carry *ant(6)-Ie* genes, resistance to STR might be determined by RPSL mutation since the adenylyl transferase coding sequence is truncated and most probably not functional. In addition, there was no contribution to this phenotype from efflux pump activity, as deduced by the lack of any effect on MIC by PAβN treatment (**Figure 1**).

A group of six *C. jejuni* and one *C. coli* isolates from humans that expressed low-level STR-resistance, did not contain any of the screened determinants and presented a strong decreased MIC to STR in the presence of PAβN (**Figure 1**). Thus, efflux pump activity must be responsible for low-level STR resistance of these strains, similarly to *Mycobacterium tuberculosis* where the effect of outward transporters is known to increase modestly the MIC for STR (Spies et al., 2008). At least three different efflux pump systems have been shown to be up-regulated in *Campylobacter* strains resistant to a broad range of antimicrobials (Lin et al., 2005; Akiba et al., 2006; Jeon et al., 2011), so they could be candidates for determinants to the low level STR resistance. In addition, treatment with PAβN produced a strong effect on MIC of *Campylobacter* isolates carrying *ant(6)-I* genes, mostly for those with *ant(6)-Ia* or *ant(6)-Ib* as unique resistance determinants (**Figure 1**). This observation might indicate that, among human isolates analyzed in this work, the only functional adenylyl transferase gene is *ant(6)-Ie* and that even these isolates require efflux pump activity to support the medium-level of resistance. Treatment of *ant(6)-Ie* carrying strains with PAβN reduces their STR MIC to low-level resistance, which might correspond to their *in vivo* expression level. Synergic effects of efflux pumps have been evidenced in *Campylobacter* with resistance determinants for quinolones and macrolides, *gyr*A and 23S rRNA gene mutations, respectively (Luo et al., 2003; Cagliero et al., 2006; Corcoran et al., 2006). Indeed, three *Campylobacter* isolates showing low-level resistance to STR were also found to have low-level resistant to CIP and TET (**Figure 1**), lacked the *gyrA* C-257-T mutation conferring low susceptibility to fluoroquinolones

(Hormeño et al., 2016) and also *tetO,* the major TET resistant determinant in this species (not shown, authors' personal communication). A weak overexpression of efflux pump activity might be involved in the antimicrobial resistance phenotype of these strains.

The set of primers described in this work allows specific detection of the three *ant(6)-I* genes described in *Campylobacter*, including those belonging to *ant(6)-Ie* and encoding a new subfamily of aminoglycoside O-nucleotidyltransferases (**Figure 2**) that provides functional information for hundreds of orthologs annotated as hypothetical proteins, mainly from *Campylobacter* and related organisms like *Helicobacter*. In addition, the molecular and antimicrobial resistance typing of *Campylobacter* isolates expressing ANT(6)-Ie has revealed a spread pathway for this zoonotic pathogen between cattle and humans.

AUTHOR CONTRIBUTIONS

SP and AQ conceived and designed the study. LH, MU-R, GP, CB, and DF-C acquired the samples and data. LH, MU-R, GP, DF-C, and MC performed the laboratory analysis. SV, SP, LD, MC, and AQ analyzed and interpreted the data. MC and AQ wrote the manuscript. All authors have approved the final article.

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Reduction of Antibiotic Resistant Bacteria During Conventional and Advanced Wastewater Treatment, and the Disseminated Loads Released to the Environment

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The occurrence of new chemical and microbiological contaminants in the aquatic environment has become an issue of increasing environmental concern. Thus, wastewater treatment plants (WWTPs) play an important part in the distribution of so-called new emerging pathogens and antibiotic resistances. Therefore, the daily loads released by the WWTP were calculated including a model system for the distribution of these loads within the receiving water body. UV-, as well as ozone-treatment in separate or in combination for wastewater treatment were under investigation aiming at the reduction of these loads. Here, the impact of these treatments on the DNA integrity via antibody staining and PCR efficiencies experiments were included. All three facultative pathogenic bacteria [enterococci (*23S rRNA*), *Pseudomonas aeruginosa (ecfX*), and *Escherichia coli* (*yccT*)] and seven clinically relevant antibiotic resistance genes (ARGs) (*mecA* (methicillin resistance gene), *ctx-M32* (β- lactame resistance gene), *ermB* (erythromycine resistance gene), bla_{TEM} (β- lactame resistance gene), *sul1* (sulfonamide resistance gene), *vanA* (vancomycin resistance gene), and *intI1* (Integrase1 gene) associated with mobile genetic elements were detected in wastewaters. Different reduction efficiencies were analyzed during advanced wastewater treatments. ARGs were still found to be present in the effluents under the parameters of 1.0 g ozone per g dissolved organic carbon (DOC) and 400 J/m², like *ctx-M32, ermB,* bla_{TEM}, *sul1*, and *intI1.* Especially UV radiation induced thymidine dimerization which was analyzed via antibody mediated detection in the metagenome of the natural wastewater population. These specific DNA alterations were not observed during ozone treatment and combinations of UV/ozone treatment. The dimerization or potential other DNA alterations during UV treatment might be responsible for a decreased PCR efficiency of the 16S rRNA amplicons (176, 490, and 880 bp fragments) from natural metagenomes compared to the untreated sample. This impact on PCR efficiencies was also observed for the combination of ozone and UV treatment.

Keywords: antibiotic resistance, wastewater treatment, ozonation, UV irradiation, DNA damage, qPCR, modeling, daily discharge

INTRODUCTION

Municipal wastewater treatment plants (WWTPs) are already identified as sources of nutrients, inorganic and organic pollutants as well as antibiotic resistant bacteria (ARB) and resistance genes (ARGs) (Guo et al., 2013; Michael et al., 2013; Rizzo et al., 2013; Hembach et al., 2017). Some ARB can be removed through conventional wastewater treatment processes (Guardabassi et al., 2002; Da Costa et al., 2006), but there are still large numbers that survive in the effluent (Pruden et al., 2006; Hembach et al., 2017). As a consequence ARB and ARGs are released and widely distributed in the environment (Kim and Carlson, 2007; Czekalski et al., 2012; Alexander et al., 2015). The hygienic quality of receiving waters affected by WWTP effluents are of high relevance, especially by water reuse. For example, the European Urban Wastewater Treatment Directive (Directive, 1991) advised that "treated wastewater shall be reused whenever appropriate" under the requirement of "minimizing the adverse effect on the environment" which is defined as the protection of the environment from the adverse effects of wastewater discharges. It is important to determine the daily discharges of WWTPs which are released into the receiving waters when it's reused for crop irrigation or used as raw water reservoir. With the goal to interrupt dissemination pathways, advanced technologies have to be identified which are able to reduce the bacterial load and minimize the risk of WWTP effluents for subsequent water reuse or human health.

Therefore, several wastewater treatment options are discussed for their capability to reduce the ARB and ARG in the final effluent of WWTPs to achieve an adequate water quality (Norrby et al., 2009; WHO, 2014; Ventola, 2015). Still, a coherent assessment concept is missing to prove the success of reduction efficiency of microbial parameters. Since ozone is frequently used to remove chemical micro-pollutants (Lee and von Gunten, 2010; Ruel et al., 2011), and UV irradiation was reported to damage nucleic acids in bacterial cells (McKinney and Pruden, 2012) and reduce ARG abundances in wastewater (Munir et al., 2011; Hu et al., 2016), this study tightly focuses on the reduction of antibiotic resistant bacteria during conventional and advanced wastewater treatment. Ozonation is described to be an efficient process to remove organic micro-pollutants and also considered adequate to inactivate bacteria via production of highly reactive radicals (Hollender et al., 2009; Zimmermann et al., 2011; Dodd, 2012; Lüddeke et al., 2015; Zhuang et al., 2015). A previous study reported a selection of a robust bacterial population via ozonation, which is characterized by a high GC-content of their genomes (Alexander et al., 2016). Here, pseudomonads including *P. aeruginosa* containing GC-contents >60% (Lee et al., 2006; Hyatt et al., 2010) were identified as ozone robust. The germicidal effects of UV light is inducing alterations on DNA, RNA, and proteins by absorbing irradiation at the respective wavelength (absorption max. for DNA 260 nm, absorption min. 280 nm) (Jungfer et al., 2007; Süß et al., 2009). UV radiation is also known to accelerate horizontal gene transfer (HGT) (Aminov, 2011) by mobile genetic elements (MGEs), which is considered as the main factor driving resistome alteration in aquatic habitats (Chao et al., 2013). This advanced wastewater treatment technologies induce HRT due to the activation of different repair mechanisms involved in dissemination of ARGs. The present study shows the effect of ozone treatment (1 g ozone per g DOC), UV treatment (400 J/m²), and the combination (400 J/m² + 1 g ozone per g DOC) on facultative pathogenic bacteria and ARGs present in the wastewater of a large scale WWTP, as well as the impact of these advanced wastewater treatment technologies on the bacterial DNA integrity. Furthermore, we calculate the daily discharges of facultative pathogenic bacteria and antibiotic resistance genes into the adjacent receiving river and simulate different flow rate scenarios. Modeling approaches illustrate the dispersion of the different targets along the receiving river sides, which might be important for reuse approaches in downstream areas.

MATERIALS AND METHODS

Sampling

At a large scale WWTP (440,000 population equivalents; average sewage quantity 112,000 m^3/day) the inflow, conventionally treated wastewater and the final effluent, as well as advanced technologies using either an UV system apparatus (Collimated Beam Device) designed by the company with a mercury low pressure lamp (254 nm) (NLR2036) (Xylem Services GmbH, Herford, Germany), the ozone system type OCS-GSO30 by WEDECO or a combination of both techniques on conventionally treated wastewater were under investigation. According to the turbidity of the water sample the UV intensity was adjusted to 400 J/m². Ozone treatment was adjusted to 1 g ozone per 1 g DOC according to the dissolved organic carbon and a retention time of ∼5 min (flow rate ca. 7 m^3/h). This ozone concentration was specified by the operation company for further reduction of the organic trace substances of treated wastewater. Grab water samples were taken from the sampling points at four sampling campaigns (09/2016, 03/2017, 07/2017, and 10/2017). The wastewater samples were filtered by vacuum filtration on polycarbonate membranes (Ø 47 mm, pore size 0.2 µm, Whatman Nucleopore Track-Etched Membranes, Sigma-Aldrich, Munich, Germany) using 200 to 250 mL of the water samples. By using propidium mono azid (PMA, 25 µM) prior to DNA extraction according to Jäger et al. (2018), the evaluation of disinfection processes can be limited to viable cells with intact cell membranes and an overestimation by molecular biology methods can be avoided (Nocker et al., 2007a,b). A recent study revealed that PMA treatment in wastewater samples is a suitable tool to focus on the viable part of the population. In this study, the authors were focusing on the indicator bacteria *E. coli* and enterococci and showed no significant differences between the cultivation-based approaches and the PMA-qPCR experiments, but there were significant differences between the culture-based method and qPCR experiments without PMA treatment (Li et al., 2014; Jäger et al., 2018). Possible wastewater matrix effects on the PMA efficiencies should be controlled with internal standard experiments and the PMA concentrations should become adjusted to the wastewater characteristic of state. This was done previously for this study.

DNA Extraction for Quantitative PCR Analysis

DNA was extracted using the FastDNA™ Spin Kit for soil (MP Biomedicals, Illkirch, France). The membranes of the filtered wastewater samples were directly used for DNA extraction and were placed in the Lysing Matrix E tube for mechanical cell disruption. The further DNA extraction steps were performed following the manufacturer's protocol. The concentration of the extracted DNA was measured by using the Qubit™ 3.0 (Thermo Fisher Scientific, Nidderau, Germany).

Quantitative PCR Analysis

SYBR Green qPCR experiments were performed on the Bio-Rad Cycler CFX96 (CFX96 Touch™ Deep Well Real-Time PCR Detection System, Bio-Rad, Munich, Germany) and the analysis was done using the manufacturer's software (Bio-Rad CFX Manager Software). All samples were measured in technical duplicates by qPCR. The reaction mixture consisted of 1 µL template DNA, 1 µL Primer FW (10 µM), 1 µL Primer Rev (10 µM), 10 µL Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher scientific, Nidderau, Germany). Nuclease-free water (Ambion, Life technologies, Karlsbad, Germany) was added to adjust a total volume of 20 µL. The used thermocycler profile consisted of 1 cycle at 95°C for 10 min for DNA polymerase activation, followed by 40 cycles consisting of 95°C for 10 s, and 60°C for 30 s for primer annealing, and elongation. A melting curve, ranging from 60 to 95°C (0.5°C/s), was performed to confirm the specific amplicon.

Calibration curves were generated using extracted DNA from the different reference bacteria, i.e., facultative pathogenic bacteria carrying the respective resistance gene using the DNA extraction kit for soil (MP Biomedical, Illkrich, France). A regression line was made for each tested gene by using serial dilutions of the extracted DNA of the corresponding reference strain to calculate the gene specific cell equivalents (Hembach et al., 2017; Rocha et al., in press). The primer systems and the calculation of the cell equivalents were done based on the already known genome sizes of the retference bacteria and are listed in **Supplementary Information Table 1**. The PMA-treatment was performed prior to DNA extraction to consider the viable fraction of the wastewater sample (Jäger et al., 2018). The Ct–values from the wastewater samples were adjusted to the corresponding regression line and then normalized to 100 mL of filtered wastewater to show the different reduction efficiencies of absolute abundance within the surviving population of the wastewater samples.

Detection of DNA Damages via PCR

To analyze DNA damages, extracted DNA originating from the different sampling points were used in PCR experiments to distinguish the polymerase efficiency, as described by Süß et al. (2009). Therefore, different 16S rRNA amplicons (176, 490, and 880 bp) were investigated and afterwards separated by gel electrophoresis to distinguish the light units intensities via a F1 Lumi-Imager workstation (Roche Diagnostics) using the included Lumi-Imager software (LumiAnalyst 3.1). Afterwards the light units were determined and normalized to the control. Therefore, the amplicons were separated by a 2% w/v agarose gel electrophoresis and the light units of each amplicon were determined and normalized to their corresponding amplicon of the untreated wastewater sample so that the control results in a value of 1, and the other values represent the light units of the corresponding band in the agarosegel according to the control band. In each PCR reaction 2.5 µL Buffer (10x), 0.5 µL dNTPs (10 µM), 0.25 µL of each Primer (40 µM), 0.125 µL TaqPolymerase and 1 ng/µL template were used and the volume was adjusted to 25 µL by adding water. The thermoprofile consists of 3 min at 95°C followed by 25-times 95°C for 30 s, 56°C for 1 min, and 72°C for 2 min. The last step was an extended elongation step with 72°C for 7 min. Afterwards the samples were cooled down to 4°C.

Detection of DNA Damages via Immunological Assay

For the DNA damage analyses with antibodies samples were directly mixed with RNA protect to stop any further degradation of the DNA. As control sample untreated wastewater was used. For further processing the samples were spotted on a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) using a slot-blot apparatus (Slot-Blot R Microfiltration Apparatus, Bio-Rad, Munich, Germany) connected to a vacuum pump. Triplicates of each sample were tested using 200 µL per slot. Lysis of the bacterial cells was done directly on the nylon membrane by adding 500 µL of lysing and denaturation solution (1.5M NaCl, 0.5M NaOH, pH 13) and incubated for 20 min. This step was repeated three times. Afterwards the solution was removed by vacuum filtration followed by two neutralization steps with 500 µL neutralization solution [1.5M NaCl, 0.5M Tris/HCl (pH 7.2), 1 mM EDTA (pH 8.0)] Then a washing step with 300 µL TBS (0.5M Tris/HCl, 1.5M NaCl, pH 7.5) was performed. Afterwards the nylon membrane was removed from the apparatus and dried for 15 min on a clean filter paper. The immunoreaction was done in a hybridization tube continuously rotating starting with a blocking reaction with 5% non-fat milk solution at room temperature (RT) for 1 h. This was followed by the binding of the primary antibody (anti-CPD or anti-6–4 PP) 1:2,000 diluted in 5% non-fat milk solution for 30 min at 37°C. The incubation of the secondary antibody was performed at 37°C for 1 h. Two washing steps with TTBS (TBS + 1/100 Tween 20) were performed between the treatments. Afterwards two final washing steps with TBS were performed. In addition to the in the protocol mentioned antibodies anti-CPD or anti-6–4 PP (Cosmo Bio Co., Tokyo, Japan), which is based on Kraft et al. (2011), here, a different secondary antibody IgG-AP (Sigma-Aldrich, Munich, Germany) was used. Before developing the blot with the alkaline phosphatase reagent, the membrane was equilibrated with a detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) for 5 min at RT. The chemiluminescence detection (CSPD ready to use, DIC High Prime DNA labeling and detection Starter Kit II, Roche) was done at the F1 Lumi-Imager workstation (Roche Diagnostics) using the Lumi-Imager software (LumiAnalyst 3.1).

Calculation of Daily Charges of ARB and ARGs

For the calculation of the daily charges the annual mean discharge of the WWTP was used (1.165 m^3/s), according to

TABLE 1 | Daily load situation of a municipal wastewater treatment plant effluent.

		Bacterial concentration at the WWTP		Bacterial concentration within the river at different water levels		
		Daily discharges (24 h)	Discharge per second	Low water (Q22)	Mean water (Q124)	Flood water (HQ20)
				(22 m^3/s)	(124 m^3/s)	(994 m^3/s)
	Gene	[Cell equivalents/24 h]	[Cell equivalents/m^3]	[Cell equivalents/m^3]	[Cell equivalents/m^3]	[Cell equivalents/m^3]
Eubacteria	*16S rRNA*	1.49E+18	1.72E+13	7.84E+11	1.39E+11	1.73E+10
Enterococci	*23S rRNA*	1.40E+13	1.62E+08	7.36E+06	1.31E+06	1,63E+05
P. aeruginosa	*ecfX*	6.19E+10	7.16E+05	3.26E+04	5.78E+03	7.21E+02
E. coli	*yccT*	1.97E+13	2.28E+08	1.04E+07	1.84E+06	2.30E+05
Cefotaxime resistance gene	*ctx-M32*	2.49E+13	2.88E+08	1.31E+07	2.32E+06	2.89E+05
Erythromycine resistance gene	*ermB*	2.22E+14	2.57E+09	1.17E+08	2.08E+07	2.59E+06
β- Lactame resistance gene	bla_{TEM}	2.80E+14	3.24E+09	1.47E+08	2.61E+07	3.26E+06
Sulfonamide resistance gene	*sul1*	4.97E+15	5.76E+10	2.62E+09	4.64E+08	5.79E+07
Integrase 1 gene	*intI1*	1.54E+15	1.78E+10	8.10E+08	1.44E+08	1.79E+07

Shown are the calculated cell equivalents/24 h of the wastewater treatment effluent, as well as the calculated cell equivalents/m^3 at different water levels for the measured parameters.

the information by the operator of the WWTP. The obtained qPCR data given in cell equivalents per 100 mL were transformed to cell equivalents per m^3 and multiplied with 86400 s (24 h) (formula 1).

Formula 1: Calculation of the discharge of the WWTP within 24 h given in cell equivalents/ 24 h.

$$\frac{\text{cell equivalents}}{\text{m}^3} \times \text{annual mean discharge} \left[\frac{\text{m}^3}{\text{s}}\right] \times 24\ \text{h}\ [\text{s}] = \frac{\text{cell equivalents}}{24\ \text{h}}$$

$$\frac{\text{cell equivalents}}{\text{m}^3} \times 1.165\ \frac{\text{m}^3}{\text{s}} \times 86400\ \text{s} = \frac{\text{cell equivalents}}{24\ \text{h}}$$

For the calculation of the cell equivalents in the river regarding the dilution factor of different water levels, the formula 2 was used. For the river Danube low water is indicated by a flow rate of 22 m^3/s, mean water by 124 m^3/s, and flood water by 994 m^3/s.

Formula 2: Calculation of the concentration within the river system at different water level scenarios (low water, mean water, and flood water).

$$\left(\frac{\text{cell equivalents (effluent)}}{\text{m}^3} \times \text{annual mean discharge} \left[\frac{\text{m}^3}{\text{s}}\right]\right) \div \text{water level} \left[\frac{\text{m}^3}{\text{s}}\right] = \frac{\text{cell equivalents (river)}}{\text{m}^3}$$

$$\left(\frac{\text{cell equivalents (effluent)}}{\text{m}^3} \times 1.165\ \frac{\text{m}^3}{\text{s}}\right) \div 22\frac{\text{m}^3}{\text{s}} = \frac{\text{cell equivalents (river)}}{\text{m}^3}$$

Modeling of the Distribution Within the Receiving Body (River Danube)

A steady state and transient hydraulic 2D-water flow model (Hydrodynamic Wave Propagation Model HDWAM) originally developed by the Aquantec GmbH to assess and manage flood risks was used in this study. HDWAM is a one- and two-dimensional hydraulic model. A finite-volume discretization is applied to the diffusive wave equations and an implicit scheme is used for time integration (Krauter, 2002).

HDWAM is extended by a water quality module (GQSM) in order to simulate the dispersal of antibiotic resistance bacteria/ genes (ARB/G). The transport of quality parameters in 2D-compartments in the GQSM is described by the following partial differential equation (formula 3).

Formula 3: Partial differential equation describing the transport of quality parameters in 2D-compartments in the GQSM.

$$\frac{\partial hC_i}{\partial t} + \frac{\partial q_x C_i}{\partial x} - \frac{\partial}{\partial x}\left(hD_\tau \frac{\partial C_i}{\partial x}\right) + \frac{\partial q_y C_i}{\partial y} - \frac{\partial}{\partial y}\left(hD_\tau \frac{\partial C_i}{\partial y}\right) - \frac{1}{h}\sum_{j=1}^{nzu} q_{zu,j} C_{zu,j,i} + \frac{C_i}{h}\sum_{j=1}^{nab} q_{ab,j} = 0$$

h water depth [m]
q_x specific flow rate in x-direction [m^2/s]
q_y specific flow rate in y-direction [m^2/s]
C_i concentration of quality parameter i [mass/m^3, C°, …]
D_τ turbulent dispersion coefficient [m^2/s]
nzu number of external inflow by coupling
$q_{zu,j}$ external specific inflow j [m^2/s]
$C_{zu,j,i}$ concentration of quality parameters i in external inflow j [mass/m^3, C°, …]
nab number of external outflow by coupling
q_{ab} external specific outflow [m^2/s]

The turbulent viscosity can approximately be determined by the depth-averaged parabolic model (formula 4).

Formula 4: The depth-averaged parabolic model to determine the turbulent viscosity.

$$\mu_\tau = c_\mu \sqrt{g h I_E} h$$

g Gravitational constant [m/s²]
I_E Energy gradient [-]
c_μ Dimensionless coefficient for characterization of the riverbed [Natural riverbeds are characterized by c_μ between 0.3 (riverbed with low roughness) and 0.9 (riverbed with high roughness)].

The required finite element mesh (FE-mesh of the 2D-hydraulic model HydroAs-2D) for the part of the Danube River with the WWTP is placed at disposal by courtesy of the water authority Donauwörth (© Wasserwirtschaftsamt Donauwörth, www.wwa-don.bayern.de accessed on March 2018). The FE-mesh reaches from Danube-km 2,583 up to Danube-km 2,557. The FE-mesh was revised by Aquantec in order to make the mesh suitable for the program system HDWAM. A part of the FE-mesh was cut out, from Danube-km 2,581.43 (downstream the barrage Böfinger Halde) up to Danube-km 2,574.67 (downstream the barrage Leibi). The revised FE-mesh includes the floodplain which is flooded in case of a HQ_{20}. The part of the FE-mesh used for simulations with the program HDWAM consists of 20,039 knots and 29,742 elements.

The dispersal of different ARB and ARGs is simulated with the 2D-hydraulic approach of HDWAM for steady state scenarios ranging from low water level (gauge Neu-Ulm 22 m^3/s), medium water level (124 m^3/s) up to more or less an HQ_{20} (994 m^3/s) flood. Depending on the flow conditions the dispersal stays in the riverbed itself or extends to the floodplain.

Statistical Evaluation

Box plot graphs were chosen to illustrate the distribution of the measured values using the median values and the quartiles. Therefore, the median values of each sampling campaign were used, resulting in four median values. For the statistical analyses these values were used to calculate the different p-values to show significant differences between the treatments. In order to decide which statistical test should be used for determining the significance the data were first analyzed for their normal distribution using the Shapiro-Wilk test. In most of the cases the values for the different detected targets were normally distributed. Therefore, the t-test was applied to demonstrate the significance, which is also present with the illustrated figures. In some cases the data were not normally distributed and therefore the Mann-Whitney test was used to indicate significant differences between the samples.

RESULTS AND DISCUSSION

Conventional Wastewater Treatment and Its Impacts on Facultative Pathogenic Bacteria and ARGs

To determine the occurrence of facultative pathogenic bacteria and ARGs during the conventional wastewater treatment process at the WWPT volume based qPCR data were analyzed at three processing steps. Samples of the influent, activated sludge treatment in combination with sedimentation (biological treatment), and the final effluent were under investigation, firstly (**Figure 1A**).

The abundances of specific marker genes representing specifically facultative pathogenic bacteria and ARGs within the population were normalized to 100 mL wastewater volumes. The used primer sequences are listed in **Supplementary Information Table 1**. Quality controls were performed as described previously. The selection of the facultative pathogenic bacteria reflects their clinical relevance and their association with wastewaters. There is no regulation or guideline for the presence of such bacteria in municipal wastewaters in Germany, but for other European countries. The regulations of Spain, Cyprus, France, Greece, and Italy have selected *Escherichia coli* as a surrogate for facultative pathogenic bacteria, where also coliforms were studied previously in contaminated waters (Ashbolt et al., 2001). Nevertheless, it became obvious that some facultative pathogenic bacteria like *P. aeruginosa* released by WWTPs did not behave like indicator bacteria in susceptibility for oxidative treatment and regrowth capacities in downstream aquatic environments (Lüddeke et al., 2015; Alexander et al., 2016). Therefore, the following taxonomic marker genes [*16S rRNA* (*Eubacteria*), *23S rRNA* (enterococci), *ecfX* (*P. aeruginosa*), and *yccT* (*E. coli*)] were used for quantification via qPCR. In addition six ARGs (*mecA* (methicillin resistance gene), *ctx-M32* (β- lactame resistance gene), *ermB* (erythromycine resistance gene), bla_{TEM} (β- lactame resistance gene), *sul1* (sulfonamide resistance gene), *vanA* (vancomycin resistance gene), and *intI1* (Integrase1 gene) were used to quantify the load factor at the mentioned sampling points of the conventional WWTP. These antibiotic resistance genes were chosen due to their different occurrence in WWTPs (Hembach et al., 2017). The frequently found antibiotic genes (e.g., bla_{TEM}, *ermB*, *sul1*, and *intI1*) are suitable tools to show the reduction efficiencies of the different treatment steps. Furthermore, less frequently detected genes were included into the analysis to see if these genes will be effectively reduced during advanced treatments or if they will be still present after the treatments. These used gene targets are considered as suitable parameters for wastewater quality (Berendonk et al., 2015).

The results are illustrated in box plot graphics with medians, standard deviations, and minimum/maximum values of four sampling periods (**Figure 2**). Median values of the cell equivalents were used for the calculations of the reduction efficiencies. In all cases the measured cell equivalents per 100 mL were highest in the influent samples of the WWTP. A reduction due to the conventional treatment ranging from 1.1 to 3.4 orders of magnitudes (log units) can be observed for all of the tested taxonomic and resistance genes. In case of the taxonomic marker genes the highest reduction was measured for enterococci with 1.51×10^7 cell equivalents/100 mL in the inflow to 6.27×10^3 cell equivalents/100 mL after the conventional treatment (i.e., 3.4 log units reduction). The lowest reduction was observed for *P. aeruginosa*. Here, a reduction of only 2.2 logs, from 1.70×10^4 cell equivalents/100 mL to 9.89×10^1 cell equivalents/100 mL was analyzed. The abundance of *E. coli* was decreased from 1.88 ×

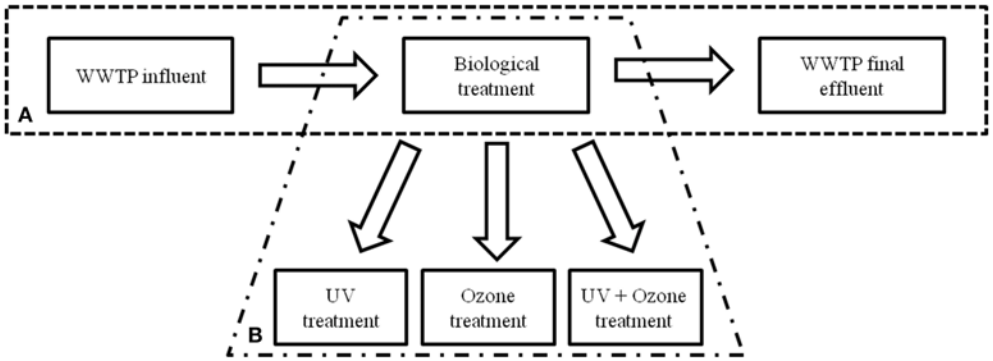

FIGURE 1 | Schematic illustration of the WWTP processes performed at the WWTP under investigation. **(A)** Conventional treatment with biological treatment (activated sludge and sedimentation tank) and **(B)** installation of semi-industrial advanced technologies.

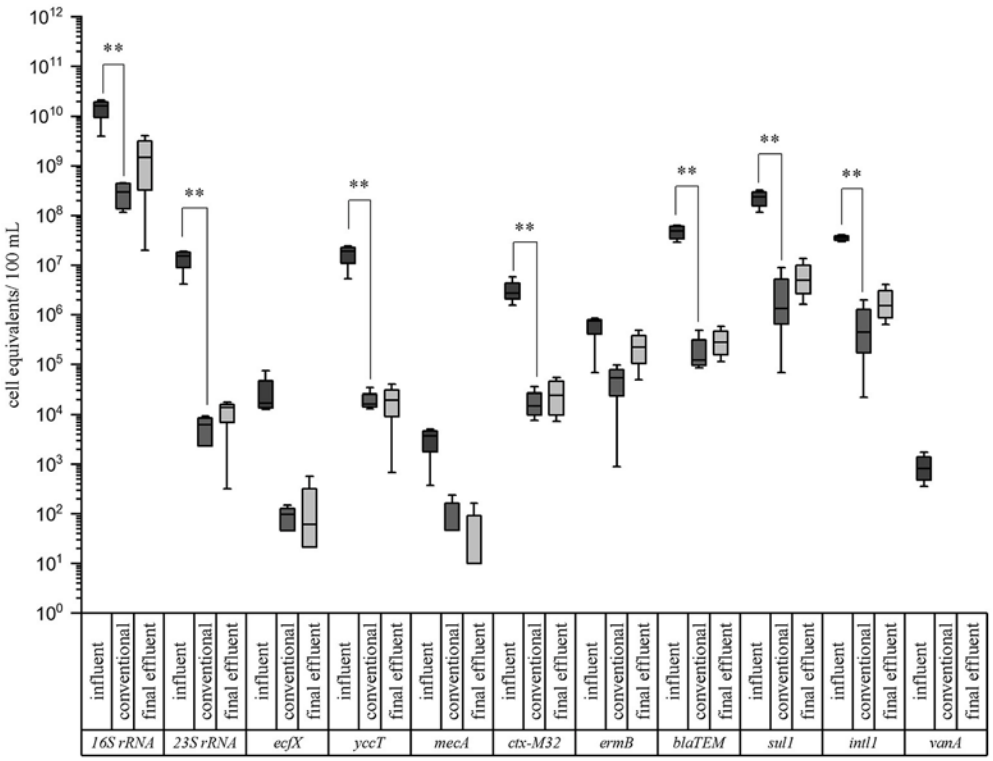

FIGURE 2 | Box plot graphs of the qPCR analyses targeting taxonomic and antibiotic resistance gene markers in wastewater samples of a municipal WWTP. Data are given for the influent, conventional (activated sludge with sedimentation), and effluent samples. Median values, standard deviations, and minimum/maximum values from 4 sampling periods are given. Significance is given by *t*-test calculation and is shown by asterisks (*t*-test; $^{**}p < 0.05$, $^{*}p < 0.1$).

10^7 to 1.64×10^4 cell equivalents/100 mL after the conventional treatment, resulting in a reduction of 3.1 logs. No significant differences occurred between the conventional treatment and the final effluent.

In case of the ARGs, the highest reduction was determined for ß-lactamase gene bla_{TEM} (2.6 log units) and vancomycin resistance gene *vanA* (2.9 log units; < LOD), which was not detectable after conventional treatment. More specifically, the β-lactame resistance gene (bla_{TEM}) was reduced from 4.82×10^7 cell equivalents/100 mL in the influent to 1.22×10^5 cell equivalents/100 mL after the conventional treatment. The *ctx-M32* and *sul1* resistance genes were reduced from 2.73×10^6 to 1.50×10^4 and from 2.35×10^8 to 1.33×10^6 cell equivalents/100 mL after conventional treatment, respectively. The lowest reduction showed *ermB* gene, coding for the erythromycin resistance, with 1.1 log units. Here, the abundance was decreased from 7.51×10^5 cell equivalents/100 mL in the influent to 5.37×10^4 cell equivalents/100 mL after the

conventional treatment. Significant differences between the influent and the conventional treatment (*t*-test; $^{**}p < 0.05$, $^{*}p < 0.1$) could be calculated for these mentioned genes showing no differences in their significance using the student's *t*-test or the Mann-Whitney test in case of not normally distributed data. Also no significant differences were observed between the conventional treatment and the final effluent. Furthermore, it became obvious that the *P. aeruginosa* gene marker (*ecfX*) and some antibiotic resistance genes *mecA*, and *ermB* were not significantly reduced by the biological treatment using the student's *t*-test. Using the Mann-Whitney test *ecfX* and *mecA* showed a significant reduction. The vancomycin resistance gene, directed against an antibiotic of last choice, was only detected in the influent samples. Nevertheless it became evident that the activated sludge with sedimentation didn't increases the abundances of facultative pathogenic bacteria as well as ARGs. Furthermore the abundances of the gene markers didn't changed significantly from the outflow of the biological treatment to the effluent sampling point. Comparing our data with a previous study of Czekalski et al. (2012), similar cell equivalents per 100 mL or gene copies were measured for the *16S rRNA* representing the total bacterial community and the *sul1* gene coding for the sulfonamide resistance. Other studies like Munir et al. (2011), and Alexander et al. (2015) revealed some differences in gene abundances. These differences may arise from several points, like regional differences, influences of industries and hospitals on the WWTP, as well as different wastewater treatment processes at the WWTPs.

Based on the collected qPCR data showing the presence of facultative pathogenic bacteria marker genes and ARGs in the final effluent of the WWTP (**Figure 2**), the cell equivalents per 100 mL were converted into cell equivalents per m^3. For the calculations of the daily charges via the WWTP effluent, these values were multiplied with the annual mean discharge of 1.165 m^3/s resulting in the amount of released cell equivalents per second and afterwards multiplied with 86400 s to obtain the amount of cell equivalents released within 24 h (**Table 1**). Furthermore, calculations regarding the dilution factor of different water level scenarios of the receiving river Danube were performed using the obtained cell equivalent per m^3 data and flow rates of the river for low, mean, and flood waters (**Table 1**). Furthermore, the calculation of the distribution and dilution within the receiving system allows estimating these risks of dissemination of facultative pathogenic bacteria and antibiotic resistances in downstream bulk water systems used for possible water reuse processes including drinking water conditioning. More specifically, the consideration of scenarios like flood water events are important where facultative pathogenic bacteria and ARGs may be discharged into floodplains and will be further spread into the environment.

Table 1 describes the 24 h discharges with the highest calculated values for *Eubacteria* as a marker gene for all bacteria followed by *E. coli* and enterococci in a similar range of 10^{13} orders of magnitude present in the WWTP effluent. *P. aeruginosa* was calculated with 2 orders of magnitudes less (10^{11} log units). In case of the ARGs the daily loads range from 10^{10} order of magnitudes for the methicillin resistance gene to 10^{15} log units for the sulfonamide resistance gene. The class-1 specific integron gene *intI1* representing a mobile genetic element for resistance genes was also found to be present in high abundances of 10^{15} log units. The vancomycin resistance gene (*vanA*) was not detected in the final effluent of the WWTP and is therefore not listed in **Table 1.** Within the river system dilution effects could be calculated. In case of low water events, a dilution effects up to 1.3 orders of magnitude could be calculated. For mean water, and flood water these dilution effects reached values of 2.1 and 3.0 log units, respectively.

With the help of the real quantification data from qPCR analyses and the load calculation equations (see chapter 2.6) the burden of one rivers system impacted by only one WWTP became visible. This calculation did not reflect the already present charges with facultative pathogenic bacteria and antibiotic resistance genes from upstream scenarios, where other entries from additional WWTPs or rain overflow basins at heavy rain seasons impacts the microbial quality of the river system. In consequences, the real burden with facultative pathogenic bacteria and ARGs are expected to be higher even at flood scenarios.

Impact of Advanced Wastewater Treatment Technologies on Facultative Pathogenic Bacteria and ARGs

Different advanced wastewater treatment technologies, i.e., UV irradiation, ozone treatment, and the combination of UV with ozone treatment on conventionally treated wastewater (after activated sludge with sedimentation) were under investigation (**Figure 1B**). Here, the same taxonomic and antibiotic resistance gene markers were used for qPCR analyses (**Supplementary Information Table 1**). The vancomycin resistance gene (*van*A) was not analyzed because of its absence after conventional treatment. The biological treated wastewater, i.e., activated sludge treatment followed by sedimentation, was used as reference value (control) for the different reduction efficiencies during the advanced wastewater treatments. In **Table 2** the median values calculated for the box plot graph (**Figure 3**) were used to determine the reduction efficiencies of the different treatment technologies.

In case of the taxonomic marker genes all three facultative pathogenic bacteria were detectable after conventional treated wastewater. The abundance of the viable fraction after PMA treatment ranged from 9.89×10^1 cell equivalents per 100 mL for *P. aeruginosa* (*ecf*X) to 1.50×10^4 cell equivalents per 100 mL for *E. coli* (*yccT*). The abundance of enterococci (enterococci specific 23S rRNA) and the overall bacterial load (16S rRNA) were determined with 6.27×10^3 cell equivalents per 100 mL and 2.94×10^8 cell equivalents per 100 mL, respectively (**Figure 3**). In case of the antibiotic resistance genes, the measured cell equivalents per 100 mL ranged from 1.33×10^6 cell equivalents per 100 mL for *sul1* to 1.50×10^4 cell equivalents per 100 mL for *ctx-M32*. The abundances of intI1, bla_{TEM} and *ermB* showed values of 4.42×10^5, 1.22×10^5, and 5.37×10^4 cell equivalents per 100 mL, respectively. The abundance of the methicillin resistance gene was determined with 4.70×10^1 cell equivalents per 100 mL. As

TABLE 2 | Reduction efficiencies of advanced wastewater treatment technologies on taxonomic and antibiotic resistance gene markers.

Target	Control	UV treatment		Ozone treatment		Combination	
	Absolute abundance	Absolute abundance	Reduction (–) Increase (+)	Absolute abundance	Reduction (–) Increase (+)	Absolute abundance	Reduction (–) Increase (+)
	[Cell equivalents/ 100 mL]	[Cell equivalents/ 100 mL]	[%]	[Cell equivalents/ 100 mL]	[%]	[Cell equivalents/ 100 mL]	[%]
16S	2.94E+08	9.04E+07	−69.3%	4.65E+06	−98.4%	5.47E+06	−98.1%
23S	6.27E+03	3.61E+03	−42.4%	1.91E+01	−99.7%	9.92E+01	−98.4%
ecfx	9.89E+01	7.50E+01	−24.1%	0.00E+00	<LOD	0.00E+00	<LOD
yccT	1.50E+04	1.09E+04	−27.4%	1.14E+02	−99.2%	1.57E+02	−99.0%
mecA	4.70E+01	0.00E+00	<LOD	0.00E+00	<LOD	0.00E+00	<LOD
ctxM32	1.50E+04	5.05E+04	236.3%	2.17E+03	−85.5%	2.38E+03	−84.1%
ermB	5.37E+04	3.75E+04	−30.2%	1.01E+03	−98.1%	1.07E+03	−98.0%
bla_{TEM}	1.22E+05	1.83E+05	50.1%	1.10E+04	−91.0%	1.12E+04	−90.8%
sul1	1.33E+06	9.33E+05	−29.9%	6.83E+04	−94.9%	5.53E+04	−95.8%
intl1	4.42E+05	2.43E+05	−44.9%	2.34E+04	−94.7%	4.61E+03	−99.0%

The abundances and reduction efficiencies of conventional treated wastewater (control), UV treated wastewater at 400 J/m² (UV treatment), ozone treated wastewater with 1 g ozone/g DOC (ozone treatment) and the combination of UV and ozone treatment (combination) are illustrated.

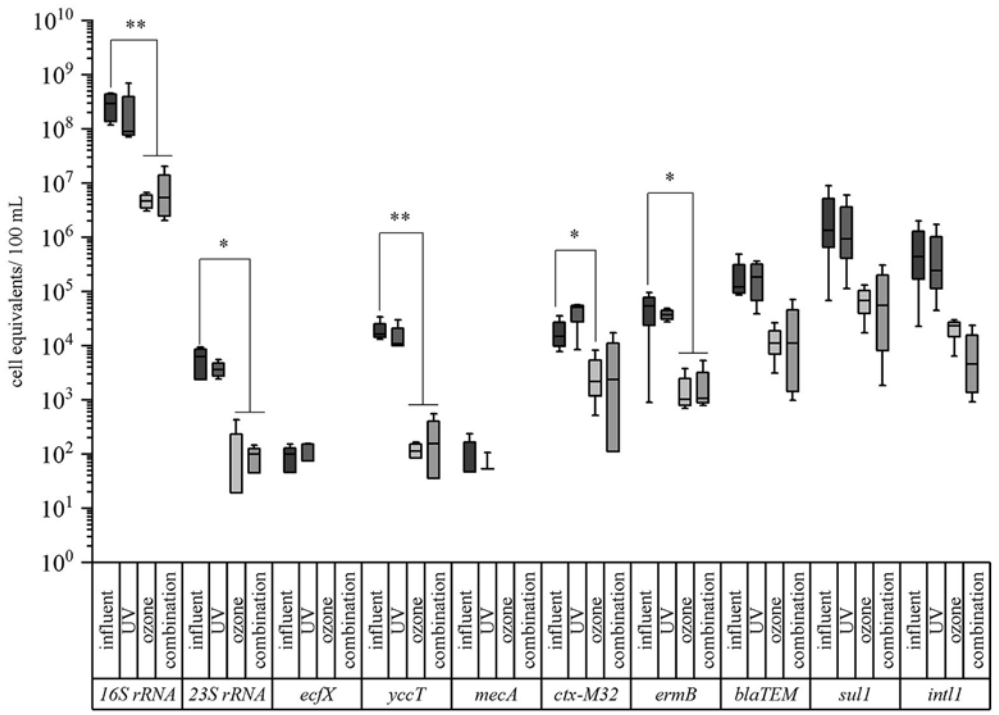

FIGURE 3 | Box plot graphs of the qPCR analyses targeting taxonomic and antibiotic resistance gene markers in advanced treated wastewater samples of a municipal WWTP. Data are given for the conventional treatment (activated sludge with sedimentation, influent), UV treated samples (400 J/m²), ozone treated samples (1 g ozone/ g DOC), and the combined treatment of UV and ozone (combination). Median values, standard deviations, and minimum/maximum values from 4 sampling periods are given. Significance is given by *t*-test calculation and is shown by asterisks (*t*-test; $^{**}p < 0.05$, $^{*}p < 0.1$).

reference for the determination of the reduction efficiencies of the different treatments the conventional treated wastewater was taken into consideration.

UV treatment resulted in a reduction of the abundance of all taxonomic marker genes ranging from 24.1, 27.4, 42.4, to 69.3% for *P. aeruginosa, E. coli,* enterococci, and 16S rRNA gene,

respectively (**Table 2**, **Figure 3**). Similar reduction efficiencies were detectable for *sul1*, *ermB*, and *intI1* showing reduction efficiencies of 29.9, 30.2, and 44.9%, respectively. The cell equivalents per 100 mL were reduced to 9.33×10^5, 3.75×10^4, and 2.43×10^5, respectively. In contrast the antibiotic resistance genes bla_{TEM} and *ctx-M32* showed an increase in their abundance after the UV treatment. No significant differences could be calculated neither with the student's *t*-test nor with the Mann-Whitney test between the influent samples and the UV treated samples.

UV treatment referring to wastewater treatment technologies seems not to be very effective. Also other studies report that reduction efficiencies could vary between 0.5 and 3.0 log units of gene copies/ 100 mL depending on the used fluences, as well as on the investigated resistance genes. It is reported that *tetA* and *ampC* genes are more resistant to UV treatment compared to *mecA* or *vanA* resistance genes (McKinney and Pruden, 2012). Furthermore, the complex wastewater matrix could influence the reduction efficiencies due to the high turbidity of the wastewater samples so that the UV light cannot interpenetrate the wastewater (Zhuang et al., 2015).

Ozone treatment resulted for all tested taxonomic marker genes in reduction efficiencies between 98.4% in case of the 16S rRNA gene to below the detection limit. *E. coli* and enterococci showed reductions of their abundance of 99.2% to 1.14×10^2 cell equivalents per 100 mL and of 99.7% to 1.91×10^1 cell equivalents per 100 mL. In case of *P. aeruginosa* with a relative low burden at the reference point (after biological treatment) qPCR measures were below the detection limit (**Table 2**, **Figure 3**). The ozone treatment showed for all tested antibiotic resistance genes reductions ranging from 85.5 to 98.1%. The methicillin resistance gene (*mecA*) wasn't detectable after the ozone treatment. The strongest reduction was measured for the erythromycin resistance gene (*ermB*) by 98.1% to 1.01×10^3 cell equivalents per 100 mL. The sulfonamide resistance gene (*sul1*) was reduced to 6.83×10^4 cell equivalents per 100 mL resulting in a reduction of 94.9% followed by the integrase 1 gene (*intI1*) with a reduction in percentage of 94.7%. The abundance of the ß-lactame resistance gene (bla_{TEM}) was reduced to 1.10×10^4 cell equivalents per 100 mL (reduction of 91%). The abundance of the cefotaxime resistance gene (*ctx-M32*) showed a reduction of its abundance to 2.17×10^3 cell equivalents per 100 mL (reduction of 85.5%). Significant differences between the influent and the ozone treated wastewater could be calculated with the student's *t*-test for all tested parameters except the enterococci specific marker gene (*23S rRNA*) gene and the erythromycin resistance gene (*ermB*). Here, the data were not normally distributed and the Mann-Whitney test was applied for statistical analysis.

The ozone treatment was able to reduce all the investigated antibiotic resistance genes. In contrast to the chemical micro-pollutants, which are discussed to become reduced to 80% during ozone treatment, microbiological hazardous contamination should be reduced to percentages of at least 99% to avoid any regrowth, afterwards. An advantage of the ozone treatment is it's applicability to microbiology reduction or elimination in parallel with the reduction or transformation of micro-pollutants. It has to be stated that the disinfection efficiency of ozone depends on the ozone concentration, the contact time, and water quality. Especially, dissolved organic carbon (DOC), suspended solids (SS), and particulate matter from activated sludge should be considered during ozonation (Lazarova, 2013; Czekalski et al., 2016; Pak et al., 2016). The used hydraulic retention time of the wastewater was arranged with 5 min. Both, ozone concentration and hydraulic retention time are parameters with could be adapted to increased elimination impacts on bacteria carrying antibiotic resistance genes. In this context unwanted chemical by-products like bromide should not become transformed by elevated ozone concentrations as previously mentioned (von Gunten and Hoigne, 1994; von Gunten, 2003; Lee and von Gunten, 2010).

In addition, the potential mutation of DNA after ozone exposure and toxic transformation products (e.g., bromate and nitrosamines) should be noted. Biological filtration with sand or activated charcoal is frequently recommended after ozonation to avoid the release of newly transformed unwanted compounds to the downstream environments. But, these filter systems bear the risk of microbial regrowth of facultative pathogenic bacteria or ARGs. Hence the ozone treatment should become adjusted to remove bacterial loads in sufficient high efficiencies.

The combination of UV and ozone treatment also revealed high percentages of reduction for all tested bacteria. The relative abundance of *E. coli* could be reduced from 1.50×10^4 cell equivalents per 100 mL to 1.57×10^2 cell equivalents per 100 mL and enterococci were reduced from 6.27×10^3 to 9.92×10^1 cell equivalents per 100 mL, resulting in 99.0 and 98.4% reduction of these bacteria within the surviving population. The eubacterial fraction (16S rRNA gene) was reduced by 98.1% and *P. aeruginosa* again was not detectable after the combined treatment (**Table 2**, **Figure 3**). Also the combination of UV and ozone treatment led to a reduction for all tested antibiotic resistance genes from 84.1% up to 99.0%. Here, the abundance of the integrase 1 gene (*intI1*) could be detected with 4.61×10^3 cell equivalents per 100 mL resulting in 99.0% reduction. The erythromycin resistance gene (*ermB*) was reduced to 1.07×10^3 cell equivalents per 100 mL (98.0% reduction) followed by the sulfonamide resistance gene (*sul1*), which was detected with an abundance of 5.53×10^4 cell equivalents per 100 mL resulting in 95.6% reduction. The ß-lactame resistance gene (bla_{TEM}) showed a reduction of 90.8% with a detectable abundance of 1.12×10^4 cell equivalents per 100 mL. The abundance of the cefotaxime resistance gene (*ctx-M32*) was detected with 2.38×10^3 cell equivalents per 100 mL resulting in a reduction of 84.1%. The methicillin resistance gene (*mecA*) wasn't detectable after the combined treatment.

Significant differences between the influent and the UV and ozone treated wastewater could be calculated with the student's *t*-test for all tested parameters except for the erythromycin resistance gene (*ermB*). Here, the data were not normally distributed and the Mann-Whitney test was applied for statistical analysis.

The combination of UV and ozone treatment under the given conditions didn't result in a more effective reduction compared to ozone treatment. This might be due to the particulate material which might be still present after the ozone treatment so that

the UV light was not able to interpenetrate the ozone treated wastewater. It would be possible that at further processing steps (e.g., after particle removal via filtration steps) the UV treatment might be a very suitable method to eliminate the residual contaminations. In consequence, adjustments to ozone treatment which achieve a high elimination rate of ARBs and ARGs should have high priority for the application in WWTPs. As mentioned before ozone contact times with an adapted hydraulic retention time at the ozone facility might a possible way to increase the elimination rates.

As previously described ozone treatment is based on radical ion production. Hence, ozone could also induce oxidative stress responses in surviving wastewater populations. It is known, that the impact of ozone given to wastewaters depends on many biotic and abiotic factors like bacteria densities, chemical load, and also suspended solids concentration. This implicates that sub-lethal effects on bacteria can occur promoting stress responses, population shifts, and bacterial selection processes. Dwyer et al. (2009) described the formation of reactive oxygen species (ROS) impacting the metabolism of bacteria. The triggered SOS response contributed to resistance development and the adaptation process would account for an increased robustness toward ROS of affected bacteria. Furthermore, the presence of anti-oxidative mechanisms in different species may lead also to different dynamics in the reduction efficiency of oxidative treatments (Dwyer et al., 2009; Alexander et al., 2016). The efficiencies of the different advanced treatment processes might also depend on the microorganisms carrying the mentioned antibiotic resistance genes. The presence of the genes are not limited to one specific bacterium, but can also be transferred to other so far uncharacterized bacteria from the wastewater population. Therefore, it's difficult to estimate the accessibility of disinfectants (ozone) or physical measurements (UV) on mixed communities in natural habitats. Most of the analyzed ARGs are located on mobile genetic elements described for horizontal gene transfer (HGT). Other studies have shown, that there is a secondary effect of bactericidal antibiotics besides their drug target-specific interaction within bacteria (Kohanski et al., 2007, 2010). There, sub-lethal concentrations of bactericidal antibiotics were used to stimulate the formation of intracellular, highly reactive hydroxyl radicals, which contribute to the killing efficiency of bactericidal antibiotics. The induction of oxidative stress by bactericidal antibiotics may induce sub-lethal stress response mechanisms in bacteria that deal not only with the adaptation to the original drug target (antibiotic resistance development), and oxidative damage-associated responses (e.g., *rec*A response). Bacteria which experienced these stress signals, responded, and survived. Therefore, they have a considerable advantage in surviving oxidative wastewater treatments (Alexander et al., 2016). In consequence, higher ozone concentration as proposed to increase the biocidal impacts during advanced wastewater treatment might a good strategy to avoid sub-lethal or selective side effects of ozone in certain bacteria of wastewater populations. Here, we focused on the absolute abundance of bacteria in 100 mL of wastewater. For visualizing changes of the relative abundance within the surviving population caused by these advanced wastewater treatments a normalization to 100 ng DNA would be possible and was shown in previous poplications of the group (Alexander et al., 2016; Jäger et al., 2018).

Influence of Advanced Wastewater Treatment Technologies on DNA Lesions

To investigate the occurrence of DNA lesions after the advanced treatments, different assays were performed. Here, antibody based detection systems against CPDs and 6-4 PPs DNA alterations, as well as PCR elongation experiments were performed (Süß et al., 2009; Kraft et al., 2011).

In case of the antibody based approach, the occurrence of cyclobutane pyrimidine dimers, as well as 6-4 photoproducts in the different treated wastewater samples was analyzed. Here, both DNA lesions could be detected in samples, which were treated with UV intensity of 400 J/m² but neither in the untreated, nor in the samples which were treated with ozone (**Figure 4**). Increasing the spotted volume of samples which were treated with ozone or a combination of UV and ozone did not result in a detectable signal (data not shown).

To complement the pyrimidine dimer analysis, PCR efficiency experiments with different sized 16S rRNA amplicons were performed according to Süß et al. (2009). In the first sampling campaign the 176 bp amplicon of the 16S rRNA gene showed a reduction of polymerase efficiency compared to the untreated control after UV treatment, whereas for the ozone treatment no PCR efficiency reduction was detectable (**Table 3**). The combination of UV and ozone treatment showed a small decrease in polymerase efficiency. In case of the 490 bp amplicon, polymerase efficiencies were decreased for all different treatment types. For the 880 bp amplicon the strongest reduction in polymerase efficiency could be detected after the UV treatment and after the combined treatment, whereas ozone didn't lead to a reduction in the PCR efficiency. These results underline the strong impact of UV irradiation on the DNA integrity of bacteria which might impact the mutation rates since 16S rDNA amplicons are representatives of the total bacterial genome. In consequence sub-lethal changes in the DNA integrity might be responsible for newly introduced mutations and might be responsible for bacteria evolution including antibiotic resistance.

The second sampling campaign resulted for the 176 bp amplicon in reduced efficiencies of 0.21, 0.11, and 0.1 for UV,

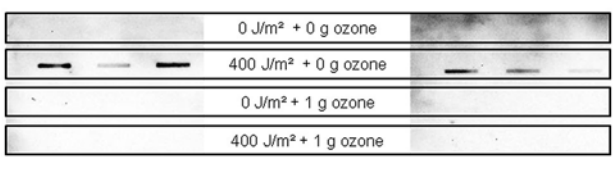

FIGURE 4 | Detection of cyclobutane pyrimidine dimers **(left)** and 6-4 photoproducts **(right)** at 400 J/m² UV and/or 1 g ozone per g DOC with an immunological slot-blot assay.

ozone, and the combined treatment, respectively. For the 490 bp amplicon no reduction in efficiency was detectable after UV treatment. After the ozonation and the combination of UV and ozone treatment a reduction of the polymerase efficiency was detectable (0.71 and 0.23). No effects could be seen for the 880 bp amplicon after UV or ozone treatment. Only the combination resulted in a weaker polymerase efficiency of 0.36 (**Table 3**). In consequence, these DNA lesions occur randomly within different regions of the genome. Therefore, there is some variability in the frequency of occurrence of these DNA lesions within the different amplicons, which has different effects on PCR efficiencies.

The PCR based experiments showed that DNA lesions are present after the combined treatment of UV and ozone, but there are no pyrimidine dimers detectable via the immunological assay. Also in the ozone treated samples no pyrimidine dimers were detected by the chemiluminescence measurements, whereas, DNA alterations were detectable in the PCR efficiency experiments. This might be an effect induced by the ozone reaction with the DNA molecule, which results in other types of DNA lesions compared to UV treatment. It is reported, that the kinetics of ozone molecules are higher for thymine (rate constant 3.4×10^4 L*mol^{-1} s^{-1}) than for guanine, cytosine, or adenine (Alexander et al., 2016) and that the thymine reacts with the ozone at the position of the methyl group at the C(5)-C(6) double bond, which has a noticeable effect on the rate of reaction (Flyunt, 2007). The oxidation at positions C(5) and C(6) may inhibit the dimer formation and therefore no CPDs and 6-4 PPs were detectable via the immunological assay.

These different degrees of DNA changes induced by UV-irradiation, as well as ozone-treatment especially at sub-lethal levels are known to trigger repair mechanisms in bacteria like *rec*A gene expression (Jungfer et al., 2007), which is a key regulator for recombination events and, therefore, can lead to an increased mutation rate and uptake/incorporation of extracellular DNA. This promotes the HGT, which is one of the main factor in resistome evolution in aquatic habitats (Fall et al., 2007; Aminov, 2011; Chao et al., 2013). Recombination events can also promote adaptation processes as well as the evolution of bacteria and ARGs. Again, elevated ozone concentration or adapted hydraulic retention times might help to suppress these unwanted side-effects in bacteria driving HGT or antibiotic resistance evolution.

Hydraulic Simulations of Dispersal of Several ARB and ARGs in the Danube Downstream of WWTP

For three bacteria and three resistance genes listed in **Table 4** 2D-hydraulic simulations with the Hydrodynamic Wave Propagation Model (HDWAM) have been conducted in order to determine the dispersal of the microbiological parameters. Simulations were done with steady state runoff in the river Danube of 22, 124, and 994 m^3/s.

As an example, the **Figures 5**, **6** show the concentration of *E. coli* at several knots of a cross section of the river Danube from 22 m to about 3,000 m downstream of the outlet of the WWTP. The simulated input from the WWTP is 1.165 m^3/s with a concentration of *E. coli* in the WWTP outlet of 9.20×10^8 cell equivalents/m^3. The runoff of the river Danube is simulated with steady state flow conditions of 22 m^3/s (**Figure 5**) and 994 m^3/s (**Figure 6**).

The runoff of 22 m^3/s stays in the riverbed itself. The maximum concentration of *E. coli* with a cell equivalent of $\sim 2.21 \times 10^8$ is calculated at 22 m downstream of the WWTP. According to the results of the hydraulic model after about 3,000 m downstream of the outlet of WWTP the concentration of *E. coli* is more or less evenly distributed across the river Danube with an average concentration of *E. coli* of about 4.63×10^7 cell equivalents/m^3.

At a runoff of 994 m^3/s the maximum concentration is about 5.22×10^6 cell equivalents/m^3 near the inflow point of the WWTP. The inflow point of the WWTP to the river is situated several meters from the right riverbank toward the left riverbank

TABLE 4 | Concentration of bacteria and resistance genes in the outlet of WWTP which were used as input for the simulation with the hydraulic program HDWAM.

Facultative pathogenic bacteria	**Outlet WWTP [cell equival./m^3]**
Escherichia coli	9.20E+08
Enterococcus spp.	6.00E+07
Pseudomonas aeruginosa	6.43E+06
Antibiotic resistance genes	
Sulfonamide resistance gene (*sul1*)	3.20E+10
β- Lactame resistance gene (bla$_{TEM}$)	3.83E+09
Erythromycine resistance gene (*ermB*)	2.88E+09

TABLE 3 | Detection of DNA damages via PCR experiments.

	Sampling campaign 1			**Sampling campaign 2**		
	176 bp amplicon	**490 bp amplicon**	**880 bp amplicon**	**176 bp amplicon**	**490 bp amplicon**	**880 bp amplicon**
Control	1.0	1.0	1.0	1.0	1.0	1.0
UV treatment	0.62	0.69	0.47	0.21	0.99	0.98
Ozone treatment	1.5	0.76	1.0	0.11	0.71	0.93
Combination	0.92	0.87	0.76	0.1	0.23	0.36

The quantified light units of the different treatments are normalized to the corresponding amplicon of the conventionally treated wastewater (control). The amplicons were separated by agarose gel electrophoresis and the light units (LU) of each amplicon were determined and normalized to their corresponding amplicon of the untreated wastewater sample.

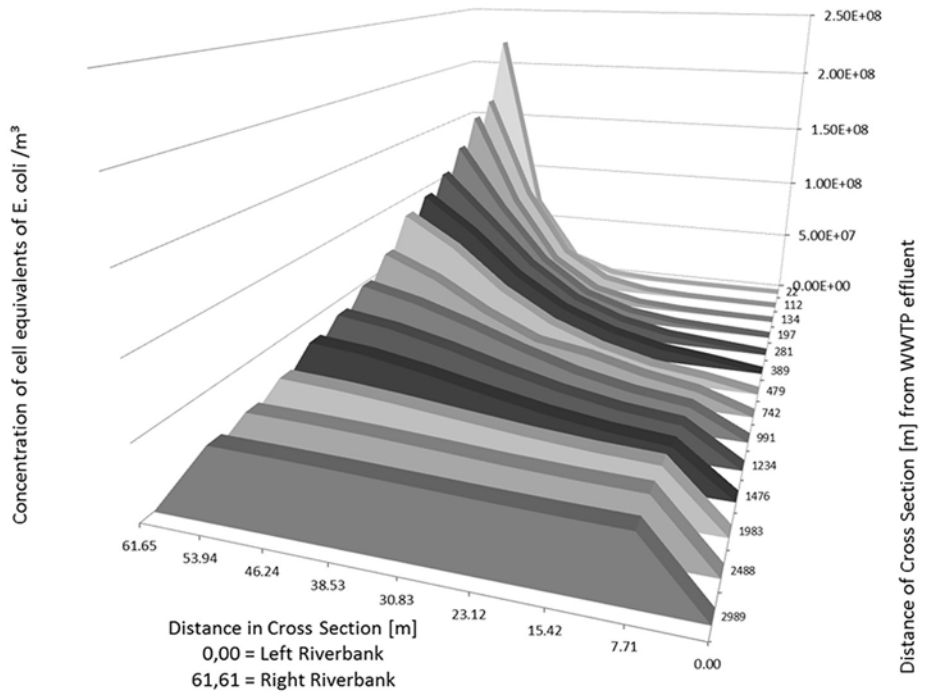

FIGURE 5 | Distribution of calculated (2D-HDWAM) concentration of *E. coli* in the river Danube downstream of the WWTP for different cross sections, 9.20×10^8 cell equivalents/m^3 in outlet of WWTP, discharge of Danube at 22 m^3/s (NQ).

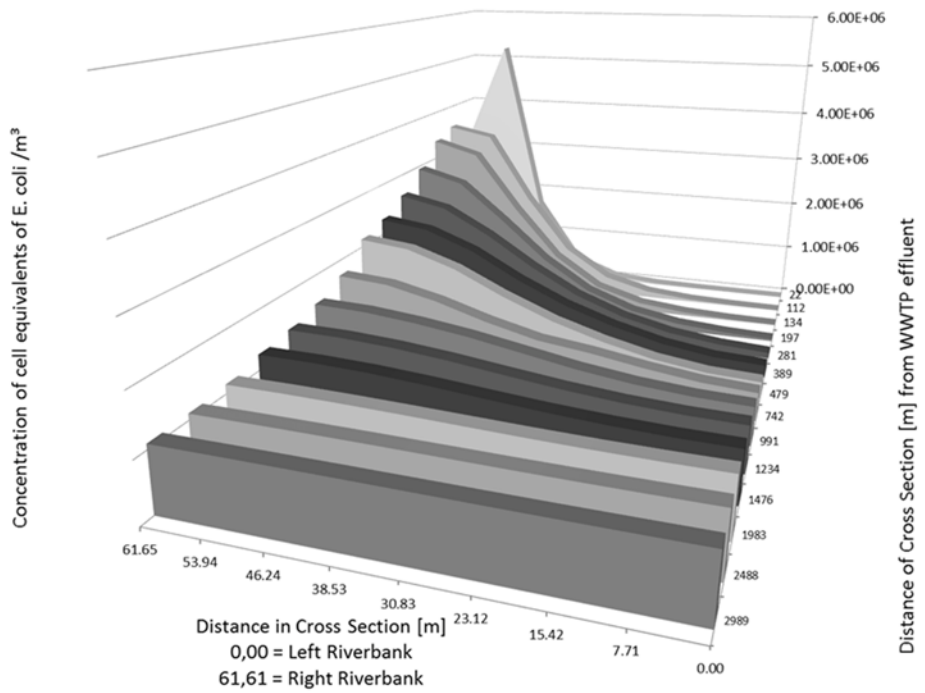

FIGURE 6 | Distribution of calculated (2D-HDWAM) concentration of *E. coli* in the river Danube downstream of the WWTP for different cross sections, 9.20×10^8 cell equivalents/m^3 in outlet of WWTP, discharge of Danube at 994 m^3/s (HQ20).

TABLE 5 | Calculated (2D-HDWAM) concentration of cell equivalents of *E. coli* for cross sections of the river Danube from outlet of WWTP downstream to 3,000 m.

		Position of cross section across the Danube [m]; inflow point of WWTP situated at right riverbank steady state runoff of Danube at 994 m^3/s								
		0.00 Left riverbank	**7.71**	**15.42**	**23.12**	**30.83**	**38.53**	**46.24**	**53.94**	**61.65 Right riverbank**
Downstream distance from WWTP outlet [m]	22	6.956E+02	1.445E+03	6.064E+03	2.373E+04	9.622E+04	4.303E+05	1.681E+06	5.223E+06	2.879E+06
	112	7.215E+03	1.601E+04	4.472E+04	1.272E+05	3.626E+05	9.348E+05	2.024E+06	3.369E+06	3.547E+06
	134	1.310E+04	2.621E+04	6.593E+04	1.664E+05	4.313E+05	1.021E+06	2.005E+06	3.130E+06	3.379E+06
	197	3.854E+04	7.090E+04	1.438E+05	3.102E+05	6.450E+05	1.219E+06	1.984E+06	2.703E+06	2.947E+06
	281	1.087E+05	1.655E+05	2.837E+05	5.042E+05	8.450E+05	1.338E+06	1.913E+06	2.391E+06	2.582E+06
	389	2.236E+05	2.720E+05	4.107E+05	6.418E+05	9.484E+05	1.356E+06	1.843E+06	2.184E+06	2.310E+06
	479	2.936E+05	3.389E+05	4.685E+05	6.858E+05	9.638E+05	1.330E+06	1.756E+06	2.053E+06	2.155E+06
	742	4.961E+05	5.535E+05	6.752E+05	8.265E+05	9.427E+05	1.168E+06	1.444E+06	1.649E+06	1.720E+06
	991	6.701E+05	7.032E+05	7.819E+05	9.057E+05	1.071E+06	1.236E+06	1.362E+06	1.454E+06	1.487E+06
	1,234	7.983E+05	8.189E+05	8.725E+05	9.582E+05	1.068E+06	1.185E+06	1.278E+06	1.335E+06	1.350E+06
	1,476	8.873E+05	9.020E+05	9.404E+05	9.999E+05	1.078E+06	1.161E+06	1.227E+06	1.265E+06	1.275E+06
	1,983	1.002E+06	1.006E+06	1.020E+06	1.044E+06	1.076E+06	1.108E+06	1.135E+06	1.153E+06	1.158E+06
	2,488	1.044E+06	1.046E+06	1.052E+06	1.063E+06	1.078E+06	1.091E+06	1.101E+06	1.107E+06	1.108E+06
	2,989	1.062E+06	1.062E+06	1.065E+06	1.071E+06	1.078E+06	1.085E+06	1.090E+06	1.093E+06	1.093E+06

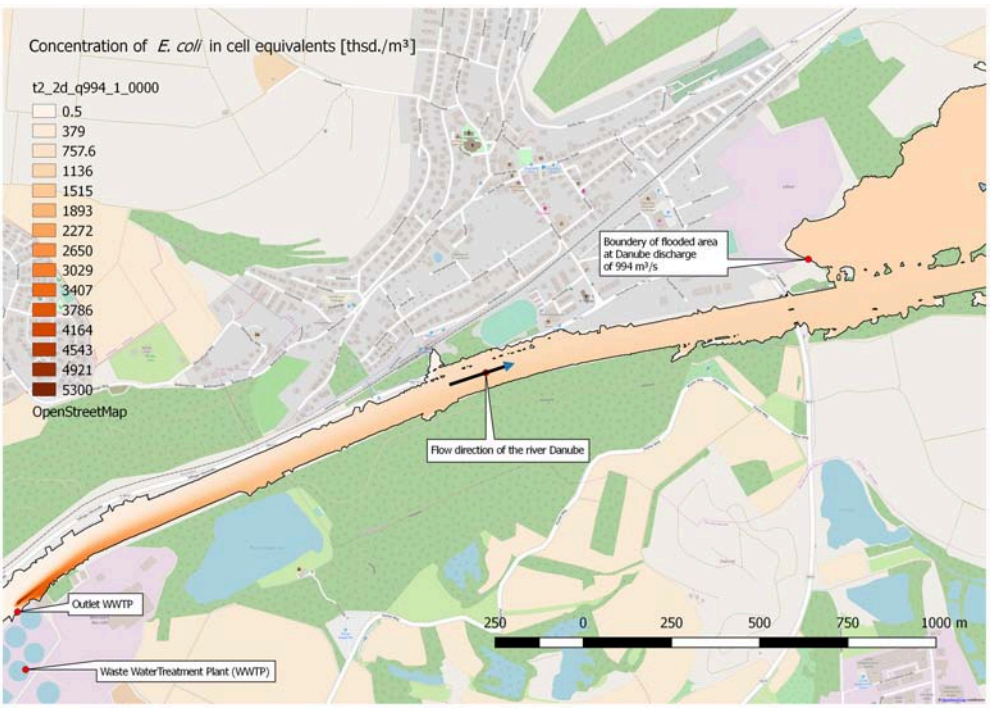

FIGURE 7 | Distribution of calculated (2D-HDWAM) concentration of *E. coli* in the river Danube downstream of the WWTP, 9.20×10^8 cell equivalents/m^3 in outlet of WWTP, discharge of Danube at 994 m^3/s (HQ20), interpolated results.

(**Table 5**, bold numbers), not directly at the riverbank. Therefore, the concentration at the cross section 22 m is the highest in the point 53.94 m (left riverbank is 0.00 m). Further downstream the cell equivalents mix and in the following cross sections the concentration decreases from the right (61.65 m) to the left river bank (0.00 m) (**Table 5** and **Figure 6**). Similar to the simulation with a runoff of 22 m^3/s in the river Danube there is a more or less evenly distribution of *E. coli* across the Danube after about 3,000 m with an average concentration of about 1.08×10^6 cell equivalents/m^3.

The runoff of 994 m^3/s in the Danube, and with it *E. coli* with a concentration of about 1.08×10^6 cell equivalents/m^3,

spreads also to parts of the Danube floodplain. **Figure 7** shows the maximum extend of the flooding and the concentration of *E. coli* at a steady state runoff in the Danube of 994 m^3/s. The stretch ranges from the outlet of the WWTP to about 3,500 m downstream. In consequence, the concentration of *E. coli* in the flooded area of the river Danube floodplain is at about 1.08×10^6 cell equivalents/m^3.

CONCLUSION

It was shown that a large WWTP (400.000 p.e.) plays an important part in the distribution of facultative pathogenic bacteria and antibiotic resistances after conventional treatment. The calculation of the daily loads of the WWTP and the consideration of dilution factors of different water level scenarios of the receiving river underline the high burden situations in the adjacent aquatic environment.

Molecular biology analyses revealed that the overall bacterial load and the majority of other clinically relevant bacterial targets were reduced during ozone/UV treatment using semi-industrial facilities, but not eliminated. Antibiotic resistance genes were still found to be present in the effluents under the adjusted parameters within the surviving population. In addition, the occurrence of DNA alterations like CPDs and 6-4 PPs, which were shown to be induced during UV treatment, as well as DNA lesions induced by ozonation might up-regulate specific DNA repair mechanisms like *recA* activities, which are known to enhance horizontal gene transfer, but also mutations rates. Both contribute also to antibiotic resistance evolution and the risk potential in aquatic environments.

Furthermore, the model of the distribution within the river system, which based on data from a conventional working, full-scaled WWTP, showed that a homogenous distribution is achieved after just a few kilometers. The model systems also showed the impacts on downstream river locations used for indirect water reuse or raw water source for drinking water conditioning. Especially at flood water events, facultative pathogenic bacteria and ARGs may be discharged into floodplains. Therefore, it is important to minimize the risk of contamination for the environment and the public health by using advanced treatment technologies to reduce the bacterial load and ARGs at WWTPs.

Further advanced treatment options are also available which may be suitable for reducing the bacterial load in WWTPs like the ultrafiltration. But these technologies might not be able to reduce other micro-pollutants. Therefore, a combination of different methods may lead to an adequate reduction of all types of pollution. Therefore, to the already available guidelines for the removal of chemical pollutants at WWTPs it is necessary to develop additional or adjusted strategies and guidelines adapted for the removal of microbial contaminants in wastewater, including facultative pathogenic bacteria and ARGs.

AUTHOR CONTRIBUTIONS

TS coordinated and organized the experiment. TJ, NH, and JA performed the experiments and generated the scientific data. CH arranged local support at the municipal wastewater treatment plants, executed sampling procedures, and provided WWTP specific data. AW provided the equipment of advanced treatment techniques. CE and GK carried out the calculations and simulations of the computer-based model.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02599/full#supplementary-material

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Linoleic Acids Overproducing *Lactobacillus casei* Limits Growth, Survival, and Virulence of *Salmonella* Typhimurium and Enterohaemorrhagic *Escherichia coli*

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Probiotics, particularly lactic acid bacteria, are biologic agents which limit the growth, virulence, and survival/colonization of various enteric bacterial pathogens and serve as potential alternatives to antibiotics. Mechanisms that contribute to this antimicrobial effect include producing bioactive metabolites/acids, increasing nutrient and receptor-mediated competition, and modulating gut microbiome ecology. However, these functions of common probiotic strains are limited due to the finite quantity of metabolites they produce and their total number in the gut ecosystem. Conjugated linoleic acids (CLAs), critical metabolites of *Lactobacillus*, have multiple beneficial effects on human health including anti-carcinogenesis, anti-inflammation, anti-oxidation, and anti-pathogenicity. In this study, we aim to overexpress the myosin cross-reactive antigen gene (*mcra*) in *Lactobacillus casei* (LC) to enhance the production of CLA and investigate its effectiveness against enteric bacterial pathogens, specifically *Salmonella enterica* serovar Typhimurium (ST) and enterohaemorrhagic *Escherichia coli* (EHEC). By inserting *mcra* in *L. casei*, we generated LC-CLA and found the total linoleic acid production by an individual bacterial cell was raised by 21-fold. The adherence ability of LC-CLA on human epithelial cells increased significantly and LC-CLA competitively excluded both ST and EHEC in a mixed-culture condition. Furthermore, LC-CLA significantly altered the physicochemical properties, biofilm formation abilities, interactions with host cells of both ST and EHEC, and triggered anti-inflammatory activities of host cells. These findings offer insights on applying a genetically engineered probiotic to control gut intestinal infections caused by ST and EHEC and prevent foodborne enteric illness in human.

Keywords: lactic acid bacteria, foodborne enteric bacterial pathogens, conjugated linoleic acid, anti-pathogenesis, anti-inflammation

INTRODUCTION

Human enteric microbial infections are principally characterized by diarrhea with or without other complications/consequences, which causes approximately 4–6 million deaths annually and possesses huge economic burden worldwide (Viswanathan et al., 2009; Christou, 2011). The dominant causative agents of enteric bacterial diseases include *Salmonella*, enterohaemorrhagic *Escherichia coli* (EHEC), *Campylobacter*, *Listeria monocytogenes,* and *Shigella* (Viswanathan et al., 2009; Mor-Mur and Yuste, 2010; Forsythe, 2016; Huang et al., 2016). These enteric bacterial pathogens are typically acquired through contaminated foods and water; therefore, risk is always associated with these foodborne diseases for everyone living on this planet. The Center for Disease Control and Prevention (CDC) estimated that in the United States alone, 48 million illnesses (approximately 1 in 6 Americans), more than 128 thousand hospitalizations, and thousands of deaths are caused by foodborne infections each year (Hoffmann et al., 2012; Adams et al., 2015, 2016, 2017). The most predominant causative foodborne infectious agents, including *Salmonella enterica* serovar Typhimurium (ST) and EHEC, commonly colonize in farm animals' guts, and during normal food production or processing, these pathogens often cross-contaminate meat products (Peng et al., 2014, 2016, 2018b; Salaheen et al., 2016b, 2017).

Probiotics, as bio-agents, can be considered the priority in prevention and control of foodborne bacterial pathogen-induced enteric illness (Amalaradjou and Bhunia, 2012; Hayes and Vargas, 2016; Peng and Biswas, 2017; Peng et al., 2018a). Through colonizing the host's gastrointestinal (GI) tract, these beneficial bacteria ferment or metabolize undigested dietary components; after reaching the small and large intestine, the probiotics generate/release a tremendous treasury of secondary metabolites (byproducts), most of which are associated with multiple health benefits (Flint et al., 2012; Marcobal et al., 2013). Functional metabolites from probiotics generally include bio-active polypeptides, with antimicrobial and immune-modulatory properties, as well as vitamin B, which is essential for mammalian cells in metabolism and reproduction (Stanton et al., 2005). The major byproducts of probiotics are lipid molecules, like fatty acids especially short chain fatty acids and poly-unsaturated fatty acids with various isomers (Serini et al., 2009; Louis et al., 2014). The mixed concentration of by-produced lipid molecules in human colon is approximately 50–150 mM, and these beneficial lipid molecules are active and help modulate the host's immune responses (Louis et al., 2014).

Among these functional fatty acids, linoleic acid (LA) is one of the most crucial beneficial metabolites produced from microbial sources, including *Bifidobacterium*, *Lactobacillus*, and *Lactococcus* (Rizos et al., 2012). The mixture of positional and geometric isomers of LA (C18:2, c9, c12), as conjugated linoleic acids (CLA), distinguishes it from other fatty acids because of its wide range of benefits on host health, including anti-carcinogenesis, anti-inflammation, and anti-pathogenicity (Lee et al., 2006; Benjamin and Spener, 2009; O'Shea et al., 2012; Yang et al., 2015). Bacteria that originate from dairy and human/animal intestines, specifically lactobacillus, including LA, *L. acidophilus*, *L. plantarum,* and *L. rhamnosus,* are known as predominant CLA producing strains (Van Nieuwenhove et al., 2011); however, their CLA productivity varies and is usually limited by multiple factors, including temperature, oxygen availability, substrate concentration, etc. (Pandit et al., 2012). A number of researchers, including our lab, are focusing on stimulating the productivities of LA and CLA from microbial sources especially probiotics both at the level of the human intestine and the industry production level (Peng and Biswas, 2017).

Through our previous research, we observed relatively intense antimicrobial activities of LA against enteric bacterial pathogens such as ST and EHEC (Peng et al., 2015c). However, the LA productivity (conversion ratio) of LC remains relatively low as 4.8%. In contrast, although *L. rhamnosus* possesses the highest CLA conversion rate among all active *Lactobacillus* species, it has a relatively low anti-pathogen activity (Van Nieuwenhove et al., 2011). In this study, we cloned and over-expressed the *mcra* (myosin-cross-reactive antigen) gene, encoding linoleate isomerase, from *L. rhamnosus* GG into LA, and aimed to examine the role of this novel probiotic in limitation and control of enteric pathogenic bacteria.

MATERIALS AND METHODS

Bacterial Strain and Their Growth Conditions

Probiotic strains, *Lactobacillus casei* ATCC 334 (LC-WT) and *L. rhamnosus* GG ATCC 53103, were purchased from American Type Culture Collection (ATCC, VA, United States). *Lactobacillus* strains were grown on De Man, Rogosa and Sharpe (MRS) (EMD Chemicals Inc., Gibbstown, NJ, United States) agar at 37°C for 24 h in the presence of 5% CO_2 (Forma^{TM} Scientific CO_2 water jacketed incubator, Thermo Fisher Scientific, Waltham, MA, United States). Enteric bacterial pathogens *Salmonella enterica* serovar Typhimurium (ATCC 14028) (ST) and enterohemorrhagic *Escherichia coli* EDL933 (ATCC 700927) (EHEC) were grown on LB agar (EMD Chemicals Inc., Gibbstown, NJ, United States) for 18 h at 37°C under aerobic conditions (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, United States).

Cell Lines and Culture Conditions

Human epithelium cells (INT407, ATCC CCL-6) were purchased from ATCC and cultured at standard condition (37°C, 5% CO_2, 95% humidity) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 100 μg/mL gentamicin (HyClone Laboratories Inc., Logan, UT, United States). The cultured cells were seeded at approximately 2×10^5 cells/mL/well into 24-well tissue culture plates (BD Falcon, Franklin Lakes, NJ, United States) to reach 80–90% confluence monolayer at standard condition for cell adhesion assay. The post-confluent INT-407 cell monolayers were rinsed with PBS and stabilized in antibiotic-free DMEM for 1 h prior to the invasion assay.

Human macrophage cell line (U937, ATCC CRL3253) was purchased from ATCC and grown at standard condition in RPMI-1640 Medium supplemented with 10% FBS and 100 μg/mL gentamicin. An aliquot of 6 mL cell suspension containing 1×10^6 cells were transferred into 25 cm^2 flask (Greiner Bio-One, Monroe, NC, United States) and cultured at standard condition for 24–30 h. After time, the cell monolayer was washed for three times with RPMI for further bacterial infection.

Over-Expression of Myosin-Cross-Reactive Antigen Gene (*mcra*) in *L. casei* and LC-CLA Development

Plasmid pJET and *E. coli* DH5α were purchased from Thermo Fisher Scientific (Waltham, MA, United States), pDS132 and *E. coli* β2155 were donated by Dr. Fidelma Boyd (Delaware University, Newark, DE, United States), and pMSP3535 were purchased from Addgene (Cambridge, MA, United States). LC-WT and *L. rhamnosus* GG (ATCC 53103) were harvested from overnight culture in MRS broth, followed by three times sub-culture on MRS agar plate at 37°C for 24 h in the presence of 5% CO_2 incubator.

The entire cloning design was summarized in **Figure 1**. Briefly, the 1750 bp *mcra* from *L. rhamnosus* GG was PCR amplified and ligated into pJET vector through blunt-end cloning. Aliquot of 250 μL *E. coli* DH5α bacterial suspension in cold 50 mM $CaCl_2$ was mixed with 10 μL ligated product (pJET-*mcra*) for 10 min incubation on ice, followed by 50 s incubation at 42°C in water bath. After further 2 min incubation on ice, 250 μL LB broth was added into bacteria-plasmid mixture for 10 min incubation at room temperature followed by selection on LB agar with 100 μg/mL ampicillin for transformation. The *E. coli* DH5α-expressed *mcra* was double-excised from pJET-*mcra* with BamHI and XbaI and then ligated into pMSP3535 vector at 16°C overnight. Following the same condition, pMSP3535-*mcra* was further transformed into *E. coli* DH5α and mixed with LC-WT at ratios of 1:1, 1:5, and 1:10 (donor cells: recipient cells) for bacterial mating. The *L. casei*-pMSP3535 was harvested through consecutive sub-culture and selection on MRS agars containing 300 μg/mL erythromycin at 37°C under micro-aerophilic condition (Tabashsum et al., 2018).

Removal of Antibiotic-Resistance Marker and *mcra* Chromosomal Recombination

The pMSP3535-*mcra* was isolated using Plasmid Mini Kit (Qiagen, Germantown, MD, United States). The gene sequence of *mcra* linked with transcription promoter P_{nis} was amplified by PCR using pMSP3535-*mcra* as the template. The upstream homologous arm *upp1* (208 bp) and downstream homologous arm *upp2* (211 bp) concatenated with Xba1 and Sac1 linkers were also PCR amplified using LC-WT genomic DNA as the template. Ligation of *upp1-mcra-upp2* was performed by PCR programmed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. After pJET blunt-end cloning, pJET-*upp1-mcra-upp2* and pDS132 were double-digestion with Xba1 and Sac1, followed by sticky-end ligation for overnight at 16°C. The pDS132-*upp1-mcra-upp2* was then transformed into *E. coli* β2155 following the same method described above but with 0.3 mM DAP selection. The transformed *E. coli* β2155 was mixed with overnight cultured LC-WT at ratio of 1:1, 1:5, and 1:10 (donor cells: recipient cells) for bacterial mating. Aliquot of 1 mL of the mixed bacterial suspension was spread on MRS agar plate with 0.3 mM DAP, followed by 5 h incubation at 37°C under micro-aerophilic condition. The *L. casei*-pDS132 was harvested through sub-culture and selection on MRS agar with 30 μg/mL chloramphenicol. Individual bacterial colony was consecutively sub-cultured in fresh MRS broth and selected on MRS agar containing 100 μg/mL 5-fluorouracil (5-FU) for *upp1-mcra-upp2* chromosomal homologous recombination. Finally, the *mcra* chromosomal recombinant *L. casei* mutant was harvested and named it as LC-CLA.

Co-culturing of *Lactobacillus* Strains With ST and EHEC

The survival and growth conditions of either ST or EHEC in the mixed culture with wild-type *L.* casei (LC-WT) or and mutant (LC-CLA) strains were investigated based on our previously described approach (Peng et al., 2015b). Briefly, bacterial cells from overnight agar plates were collected in 10 mL PBS using 10 μL sterile disposable loops. Each concentrated bacterial suspension was adjusted using PBS and measured by LAMBDA BIO/BIO+ spectrophotometer (PerkinElmer, Beaconsfield, United Kingdom) for adjusting the bacterial concentration to approximately 7 log CFU/mL. Aliquots of 400 μL adjusted bacterial suspension were added to sterilized test tubes containing 3.2 mL DMEM with 10% FBS and then incubated at 37°C for different time points (0, 2, 4, 8, 24, 48, and 72 h). After incubation, serial dilutions were performed in PBS, and then plated on agar plates (MRS agar for *L. casei*, LB agar for *S.* Typhimurium and EHEC) in triplicate, followed by incubation for 18 h at 37°C for growth. Bacterial CFUs were counted afterwards and results were expressed in unit of bacterial log CFU/mL as the average number from triplicate assays.

Evaluation of Physicochemical Properties and Biofilm Formation of ST and EHEC

Both ST and EHEC were cultured at 37°C for 18 h and the cell surface hydrophobicity of both pathogens was determined following method previously described by Peng et al. (2015c). The interactions between bacteria cell surfaces were determined by the auto-aggregation assay according to Ahn et al. (2014) in triplicate using Multiskan microplate reader (Thermo Fisher Scientific, Waltham, MA, United States), and the enteric bacterial cell injury induced by *Lactobacillus* strains was evaluated according to the overlay method previously described by Ahn et al. (2014) in triplicate using Trypticase soy (TSA) agar and XLD- or MacConkey-overlaid TSA agar.

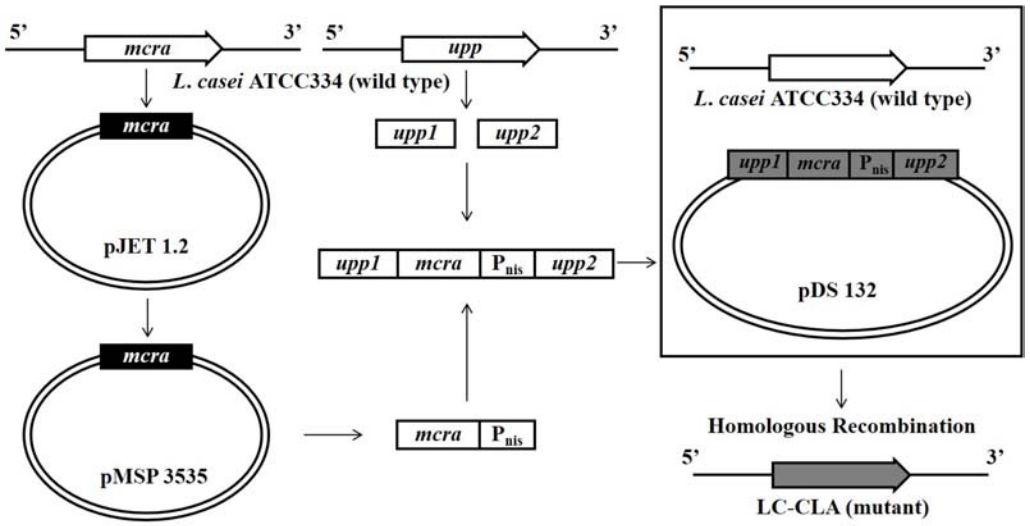

FIGURE 1 | Over-expression of *mcra* in LC-WT and chromosomal recombination constructing LC-CLA.

The bacterial biofilm formation was determined according to Salaheen et al., 2016a) with brief modifications. Both ST and EHEC were inoculated at approximately 5×10^5 CFU/mL in 6-well plates (Corning, NY, United States) containing 22 mm × 22 mm glass slides and LB broth at 37°C without shaking. At 24, 48, and 72 h point, the glass slides were rinsed with PBS for five times, and bacterial cells were scrapped from glass slides followed by serially diluted for plating on LB agar.

Scanning Electron Microscopic Analysis of Bacterial Cell Morphology

The ST and EHEC bacteria cells were harvested from overnight cultures and collected through 0.22 μm filter membranes. The bacteria cells were then fixed by submersing in 0.25% glutaraldehyde for 1 h (Kihm et al., 1994). The filter membranes were washed three times in sterile DI water followed by dehydration through sequential immersing the membranes in 10, 20, 50, 75, 90, and 100% (v/v) aqueous solutions of absolute ethanol. Filter membranes were then stored under anhydrous calcium sulfate overnight. To observe the morphology of the cells under SEM, the bacterial cells were sputter-coated with gold for Hitachi SU-70 FEG Scanning Electron Microscope (Hitachi Ltd., Japan) at an accelerating voltage of 5 kV.

Adhesion and Invasion Assay

The cultured mammalian cell adhesion and invasion assays were carried out in triplicates following the method described previously by Peng et al. (2015b) with some modification. We used MOI = 1:100 of host cell and bacterial CFU for both ST and EHEC on INT407 cells in triplicate wells *ex vivo*. The INT407 cells grown in 24-well plate with 800 μL DMEM were pretreated with 100 μL DMEM (control), *L. casei* CFCSs, or 2×10^8 CFUs *L. casei* bacterial cells, separately for 1 h, with each treatment in triplicate. A 100 μL aliquot of *S.* Typhimurium or EHEC PBS bacterial suspension with MOI = 100 (2×10^8 CFUs) was inoculated into triplicate wells. Afterwards, the infected cells were incubated at standard condition for another 2 h, and then followed by three times washing with DMEM. The cell monolayers were lysed with 0.1% Triton X-100 for 15 min, serial diluted, and plated on agar plates (MRS agar for *L. casei*, LB agar for *S.* Typhimurium and EHEC) to estimate the adhesive bacterial CFU. To measure bacterial cell invasive activity, DMEM washed cell monolayers after 2 h bacterial infection was incubated in DMEM containing 10% FBS supplemented with 250 μg/mL gentamicin for 1 h, then followed by three times DMEM washing, Triton X-100 lysis, serial dilution, and eventually plating on agar plates mentioned above.

Simulation of Enteric Bacterial Inflammation in Human Macrophage Cells

Enteric bacterial pathogen ST that provoke inflammation in human gut intestine was cultured on LB agar plate for 18 h and collected in PBS to be adjusted in approximately 1×10^9 CFU/mL. A 100 μL aliquot of bacterial suspension, containing approximately 1×10^8 CFU was inoculated into triplicate 25 cm^2 flasks containing U937 cell monolayer (approximately 10^6 host cells/flask). In the test flasks, 500 μL overnight (18 h) cell-free cultural supernatants (CFCSs) from *L. casei* (LC-WT and LC-CLA) strains in DMEM with 10% FBS were added during ST infection period. The infected monolayers were incubated for 24 h at standard condition, followed three times washing with ice-cold PBS for RNA extraction.

Quantitative RT-PCR for Evaluation of Gene Expressions

Extraction of RNA from bacterial cells and human macrophage cell line, the cDNA synthesis, and the qRT-PCR were performed in triplicate according to the method described

TABLE 1 | Primers used for RT-qPCR analysis of EHEC and *S.* Typhimurium.

Bacteria	Gene	Primer Sequence (5′–3′)	Function
EHEC	*gapA*	F: ACTTCGACAAATATGCTGGC	Housekeeping gene
		R: CGGGATGATGTTCTGGGAA	
	eaeA	F: CCCGAATTCGGCACAAGCATAAGC	Attaching and effacing
		R: CCCGAATCCGTCTCGCCAGTATTCG	
	espA	F: GTTTTTCAGGCTGCGATTCT	Type III secretion protein
		R: AGTTTGGCTTTCGCATTCTT	
	espB	F: GCCGTTTTTGAGAGCCAGAA	Type III secretion protein
		R: AAAGAACCTAAGATCCCCA	
	espD	F: AAAAAGCAGCTCGAAGAACA	Type III secretion protein
		R: CCAATGGCAACAACAGCCCA	
	ler	F: ACTTCCAGCCTTCGTTCAGA	Locus of Enterocyte
		R: TTCTGGAACGCTTCTTTCGT	Effacement regulator
	tir	F: GCTTGCAGTCCATTGATCCT	Translocated intimin receptor
		R: GGGCTTCCGTGATATCTGA	
	50S ribosomal protein L5	F: GTAGTACGATGGCGAAACTGC	House keeping gene
		R: CTTCTCGACCCGAGGGACTT	
S. Typhimurium	*hilA*	F: TATCGCAGTATGCGCCCTTT	Transcriptional regulator
		R: CAAGAGAGAAGCGGGTTGGT	
	hilC	F: AATGGTCACAGGCTGAGGTG	Transcriptional regulator
		R: ACATCGTCGCGACTTGTGAA	
	hilD	F: CTCTGTGGGTACCGCCATTT	Transcriptional regulator
		R: TGCTTTCGGAGCGGTAAACT	
	invA	F: CGCGCTTGATGAGCTTTACC	Invasion protein
		R: CTCGTAATTCGCCGCCATTG	
	invC	F: GCTGACGCTTATCGCAACTG	Type III secretion system ATPase
		R: GGCGGTGCGACATCAATAAC	
	invF	F: TCGCCAAACGTCACGTAGAA	Transcriptional regulator
		R: CATCCCGTGTATAACCCCCG	
	invG	F: CGAATGACGCCAGCTGTTC	Invasion protein
		R: TGCGTCAGGCGTCGTAAA	
	invH	F: GGTGCCCCTCCCTTCCT	Invasion lipoprotein
		R: TGCGTTGGCCAGTTGCT	
	orgA	F: AGGCAGGGAGCCTTGCTT	Oxygen- regulated invasion protein
		R: CCCTGATGCATTGCCAAAA	
	orgB	F: ACCATCCCGAAACGCTTTTA	Oxygen- regulated invasion protein
		R: TTGCCCCTCAGGCTTATCG	
	prgH	F: TGAACGGCTGTGAGTTTCCA	Type III secretion protein
		R: GCGCATCACTCTGACCTACCA	
	prgI	F: GGTCTATGGAAACGGACATTGTC	Type III secretion protein
		R: CGCCGAACCAGAAAAAGC	
	prgK	F: GGGTGGAAATAGCGCAGATG	Type III secretion lipoprotein
		R: TCAGCTCGCGGAGACGATA	
	sipA	F: CGTCTTCGCCTCAGGAGAAT	Cell invasion protein
		R: TGCCGGGCTCTTTCGTT	

(Peng et al., 2017). The PCR reaction mixture containing 10 μL PerfeCTa SYBR Green Fast Mix (Quanta Biosciences, Beverly, MA, United States), 2 μL of each 100 nM primer (listed in **Tables 1**, **2**), 2 μL of cDNA (10 ng), and 4 μL of RNase-free water was amplified using an Eco Real-Time PCR system with 30 s denaturation at 95°C, followed by 40 cycles of 95°C for 5 s, 55°C for 15 s, and 72°C for 10 s. All the relative transcription levels of target genes were estimated by comparative fold change. The C_T values of genes were normalized to the housekeeping/reference gene (listed in **Tables 1**, **2**), and the relative expression levels of target genes were compared between control and treatment. The fold change in terms of expression of each individual target gene was calculated as $\Delta\Delta C_T = [C_T(\text{target mRNA})\text{-}C_T(\text{reference mRNA})]_{\text{treatment}} - [C_T(\text{target mRNA})\text{-}C_T(\text{reference mRNA})]_{\text{control}}$ (Livak and Schmittgen, 2001). Quantitative RT-PCR was carried out in triplicate.

TABLE 2 | Primers used for RT-qPCR analysis of U937 cells cytokine genes.

Gene	Primer	Sequence (5′–3′)	Function
18srRNA	Forward	ATCCCTGAAAAGTTCCAGCA	Housekeeping gene
	Reverse	CCCTCTTGGTGAGGTCAATG	
IL-1β	Forward	GCCATGGACAAGCTGAGGAAG	Inflammatory cytokine gene
	Reverse	GTGCTGATGTACCAGTTGGG	
IL-6	Forward	GAACTCCTTCTCCACAAGCG	Pro-/Anti-inflammatory cytokine gene
	Reverse	TTTTCTGCCAGTGCCTCTTT	
IL-10	Forward	AGCAGAGTGAAGACTTTCTTTC	Anti-inflammatory cytokine gene
	Reverse	CATCTCAGACAAGGCTTGG	
IL-12	Forward	AATGTTCCCATGCCTTCACC	Pro-inflammatory cytokine gene
	Reverse	CAATCTCTTCAGAAGTGCAAGGG	
IL-23	Forward	GACACATGGATCTAAGAGAAGAG	Inflammatory cytokine gene
	Reverse	AACTGACTGTTGTCCCTGAG	
TGF-β	Forward	CTTGCTGTCCTCCTCTGCAC	Anti-inflammatory cytokine gene
	Reverse	TCACTGGGGTCAGCACAGAC	
TNFα	Forward	CAGAGGGAAGAGTTCCCCAG	Inflammatory cytokine gene
	Reverse	CCTTGGTCTGGTAGGAGACG	
CXCL-8	Forward	CTGCGCCAACACAGAAATTA	Inflammatory chemokine gene
	Reverse	ATTGCATCTGGCAACCCTAC	

Statistical Analysis

All the data were analyzed by the Statistical Analysis System software. The one-way analysis of variance followed by Tukey's test was applied to determine the significant differences of bacterial counts, physicochemical values, and virulent gene expression levels among the control and treatments based on a significant level of 0.05.

RESULTS

Phenotypical Characterization of LC-CLA

In comparison with LC-WT, LC-CLA maintained their *in vivo* growth/survival rate during exponential, stationary and death phases up to 96 h (**Figure 2A**) and remarkably ($p < 0.05$) improved their host cell adhesion ability onto human epithelial (INT-407) cells *ex vivo* (**Figure 2B**). The INT-407 cell-attached amount of LC-CLA was found to be significantly higher at 4 and 24 h of incubation comparing with LC-WT. In addition, the genetically engineered probiotic strain LC-CLA induced significant ($p < 0.05$) up-regulation on *mcra* (linoleate isomerase gene) mRNA level expression identified by qPCR; with HPLC-MS/MS analysis, we also detected fold increment in relative total linoleic acids per 1 mL overnight cultural supernatant as well as even higher fold boost in relative total linoleic acids per bacterial cell (**Table 3**).

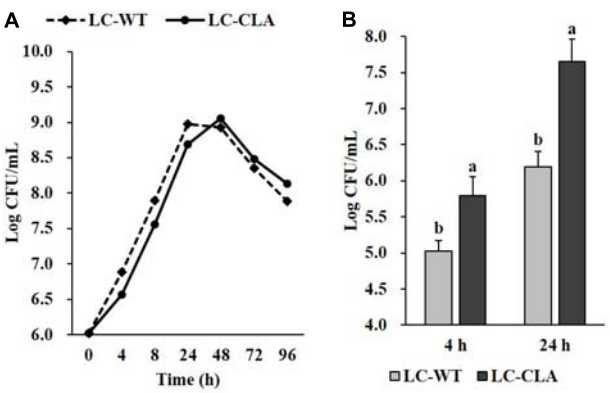

FIGURE 2 | Phenotypic characterization of LC-CLA. The comparative growth of 96 h **(A)** and *ex vivo* adherence on human epithelial cells at 4 and 24 h **(B)** were examined in triplicate and compared between LC-WT and LC-CLA. Bars indicate average ± standard deviation from parallel trials. Letters ('a' and 'b') indicate significantly different between LC-WT and LC-CLA on host cell adherence over three biological repetitions at $p < 0.05$.

Competitive Exclusion of Enteric Bacterial Pathogens, ST and EHEC

Probiotic *Lactobacillus* (LC-WT or LC-CLA) strains and enteric bacterial pathogens (ST or EHEC) were grown in mixed-cultured condition *in vitro* to investigate their competitive survival ability through competition between them in both short (4 and 8 h) and long (up to 72 h) period of time. The competitive inhibitory abilities of both LC-WT and LC-CLA against ST or EHEC were shown in **Figure 3**. Specifically, LC-CLA rapidly started to phase out both enteric bacterial pathogens with significantly ($p < 0.01$) higher loads of ST and EHEC reduction during the first 8 h incubation comparing with LC-WT. Overall, LC-CLA competitively exclude ST at 72 h and EHEC at 48 h.

Metabolites From LC-CLA in Combating Against Enteric Bacterial Pathogens

Overnight CFCSs from both LC-WT (CFCS1) and LC-CLA (CFCS2), in terms of initial inoculum of 10^6 CFU/mL

TABLE 3 | Relative expression level of *mcra* and relative production rate of linoleic acids in fold-change*.

Strain	Genotype	*mcra* mRNA expression[1]	RTLA[2] per mL supernatant	RTLA[2] per bacterial cell
LC-WT	Wild type	1.00	1.00	1.00
LC-CLA	*mcra* over-expressed	7.15 ± 1.76	4.48 ± 0.59	21.06 ± 1.33

*Gene expression level and metabolite production rate is standardized with LC-WT.
[1]mcra expression fold change was calculated based on 16S rRNA as reference gene.
[2]Relative Total Linoleic Acids based on HPLC-MS/MS analysis.

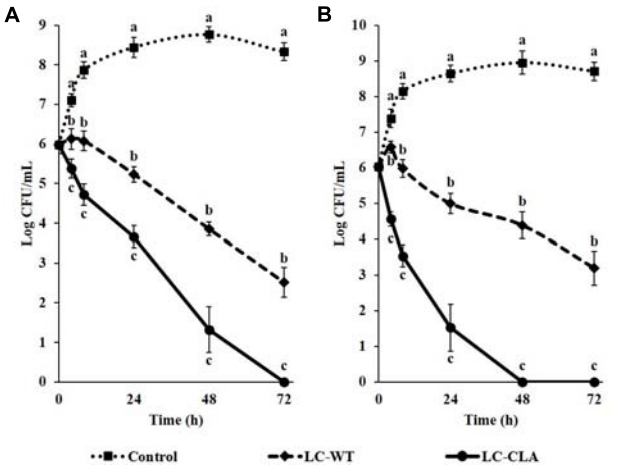

FIGURE 3 | Competitive exclusion of enteric bacterial pathogens by either LC-WT or LC-CLA. Comparative growth of ST **(A)** and EHEC **(B)** in single-culture or mix-culture with LC-WT or LC-CLA over 72 h was evaluated in triplicate. Bars indicate average ± standard deviation from parallel trials. Different letters ('a' through 'c') at single time point are significantly different in growth of ST or EHEC among control and treatments over three biological repetitions at $p < 0.05$.

overnight probiotic culture, were collected for examination the antimicrobial activities of their secreted byproducts. Comparing with negative control (only medium), both CFCSs from LC-WT and LC-CLA strains inhibited the growth of both pathogens, ST and EHEC, however, CFCS2 from LC-CLA showed more intensive effects (**Figure 4**). To be specific, CFCS2 reduced notably ($p < 0.01$) higher loads of ST and EHEC in the early stage at 4 and 8 h compared with CFCS1. The inhibitory activity of CFCS1 was attenuated after 24 h, whereas metabolites from LC-CLA exhibited a stable antimicrobial activity after 24 h, which ruled out all survival ST at 72 h and EHEC at 48 h.

Alterations in Physicochemical and Morphological Properties of ST and EHEC

The produced metabolites from both LC-WT and LC-CLA in CFCSs alter multiple physicochemical properties of both pathogens, ST and EHEC (**Table 4**). For example, CFCS1 decreased bacterial surface hydrophobicity of ST and EHEC, whereas CFCS2 exhibited more profound effectiveness in significantly lowering hydrophobicity of both pathogens (**Table 4**). Following the same trend, metabolites produced by LC-CLA in CFCS2 significantly reduced bacterial auto-aggregation activities of both ST and EHEC compared with metabolites from LC-WT. Similarly, we found that CFCS2 could intensify the effect of bacterial cell wall disruption of both ST and EHEC.

The bacterial cell morphology of ST/EHEC treated with CFCSs collected from LC-WT (CFCS1) or LC-CLA (CFCS2) was examined by scanning electron microscopy (**Figure 5**). Comparable ST and EHEC cells were observed for morphological changes including elongation, shrinkage, and swelling during the treatment with CFCS1 (**Figures 5A2,A3,B2,B3**). Much more pronounced alterations in the bacterial cell morphology were also observed when the cells were treated with CFCS2, for example, enormous outer membrane disruption and immense bacterial perforation (**Figures 5A4,A5,B4,B5**).

Effect on Biofilm Formation by ST and EHEC

The biofilm formation abilities of ST and EHEC in absence or presence of CFCSs from both LC-WT and LC-CLA are showed in **Figure 6**. At 24, 48, and 72 h incubation under the inhibitory pressure of LC-CLA secreted metabolites in CFCS2, the biofilm formation of ST was significantly ($p < 0.05$) suppressed. Whereas CFCS1 from LC-WT exhibited less inhibitory effects and failed to decrease the ability of ST to form a biofilm significantly after 72 h of incubation. The biofilm formation ability of EHEC was also significantly ($p < 0.05$) restrained at 24 h treatment with CFCS2 from LC-CLA. At 48 and 72 h, both CFCS1 and CFCS2 exhibited significant reduction on EHEC biofilm formation.

Disruption on Host Cells-ST/EHEC Interactions

The host cell-ST or -EHEC interactions were evaluated based on their adhesion to and invasion into human epithelial (INT-407) cells (**Figure 7**). With pre-treated of LC-WT, the cell adhesive and invasive abilities of ST were significantly ($p < 0.05$) reduced. In the same investigation, host cells pretreated with LC-WT also decreased the adherence abilities of EHEC, but more effective performance was observed when INT-407 cells were allowed to pre-colonize with LC-CLA. The adhesive and invasive activities of ST were suppressed by 99.58 and 99.34% separately, by LC-CLA. Similarly, the pre-colonized LC-CLA also reduced EHEC host cell adhesion capabilities by 99.10.

Correspondingly, the pre-treatments of ST and EHEC with CFCSs collected from both LC-WT and LC-CLA displayed

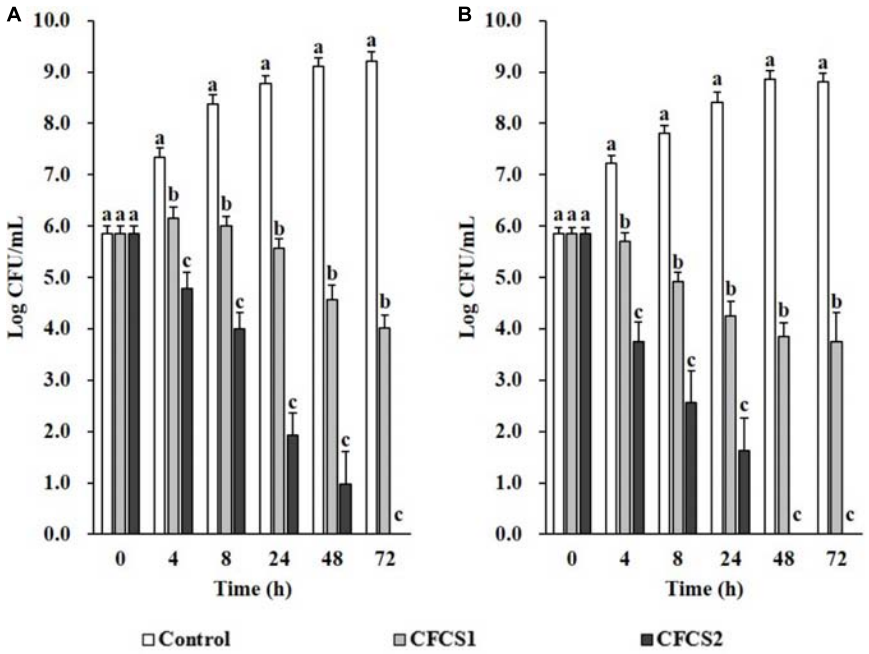

FIGURE 4 | Antimicrobial activities of LC-WT and LC-CLA metabolites on EHEC and ST growth and survival abilities. Inhibitory effects of CFCSs from LC-WT (CFCS1) or LC-CLA (CFCS2) were detected on growth of ST **(A)** and EHEC **(B)** over 72 h from triplicate biological experiments. Bars indicate average ± standard deviation from parallel trials. Different letters ('a' through 'c') at single time point are significantly different in growth of ST or EHEC among control and treatments over three biological repetitions at $p < 0.05$.

TABLE 4 | Physicochemical properties of ST and EHEC with CFCS treatments.

Treatment	Hydrophobicity (%)		Auto-aggregation (%)		Injured bacterial cells (%)	
	ST	EHEC	ST	EHEC	ST	EHEC
Control	$18.01 \pm 0.32^{a*}$	14.56 ± 0.83^{a}	14.72 ± 0.41^{a}	6.79 ± 0.91^{a}	19.80 ± 1.79^{c}	16.98 ± 4.18^{c}
LC-WT	10.85 ± 0.35^{b}	11.39 ± 0.77^{b}	8.65 ± 0.32^{b}	5.24 ± 0.29^{a}	30.92 ± 5.55^{b}	38.28 ± 2.74^{b}
LC-CLA	6.32 ± 0.43^{c}	4.35 ± 0.65^{c}	5.11 ± 0.41^{c}	3.02 ± 0.55^{b}	42.84 ± 2.64^{a}	50.64 ± 4.15^{a}

**Means with different letters (a–c) in individual column are significantly different at $p < 0.05$ between control and treatments.*

significant effects on their interactions/infections with INT-407 cells. Specifically, metabolites in CFCS collected from LC-WT, CFCS1 restricted the adherence activities of both ST and EHEC as well as invasive activity of ST on INT-407 cells. Whereas, CFCS2, collected from LC-CLA, altered the interaction between INT-407 cells and ST/EHEC intensively ($p < 0.01$) by decreasing 99.66% ST and 98.53 EHEC adhesion, respectively. In the same experiment, CFCS2 reduced the invasion ability of ST by 99.15% into INT-407 cells, respectively.

Down-Regulation on Expression of Bacterial Virulence Genes by CFCSs

The relative expression levels of multiple ST/EHEC virulence genes were found to be significantly ($p < 0.05$) down-regulated with CFCSs from both LC-WT and LC-CLA based on qPCR analysis, among which, the suppressive effects from CFCS2 were detected to be more intensive than CFCS1 (**Figure 8**). For ST, CFCS2 collected from LC-CLA notably ($p < 0.01$) down-regulated the expression of transcriptional regulator genes *hilA*, *hilC*, *hilD*, and *invF* by various fold. Similarly, the expression levels of effector genes *invA*, *invG*, *invH*, and *prgK* were also significantly ($p < 0.01$) suppressed by CFCS2. Whereas, insignificant fold changes were detected in relative expression levels of *invC*, *prgH*, *prgI*, and *sipA* when the cells were treated with either CFCS1 or CFCS2. For EHEC, eight virulence genes were investigated in this study, among which only effector gene *tir* kept conservative under the pressure of both CFCSs treatment. CFCS2 effectively ($p < 0.01$) down-regulated the expression levels of regulator gene *ler* as well as other effector genes including *eaeA*, *espA*, *espB*, and *espD*.

Anti-inflammatory Effects of LC-CLA

Metabolites secreted by both *Lactobacillus* (LC-WT and LC-CLA) strains managed to induce anti-inflammatory effects on ST-induced human macrophage (U937) cells by down-regulating pro-inflammatory cytokine genes and up-regulating

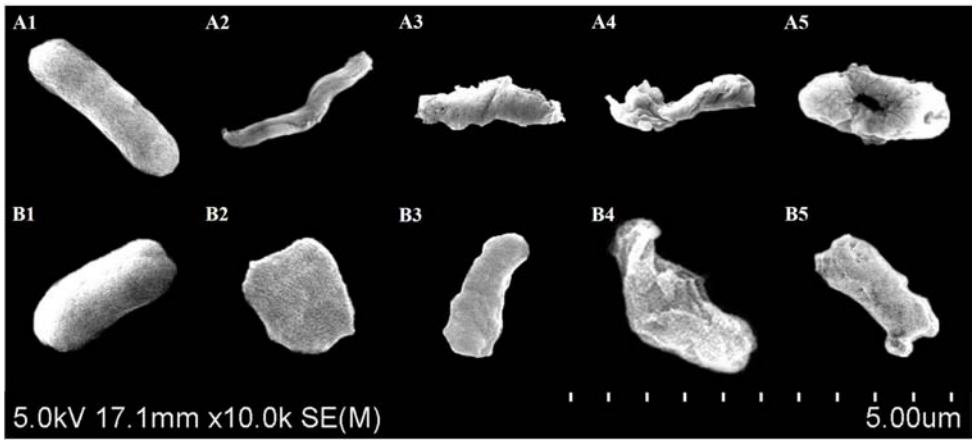

FIGURE 5 | Scanning electron microscopy for bacterial cell morphology. Comparable ST **(A)** and EHEC **(B)** morphology was observed and compared between control **(A1,B1)**, CFCS1 treatment **(A2,A3,B2,B3)**, and CFCS2 treatment **(A4,A5,B4,B5)**.

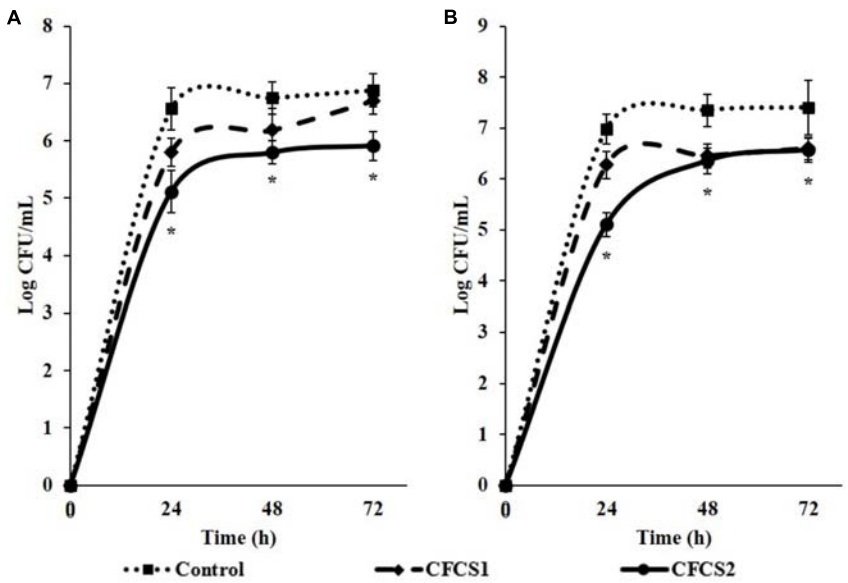

FIGURE 6 | Reduction of EHEC and ST biofilm formation in the presence of either LC-WT or LC-CLA or CFCSs collected from LC-WT and LC-CLA. Comparative biofilm formation of ST **(A)** and EHEC **(B)** under pressure of CFCS from either LC-WT (CFCS1) or LC-CLA (CFCS2) over 72 h was investigated in triplicate. Bars indicate average ± standard deviation from parallel trials. Asterisks (*) at single time point are significantly different in biofilm formation of ST or EHEC among control and treatments over three biological repetitions at $p < 0.05$.

anti-inflammatory cytokine genes (**Figure 9**). In detail, CFCS1 collected from LC-WT suppressed the expression levels of IL-1β, CXCL-8 (IL-8), IL-12, and TNF-α genes by 3.3-, 3.0-, 3.0-, and 4.8-fold, respectively, and at the same experiment, it raised the expression levels of IL-10 and TGF-β genes by 4.4- and 2.5-fold, respectively. Whereas, negligible differences in fold change were observed on IL-6 and IL-23 genes expression. On the other side, CFCS2 containing metabolites released from LC-CLA impressively amplified the anti-inflammatory activities, by which relative expression levels of pro-inflammatory cytokine IL-1β, IL-8, IL-12, IL-23, and TNF-α genes were all significantly ($p < 0.01$) down-regulated by 7.7-, 5.2-, 6.0-, 1.6-, and 6.7-fold, respectively; whereas relative expression levels of anti-inflammatory cytokine IL-10 and TGF-β genes were significantly ($p < 0.01$) up-regulated by 8.0- and 5.9-fold.

DISCUSSION

Probiotics, prebiotics, or a combination of the two, referred to as synbiotics, have emerged as a promising alternative treatment for enteric bacterial infections (Vyas and Ranganathan, 2012; Hardy et al., 2013; Pandey et al., 2015; Peng et al., 2015c; Salaheen et al., 2015). To improve and maintain the host's gut health,

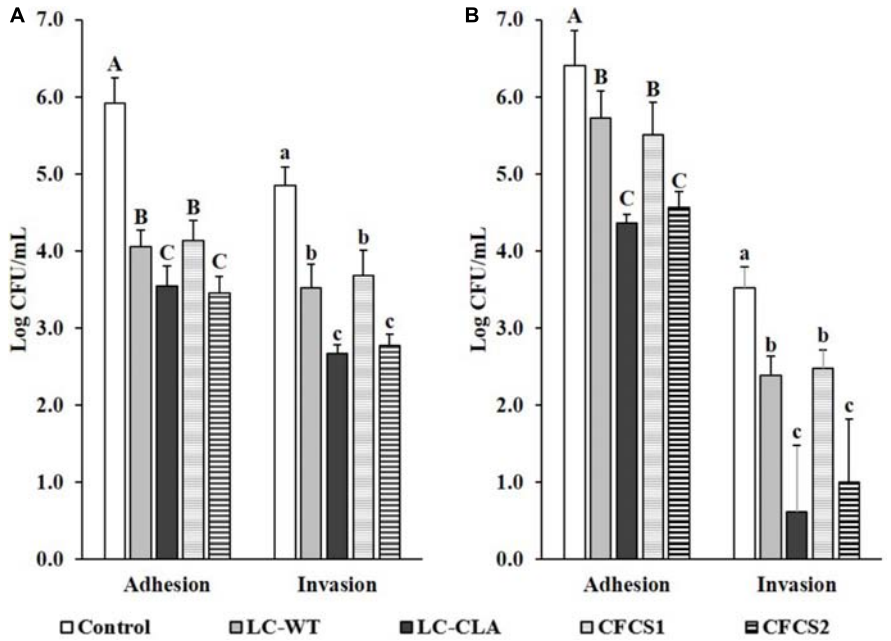

FIGURE 7 | Effect of LC-CLA in interfering with enteric bacterial pathogen-cell interactions. Human epithelial cell adhesive and invasive activities of ST **(A)** and EHEC **(B)** with pre-treatment of either *L. casei* or CFCSs from *L. casei* strains were examined in triplicate. A constant MOI = 100 was applied in each sub-figure. Bars indicate average ± standard deviation from parallel trials. Different letters 'A'–'C' and 'a'–'c' within each bacterial pathogen are significantly different among control and treatments for cell bacterial adhesion and invasion separately over three biological repetitions at $p < 0.05$.

the beneficial effects of probiotics depend largely upon the total quantity and type of functional metabolites they can produce. In our recent studies, we found several prebiotic-like components in cocoa and peanuts facilitated *L. casei* in producing more linoleic acids and outcompeting major foodborne bacterial pathogens, including ST and EHEC (Salaheen et al., 2014; Peng et al., 2015a,b). Based on these findings, we have overexpressed the *mcra* encoding Linoleate isomerase in LC-WT to verify the ability of the genetically modified strain, LC-CLA, in combating enteric bacterial infection *ex vivo* based on the cell culture model.

As discussed in previous studies, the myosin-cross-reactive antigens, which are present across a wide range of taxa, including *Lactobacillus*, not only take responsibility in linoleic acid construction and isomerization (Kishino et al., 2011; O'connell et al., 2013; Yang et al., 2014), but also have been revealed to contribute in bacterial stress-tolerance, blood-survival, and host cell interactions (O'Flaherty and Klaenhammer, 2010; Volkov et al., 2010; Chen et al., 2016). In this study, accordingly, in comparison to LC-WT, the *mcra* overexpressed LC-CLA was found with prominently higher production of total linoleic acids, fitter growth patterns, though not statistically significant, and remarkably improved epithelial adhesion *ex vivo* especially on INT-407 cells.

Though they assist in the development of healthy gut microbiota and the maintenance of cardiovascular health, prebiotic or prebiotic-like components, contain functional foods such as peanuts and cocoa. Therefore these symbiotic combinations are not entirely ideal for antimicrobial use in long term application or in specific populations due the cost of these foods, their potential to induce allergic reactions, the ability of beneficial and pathogenic microbes to use them as an uncontrolled source of nutrients, and their limited bio-availability (Hasler, 2002; Badrie et al., 2015; Feeney et al., 2016). Therefore, the genetically engineered probiotic in our research, being self-sufficient, stands out in supply of increased bio-active byproducts devoid of any prebiotic.

As previously reported by Peng et al. (2015c), *Lactobacillus,* by releasing antimicrobial components like organic acids, hydrogen peroxide, and poly-peptides, outcompete pathogenic bacteria in a time-dependent manner. In this study, LC-CLA exhibited even stronger effects against ST and EHEC than by LC-WT in mix-culture competitive exclusion, and the CFCS2 collected from LC-CLA also showed an extensive growth inhibition effect on both pathogens, through inducing bacterial cell membrane damage. The outcomes are also supported by the previous findings on anti-pathogenic activities in CLA (Hontecillas et al., 2002; Bhattacharya et al., 2006; Meraz-Torres and Hernandez-Sanchez, 2012). Furthermore, we also surprisingly observed that due to over-expression of *mcra* in LC, LC-CLA induced significant alterations on several physiochemical properties of ST/EHEC, including surface hydrophobicity, auto-aggregation, bacterial cell morphology, and biofilm formation. The over-produced LA in LC might have induced these changes since they were suggested to interact with cytoplasmic membrane of bacterial pathogens and further disrupt phospholipid or extracellular polysaccharides (Peng and Biswas, 2017), both of which are crucial factors for bacterial physicochemical properties as well as biofilm formation (Vu et al., 2009; Renner and Weibel, 2011).

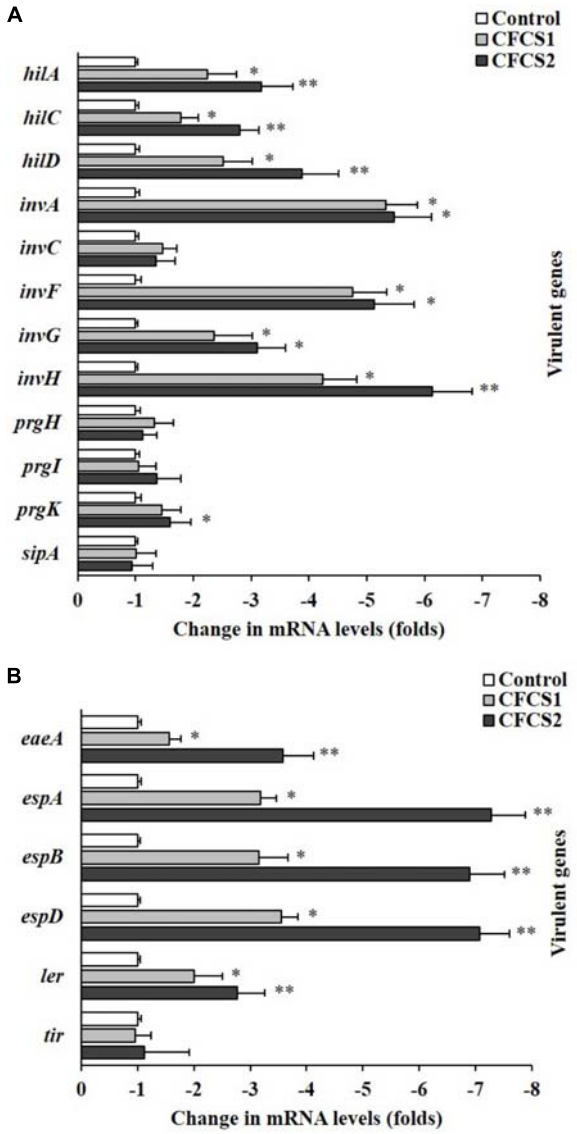

FIGURE 8 | Role of LC-CLA in suppression of EHEC and ST virulence genes. The relative expression of T3SS-related virulence genes from ST **(A)** and EHEC **(B)** under pressure of CFCSs from *L. casei* strains was investigated in triplicate. The relative transcription levels are in the form of comparative fold change with control being 1.0. Bars indicate average ± standard deviation from parallel trials. Asterisks '*' and '**' indicate the significant difference in each individual virulence gene expression among control and treatments over three biological repetitions at $p < 0.05$ and $p < 0.01$ separately.

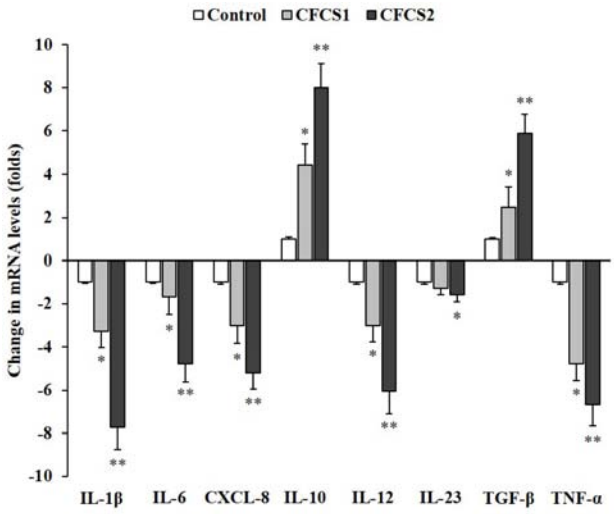

FIGURE 9 | Anti-inflammatory effects of LC-CLA on human macrophage cells. The relative expression of ST-induced macrophage (anti-)inflammatory cytokine genes with treatment of CFCSs from *L. casei* strains was investigated in triplicate. The relative transcription levels are in the form of comparative fold change with control being 1.0. Bars indicate average ± standard deviation from parallel trials. Asterisks '*' and '**' indicate the significant difference in each individual cytokine gene expression among control and treatments over three biological repetitions at $p < 0.05$ and $p < 0.01$ separately.

Specific virulence genes of ST/EHEC involved in Type-3 secretion (T3SS) were significantly down-regulated in the presence of the secreted metabolites in CFCS2 collected from LC-CLA. These genes include invasion regulator genes and effector genes, especially *eaeA*, that functions in EHEC A/E and *invH* encoding ST invasion lipoprotein. In fact, several research groups have also previously reported the dose-dependent activities of poly-unsaturated fatty acids in regulation of *Salmonella* and *E. coli* (Cardenal-Muñoz and Ramos-Morales, 2011; Nakamura et al., 2012); however, the conclusion remains to be ambiguous and bears little correlation with bacterial infections (Peng and Biswas, 2017). The repressed virulence genes and the disrupted bacterial physicochemical properties of ST and EHEC by LC-CLA served as identical indicators for the attachment of pathogens on host cells. It further supported the *ex vivo* reduction of ST/EHEC-host cell interactions excluding the negligible toxic effect of gentamycin on bacteria (Peng et al., 2015c). Through competitively occupying INT-407 cell surface receptor-like molecules (Bernet et al., 1994; Matsuo et al., 2012; Peng et al., 2015c) and enhancing the regulation of these two bacterial pathogens via the increased production of linoleic acids (Belury, 2002; Hontecillas et al., 2002; Yang et al., 2017), LC-CLA stands out with strong inhibitory actions against enteric bacterial pathogens. Though 1 h probiotic pre-occupation and 2 h pathogenic infection was investigated in this study, further research targeting up to 72 h ST/EHEC infections could be favorable in revealing the long-term preventive effects of LC-CLA.

Finally, extensive anti-inflammatory effects of LC-CLA were presented *ex vivo* on human macrophage cells. In accordance with previous studies on linoleic acids (Albers et al., 2003; Akahoshi et al., 2004; Tricon et al., 2004), we also detected a reduction in levels of pro-inflammatory cytokines/chemokines including TNF-α, IL-1β, IL-6, CXCL-8, and IL-12 in this study. Moreover, we identified the up-regulation of anti-inflammatory cytokine IL-10 and TGF-β genes as well, the two cytokines of which were believed to induce inhibition on T_h cells activation (Gorelik and Flavell, 2002; Gorelik et al., 2002;

Hsieh et al., 2012). The activated macrophage cells bearing bacterial pathogen challenges normally produce and release IL-12 for activation of T_h1 cells and further induces INF-γ, TNF-α, and IL-12 production (Romagnani, 1999; Dong and Flavell, 2001; Bassaganya-Riera et al., 2003; Kidd, 2003), which explained the significantly elevated expressions of TNF-α and IL-12 genes with ST infections. LC-CLA in secreting auxiliary amounts of CLA, ameliorated the ST infection-induced gut inflammatory responses by suppressing T_h1 cells through reducing IL-12 and pathogenic T_h17 cells through reducing IL-1β (Acosta-Rodriguez et al., 2007; Monteleone et al., 2009; Cosmi et al., 2014). Most importantly, the anti-inflammatory activities of linoleic acids have not been documented to impair any gut immunity against enteric bacterial pathogen infections (Turnock et al., 2001; Peng and Biswas, 2017).

CONCLUSION

Findings from this study herald a new era, wherein non-traditional preventive strategies through using functional probiotics could become applicable in defense against enteric bacterial pathogens specifically *Salmonella* and pathogenic *E. coli,* regardless of altering the normal gut microbiota. LC-CLA with *mcra* gene over-expression managed to adhere efficiently on human epithelial cells and secret larger amounts of linoleic acids. By this pathway for combating ST and EHEC infections, the effective probiotic strain competitively excluded their growth *in vitro*, altered their physicochemical properties, as well as biofilm formation abilities, reduced their interactions to host cells *ex vivo*, and attenuated the host cell inflammatory process induced by enteric bacterial pathogens. The development and implementation of such novel, cost-effective, and simple-to-use genetically engineered probiotics, independent of prebiotics or prebiotic-like functional food ingredients, is promising to open a new avenue in prevention and treatment of *Salmonella* and pathogenic *E. coli* provoked GI infections and in improving gut health where antibiotic therapy could be limited, and helpful in avoiding negative consequences of antibiotic therapy.

AUTHOR CONTRIBUTIONS

MP designed the work, conducted experiments, interpreted and analyzed data, ensure the integrity of the work, and drafted and revised the manuscript. ZT performed the experiments and ensured the accuracy of the work. PP and CB conducted part of the experiments and acquired data. DB contributed to the conception and design of the research, and ensured both the accuracy and integrity of the work, and critically revised and approved the final manuscript for submission and publication.

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Antibiotic-Resistant Bacteria in Greywater and Greywater-Irrigated Soils

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This study represents the first systematic attempt to evaluate antibiotic-resistant bacteria (ARB) occurrence in treated greywater and the potential spread of these bacteria from the greywater to greywater-irrigated soil. Treated greywater from three recirculating vertical flow constructed wetlands, each located in a household in the central Negev Desert, Israel, was surveyed. The presence of antibiotic-resistant bacteria in raw and treated greywater was investigated with culture and molecular methods, as well as their presence in the corresponding treated-greywater-irrigated soils. Additionally, the effectiveness of chlorination to prevent the spread of ARB was tested. The total count of tetracycline-resistant bacteria significantly increased in the treated greywater, likely due to their concentration on the filter matrix of the treatment systems. Twenty-four strains of tetracycline-resistant bacteria were isolated and identified at the genus level by 16Sr RNA gene sequencing. All the tetracycline-resistant bacteria showed high resistance traits, and some of them presented multiple antibiotic resistances. Six tetracycline resistance genes (coding for efflux and ribosomal resistance mechanisms) and five β-lactamase genes were detected. In 14 of the isolated strains, the gene *tet*39, which is phylogenetically related to both environmental and clinical strains, was identified. All the *tet*39 resistant bacteria were positive to at least one of the β-lactamase genes tested. Chlorination was found to be an efficient method to reduce ARB in treated greywater. We concluded that disinfection of treated greywater may reduce the risks not only from the potential presence of pathogens but also from the presence of ARB and antibiotic resistance genes.

Keywords: greywater, antibiotic resistance, tetracycline, irrigation, recirculating vertical flow constructed wetland

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INTRODUCTION

The modern lifestyle requires a large quantity of potable water and generates large amounts of wastewater (Eriksson et al., 2002; Schacht et al., 2016). This, in combination with dwindling water resources worldwide, has led to increasing interest in wastewater reuse in many parts of the world, including both industrialized and developing countries (Eriksson et al., 2002).

One method of conserving water, on the local scale, is by recycling greywater (GW) for irrigation (Gross et al., 2007). Greywater is defined as domestic wastewater that excludes wastewater from toilets and typically includes water from baths, showers, hand basins, and washing machines

(Jefferson et al., 2000; Gross et al., 2007; Ghaitidak and Yadav, 2015). Greywater constitutes 50–80% of the total household wastewater, and its recycling can reduce potable water use by up to 50% (Gross et al., 2007). In recent years, there has been an increase in the use of GW for various purposes such as toilet flushing, landscaping, and garden irrigation (Gross et al., 2015).

It has been well-established that raw GW is contaminated with pathogens (although less than "full" domestic wastewater) and other chemical contaminants and thus should be treated before reuse (James et al., 2016). Potential health risks associated with the spread of pathogenic organisms through the use of treated GW are critical issues (Benami et al., 2016). In fact, a number of pathogens are occasionally found in raw GW (RGW), including fecal coliforms, fecal enterococci, fecal streptococci, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, among others (Benami et al., 2016). Interestingly contradicting results regarding increasing levels of fecal coliforms in soils following long term greywater irrigation were reported (Casanova et al., 2001; Benami et al., 2016). While Casanova reported on significant increase in fecal coliforms, Benami et al. (2013) reported no such differences.

Another source of recent concern is the spread of antibiotic-resistant bacteria from GW, as well as the evolution and propagation of antibiotic-resistant microorganisms (Rizzo et al., 2013; Berendonk et al., 2015). The intensive use of antibiotics for human medical, veterinary, and agricultural purposes results in their continuous release into the environment (Rizzo et al., 2013), with the primary concern of the development of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB), which reduce the therapeutic potential against human and animal pathogens (Kemper, 2008; Zhang X. X. et al., 2009).

The presence of ARB and ARGs, even at very low levels in the household garden, may represent a high risk to human health through the spread of antibiotic resistance, especially if humans have high exposure to places where ARB are present (e.g., food crops cultivated in GW-irrigated fields). ARGs may persist in the environment, and even worse, they can be spread to other bacteria including human commensals or pathogens of clinical relevance, through the horizontal gene transfer (HGT) of mobile genetic elements (Christou et al., 2017).

The dissemination of ARB and ARGs is an alarming problem because it has been demonstrated that intrinsic antibiotic resistance might have been selected in the course of bacterial evolution, even without antibiotic selective pressure, for covering functions other than antibiotic resistance (Alonso et al., 2001). For example, it was shown that non-antibiotic biocidal compounds such as triclosan in greywater increase the prevalence of ARB in the soil microcosm (Harrow et al., 2011).

Nevertheless, recent studies demonstrated that irrigation with treated municipal wastewater does not seem to impact antibiotic resistance levels in the soil microbiome (Gatica and Cytryn, 2013). Thus, our initial hypothesis was that greywater doesn't harbor ARB and that treated GW will not increase the abundance of ARB in TGW irrigated soil. However, there is still a lack of evidence about the potential efficacy of actual GW treatment before reuse on ARB abundance and the potential contribution of GW irrigation to the spread of ARB. Understanding the dynamics of ARB and ARGs in the urban water cycle is an increasingly important goal as antibiotic resistance is recognized as one of the most significant human health challenges of the Twenty-first century (WHO, 2012; Voolaid et al., 2018).

Therefore, the objectives of this work were to investigate the prevalence of ARB and ARGs in raw and biologically treated GW, as well as their presence in the corresponding treated-GW-irrigated soils. We also tested the effect of chlorination on the survival of ARB. Specifically, we focused on tetracycline-resistant bacteria because tetracyclines were the first primary group to which the term "broad spectrum" was applied. For their spectrum of activity, their relative safety, and their low cost, tetracyclines have been used widely across the globe for clinical and non-clinical uses and, are the fifth most consumed antibiotics in the world (Van Boeckel et al., 2014). Furthermore, tetracycline resistance bacteria are widespread in treated wastewater from Israel leading us to believe that they present also in greywater (Gatica and Cytryn, 2013).

MATERIALS AND METHODS

Location and Sampling

Raw and treated GW from three different households in the central Negev Desert, Israel (30°51′05″ N 34°47′00″ E) were monitored. GW treatment was done by a recirculating vertical flow constructed wetland (RVFCW) as described by Gross et al. (2007) and the system layout and operation parameters are presented in **Figure S1**. The three systems were selected since they have been working now for over 7 years and the treated greywater TGW is used continuously for irrigation at this time in parallel to freshwater irrigated controls. All households contain kids of different ages. TGW samples were collected routinely and analyzed for physicochemical parameters by standard methods (**Table S1**) as well as ARB and ARGs (as described below). From each household, 100 mL of water (raw and treated) were taken and placed in two sterile 50-mL falcon plastic tubes. Ten tuff gravel pieces with an average weight of 8 g were taken from the upper surface layer of the RVFCW bed for ARB and ARG biofilm analyses. Similarly, ARB and ARGs were monitored in freshwater- and treated-GW-irrigated soils. Duplicate soil samples from each location (15 g of soil at 5 cm depth) were collected twice, in January and March 2017. All samples were immediately transported to the laboratory and analyzed within a few hours.

Isolation and Count of Tetracycline-Resistant Bacteria From Water, Filter Bed, and Soil

A modified PTYG broth (peptone, tryptone, yeast extract, glucose) was used at 10% of the original strength and without sodium thioglycollate (Atlas, 2010). The PTYG media were solidified by using 15 g L^{-1} of bacteriological agar (Difco, Franklin Lakes, NJ, USA). For bacterial extraction, 3 g of soil was suspended in 10 mL of the sterile PTYG broth and then shaken for 5 min on an orbital shaker at 200 RPM at 25°C. The solids settled for 5 min, and 100 μL of the supernatant was used to

prepare the dilutions. The dry weight of the soil and the tuff gravel was obtained after drying for 24 h at 65°C. The supernatant from this slurry was used for dilutions, counting, and microbial isolation.

For the isolation of tetracycline-resistant bacteria, 0.1 mL of the serial dilutions of the different samples [raw greywater (RGW), biofilm (BF), treated greywater (TGW), greywater-irrigated soil (TS) and freshwater-irrigated soil (US)] was spread in duplicate with a sterile disposable Drigalski spreader on the agar surface of two different types of plates: the control containing only the medium PTYG and the second containing PTYG + tetracycline (20 mg L^{-1} Sigma Park Rabin, Rehovot, Israel). The CLSI guidelines (2014) were used as a benchmark for isolating tetracycline-resistant bacteria. Accordingly, isolates with MIC values of Tetracycline at ≥ 16 μg mL^{-1} are regarded as resistant, and thus we applied a concentration of 20 μg mL^{-1} in our isolation plates.

For both the control and the treated samples of water, soil, and biofilm, suitably diluted samples were inoculated in the respective plates and were incubated at 25°C for 48 h and at 37°C for 24 h. After the incubation period, the colonies were counted. The percentage of tetracycline-resistant bacteria was obtained from the ratio between the colony count on the plates containing tetracycline and the colony count on the control plates.

From the isolation plate containing tetracycline, colonies with distinct morphologies were taken with a sterile loop and streaked on a fresh plate of PTYG + tetracycline (20 mg L^{-1}) and cycloheximide (20 mg L^{-1} Sigma Park Rabin, Rehovot, Israel). After an incubation period of 48 h for the bacteria incubated at 25°C and 24 h for the bacteria incubated at 37 °C, all strains were purified by streaking them twice on a fresh sterile plate of PTYG + antibiotic. The isolates were stored at −80°C in glycerol (25%), PTYG, and tetracycline (20 mg L^{-1}).

Tap Water Analysis

To confirm that the tetracycline-resistant bacteria did not originate from the tap water, 0.1-mL samples of tap water, collected from the three different households, were spread on the surface of the agar plate with or without tetracycline (20 mg L^{-1}).

Identification of Tetracycline-Resistant Bacteria

The isolated tetracycline-resistant bacteria ($n = 24$) were identified at the genus level by 16S rRNA gene sequencing by Hy Laboratories Ltd. (Rehovot, Israel). Following DNA isolation, the first ∼800 bp region of the 16S rRNA gene was amplified by PCR, and the resulting amplicon was sequenced using an ABI3730xl genetic analyzer and BigDye V1.1 chemistry, according to the manufacturer's instructions. The obtained sequence was analyzed using sequencing analysis software (Applied Biosystems v5.4) and compared with archived NCBI sequences for gene identification. Sequences of 16S rRNA genes were deposited in Genbank with accession numbers from MH090940 to MH090963. Nucleotide sequences were aligned and compared, and were then used to infer a phylogenetic tree with MEGA7.0.14 (Kumar et al., 2016).

The Growth of Tetracycline-Resistant Bacteria in the Presence of Chlorine

To evaluate the possible effect of chlorine on the viability of tetracycline-resistant strains, the growth of *Serratia spp*. strains, an opportunistic pathogen, isolated from SYS3, was examined in the absence (control group) and the presence of 2 mg L^{-1} of free chlorine as NaClO. The initial culture was about 1×10^6 CFU mL^{-1} that was incubated in treated greywater at either 25°C or 37 °C. The suspension sampled was diluted hourly, and then 10 μL was spotted on the plate. The colonies in the spots were counted after 24 h of incubation under a magnifying glass.

Multiple Resistances

The isolated tetracycline-resistant bacteria were also evaluated for possible multiple resistances to three different antibiotics (all from Sigma): amoxicillin (β-lactams), ciprofloxacin (fluoroquinolones), and kanamycin (aminoglycosides). The bacteria were streaked on PTYG agar plates containing 20 mg L^{-1} of each one of the three antibiotics. The plate was incubated at 25°C (for the bacteria isolated at 25°C) and 37°C (for the bacteria isolated at 37 °C).

Minimum Inhibitory Concentration (MIC)

For all the isolated tetracycline-resistant bacteria, the MIC of tetracycline was tested based on the broth microdilution protocol (Wiegand et al., 2008). In addition, the isolates that were shown to be also able to grow in the presence of 20 mg L^{-1} of amoxicillin, ciprofloxacin and kanamycin were tested for the MICs of these three antibiotics. Filtered (0.22 μm) stock solutions of antibiotics (0.5 mg/ml) were dissolved in distilled water. Strains from glycerol stocks were inoculated in PTYG and incubated overnight. After 12 h, the optical density (OD) of the samples was measured, and the bacterial cultures were diluted to an OD of 0.1 (corresponding to about 5.7×10^7 CFU mL^{-1}), and then 50 μL was used for MIC determination.

The tetracycline MIC was tested at concentrations ranging from 100 to 350 μg mL^{-1} for the bacteria isolated at 37°C and from 100 to 500 μg mL^{-1} ml for the bacteria isolated at 25 °C. The other three antibiotics were tested at concentrations ranging from 50 to 300 μg mL^{-1}. For the experiment, multiple sterile 48-well plates (Costar, Corning, NY, USA) were used. In each plate, the wells of the first column were used as a negative control and contained only 500 μl of the PTYG medium; the wells of the second column were used as a positive control and contained 450 μL of PTYG and 50 μL of the tested strain; the remaining wells were used as a test group and contained 450 μL of PTYG to which was added six different antibiotic concentrations. The test was performed in duplicate. The OD of 600 nm at time zero and after 12 h was measured with a multi-plate reader (Infinite® 200 PRO, Tecan Männedorf, Switzerland). The % inhibition of all samples was calculated, using the following formula:

$$\%\ inhibition = \frac{OD\ positive\ control - OD\ given\ concentration}{OD\ positive\ control - OD\ negative\ cobtrol} \times 100$$

To determine the MIC value (μg mL^{-1}), the following criterion was used: between wells with no bacterial growth, the one with the lowest antibiotic concentration indicates the MIC value. The

results were reported in the following way: the values preceded by the sign ≤ indicate that the microorganism growth was inhibited by the lowest concentration of the antibiotic used for the test, while values preceded by the sign ≥ indicate that growth was not inhibited by the higher concentrations of the antibiotic tested.

DNA and Plasmid Extraction

Nucleic acid extraction from an overnight culture of each strain in PTYG plus tetracycline (20 mg L^{-1}) was performed using a GenElute Bacterial Genomic DNA kit (Sigma) following the manufacturer's protocol. The concentration and quality of the DNA were determined by spectrophotometric analysis and agarose gel electrophoresis. For the spectrophotometric analysis, the NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) was used. Electrophoresis visualization of DNA was performed on 0.8% of agarose stained with Gel Red (Biotium, Fremont, CA, USA).

Positive controls of β-lactamase genes were cloned in different plasmids. The blaOXA2 and 10 were synthesized and cloned by Syntezza Bioscience Ltd. (Jerusalem, Israel) on a vector pUC57 (Rocha et al., 2018) provided by Dr. Eddie Cytryn (The Institute of Soil, Water, and Environmental Science, Volcani Center, Israel); the CTX- M32 and blaTEM genes were cloned on a pNORM kindly provided by Christophe Merlin (the University of Lorraine, Laboratory of Physical Chemistry and Microbiology for the Environment, Nancy, France); the blaSHV from the amoxicillin-resistant *K. pneumoniae* strain G-A-TGW (MG982455.1) was cloned in a pJET vector.

PCR Analyses

For the presence of β-lactamases, five genes were evaluated, including blaTEM, blaCTXM-32, and blaSHV that belong to the class A of β -lactamase, and blaOXA-2 and blaOXA-10 that belong to the class D of β-lactamase. For tetracycline, six genes were evaluated: *tet*39, *tet*A, and *tet*B (efflux), and *tet*M, *tet*Q, and *tet*W (ribosomal).The primers and sizes of the PCR products are presented in **Table 1**. The PCR conditions appear in the **Table S2**.

All the positive *tet*39 PCR products were purified and sequenced by Macrogen (Amsterdam, the Netherlands). Sequences of *tet*39 were deposited in Genbank with the accession numbers MH106412 to MH106425. Nucleotide sequences were aligned and compared, and then were used to infer a phylogenetic tree with MEGA7.0.14 (Kumar et al., 2016).

Statistical Analysis

The result of the bacterial count was plotted in histograms and box plots demonstrating means and standard deviation. The differences in the total bacterial count (TC) and the tetracycline-resistant bacterial count (TRBC) were compared by an analysis of variance (ANOVA) with $p < 0.05$ for significance, using Past 3.19 Software (Hammer et al., 2001).

RESULTS

Tetracycline-Resistant Bacteria Quantification and Isolation

Tetracycline-resistant bacteria were not isolated in tap water in any of the households. This study showed that the two isolation temperatures did not cause a significant difference ($p > 0.05$) in the total bacterial count in the different sampling locations (water, biofilm, and soil), but they did play a significant role ($p < 0.05$) in the tetracycline-resistant bacterial count (TRBC). The total bacterial count and the TRBC in all three examined systems in all sampling locations are presented in **Figure 1**. The total bacterial count in the RGW level of SYS1 was about an order of magnitude higher than in the other systems at both incubation temperatures. By contrast, the TRBC was lower and no significant differences ($p > 0.05$) were detected at the 25°C isolation temperature for the three households, while only for the SYS3 system was a detectable level of tetracycline-resistant bacteria found at 37°C.

In the soil irrigated with freshwater and with treated greywater, the total microbial counts at 25°C and 37°C were 5 Log CFU g^{-1} (**Figure 2**). Detectable levels of tetracycline-resistant bacteria were found only in the SYS2 (at 25°C) and SYS3 (at both incubation temperatures) systems.

The RVFCW systems were characterized by lower levels of total bacteria on the filter bed biofilm (average 8.50×10^4 CFU/g^{-1}) and while for SYS2, no tetracycline-resistant bacteria were detected, the SYS1 and SYS3 biofilm communities were characterized by resident tetracycline-resistant populations of between 2 and 4 Log CFU g^{-1}. TGW still retained a significant level of tetracycline-resistant bacteria, since in all systems, about 1.12×10^4 CFU mL^{-1} was present. Strongly significant differences ($p < 0.01$) were observed in the TRBC in TGW between the three systems, and in the SYS3 system, the highest level of tetracycline-resistant bacteria was found at 25°C (**Figure 3A**). Only in the SYS3 system were tetracycline-resistant bacteria isolated at both temperatures in TGW. In particular, it is possible to observe an increase in tetracycline-resistant bacteria at 25°C in treated greywater compared to raw greywater and biofilms (**Figure 3B**).

Identification of Tetracycline-Resistant Bacteria

Twenty-four species of tetracycline-resistant bacteria were identified at the genus level by 16S rDNA sequencing by Hy Laboratories Ltd. (**Table 2**). Even if the 16S rRNA gene was not sufficient to precisely identify a bacterial strain at the species level, we reported, in the results and the table, references to the relative species sequences that are closest to our strain. Only four of the tetracycline-resistant bacteria isolated were Gram-positive (17%), while the others were all Gram-negative (**Figure S2**). The isolated and characterized bacterial strains mainly belonged to the genera of *Serratia* (29%) and *Acinetobacter* (25%), while 62.2% of the isolated tetracycline-resistant bacteria belong to the class of *Gamma-Proteobacteria*.

TABLE 1 | PCR primers that were used in the work for screening of isolated strains.

Target gene	Primer name	Sequence (5′-3′)	Amplicon size (bp)	References
blaSHV	bla-SHV- F	CGCTTTCCCATGATGAGCACCTTT	110 bp	Xi et al., 2009
	bla-SHV-R	TCCTGCTGGCGATAGTGGATCTTT		
blaTEM	qblaTEM-F	TTCCTGTTTTTGCTCACCCAG	113 bp	Muyzer et al., 1993
	qblaTEM-R	CTCAAGGATCTTACCGCTGTTG		
blaCTX-M32	CTXM-F	CTATGGCACCACCAACGATA	156 bp	Bibbal et al., 2007
	CTXM-R	ACGGCTTTCTGCCTTAGGTT		
blaOXA-2	OXA-2 F	AAGAAACGCTACTCGCCTGC	478 bp	Bert et al., 2002
	OXA-2 R	CCACTCAACCCATCCTACCC		
blaOXA-10	OXA-10 F	TCAACAAATCGCCAGAGAAG	276 bp	Bert et al., 2002
	OXA-10 R	TCCCACACCAGAAAAACCA		
*tet*M	TetM-F	ACAGAAAGCTTATTATATAAC	171 bp	Aminov and Mackie, 2001
	TetM-R	TGGCGTGTCTATGATGTTCAC		
*tet*Q	TetQ-F	AGAATCTGCTGTTTGCCAGTG	169 bp	Aminov and Mackie, 2001
	TetQ-R	CGGAGTGTCAATGATATTGCA		
*tet*W	TetW-F	GAGAGCCTGCTATATGCCAGC	168 bp	Aminov and Mackie, 2001
	TetW-R	GGGCGTATCCACAATGTTAAC		
*tet*A	Tet A-F	GCGCGATCTGGTTCACTCG	164 bp	Aminov et al., 2002
	Tet A-R	AGTCGACAGYRGCGCCGGC		
*tet*B	Tet B- F	TTGGTTAGGGGCAAGTTTTG	659 bp	Fan et al., 2007
	TetB- R	GTAATGGGCCAATAACACCG		
*tet*39	tet(39)-F	CTCCTTCTCTATTGTGGCTA	701 bp	Adelowo and Fagade, 2009
	tet(39)-R	CACTAATACCTCTGGACATCA		

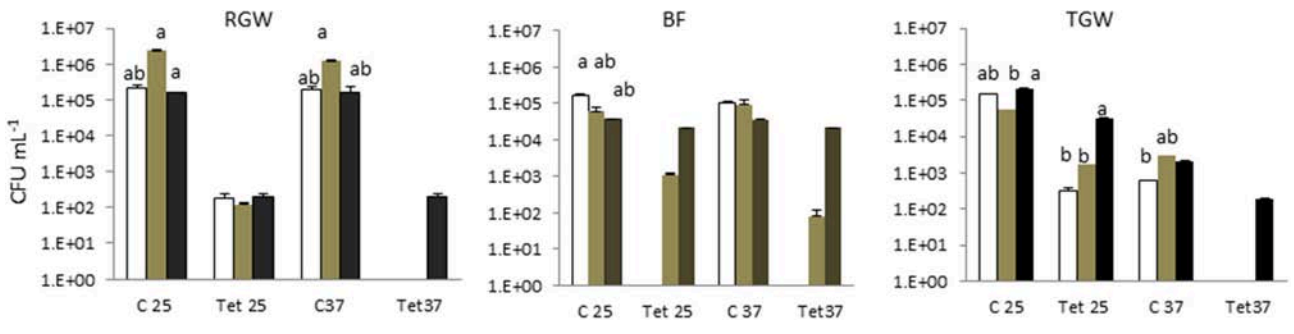

FIGURE 1 | Total bacterial count (TBC) and tetracycline (tet)-resistant bacterial count (TRBC) in the three examined systems in all sampling locations. SYS1 □, SYS 2 ■, SYS 3 ■. RGW (raw greywater); BF (biofilm); TGW (treated greywater). C25: total bacterial count (TC), incubation at 25°C. Tet25, tetracycline-resistant bacterial count (TRBC), incubation at 25°C; C37, TC, incubation at 25°C; Tet37, TRBC, incubation at 37°C. Error bars represent standard deviation of duplicate plate counts for each of the three examined systems in all sampling location. $^{a, ab, b}$ superscript letters indicate significant differences ($P < 0.05$) between the three systems.

Effect of Chlorination on ARB Survival

We selected two *Serratia* strains with high tetracycline resistance as indicators for the chlorination effectiveness of the TGW. The results show that the two strains of *Serratia* isolated from RGW and BF (**Figure 4**) were able to survive despite having been exposed to a high concentration of chlorine (2 mg L^{-1}). In particular, the growth of the *Serratia* strain isolated from RGW at 37°C was inhibited after the second hour, while the *Serratia* strain isolated from the biofilm samples at 25°C decreased only by 90% (10% survival) after 4 h in comparison to the non-chlorinated control.

Minimum Inhibition Concentration (MIC)

The MIC's results were compared with the epidemiological cut-off values for resistance (ECOFFs) established by EUCAST: all the microorganism with acquired resistance showed higher MIC values than the epidemiological cut-off value, so according to EUCAST all the microorganism were very resistant to antibiotics (http://www.eucast.org/mic_distributions_and_ecoffs). To highlight the different resistance levels of isolated microorganisms, according to the obtained MIC values (μg mL-1), five different resistance levels were identified for the isolated strains, as follows: sensitive (S): MIC values between

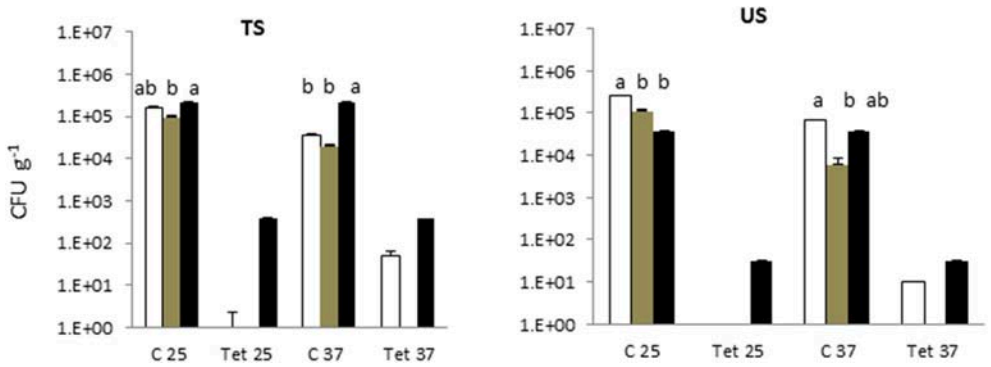

FIGURE 2 | Total bacterial count (TBC) and tetracycline (tet)-resistant bacterial count (TRBC) in the in the soils of the three examined systems in all sampling locations. SYS1 □, SYS 2 ■, SYS 3 ■. TS (greywater irrigated soil); US (freshwater-irrigated soil). C25, total bacterial count (TC), incubation at 25°C. Tet25, tetracycline-resistant bacterial count (TRBC), incubation at 25°C; C37, TC, incubation at 25°C; Tet37, TRBC, incubation at 37°C. Error bars represent standard deviation of duplicate plate counts for each of the three examined systems in all sampling location. $^{a, ab, b}$ superscript letters indicate significant differences ($P < 0.05$) between the three systems.

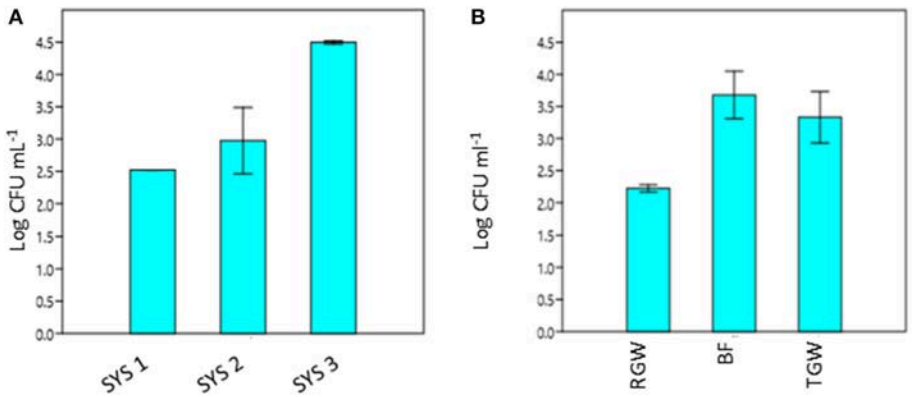

FIGURE 3 | Tetracycline-resistant bacterial count (TRBC) at 25°C from treated greywater (TGW) from the three systems **(A)** and in RGW, biofilm and TGW samples from the three systems **(B)**. RGW, raw greywater; BF, biofilm; TGW, treated greywater; Log CFU mL^{-1}, tetracycline-resistant bacterial count (TRBC). Error bars indicate SD of duplicate plate counts from a single system **(A)** and from each sampling point **(B)**.

0 and 50 μg mL^{-1}; low resistance (L): MIC values between 50 and 100 μg mL^{-1}; medium resistance (M): MIC values between 150 and 350 μg mL^{-1}; high resistance (H): MIC values between 350 and 500 μg mL^{-1}; and very high resistance (VH): MIC values higher than 500 μg mL^{-1}. Based on this classification, most of the tetracycline-resistant bacteria isolated were considered to have medium resistance (**Table 3**). Only one *Serratia* strain (TW5) isolated from the TGW of SYS3 had a lower resistance (L).

Five strains (SYS3-TW4, SYS3-RW9, SYS3-TS11, SYS3-US12, and SYS3-BF15) were able to grow at higher tetracycline concentrations than those tested (≥ 500 μg/ml), so they have a very high resistance (VH). Five strains (SYS3-TW5, SYS1-RW7, SYS3-RW8, TWRW9, and SYS3-TS11) were also shown to be resistant to the other three tested antibiotics. It has been observed that tetracycline-resistant bacteria were more sensitive to ciprofloxacin than to amoxicillin and kanamycin. Nine tetracycline-resistant bacteria (SYS2-TW3, SYS1-RW6, SYS3-RW8, SYS1-TS10, SYS3-S11, SYS2-BF13, SYS3-TW16, SYS3-RW18, and SYS3-BF24) showed a medium resistance to amoxicillin, and two (SYS3-RW8 and SYS3-TS11) of them were also able to survive at higher concentrations of kanamycin (higher than 300 mg L^{-1}).

Tetracycline Resistance Gene Characterization

Based on the PCR analysis, none of the isolated resistant strains were positive for the *tet*A or *tet*B (efflux) or for the *tet*M, *tet*Q, or *tet*W (ribosomal) genes (**Table 4**). It was found that 58% of the tetracycline-resistant isolates were positive for *tet*39, all isolated at 25°C. All the tetracycline-resistant bacteria were also positive for at least one of the β-lactamase genes tested. In particular, 79% were positive for blaTEM, 58% were positive for blaCXTM-32, 67% were positive for blaOXA-2, 12.5% for blaOXA-10 and only 8% for blaSHV.

TABLE 2 | Identification of isolated tetracycline-resistant strains by 16SrDNA sequencing.

Strain[1]	16s rDNA	GenBank acc. num.	Closest relative species[b]	GenBank acc. Num.
SYS1-TW1	*Acinetobacter* sp.	MH090940.1	*Acinetobacter tjernbergiae*	NR117629.1
SYS1-TW2	*Acinetobacter* sp.	MH090941.1	*Acinetobacter tjernbergiae*	KM070562.1
SYS2-TW3	*Acinetobacter* sp.	MH090942.1	*Acinetobacter tjernbergiae*	KR094129.1
SYS3-TW4	*Acinetobacter* sp.	MH090943.1	*Acinetobacter junii*	AM184300.1
SYS3-TW5	*Serratia* sp.	MH090946.1	*Serratia marcescens*	CP018925.1
SYS1-RW6	*Acinetobacter* sp.	MH090944.1	*Acinetobacter junii*	AM184300.1
SYS1-RW7	*Serratia* sp.	MH090947.1	*Serratia marcescens*	CP018924.1
SYS3-RW8	*Elizabethkingia* sp.	MH090953.1	*Elizabethkingia meningoseptica*	MG982467.1
SYS3-RW9	*Serratia* sp.	MH090948.1	*Serratia marcescens*	HG738868.1
SYS1-TS10	*Achromobacter* sp.	MH090954.1	*Achromobacter insolitus*	CP026973.1
SYS3-TS11	*Lysobacter* sp.	MH090955.1	*Lysobacter enzymogenes*	AP014940.1
SYS3-US12	*Acinetobacter* sp.	MH090945.1	*Acinetobacter junii*	AM184300.1
SYS2-BF13	*Chryseobacterium* sp.	MH090956.1	*Chryseobacterium* sp.	JQ582957.1
SYS3-BF14	*Serratia* sp.	MH090949.1	*Serratia marcescens*	CP026702.1
SYS3-BF15	*Stenotrophomonas* sp.	MH090958.1	*Stenotrophomonas maltophilia*	MG982475.1
SYS3-TW16	*Serratia* sp.	MH090950.1	*Serratia marcescens*	MG982466.1
SYS3-RW17	*Serratia* sp.	MH090951.1	*Serratia marcescens*	EU048327.1
SYS3-RW18	*Serratia* sp.	MH090952.1	*Serratia marcescens*	CP026702.1
SYS1-TS19	*Chryseobacterium* sp.	MH090957.1	*Chryseobacterium lathyri*	KY933466.1
SYS1-US20	*Rummeliibacillus* sp.	MH090960.1	*Rummeliibacillus stabekisii*	CP014806.1
SYS2-BF21	*Bacillus* sp.	MH090961.1	*Bacillus cereus*	MF355368.1
SYS2-BF22	*Bacillus* sp.	MH090962.1	*Bacillus cereus*	MF355367.1
SYS2-BF23	*Bacillus* sp.	MH090963.1	*Bacillus cereus*	MF800922.1
SYS3-BF24	*Stenotrophomonas* sp.	MH090959.1	*Stenotrophomonas maltophilia*	MG982475.1

[a] *The strain ID represent the system number, the isolation location and isolate number. Raw greywater-RW, Treated grey water-TW, Biofilm on filter-BF, soil irrigated with treated grey water TS, soil irrigated with freshwater- US.*
[b] *All closest relative species showed 99% sequence homology.*

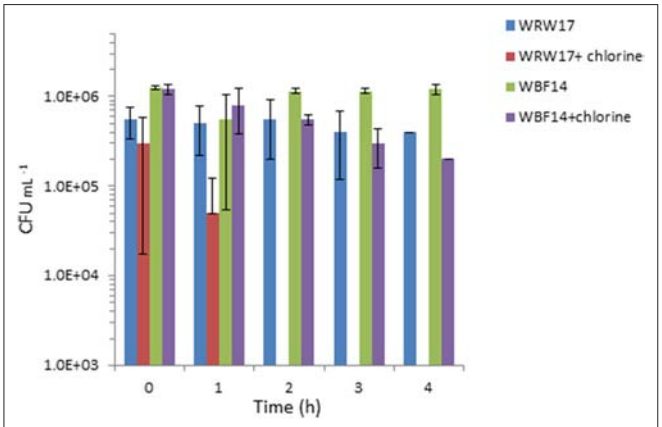

FIGURE 4 | Effect of chlorination (2 mg L^{-1} NaClO) of treated greywater on the survival of two strains of isolated *Serratia* (SYS3-RW17 and SYS3-BF14). T_0 represents the initial contact time with the chlorine. After 2 h, WRW17+chlorine counts were "0".

Sequencing of the *tet*39 Gene

Because *tet*39 (conferring resistance via an active efflux pump) was found to be the most abundant resistance gene determinant, its PCR amplicons were sequenced for a better understanding. The results revealed that two different genotypes belonging to two clusters (cluster A and cluster B) were randomly observed among *tet*39 resistance bacteria (**Figure 5**). In both clusters, the *tet*39 sequences were very similar across different genera.

DISCUSSION

The need to treat greywater before reuse at a local scale led to the development of a small on-site RVFCW bioreactor that is effective in removing chemical and biological contaminants (see **Table S2**) (Gross et al., 2007). However, the possible risk of spreading opportunistic pathogens after the treatment was also considered (Benami et al., 2016). Because of the proximity between the treatment units and the point of greywater reuse, it is also important to investigate other microbiological factors such as antibiotic resistance in the treated greywater's microbial community.

The presence of antibiotic-resistant genes (ARGs), such as tetracycline and beta-lactam resistance genes, have been reported in wastewater (Szczepanowski et al., 2009; Karkman et al., 2017; Voolaid et al., 2018), but the current study represents the first investigation on ARB in greywater. Previous studies on ARB in municipal wastewater (Huang et al., 2012; Harnisz et al., 2015) reported tetracycline-resistant bacterial levels in the range

TABLE 3 | Minimum inhibitory concentration (MIC) of the tested antibiotics on the tetracycline-resistant bacterial strain.

Strain	Microrganism[a]	MIC (ug mL^{-1})			
		Tet	Amox	Kana	Cipro
SYS1-TW1	*Acinetobacter tjembergiae*	300	S	S	S
SYS1-TW2	*Acinetobacter tjembergiae*	250	S	S	S
SYS2-TW3	*Acinetobacter tjembergiae*	250	150	S	S
SYS3-TW4	*Acinetobacter junii*	≥500	S	S	S
SYS3-TW5	*Serratia marcescens*	50	150	≤50	100
SYS1-RW6	*Acinetobacter junii*	250	S	≤50	≤50
SYS1-RW7	*Serratia marcescens*	400	≤50	≤50	≤50
SYS3-RW8	*Elizabethkingia endophytica*	150	≥300	≥300	≤50
SYS3-RW9	*Serratia marcescens*	≥500	80	100	≤50
SYS1-TS10	*Achromobacter insolitus*	250	150	200	S
SYS3-TS11	*Lysobacter enzymogenes*	≥500	≥300	200	≤50
SYS3-US12	*Acinetobacter junii*	≥500	S	S	S
SYS2-BF13	*Chryseobacterium* sp.	300	≥300	≥300	S
SYS3-BF14	*Serratia marcescens*	300	S	150	≤50
SYS3-BF15	*Stenotrophomonas maltophilia*	≥500	S	200	≤50
SYS3-TW16	*Serratia marcescens*	≥350	150	S	S
SYS3-RW17	*Serratia marcescens*	≥350	50	S	S
SYS3-RW18	*Serratia marcescens*	350	150	<50	S
SYS1-TS19	*Chryseobacterium lathyri*	≥350	S	150	100
SYS1-US20	*Rummeliibacillus stabekisii*	300	S	S	S
SYS2-BF21	*Bacillus cereus*	300	S	S	S
SYS2-BF22	*Bacillus cereus*	350	S	S	S
SYS2-BF23	*Bacillus cereus*	300	S	S	S
SYS3-BF24	*Stenotrophomonas maltophilia*	300	150	S	S

[a] *The names refer to the closest relative species identified by 16SrDNA sequencing (**Table 2**).*

Tet, Tetracycline; Amox, Amoxicillin; Kana, Kanamycin; Cipro, Ciprofloxacin.

≤ = microorganism growth was inhibited by the lowest concentration of the antibiotic tested.

≥ = microorganism growth was not inhibited by the higher concentration of antibiotic tested.

Green, Sensitive; Yellow, Lower (< 50 to 100 μg mL^{-1}); Orange, Medium (from 150 to 350 μg mL^{-1}); Red, High (from 350 to 500 μg mL^{-1}); Dark red, Very High (≥500 μg mL^{-1}).

of 10^2-10^3 CFU mL^{-1}, and their findings are consistent with our present results for treated greywater (TGW) in the SYS1 and SYS2 systems at 25°C. In SYS3 system, the tetracycline-resistant bacterial count (TRBC) was higher than in SYS1 and SYS2 systems (**Figure 3A**). These differences may be related to many factors such as health status of inhabitants, age, number or lifestyles. Our study, however, did not examine these parameters, so the origin of the TRBC remains uncertain. Our study also examined whether the irrigation caused a buildup of resistance in the soil; it is worth noting that a significant difference in the TRBC was not observed ($p > 0.05$) in the freshwater and treated greywater irrigated soils (**Figure 1**). These findings are in agreement with previous studies which demonstrated that irrigation with wastewater does not seem to impact antibiotic resistance levels in the soil microbiome (Gatica and Cytryn, 2013). However, we cannot exclude the greywater as a possible source of the tetracycline-resistant bacteria even if the existence of tetracycline-resistant strains in both treated and untreated soil could prove that the greywater is not the only contamination source. We also need to consider the possibility of a cross- or direct contamination caused by humans or animals that could contribute to the spread of ARB, bypassing the irrigation water route. A recent publication regarding the contribution of treated effluents to the soil resistome stated that while antibiotic resistance levels in soil are increased temporally by land application of wastes, their persistence is not guaranteed and is, in fact, variable, and often contradictory, depending on the application site (Pepper et al., 2018).

In all three systems, we observed a significant ($p < 0.05$) increase in the TRBC at 25°C in BF and in TGW compared to RGW (**Figure 3B**). Bacteria retained inside the filters could be the explanation for this observation. The RVFCW can be considered as a biofilm-based wastewater treatment system such as a trickling filter wastewater treatment. Balcázar et al. (2015) proposed, based on many studies, that environmental biofilms are true reservoirs of ARGs. Thus, a concentration effect within the system is a possible explanation for the presence of tetracycline-resistant bacteria in the treated water. In contrast, however, a recent study that compared abundances of ARGs in activated sludge and a trickling filter suggested that there is no difference in the prevalence of ARG mobilization in the treated effluents (Petrovich et al., 2018).

TABLE 4 | Occurrence of tetracycline and β-lactamase resistance genes in the bacterial isolates, determined by PCR.

				Beta-lactamase		Tetracycline[b]	
Strain	**Microorganism[a]**	**blaTEM**	**BlaCTM_32**	**blaSHV**	**blaOXA-2**	**blaOXA-10**	**Tet(39)**
SYS1-TW1	*Acinetobacter tjembergiae*	+	−	−	−	−	+
SYS1-TW2	*Acinetobacter tjembergiae*	+	−	−	+	−	+
SYS2-TW3	*Acinetobacter tjembergiae*	+	+	−	−	−	+
SYS3-TW4	*Acinetobacter junii*	+	−	−	+	−	+
SYS3-TW5	*Serratia marcescens*	+	−	−	−	−	+
SYS1-RW6	*Acinetobacter junii*	+	+	−	+	−	+
SYS1-RW7	*Serratia marcescens*	−	−	−	−	−	+
SYS3-RW8	*Elizabethkingia endophytica*	+	+	−	+	−	+
SYS3-RW9	*Serratia marcescens*	+	−	−	+	−	+
SYS1-TS10	*Achromobacter insolitus*	+	+	−	+	−	+
SYS3-TS11	*Lysobacter enzymogenes*	+	+	−	+	−	−
SYS3-US12	*Acinetobacter junii*	+	−	−	+	+	+
SYS2-BF13	*Chryseobacterium* sp.	+	−	+	−	−	+
SYS3-BF14	*Serratia marcescens*	+	+	+	+	−	+
SYS3-BF15	*Stenotrophomonas maltophilia*	+	+	−	+	−	+
SYS3-TW16	*Serratia marcescens*	−	−	−	+	−	−
SYS3-RW17	*Serratia marcescens*	+	+	−	+	−	−
SYS3-RW18	*Serratia marcescens*	+	+	−	+	−	−
SYS1-TS19	*Chryseobacterium lathyri*	−	+	−	+	−	−
SYS1-US20	*Rummeliibacillus stabekisii*	+	+	−	+	−	−
SYS2-BF21	*Bacillus cereus*	+	+	−	+	−	−
SYS2-BF22	*Bacillus cereus*	−	−	−	−	−	−
SYS2-BF23	*Bacillus cereus*	+	+	−	−	+	−
SYS3-BF24	*Stenotrophomonas maltophilia*	−	+	−	−	+	−

[a] *The names refer to the closest relative species identified by 16SrDNA sequencing (**Table 1**).*
[b] *Only tet39 is reported among the six tet genes examined, since the strains were all negative for the other five genes.*

In our study, it was not possible to make a comparison at 37°C between the TRBCs in the three systems, since at 37°C, the tetracycline-resistant bacteria were isolated only in the number three system. We hypothesize that the resistant bacteria that grow best at 37°C represent enteric or fecal microorganisms. The low detection of these bacteria agrees with the effective removal of fecal coliform to the level of 2 CFU per 100 ml in the examined RVFCW after disinfection (Benami et al., 2016). Our results suggest that these three systems were unable to prevent ARB survival after greywater treatment, and to achieve this goal, additional treatment methods need to be included, such as the use of chlorine or UV disinfection. Our results showed that chlorination was effective in immediately inactivating three out of five tested isolated *Serratia* strains, so ARB removal could be a possible solution, even if some strains can survive a longer contact time (**Figure 3**). As reported in the literature, conflicting results still exist concerning ARB removal by chlorination (Yuan et al., 2015). Some researchers reported effective ARB reduction using this method (Huang et al., 2011), whereas other results indicated that chlorination did not significantly reduce ARB (Munir et al., 2011).

The isolated and characterized bacterial strains belonged mainly to the genera *Serratia* sp. (29%) and *Acinetobacter* sp. (25%). As previously mentioned, *Acinetobacter* sp. is a particularly suitable genus for monitoring antibiotic resistance in the environment; in fact, until recently, bacterial screening of WWTP influents and effluents usually focused on *Acinetobacter* spp. (Zhang Y. et al., 2009; Voolaid et al., 2018). Similarly to our case, in a previous study on treated wastewater, tetracycline-resistant strains of *Serratia marcescens* and *Acinetobacter spp*. were isolated (Harnisz et al., 2015). In that case, the simultaneous presence of two resistance determinants, *tet*(A) and *tet*(B), was documented, while in our experiment, all the tetracycline-resistant strains were negative for these two genes. It must be noted that a fecal indicator bacteria survey was not conducted since other authors already tested the same systems for this purpose (Benami et al., 2013).

According to our classification, 62.5% of the bacteria showed at least a medium resistance to tetracycline. It should be mentioned that according to EUCAST's epidemiological cut-off values for AR (ECOFFs) (The European Committee on Antimicrobial Susceptibility Testing, 2018) MIC levels above $>100\,g\ mL^{-1}$ are already considered a high resistance trait. Thus, the possible spread of high dose antibiotic resistance determinants in the environment in which greywater is used for irrigation is worthy of concern.

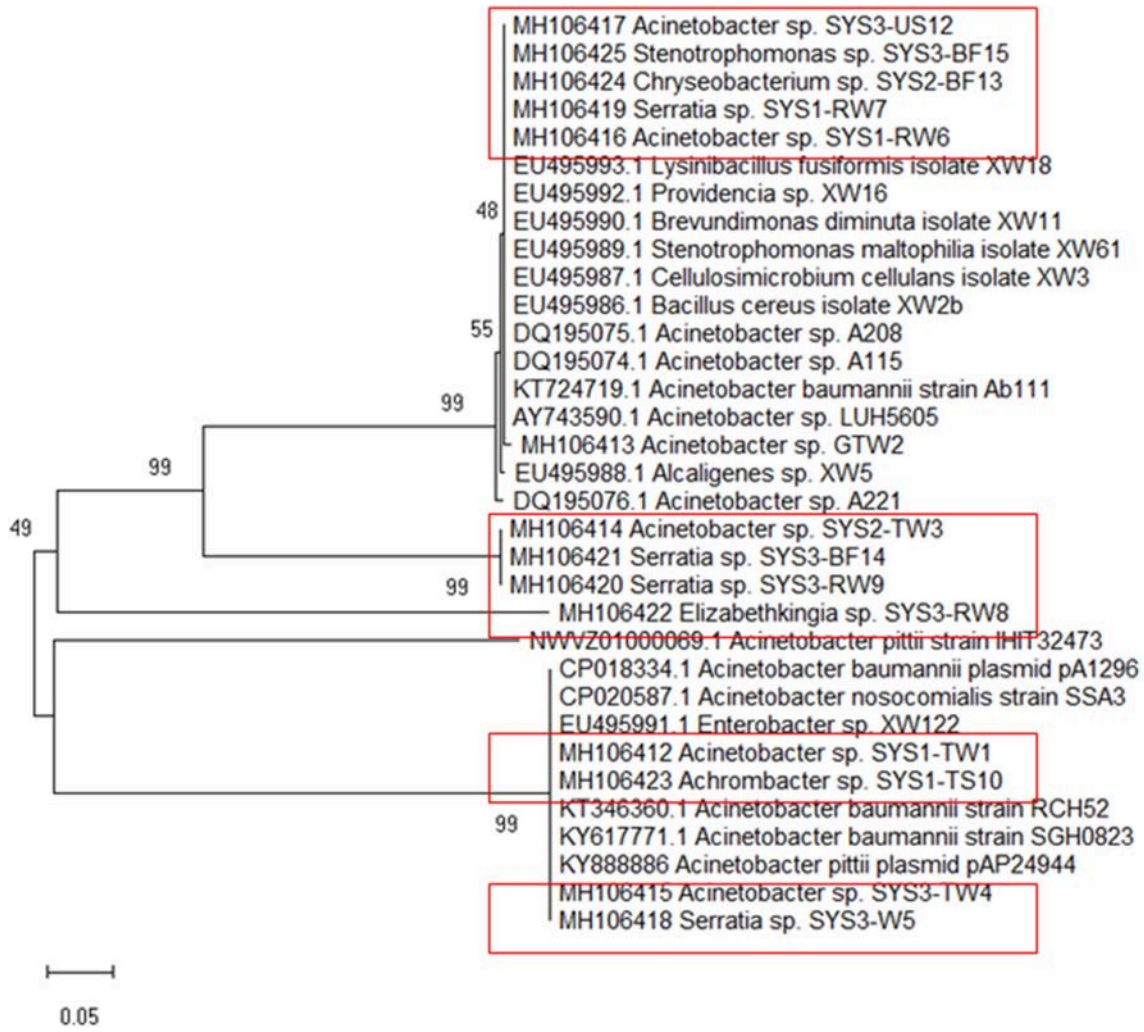

FIGURE 5 | Phylogenetic tree of *tet*39 genes sequenced from the positive strains (in box) was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Kumar et al., 2016).

In this study, tetracycline-resistant bacteria were positive only for *tet*39, and this confirmed the fact that even if *tet*39 remains closely associated with *Acinetobacter* spp., its plasmid location should enable dissemination to other species (Coyne et al., 2011). Initially, it was thought that the *tet*39 gene was one of the efflux genes unique to environmental bacteria (Roberts, 2011), but then it was understood that this gene, associated with mobile elements, could be transferred back and forth between environmental and non-environmental bacteria.

We focused our work on six tetracycline resistance genes from the 46 genes currently known. They were the *tet*A, *tet*B, and *tet*39 (efflux), and *tet*M, *tet*Q and *tet*W (ribosomal) genes that are the most common tetracycline resistance genes among Gram-negatives (Zhang Y. et al., 2009; Roberts, 2011; Roberts et al., 2015a). The search of all the genes that determine the tetracycline resistance in the isolated bacteria falls beyond the scope of the manuscript. Other surveys of tetracycline resistance genes focused on covering the most common genes (Henriques et al., 2008; Nikolakopoulou et al., 2008; Tao et al., 2010; Harnisz et al., 2015).

We also need to consider that the Gram-negative efflux genes are widely distributed and generally associated with large plasmids, most of which are conjugative, which often carry other antibiotic resistance genes. This phenomenon contributed to the dramatic increase of the multiple-drug-resistant bacteria over the last 40 years (Chopra and Roberts, 2001). Many of the tetracycline efflux resistance genes are found mostly in environmental strains but can also be found in bacteria associated with humans and animals (Roberts, 2011).

It is noteworthy that the majority of the isolated strains shared *tet*39, independent of the source (water, biofilm, and soil) and the location (SYS1, 2, and 3), even if we cannot exclude the possibility that isolated strains may harbor other tetracycline resistance genes. It has been reported that both Gram-positives and more than 10% of Gram-negatives could carry multiple tetracycline resistance genes (Roberts, 2005); thus it is essential to specify that

the different tetracycline genes can have either the same mode of action (efflux or ribosomal protection) or different modes of action (efflux and ribosomal protection), as do the pathogenic and opportunistic species (Chopra and Roberts, 2001).

All the isolated *tet*39 resistance bacteria were positive for at least one of the β-lactamase genes tested. In fact, the majority of the 30 tetracycline resistance efflux genes are usually associated with plasmids (Roberts, 2005) that often carry other antibiotic resistance genes (such as those that confer aminoglycoside, β-lactam resistance), heavy metal resistance genes, or pathogenic factors such as toxins (Chopra and Roberts, 2001). Therefore, it indicates the increasing possibility of multidrug resistance and environmental dissemination.

Except for two isolates, beta-lactam resistance genes have been found in all amoxicillin-susceptible bacteria, confirming the fact that even low concentrations of antibiotics can result in the selection of ARGs. This makes it very difficult to establish a safe concentration of an antibiotic compound in wastewater (Karkman et al., 2017).

The isolated strains showed *tet*39 sequences belonging to two different genotypes separated in two distinct clusters (A and B) by cluster analysis (**Figure 4**). It must be highlighted that the genotype associated with cluster A in this study has been identified in bacteria belonging to different genera (*Bacillus* sp., *Acinetobacter* sp., *Stenotrophomonas* sp.) isolated from environmental samples, mostly of aquatic origin (Agersø and Guardabassi, 2005; Adelowo and Fagade, 2009; Roberts and Schwarz, 2015b; Hamidian et al., 2016). Additionally, the genotype associated with cluster B in this study has been identified in bacteria isolated from clinical samples such as blood or sputum Adelowo and Fagade, 2009; Hamidian et al., 2016; Yoon et al., 2017). In our study, *tet*39 resistance bacteria harbored both genotypes (environmental and clinical), independent of source and isolation temperature. This observation demonstrates the possible transfer of the *tet*39 gene between bacteria of clinical and environmental origin.

It is interesting to note that the *tet*39 gene from bacteria of clinical origin was not present among the samples isolated from the SYS3, but given the limited number of samples, we cannot be entirely confident of its absence.

Among the *tet*39 resistance bacteria belonging to the two different clusters, there were no significant differences between the tetracycline's MIC values. This is most likely due to the fact that other genes are involved in the resistance to tetracycline; indeed, as previously noted, all Gram-positives and more than 10% of Gram-negatives could carry multiple tetracycline genes (Roberts, 2005).

CONCLUSIONS

The ARB isolated in this study were not obligatory pathogens. The fact that *tet*39 was the dominant resistance gene may arise from its broad host range. Like other biological wastewater treatment systems, the RVFCW system does not remove all of the ARB present in the raw greywater. Most likely, the filter bed biofilm of the system contributed to the ARB community in the treated effluents. Thus, additional treatment methods such as chlorination need to be included in this system to minimize the ARB numbers in the effluent. Interestingly, the ARB abundance in the TGW-irrigated soil and the freshwater-irrigated soil did not alter, suggesting that ARB did not accumulate in the TGW-irrigated soil.

To safely eliminate ARB from greywater, further studies should be carried out to understand how the transfer of ARGs occurs. Of particular importance is the determination of whether specific compounds abundant in greywater (e.g., detergents) lead to resistance evolution. This work dealt with the detection of Tetracycline-resistant bacteria and Tetracycline resistant genes on greywater, in the system and the soil also evaluating the potential multiple resistance. Preliminary genetic relations between the tet39 genes isolated showed a possible exchange between clinical and environmental strains. However, further study needs to be done to understand the clonal relations between the isolates better understand the clonal relations between the isolates and strengthen our results.

AUTHOR CONTRIBUTIONS

ET performed the experiments, analyzed the data, and wrote a draft manuscript. LB analyzed the data and supervised the writing of the draft manuscript. AG aided in interpreting the results and worked on the manuscript. ZR conceived the study and was in charge of the overall direction and planning, as well as writing the manuscript.

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SUPPLEMENTARY MATERIAL

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An Insight Into the Potentiation Effect of Potassium Iodide on aPDT Efficacy

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Antimicrobial photodynamic therapy (aPDT) is gaining a special importance as an effective approach against multidrug-resistant strains responsible of fatal infections. The addition of potassium iodide (KI), a non-toxic salt, is recognized to increase the aPDT efficiency of some photosensitizers (PSs) on a broad-spectrum of microorganisms. As the reported cases only refer positive aPDT potentiation results, in this work we selected a broad range of porphyrinic and non-porphyrinic PSs in order to gain a more comprehensive knowledge about this aPDT potentiation by KI. For this evaluation were selected a series of *meso*-tetraarylporphyrins positively charged at meso positions or at β-pyrrolic positions and the non-porphyrinic dyes Methylene blue, Rose Bengal, Toluidine Blue O, Malachite Green and Crystal Violet; the assays were performed using a bioluminescent *E. coli* strain as a model. The results indicate that KI has also the ability to potentiate the aPDT process mediated by some of the cationic PSs [**Tri-Py(+)-Me**, **Tetra-Py(+)-Me**, **Form**, **RB**, **MB**, **Mono-Py(+)-Me**, **β-ImiPhTPP**, **β-ImiPyTPP**, and **β-BrImiPyTPP**] allowing a drastic reduction of the treatment time as well as of the PS concentration. However, the efficacy of some porphyrinic and non-porphyrinic PSs [**Di-Py(+)-Me *opp***, **Di-Py(+)-Me *adj***, **Tetra-Py**, **TBO**, **CV**, and **MG**] was not improved by the presence of the coadjuvant. For the PSs tested in this study, the ones capable to decompose the peroxyiodide into iodine (easily detectable by spectroscopy or by the visual appearance of a blue color in the presence of amylose) were the most promising ones to be used in combination with KI. Although these studies confirmed that the generation of $^1O_2$ is an important fact in this process, the PS structure (charge number and charge position), aggregation behavior and affinity for the cell membrane are also important features to be taken in account.

Keywords: antimicrobial photodynamic therapy, cationic porphyrins, phenothiazines, xanthenes, potassium iodide, bioluminescent *E. coli*

INTRODUCTION

Antibiotics are among the most commonly prescribed drugs used in both human medicine and in farm animals, resulting in the selection of multiple drugs resistant (MDR) bacteria (Economou and Gousia, 2015; O'Neill, 2016). Infections with resistant bacteria are difficult to treat, causing severe illness and requiring costly and sometimes toxic alternatives, such as antibiotics of last resort.

Drugs of last resort, such as vancomycin against Gram-positive bacteria and colistin against Gram-negative bacteria, have been the most reliable therapeutic agents against MDR bacteria. However, bacterial strains resistant to these antibiotics have been isolated worldwide (Levine, 2006; Wang et al., 2018). This resistance can result from a chromosomal gene mutation, but comes mainly from horizontal transfer from external gene sources (Chambers and DeLeo, 2009; DeLeo et al., 2010; Gardete and Tomasz, 2014; Gao et al., 2016). The development of novel antibiotics is not likely to solve the problem and it is probably only a matter of time until they will be also ineffective. Bacteria will inevitably find ways of resisting to the conventional antibiotics, which is why alternative approaches are urgent.

Antimicrobial photodynamic therapy (aPDT) can be a very promising alternative to antibiotic treatment namely in localized infections (Dai et al., 2010). aPDT involves the use of a photosensitizer (PS) which in the presence of visible light and oxygen produces reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$). These species are responsible for the oxidation of several cellular components conducting to rapid cell inactivation. This approach presents some advantages when compared with the use of antibiotics, such as being efficient independently of the microorganism antibiotic resistance profile (Jori et al., 2011), does not induce the development of resistance, even after several cycles of treatment (Giuliani et al., 2010; Tavares et al., 2010; Costa et al., 2011) and can be applied with efficacy against Gram-negative and Gram-positive bacteria. aPDT is considered more effective against Gram-positive bacteria due to their highly permeable cell walls allowing the easy diffusion of neutral, positive and negative charged PS into the cell. However, the impermeable external membrane of Gram-negative bacteria cell wall limits the anionic or neutral-charge PSs entrance (Minnock et al., 2000). This limitation is overcome by the use of cationic PS. These PSs are able to bind and penetrate into the cell wall by the "self-promoted uptake pathway" (Hancock et al., 1991; Merchat et al., 1996). Nevertheless, neutral PSs or PSs with low number of charges can be effective against this type of bacteria by coupling or combining them with positively charged entities such as poly-L-lysine, polyethylenimine and polymyxin B nonapeptide that act as membrane disruptors (Nitzan et al., 1992; Helander et al., 1997; Lounatmaa et al., 1998; Soukos et al., 1998). Ethylenediaminetetraacetic acid (EDTA) is also commonly used to destabilize the native organization of Gram-negative wall (Yoshimura and Nikaido, 1985; Jori et al., 2006). It has also been shown that different organic salts can improve the efficiency of aPDT against Gram-negative bacteria (Huang et al., 2012; Kasimova et al., 2014). Recently, some studies have demonstrated that aPDT can be potentiated by addition of several different inorganic salts, such as sodium bromide (Wu et al., 2016) sodium azide (Huang et al., 2012; Kasimova et al., 2014), sodium thiocyanate (St Denis et al., 2013) and potassium iodide (Vecchio et al., 2015; Zhang et al., 2015; Freire et al., 2016; Huang et al., 2016, 2017, 2018a,c; Hamblin, 2017; Reynoso et al., 2017; Wen et al., 2017). In fact, the addition of iodide has been shown to improve the efficiency of aPDT in several animal models of localized infection. This salt is non-toxic and is an approved drug for antifungal therapy (Hamblin, 2017). The studies involving the use of KI demonstrate that the combination of this salt with neutral porphyrins, fullerenes and other dyes gives rise to higher microbial inactivation rates when are compared to the use of the PSs alone. KI was firstly studied as potentiator of aPDT mediated by a C_{60} fullerene bisadduct (Zhang et al., 2015). The results showed that KI potentiated the ultraviolet A (UVA) or the white light-mediated killing of Gram-negative bacteria *Acinetobacter baumannii*, Gram-positive methicillin-resistant *Staphylococcus aureu*s and fungal yeast *Candida albicans*, increasing the effect in 1–2 logs. This extra killing effect was also observed *in vitro* and *in vivo* using a mouse model with an infected skin abrasion (Zhang et al., 2015). These promising results conducted to new studies concerning the mechanism of action involved. The KI effect using Methylene Blue (MB) as PS in the photoinactivation of *Escherichia coli* and *S. aureus* was also evaluated (Vecchio et al., 2015). The results showed that the addition of KI increased the bacterial killing in 4 and 2 logs for *S. aureus* and *E. coli*, respectively, in a dose-dependent manner. The authors also affirmed that the KI potentiator effect in these aPDT studies mediated by MB was probably due to the formation of reactive iodine species that were quickly produced with a short lifetime (Vecchio et al., 2015). Since then, some other examples of the potentiation of aPDT effect using combinations of PSs and KI were reported. For instance, MB and new methylene blue (NMB) were studied in the photoinactivation of oral *C. albicans* infection in a mouse model (Freire et al., 2016), Photofrin in the photoinactivation of several Gram-negative bacteria (Huang et al., 2017), BODIPY dyes in the photoinactivation of *S. aureus*, *E. coli*, and *C. albicans* (Reynoso et al., 2017). This approach was also efficient in aPDT of Gram-negative and Gram-positive bacteria mediated by Rose Bengal (Wen et al., 2017) and fullerenes (Huang et al., 2018b). Interestingly, an anionic porphyrin in the presence of KI was able to photoinactivate *E. coli* (Huang et al., 2018a). The combination of MB and KI was also efficient to treat an urinary tract infection in a female rat model (Huang et al., 2018c). All these reports helped to elucidate the mechanism of action of KI potentiation. It was proposed that the extra killing effect is caused by several parallel reactions initiated by the reaction of $^1O_2$ with KI producing peroxyiodide (**Figure 1**), that can suffer further decomposition by two different pathways, which are dependent on the degree of binding of the PS to the microbial cells (Vecchio et al., 2015; Zhang et al., 2015; Freire et al., 2016; Gsponer et al., 2016; Hamblin, 2017; Huang et al., 2017, 2018a; Kashef et al., 2017; Reynoso et al., 2017; Wen et al., 2017). One of the pathways involves the formation of free iodine (I_2/I_3^-) and hydrogen peroxide (H_2O_2). Free iodine can kill microbial cells when generated in solution but needs to reach a sufficient threshold concentration to be microbicidal. The amount of free iodine produced depends on the amount of $^1O_2$ produced, but also on the concentration of iodide anion present in solution (**Figure 1**). The other one involves a homolytic cleavage process producing reactive iodine radicals ($I_2^{\cdot-}$), which are much more toxic if generated very close to the target cells since these radicals have short diffusion distance (**Figure 1**).

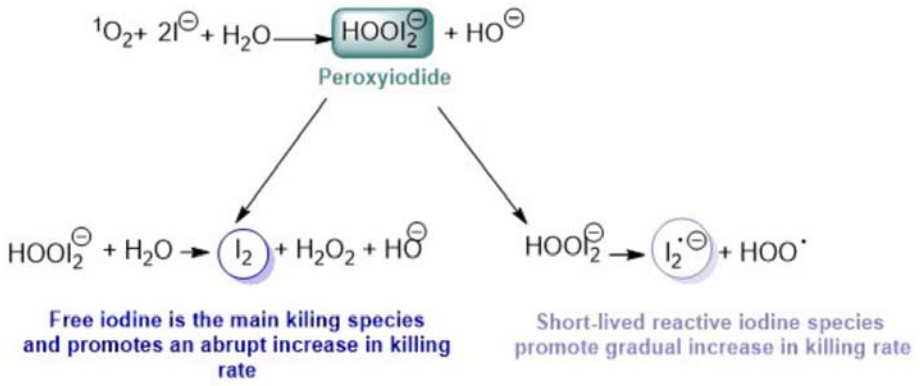

FIGURE 1 | Schematic representation of the decomposition of peroxyiodide produced by the reaction of $^1O_2$ and KI (elaborated according with the literature – Vecchio et al., 2015; Zhang et al., 2015; Freire et al., 2016; Gsponer et al., 2016; Hamblin, 2017; Huang et al., 2017, 2018a; Kashef et al., 2017; Reynoso et al., 2017; Wen et al., 2017).

The microbial killer role of the two species can be distinguished by observing the killing microbial curve profile. When the principal contribution for the killing is the free iodine, the curves assumes an abrupt threshold value. On the other hand, a gradual killing curve can be observed when the short-lived reactive iodine species are the mainly killing species (Huang et al., 2018a).

Until now, the literature survey only reported combinations of PSs and KI with a positive aPDT potentiation. Additionally, the possibility of extending the approach to cationic porphyrins was not evaluated. Consequently, in this work, in order to gain a more comprehensive knowledge about this type of potentiation, we decided to assess the effect of KI in the presence of a broad range of cationic porphyrinic and non-porphyrinic dyes as PSs (**Figure 2**). To achieve this objective and considering the high number of assays required to evaluate the different combinations of PSs with KI, the assays were performed using a bioluminescent *E. coli* strain as a bacterial model. It is well known that the bioluminescence approach can provide a sensitive and innocuous way to detect the viability state of microorganisms. Compared to the conventional plating count methodology, the use of bioluminescent strains in aPDT allows to monitor the process in real-time and it is a sensitive and cost-effective methodology to evaluate this effect. Moreover, the strong correlation between CFU and bioluminescent signal of the bioluminescent *E. coli* used in this work has already been proved and described (Alves et al., 2008, 2011a,b).

The structures of the selected PSs summarized in **Figure 2** comprise: (i) the five structurally related *meso*-tetraarylporphyrins with one [**Mono-Py(+)-Me**], two [**Di-Py(+)-Me *opp*** and **Di-Py(+)-Me *adj***], three [**Tri-Py(+)-Me**], and four [**Tetra-Py(+)-Me**] positives charges and a formulation (**Form**) based on these porphyrins; (ii) the three β-substituted porphyrins β-**ImiPhTPP**, β-**ImiPyTPP**, and β-**BrImiPyTPP** bearing positively charged imidazole units; and (iii) the non-porphyrinic dyes – methylene blue (**MB**), Rose Bengal (**RB**) and Toluidine Blue O (**TBO**), crystal violet (**CV**) and malachite green (**MG**).

In the selection of these three series of PSs was considered their different photoinactivation profile toward *E. coli* and their mechanism of action (Type I and Type II).

For the *meso*-tetraarylporphyrins with positive charges at the *meso* position the studies already performed demonstrated that their photodynamic efficiency was dependent on charge number, charge distribution, aggregation behavior and molecular amphiphilicity and the order of their efficacy was: **Mono-Py(+)-Me** < **Di-Py(+)-Me *opp*** < **Di-Py(+)-Me *adj*** < **Tetra-Py(+)-Me** < **Tri-Py(+)-Me**. Additionally, a formulation (**Form**) constituted by a non-separated mixture of Mono-Py(+)-Me (19%), Di-Py(+)-Me *opp* and Di-Py(+)-Me *adj* (20%) Tri-Py(+)-Me (44%) and Tetra-Py(+)-Me (17%) was also studied. This mixture has already proved to be efficient in the photoinactivation of *S. aureus*, *E. coli* and *Pseudomonas syringae* pv. *actinidiae* and is considered an excellent alternative to the highly efficient **Tri-Py(+)-Me** since the production costs and also the production time was reduced significantly (Marciel et al., 2018; Martins et al., 2018). The neutral 5,10,15,20-tetra-(4-pyridyl)porphyrin (**Tetra-Py**) precursor of the positively charged **Tetra-Py(+)-Me** was also included.

For the *meso*-tetraarylporphyrins with a positive charge at the *beta*-pyrrolic position (β-**ImiPhTPP**, β-**ImiPyTPP**, and β-**BrImiPyTPP**) a different efficacy profile in photoinactivation of *E. coli* at concentrations of 20 μM was observed in previous studies; however, at 5.0 μM none of the three PSs caused a significant decrease in bacterial activity (Moura et al., 2019).

Although porphyrins and porphyrins analogs comprise most of the PSs used in aPDT, several non-porphyrinic chromogens exhibit photodynamic activity (Ormond and Freeman, 2013). Thus, for this study were selected good $^1O_2$ generators with positive charges that already proved their photodynamic efficiency in clinical trials such as the phenothiazinium salts **MB** and **TBO** (Abrahamse and Hamblin, 2016). In this study were also included two photoactive dyes that act mainly through type I mechanism (with lower $^1O_2$ production rates), the **CV** and **MG**. In this evaluation the study was extended to the xanthene derivative **RB**. Combinations of KI with **RB** and with **MB** were

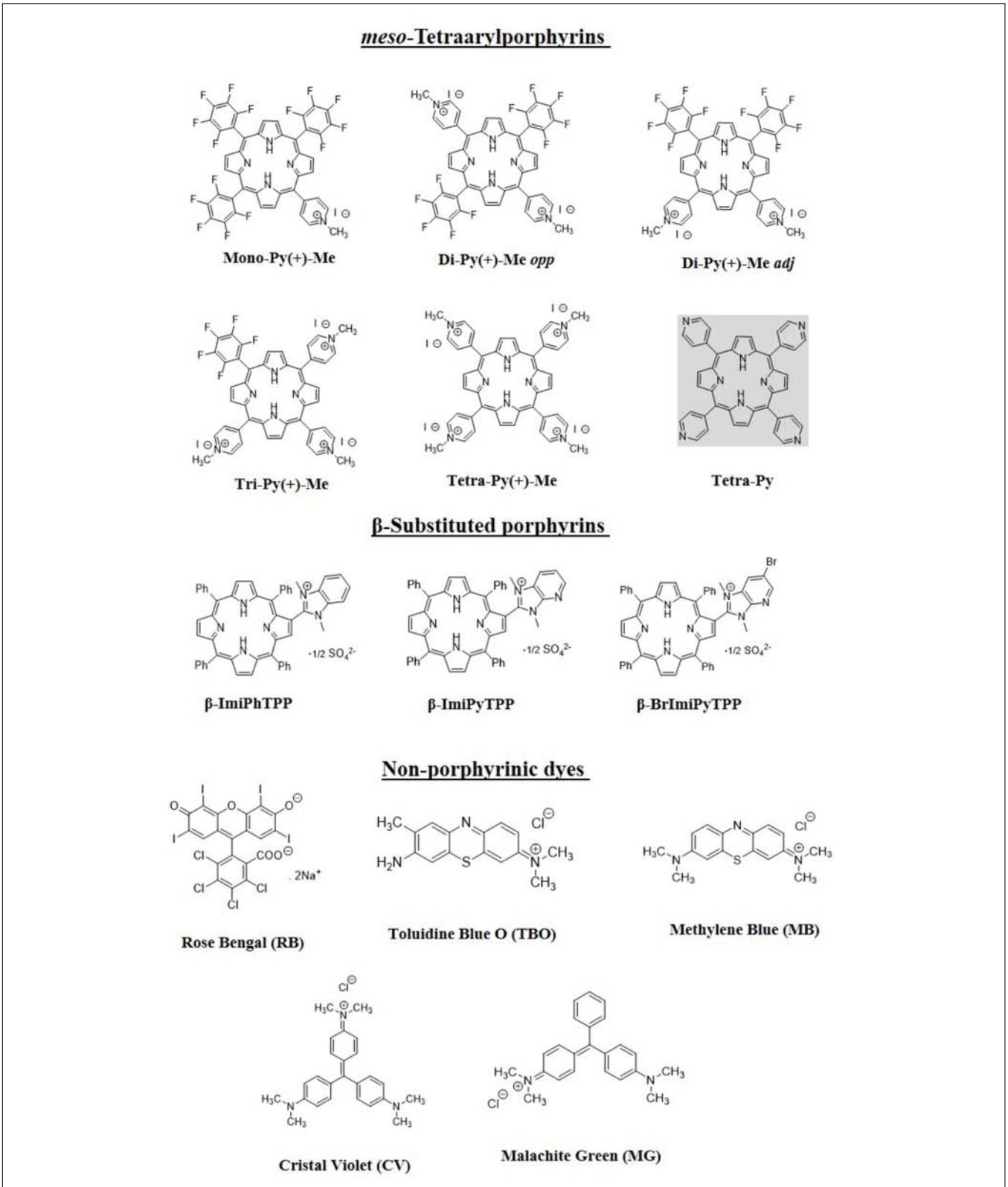

FIGURE 2 | Structures and acronyms/abbreviations of the PSs used in this study.

already studied and were introduced in this work to corroborate our results (Vecchio et al., 2015; Wen et al., 2017).

MATERIALS AND METHODS

Photosensitizers: Stock Solutions and UV-Vis Spectra

Stock solutions of each porphyrin were prepared at 500 μM in dimethyl sulfoxide (DMSO) and stored in the dark. Stock solutions of non-porphyrinic dyes were prepared at 500 μM in phosphate buffer solution (PBS) and stored in the dark.

The porphyrins 5-(1-methylpyridinium-4-yl)-10,15,20-tris(pentafluorophenyl)-porphyrin mono-iodide [**Mono-Py(+)-Me**], 5,15-bis(1-methylpyridinium-4-yl)-10,20-bis(pentafluorophenyl)porphyrin di-iodide [**Di-Py(+)-Me *opp***] 5,10-bis(1-methylpyridinium-4-yl)-15,20-bis(pentafluorophenyl)-porphyrin di-iodide [**Di-Py(+)-Me *adj***], 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide [**Tri-Py(+)-Me**] and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide [**Tetra-Py(+)-Me**], the formulation (**Form**) of the non-separated porphyrins Mono-Py(+)-Me (19%), Di-Py(+)-Me *opp* and Di-Py(+)-Me *adj* (20%) Tri-Py(+)-Me (44%) and Tetra-Py(+)-Me (17%) and the neutral 5,10,15,20-tetra-(4-pyridil)porphyrin (**Tetra-Py**) were synthetized according with the literature (Simões et al., 2016; Marciel et al., 2018; Martins et al., 2018). The preparation of the mono-cationic porphyrins β-**ImiPhTPP**, β-**ImiPyTPP**, and β-**BrImiPyTPP** bearing an imidazole ring at the β-pyrrolic position were synthetized according with a procedure developed in our laboratory (Moura et al., 2019), Crystal Violet (**CV**) was purchased from Merck, Rose Bengal (**RB**) from Fluka AG, Malachite Green (**MG**) from Riedel-de-Haën^{TM}, Methylene Blue (**MB**) and Toluidine Blue O (**TBO**) from Acros Organics. The UV-Vis spectra of the PSs are presented in **Supplementary Figure S2** (see Supporting Information).

Light Sources

The potentiation of aPDT effect between the PS and KI was evaluated by exposing the bacterial suspension in the presence of each combination to a set of fluorescent PAR lamps which is constituted by 13 fluorescent lamps OSRAM 21 of 18 W each, PAR white radiation (380–700 nm) at an irradiance of 25 W m^{-2}. All the irradiances were measured with a Power Meter Coherent FieldMaxII-Top combined with a Coherent PowerSens PS19Q energy sensor.

Bacterial Strains and Growth Conditions

The genetically transformed bioluminescent *E. coli* Top10 (Alves et al., 2011b) was grown on Tryptic Soy Agar (TSA, Merck) supplemented with 50 mg mL^{-1} of ampicillin (Amp) and with 34 mg mL^{-1} of chloramphenicol (Cm). Before each assay, one isolated colony was transferred to 10 mL of tryptic soy broth medium (TSB, Merck) previously supplemented with Amp and Cm and was grown overnight at 25°C under stirring (120 rpm). An aliquot was transferred into 10 mL TSB under the same growth conditions till stationary growth phase was achieved. An optical density at 600 nm (OD_{600}) of 1.6 ± 0.1 corresponded to ≈10^8 colony forming units (CFU) mL^{-1}.

The correlation between CFU mL^{-1} and the bioluminescent signal (in RLUs) of bioluminescent *E. coli* strain was evaluated. A fresh overnight bacterial culture was serially diluted (10^{-1} to 10^{-9}) in PBS. Non-diluted and diluted aliquots were pour-plated on TSA medium (0.5 mL) and, simultaneously, were read on a luminometer (0.5 mL) (TD-20/20 Luminometer, Turner Designs, Inc., Madison, WI, United States) to determine the bioluminescence signal. The results obtained are presented in **Supplementary Figure S1** (see Supporting Information).

Antimicrobial Photodynamic Therapy (aPDT) Procedure

Bioluminescent *E. coli* culture was grown overnight and was tenfold diluted in PBS (pH 7.49), to a final concentration of ∼10^8 CFU mL^{-1}, which corresponds approximately to 10^8 RLU. The bacterial suspension was equally distributed in 50 mL sterilized and acid-washed beakers.

Bioluminescence Monitoring

All the experiments were carried out under PAR white light (380–700 nm) and the *E. coli* bioluminescence signal was measured in the luminometer at different times of light exposure. The assays were finished whenever the detection limit of the luminometer was achieved (*c.a* 2.3 log). Light control (LC), dark control (DC), and KI control, were also evaluated as described below.

Evaluation of the Inorganic Salt Effect on Tetra-Py(+)-Me Photodynamic Action

The first experiments were performed in order to assess the effect of different inorganic salts in the inactivation of *E. coli* through aPDT approach using the tetracationic porphyrin **Tetra-Py(+)-Me,** extensively studied in bacterial photoinactivation processes (Alves et al., 2008; Tavares et al., 2011; Simões et al., 2016). The selected inorganic salts were KI, NaI, KCl, NaCl, and NaBr and the assays were conducted with 50 mM of each salt and 5.0 μM of **Tetra-Py(+)-Me**. All the inorganic salts were purchased from Sigma-Aldrich (St. Louis, MO, United States) and stock solutions were prepared at 500 mM in PBS immediately before each experiment.

The assays were carried out by exposing the bioluminescent *E. coli* suspension to **Tetra-Py(+)-Me** at 5.0 μM with each salt added from the stock solution to achieve the final concentrations of 50 mM. Simultaneously, the following different controls were performed: one light control (LC) that contained a bacterial suspension exposed to the same light conditions as the samples, and dark controls (DC) that comprised a bacterial suspension incubated with the PS at 5.0 μM and with the distinct salts at 50 mM. DC were protected from light during all the procedure. The samples and controls were protected from light with aluminum foil and remained in the dark for 15 min to promote the porphyrin binding to *E. coli* cells before irradiation. Then, both samples and controls were exposed to the PAR

white light at 25 W m^{-2} under stirring (120 rpm) and placed on a tray; the beaker bottoms were covered with water to maintain the samples at constant temperature (25°C). Finally, aliquots of 0.8 mL of samples and controls were collected at different times of light exposure and the bioluminescence signal was measured in the luminometer. Three independent experiments with two replicates were performed and the results were averaged.

Evaluation of the Antimicrobial Effect in the Presence of Different PSs and KI

The assays were carried out by exposing a final volume of 10 mL of a bioluminescent *E. coli* suspension to each PS at 5.0 μM and combinations of each PS at 5.0 μM and KI concentrations at 50 and 100 mM and for **RB**, **CV**, **MG** also at 25 mM. The samples were protected from light with aluminum foil and incubated in the dark for 15 min. Light and dark controls were also carried out simultaneously with the aPDT procedure: the light controls (LC) comprised a bacterial suspension and a bacteria suspension with KI at 100 mM exposed to the same light protocol; and the dark control (DC) comprised a bacterial suspension incubated with the PSs at 5.0 μM and KI at the higher concentration tested (100 mM) protected from light. The aPDT treatment was performed as described above. Three independent experiments with two replicates were performed and the results were averaged.

Detection of Iodine Formation

In a 96 wells microplate, appropriate volumes of each PS at 5.0 μM (1 μL) and combinations of each PS at 5 μM (1 μL) and KI at 100 mM (2 μL) were added to each well and irradiated with PAR white light at 25 W m^{-2}. The generation of iodine was monitored by reading the absorbance at 340 nm at irradiation times 0, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min. As positive control it was used Lugol's solution diluted to 1:1000.

Another simple assay to detect iodine was also performed, for the different combinations of PS and KI, in the presence of amylose due to the well-known formation of a strong blue complex when these two species are present (Luallen, 2017). So, to the beakers containing a starch solution at a concentration of 2 mg L^{-1}, it was added each PS at 5 μM and KI at 100 mM. The samples were incubated in the dark for 15 min and afterward were exposed continuously and under stirring (120 rpm) to the same light source used in the aPDT assays. The color change was registered and photographed at different times of irradiation for each sample. At the same time, the following control assays were performed: PS + light; KI + light, PS + KI under dark.

Statistical Analysis

Three independent experiments with two replicates per assay for each condition were done. The statistical analysis was performed with GraphPad Prism. Normal distributions were checked by the Kolmogorov–Smirnov test and the homogeneity of variance was verified with the Brown Forsythe test. ANOVA and Dunnet's multiple comparison tests were applied to assess the significance of the differences between the tested conditions. A value of $p < 0.05$ was considered significant.

RESULTS

The effect of KI for each series of PSs toward *E. coli* was evaluated using the same concentration of PS (5.0 μM) and KI concentrations of 50 and 100 mM (unless other concentrations were mentioned) under PAR white light at an irradiance of 25 W m^{-2}. These KI concentrations were selected considering the ones referred in similar studies and knowing that higher concentrations can limit the combined protocol application in clinic area due to osmotic stress. The PS, **TetraPy(+)-Me,** was selected to confirm the benefic effect of KI among other inorganic salts (NaI, NaCl, KCl, and NaBr). This well-known tetracationic porphyrin is extensively studied in bacterial photoinactivation processes and is considered an excellent reference when the efficacy of different cationic porphyrins are compared (Alves et al., 2008; Tavares et al., 2011; Simões et al., 2016). Low light doses ranging from 1.5 to 36 J/cm^2 emitted by a fluorescent lamp set (380–700 nm) were selected based on their efficacy to inactivate a large range of microorganisms (Marciel et al., 2017; Moura et al., 2019). Additionally, this light source was able to accomplish the required overlap between PS absorption and light setup emission spectrum (see **Supplementary Figure S2**; Costa et al., 2010; Cieplik et al., 2015).

Evaluation of the Salt Effect on Tetra-Py(+)-Me Photodynamic Efficiency

The results presented in **Figure 3** show that the photoinactivation pattern of *E. coli* in the presence of **Tetra-Py(+)-Me** is strongly dependent on the anion used.

The results clearly indicate that when combinations of **Tetra-Py(+)-Me** with KI and NaI were used, a reduction of the bioluminescence signal of *c.a.* 4 log was observed after 30 min of irradiation. In the case of NaBr, KCl and NaCl no potentiation on

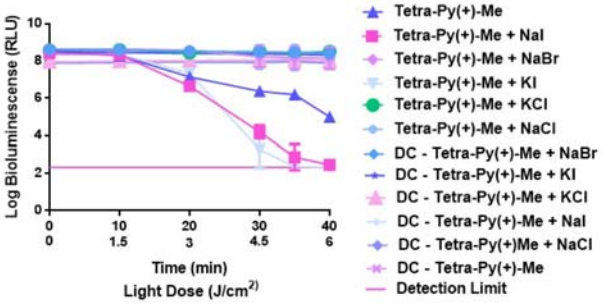

FIGURE 3 | Survival of bioluminescent *E. coli* during aPDT with **Tetra-Py-(+)-Me** at 5.0 μM and 50 mM of KI, NaI, KCl, NaCl, and NaBr after irradiation with PAR white light (380–700 nm) at an irradiance of 25 W m^{-2} for 40 min. The values are expressed as the three independent experiments; error bars indicate the SD and in some cases are collapsed with the symbols.

the aPDT effect was detected. Light and dark controls showed no significant variation in the bioluminescence produced by *E. coli*.

Evaluation of the KI Effect on the Photodynamic Action of *Meso*-Tetraarylporphyrins Bearing One to Four Positive Charges

The effects of KI at 50 and 100 mM in the photodynamic action of **Mono-Py(+)-Me**, **Di-Py(+)-Me *opp***, **Di-Py(+)-Me *adj***, **Tri-Py(+)-Me**, and **Tetra-Py(+)-Me** toward *E. coli* are summarized in **Figure 4**.

In the cases of the LCs (Bacteria and bacteria + KI irradiated) and DC (bacteria + PS + KI in the dark) no decrease in *E. coli* bioluminescent signal was detected. These results indicate that the viability of this recombinant bioluminescent bacterium was not affected by irradiation, by the presence of the salt or by any of the tested combinations of PS + KI in the dark.

The results shown in **Figure 4A** for the monocationic porphyrin [**Mono-Py(+)-Me**] demonstrated that its low efficacy is strongly improved by the presence of KI; the poor activity of this PS toward *E. coli* was previously related with its low water solubility leading to aggregation and, consequently, to low $^1O_2$ generation. Under the conditions used in these assays this porphyrin maintained its low efficacy causing a decrease on *E. coli* bioluminescence signal of 0.9 log ($p < 0.0001$) after 240 min of irradiation. However, the addition of KI at 50 mM and 100 mM potentiated the effect of this mono-cationic porphyrin, causing bioluminescent signal reductions of *c.a.* 3.5 and 5.5 log ($p < 0.0001$) after 150 min of irradiation.

The dicationic porphyrins **Di-Py(+)-Me *opp*** and **Di-Py(+)-Me *adj*** without the presence of the coadjuvant promoted similar effects on the reduction (*c.a.* 6 log after, respectively, 150 and 120 min of irradiation) of *E. coli* bioluminescence signal (**Figures 4B,C**). However, when these two isomers were combined with KI the results obtained were significantly different. The combination of **Di-Py(+)-Me *adj*** with KI at 50 and 100 mM produced similar results in the photoinactivation of bioluminescent *E. coli* and no improvement in aPDT efficiency was detected (**Figure 4C**). In fact, in the last irradiation time, there were no significant differences in the *E. coli* bioluminescence signal promoted by **Di-Py(+)-Me *adj*** and the two combinations of **Di-Py(+)-Me *adj*** + KI. In the case of **Di-Py(+)-Me opp** (**Figure 4B**) the presence of KI (at 50 and 100 mM) led to a significant reduction on its efficacy. The maximum inactivation achieved for the combination of this PS with 100 mM of KI was 1.7 log ($p < 0.0001$).

The **Tetra-Py(+)-Me** and **Tri-Py(+)-Me** were the most efficient porphyrins in the photoinactivation of bioluminescent *E. coli*, which is also in accordance with the literature (Simões et al., 2016). These porphyrins, when acting by themselves, showed to be potent PSs for the inactivation of bioluminescent *E. coli*, demanding short irradiation times (*c.a.* 70 min) to achieve total photoinactivation of this Gram-negative bacterium (**Figures 4D,E**). The combination of these PSs with KI at 50 and 100 mM increased dramatically the effect of these PSs in the photoinactivation of bioluminescent *E. coli* (**Figures 4D,E**). In the case of **Tri-Py(+)-Me**, it was observed an abrupt decrease in *E. coli* viability after 30 and 10 min of irradiation when the combinations of this PS with 50 mM and 100 mM of KI were used, respectively (**Figure 4D**). This sharp decrease was also observed for the combination of **Tetra-Py(+)-Me** and KI; after 30 and 10 min of irradiation no bioluminescent signal was detected for combinations **Tetra-Py(+)-Me** +KI 50 mM and **Tetra-Py(+)-Me** +KI 100 mM, respectively.

These results prompted us to study the effect of KI in the aPDT efficiency of the porphyrinic formulation (**Form**) described as an excellent alternative to the highly efficient **Tri-Py(+)-Me,** as it was mentioned above. The results summarized in **Figure 4F** show that this formulation at 5 μM in the absence of the coadjuvant and after 60 min of irradiation, promoted a decrease in the bioluminescence signal of *E. coli* of 4 log ($p < 0.0001$) (**Figure 4F**). When the assays were repeated in the presence of KI at 50 mM a more pronounced decrease in *E. coli* viability was detected after 40 min of irradiation, reaching the detection limit of the luminometer after 60 min. This rapid decrease in the viability of this bacterium occurred even sooner, after only 20 min of irradiation, when KI was used at 100 mM.

In order to check if the presence of positive charges is a required feature for the combination of KI with this series of porphyrins, the efficacy of the neutral 5,10,15,20-tetra (4-pyridyl)porphyrin (**Tetra-Py**) was evaluated in the presence of this salt at 50 and 100 mM. In **Figure 4G** are summarized the results obtained and it was verified that the low efficacy of this neutral porphyrin was not improved by the presence of the salt, suggesting that when an increment effect was observed in the presence of KI in this series of porphyrins, the presence of at least one positive charge is mandatory.

Evaluation of the KI Effect on the Photodynamic Action of Porphyrin Derivatives Bearing Cationic Imidazole Units at the β-Pyrrolic Position

The results obtained in the photoinactivation of bioluminescent *E. coli* with the monocationic porphyrins β-**ImiPhTPP**, β-**ImiPyTPP**, and β-**BrImiPyTPP** bearing an imidazole moiety at the β-pyrrolic position, both in the absence and in the presence of KI are presented in **Figure 5**. The low activity of these porphyrins at 5.0 μM in the photoinactivation of bioluminescent *E. coli* was improved in the presence of KI, although the inactivation increment was different. The combination of β-**BrImiPyTPP** and β-**BrImiPhTPP** with KI at 100 mM promoted a significant positive effect in the photoinactivation of *E. coli* with an increment on the bioluminescent reduction of 1.3 and 1.1 log for β-**ImiPhTPP** and β-**BrImiPyTPP** ($p < 0.0001$), respectively, after 240 min of irradiation when compared with the effect of these PSs in the absence of KI (**Figures 5A,C**).

A different profile was observed for porphyrin derivative β-**ImiPyTPP**. The best results were obtained with the combination of this PS with 100 mM of KI, promoting a significant decrease in *E. coli* viability (**Figure 5B**). The bioluminescence signal reduction reached the method detection limit after 240 min; when compared with the effect of these PS in

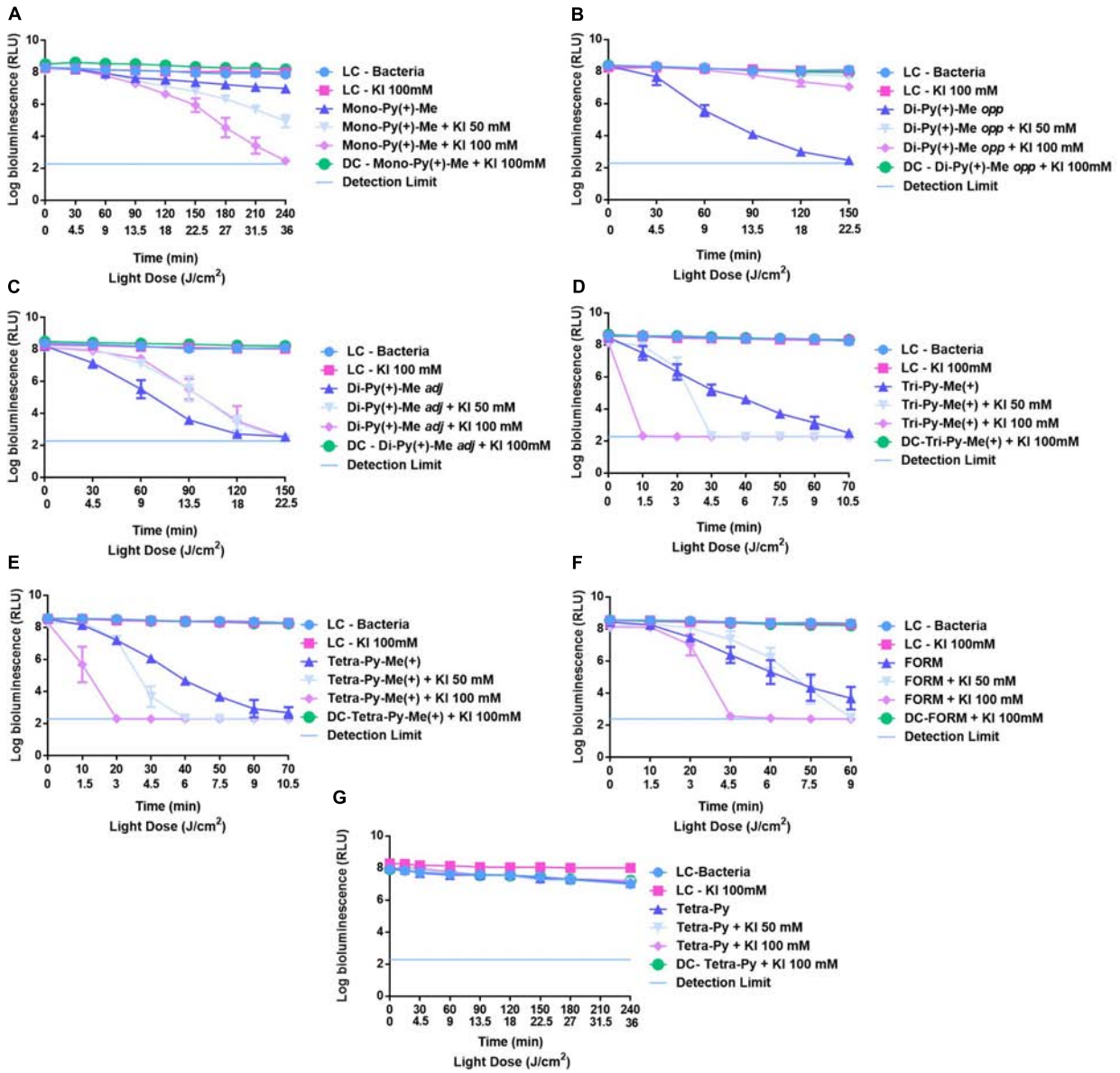

FIGURE 4 | Survival of bioluminescent *E. coli* during aPDT assays in the presence of **Mono-Py(+)-Me (A)**, **Di-Py(+)-Me *opp* (B)**, **Di-Py(+)-Me *adj* (C)**, **Tetra-Py(+)-Me (D)**, **Tri-Py(+)-Me (E)**, **Form (F)**, and **Tetra-Py (G)** at 5.0 μM alone and combined with KI at 50 and 100 mM. The values are expressed as the three independent experiments; error bars indicate the SD.

the absence of KI an increment on the bioluminescent reduction of 5.3 log in cell viability was observed ($p < 0.0001$).

Evaluation of the KI Effect in the Photodynamic Action of Non-porphyrinic Dyes

In **Figure 6** are summarized the effects of KI at 50 and 100 mM in the photodynamic inactivation of *E. coli* when using **RB** (A), **TBO** (B), **MB** (C), **CV** (D), and **MG** (F). Combinations of **RB** (**Figure 6A**) and **MB** (**Figure 6C**) at 5.0 μM and KI showed to have a potential effect in the photodynamic inactivation of *E. coli*, causing marked reductions in the *E. coli* viability when compared with the results obtained with these dyes alone. The PS **RB**, when acting alone, promotes a decrease of 1.3 log ($p < 0.0001$) in *E. coli* viability after 150 min of irradiation. When combined with KI, an efficient decrease in bioluminescent signal of *E. coli* was observed, even when KI at 25 mM was used. At this concentration, the combination of **RB** 5.0 μM + KI 25 mM, caused a sharp decrease in the *E. coli* viability after 90 min of irradiation, reaching the detection limit of the luminometer after 120 min. This marked effect was also observed when **RB** was combined with 50 mM of KI, but it was with the combination of **RB** 5.0 μM+ KI 100 mM that this effect became more noteworthy; after 20 min

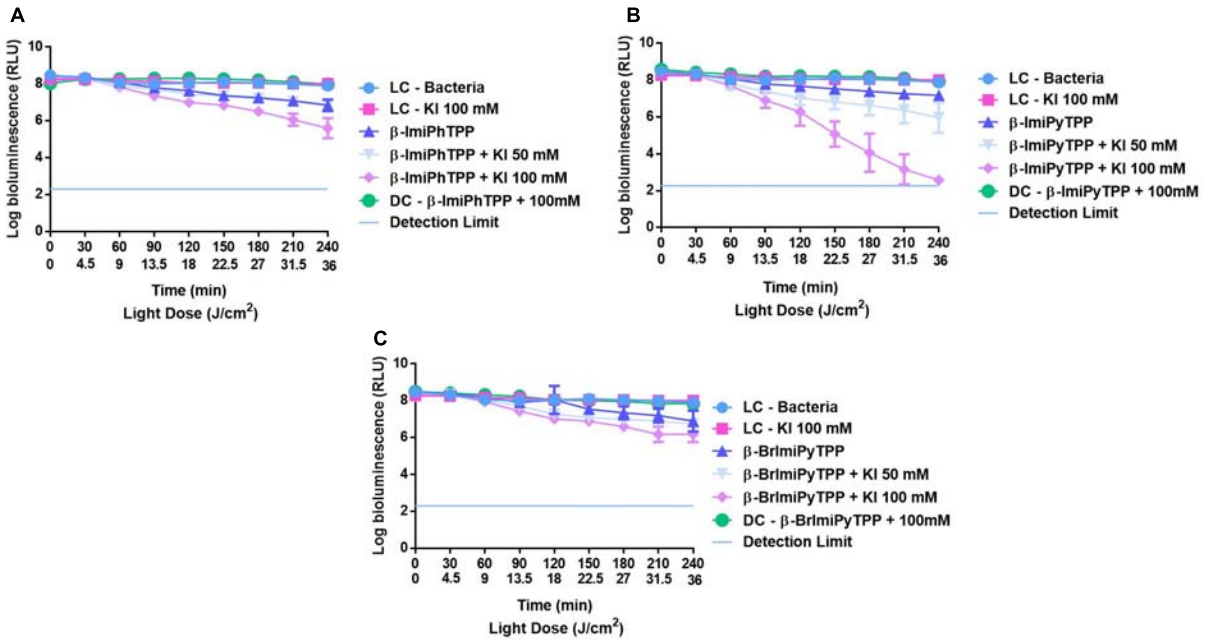

FIGURE 5 | Survival of bioluminescent *E. coli* during aPDT assays in the presence of mono-cationic porphyrins **β-ImiPhTPP (A)**, **β-ImiPyTPP (B)**, and **β-BrImiPyTPP (C)** at 5.0 μM alone or combined with KI at 50 and 100 mM. The values are expressed as the three independent experiments; error bars indicate the SD.

of irradiation it was observed a decrease of 6 log ($p < 0.0001$) in *E. coli* viability and after 30 min no bioluminescent signal was observed.

A similar profile was observed with combinations of **MB** at 5.0 μM and KI. In the absence of KI, **MB** caused a decrease in the bioluminescence signal of *E. coli* of 5.5 log ($p < 0.0001$) after 180 min of irradiation, but when combinations of this PS with KI were used, an efficient decrease in the viability of this bacterium was also observed, after 30 and 60 min of irradiation, with KI at 100 and 50 mM, respectively.

In the cases of **TBO**, **CV**, and **MG**, a potentiation of their photodynamic action mediated by the presence of KI was not observed. In fact, **TBO** when acting alone at 5.0 μM revealed to be an excellent PS for the inactivation on bioluminescent *E. coli*, promoting a remarkable decrease in the bioluminescent signal of 6 log ($p < 0.0001$) after 60 min of irradiation. In the presence of KI, this reduction was only observed after 90 min of irradiation.

CV when acting alone caused a decrease in the bioluminescent signal of 3.2 log ($p < 0.0001$), however, in the presence of KI at 25, 50, and 100 mM the decrease did not go beyond 1.4, 2.2, and 2.7 log ($p < 0.0001$), respectively.

In the case of **MG** no significant effect was observed in the *E. coli* viability either when this dye was used alone or combined with KI.

Detection of Iodine Formation Mediated by the PS

In order to clarify if the photodynamic improvement was related with the iodine generation from KI by the PS, the different PSs (5.0 μM) were irradiated both in the absence and in the presence of that coadjuvant at 100 mM. To verify the generation of iodine, the absorbance at 340 nm was read after 0, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min of irradiation. The results obtained are summarized in **Figure 7**.

The results had shown that the combination of KI with **Tri-Py(+)-Me**, **Tetra-Py(+)-Me**, and **Form** causes a higher production of I_2, leading to a sharp increase in absorbance at 340 nm in the first 20 min of irradiation. On the other hand, the combination of KI with **Mono-Py(+)-Me**, **Di-Py(+)-Me *adj***, **Di-Py(+)-Me *opp*** only was able to induce a gradual increase of the absorbance at 340 nm, thus indicating the lower ability to produce I_2. The combination of **Tetra-Py** + KI did not produce I_2.

The gradual increase in the absorbance at 340 nm was also observed in the case of mono-cationic porphyrins β-**ImiPhTPP**, β-**ImiPyTPP**, and β-**BrImiPyTPP**. However, in the case of β-**ImiPyTPP**, the absolute value of absorbance at 340 nm after 40 min of irradiation was higher than the values observed for the other PSs, indicating the formation of higher amounts of I_2 in this case.

In the case of the non-porphyrinic dyes, the combination of KI with **MB** and **RB** demonstrated a higher ability to produce I_2, with a sharp increase in the absorbance at 340 nm, after 30 min of irradiation. However, combinations of **TBO**+ KI and **CV**+ KI only produced a gradual increase in the absorbance, indicating the lower capability to produce I_2. Combination of **MG**+ KI did not promote the formation of I_2.

The visual appearance of the starch solutions after different irradiation periods are presented in **Figure 8**

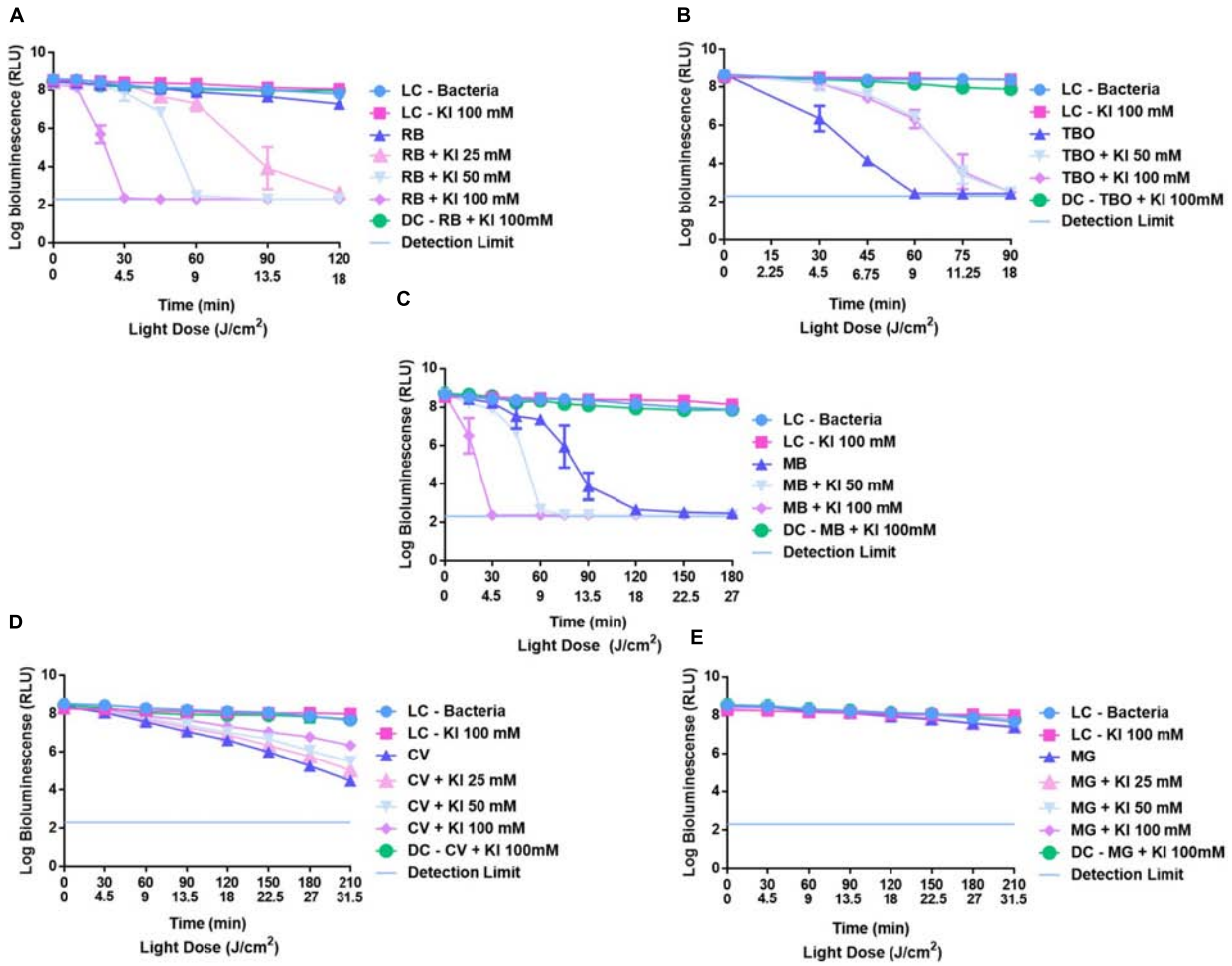

FIGURE 6 | Survival of bioluminescent *E. coli* during aPDT assays in the presence of non-porphyrinic PSs at 5.0 μM **RB (A)**, **TBO (B)**, **MB (C)**, **CV (D)**, and **MG (E)** alone and combined with KI at 25, 50, and 100 mM. The values are expressed as the three independent experiments; error bars indicate the SD.

(**Supplementary Tables S1**–**S3**) and the results corroborated that the time required for the formation of the complex between amylose and iodine was dependent on the PS used. In the presence of **Tri-Py(+)-Me**, **Tetra-Py(+)-Me**, and **Form**, the formation of the dark color (**Supplementary Table S1**) appeared just after 2–4 min of irradiation, while for **Di-Py(+)-Me *adj*** the iodine-amylose complex was observed after 45 min of irradiation. The formation of the colored complex was not observed for the neutral **Tetra-Py** after 240 min of irradiation and for **Mono-Py(+)-Me** and **Di-Py(+)-Me *opp*** after 75 min of irradiation a slight darkening of the solution was observed.

For the mono-cationic porphyrins β-**ImiPhTPP**, β-**ImiPyTPP**, and β-**BrImiPyTPP** the formation of the deep colored complex was only observed in the presence of β-**ImiPyTPP** after 60 min of irradiation (**Supplementary Table S2**).

In the assays performed with the non-porphyrinic dyes the combinations **MB**+KI and **RB**+KI promoted the formation of the dark complex after 2–5 min of irradiation and the combination **TBO**+KI after 30 min of irradiation. The combinations of **CV** and **MG** with KI were not able to produce the iodine-amylose complex even after 240 min of irradiation (**Supplementary Table S3**).

DISCUSSION

Several studies have shown that aPDT combined with some inorganic salts, namely potassium iodide (Vecchio et al., 2015; Zhang et al., 2015; Huang et al., 2016, 2018a,b,c; Wen et al., 2017) can be potentiated. However, there is not any evidence until now that this potentiation can be observed for all types of PSs, namely cationic porphyrins. In order to gain a more comprehensive knowledge about the potentiation of aPDT by KI, a broad range of PSs were tested in this study.

We started our study by selecting the most effective salt and using as PS the widely studied tetracationic porphyrin 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (**Tetra-Py^{+}-Me**), which is frequently used as standard in aPDT studies. This can be considered a reference for all porphyrinic PSs,

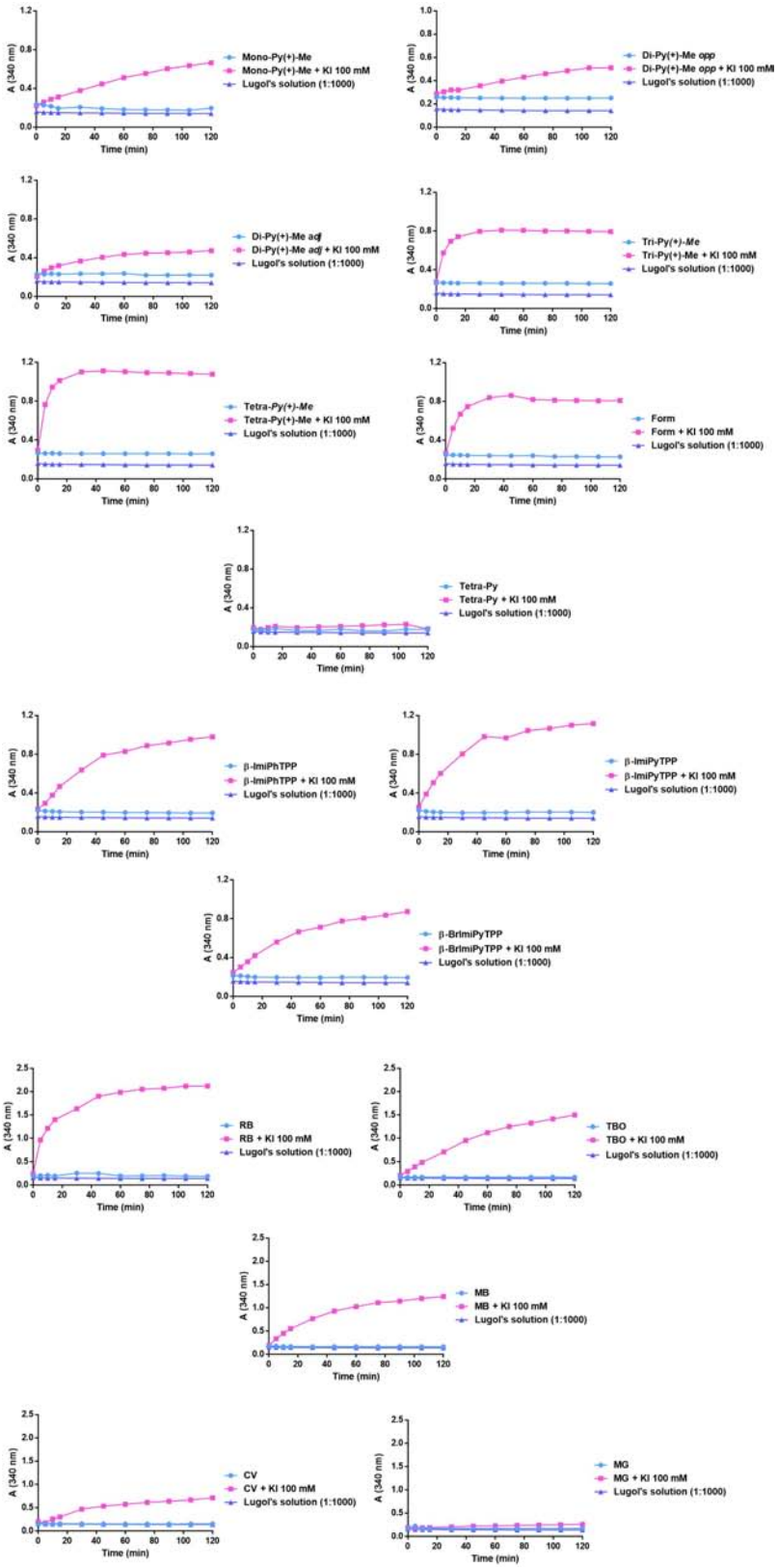

FIGURE 7 | Monitoring of the formation of iodine at 340 nm after different irradiation periods in the presence of each PS at 5.0 μM and combinations of each PS at 5.0 μM and KI at 100 mM.

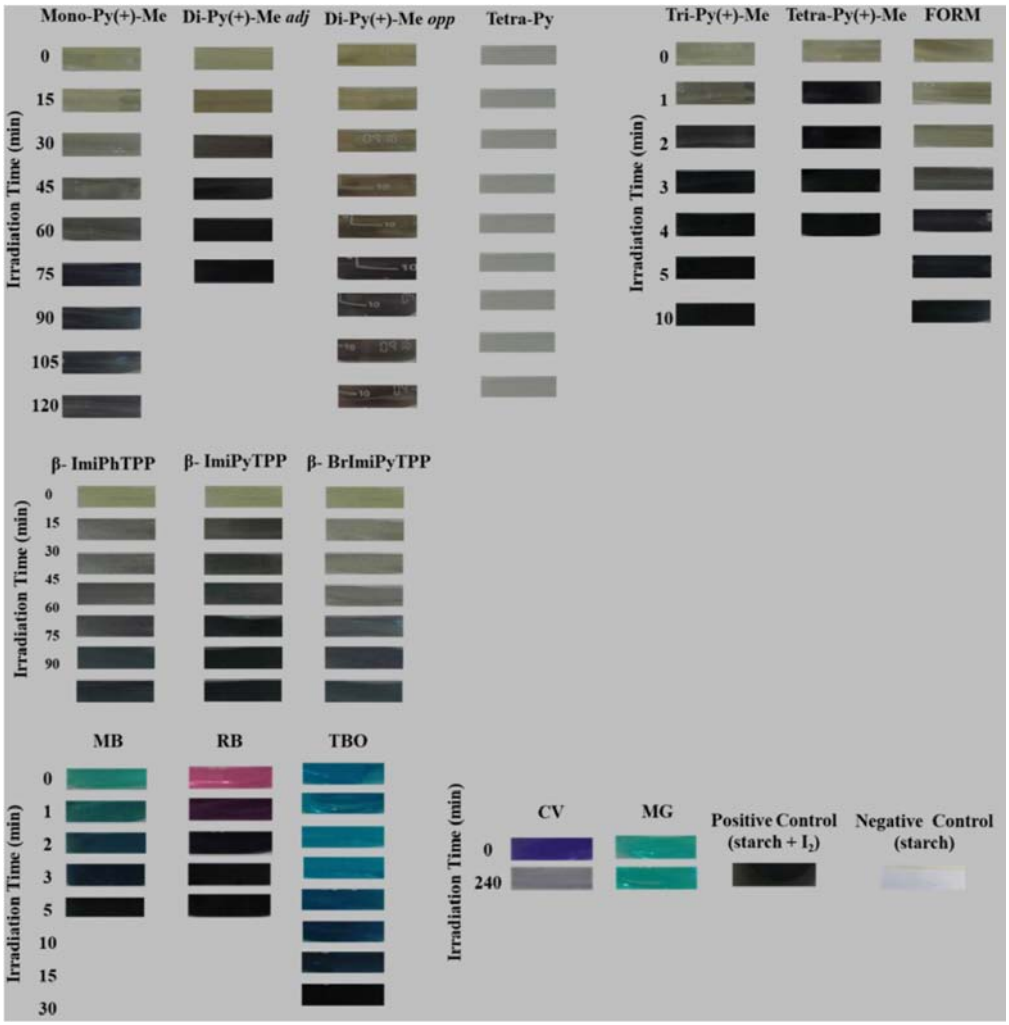

FIGURE 8 | Visual appearance of the starch solutions after different irradiation periods in the presence of each PS at 5.0 μM and KI at 100 mM.

since this PS is extensively studied in bacterial photoinactivation processes (Alves et al., 2008; Tavares et al., 2011; Simões et al., 2016). The efficacy of bacterial inactivation by the combination of this PS and the salts KI and NaI was clearly higher than when the PS was used alone, showing that these combinations promoted an increase of the antimicrobial photodynamic efficiency of the PS. On the other hand, no effect was observed with the combinations of **Tetra-Py(+)-Me** with NaBr, KCl, and NaCl during the irradiation time. The loss of efficiency of this porphyrin in these cases could be explained by the fact that bromide and chloride ions retarded the $^1O_2$ generation, and consequently its action as PS (Keum et al., 2003; Krumova and Cosa, 2016). Therefore, it was obvious that for this PS and under the tested conditions, only salts containing I^- as counterion were capable of potentiate the antimicrobial photodynamic inactivation. Similar results were earlier observed when other PSs were tested (Hamblin, 2016). As the combinations PS + KI and PS + NaI were both effective to inactivate the *E. coli*, the potentiation of the others PSs was performed in the presence of the most studied salt KI.

Besides the difficulty of explaining which of the two proposed pathways of decomposition of peroxyiodide produced by the reaction of $^1O_2$ and I^- (see **Figure 1**) are responsible for the extra microbial killing when KI is present, it was assumed, as proposed previously in other studies, that some information can be taken by the profile of inactivation. If the inactivation curve shows a sharp decrease, free iodine is the main killing species, but if there is a more gradual increase in killing, then there is a contribution from short-lived reactive iodine species (Huang et al., 2018a). Considering the above, we tried to explain the results obtained with the two series of cationic porphyrins, including the neutral **Tetra-Py**, and with the non-porphyrinic PSs. In **Table 1** are summarized the results obtained concerning the inactivation profile observed for each combination of KI and PS at 5.0 μM in the photoinactivation of bioluminescent *E. coli*.

These results allow to classify the PSs studied as: (1) PSs in which its efficiency was potentiated by KI and it was observed a gradual decrease in the *E. coli* survival rate profile [**Mono-Py(+)-Me**, β-**ImiPhTPP**, β-**ImiPyTPP**, and β-**BrImiPyTPP**]; (2) PSs in

TABLE 1 | Results obtained in the photoinactivation of bioluminescent *E. coli* using combinations of tested PSs at 5.0 μM and KI.

	Mono-Py(+)-Me	Di-Py(+)-Me *opp*	Di-Py(+)-Me *adj*	Tri-Py(+)-Me	Tetra-Py(+)-Me	FORM	Tetra-Py	β-ImiPhTPP	β-ImiPyTPP	β-BrImiPhTPP	RB	MB	TBO	CV	MG
KI potentiate aPDT?	✓	✗	✗	✓	✓	✓	✗	✓	✓	✓	✓	✓	✗	✗	✗
KI causes a sharp decrease in the *E. coli* survival?	✗	–	–	✓	✓	✓	–	✗	✗	✗	✓	✓	–	–	–

✓, *Yes*; ✗, *No*.

which its efficiency was potentiated by KI and it was observed an abrupt decrease in the *E. coli* survival rate profile [**Tri-Py(+)-Me, Tetra-Py(+)-Me**, **Form**, **RB**, and **MB**]; and (3) PSs in which its efficiency was not potentiated by KI [**Di-Py(+)-Me *opp***, **Di-Py(+)-Me *adj***, **Tetra-Py**, **TBO**, **CV**, and **MG**].

Based on the explanations given in previous works, we can assume that the mechanism of action of the combinations of KI and the PSs **Mono-Py(+)-Me**, β-**ImiPhTPP**, β-**ImiPyTPP**, and β-**BrImiPyTPP** is probably related to the preferential decomposition of the peroxyiodide to the iodine radicals ($I_2^{\cdot-}$) that, due to their short diffusion distance, cause a gradual decrease in the photoinactivation profile. In the case of **Tri-Py(+)-Me**, **Tetra-Py(+)-Me**, **Form**, **MB** and **RB** the preferential decomposition of the peroxyiodide leads to the formation of free iodine (I_2/I_3^-), which contributes significantly for the abrupt increase observed in the photoinactivation profile of the *E. coli*. This fact was confirmed by the formation of iodine, visible by spectroscopy (**Figure 7**) and by the color alteration during the irradiation in the presence of starch (**Figure 8**): PSs that cause a sharp decrease in the *E. coli* survival rate profile revealed higher ability to produce I_2. On the other hand, the belatedly detection of I_2 was observed for PSs that cause a gradual decrease in the *E. coli* survival rate profile.

In the cases of PSs in which the efficiency was not potentiated by KI, or was even reduced, we need also to look at other factors that can likewise contribute to this behavior.

The different behavior observed with the dicationic PSs **Di-Py(+)-Me *opp*** (the efficacy was lost in the presence of KI) and **Di-Py(+)-Me *adj*** (no potentiation with KI) (**Figures 4B,C**) is probably related with their structural features since both isomers have similar capability to generate $^1O_2$ with high efficiency, as it was described by Simões et al. (2016). Consequently, it can be assumed that both compounds are able to promote the formation of peroxyiodide and its decomposition to iodine radical species ($I_2^{\cdot-}$). However, for **Di-Py(+)-Me *opp*** these radicals, with a short diffusion distance, probably were not generated close to the target cells and the depletion of $^1O_2$ by the previous reaction was responsible by losing its previous efficacy. On the other hand, for **Di-Py(+)-Me *adj*** the formation of toxic radicals in close proximity to the target cells can justify the maintenance of its efficacy. However, the toxicity under these conditions was comparable to the previous one in the absence of iodide. The different charge distribution in the two di-cationic porphyrins can explain the different behavior in the presence of KI. A study of Alves et al. (2011a) showed the massive importance of the charge distribution in these two PS efficacies. In this work, the photodynamic inactivation of *E. coli* and *Enterococcus faecalis* using the two isomeric di-cationic porphyrins with different charge distribution showed that the porphyrin with adjacent cationic groups was significantly more active (for both bacteria) than the one with the cationic groups located in opposite *meso* positions. This fact was justified by the distortion of the macrocycle induced by the electrostatic repulsion between the neighboring charged groups in the porphyrin with adjacent cationic groups (Kessel et al., 2003). So, in the case of porphyrinic PSs with cationic groups located in opposite *meso* positions, accompanied by the preferential decomposition of the

peroxyiodide to the iodine radicals, as it was observed with **Di-Py(+)-Me *opp***, the addition of KI can even impair the aPDT efficacy. With the porphyrin derivatives **Di-Py(+)-Me *adj***, **Mono-Py(+)-Me**, and β-**ImiPyTPP** the asymmetric distribution of the charge allows the radicals to reach the bacterial cells more effectively. However, the potentiation of the aPDT processes mediated by **Mono-Py(+)-Me** and β-**ImiPyTPP** in the presence of KI but not by **Di-Py(+)-Me *adj*** can also be due to the higher production of free iodine by the two first porphyrins when compared with porphyrin **Di-Py(+)-Me *adj***.

Neutral **Tetra-Py** revealed to be inefficient to photoinactivate *E. coli*, even when KI was used. This can be explained by the fact that this is a neutral PS, and consequently, is not capable to interact with the external membrane of the cell wall of this Gram-negative bacterium. Thus, even when $^1O_2$ is produced in great amounts, the cytotoxic species will never be close enough to the bacterial cells to cause damage. It is also important to refer that this porphyrin tends to aggregate in aqueous media, making it difficult to act as a PS.

CV is known to have an efficient non-radiative deactivation route producing triplet species, such as $^1O_2$, with low yield and acting mainly through an electron-transfer mechanism (Type I), which causes its bleaching (Docampo et al., 1983; Indig et al., 2000). The results clearly indicate its low efficiency in the photoinactivation of *E. coli*, either when acting alone or combined with KI. These results are justified by its poor $^1O_2$ production rates allied to its photodegradation when irradiated. Such as in the case of **CV**, it was not surprising that **MG** did not produced any effect in the photoinactivation of bioluminescent *E. coli*, since this PS dye did not produce $^1O_2$, acting only by the Type I mechanism (Zhuo, 2016). These two PSs dyes show the importance that $^1O_2$ generation has in the potentiation of aPDT processes mediated by KI. The **TBO** acts mainly by Type II mechanism and, when acting alone inactivate efficiently the bacteria, as **MB** and **RB.** However, when combined with KI, no potentiation was observed. There is, however, a study in the literature reporting the potentiation of the effect of **TBO** by KI, but in this study the **TBO** was tested at 100 μM (Ghaffari et al., 2018). In our case, the concentration of **TBO** was 20 times lower (5.0 μM). These different experimental conditions can justify the differences observed in these two studies. Nevertheless, using NaN_3 as potentiation agent, the aPDT effect of **TBO** was more effective when compared with the result without the NaN_3 (Kasimova et al., 2014). **MB** used as the reference for all non-porphyrin dyes, once is the most commonly studied antimicrobial PS in the literature and has received regulatory approval to mediate photodynamic therapy (PDT) of several infectious diseases, acts mainly trough Type II mechanism (Marotti et al., 2010; de Oliveira et al., 2014). Moreover, its aPDT potentiation when combined with KI was already described (Vecchio et al., 2015). Besides that, and according with our results, **MB** can be designated as a PS reference for evaluate the potentiation of these dyes by KI.

It remains unanswered which factor determines whether the mechanism follows *via* formation of iodine radical species (I_2^-) or *via* formation of free iodine (I_2/I_3^-). To answer this question, we cannot neglect other factors that can also contribute for the efficiency of these PSs, such as $^1O_2$ production, charge number and distribution, aggregation behavior, affinity for the cell membrane.

It is undeniable that the ability of KI to potentiate the aPDT process mediated by some cationic PSs, allows a drastic reduction of the aPDT treatment time as well as the reduction of the PS concentration. However, this potentiation is limited to some PSs and the addition of KI can even impair some PSs. This work helped to elucidate that for the series of compounds studied, the PSs capable to decompose the peroxyiodide into iodine (easily detectable by monitoring the formation of I_2 through spectroscopy or by the visual appearance of a blue color in the presence of starch) are the promising ones in terms of complementing their efficacy with the action of iodine. Although these studies confirm that the generation of $^1O_2$ is an important fact in this process, the PS structure, aggregation behavior and affinity for the cell membrane are also important features to take into account.

AUTHOR CONTRIBUTIONS

CV performed the antimicrobial photodynamic evaluations assays of all PSs, analysis of biological results and contributed to the manuscript preparation. AG performed the analysis and interpretation of the biological results and contributed to the manuscript preparation. MM and NM performed the synthesis of the porphyrin derivatives. MN and MF were responsible for the supervision of the synthesis of the PSs and contributed in the analysis and interpretation of the biological results and in the manuscript preparation. AA was responsible for the supervision and the design of the antimicrobial photodynamic experiments and contributed in the analysis and interpretation of the biological results and in the manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02665/full#supplementary-material

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Molecular Epidemiology and Risk Factors of Carbapenemase-Producing *Enterobacteriaceae* Isolates in Portuguese Hospitals: Results From European Survey on Carbapenemase-Producing *Enterobacteriaceae* (EuSCAPE)

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In Portugal, the epidemiological stage for the spread of carbapenemase-producing *Enterobacteriaceae* (CPE) increased from sporadic isolates or single hospital clones (2010–2013), to hospital outbreaks, later. Here we report data from a 6-month study performed under the European Survey on Carbapenemase-Producing *Enterobacteriaceae* (EuSCAPE). During the study period, 67 isolates (61 *Klebsiella pneumoniae* and 6 *Escherichia coli*) non-susceptible to carbapenems were identified in participant hospital laboratories. We detected 37 $bla_{KPC-type}$ (including one new variant: bla_{KPC-21}), 1 bla_{GES-5}, and 1 bla_{GES-6} plus bla_{KPC-3}, alone or in combination with other *bla* genes. Bioinformatics analysis of the KPC-21-producing *E. coli* identified the new variant bla_{KPC-21} in a 12,748 bp length plasmid. The bla_{KPC-21} gene was harbored on a non-Tn*4401* element, presenting upstream a partial IS*Kpn6* (ΔIS*Kpn6*/Δ*traN*) with the related left IR (IR_L) and downstream a truncated Tn3 transposon. PFGE and MLST analysis showed an important diversity, as isolates belonged to distinct PFGE and STs profiles. In this study, we highlighted the presence of the high-risk clone *E. coli* sequence-type (ST) 131 clade C/H30. This worldwide disseminated *E. coli* lineage was already detected in Portugal among other antibiotic resistance reservoirs. This study highlights the intra- and inter-hospital spread and possible intercontinental circulation of CPE isolates.

Keywords: carbapenemase-producing *Enterobacteriaceae*, KPC-21, EuSCAPE, Portugal, *Klebsiella pneumoniae*, *Escherichia coli*

INTRODUCTION

Carbapenems, a class of β-lactam antibiotics with wide activity, are often the antimicrobials of last resort to treat infections associated to extended-spectrum β-lactamase (ESBL)- or plasmid-mediated AmpC (PMAβ)-producing *Enterobacteriaceae* isolates (Papp-Wallace et al., 2011; Rodríguez-Baño et al., 2018). Unfortunately, carbapenem non-susceptible *Enterobacteriaceae* (CNSE) have been reported worldwide mainly because of the acquisition of carbapenemase-encoding genes (Potter et al., 2016; Codjoe and Donkor, 2018). Since the first description of a carbapenemase-producing *Enterobacteriaceae* (CPE) in Europe in the 1990s, a large variety of carbapenemases has been identified in each of the four Ambler molecular classes, mainly the KPC-type (class A), VIM-, IMP-, and NDM-types (class B), and OXA-48-type (class D) (Grundmann et al., 2017; Logan and Weinstein, 2017). CPE isolates are usually resistant to many other β-lactam and non-β-lactam antibiotics, leading to multi-resistant isolates.

In Portugal, the epidemiological stage for the spread of CPE increased from sporadic isolates or single hospital clones, from April 2010 to February 2013, to sporadic hospital outbreaks later (Albiger et al., 2015; Manageiro et al., 2015b,c). Here we report data from a 6-month prevalence study performed under the European Survey on Carbapenemase-Producing *Enterobacteriaceae* (EuSCAPE) with the collaboration of different Portuguese Laboratories.

MATERIALS AND METHODS

Bacterial Isolation, Antibiotic Susceptibility, and Molecular Characterization

This study included a total of 104 clinical isolates (94 *Klebsiella pneumoniae* and 10 *Escherichia coli*) collected from November 2013 to April 2014 in 10 Portuguese hospitals. The first ten consecutive and non-replicated CNSE isolates obtained during this period, in each hospital, from blood, lower respiratory tract secretions, urine, puncture fluids, and wound secretions, of single patients, were sent to the National Reference Laboratory, in Lisbon, and were considered. Successive carbapenem-susceptible isolates of the same species were also preserved as controls whenever possible, accordingly to EuSCAPE protocol (Grundmann et al., 2017). Overall, 67 CNSE (61 *K. pneumoniae* and 6 *E. coli*) and 37 controls (33 *K. pneumoniae* and 4 *E. coli*) were analyzed.

In the context of the EuSCAPE study, all data were anonymized and collected in accordance with the European Parliament and Council decisions on the epidemiological surveillance and control of communicable disease in the European Community (Eur-Lex-31998D2119, 1998; Eur-Lex-32000D0096, 2000).

Antibiotic Susceptibility and Molecular Characterization of Antimicrobial Resistance

Antimicrobial susceptibility was performed by disk diffusion method for 15 antibiotics (**Table 1**), and by broth microdilution method for tigecycline and colistin, using EUCAST guidelines[1]. Clinical isolates with resistance or with decreased susceptibility to ertapenem were considered presumptively CPE. Isolates were considered multidrug resistant when presenting reduced susceptibility to three or more structurally unrelated antibiotics.

PCR and sequencing were applied to detect and identify the main CPE (bla_{KPC} and bla_{GES} from class A; bla_{IMP}, bla_{VIM}, and bla_{NDM} from class B; and bla_{OXA-48} from class D)-, ESBL (bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}) – and PMAβ (bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{LAT}, bla_{ACT}, bla_{MIR}, bla_{DHA}, bla_{MOR}, bla_{ACC})-encoding genes, as previously described (Manageiro et al., 2015b). Plasmid-mediated colistin resistance-encoding genes (*mcr*-type) were also investigated (Manageiro et al., 2017).

Transfer Experiments

Transferability of bla_{KPC-21} from *E. coli* UR19829 was performed by broth mating out assays using sodium azide-resistant *E. coli* J53 as a recipient strain, and by transformation, as previously described (Manageiro et al., 2015b, 2017).

Molecular Typing

Clonal relatedness of 67 CNSE isolates was investigated by pulsed-field gel electrophoresis (PFGE) as previously described (Manageiro et al., 2017). Genetic diversity of the *K. pneumoniae* (n = 10, i.e., 1 representative of each PFGE cluster) and *E. coli* (n = 10) isolates was investigated by multilocus sequence typing (MLST) (Manageiro et al., 2015b). *E. coli* sequence type (ST) subclones were also analyzed on the basis of the *E. coli fimH* gene (Manageiro et al., 2015a).

Genomic Characterization of KPC-21-Producing *E. coli*

KPC-21-producing *E. coli* was genotypically characterized by whole-genome sequencing (WGS) as previously described (Manageiro et al., 2017). The assembled contigs were analyzed and studied for the presence of antibiotic resistance- and virulence-encoding genes, multi-locus sequence types, *fim* type, serotype, plasmid replicon types, and insertion sequences (ISs) using bioinformatics tools from the Center for Genomic Epidemiology[2] and ISsaga (Varani et al., 2011).

The pUR19829-KPC21 plasmid structure was constructed based on the genetic organization of the closest plasmid

[1] http://www.eucast.org/clinical_breakpoints/

[2] https://cge.cbs.dtu.dk/services/

sequences obtained by BLASTn, provided by NCBI[3], followed by contig neighbor's prediction from assembly information.

Statistical Analysis

OpenEpi software, version 3.01 was used for statistical analysis (Sullivan et al., 2009). Fisher exact test was used to assess differences in clinical and epidemiological risk factors for control and CNSE-carrying patients. One-tailed P values of ≤ 0.05 were considered to be statistically significant. Associations were determined by calculation of odds ratios with 95% confidence intervals.

Nucleotide Sequence Accession Number

The new bla_{KPC-21} nucleotide sequence was submitted to the NCBI GenBank Database with accession number NG_049254 and the complete plasmid sequence of pUR19829-KPC21 with accession number MH133192.

RESULTS AND DISCUSSION

During the study period, 67 isolates (61 *K. pneumoniae* and 6 *E. coli*) CNSE were identified in nine of the 10 Hospital Laboratories, with a non-susceptibility rate for meropenem and imipenem of 64 and 59%, respectively, for *K. pneumoniae*, and of 100% for *E. coli*. As expected, when compared with the control isolates, CNSE presented higher level of non-susceptibility to all antibiotic classes tested (**Table 1**). Colistin and tigecycline MIC_{50} values for CNSE were similar than those obtained for control isolates. Eleven out of the 104 (16.3%) isolates were colistin resistant, without the presence of the plasmid-mediated *mcr-1* or *mcr-2* gene. However, MCR-1 determinant was already identified in different reservoirs in Portugal, such as vegetables, animals and humans (Jones-Dias et al., 2016; Beyrouthy et al., 2017; Kieffer et al., 2017).

Thirty-eight (56.7%) isolates (36 *K. pneumoniae*, 2 *E. coli*) were confirmed to be CPE; we identified 36 $bla_{KPC-type}$ (including one new variant: bla_{KPC-21}), 1 bla_{GES-5}, and 1 bla_{GES-6} plus bla_{KPC-3}, alone or in combination with other *bla* genes (**Supplementary Figure S1**). The remaining 29 isolates were non-susceptible to carbapenems possibly due to porins deficiency with association of PMAβ (CMY-2 and DHA-1) and/or ESBL (mainly CTX-M-15) production (Martínez-Martínez, 2008).

The new bla_{KPC-21} gene differed from bla_{KPC-2} by one point mutation that leads to the amino acid substitution Trp105Arg; this position is involved in the binding and maintaining of the KPC catalytic activity (Papp-Wallace et al., 2010). *In silico* typing revealed an KPC-21-producing *E. coli* belonging to ST131 clade C/H30, associated with the fimbriae-encoding *fimH* allele 30, which become the most dominant lineage since the 2000s

[3]http://blast.ncbi.nlm.nih.gov/Blast.cgi

TABLE 1 | Antimicrobial susceptibility of 67 (61 *K. pneumoniae* and 6 *E. coli*) CNSE and 37 (33 *K. pneumoniae* and 4 *E. coli*) control isolates.

Antibiotic	*K. pneumoniae*				*E. coli*			
	Control (*n* = 33)		CNSE (*n* = 61)		Control (*n* = 4)		CNSE (*n* = 6)	
	IR (%)	S (%)	IR (%)	S (%)	IR (%)	S (%)	IR (%)	S (%)
Ampicillin	100	0	100	0	100	0	100	0
Amoxicillin/Clavulanate	30	70	89	11	25	75	100	0
Piperacillin/Tazobactam	58	42	98	2	25	75	100	0
Cefotaxime	30	70	92	8	25	75	100	0
Ceftazidime	36	64	95	5	25	75	100	0
Cefepime	36	64	90	10	25	75	100	0
Aztreonam	30	70	92	8	25	75	100	0
Imipenem	0	100	59	41	0	100	100	0
Meropenem	0	100	64	36	0	100	100	0
Ertapenem	0	100	100	0	0	100	100	0
Ciprofloxacin	36	64	69	31	25	75	100	0
Gentamicin	21	79	64	36	50	50	83	17
Tobramycin	33	67	74	26	50	50	83	17
Amikacin	0	100	18	82	0	100	17	83
SXT	33	67	90	10	50	50	67	33
Colistin*	6	94	11	89	0	100	0	100
MIC_{50}	1		1		1		1	
MIC_{90}	2		4		2		2	
Tigecycline*	39	61	56	44	0	100	33	67
MIC_{50}	1		2		0.5		0.5	
MIC_{90}	4		4		1		4	

**Microdilution method.*

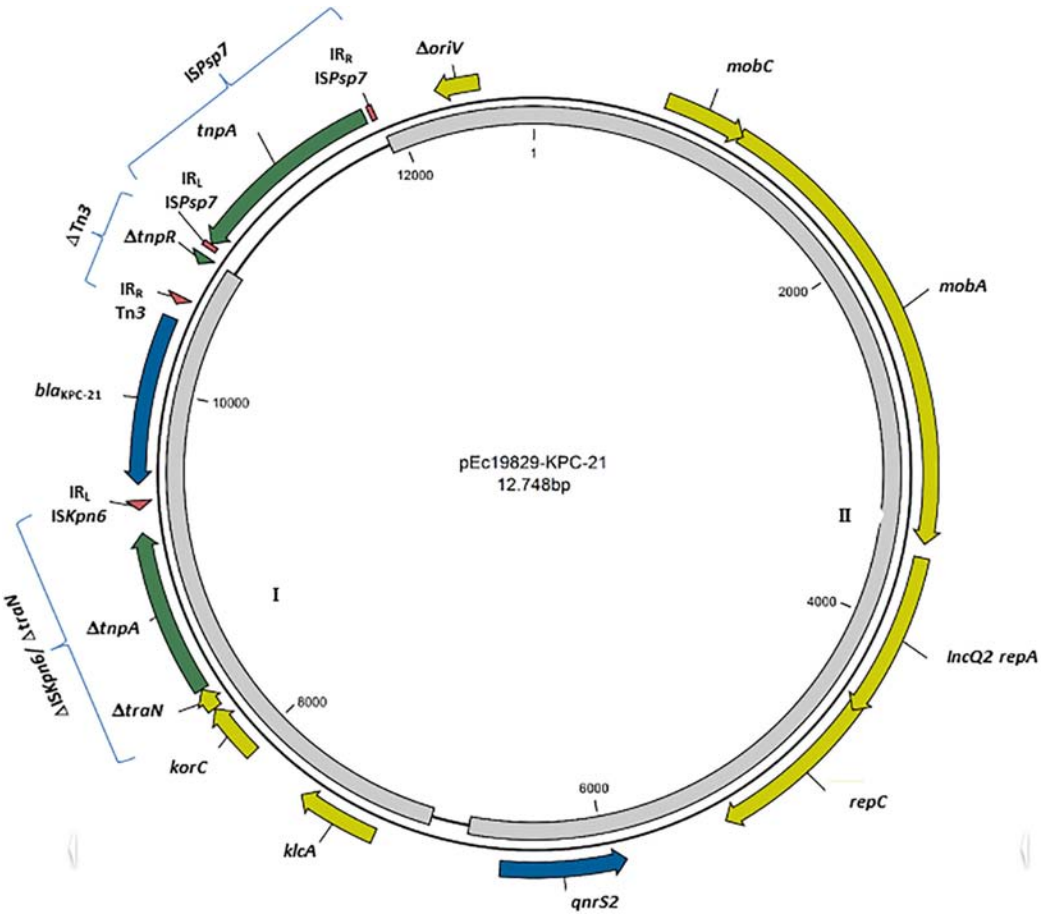

FIGURE 1 | Schematic representation of KPC-21-harboring plasmid (pUR19829-KPC21). Genes are denoted by arrows. Blue, antibiotic resistance genes; Green, mobile genetic elements; Yellow, plasmid mobilization genes and replication origin. Right and left inverted repeats (IR_R and IR_L) are indicated as red triangles. Gray regions 1: 99.97% of identity with KX756453, and Gray region 2: >99.9% sequence identity to KT896499.

(Nicolas-Chanoine et al., 2014; Pitout and DeVinney, 2017). Moreover, bioinformatics analysis of the KPC-21-producing *E. coli* identified this variant in a 12,748 bp length plasmid, with a mean coverage of 580-fold and GC content of 58.5% (**Figure 1**).

Dissemination of bla_{KPC} has been mainly supported by the horizontal transfer of Tn4401-type transposon, which harbors *tnpA* encoding a transposase, *tnpR* encoding resolvase, and two insertion sequence elements (ISKpn7 and ISKpn6) bracketing the bla_{KPC} gene (Cuzon et al., 2011). In this study, the bla_{KPC-21} gene was harbored on a non-Tn*4401* element (Chen et al., 2014), presenting upstream a partial IS*Kpn6* (ΔIS*Kpn6*/Δ*traN*) with the related left IR (IR_L) and downstream truncated Tn3 transposon downstream (**Figure 1**). This region has 99.97% of identity with pKP1194a, a plasmid carried by a hospital-associated KPC-2-producing *K. pneumoniae* isolated in Brazil (Accession number KX756453) (**Figure 1**- gray region I); this suggest an intercontinental circulation of isolates and mobile genetic elements (MGE), and the consequent need of concerted actions against the spreading of antibiotic resistance, at a worldwide level. The pUR19829-KPC21 enclosed also an intact IS*Psp7* element, an insertion sequence from IS*30* family, firstly described in *Pseudomonas* spp. (Szuplewska et al., 2014). Furthermore, the pUR19829-KPC21 backbone contained a region coding for plasmid replication (IncQ2 *repA*, *repC*), and mobilization (*mobA, mobC*), showing >99.9% sequence identity to the corresponding regions of pKPSH169, a 7.7 Kbp *qnrS2*-harboring IncQ plasmid identified in municipal wastewater treatment facilities in Israel (Accession number KT896499) (**Figure 1**- gray region II); this similarity highlights the high level of promiscuity of isolates between clinical settings and environment, where both reservoirs play a role in the antibiotic resistance dissemination (Stokes and Gillings, 2011). However, the lack of conjugative elements or an *oriT* region, associated with the presence of a truncated *oriV* region (**Figure 1**) suggests that pUR19829-KPC21 plasmid is nonmobilizable (Smillie et al., 2010). This fact is corroborated by the absence of a successful plasmid conjugation or transformation.

The variables used in the evaluation of risk factors for infection or colonization of patients with CNSE or control

TABLE 2 | Evaluation of risk factors for patients with infections caused by carbapenem susceptible or CNSE bacteria.

Variables	CNS isolates (no.)	Control isolates (no.)	OR	95% CI	*P* value
Region of patient admission					
North	20	13	0.7874	0.3094–2.038	0.3666(*P*)
Center	11	1	6.977	0.9398–312.7	0.03098
LVT	36	23	0.7092	0.2848–1.726	0.2670(*P*)
Patient age					
≤18 years old	3	1	1.68	0.1294–91.01	0.5522
19–64	22	12	1.018	0.401–2.66	0.5727
≥65	37	18	1.299	0.5389–3.146	0.3305
Unknown	5	6	–	–	–
Patient gender					
Female	31	18	0.9098	0.3769–2.197	0.4885(*P*)
Male	35	14	1.787	0.7359–4.453	0.1142
Unknown	1	5	–	–	–
Bacteria vs. host infection					
Colonization	0	2	0	0.0–2.912	0.1243(*P*)
Infection	28	22	0.493	0.1989–1.194	0.06393(*P*)
Unknown	39	13	–	–	–
Type of infection					
Community Onset	26	15	0.9307	0.3804–2.305	0.5127(*P*)
Hospital Acquisition	31	11	2.022	0.8072–5.311	0.07451
Unknown	10	11	–	–	–
Local of infection					
Urinary tract infection	39	21	1.061	0.4334–2.574	0.5242
Blood infection	8	4	1.117	0.2737–5.463	0.5679
Pus production with bacteria	7	1	4.155	0.5004–194.5	0.1502
Lower respiratory tract infection	4	3	0.7219	0.1148–5.215	0.4825(*P*)
Other infections	8	2	2.356	0.4353–24	0.2365
Unknown	1	6	–	–	–
ESBL production					
Positive	37	8	4.406	1.66–12.87	0.0007772
Negative	30	29	0.227	0.0777–0.6024	0.0007772 (*P*)
Total	**67**	**76**			

OR, odds ratios; CI, 95% confidence intervals. (P) indicates a one-tail P-value for protective or negative association. One-tailed P values of ≤ 0.05 are underlined. LVT, Lisbon and Tagus Valley.

isolates are present in **Table 2**. When compared to the 37 control strains, only ESBL-production and the patient admission at a hospital in the center of Portugal were significantly associated with CNSE isolates in the period of the study. In the era of ESBL-producing *Enterobacteriaceae*, the antibiotic regimens suggested for severe health-associated infections are necessarily based on carbapenems (Rodríguez-Baño et al., 2018). Unfortunately carbapenem use has being described as a risk factor for CPE acquisition, only preceded by the use of medical devices (van Loon et al., 2018). In addition, the present study attests that Portugal, during the period of the study, has a different CNSE geographical distribution with the center of Portugal significantly associated with carbapenem non-susceptibility. This fact corroborates previous studies which indicated that in Portugal, in 2015, only sporadic isolates or single hospital cases were described (Albiger et al., 2015).

PFGE and MLST analysis showed an important diversity, with isolates belonging to distinct PFGE and STs (**Supplementary Figure S1**). With respect to *K. pneumoniae* (**Supplementary Figure S1A**), a total of 10 clusters and 25 unique PFGE profiles were generated using XbaI, indicating the that the circulating clones in that period were genetically diverse. However, carbapenemase-producing *K. pneumoniae* isolates were more clonal (six PFGE clusters including 69.4% of these isolates) than non-carbapenemase-producing *K. pneumoniae* (four PFGE clusters including 50.0% of these isolates). As shown in **Supplementary Figure S1**, both CNSE species showed intra- and inter-hospital spread (e.g., PFGE clusters KpI and KpIX), with some hospital-specific clones (e.g., PFGE clusters KpIV and KpVIII). However, as also showed in Spain in other EuSCAPE study (Esteban-Cantos et al., 2017), the carbapenem-non-susceptible *K. pneumoniae* population was more clonal than the carbapenem-susceptible population (data not shown). Ten different MLSTs were detected among carbapenemase-producing (ST14, ST15, ST45, ST231, and ST1513) and non-carbapenemase-producing (ST11, ST17, ST348, and ST395) *K. pneumoniae* isolates. At our knowledge,

this is the first description of ST17, ST395, and ST1513 *K. pneumoniae* in Portugal (Manageiro et al., 2015b; Rodrigues et al., 2016; Vubil et al., 2017). Noteworthy, the GES-5 enzyme was detected in a ST231 *K. pneumoniae* isolate as previously reported in Portugal, but in the same hospital, which shows its capacity to maintain in clinical settings due to the selection pressure of this environment (Manageiro et al., 2015b). Furthermore, ST45 was recently the cause of a hospital-based outbreak caused by multidrug-resistant, KPC-3- and MCR-1-producing *K. pneumoniae* in Portugal (Mendes et al., 2018).

The high-risk clone carbapenemase-positive *K. pneumoniae* ST258 was not detected in this study or among clinical carbapenemase-producing *K. pneumoniae* isolates in Portugal (Manageiro et al., 2015b; Rodrigues et al., 2016; Vubil et al., 2017). However, concerning carbapenem-non-susceptible *E. coli*, besides the six different PFGE unique profiles, the isolates belongs all but two (ST405-*fimH27* and ST23-*fimH35*) to the ST131 clade C/H30 high-risk clone disseminated worldwide (**Supplementary Figure S1B**) (Woodford et al., 2011; Pitout and DeVinney, 2017). Noteworthy, this clone was already detected in Portugal among other antibiotic resistance reservoirs, such as in an *E. coli* strain isolated from a dolphin housed at a Zoo Park (Manageiro et al., 2015a); in dogs and cats with urinary tract infection (Marques et al., 2018); and in *E. coli* strains from wastewater and gulls (Varela et al., 2015). Again, this shows that clinical settings and different environmental compartments may be considered communicating vessels through which bacteria and resistance genes are able to flow (Stokes and Gillings, 2011).

Portugal was one of the EuSCAPE participating countries that presented higher proportions of KPC-positive *K. pneumoniae* (Grundmann et al., 2017). The percentage of carbapenem non-susceptible *K. pneumoniae* was low in invasive infections in the study period [2.4%, EARS-Net 2013][4]. However, although the consumption of carbapenems has declined by 13.3% between 2012 and 2016 (PPCIRA, 2017), Portugal is reporting since 2013 a significant increasing trend of carbapenem non-susceptible *K. pneumoniae* [6.4%, EARS-Net 2016][4]. The number of inter-institutional transmission is also increasing (Glasner et al., 2013; Albiger et al., 2015), being *K. pneumoniae* the principal cause of bacterial health-associated infections in Portugal, as in other European countries (ECDC, 2013). Of concern is the fact that KPC-producing organisms cause infections with high morbidity and mortality (Porreca et al., 2018; Rodríguez-Baño et al., 2018). These results reinforces that reducing antibiotic use alone is likely insufficient for reversing resistance (Lopatkin et al., 2017). We strongly believe that the chain of transmission of isolates and genes in clinical settings will be reduced or broken, especially with containment measures rigorously implemented and followed at local level.

[4]https://ecdc.europa.eu/

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AUTHOR CONTRIBUTIONS

VM designed the study, performed the molecular experiments, bioinformatics analysis, analyzed the data, and wrote the manuscript. RR, IBM, and EF performed the microbiological and molecular experiments, and analyzed the data. DAS and LV performed Illumina genome sequencing experiments. The Network EuSCAPE-Portugal participants acquired laboratory data. MC designed the study, wrote and reviewed the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02834/full#supplementary-material

FIGURE S1 | Pulsed-field gel electrophoresis (PFGE) dendrogram and genetic relatedness of 61 *K. pneumoniae* **(A)** and 6 *E. coli* **(B)** CNSE isolates. Isolate number, hospital code, year of isolation, carbapenems antibiotic susceptibility, detected carbapenemases, extended-spectrum β-lactamases (ESBL), inhibitor resistant SHV (IRS), and plasmid-mediated AmpC (PMAβ), Multilocus sequence typing (MLST) for selected isolates and PFGE profile types are shown. These profiles, from 0001 to 0035, were defined as forming clusters KpI to KpX, for *K. pneumoniae*, and from 0001 to 0006 for *E. coli*. For *E. coli* isolates, *fim*-type is also shown.

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Combined Antibacterial Effects of Goat Cathelicidins With Different Mechanisms of Action

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Being essential components of innate immune system, animal antimicrobial peptides (AMPs) also known as host-defense peptides came into sharp focus as possible alternatives to conventional antibiotics due to their high efficacy against a broad range of MDR pathogens and low rate of resistance development. Mammalian species can produce a set of co-localized AMPs with different structures and mechanisms of actions. Here we examined the combined antibacterial effects of cathelicidins, structurally diverse family of host-defense peptides found in vertebrate species. As a model we have used structurally distinct cathelicidins expressed in the leukocytes of goat *Capra hircus*. The recombinant analogs of natural peptides were obtained by heterologous expression in bacterial system and biological activities as well as the major mechanisms of antibacterial action of the peptides were investigated. As the result, the marked synergistic effect against wide panel of bacterial strains including extensively drug-resistant ones was observed for the pair of membranolytic α-helical amphipathic peptide ChMAP-28 and Pro-rich peptide mini-ChBac7.5Nα targeting a bacterial ribosome. ChMAP-28 was shown to damage the outer bacterial membrane at sub-inhibitory concentrations that could facilitate Pro-rich peptide translocation into the cell. Finally, resistance changes under a long-term continuous selective pressure of each individual peptide and the synergistic combination of both peptides were tested against *Escherichia coli* strains. The combination was shown to keep a high activity after the 26-days selection experiment in contrast to mini-ChBac7.5Nα used alone and the reference antibiotic polymyxin B. We identified the point mutation leading to amino acid substitution V102E in the membrane transport protein SbmA of the mini-ChBac7.5Nα-resistant strain obtained by selection. The experiments revealed that the presence of sub-inhibitory concentrations of ChMAP-28 restored the activity of mini-ChBac7.5Nα against this strain and clinical isolate with a weak sensitivity to mini-ChBac7.5Nα. The obtained results suggest a potential medical application of synergistic combinations of natural cathelicidins, which allows using a lower therapeutic dose and minimizes the risk of resistance development.

Keywords: antimicrobial peptide, cathelicidin, goat, proline-rich peptide, synergy, extensively drug-resistant, immune system

INTRODUCTION

Over recent years, a growing number of bacterial species became resistant to clinically significant antibiotics. Host defense antimicrobial peptides (AMPs) came into sharp focus as possible alternatives to conventional antibiotics due to their high efficacy against a broad range of multiple drug-resistant pathogens, a rapid membranolytic mode of action and, as consequence, a low risk of resistance development. Cathelicidins, one of the major groups of animal AMPs, are known to be the key molecular factors of innate immunity of most vertebrate species, from hagfish to human (Kościuczuk et al., 2012). Along with direct antimicrobial action, these peptides possess immunomodulatory activities, such as inhibition of apoptosis, cytokine stimulating, lipopolysaccharide (LPS) neutralizing, promotion of wound healing, and regulation of adaptive immune responses. All the above suggest that these compounds can be prototypes for novel therapeutics with complex mechanism of action (Steinstraesser et al., 2011). The precursors of cathelicidins are produced in immune and epithelia cells and contain the N-terminal part of 99–114 amino acid residues which is known as the cathelin domain. This structure is highly conserved among vertebrates, whereas the C-terminal domain, encoding the mature peptide, shows substantial heterogeneity. Interestingly, the cathelin domain does not exhibit a protease inhibitory function regardless of its high structural similarity to cystatins (Pazgier et al., 2013). Therefore, the question why the cathelin domain is highly conserved among vertebrate cathelicidins is still open. It is believed that the precursor proteins could play a role in the secretion, intracellular trafficking as well as prevent cytotoxicity of mature peptides and their proteolytic degradation. The potential toxicity of cathelicidins is also controlled by their compartmentalization in cytoplasmic granules of immune cells. In case of contact with pathogens AMPs are activated by fusion of procathelicidin-containing specific granules (or large granules of ruminant neutrophils) with the elastase/proteinase 3-containing azurophil granules and either the cytoplasmic membrane or phagosome (Graf et al., 2017). Secondary structures of mature cathelicidins include α-helices, β-hairpins, and extended linear regions enriched with Trp or Pro residues. Interestingly, neutrophils of some artiodactyls, including goats, do not contain defensin-like AMPs (Zhao et al., 1999), suggesting a key role of cathelicidins in the protection of these animals against pathogens. Study of artiodactyl cathelicidins can provide new molecular insight into their role in the host defense.

A number of studies on the synergy between AMPs and conventional antibiotics have been performed over the last years (Cassone and Otvos, 2010; Reffuveille et al., 2014; Simonetti et al., 2014; Ribeiro et al., 2015; de la Fuente-Núñez et al., 2016; Lázár et al., 2018). In contrast, the synergy between AMPs is not well investigated although this phenomenon might contribute to understanding of substantial peptide diversity at any host anatomic site. In most cases the repertoire of structurally diverse animal AMPs make possible both disturbing the membrane integrity of pathogenic microorganisms and inhibiting a number of metabolic processes via interaction with intracellular targets. Such a complex mechanism of action appears to prevent the development of resistance to AMPs. The present work is aimed to examine combined antibacterial effects of structurally distinct cathelicidins expressed in leukocytes of the domestic goat *Capra hircus*. Previously, we have isolated two novel AMPs mini-bactenecins, designated as mini-ChBac7.5Nα and mini-ChBac7.5Nβ, from leukocytes of the domestic goat (Shamova et al., 2016). These peptides are N-terminal fragments (22 and 21 aa, respectively) of the hypothetic ChBac7.5 peptide also classified as cathelicidin-3. Being Pro-rich AMPs, mini-bactenecins are thought to target intracellular structures such as the 70S ribosome and/or heat shock protein DnaK (Graf et al., 2017). In the study, we investigated a biological significance of the PRPRPR fragment localized at the C-terminus of mini-ChBac7.5Nα. For this purpose, a comparative testing of the wild-type peptide and its short derivative termed as mini-ChBac7.5Nα(1–16) was carried out. Earlier, bovine Bac7(1–16) was shown to be the minimal fragment of the native 60-residue peptide Bac7 displaying both antimicrobial activity in broth microdilution tests and ability to inhibit protein synthesis *in vitro* (Benincasa et al., 2004; Seefeldt et al., 2016). Along with mini-ChBac7.5Nα, the previously not investigated *C. hircus* myeloid AMP cathelicidin-6, designated as ChMAP-28, was chosen as the second component of the model system. The peptide primary structure was deduced from the deposited in GenBank mRNA sequence (AJ243126.1) coding the appropriate precursor protein. The novel cathelicidin has relatively high homology with the α-helical bovine cathelicidin BMAP-27 (**Figure 1**). ChMAP-28 contains eleven basic amino acid residues (Arg, Lys, His). As goat leukocytes were shown to simultaneously express mRNA for both cathelicidin-3 and -6 (Zhang et al., 2014), we supposed that the peptides were co-localized in the cells and could act synergistically during the immune response. The combined antibacterial effects of the goat cathelicidins were studied by a checkerboard titration method against a set of bacterial strains including the "ESKAPE" pathogens. The role of each cathelicidin in the synergistic cooperation and their predominant mechanisms of action were elucidated. Finally, antibacterial activity changes under a long-term continuous selective pressure of the individual peptides and their combination were investigated against *Escherichia coli* strains.

MATERIALS AND METHODS

All the bacterial strains used in this study are listed in **Table 1**. The clinical isolates were collected and provided by Sechenov First Moscow State Medical University hospital and Solixant LLC (Moscow, Russia). The resistant to conventional antibiotics strains were defined as extensively drug resistant (XDR) according to (Magiorakos et al., 2012).

Expression and Purification of the Antimicrobial Peptides

The recombinant plasmids for expression of the goat cathelicidins were constructed with the use of pET-based vector as described previously (Panteleev and Ovchinnikova, 2017).

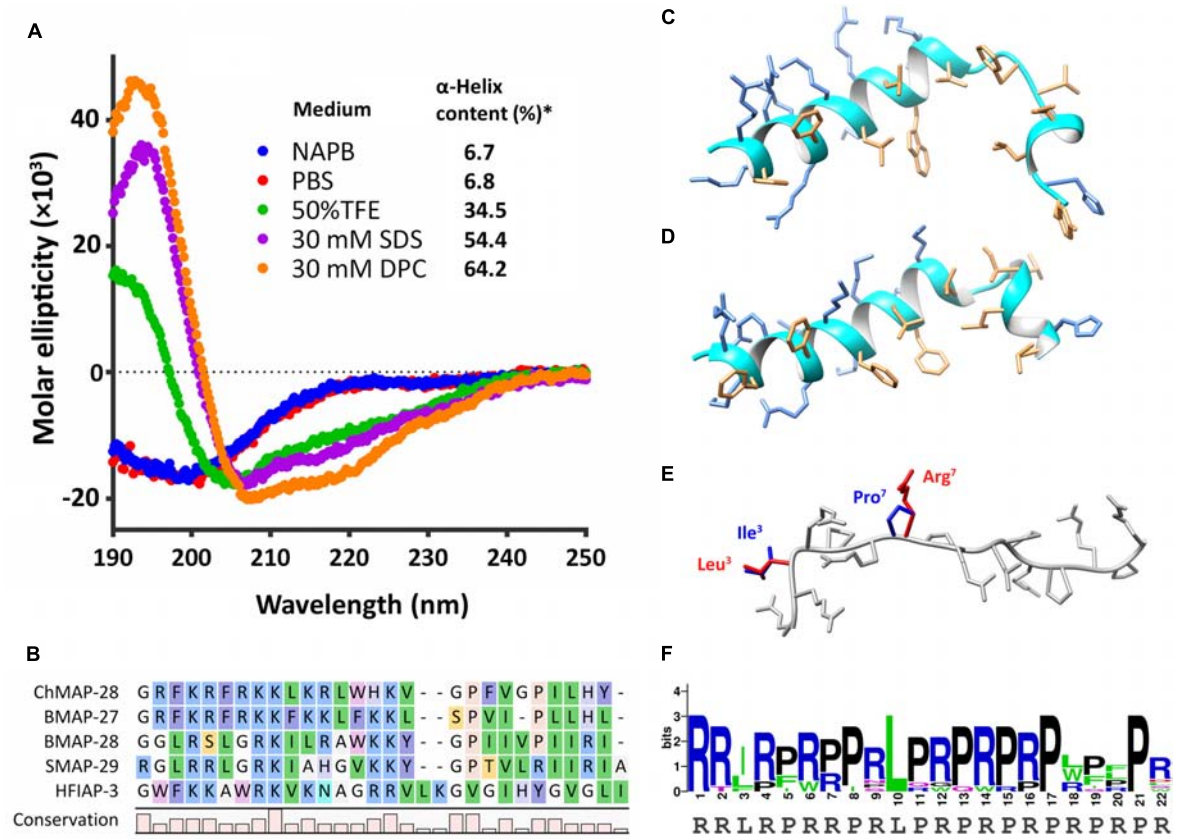

FIGURE 1 | Structure analysis of goat cathelicidins. **(A)** CD-spectra of ChMAP-28 in 10 mM sodium phosphate buffer (NAPB, pH 7.4), phosphate-buffered saline (PBS, pH 7.4), 50% TFE, 30 mM SDS micelles, 30 mM DPC micelles. *The CONTINLL program (Provencher and Glöckner, 1981) was used for data analysis. **(B)** Alignment of the mature ChMAP-28 with α-helical bovine, sheep, and hagfish cathelicidins. **(C)** Spatial structure of ChMAP-28 was simulated in the MODELLER software (Sali and Blundell, 1993) by homology modeling on the basis of the NMR structure **(D)** of BMAP-27 (PDB 2KET) serving as a template. **(E)** Spatial structure of the mini-ChBac7.5Nα(1–16) fragment was modeled and overlaid on the basis of the crystal structure of the Bac7(1–16) bound to bacterial 70S ribosome (PDB 5F8K). Varying residues are marked with red for goat cathelicidin and blue for bovine cathelicidin. The structures were visualized by the Chimera software (Pettersen et al., 2004). **(F)** Amino acid frequency in mini-ChBac7.5Nα and its orthologs from mammalian species. The graph was plotted using the WebLogo server.

The target peptides were expressed in *E. coli* BL21 (DE3) as chimeric proteins that included 8 × His tag, the *E. coli* thioredoxin A with the M37L substitution (TrxL), methionine residue, and a mature cathelicidin. The ChMAP-28 amino acid sequence was translated from mRNA for the corresponding precursor protein (GenBank: AJ243126.1) as a 27-residue peptide without the C-terminal glycine, a common amidation signal in cathelicidins. The transformed *E. coli* BL21 (DE3) cells were grown up to OD_{600} 1.0 at 37°C in lysogeny broth (LB) containing 20 mM glucose, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$, 100 μg/ml of ampicillin and then were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.3 mM. The cells were cultivated for 5 h at 30°C with intense agitation. Then the cells were pelleted by centrifugation and sonicated in immobilized metal affinity chromatography (IMAC) loading buffer containing 6 M guanidine hydrochloride. The clarified lysate was applied on a column packed with Ni Sepharose (GE Healthcare). The recombinant protein was eluted with the buffer containing 0.5 M imidazole. Then the eluate containing the fusion protein was acidified (up to pH 1.0) and cleaved by 100-fold molar excess of cyanogen bromide over methionine for 20 h at 25°C in the dark. The reaction products were lyophilized, dissolved in water, titrated to pH 5.0, and loaded on a semi-preparative Reprosil-pur C18-AQ column (10 mm × 250 mm, 5-μm particle size, Dr. Maisch GmbH). Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed with a linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. The peaks were monitored at 214 and 280 nm. The collected fractions were analyzed by MALDI-TOF mass-spectrometry using Reflex III instrument (Bruker Daltonics). The fractions containing the target peptides were lyophilized and dissolved in water. The synthetic melittin (>98% pure) was kindly provided by Dr. Sergey V. Sychev (M.M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russia). The recombinant tachyplesin-1 was obtained as described previously (Panteleev and Ovchinnikova, 2017). The peptides concentrations were estimated using UV absorbance.

TABLE 1 | Bacterial strains used in this study.

Bacterial strain	Characteristics (source, antibiotic resistance)
Micrococcus luteus B-1314	Laboratory strain (VKM collection)
Bacillus subtilis B-886	Laboratory strain (VKM collection)
Enterococcus faecalis ATCC 29212	Laboratory strain (ATCC collection)
Staphylococcus aureus ATCC 29213	Laboratory strain (ATCC collection)
Staphylococcus aureus 209P	Laboratory strain (ATCC collection)
Escherichia coli DH10B	Cloning strain (Invitrogen)
Escherichia coli BL21 (DE3)	Expression strain (Novagen)
Escherichia coli BL21 Star (DE3)	Expression strain (Novagen)
Escherichia coli ML-35p	Laboratory strain (ATCC collection)
Escherichia coli C600	Laboratory strain (ATCC collection)
Escherichia coli (XDR CI 1057)	Extensively drug resistant clinical isolate (urine, urinary tract infection; ESBL+)
Escherichia coli (CI 214)	Clinical isolate (urine, acute pyelonephritis)
Klebsiella pneumoniae (CI 287)	Clinical isolate*
Klebsiella pneumoniae (XDR CI 1056)	Extensively drug resistant clinical isolate (urine, urinary tract infection; ESBL+)
Enterobacter cloacae (XDR CI 4172)	Extensively drug resistant clinical isolate* (MBL+)
Acinetobacter baumannii (XDR CI 2675)	Extensively drug resistant clinical isolate* (MBL+)
Pseudomonas aeruginosa PAO1	Laboratory strain (ATCC collection)
Pseudomonas aeruginosa (XDR CI 1049)	Extensively drug resistant clinical isolate (urine, kidney stone disease; MBL+)
Proteus mirabilis (XDR CI 3423)	Extensively drug resistant clinical isolate* (MBL+)

*CI, clinical isolate; *, no data available on strain source; XDR, extensively drug resistant strain; ESBL+, extended spectrum beta-lactamase producing strain; MBL+, metallo-beta-lactamase producing strain.*

Circular Dichroism Spectroscopy and Structure Analysis

Secondary structures of the cathelicidins were analyzed in different environments by circular dichroism spectroscopy (CD) with the use of Jasco J-810 instrument (Jasco) at 25°C. The experiment was performed in 10 mM sodium phosphate buffer (NAPB, pH 7.4), phosphate-buffered saline (PBS, pH 7.4), 50% TFE (Sigma), 30 mM DPC (Anatrace) micelles, and 30 mM SDS (Sigma) micelles. Final concentrations of the peptides were of 300 μM. Four consecutive scans were performed and averaged, followed by subtraction of the blank spectrum of the solvent. The CONTINLL program was used for data analysis (Provencher and Glöckner, 1981). Homology modeling was performed by MODELLER software (Sali and Blundell, 1993). The spatial structures were visualized by Chimera software (Pettersen et al., 2004).

Hemolysis and Cytotoxicity Assay

Hemolytic activity of the peptides was tested against the fresh suspension of human red blood cells (hRBC) using the hemoglobin release assay as described previously (Panteleev et al., 2016). Three experiments were performed with the hRBC from blood samples obtained from independent donors. The obtained data were represented as average means with standard deviations. The cytotoxicity of the peptides against HEK293T (transformed human embryonic kidney cells) and HEF (human embryonic fibroblasts) cell lines was studied using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay according to (Panteleev and Ovchinnikova, 2017). Three independent experiments were performed for each peptide. Half maximal inhibitory concentration (IC_{50}) values were estimated as described previously (Kuzmin et al., 2018).

Antimicrobial Assay

Antimicrobial assay was performed as described previously (Panteleev et al., 2017b). Briefly, mid-log phase bacterial test cultures were diluted with the 2× Mueller-Hinton broth (MH, Sigma) supplemented with 1.8% NaCl or without it so that to reach a final cell concentration of 10^6 CFU/ml. 50 μl aliquots of the obtained bacterial suspension were added to 50 μl of the peptide solutions serially diluted with 0.1% water solution of bovine serum albumin (BSA) in 96-well flat-bottom polystyrene microplates (Eppendorf #0030730011). After incubation at 37°C and 900 rpm for 24 h the minimum inhibitory concentrations (MIC) were determined as the lowest peptide concentrations that prevented growth of a test microorganism observed as visible turbidity. The results were expressed as the median values determined on the basis of at least three independent experiments performed in triplicate.

Checkerboard Assay

The peptides were twofold serially diluted with 0.1% BSA in 96-well microplates (Eppendorf #0030730011). Then, the peptide solutions were mixed in the new test plate crosswise in such a way that the resulting checkerboard contained each combination of the cathelicidins in eight doubly increasing concentrations, with wells containing the highest concentration of each peptide at opposite corners (Berditsch et al., 2015). Then, the antimicrobial assay was performed as described in the previous section. MICs were defined as the lowest concentrations of the peptides (when used individually or in the mix with another peptide at a sub-inhibitory concentration) that completely inhibited bacterial growth. The results were expressed as the median values determined on the basis of three independent experiments performed in duplicate. Estimation of synergistic effects of different cathelicidins was performed by calculating the fractional inhibitory concentration index (FICI) according to the equation: $FICI = [À]/MIC_{À} + [B]/MIC_B$, where $MIC_{À}$ and MIC_B are the MICs of the individual substances, while [A] and [B] are the MICs of A and B when used together. A synergistic effect was defined at a $FICI \leq 0.5$.

Biofilm Assay

Biofilm formation assay was performed as described previously (Panteleev et al., 2017a) with some modifications. Different *E. coli* strains and cultivating conditions were preliminary tested to achieve a strong biofilm formation (**Supplementary Figure S2**). The *E. coli* CI 214 cells were incubated in the trypticase soy broth (TSB) for 16 h at 37°C and then were diluted 150-fold with the 2×M9 minimal medium supplemented with 50 mM

glucose, 10 μM thiamine, 2 mM $MgSO_4$, 1 mM $CaCl_2$, and the trace metals mixture. 50 μl of the obtained bacterial suspension were added to 50 μl aliquots of the peptide solutions serially diluted with sterilized water in 96-well microplates (Eppendorf #0030730011). The plates were incubated at 32°C with gentle agitation (120 rpm) for 24 h to allow biofilm formation. Then, planktonic (unattached) cells were transferred into the new 96-well plate and OD_{620} of the cell culture was measured with the use of a microplate reader. The wells of the former plate were washed with PBS twice, and the formation of sessile biofilms was analyzed by crystal violet (CV) staining. Briefly, 160 μl of 0.1% crystal violet (CV, Sigma) solution was transferred to each well. The plates were incubated at 25°C for 40 min and rinsed with distilled water to remove an excess of CV. Then the samples were dried for 10 min, and 160 μl of 96% ethanol (v/v) was added to the wells so that to dissolve the CV. 40 min later, the obtained extracts were transferred to a new 96-well plate. The absorption at 570 nm was measured with the use of a microplate reader. The experimental data were obtained from at least three independent experiments performed in triplicate. The results were reported relative to untreated bacteria served as a control. The results were analyzed using the GraphPad Prism 6.0 software.

Resistance Induction Experiments

Resistance induction experiments were performed using the previously described method (Chernysh et al., 2015) with some modifications. Briefly, on day 1, the overnight culture of wild-type bacteria was diluted with the 2× MH broth containing 1.8% NaCl so that to reach a final cell concentration of 10^6 CFU/ml. 50 μl aliquots of the obtained bacterial suspension were added to 50 μl of the peptide solutions serially diluted with 0.1% water solution of BSA in 96-well microplates (Eppendorf #0030730011). After incubation at 37°C and 900 rpm for 22 ± 2 h, MICs were determined as described above. For each subsequent daily transfer, 5 μl of the inoculum taken from the first well with a sub-inhibitory drug concentration were diluted with 1 ml of the fresh 2× MH broth supplemented with 1.8% NaCl. Then, 50 μl of this suspension were sub-cultured into the next passage wells containing 50 μl aliquots of the peptide at concentrations from 0.25× to 16× of the current MIC of each agent. 26 repeated passages in the presence of antimicrobial agents were made for each bacterial strain during the experiment. Typically, the experiment was finished when the bacterial culture became resistant to antibiotic polymyxin B (Applichem) used as a control. Finally obtained cell cultures were passaged five times in the absence of antimicrobial agent to confirm that the acquired resistance is stable. Control serial passages in the absence of the agent were also performed. The obtained cultures showed unchanged MICs against antibacterial agents.

Bacterial Membranes Permeability Assay

To examine an ability of the peptides to affect the barrier function of outer and inner membranes of Gram-negative bacteria, we slightly modified the previously described procedure (Shamova et al., 2016) with the use of the *E. coli* ML-35p strain constitutively expressing cytoplasmic β-galactosidase and lacking lactose permease, and also containing β-lactamase in the periplasmic space. The state of the *E. coli* ML-35p outer and cytoplasmic membranes was assessed based on their permeability to chromogenic markers nitrocefin (Calbiochem-Novabiochem) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG, AppliChem) which are the β-lactamase and β-galactosidase substrates, respectively. The cells were incubated in the TSB medium at 37°C for 16 h, washed three times with 10 mM sodium phosphate buffer (pH 7.4) to remove residual growth media, diluted to the concentration of 2.5×10^8 CFU/ml. The experiments were performed in 10 mM sodium phosphate buffer with or without 0.9% NaCl. The final concentration of *E. coli* ML-35p cells was of 2.5×10^7 CFU/ml. The concentrations of ONPG and nitrocefin were of 2.5 mM and 20 μM, respectively. Peptide samples were placed in the wells of a 96-well plate with non-binding surface (NBS, Corning #3641), and the optical density (OD) of the solution rising due to the appearance of the hydrolyzed nitrocefin or ONPG was measured at 540 and 405 nm, respectively, using the Multiskan EX microplate reader (Thermo Fisher Scientific). The final volume in each well was 200 μl. The experiments were performed at 37°C under stirring at 300 rpm. Control experiments were performed under the same conditions without addition of a peptide. Three independent experiments were performed, and the curve pattern was similar for all three series.

Flow Cytometry

The *E. coli* ML-35p cells were incubated in the TSB medium for 16 h at 37°C and washed as described above. Bacterial cell suspensions were then incubated for 1 h at 37°C with peptides at different concentrations prepared by twofold serial dilution. The assay was performed in the 96-well NBS microplates in 10 mM sodium phosphate buffer with or without 0.9% NaCl (pH 7.4). Then, SYTOX green (Life Technologies) was added to the treated cells at a final concentration of 2.5 μM and incubated for 10 min at room temperature in the dark. The SYTOX green does not penetrate live cells, but once inside the cell it binds to nucleic acids resulting in more than 500-fold enhancement of fluorescent emission. The fluorescence of the bacterial suspensions diluted 5-fold with PBS was measured (λ_{Exc} = 488 nm, λ_{Em} = 530 nm) by NovoCyte flow cytometer (ACEA Biosciences). For each sample 10^5 events were recorded. Fluorescence signals were expressed as a percentage of two distinct cell groups: (1) healthy and partially damaged cells were deemed as totaling from 10^2 to 10^5 range of detection at 530 nm; (2) completely permeabilized (dead) cells were deemed as amounting $\geq 10^5$ range of detection at 530 nm. Two independent experiments were performed, and the similar results were obtained.

Cell-Free Protein Expression Assay

The cell lysate used for translation inhibition assay was prepared using the *E. coli* BL21 Star (DE3) cell culture grown at 30°C in the 2x YTPG liquid medium (1% yeast extract, 1.6% tryptone, 0.5% NaCl, 22 mM NaH_2PO_4, 40 mM Na_2HPO_4, 0.1 M glucose). The chromosome of DE3 strains contains a gene encoding T7 RNA polymerase under control of the *lacUV5* promoter. The bacterial culture was grown to OD_{600} 0.8–1.0, then T7 RNA polymerase gene was induced by adding 0.2 mM IPTG. Bacteria were harvested at OD_{600} 5.0–6.0 by centrifugation (3000 *g*,

30 min, 4°C). The bacterial pellet was washed three times by suspending it in four volumes of wash buffer (10 mM Tris-acetate buffer, pH 8.2, 60 mM potassium glutamate, 14 mM magnesium acetate, 1 mM DTT), then resuspended in one volume of the same buffer (1 ml per 1 g of wet cell mass) and disrupted by sonication at 5–15°C. The total cell lysate was centrifuged at 15000 *g* (30 min, 4°C). The supernatant was split into aliquots and stored at −70°C.

In order to investigate the effect of AMPs on the translation process, the peptides were added to a cell-free protein synthesis (CFPS) reaction mix with a plasmid encoding EGFP variant (F64L, S65T, Q80R, F99S, M153T, and V163A) under a control of the T7 promoter. The reaction mix consisted of the following components: 1.2 mM ATP, 0.8 mM UTP, 0.8 mM GTP, 0.8 mM CTP, 2 mM of each of 20 proteinogenic amino acids, 1.5 mM spermidine, 1 mM putrescine dihydrochloride, 0.06647 mM calcium folinate, 170 ng/ml tRNA from the *E. coli* MRE 600 strain, 0.33 mM NAD, 120 mM HEPES-KOH (pH 8.0), 10 mM ammonium glutamate, 175 mM potassium glutamate, 60 mM glucose, 15 mM magnesium glutamate, 2% PEG 8000, 25% *E. coli* BL21 Star (DE3) cell lysate, 10 ng/μl plasmid DNA. The reaction volume was 50 μl. The peptides were dissolved in PBS with the addition of 0.1% BSA. Streptomycin and erythromycin were used in the positive control reactions. Fluorescence of the sample without inhibitor was set as the 100% value. The reaction proceeded for 1.5 h in 96-well clear flat-bottom black polystyrene microplates (Corning #3340) sealed with Parafilm in a plate shaker (30°C, 900 rpm). Fluorescence of the synthesized EGFP was measured with the microplate reader AF2200 (λ_{Exc} = 488 nm, λ_{Em} = 510 nm). The experimental data were obtained from at least three independent experiments. IC_{50} values were determined by interpolation from non-linear regression curves using the GraphPad Prism 6.0 software.

Electrophoretic Mobility Shift Assay

The peptides binding to DNA was examined by electrophoretic mobility shift assay (EMSA) according to the previously described protocol (Panteleev et al., 2016). Briefly, the plasmid pUC19 was incubated with the tested peptides at increasing concentrations in the binding buffer containing 10 mM Tris-HCl (pH 8.0), 50 μg/ml BSA, 5% glycerol, 1 mM DTT, 150 mM NaCl, 20 mM KCl, and 1 mM EDTA, at 25°C for 30 min. Then, the samples were analyzed by electrophoresis in 0.8% agarose gel. The DNA migration was detected by means of the ethidium bromide fluorescence tracking. The DNA-peptide (w/w) ratios were of 1:0 (negative control), 4:1, 2:1, 1:1, 1:2, respectively.

Genetic Analysis of Bacterial Strains

The *sbmA* and *yaiW* genes encoding the *E. coli* inner or outer membrane proteins, respectively, as well as a regulatory part of their common operon were amplified by polymerase chain reaction (PCR) using gene-specific primers (**Supplementary Figure S3**). Individual bacterial colonies of the tested strain were picked up from Petri dish and used as a template for PCR. The following components were mixed for the PCR: 2 μl of 10× Encyclo buffer (Evrogen), 0.4 μl of 50× Encyclo DNA polymerase (Evrogen), 10 μM forward primer, 10 μM reverse primer, 0.2 mM dNTPs, bacterial cells on inoculation loop, and water diluting to the total volume of 20 μl. Amplification was carried out on a thermocycler using: initial denaturation (95°C, 10 min), 25 amplification cycles (94°C, 30 s; 55°C, 40 s; 72°C, 90 s), and final elongation (72°C, 10 min). The products were separated by electrophoresis on 1.5% agarose gel (4 V/cm) and visualized on a UV trans-illuminator. The PCR products were purified from agarose gel and inserted into pGEM-T vector (Promega). The ligation products were transformed into the chemically competent *E. coli* DH10B cells. Plasmid DNA was isolated from overnight cultures of single white colonies on LB agar plates supplemented with ampicillin (100 μg/ml), using Plasmid Miniprep kit (Evrogen). The plasmids were sequenced on both strands using the ABI PRISM 3100-Avant automatic sequencer (Applied Biosystems). At least two independent experiments were performed with each strain to prove the obtained results.

RESULTS

Expression and Purification of the Recombinant Peptides

Natural goat cathelicidins do not undergo significant post-translational modifications, therefore heterologous expression in *E. coli* of the peptides fused with a carrier protein seems to be a reasonable approach for their production. The goat cathelicidins were produced using the same protocol. To facilitate the purification process and improve final yield, the recombinant peptides were obtained as fusion proteins with the N-terminal 8×His tag and thioredoxin A which was approved to be an effective carrier protein for different peptide scaffolds having antibacterial activity (Li, 2011). The peptides were purified by a downstream process including IMAC of the clarified total cell lysate, cleavage of the fusion protein with cyanogen bromide, and fine purification by RP-HPLC (**Supplementary Figure S1**). Final yields of ChMAP-28, mini-ChBac7.5Nα, and mini-ChBac7.5Nα(1–16) were 3.4, 9.2, and 7.5 mg per 1 l of the culture medium, respectively. The obtained recombinant cathelicidins were analyzed by MALDI-TOF mass-spectrometry. The measured m/z values of the cathelicidins matched the corresponding calculated molecular masses (**Supplementary Table S1**).

Secondary Structure of Goat Cathelicidins

In this study, CD spectroscopy was used to analyze the secondary structure of the goat cathelicidin ChMAP-28. As shown in **Figure 1A**, the CD spectra of ChMAP-28 dissolved in phosphate buffer or phosphate-buffered saline showed a negative peak at the wavelength of 200 nm, which indicated that it mainly adopted random coil conformation. Therefore, the above conditions do not facilitate the peptide folding. In contrast, the CD spectra of ChMAP-28 interacted with SDS or DPC micelles showed a strong positive peak at 195 nm, and two negative peaks at 208 and 220 nm, which indicated that ChMAP-28 mainly adopted α-helix secondary structures in hydrophobic environments.

Indeed, the peptide has a relatively high homology to known α-helical cathelicidins from *Bos taurus*: 61% sequence identity with BMAP-27 and 42% – with BMAP-28 (**Figure 1B**). We performed homology modeling based on the BMAP-27 structure to visualize a probable spatial structure of ChMAP-28 in membrane-mimicking environment (**Figures 1C,D**). Significant homology between mini-ChBac7.5Nα and the N-terminal fragment of Bac7 suggests their similar structure, thus mini-ChBac7.5Nα was not analyzed by CD-spectroscopy. It is assumed that mini-ChBac7.5Nα, alike the peptide Bac7(1–16), adopts extended structures within the bacterial ribosomal exit tunnel (**Figure 1E**). Generally, mini-ChBac7.5Nα and its orthologs from mammalian species (artiodactyls and cetaceans) recorded in the Genbank showed a relatively high homology, especially between sequences at the N-terminal and central parts of the peptides (**Figure 1F**). Interestingly, recent studies revealed that the Bac7 homolog, isolated from the bottlenose dolphin *Tursiops truncatus* and designated as Tur1B, was enriched with Trp residues and displayed rather modest inhibitory effect on bacterial translation (Mardirossian et al., 2018).

Cytotoxic Properties of Goat Cathelicidins

To estimate cytotoxic effect of the cathelicidins, human red blood cells (hRBC) as well as adhesive cell lines of human embryonic fibroblasts (HEF) and human embryonic kidney cells (HEK293T) were used. Melittin known as a potent cytolytic peptide was used as a positive control. It is known that most Pro-rich AMPs have no pronounced toxicity to mammalian cells. Earlier, we showed that cytotoxicity of mini-bactenecins at concentrations up to 30 μM against a set of mammalian cell lines after 24 h was quite modest (Shamova et al., 2016). However, the peptides are not completely non-toxic. The data analysis revealed that both mini-ChBac7.5Nα and its shortened analog showed cytotoxic activity against mammalian cell lines at concentrations >25 μM (**Figure 2**). Mini-ChBac7.5Nα almost lacked hemolytic activity and lysed only 2% of red blood cells at the concentration of 100 μM. In contrast, a half maximal hemolysis concentration (HC_{50}) of ChMAP-28 was of ∼100 μM, and the peptide had the IC_{50} against HEK293T cells of ∼3.5 μM. Interestingly, its bovine ortholog BMAP-28 possessed the IC_{50} against murine 3T3 cells and HC_{50} of <3.75 and ∼20 μM, respectively (Ahmad et al., 2009). Melittin was proved to be significantly more toxic than α-helical cathelicidins and completely damaged all the cells tested at concentrations of <2.5 μM.

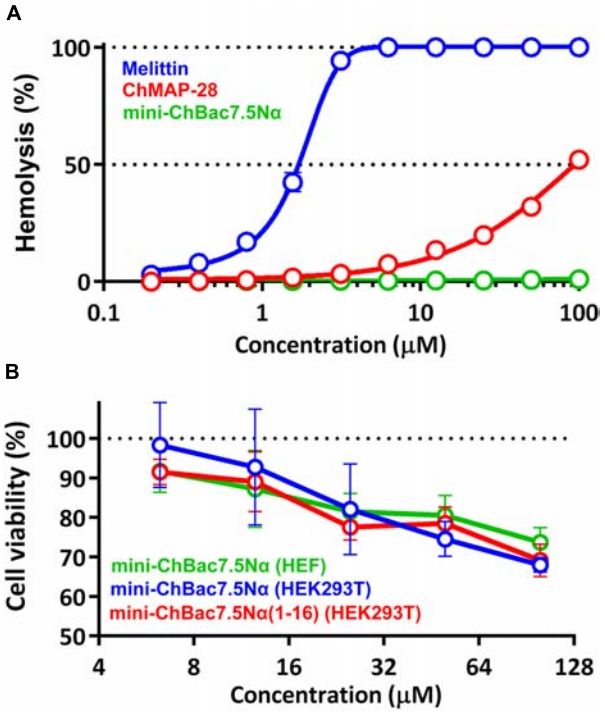

FIGURE 2 | (A) Hemolytic activity of the goat cathelicidins and melittin after 1.5 h incubation (hemoglobin release assay). **(B)** Cytotoxicity of mini-ChBac7.5Nα and its shortened analog toward HEK293T (transformed human embryonic kidney cells) and HEF (human embryonic fibroblasts) cells after 24 h incubation (MTT-assay). Three independent experiments were performed with each peptide.

Antimicrobial Activity of Goat Cathelicidins

Amphiphilic AMPs are known to be adsorbed on plastic surfaces (Wiegand et al., 2008). For these reason, serial dilutions of the peptides were performed in the presence of BSA in the growth medium in order to minimize this effect. MICs of goat cathelicidins and melittin against Gram-positive and Gram-negative bacteria are presented in **Table 2**. It was reported that Pro-rich AMPs have high antimicrobial activity against Gram-negative bacteria and are less active or inactive against most Gram-positive bacteria. In whole, our results confirmed this. It is noteworthy that the medium formulation as well markedly affects the activity values of Pro-rich AMPs. Antibacterial activities of some insect Pro-rich AMPs was low when tested in the presence of a salt, which might inhibit absorption of the peptides to the bacterial surface (Gennaro et al., 2002). Therefore, the salt influence on the antibacterial activity was investigated in this study. Indeed, the presence of 0.9% NaCl resulted in several-fold decrease in the activity of mini-bactenecins against all the strains tested. The shortened analog mini-ChBac7.5Nα(1–16) was shown to be less active and more salt-sensitive as compared with the wild-type mini-ChBac7.5Nα. Interestingly, antibacterial activities of the peptides were similar when tested in a salt-free medium against Gram-negative bacteria *E. coli, Acinetobacter baumannii, Klebsiella pneumoniae,* and *Enterobacter cloacae*. In contrast to the other tested strains, these bacteria have the ABC transport system based on the homodimeric cytoplasmic membrane protein SbmA. In *E. coli* the cytoplasmic membrane protein SbmA and outer membrane lipoprotein YaiW participate in transport of some Pro-rich AMPs and bacteriocins (Arnold et al., 2014). Mutation or deletion of either SbmA or YaiW significantly decreased the ability of the Bac7 to internalize, and significantly reduced susceptibility to the peptide (Arnold et al., 2014). Our results

TABLE 2 | Antibacterial activity of goat cathelicidins and melittin.

Strain	*sbmA/yaiW***	Minimum inhibitory concentration (μM)*							
		Melittin		ChMAP-28		mini-ChBac7.5Nα		mini-ChBac7.5Nα(1–16)	
		Without NaCl	0.9% NaCl	Without NaCl	0.9% NaCl	Without NaCl	0.9% NaCl	Without NaCl	0.9% NaCl
M. luteus B-1314	–/–	0.25	0.5	0.25	0.5	0.125	0.5	1	8
B. subtilis B-886	–/–	0.5	0.5	0.25	1	0.25	4	1	>32
E. faecalis ATCC 29212	–/–	1	1	4	>8	>32	>32	>32	>32
S. aureus ATCC 29213	–/–	1	1	1	2	8	>32	>32	>32
S. aureus 209P	–/–	2	16	0.06	0.5	2	16	8	>32
E. coli C600	+/+	4	8	0.06	0.125	2	4	2	16
E. coli ML-35p	+/+	2	8	0.06	0.06	0.5	4	0.5	8
P. aeruginosa PAO1	–/–	4	8	0.25	1	2	>32	16	>32
A. baumannii (XDR CI 2675)	+/–	2	8	0.03	0.25	2	>32	4	>32
K. pneumoniae (CI 287)	+/+	4	16	0.125	0.5	4	16	4	>32
E. cloacae (XDR CI 4172)	+/+	2	8	0.125	0.25	1	4	1	>32

*Antibacterial testing was performed in the Mueller-Hinton broth at 37°C. **The presence of genes encoding the membrane transporters SbmA and YaiW that affect sensitivity to proline-rich antimicrobial peptides (Arnold et al., 2014).

indicated that the presence of the C-terminal fragment PRPRPR did not influence the efficiency of the peptide translocation via SbmA transporter in a salt-free medium, but could play a key role when acting against SbmA-deficient bacteria (e.g., Gram-positive bacteria) or applying in the presence of a salt. Previous study of the Pro-rich pig cathelicidin PR-39 revealed that an activity of the full length peptide was hardly affected by 100 mM NaCl while the shortened peptide derivatives lacked most of their antimicrobial properties under the same conditions (Veldhuizen et al., 2014). It is likely that the observed effect occurs due to electrostatic interactions between positively charged peptides and negatively charged bacterial membranes. Antimicrobial activity of mini-bactenecins seems to be a function of a total charge of the peptide rather than of a charge density and overall hydrophobicity, since mini-ChBac7.5Nα(1–16) has both higher charge-to-length ratio and longer retention time in reversed-phase HPLC (**Supplementary Figure S1**) than the wild-type mini-ChBac7.5Nα. A total charge could be important at initial stages of Pro-rich AMPs interaction with bacteria, i.e., during the primary electrostatic attraction followed by displacement of divalent cations cross-bridging LPS on the cell surface, that destabilized the membrane and led to the peptide self-promoted uptake.

Cathelicidin ChMAP-28 exhibited significantly more potent antibacterial activity (≥16-fold higher) than melittin against most strains tested. ChMAP-28 was shown to be less sensitive to high ionic strength as compared with mini-bactenecins. ChMAP-28 and last line antibiotics polymyxin B and meropenem were tested against extensively drug resistant clinical isolates of Gram-negative bacteria which belong to "ESKAPE" pathogens: *E. coli, K. pneumoniae, A. baumannii, P. aeruginosa, E. cloacae, P. mirabilis* (**Supplementary Table S2**). Generally, ChMAP-28 exhibited a potent antimicrobial activity comparable with that or even higher than that of the above mentioned control antibiotics. The peptide was shown to effectively kill all the bacteria including polymyxin- and meropenem-resistant strains, thus arguing against cross-resistance to the peptide.

Synergy Between Goat Cathelicidins

Antimicrobial activity of most Pro-rich AMPs including mini-bactenecins is reduced at physiological salt concentrations. In view of this, interaction with other co-localized membrane-active molecules may enhance or restore the activity of Pro-rich AMPs. To check the assumption, antibacterial effects of the combination of the goat cathelicidins mini-ChBac7.5Nα and ChMAP-28 were evaluated in the medium containing a physiological concentration of NaCl (**Table 3**). A set of Gram-negative bacterial species and one Gram-positive strain *S. aureus* 209P sensitive to mini-bactenecins were used as the test microorganisms. To reduce adsorption of AMPs on plastic surfaces while testing antimicrobial activity *in vitro,* we used 0.1% BSA for serial dilutions (Wiegand et al., 2008; Bolosov et al., 2017). In combination with ChMAP-28 at sub-inhibitory concentrations, mini-ChBac7.5Nα exhibited antimicrobial activity with more than fourfold decreased MIC values that led to FICI values of ≤0.375 against different *E. coli* strains. The peptides showed a strong synergistic effect against *K. pneumoniae, E. cloacae*, *A. baumannii* with at least an eightfold decrease in MICs for both agents and FICI values of 0.25, 0.25, and 0.133, respectively. Interestingly, the presence of ChMAP-28 either completely restored or slightly increased the activity of mini-ChBac7.5Nα as compared with that evaluated in a salt-free medium against these bacterial strains (**Table 2**), including the clinical isolate of *E. coli* CI 214 with a weak sensitivity to mini-ChBac7.5Nα (the MIC values were of 4 μM in a salt-free medium and >64 μM in the presence of 0.9% NaCl). As described above, all the mentioned strains normally have the SbmA transport system. It suggests that ChMAP-28 acting at sub-inhibitory concentrations may promote translocation of

TABLE 3 | Synergy between goat cathelicidins ChMAP-28 and mini-ChBac7.5Nα.

Strain	ChMAP-28			mini-ChBac7.5Nα			FICI*	Synergy
	MIC_A	[A]	FIC_A	MIC_B	[B]	FIC_B		
E. coli BL21 (DE3)	0.06	0.015	0.25	8	1	0.125	0.375	Yes
E. coli ML-35p	0.06	0.008	0.125	4	1	0.25	0.375	Yes
E. coli C600	0.125	0.015-0.03	0.125-0.25	4	1	0.25	0.375-0.5	Yes
E. coli (XDR CI 1057)	0.125	0.008	0.063	8	2	0.25	0.313	Yes
E. coli (CI 214)	0.06	0.015	0.25	>64	4	0.031	0.281	Yes
E. cloacae (XDR CI 4172)	0.25	0.03	0.125	4	0.5	0.125	0.25	Yes
K. pneumoniae (CI 287)	0.5	0.06	0.125	16	2	0.125	0.25	Yes
A. baumannii (XDR CI 2675)	0.25	0.03	0.125	>32	0.5	0.008	0.133	Yes
P. aeruginosa PAO1	0.125	0.06	0.5	>32	8	0.125	0.625	No
S. aureus 209P	0.5	0.03	0.063	16	2	0.125	0.188	Yes

**The estimation of synergistic effects between cathelicidins was performed by calculating the fractional inhibitory concentration index (FICI) according to the equation: $FICI = FIC_A + FIC_B = [À]/MIC_À + [B]/MIC_B$, where $MIC_À$ and MIC_B are the MICs of individual peptides, while [A] and [B] are the MICs of A and B when used together. A synergistic effect was defined at a $FICI \leq 0.5$.*

mini-ChBac7.5Nα through the outer membrane, which is an obstacle to Pro-rich AMPs when electrostatic interactions are affected by increased ionic strength. Inside the periplasmic space mini-ChBac7.5Nα can effectively use cytoplasmic membrane transporters to get into the cell. At the same time, ChMAP-28 did not restore the activity of mini-ChBac7.5Nα against *P. aeruginosa*, and no synergy was observed. These findings are consistent with the previous study that revealed the lack of synergy between Pro-rich AMPs and the membranolytic peptide CRAMP while testing antimicrobial activity against *P. aeruginosa* (Knappe et al., 2016). Surprisingly, a pronounced synergistic effect was observed against *S. aureus* 209P with MICs of both peptides almost identical to those measured in a salt-free medium, thus resulting in FICI of 0.188. This observation allowed us to speculate that some Gram-positive bacterial strains might have transport systems for Pro-rich AMPs. On the other hand, the presence of Pro-rich AMPs could interact with the structures of cell wall teichoic acids, the anionic glycopolymers, and thereby helped ChMAP-28 molecules to reach lipid bilayer.

Analysis of Membrane-Permeabilizing Activity

Antimicrobial peptides can realize their biological functions by damaging membrane integrity and specifically inhibiting intracellular processes. One of the most important objectives in functional study of AMPs is to elucidate a mechanism of their antimicrobial action. The effect of the goat cathelicidins on *E. coli* ML-35p membrane integrity was characterized by monitoring both the SYTOX Green uptake by flow cytometry and permeability to chromogenic markers – ONPG and nitrocefin. The membranolytic peptide melittin was used as a positive control. The flow cytometry data show that mini-ChBac7.5Nα does not influence the *E. coli* cytoplasmic membrane integrity regardless of salt concentration (**Figure 3A**). This is in agreement with our previous data (Shamova et al., 2016). In contrast to the longer peptide Bac7(1–35) (Podda et al., 2006), mini-ChBac7.5Nα did not significantly damage membranes at higher concentrations than the MIC values. The cathelicidin ChMAP-28 was shown to damage bacterial membrane at nanomolar concentrations that led to the appearance of bacterial subpopulations with increased fluorescence intensity by one or two orders of magnitude vs. a control (**Figure 3B**). These shifted peaks on the graph may represent cells with qualitatively different grades of membrane damage.

Synergy between two different AMPs could result from either facilitation of translocation of one of them into the cell by another peptide or cooperative augmentation of the membrane damage, as was shown for cathelicidins and defensins (Nagaoka et al., 2000). To decide between these scenarios, a comparative analysis of the ability of the cathelicidins to disrupt the integrity of inner and outer bacterial membranes was conducted in a wide range of concentrations. Interestingly, mini-ChBac7.5Nα was shown to effectively damage outer membrane in a salt-free environment (**Figure 4A**). However, the addition of 0.9% NaCl reduced the activity to a modest effect at 8–32 μM (**Figure 4B**), that could explain a weak antibacterial activity of mini-bactenecins in the presence of salt (**Table 2**). Translocation of mini-ChBac7.5Nα into periplasmic space likely depends on ability to disrupt the outer membrane, which becomes an impassable barrier in the presence of NaCl. At the same time, ChMAP-28 was proved to damage the outer membrane in a salt-containing medium at concentration of 0.008 μM (**Figure 4C**) that was equal to the fractional MIC of the synergy combination with mini-ChBac7.5Nα (see **Table 3**). The data presented in **Figure 4D** allowed us to rule out the effect of potentiating the cytoplasmic membrane permeabilization: in most cases the presence of mini-ChBac7.5Nα did not significantly affect or even decreased the ability of ChMAP-28 to damage the membrane. The same was true when we tested the peptide mixtures on the *E. coli* ML-35p outer membrane (graphical data not shown). Taken together, these results suggest that ChMAP-28 at sub-inhibitory concentrations promotes translocation of mini-ChBac7.5Nα into the periplasmic space rather than enhances its membrane activity.

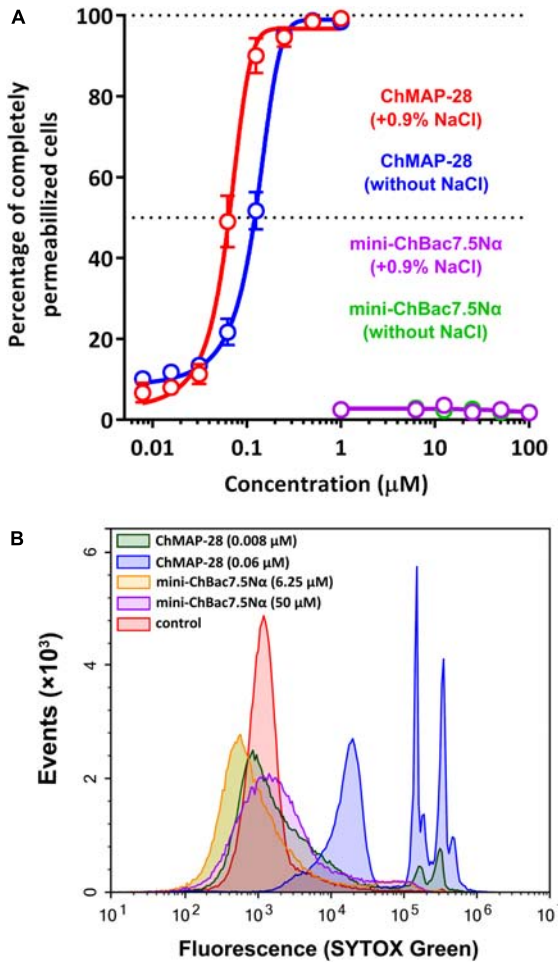

FIGURE 3 | Flow cytometry analysis of the SYTOX Green uptake in *E. coli* ML-35p cells after 60 min treatment with goat cathelicidins. **(A)** The graph showing the effect of the peptides at different concentrations. The experiment was performed in duplicate, with the plotted points representing the mean value ± SD. **(B)** Analysis of bacterial cell populations after treatment in the presence of 0.9% NaCl.

Inhibition of *in vitro* Protein Synthesis in *E. coli*

Taking into account the reported data on the mechanism of action of proline-rich AMPs and the inability of mini-ChBac7.5Nα to disrupt cytoplasmic membrane integrity, we tested an ability of this peptide and other antimicrobial compounds to inhibit protein biosynthesis *in vitro*. The experiment was carried out using the bacterial cell-free protein synthesis system expressing the green fluorescent protein (GFP). The results obtained for streptomycin with IC_{50} value of 0.2 μM and a full inhibition of >1 μM correspond with the published data (Krizsan et al., 2014) (**Figure 5A**). IC_{50} for mini-ChBac7.5Nα was of ∼1 μM which is comparable to that of conventional inhibitors of bacterial translation – streptomycin and erythromycin. Apart from that, the values of IC_{50} for mini-ChBac7.5Nα were similar to those of its homologs – the Bac7 fragments (Seefeldt et al., 2016), and also to the previously determined MICs against *E. coli* (see **Table 2**). It should be noted that the mini-ChBac7.5Nα(1–16) fragment inhibits biosynthesis twice less effectively than the wild-type mini-ChBac7.5Nα that might account for the reduction of antibacterial activity. Interestingly, the cathelicidin ChMAP-28 also affects protein biosynthesis, but at much higher concentrations than its MIC. It seems that ChMAP-28 ability to inhibit translation is due to a non-specific interaction with nucleic acids. This assumption is supported by the fact that tachyplesin-1, which is known to bind DNA (Yonezawa et al., 1992), demonstrates a comparable level of inhibition. For several cationic AMPs, e.g., for indolicidin (bovine tryptophan-rich cathelicidin), binding to DNA is considered to be one of the mechanisms of their antimicrobial action. AMP-DNA binding induces aggregation and interferes with the process of replication (Hsu et al., 2005). It was shown that both goat cathelicidins bound plasmid DNA at a mass ratio of 1:1 (**Figures 5B,C**). In addition, ribosome-binding is supposed to be the main factor responsible for bacterial growth inhibition by the *Bos taurus* cathelicidin Bac7. Comparing our results with known data on Bac7 and bearing in mind a high homology degree between mini-ChBac7.5Nα and the N-terminal fragment of Bac7, we assume that the main target for mini-ChBac7.5Nα is also the 70S ribosome. Data obtained allow us to conclude that two goat cathelicidins – ChMAP-28 and mini-ChBac7.5Nα possess essentially different mechanisms of antimicrobial action: ChMAP-28 preferentially acts by increasing cytoplasmic membrane permeability, while mini-ChBac7.5Nα specifically inhibits bacterial translation.

Anti-biofilm Activity of Goat Cathelicidins

The biofilm formation raises difficulties for therapy of bacterial infectious diseases due to the resistance to conventional antibiotics. Notably, the biofilms can colonize abiotic objects such as surfaces of medical devices and instruments and also be localized in host-organism tissues. Development of compounds that could prevent adhesion of microorganisms to the surfaces and therefore block the formation of biofilms is one of the key problems of modern medicine. In the present work, we investigated whether the synergistic combination of different goat cathelicidins prevent formation of biofilms. The strain *E. coli* CI 214 isolated from urine in acute pyelonephritis was proved to be a strong biofilm producer when cultivated in minimal growth medium (**Supplementary Figure S2**). It is important to notice that this strain has comparatively low sensitivity to mini-ChBac7.5Nα (**Table 2**). All the compounds demonstrated high activity, and at concentrations suppressing planktonic bacterial growth (MIC) the biofilm formation was not observed (**Figure 6**). Complete inhibition of both planktonic and biofilm growth by ChMAP-28, mini-ChBac7.5Nα, and their combination was achieved at concentrations of 1, 32, and (0.125 + 8) μM, respectively. Therefore, the synergy effect consisting in the complete inhibition of *E. coli* was shown with the FICI value of 0.375. The MIC values shown by antibiotic polymyxin B agreed well with those reported earlier when tested against *P. aeruginosa* PAO1 (Panteleev et al., 2017a). It is noteworthy that reduction of biofilm formation with sub-inhibitory concentrations of

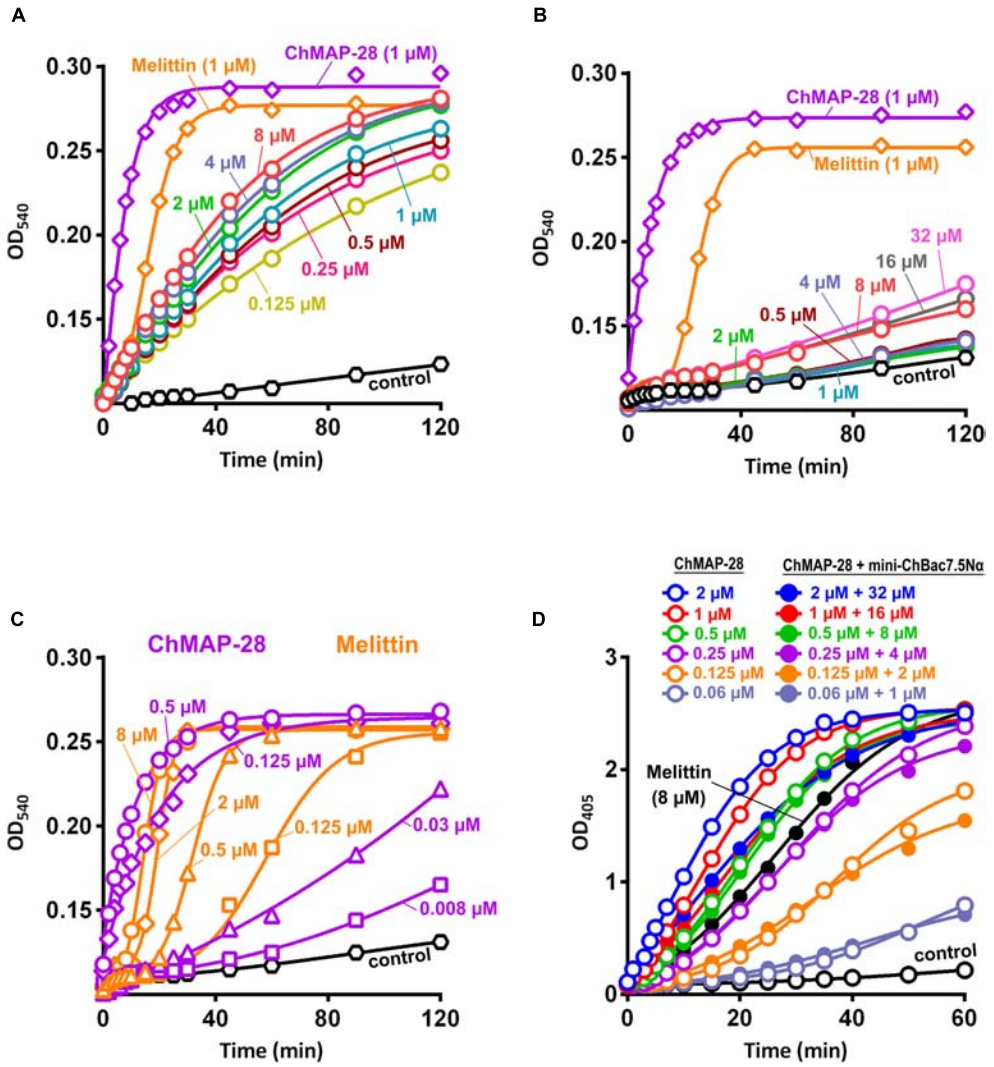

FIGURE 4 | Kinetics of changes in *E. coli* ML-35p outer and cytoplasmic membrane permeability measured with the use of chromogenic markers – the products of nitrocefin (OD_{540}) and ONPG (OD_{405}) hydrolysis, respectively. Outer membrane permeability resulting from incubation of bacteria with mini-ChBac7.5Nα at various concentrations (from 0.125 to 32 μM, highlighted with colors) in the absence **(A)** or in the presence **(B)** of 0.9% NaCl. Melittin and ChMAP-28 at concentration of 1 μM were used as positive control samples. **(C)** Analysis of outer membrane permeability resulting from incubation with ChMAP-28 or melittin in the presence of 0.9% NaCl. **(D)** Comparative analysis of cytoplasmic membrane permeability resulting from incubation with the individual ChMAP-28 or with its combinations with mini-ChBac7.5Nα. Melittin at concentration of 8 μM was used as a positive control. Three independent experiments were performed, and the curve pattern was similar for the three series.

mini-ChBac7.5Nα was followed by a significant stimulation (1.5–2-fold) of planktonic growth as compared with a control. The effect of ChMAP-28 and the combination of the peptides was less pronounced. At concentrations up to 1/16× MIC the peptides inhibited biofilm growth by more than twofold. Presumably, the peptides could prevent an adhesion of bacteria to the plate surface.

Development of Resistance to Goat Cathelicidins

Capacity of the synergistic combination of the goat cathelicidins to prevent bacterial resistance was investigated. Natural combinations of different AMPs from insects, in contrast to individual peptides and small antibiotic molecules, were proved to prevent resistance development in bacteria (Chernysh et al., 2015). Such approach allows using a lower therapeutic dose of AMPs showing synergy with each other. Two *E. coli* strains (XDR CI 1057 and ML-35p) were subjected to the resistance development test by subsequent culturing in the presence of ChMAP-28, mini-ChBac7.5Nα, or the synergistic combination of the peptides, as well as antibiotic polymyxin B at increasing concentrations. The method used in this study allows to monitor MIC values after each transfer. The 2048- and 128-fold increases in MIC values were registered in the bacterial strains XDR CI 1057 and ML-35p, correspondingly, subjected to selection by polymyxin B after 25 passages (**Figure 7**). The *E. coli* XDR CI

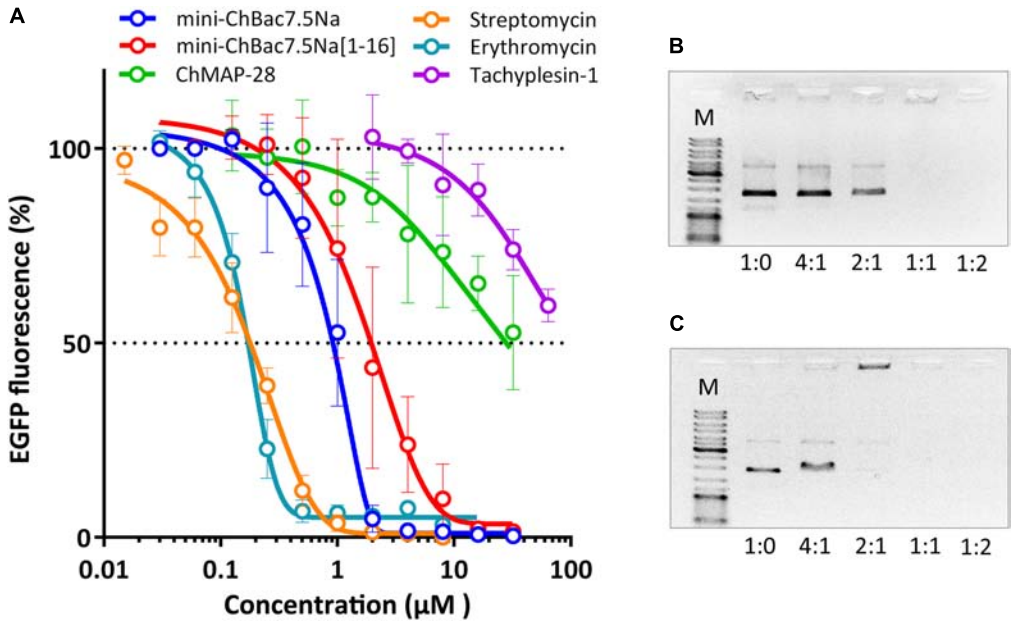

FIGURE 5 | Effects of goat cathelicidins, tachyplesin-1, and conventional antibiotics at different concentrations on the fluorescence resulting from the *in vitro* translation of EGFP with the use of *E. coli* BL21 (DE3) Star cell extract **(A)**. Data are the mean ± SD of at least three independent experiments performed in triplicate. ChMAP-28 **(B)** and mini-ChBac7.5Nα **(C)** binding to DNA was examined by electrophoretic mobility shift assay (EMSA). Various amounts of the peptides were incubated with 100 ng of the pUC19 plasmid DNA, and DNA binding was assessed by the peptide influence on the electrophoretic mobility of DNA. DNA-to-peptide weight ratios are indicated on the horizontal axis. Lane M shows the DNA molecular size marker (500–10,000 bp).

1057 resistance was developed much earlier, resulting in the MIC value of >256 μM. In both cases, an exponential increase of MICs up to 16-fold was observed as the first step of resistance formation. Susceptibility of *E. coli* ML-35p to mini-ChBac7.5Nα decreased only 4-fold over the whole experiment, and no regular MIC changes were observed. Interestingly, 64-fold increases in MIC value (>256 μM) was registered just after eight passages in the bacterial strain XDR CI 1057 subjected to selection by mini-ChBac7.5Nα, and detectable MIC changes became visible after two initial transfers. Considering that the highest peptide concentration in the experiment was of 256 μM, we cannot exclude that actual MIC was beyond this value. The resistance to mini-ChBac7.5Nα was stable, as a serial passage over five steps in the absence of the peptide did not change the MICs. In contrast, the MICs of ChMAP-28 against both strains increased only twofold after 26 passages. The same was true for the mixture of cathelicidins. Then, resistant strains were analyzed for cross-resistance to other agents tested. The MICs of all the tested antimicrobial agents before and after selection are presented in **Table 4**. No differences in MICs before and after 26 passages without antimicrobial agents were observed. Susceptibility of the strain to mini-ChBac7.5Nα acquired after incubation with the synergy combination was similar to that of the control strains, thus arguing the presence of membrane active component prevented formation of resistance against Pro-rich AMP. This also suggests that any resistance mechanisms to mini-ChBac7.5Nα developed in our experiment were associated with modification of membrane transporter system but not with mutations of intracellular targets. Notably, we did not observe any cross-resistance of the strains incubated in the presence of cathelicidins to antibiotic polymyxin B used as a control. In contrast, a considerable resistance to mini-ChBac7.5Nα was detected in the polymyxin-resistant strain obtained after selection. The resistance to polymyxins in Gram-negative bacteria can be mediated by modifications of LPS structure and cell surface charge (Soon et al., 2011). It is very likely that such modifications may influence the mini-ChBac7.5Nα activity due to its high dependence on electrostatic interactions and a low hydrophobicity of the peptide.

Analysis of Mini-ChBac7.5Nα-Resistant Strain Obtained After Selection Experiment

First, we analyzed an influence of NaCl at physiological concentration on antimicrobial activities of the goat cathelicidins against the *E. coli* XDR CI 1057 wild type strain cultivated without an antimicrobial agent and served as a control and against the strain resistant to mini-ChBac7.5Nα. Both mini-ChBac7.5Nα and its analog mini-ChBac7.5Nα(1–16) were predictably inactive against the resistant strain in the presence of 0.9% NaCl. Surprisingly, mini-ChBac7.5Nα completely restored the activity against the resistant strain with the MIC value of 1 μM when tested in the absence of salt, while the activity of mini-ChBac7.5Nα(1–16) was decreased by eightfold as compared with the wild type strain (see **Table 5**). It is known that the ABC-transporter SbmA is essential for the Pro-rich AMPs

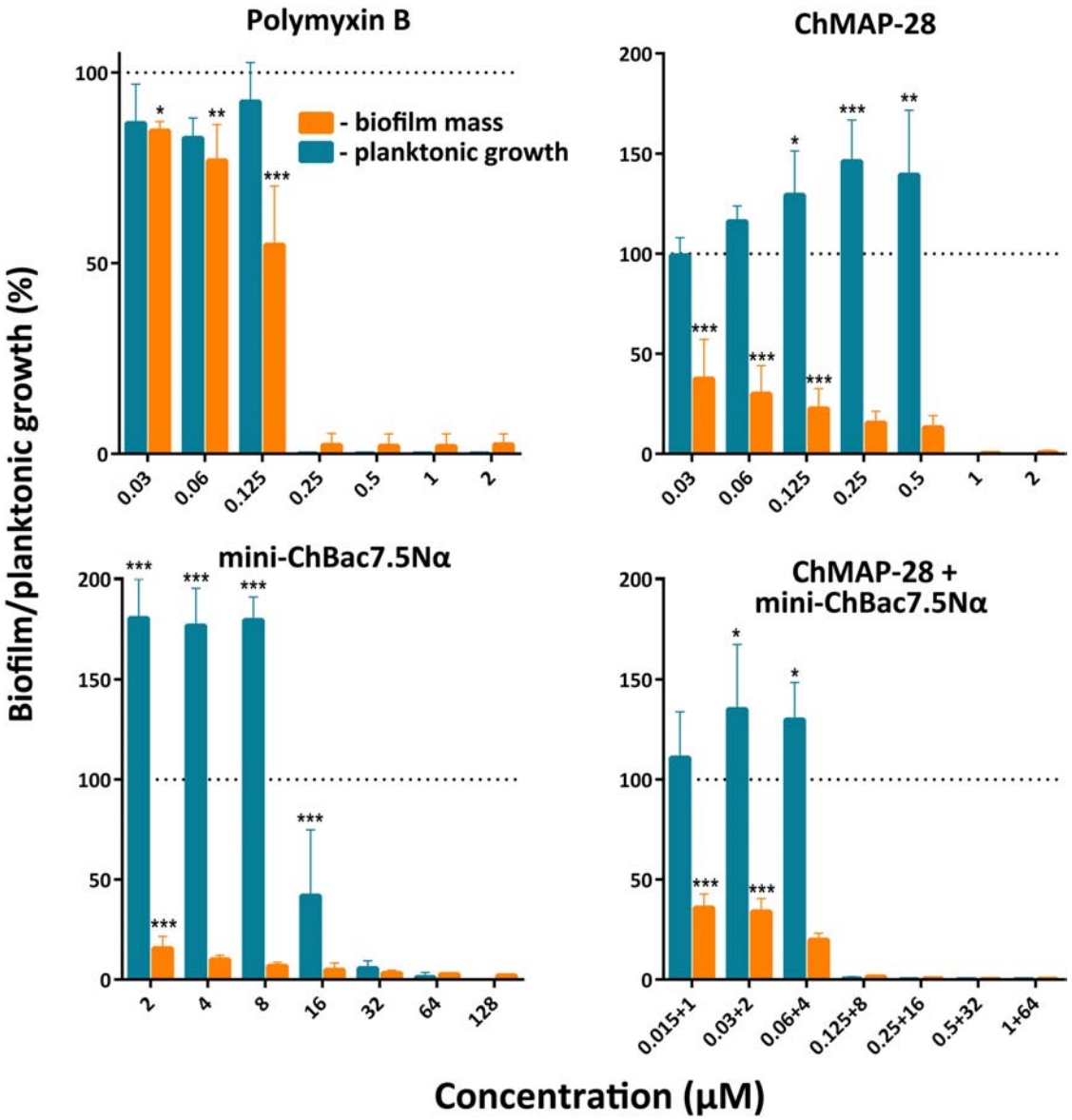

FIGURE 6 | Effect of individual goat cathelicidins ChMAP-28 or mini-ChBac7.5Nα, their synergistic combination, and polymyxin B at different concentrations including sub-inhibitory MICs on planktonic cell growth and biofilm formation of *E. coli* clinical isolate. Biofilm formation was assessed by the colorimetric crystal violet-based technique. The results are expressed as percentage of the planktonic growth or the formed biofilm by reference to an untreated control taken as 100%. Data are the mean ± SD of at least three independent experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significantly different compared to the control.

uptake and thus is crucial for their activity (Schmidt et al., 2016). Therefore, the SbmA transporter seems to be a resistance factor. Notably, the antibacterial activity of the Bac7 N-terminal fragments were also shown to be decreased by four–eightfold when tested against the SbmA-deficient *E. coli* strain in the MH medium without salt (Mattiuzzo et al., 2007; Guida et al., 2015). The outer membrane lipoprotein YaiW cotranscribed with SbmA was also shown to influence the activity of the Bac7 N-terminal fragments suggesting involvement of this protein in the SbmA-mediated uptake of the peptide (Arnold et al., 2014). To check the functionality of both proteins, analysis of the *sbmA-yaiW* gene regions of *E. coli* strains was performed (**Supplementary Figure S3A**). PCR analysis revealed that amplicon lengths for both *sbmA* and *yaiW* genes of the mini-ChBac7.5Nα-resistant *E. coli* strain were identical to those of the control strain (**Supplementary Figure S3B**). This proves the absence of any notable insertions or deletions in the genes. Earlier, a 600 bp insertion was identified in *sbmA* gene of the *E. coli* strain resistant to Pro-rich AMP apidaecin 1b (Schmidt et al., 2016). All the PCR-products were sequenced, and no difference in a regulatory part of *sbmA* operon of the control and resistant strains tested was found (data not shown). Also,

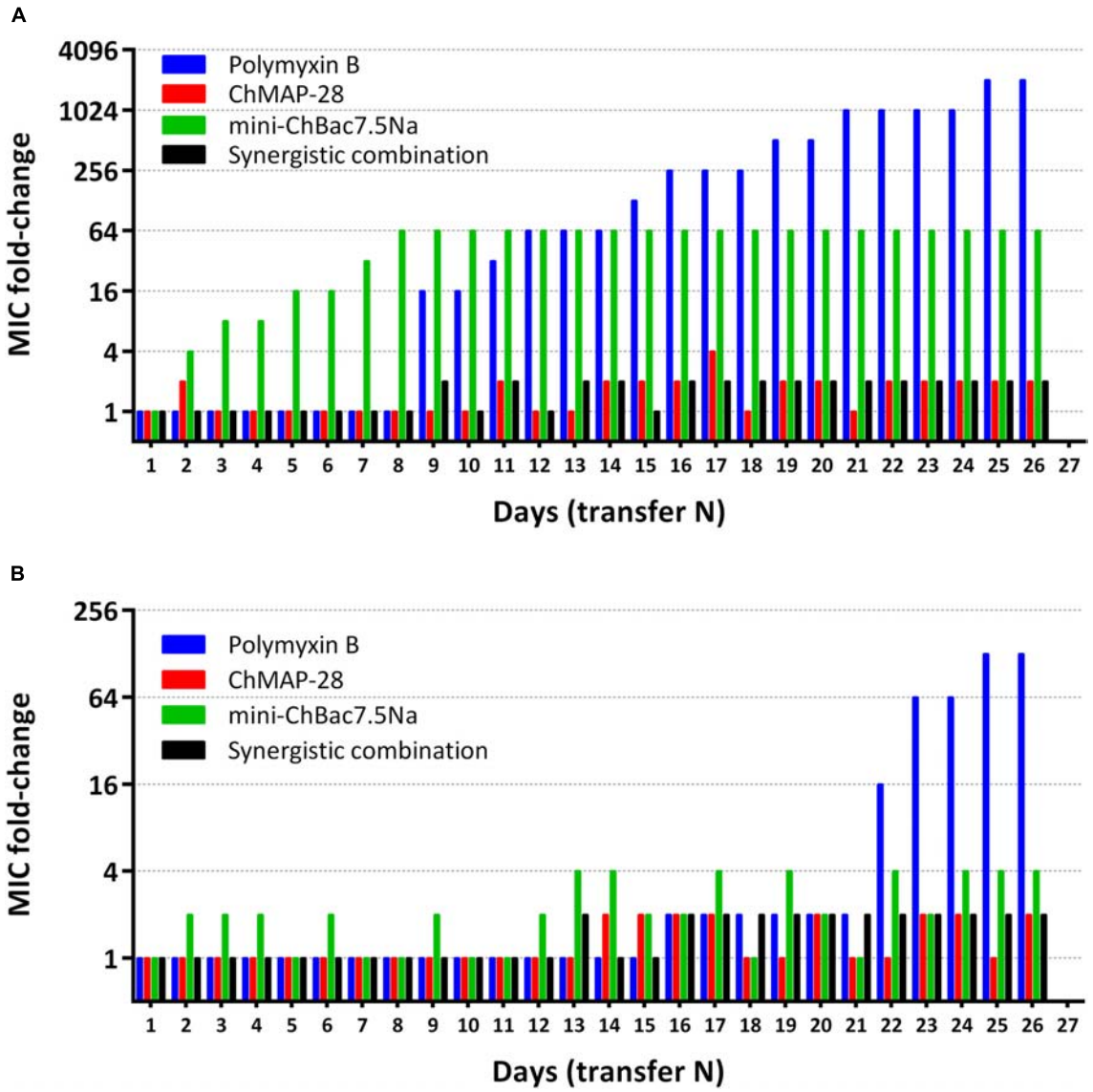

FIGURE 7 | Minimum inhibitory concentrations (MIC) changes in bacterial strains *E. coli* XDR CI 1057 **(A)** and *E. coli* ML-35p **(B)** exposed to selection by individual goat cathelicidins ChMAP-28 (MIC value at transfer "1" = 0.125 μM) or mini-ChBac7.5Nα (MIC value at transfer "1" = 8 μM), the synergistic combination of ChMAP-28 + mini-ChBac7.5Nα (MIC value at transfer "1" = 0.03 + 2 μM, respectively), and the reference antibiotic polymyxin B (MIC value at transfer "1" = 0.125 μM). The experiment was performed in the Mueller-Hinton broth supplemented with 0.9% NaCl at 37°C. 26 repeated passages (transfer N) in the presence of antimicrobial agents were made for each bacterial strain during the experiment.

there was no significant difference in the amino acid sequence of YaiW lipoprotein of all the *E. coli* strains tested in this study. The only difference was in the signal peptide mutation (V15A) as compared with *E. coli* BL21 strain. It should be noted that this mutation is quite common among other *E. coli* strains presented in Genbank. Analysis of SbmA revealed the single point mutation V102E in the mini-ChBac7.5Nα-resistant strain as compared with the control one (**Supplementary Figure S4**). The SAR analysis of SbmA demonstrated that the strains bearing the single mutations (V102G, F219G, or E276G) had a null phenotype for SbmA transport functions (Corbalan et al., 2013). In particular, the *E. coli* V102G mutant strain was almost insensitive to the Bac7(1–16) with the MIC of 156 μM. The residues V102 and F219 are likely involved in the homodimer formation (Corbalan et al., 2013). Apparently, the mutation V102E inactivates SbmA in the strain obtained in this study.

DISCUSSION

First known Pro-rich AMPs (apidaecins, bactenecins) were identified 30 years ago in insects and mammals, respectively (Casteels et al., 1989; Gennaro et al., 1989). Mechanism of a typical Pro-rich AMP action against Gram-negative bacteria

TABLE 4 | Antibacterial activity of goat cathelicidins and polymyxin B against *E. coli* strains obtained after selection experiment.

Strain	Minimum inhibitory concentration (μM)		
	Polymyxin B	ChMAP-28	mini-ChBac7.5Nα
E. coli XDR CI 1057 (0 days)	0.125	0.125	8
E. coli XDR CI 1057 (26 days without antimicrobial agent)	0.125	0.125	8
E. coli XDR CI 1057 (26 days with polymyxin B)	>128	0.25	64
E. coli XDR CI 1057 (26 days with mini-ChBac7.5Nα)	0.125	0.125	>128
E. coli XDR CI 1057 (26 days with synergy combination)	0.06	0.125	8

is accomplished via several steps: (1) electrostatic interaction between negatively charged components of the outer membrane a positively charged peptide; (2) crossing of the outer membrane and getting into the periplasmic space by self-promoted uptake or the membrane damage; (3) translocation by the transporter proteins into the cytosol; (4) interaction with the 70S ribosome. Being the C-terminal part of a large carrier protein, apidaecins were proved to retain the ability to effectively inhibit the growth of bacterial cells during heterologous expression in *E. coli* (Taguchi et al., 1994). Unlike in apidaecins, it is the N-terminus that important for manifestation of the activity of mammalian Bac7-related peptides whereas the C-terminus appears to be variable and less significant (Graf et al., 2017). Therefore, in the present work the Pro-rich mini-bactenecins were expressed as a C-terminal part of the modified thioredoxin A so that to block the active N-terminus. Here, we showed that the protein biosynthesis inhibition is a predominate mechanism of the *Capra hircus* mini-bactenecins action. The membrane activity of the peptides consists in a salt-dependent effect on the outer membrane of Gram-negative bacteria. It should be noted that goat Pro-rich cathelicidins are not completely devoid of toxicity toward mammalian cells. Minor hemolytic activity implies the absence of membranolytic effect on mammalian membranes. However, a linear increase of cytotoxicity toward both HEF and HEK293T cell lines at concentrations up to 100 μM suggests a non-lytic penetration into the cell followed by interaction with an intracellular target. Indeed, the bovine Bac7(1–35) was proved to inhibit eukaryotic translation with the use of the rabbit reticulocyte lysate system (Seefeldt et al., 2016). Pro-rich AMPs are able to interact with several targets within bacterial cells, and therefore probability of the spontaneous resistance emergence might be rather low. The advantage of Pro-rich AMPs as compared with known conventional antibiotics targeting ribosome is an ability to simultaneously occupy several functional sites of the 50S subunit (Gagnon et al., 2016), and the modifications in rRNA does not necessarily lead to the resistance. Interestingly, mutations in the ribosome that confer resistance to erythromycin result in cross-resistance to insect Pro-rich AMPs, but not to mammalian Bac7 orthologs (Gagnon et al., 2016; Mardirossian et al., 2018). Nevertheless, the "Achilles' heel" of most Pro-rich AMPs is the dependence on specific transport systems when getting into the bacterial cell. Moreover, an inactivation of the transport protein SbmA can reduce activity of some Pro-rich AMPs without an obvious fitness cost for the bacteria (Pränting et al., 2008). Therefore, it is surprising that many organisms produce Pro-rich AMPs to fight bacteria. The capacity for preventing resistance development appears to be a feature of the panel of AMPs as a part of whole immune system, but not of individual peptides (Chernysh et al., 2015). In particular, it is likely that membranolytic agents, e.g., α-helical amphipathic AMPs, can promote translocation of Pro-rich peptides into bacterial cell. The α-helical mammalian cathelicidins are known to have a wide spectrum of antimicrobial activity and a comparatively high toxicity as the result of moderate cell selectivity. Combined antibacterial effects between AMPs should be thoroughly investigated, as the results may explain a high efficacy of the AMP-based defense. Identification of synergistic combinations of AMPs may help to decrease effective concentrations of active molecules (Yan and Hancock, 2001), extend their spectrum of action (Lüders et al., 2003), and prevent the resistance formation (Chernysh et al., 2015). The last-mentioned could occur while using individual AMPs (Anaya-López et al., 2013). To date, only a few studies on synergy between co-localized AMPs have been performed (Singh et al., 2000; Schmitt et al., 2012).

In this study, structurally distinct goat cathelicidins – Pro-rich mini-ChBac7.5Nα and α-helical ChMAP-28 were used as the model system of defense peptides with the same localization, more specifically, in leucocytes. In contrast to the non-lytic mini-ChBac7.5Nα, cathelicidin ChMAP-28 was shown to be potent antibacterial agent with an extremely fast membrane disruption kinetics. Mini-bactenecins possess a moderate antibacterial activity which strongly depends on the ionic composition of the test medium. Thus, the presence

TABLE 5 | Effect of salt on activity of goat cathelicidins against *E. coli* strain obtained after 26 days selection in the presence of mini-ChBac7.5Nα.

Strain	Minimum inhibitory concentration (μM)					
	ChMAP-28		mini-ChBac7.5Nα		mini-ChBac7.5Nα(1-16)	
	Without salt	With 0.9% NaCl	Without salt	With 0.9% NaCl	Without salt	With 0.9% NaCl
E. coli XDR CI 1057 (26 days without antimicrobial agent)	0.125	0.125	1	8	2	16
E. coli XDR CI 1057 (26 days with mini-ChBac7.5Nα)	0.125	0.125	1	>256	16	>256

of 0.9% NaCl results in at least several-fold decrease in the activity of mini-bactenecins against all the tested bacterial strains. The obtained data indicate a synergy between the cathelicidins against a wide range of Gram-negative bacterial species including XDR causative agents of hospital-acquired infections. Importantly, the synergistic effect was shown against Gram-negative bacteria which normally have the SbmA transport system. Earlier, it was supposed that Pro-rich AMPs cross the outer membrane of Gram-negative bacteria and then are actively transported by SbmA into the cytoplasm (Krizsan et al., 2015). Here, mini-ChBac7.5Nα was shown to effectively damage outer membrane, while the addition of 0.9% NaCl minimized the activity. Antibacterial activity of mini-bactenecins is inhibited in the presence of salt, and the electrical double layer around the cell seems to be a key barrier on the way into the cell of highly charged and relatively hydrophilic mini-ChBac7.5Nα. At the same time, ChMAP-28 can damage the outer membrane acting at nanomolar concentrations, which corresponds to fractional MICs at synergy combinations with mini-ChBac7.5Nα (see **Table 3**). It is important to note that the presence of mini-ChBac7.5Nα does not increase the permeability of both inner and outer membrane of *E. coli* caused by ChMAP-28. A similar effect was shown earlier when the synergy between fish histone derivatives and the membranolytic AMP pleurocidin was studied (Patrzykat et al., 2001). Taken together, the obtained data suggest that ChMAP-28 at sub-inhibitory concentrations appears to promote translocation of mini-ChBac7.5Nα into the periplasmic space. Subsequently, the Pro-rich peptide crosses the cytoplasmic membrane with the participation of specific transporters and interacts with the bacterial ribosome.

Besides, AMPs are regarded as promising drug candidates for treatment of biofilms. Complete inhibition of both planktonic and biofilm growth of clinical isolates of *E. coli* by the combination of the goat cathelicidins was indicated with the FICI value of 0.375 which validates a notable synergistic effect. According to the obtained data, synergy combinations of mammalian cathelicidins might also be perspective compounds for development of antibacterial coatings for medical biomaterials and instruments.

It is known that bacteria can become resistant to individual AMPs, that in turn could induce a cross-resistance to AMP effectors of the host innate immune system, thus compromising natural host defense against pathogens (Fleitas and Franco, 2016). The resistance problem can be solved, in particular, by application of combinations of natural AMP having a complex mechanism of antibacterial action. In this paper, capacity of the synergistic combination of the goat cathelicidins for preventing bacterial resistance is reported. Selection experiments with Pro-rich AMPs were performed earlier in low-salt media (Knappe et al., 2016; Schmidt et al., 2016). Here, we used the medium containing 0.9% NaCl. As expected, the combination was shown to keep a high activity after the 26-days selection experiment in contrast to mini-ChBac7.5Nα and the reference antibiotic polymyxin B. The 64-fold increase in the MIC value (>256 μM) was registered in the XDR *E. coli* strain subjected to selection by mini-ChBac7.5Nα just after eight initial passages. Genetic analysis of the resistant strain obtained after selection revealed the single point mutation V102E in the cytoplasmic transporter SbmA as compared with the control one. In the salt-free medium the activity of mini-ChBac7.5Nα(1–16) against this strain was decreased by 8-fold as compared with the control strain subcultured without selective pressure (see **Table 5**). Earlier, it was shown that the V102G strain of *E. coli* had the same lowered sensitivity to Pro-rich AMPs as the SbmA-deleted strain (Corbalan et al., 2013). Interestingly, the activity of mini-ChBac7.5Nα against the resistant strain is restored to the wild-type level in a salt-free medium that suggests an important role of the C-terminal PRPRPR fragment for translocation across cytoplasmic membrane, together with an inhibition of the bacterial translation. In *E. coli*, some Pro-rich AMPs seems to rely exclusively on the SbmA transporter system, while others, including oncocin and Bac7(1–35) were active also in the SbmA-deficient strains, likely due to the presence of another bacterial transport system coding by the *yjiL-mdtM* gene (Runti et al., 2017). Taking into account that there is no significant difference in ability of mini-ChBac7.5Nα and its shortened analog to damage bacterial cytoplasmic membrane, the presence of C-terminal hexapeptide PRPRPR could facilitate a non-lytic translocation of mini-ChBac7.5Nα or promote an interaction of the peptide with cytoplasmic transporters different from SbmA. Nevertheless, the point mutation V102E in SbmA seems to contribute but does not provide the complete resistance to mini-bactenecins (MIC of >256 μM) in the presence of salt. Moreover, the process of the resistance formation was shown to be multistage that also suggests a complexity of the acquired resistance.

Finally, the checkerboard assay was performed to evaluate the combined effects of the cathelicidins the mini-ChBac7.5Nα-resistant *E. coli* strain. The presence of ChMAP-28 at sub-inhibitory concentrations lowered the MIC of mini-ChBac7.5Nα(1–16) from >256 μM to 16 μM while the MIC of mini-ChBac7.5Nα was reduced to 1 μM, that corresponded to their individual MICs in a salt-free medium (see **Table 5**). This proves that at nanomolar concentrations ChMAP-28 influences outer membrane permeability, rather than damages cytoplasmic membranes of bacteria. Cell surface modifications could also prevent interactions between mini-ChBac7.5Nα and bacteria in a medium with a high ionic strength. Interestingly, the MIC values of either mini-ChBac7.5Nα or mini-ChBac7.5Nα(1–16) against the resistant *E. coli* strain are very similar to those measured in the test against *P. aeruginosa*. Also, it should be noted that we did not identify any mutations which may inactivate the SbmA protein in the clinically isolated strain *E. coli* CI 214 with a weak sensitivity to mini-ChBac7.5Nα (**Supplementary Figure S4**). Hereafter, it would be necessary to gain a molecular insight into the reasons of such an increase in the *E. coli* resistance to mini-bactenecins, which could be elucidated by the use of omics-based approaches.

The obtained results suggest a potential medical application of combinations of natural cathelicidins in treating of extensively drug-resistant bacterial infections. This approach will allow using a lower therapeutic dose and minimize adverse cytotoxic effects. At the same time, goat cathelicidins potentially could be used in

medicine as individual agents. ChMAP-28 exhibits outstanding antibacterial properties, but being an α-helical AMP, which are known to be unstable to proteolysis, could be considered mainly as a topical antibiotic. The Pro-rich peptide mini-ChBac7.5Nα is also a perspective molecular scaffold for drug design. The resistance to Pro-rich AMPs can be overcome when administrated in a combination with a membrane active agent, in particular, with an amphipathic cationic peptide. Interestingly, the role of the antimicrobial agent in human bloodstream can be played by the α-helical cathelicidin LL-37. The murine ortholog of the peptide, designated as CRAMP, was shown to act synergistically with insect Pro-rich AMPs (Knappe et al., 2016). However, the absence of Pro-rich AMPs in human immune system as well as their ability to cross the blood–brain barrier (Stalmans et al., 2014) makes it necessary to thoroughly analyze their immunomodulatory and cytotoxic properties. Besides, a relatively low membrane activity against mammalian cells and the ability to inhibit protein biosynthesis make ribosome-targeting Pro-rich AMPs promising candidates for the development of new antitumor agents. Therefore, combined cytotoxic effects of goat cathelicidins toward mammalian cells should be investigated as well.

AUTHOR CONTRIBUTIONS

PP, AK, IB, AE, and SB performed the experiments. PP, AK, IB, VK, OS, AE, SB, and TO designed the experiments and analyzed data. PP, SB, and TO wrote the paper. TO contributed to the conception of the work and supervised the whole project. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02983/full#supplementary-material

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A *mcr-1*-Carrying Conjugative IncX4 Plasmid in Colistin-Resistant *Escherichia coli* ST278 Strain Isolated From Dairy Cow Feces in Shanghai, China

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Enterobacteriaceae, including *Escherichia coli*, has been shown to acquire the colistin resistance gene *mcr-1*. A strain of *E. coli,* EC11, which is resistant to colistin, polymyxin B and trimethoprim-sulfamethoxazole, was isolated in 2016 from the feces of a dairy cow in Shanghai, China. Strain EC11 identifies with sequence type ST278 and is susceptible to 19 frequently used antibiotics. Whole genome sequencing of strain EC11 showed that this strain contains a 31-kb resistance plasmid, pEC11b, which belongs to the IncX4 group. The *mcr-1* gene was shown to be inserted into a 2.6-kb *mcr-1-pap2* cassette of pEC11b. Plasmid pEC11b also contained putative conjugal transfer components, including an *oriT*-like region, relaxase, type IV coupling protein, and type IV secretion system. We were successful in transferring pEC11b to *E. coli* C600 with an average transconjugation efficiency of 4.6×10^{-5}. Additionally, a MLST-based analysis comparing EC11 and other reported *mcr*-positive *E. coli* populations showed high genotypic diversity. The discovery of the *E. coli* strain EC11 with resistance to colistin in Shanghai emphasizes the importance of vigilance in detecting new threats like *mcr* genes to public health. Detection of *mcr* genes helps in tracking, slowing, and responding to the emergence of antibiotic resistance in Chinese livestock farming.

Keywords: colistin resistance, *mcr-1*, *Escherichia coli*, IncX4 plasmid, whole genome sequence

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Abbreviations: CC, clonal complexes; CLSI, Clinical and Laboratory Standards Institute; CRE, carbapenem-resistant Enterobacteriaceae; *E. coli*, *Escherichia coli*; ESBL, extended spectrum β-lactamase; EUCAST, European Committee on Antimicrobial Susceptibility Testing; HGT, horizontal gene transfer; IRs, inverted repeats; IS, insertion sequences; MDR, multidrug-resistant; MIC, Minimum Inhibitory Concentration; MLST, Multilocus Sequence Typing; NJ, Neighbor-joining; ORFs, open reading frames; PCR, polymerase chain reaction; PEA, phosphoethanolamine; SEM, scanning electron microscope; ST, sequence type; T4CP, type IV coupling protein; T4SS, type IV secretion system; TEM, transmission electron microscope; WGS, whole-genome sequencing; XDR, extensively drug-resistant.

INTRODUCTION

Antimicrobial resistance is becoming a great challenge to public health worldwide (Laxminarayan et al., 2014). The rapid evolution of MDR Gram-negative bacteria is pushing humankind to the cusp of a post-antibiotic era. Colistin (polymyxins E) is a family of cationic polypeptide antibiotics which acts as the last line of defense in the treatment of severe bacterial infections by MDR or XDR bacteria. In particular, colistin is used to treat ESBL-producing and CRE infections (Li et al., 2006; Paterson and Harris, 2016).

Colistin resistance was assumed to be chromosomally mediated, non-transmissible and an intrinsic property of the bacteria (Olaitan et al., 2014). However, the recent discovery of the *Escherichia coli* harboring plasmid-borne colistin resistance gene *mcr-1* confirms transmission of colistin resistance by HGT (Liu et al., 2016). The MCR-1 encodes a PEA transferase that adds PEA to the lipid A of the lipopolysaccharide, leading to Gram-negative bacteria resistant to colistin (Anandan et al., 2017). This HGT mechanism of colistin resistance has alarmed the medical, media, academic and public health communities.

The global spread of the *mcr-1* gene is now evident and being documented. Currently, researchers have discovered five *mcr*-like genes, ranging from *mcr-1* to *mcr-5*, with a series of *mcr* genetic variants such as *mcr-1.2*, *mcr-1.3* . . .*mcr-1.12.* These *mcr* genes have spread to 40 countries across 5 of 7 continents in multiple ecosystems, including the environment, food, animals (e.g., pig, poultry, and cattle) and humans, and in over 11 species of Enterobacteriaceae (Schwarz and Johnson, 2016; Chen et al., 2017; Feng, 2018). Retrospective studies have shown that an isolate harboring the *mcr-1* gene had already existed in three chicken *E. coli* isolates in China from the 1980s (Shen et al., 2016). The presence of *mcr-1* in livestock is indicative of the route of *mcr-1* dissemination through the food chain and it is gravely concerning that animal-to-human transmission of MCR-1 colistin resistance has already been found in many countries.

Mobile genetic elements such as conjugative plasmids, transposons, integrons and IS are important vehicles of HGT of the *mcr-1* gene (Frost et al., 2005; Sun et al., 2018). Conjugative plasmids are the main driving force for the dissemination of the *mcr-1* gene, and the plasmids IncI2 and IncX4 are the two leading plasmid types for facilitating the global dissemination of colistin resistance (Matamoros et al., 2017; Wang et al., 2018). The *mcr-1* gene is part of an approximately 2.6-kb *mcr-1-pap2* element that contains the likely promoter regions for *mcr-1* transcription (Poirel et al., 2016; Wang et al., 2018). There are also rare cases involving chromosomally integrated *mcr-1*genes (Veldman et al., 2016; Tada et al., 2017), which are indicative of non-lineage-specific vertical dissemination of *mcr-1*.

Detection of *mcr* genes helps in the tracking, slowing, and responding to the emergence of antibiotic resistance in Chinese livestock farming. At the end of 2015, the *mcr-1*-harboring *E. coli* strain SHP45 was isolated from pigs in Shanghai (Liu et al., 2016). Also, in 2016, the colistin-resistant *E. coli* EC11 strain was isolated from cow feces collected from a commercial dairy farm. We will use WGS to outline the mechanism for acquiring and transferring colistin resistance in this strain.

MATERIALS AND METHODS

Bacterial Strains and Identification

In May 2016, we cultured *E. coli* strains from fecal samples collected from a commercial dairy farm in Shanghai, China. Samples (25 g) were dispensed in sterile plastic bags containing 225 ml of Mueller–Hinton broth and incubated at 37°C for 24 h. All samples were seeded on MacConkey agar plates with 2 μg/mL colistin and incubated at 37°C for 18 h. One putative positive *E. coli* colony per sample was selected on the basis of morphology, size, and color (peachblow), then inoculated overnight on eosin-methylene blue agar. Species were further confirmed by the amplification and sequencing of 16S rRNA, while SEM and TEM image analyses were conducted. All bacterial isolates were stored in the Luria-Bertani medium (Land Bridge, Beijing, China) with 30% glycerol at −80°C.

mcr-1 and β-Lactamase Gene Screening

Screening for the *mcr-1* gene was performed using PCR amplification and sequencing. The specific primers used to produce the 309 bp amplicon were as previously described: CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTAGGG-3′) (Liu et al., 2016). Further screening for the presence of the *mcr-2*, *mcr-3* and the main β-lactamase gene groups (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{KPC}, and bla_{NDM}) was performed by previously reported primers. In this study, all primers used are presented in **Supplementary Table S1**. Each PCR reaction system was performed in 25 μL, containing 12.5 μL of PCR Mix (Sangon Biotech, Shanghai, China), 9.5 μL of dd H_2O, 1 μL of forward and reverse primers, and 1 μL of DNA template. Finally, one *E. coli* isolate designated as *E. coli* EC11 was determined to harbor the *mcr-1* gene, and this isolate was selected to perform the follow-up experiments.

Antibiotic Susceptibility Testing

The MIC for 22 common antibiotics was determined for the isolate of *E. coli* EC11 by the broth dilution method on Mueller–Hinton broth (Oxoid, United Kingdom) following incubation at 37°C for 18–24 h. In this study, the 22 tested antibiotics we used are categorized into seven groups as shown in **Table 1**. The results were interpreted according to CLSI document M100-S25 (2015)[1] except for tigecycline and colistin, which were interpreted by the EUCAST (version 6.0)[2] guidelines. The double disk test (ceftazidime + ceftazidime/clavulanic acid and cefotaxime + cefotaxime/clavulanic acid) was performed to confirm the ESBL phenotype, and *E. coli* ATCC 25922 was used as a quality control.

[1]https://clsi.org/

[2]http://www.eucast.org/

TABLE 1 | Minimum inhibitory concentration (μg/mL) for *Escherichia coli* EC11, transconjugant EC11-T and recipient *E. coli* C600.

Type of antibiotic	Antibiotic	MIC (μg/mL)*		
		Donor *E. coli* EC11	Transconjugant E11-T	Recipient *E. coli* C600
β-lactams	Amoxicillin-clavulanic	2(S)	2(S)	2(S)
	Ampicillin	4(S)	8(S)	8(S)
	Piperacillin	2(S)	4(S)	4(S)
	Cefotaxime	<0.125(S)	0.25(S)	0.25(S)
	Ceftazidime	0.25(S)	0.5(S)	1(S)
	Cefoxitin	8(S)	4(S)	4(S)
	Cephazolin	2(S)	4(S)	4(S)
	Cefepime	<0.125(S)	0.25(S)	<0.125(S)
	Imipenem	0.5(S)	1(S)	0.5(S)
	Meropenem	<0.125(S)	<0.125(S)	<0.125(S)
Aminoglycoside	Amikacin	4(S)	4(S)	8(S)
	Gentamicin	2(S)	1(S)	1(S)
	Kanamycin	4(S)	4(S)	4(S)
Tetracycline	Tetracycline	1(S)	1(S)	1(S)
	Tigecycline	<0.125(S)	<0.125(S)	<0.125(S)
Quinolone	Ciprofloxacin	<0.125(S)	0.125(S)	<0.125(S)
	Levofloxacin	<0.125(S)	0.25(S)	0.5(S)
	Nalidixic acid	4(S)	>128(R)	>128(R)
Amino alcohol	Chloramphenicol	16(S)	8(S)	8(S)
Sulfonamide	Trimethoprim-sulfamethoxazole	8(R)	8(R)	8(R)
Cationic polypeptide	Polymyxin B	4(R)	4(R)	1(S)
	Colistin	8(R)	4(R)	1(S)

*MIC, minimum inhibitory concentration; R, resistant; I, intermediate; S, susceptible. *In vitro antimicrobial susceptibility was performed by broth microdilution method and the MICs were interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria, except for tigecycline, colistin and polymyxin B, which interpretation were performed according to the EUCAST guidelines.*

Conjugation Assay

To determine whether the colistin resistance was carried on a transferable plasmid, a conjugation experiment by filter mating assay (Smith and Guild, 1980) was performed with rifampicin-resistant *E. coli* C600 as the recipient strain. Overnight cultures of the original isolates and recipient *E. coli* C600 in LB broth were adjusted to a 0.5 McFarland standard. A 10 μl aliquot of each culture was individually added to 2 ml of fresh LB broth and then incubated at 37°C for 6 h. The original strains (20 μl) were then separately conjugated with *E. coli* C600 (60 μl) on a microporous membrane. Transconjugants were selected on MacConkey agar plates supplemented with colistin (2 μg/mL) and rifampicin (40 μg/mL), and putative transconjugants were confirmed by both PCR and an antimicrobial susceptibility test (above 22 antibiotics). The mobilization efficiency was calculated as the number of transconjugant colonies divided by the number of donor colonies (Wang et al., 2011).

Multilocus Sequence Typing

The clonal lineage of the *E. coli* EC11 strain was studied using MLST. MLST was performed as previously described (Tartof et al., 2005). The seven conserved housekeeping genes (*adk, fum*C, *gyr*B, *icd, mdh, pur*A, and *rec*A) were chosen as targets[3] and PCR fragments were sequenced. The alignments of these sequences were determined using DNAMAN software. These sequences were then analyzed using the facility provided by the above-mentioned online tool to assign allele numbers and define the ST and CC.

Furthermore, in order to explore possible genetic relationships between *E. coli* EC11 and other *E. coli* isolates harboring *mcr* reported worldwide, we performed a systematic review of the literature on *mcr* published in the NCBI-Pubmed database between November 2015 and March 2018. A phylogenetic tree was constructed using a NJ method by MEGA5.0 software, where the phylogenetic relationships among different strains were analyzed based on nucleotide differences. In addition, we conducted cluster analysis of these strains to understand the relationship between the different ST groups. The eBURST algorithm was used to group strains according to their allelic profiles by employing a user-specified group definition as well as drawing a rough sketch[4] to show the genetic relationship.

Whole Genome Sequencing

Genomic DNA of *E. coli* strain EC11 was extracted from an overnight culture using the TIANamp Bacteria DNA

[3]http://mlst.warwick.ac.uk/mlst/dbs/Ecoli

[4]http://eburst.mlst.net/v3/enter_data/single/

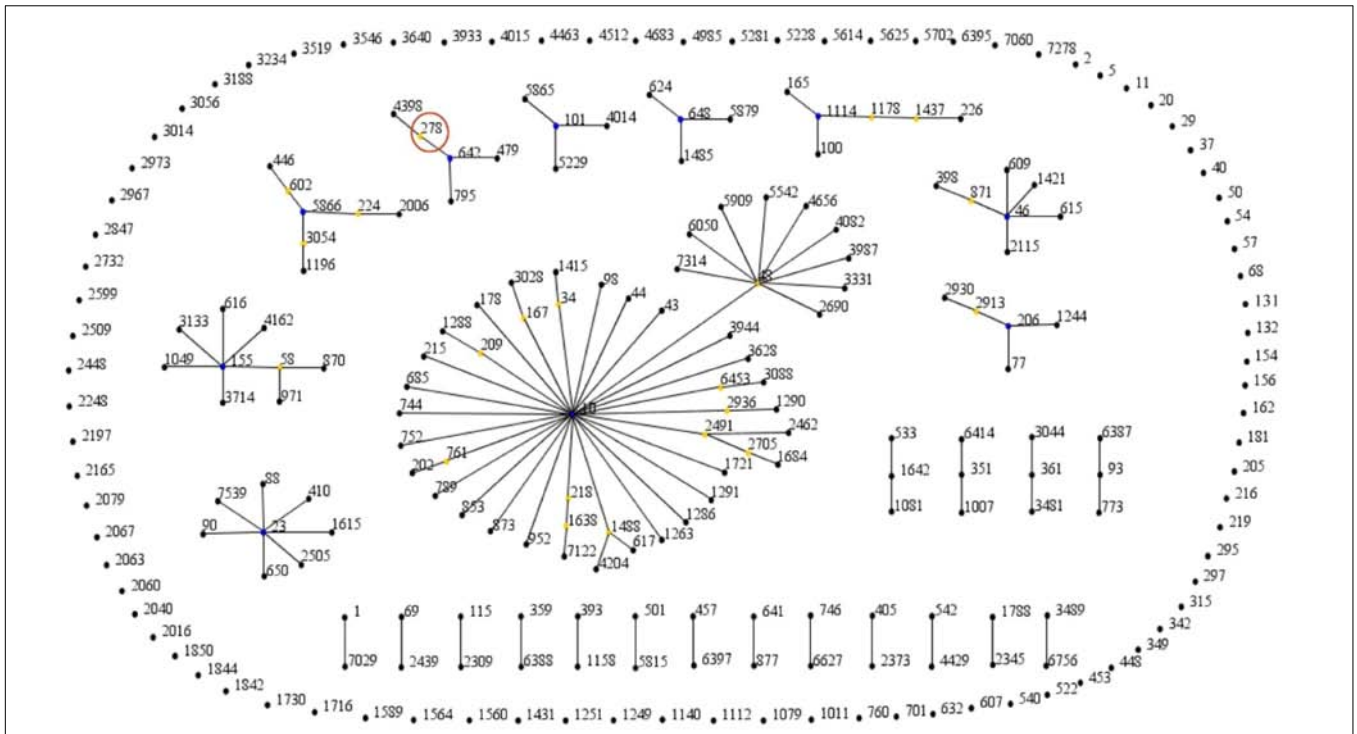

FIGURE 1 | The eBURST cluster analysis of the genetic relationships of the *mcr*-positive *Escherichia coli* strains. The analysis is based on allelic profiles of MLST data and displays clusters of linked and individual unrelated STs. The digital represents the ST type, each black node represents a sequence type, the blue nodes represent clonal ancestors, the yellow nodes represent clonal subpopulation ancestors, and the red circle represents the sequence type of *E. coli* EC11 in this study.

Kit (Tiangen Biotech Beijing Co., Ltd., China) according to manufacturer's instructions. WGS data were generated using short-read (Illumina, San Diego, CA, United States), producing 2 × 251-bp paired-end reads, and long-read (Pacific Biosciences, Menlo Park, CA, United States) technology. The raw data were assembled using SPAdesv3.9.0 (Bankevich et al., 2012). Gene prediction and annotation were done with Glimmer 3.02 and BLAST. All sequences were deposited under the Bioproject PRJNA436212. Serotypes, plasmid replicons, and *E. coli* virulence genes were identified by using SerotypeFinder1.1, PlasmidFinder1.3, and VirulenceFinder1.5, respectively, available from the Center for Genomic Epidemiology[5]. Insertion sequence (IS) elements were identified using ISfinder[6]. Additional characterization of chromosomal resistance determinants was performed using the CARD Resistance Gene Identifier[7], and ResFinder[8] was used to detect acquired resistance genes commonly located on mobile genetic elements. The sequence comparison and map generation were performed using BLAST[9] and Easyfig version 2.1 (Sullivan et al., 2011). Conjugal transfer components of the plasmids were performed using *oriT*finder (Li et al., 2018).

[5]http://genomicepidemiology.org/

[6]https://www-is.biotoul.fr/search.php

[7]https://card.mcmaster.ca/analyze/rgi

[8]https://cge.cbs.dtu.dk/services/ResFinder/

[9]http://blast.ncbi.nlm.nih.gov

RESULTS

Identification of *mcr-1*-Positive *E. coli* Isolates

In our study, out of 120 *E. coli* isolates collected from dairy cow fecal samples in May 2016 in Shanghai, only the *E. coli* isolate EC11 (**Supplementary Figures S1**, **S2**) carried the *mcr-1*gene, and none of these isolates carried *mcr-2/3* determinants or the allelic variants.

Susceptibility to Antimicrobial and Conjugative Compounds

According to EUCAST standards, the resistance cutoff of *E. coli* to colistin is 2 mg/L and the *E. coli* EC11 strain exhibited the lower level of colistin resistance (8 μg/mL) (**Table 1**). *E. coli* EC11 also showed resistance to polymyxin B, and trimethoprim-sulfamethoxazole; but it was susceptible to other 19 common antibiotics, including amoxicillin-clavulanic, ampicillin, piperacillin, cefotaxime, ceftazidime, cefoxitin, cephazolin, cefepime, imipenem, meropenem, amikacin, gentamicin, kanamycin, tetracycline, tigecycline, ciprofloxacin, levofloxacin, nalidixic acid, chloramphenicol (**Table 1**). PCR results showed that *E. coli* EC11 didn't carry the β-lactamase genes, including bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{KPC}, and bla_{NDM}. Furthermore, the double disk test suggested that *E. coli* EC11 was a non-ESBL producing isolate (**Supplementary Figure S3**).

In addition, the filter mating assays indicated that the *mcr-1*-carrying plasmid could be successfully transferred from the donor (*E. coli* EC11) to the recipient (*E. coli* C600) with an average efficiency of 4.6×10^{-5}. The MIC value of the transconjugant EC11-T to colistin was 8 μg/mL, which showed an eightfold increase when compared with the recipient *E. coli* C600 (1 μg/mL). The transconjugant *E. coli* EC11-T was also found to have resistance to nalidixic acid, trimethoprim-sulfamethoxazole and polymyxin B.

A Diversity of the *mcr-1* Positive *E. coli* Isolates

Multilocus sequence typing (MLST) showed that *E. coli* EC11 belonged to the ST278 lineage. Based on the literature review, details of the *E. coli* strains harboring *mcr* genes, including the source and year of isolation, the presence of the MDR phenotype, ST, and allelic profile, are presented in **Supplementary Table S2**. A total of 245 STs were identified among the 616 *E. coli* isolates, indicating a high degree of genotypic diversity.

The application of eBURST resolved the 245 STs into 10 clonal complexes (CC10, CC206, CC46, CC1114, CC648, CC101, CC642, CC6866, CC55, and CC23). CC10 remained the most populated clonal complex and ST10 was defined as the ancestral type of CC10 (**Figure 1**). The geographical distribution of the different STs is shown in **Supplementary Table S3**. These ST types were distributed in more than 35 cities across six continents. ST10 was isolated on five continents and China was the country where the most *mcr*-positive *E. coli* strains were found, with as many as 162 different STs being discovered.

A NJ tree representing the concatenated sequences of the seven housekeeping gene fragments in 245 *mcr*-positive *E. coli* isolates of different ST types is shown in **Figure 2**. The phylogenetic analyses revealed that *E. coli* isolates harboring *mcr* genes were distributed in different lineages, and the isolated *E. coli* EC11 was located on a single branch rather than belonging to one of the ST10 branches.

Genome Features of *E. coli* EC11 Harboring *mcr-1*

Whole gene sequencing (WGS) revealed that the serotype of the *E. coli* EC11 strain was H7. *E. coli* EC11 consisted of a chromosome and four circular plasmids (pEC11a, pEC11b, pEC11c, and pEC11d) (**Table 2**). The chromosome genome size presented 4,933,784 bp, with a G+C content of 47.6%. With an exception of the *mcr-1*, unexpectedly, any other resistance genes were not defective in EC11. WGS results revealed the *mcr-1* gene, which showed 100% BLASTn identities to the known *mcr-1* gene of the reference plasmid pHNSHP45 of *E. coli* SHP45 (Liu et al., 2016). The *mcr-1* gene was only located on plasmid pEC11b, which was 31,229 bp in length and had an average G+C content of 41.40%, encoding 38 ORFs (**Figure 3**). Using PlasmidFinder, the plasmid pEC11b had a typical IncX4 plasmid backbone encoding replication, conjugation apparatus and stability functions, and was probably responsible for the movement of the plasmid between different bacterial hosts. The type II toxin–antitoxin module *hicA/hicB* was also identified

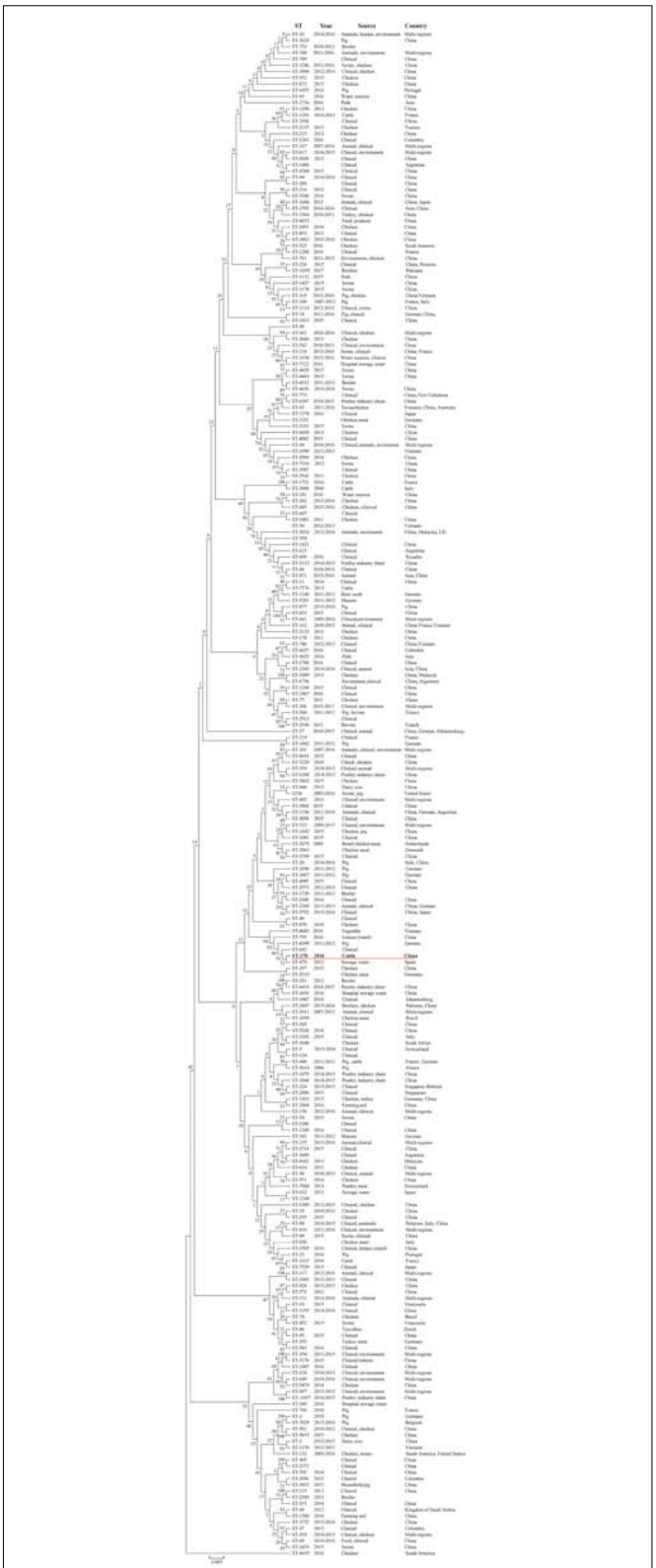

FIGURE 2 | Neighbor-joining tree of 245 concatenated sequences of *E. coli* harboring *mcr-1* from multiple sources in different countries. The numbers at the nodes represent bootstrap values based on 500 replications. In bold and red underline presented the *E. coli* EC11isolate. ST, sequence type.

TABLE 2 | General features of *E. coli* EC11 genomes.

Replicons	Accession number	Size(bp)	MLST	Plasmid typing	Antibiotic resistance	GC (%)	ORF numbers	tRNA genes	rRNA genes
Chromosome	CP027255	4,933,784	ST278	–	–	50.77	4,648	85	22
pEC11a	CP027256	103,336	–	IncFIB	–	48.08	119	0	0
pEC11b	CP027257	31,229	–	IncX4	*mcr-1*	41.74	38	0	0
pEC11c	CP027258	31,467	–	–	–	48.41	46	0	0
pEC11d	CP027259	6,812	–	ColRNAI	–	47.69	9	0	0

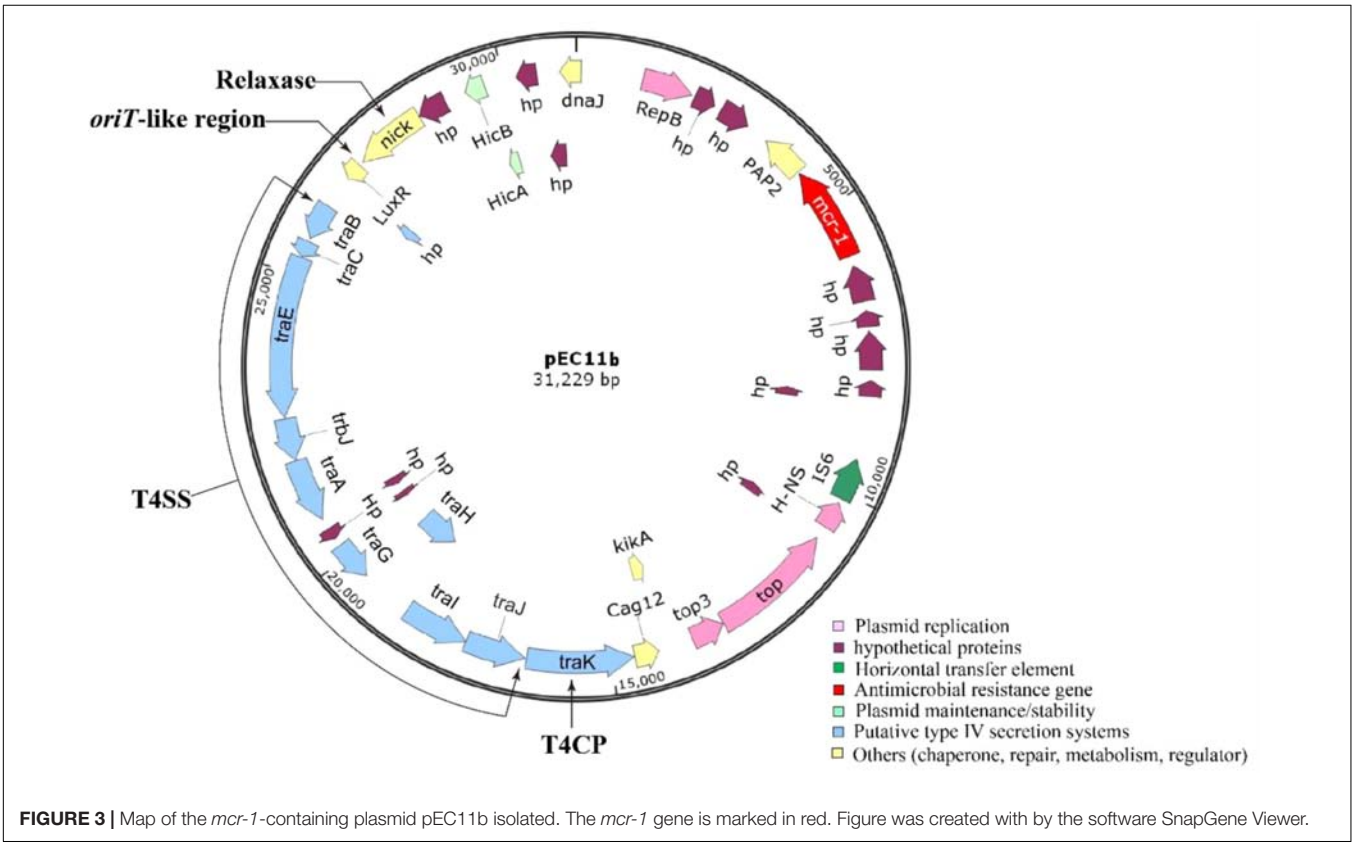

FIGURE 3 | Map of the *mcr-1*-containing plasmid pEC11b isolated. The *mcr-1* gene is marked in red. Figure was created with by the software SnapGene Viewer.

in pEC11b. The putative virulence genes, such as *gad* (coding for glutamate decarboxylase), *lpf*A (long polar fimbriae) and *iss* (increased serum survival siderophore), were found in the chromosome of *E. coli* EC11.

Genome Features of *mcr-1*-Carried Plasmid

BLASTn analysis showed that the backbone of the plasmid pEC11b (GenBank accession number CP027257.1) was strikingly similar with (the query cover of 100% and the identities 99%) other previously sequenced *mcr-1*-carrying IncX4 plasmids, such as pICBEC72H of *E. coli* (isolated in Brazil; the GenBank accession no. CP015977.1), pMCR1-IncX4 of *Klebsiella pneumoniae* (China; KU761327.1), and pNG14043 of *Salmonella* (China; KY120364) (**Figure 4**). In all, these IncX4 plasmids bearing *mcr-1* showed very high architectural conservation.

An approximately 2.6 kb *mcr-1-pap2* element was identified in the above-mentioned plasmids pEC11b, PICBEC72H, pMCR1-IncX4, and PNG14043. In addition, an IS*6* element was identified in pEC11b, IS*26* was identified in PICBEC72H and PNG14043, and *tnpA* was identified in pMCR1-IncX4 (**Figure 4**). The promoter sequences of *mcr-1* in all the aforementioned sequences were similar to that of pAf23 and pAf48 reported by Poirel et al (Poirel et al., 2016) as well as pMCR1_IncI2 and BJ10 by Zhang et al (Zhang et al., 2017) (**Supplementary Figure S4**).

The putative conjugal transfer components of pEC11b were also detected by using *oriT*finder. A *tra* gene cluster encoding a T4SS belonging to Type P was predicted on pEC11b. It encoded a relaxase (*C6C13_26300*) belonging to the MOB_P family. It also encoded a T4CP (*C6C13_26225*) belonging to the VirD4 subfamily. The *oriT*-like region (coordinate: 27,146-27,223 bp) contained a pair of 14-bp IRs (GCAGGTGAGCAAAG. . .CTTTGTTCACCTGC). This evidence confirms that the plasmid pEC11b is a conjugative plasmid.

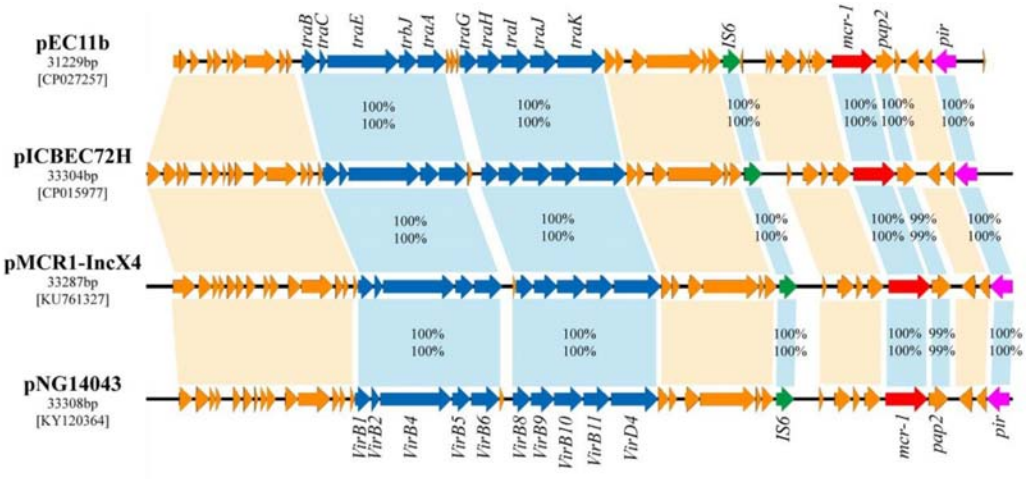

FIGURE 4 | Linear comparison of complete plasmid sequences of plasmid pEC11b from *E. coli* EC11 (this study, accession no. CP027257), plCBEC72H from *E. coli* ICBEC72H (CP015977), pMCR1-IncX4 from *Klebsiella pneumoniae* SZ04 (KU761327) and pNG14043 from *Salmonella* NG14043 (KY120364). The arrows represent the position and transcriptional direction of the ORFs. Genes associated with the type IV secretion systems are indicated by blue arrows, resistance genes are indicated by red arrows, replication initiation protein are indicated pink, while accessory genes are indicated by jacinth arrows. Insertion sequences are highlighted in green arrows. The percentages of amino acid identity (above) and nucleic acid similarity (below) are shown between the homologous genes.

DISCUSSION

Colistin has been widely used as a veterinary drug for the treatment of enterobacterial infections and as an in-feed additive to promote healthy development in food-producing animals, especially in swine and poultry production (Kempf et al., 2013, 2016). Transfer of colistin resistance among bacteria in the gastrointestinal tract of livestock animals is a probable route for the dissemination of these bacteria (Fernandes et al., 2016b; Guenther et al., 2017). These routes can be via the food chain or direct human contact with animals as well as through contamination of fresh and seawater systems (Zhang et al., 2016; Zurfuh et al., 2016). In addition, the persistence of *mcr-1* in the human gastrointestinal tract microflora provides another route for dissemination of these bacteria (Chen et al., 2017). In this study, the *mcr-1*-carrying plasmid could be conjugated into *E. coli* C600 isolates *in vitro*. The *mcr-1* gene, if present in gut microbiota, can therefore be horizontally transmitted between different species in the microbiota.

Self-transmissible IncX4-type plasmids are now accepted as key vehicles responsible for the dissemination of the *mcr-1* gene among Enterobacteriaceae worldwide (Fernandes et al., 2016a; Sun J. et al., 2017; Wang et al., 2017). In this study, we identified an IncX4-type plasmid carrying *mcr-1* in *E. coli* EC11, pEC11b, which was nearly identical to the other IncX4 plasmids bearing *mcr-1* in GenBank. IncX4 plasmid architecture is highly conserved and studies have shown similar IncX4 plasmids bearing *mcr-1* from different species. These species were isolated from different geographic locations and belonged to different STs (Sun J. et al., 2017; Wang et al., 2017). Plasmid pEC11b has four typical conjugal modules: an origin of transfer (*oriT*-like) region, a T4CP gene, a relaxase gene, and a gene cluster for the bacterial T4SS apparatus. The T4SS can act as a conjugative machine in conjugative plasmids (Cascales and Christie, 2003). These gene clusters are vital to the HGT of intra- and inter-species bacterial resistance genes (Frost et al., 2005). Also, the plasmid pEC11b contains the *mcr-1-pap2* cassette which has proven that it could be horizontally transferred into diverse plasmid replicon types (Li et al., 2016).

Multilocus sequence typing (MLST) is a powerful genetic fingerprinting technique for molecular epidemiology and population genetic studies of bacterial pathogens (Maiden et al., 1998; Urwin and Maiden, 2003; Maiden, 2006). In this study, we reported the first recorded instance of an *mcr-1* producing *E. coli* EC11 belonging to the ST278 lineage. We performed a MLST-based analysis of the *mcr*-positive *E. coli* population structure among 616 isolates collected in different laboratories in over 35 countries since 2016. The 245 STs among the 616 isolates indicate that the *mcr*-positive *E. coli* population is extremely diverse. Applying eBURST and NJ tree analyses simultaneously in this global dataset allows for better resolution in discerning the epidemiology and genetic population structure of *mcr*-positive isolates. Combined with previous studies (Matamoros et al., 2017), we speculate that the diversity in ST types of these *E. coli* strains may be related to highly promiscuous plasmids disseminating *mcr* genes. It also indicates that *mcr-1* has a huge risk of vertical transmission and may become more widespread and prevalent in the future. A ST which is highly disseminated in food, environment, animals, and human intestinal samples is ST10 (Matamoros et al., 2017; Sun P. et al., 2017). The epidemic clone ST131 (Ortiz de la Tabla et al., 2017), ST648 (Yang et al., 2016), and ST206 (Zheng et al., 2018) were reported to be the most common STs associated with various β-lactamases, including ESBLs, NDM, and KPCs, etc. Many reports indicated that bacteria carrying *mcr-1* were often

associated with ESBLs (Sun et al., 2016). In this study, *E. coli* EC11 only conferred resistance to polymyxin B, colistin, and trimethoprim-sulfamethoxazole, which are antibiotics that are extensively prescribed in veterinary medicine (Catry et al., 2015).

Currently, a number of countries have already restricted the use of colistin in animal production. China has now stopped the use of colistin as an antibiotic growth promoter (Walsh and Wu, 2016). South Africa has responded to the threat of losing colistin as an antibiotic for human health through a program to advance national stewardship of colistin across the 'One Health' platform (Mendelson et al., 2018). The discovery of the *E. coli* strain EC11 with resistance to colistin in Shanghai emphasizes the importance of vigilance in detecting new threats like *mcr* genes to public health.

CONCLUSION

In this work, we report the first case of colistin-resistant *mcr-1* gene in *E. coli* strain EC11 isolated from dairy cow feces in Shanghai, China. We show that this *E. coli* strain carrying the *mcr-1* gene can transfer resistance through HGT. This study confirms the need to monitor and survey the use of colistin and other types of antibiotics to enable proactive and effective strategies (e.g., risk assessment and risk management) for preserving the efficacy of antibiotics in the future.

Nucleotide Sequence Accession Number

The genome sequences of the chromosome and four plasmids of the *E. coli* strain EC11 were deposited as GenBank accession no. CP027255-CP027259.

AUTHOR CONTRIBUTIONS

YZ, YP, and HL conceived and supervised the study. FB designed the experiments. FB and ZZ performed the experiments. FB and XL analyzed the data. BN and XL revised the paper. PM edited the paper. FB wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02833/full#supplementary-material

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Genomic Study of a *Clostridium difficile* Multidrug Resistant Outbreak-Related Clone Reveals Novel Determinants of Resistance

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Background: *Clostridium difficile* infection (CDI) is prevalent in healthcare settings. The emergence of hypervirulent and antibiotic resistant strains has led to an increase in CDI incidence and frequent outbreaks. While the main virulence factors are the TcdA and TcdB toxins, antibiotic resistance is thought to play a key role in the infection by and dissemination of *C. difficile*.

Methods: A CDI outbreak involving 12 patients was detected in a tertiary care hospital, in Lisbon, which extended from January to July, with a peak in February, in 2016. The *C. difficile* isolates, obtained from anaerobic culture of stool samples, were subjected to antimicrobial susceptibility testing with Etest®strips against 11 antibiotics, determination of toxin genes profile, PCR-ribotyping, multilocus variable-number tandem-repeat analysis (MLVA) and whole genome sequencing (WGS).

Results: Of the 12 CDI cases detected, 11 isolates from 11 patients were characterized. All isolates were *tcdA*$^{-}$/*tcdB*$^{+}$ and belonged to ribotype 017, and showed high level resistance to clindamycin, erythromycin, gentamicin, imipenem, moxifloxacin, rifampicin and tetracycline. The isolates belonged to four genetically related MLVA types, with six isolates forming a clonal cluster. Three outbreak isolates, each from a different MLVA type, were selected for WGS. Bioinformatics analysis showed the presence of several antibiotic resistance determinants, including the Thr82Ile substitution in *gyrA*, conferring moxifloxacin resistance, the substitutions His502Asn and Arg505Lys in *rpoB* for rifampicin resistance, the *tetM* gene, associated with tetracycline resistance, and two genes encoding putative aminoglycoside-modifying enzymes, *aadE* and *aac(6′)-aph(2″)*. Furthermore, a not previously described 61.3 kb putative mobile element was identified, presenting a mosaic structure and containing the genes *ermG*, *mefA*/*msrD* and *vat*, associated with macrolide, lincosamide and streptogramins resistance. A substitution found in a class B penicillin-binding protein, Cys721Ser, is thought to contribute to imipenem resistance.

Conclusion: We describe an epidemic, *tcdA*$^{-}$/*tcdB*$^{+}$, multidrug resistant clone of *C. difficile* from ribotype 017 associated with a hospital outbreak, providing further evidence that the lack of TcdA does not impair the infectious potential of these strains. We identified several determinants of antimicrobial resistance, including new ones located in mobile elements, highlighting the importance of horizontal gene transfer in the pathogenicity and epidemiological success of *C. difficile*.

Keywords: *Clostridium difficile*, multidrug resistant clone, outbreak, resistance determinants, genomic analysis

INTRODUCTION

Clostridium difficile, recently renamed as *Clostridioides difficile* (Lawson et al., 2016), infection (CDI), is the main cause of nosocomial antibiotic-associated diarrhea in developed countries, and is prevalent in the healthcare setting. CDI incidence as well as the occurrence of outbreaks has increased dramatically in the last two decades due to the emergence of antibiotic resistant and hypervirulent strains (Freeman et al., 2010; Vindigni and Surawicz, 2015; Isidro et al., 2017). CDI usually develops in hospitalized elderly individuals when the protective colon microbiota is disrupted due to previous antimicrobial therapy (reviewed by Rupnik et al., 2009; Smits et al., 2016). Most *C. difficile* toxigenic strains produce two main virulence factors, the toxins TcdA and TcdB, encoded by genes located in the pathogenicity locus (PaLoc); some strains additionally produce a binary toxin, CDT, while others produce only TcdB (Hunt and Ballard, 2013; Chandrasekaran and Lacy, 2017).

Antibiotic resistance is frequently reported in prevalent *C. difficile* strains and is thought to play a major role in the infection and dissemination of this pathogen, as well as in the emergence of new types of epidemic clones (Spigaglia, 2016; Isidro et al., 2017). Resistance may be due to different mechanisms, such as the expression of genes located on mobile elements or specific mutations in the genes coding for the antibiotics targets (Brouwer et al., 2011; Isidro et al., 2017).

Here we describe a multidrug resistant clone from PCR ribotype 017 *C. difficile* implicated in a CDI outbreak that occurred between January and July 2016 in two surgery wards in a hospital from the Lisbon Metropolitan Area. Multilocus variable-number tandem repeat analysis (MLVA) was used to determine the genetic relatedness of the strains and whole-genome sequencing (WGS) to identify determinants of resistance.

MATERIALS AND METHODS

C. difficile Isolates

Following the CDI surveillance program, 11 stool samples from 11 CDI-positive patients, diagnosed using the C. DIFF QUIK CHEK COMPLETE®kit, were collected between January and July 2016, during an outbreak in a hospital from the Lisbon Metropolitan Area, and sent to the National Reference Laboratory for Gastrointestinal Infections, hosted in the Portuguese National Institute of Health, for laboratory-based epidemiological surveillance of CDI. As described previously, stool samples were inoculated onto ChromID *C. difficile* agar (bioMérieux, Marcy l'Etoile, France) after ethanol shock and incubated under anaerobic conditions for 48 h at 37°C (Santos et al., 2016). Total DNA was extracted with the Isolate II Genomic DNA kit (Bioline, London, United Kingdom), followed by a multiplex PCR to detect the genes *gluD*, *tcdA*, *tcdB*, *cdtA* and *cdtB* (Paltansing et al., 2007; Persson et al., 2008). An additional PCR was carried out to detect mutations in *tcdA* (Kato et al., 1999). Capillary gel-based electrophoresis PCR ribotyping was performed using Bidet primers, as previously described (Fawley et al., 2015). Patient's demographic and clinical data was collected by the infection control team of the affected hospital.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) of chloramphenicol, clindamycin, erythromycin, gentamicin, imipenem, metronidazole, moxifloxacin, rifampicin, tetracycline, tigecycline and vancomycin were determined with Etest strips (bioMérieux), according to the manufacturer's instructions. Plates were incubated under anaerobic conditions for 48 h at 37°C. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints established for *C. difficile* were used when available. For the remaining antibiotics, the Clinical and Laboratory Standards Institute (CLSI) breakpoints were used (**Table 2**).

Multilocus Variable-Number Tandem-Repeat Analysis

Multilocus variable-number tandem-repeat analysis was carried out following the method developed by van den Berg et al. to amplify the loci A6, B7, C6, E7, G8, and CDR60 (Van Den Berg et al., 2007), with an alternative reverse primer to amplify the locus G8, as previously described (Tanner et al., 2010). Each locus size was determined by capillary gel electrophoresis and the corresponding number of repeats was used to construct a minimum spanning tree using the summed absolute distance as coefficient. Isolates with a summed tandem-repeat difference (STRD) $\leq$ 10 were considered genetically related regardless the number of different loci. Clonal complexes were defined by a STRD $\leq$ 2 between two isolates that were either single or double locus variants of each other.

Whole Genome Sequencing and Bioinformatics Analysis

Three strains (A, B, and K; **Figure 1**) were selected for WGS in order to identify putative determinants of resistance and assess clonal relationship. WGS was performed as previously described (Isidro et al., 2018). Nextera XT libraries were subjected to paired-end sequencing on an Illumina Miseq platform (Illumina Inc., San Diego, CA, United States). After reads' quality analysis (FastQC v0.11.5[1]) and improvement, (Trimmomatic v0.36), draft genome sequences were *de novo* assembled using SPAdes (version 3.10.1) (Bankevich et al., 2012) followed by annotation using the RAST server[2] (Aziz et al., 2008). The PubMLST online platform[3] was used for *in silico* Multilocus Sequence Typing (MLST) and allele determination. Core-genome single nucleotide polymorphism (SNP)-based analysis was performed using Snippy v3.1[4]. Only variant sites with minimum mapping quality of 60, minimum of > 10 reads covering the variant position and > 90% reads differing from the reference genome were considered. Putative antimicrobial resistance (AMR) genes were identified using both CARD[5] and ResFinder[6] (Zankari et al., 2012; Jia et al., 2017). Prophage sequences were identified using PHASTER[7] (Arndt et al., 2016). BLASTn searches[8] against the non-redundant (nr) and wgs databases were performed to identify the presence (and similarity level) of determinants of resistance in other available genomes. The genome of strain M68 from ribotype 017 (Acc. No. NC_017175) was used as reference. Raw sequence reads of the three *C. difficile* isolates subjected to WGS were deposited in Sequence Read Archive under the Bioproject accession number PRJNA478136.

Construction of an *ermG* Inducible Strain for Heterologous Expression

To place the *ermG* gene under the control of the anhydro tetracycline-inducible *Ptet* promoter, the *ermG* gene with its ribosome-binding site (positions −12 to + 793 from the translational start codon) was PCR amplified using primers ermG850D (5′ GGATTCGGAGAGGTTAT AATGAACAAAG 3′) and ermG1660R (5′ ATAGTTTAGC GGCCGCATTTTAACTTATGCTACCCTACC 3′) and genomic DNA from strain A (**Figure 1**), isolated in January 2016, from the first outbreak patient, as the template. The resulting 810 bp-long PCR product was cleaved with EcoRI and NotI and inserted between the same sites of pAM25, to yield pMS534. pAM25 is a derivative of pRPF185 from which the *gusA* gene was removed (Fagan and Fairweather, 2011). Plasmids pRPF185 and pMS534 were introduced into *E. coli* HB101 (RP4) and the resulting strains used to transfer the plasmids, by conjugation, into *C. difficile* 630Δerm with selection for thiamphenicol resistance (15 μg/ml) as described before (Serrano et al., 2016). For induction of the *Ptet* promoter, cultures were grown in the presence of 250 μg/ml of anhydro tetracycline (Fagan and Fairweather, 2011).

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[2]http://rast.nmpdr.org/

[3]https://pubmlst.org/

[4]https://github.com/tseemann/snippy

[5]https://card.mcmaster.ca/

[6]https://cge.cbs.dtu.dk/services/ResFinder/

[7]http://www.phaster.ca/

[8]https://blast.ncbi.nlm.nih.gov/

RESULTS

C. difficile Isolates

A CDI outbreak occurred between January and July 2016 in two surgery wards of a < 500-bed tertiary care hospital. In 2015, the hospital registered a CDI incidence of 2 cases *per* 10,000 patient bed-days, while there were no cases in the two surgery wards. Twelve cases of nosocomial CDI were detected during this outbreak, 10 in the cardiothoracic surgery ward and two in general surgery ward, with the following temporal distribution: one case in January, seven in February, one in March, one in April, one in June and one in July. The patients' age ranged from 50 to 84 years and 7/12 were male. According to patient's hospital medical records, 11 of the 12 patients had received two or more classes of antibiotics in the 3 months prior to the diagnosis. Patient's demographic and clinical characteristics are summarized in **Table 1**. The isolates were recovered from 11 of the 12 cases and all belonged to ribotype 017. All were *tcdA*-negative, carrying a previously described ∼1800 bp deletion in *tcdA* (Kato et al., 1999), *tcdB*-positive and did not carry the *cdtA* and *cdtB* genes coding for the binary toxin CDT.

Antimicrobial Susceptibility

All isolates showed high level resistance to clindamycin (>256 mg/L), erythromycin (>256 mg/L), gentamicin (>256 mg/L), imipenem (>32 mg/L), moxifloxacin (>32 mg/L), rifampicin (>32 mg/L), and tetracycline (16 mg/L), being susceptible to metronidazole, vancomycin, chloramphenicol and tigecycline (**Table 2**).

TABLE 1 | Characteristics and clinical data of patients with *Clostridium difficile* infection associated with an outbreak.

Patients (*n* = 12) characteristics	Number (%)
%Males	7 (58.3%)
Mean age in years (interquartile range)	71 (64–81)
Ward	
Cardiothoracic surgery	10 (83.3%)
General surgery	2 (16.7%)
Hospital admission during the 6 previous months	4 (20%)
Antimicrobial exposure within 3-months before CDI diagnosis	11 (91.7%)
Classes of antibiotics	
Aminoglycosides	7 (58.3%)
Vancomycin	7 (58.3%)
Carbapenems	3 (25%)
Penicillins associated with clavulanic acid or tazobactam	3 (25%)
Fluoroquinolones	2 (16.7)
Cephalosporins	1 (8.3%)

		Number of repeats of each VNTR loci					
Isolate	Collection date	A6	B7	C6	E7	G8	CDR60
A	28-01-2016	2	11	20	8	32	8
B	06-02-2016	2	11	21	8	32	8
C	10-02-2016	2	11	21	8	32	8
D	18-02-2016	2	11	21	8	32	8
E	25-02-2016	2	11	15	8	32	8
F	25-02-2016	2	11	15	8	32	8
G	27-02-2016	2	11	20	8	32	8
H	01-03-2016	2	11	20	8	32	8
I	29-04-2016	2	11	15	8	32	8
J	21-06-2016	2	11	15	8	32	8
K	06-07-2016	2	11	15	8	36	8

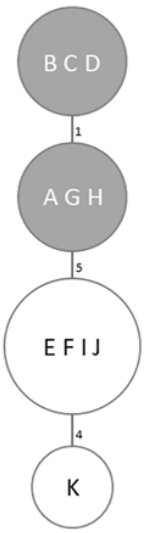

FIGURE 1 | MLVA profiles and minimum spanning tree for *Clostridium difficile* PCR ribotype 017 isolates. For the minimum spanning tree, unique MLVA types are represented by circles, and the summed tandem-repeat differences (STRD) between isolates given by the numbers between the circles. Gray shading indicates a clonal complex (isolates with a STRD of ≤2).

MLVA

Four MLVA types were identified among the studied isolates (**Figure 1**), with only one type displaying two loci differences from the remaining. Loci A6, B7, E7, and CDR60 were invariable; C6 was the most variable locus while G8 only differed in the most recent isolate (K). This isolate, from July, displayed the higher distance from the others, with a 10 tandem-repeat difference in loci C6 and G8 from the first isolate, dated from January. All isolates were genetically related and six of them, which had been collected between January 28th and March 1st, constituted a clonal complex (**Figure 1**).

Whole-Genome Sequencing Results

The 11 isolates shared a high genetic proximity, as determined by MLVA, and therefore only three, representing the outbreak period and belonging to different MLVA types, isolates A (from January), B (from February, the peak period) and K (from July), were selected for WGS (**Figure 1**). Data analysis showed the three strains belonged to the multilocus sequence type (MLST) clade 4, ST37. The pathogenicity locus (PaLoc) showed a complete *tcdB* gene (PubMLST allele 9), and a disrupted *tcdA* with a 1.8 kb deletion at the 3′ end and an early stop codon at amino acid 47, which is typical of ribotype 017. Regarding the accessory genes of the PaLoc, no mutations were found in *tcdE*, coding a holin-like protein necessary for toxin secretion, or in the putative negative regulator of toxin production *tcdC* (PubMLST allele 7). The transcriptional regulator *tcdR*, which has a frameshift mutation in the reference strain M68 (locus CDM68_RS03600) due to a deletion at nucleotide 165 that leads to an early stop codon, is in frame, and predicted as functional, in our strains.

Core-genome SNP-based analysis, using the genome of strain M68 as reference, identified a total of 35 single nucleotide variants (SNVs), of which 33 distinguished the strain M68 from the outbreak strains, being that isolates A and B had no differences between each other and isolate K had 2 SNPs distinguishing

TABLE 2 | Antimicrobial susceptibility and determinants of resistance of the 11 *Clostridium difficile* ribotype 017 isolates characterized in this study.

Antibiotic	R breakpoint (mg/L)	MIC (mg/L)	Phenotype (S/R)	Genetic determinant of resistance
Clindamycin	>4[a]	>256	R	*ermG*
Erythromycin	≥8[a]	>256	R	*ermG*
Chloramphenicol	≥32[a]	3–6	S	–
Gentamicin	≥16[b]	>256	R	*aac(6′)-aph(2′′)* and *aadE*[d]
Imipenem	≥16[a]	>32	R	Cys721Ser in PBP3[e]
Metronidazole	>2[c]	0.125–1.5	S	–
Moxifloxacin	>4[c]	>32	R	Thr82Ile in GyrA
Rifampicin	>0.004[c]	>32	R	His502Asn and Arg505Lys in RpoB
Tetracycline	≥16[a]	16	R	*tetM*
Tigecycline	>0.25[c]	0.023–0.047	S	–
Vancomycin	>2[c]	0.5–0.75	S	–

[a]Breakpoints according to the Clinical and Laboratory Standards Institute (CLSI) interpretative values for anaerobes. [b]Breakpoints according to the Clinical and Laboratory Standards Institute (CLSI) interpretative values for Staphylococcus spp. [c]Breakpoints defined by the EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing). [d]Putative mechanism of resistance in other bacterial genera. [e]Putative mechanism of resistance.

it from isolates A and B, which is consistent with nosocomial transmission.

WGS data revealed the presence of several determinants of resistance (**Table 2**). Two genes encoding putative aminoglycoside-modifying enzymes, termed *aadE* (aminoglycoside 6-adenylyltransferase) and *aac(6′)-Ie-aph(2″)-Ia* (bifunctional aminoglycoside N-acetyltransferase AAC(6′)-Ie/aminoglycoside O-phosphotransferase APH(2″)-Ia), were found in the sequenced isolates. BLASTn search against the nr database showed that *aadE* and *aac(6′)-Ie-aph(2″)-Ia*, which are homologous to the loci CDM68_RS08230 and CDM68_RS08245, respectively, in the reference strain M68, are not frequent in *C. difficile* genomes. On the other hand, they are common in other bacterial genera. The gene *aadE* is found with 100% coverage and identity in several *Campylobacter coli* genomes, as well as in a few genomes of *Campylobacter jejuni*, *Streptococcus agalactiae* and *Enterococcus faecalis*, among others. The gene *aac(6′)-Ie-aph(2″)-Ia* found in our isolates is present with 100% coverage and identity in many *Staphylococcus spp*. genomes, but also *Enterococcus spp*. and *Campylobacter spp*, among others.

The tetracycline resistance determinant *tetM* (PubMLST allele 15), homologous to the locus CDM68_RS01945 in strain M68, was also present in our isolates and was identified in the conjugative transposon Tn916 (Acc. No. KC414929).

The substitution Thr82Ile in GyrA (PubMLST allele 35), which is responsible for fluoroquinolones resistance, and two mutations in *rpoB*, leading to the amino acid substitutions His502Asn and Arg505Lys (PubMLST allele 20), both known to be associated with rifampicin resistance, were present in the three sequenced isolates.

Furthermore, we found the mutation 2162G > C in the homolog of locus CDM68_RS05670, which codes for a penicillin-binding protein (PBP), PBP3 (Isidro et al., 2018). This mutation, which leads to the amino acid substitution Cys721Ser, occurs in the PBP transpeptidase domain, the target of carbapenems action (Papp-Wallace et al., 2011).

An *ermG* gene was identified in a cluster of genes associated with macrolide, lincosamide and streptogramins (MLS) resistance that also included the genes *mefA* and *msrD*, both associated with macrolide efflux resistance, and *vat*, coding for a Streptogramin A acetyltransferase (**Figure 2**). This cluster of MLS resistance genes was found in a 61.3 kb element that interrupts the 23S rRNA (uracil-C(5))-methyltransferase encoding gene (homolog of locus CDM68_RS02190 in strain M68) and shows multiple traits associated with mobile elements likely acquired by horizontal gene transfer (HGT) (**Figure 2**). This region exhibits a mosaic structure, composed of (i) a Type I restriction-modification (RM) system, with genes coding for the subunits R (restriction), S (specificity) and M (DNA methyltransferase), (ii) an intact prophage of around 49 Kb, as detected by PHASTER, and (iii) the aforementioned cluster of MLS resistance genes, followed by a IS66 family transposase (**Figure 2**). Three other *C. difficile* genomes deposited in Genbank present this putative mobile element with >99.9% coverage and identity: the non-toxinogenic strain Z31 (ribotype 009) and strains 7499-CF/ST37 and VL_0008, both belonging to ST37 (Acc. Nos. CP013196, MPFV01000002, and CZWM01000001, respectively). Another strain, VL_0387 (Acc. No. FALC01000010), also from ST37, contains a highly similar element (also >99.9% sequence coverage and identity) but in which the region containing the *ermG* and the transposase is inverted, when comparing to the isolates from this study. Seven other *C. difficile* draft genomes (Acc. Nos. FANQ01000006, FAKJ01000001, FADL01000009, FACQ01000001, CZZV01000006, CZYY01000001, CZXE01000001) harbor a similar element (86% coverage and 98.4% sequence identity) that does not contain the MLS resistance portion, which points to the mosaic origin of this element. Likewise, the genome of *C. difficile* strain M120 (ribotype 078) exhibits a ∼40 kb region (Acc. No. NC_017174, genome position 426527–466056) with 62.8% coverage and 90.6% sequence identity with the element present in our strains, while not containing the flanking RM system nor the MLS resistance cluster.

The 61.3 kb putative mobile element has homology with other non-*C. difficile* genomes. For instance, the genomic region spanning the RM system and the prophage has a high homology with two genomes of *Thermoanaerobacter sp*., covering 70% of the element with 88% sequence identity (Acc. Nos. NC_014538 and NC_010320). The proteins coded by the RM system are common in the class *Clostridia* and are also found in *Enterococcus cecorum*. The prophage region is found with 89% sequence identity, covering 62% of the element, in the genome of *Clostridium bornimense* strain M2/40T (Acc. No. HG917868) and the cluster of MLS resistance genes is found in three genomes of *Enterococcus cecorum* with 98.5% sequence identity, covering 9% of the element (Acc. Nos. CP010060, CP010061 and CP010064).

The genes *mefA* and *msrD* present in this element are found with >99% coverage and >95% sequence identity in many bacterial species, most of which are *Streptococcus spp*., mainly *S. pneumoniae* and *S. pyogenes*, but also in *E. cecorum*, *Neisseria gonorrhoeae* and *Acinetobacter junii*, among other species. The *vat* gene is present in a few *C. difficile* genomes and is also found with >96% coverage and >91% sequence identity in several *E. cecorum*, *E. faecium* and *Streptococcus suis* genomes.

The *ermG* gene present in this element is found in multiple species with a sequence coverage and identity ≥99%, including *Lysinibacillus sphaericus* (Acc. Nos. NG_047827 and M15332), *E. cecorum* (mentioned above), *E. faecium* (Acc. No. CP003351), *Bacteroides spp*. (Acc. Nos. NG_047828, L42817, NG_047829.1 and AJ557257) and nine *C. difficile* genomes (Acc. Nos. CP013196, MPFV01000002, FALC01000010, CZWM01000001, FALZ01000014, FAIU01000023, FAES01000003, FACO01000021, FACG01000010), among which is the non-toxinogenic strain *C. difficile* Z31.

The 61.3 kb *ermG*-containing region is absent in reference strain M68 (**Figure 3**). However, the conjugative transposon Tn6194 harboring the *ermB* gene (the gene most commonly associated with MLS resistance in *C. difficile*), is present in strain M68, while being absent in all the isolates from this study.

The primer pair ermG-F (5′ TCACATAGAAAAAATAAT GAATTGCATAAG 3′) and ermG-R (5′ CGATACAAATTGT

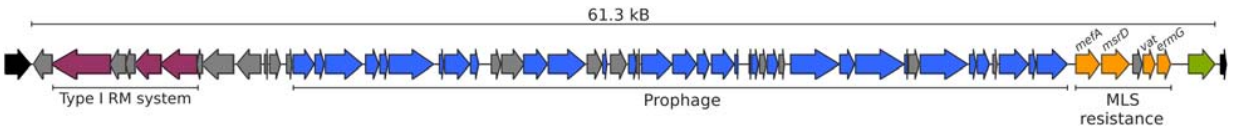

FIGURE 2 | Genetic organization of the novel *Clostridium difficile* putative mobile element harboring the *ermG* gene. Restriction-modification system genes are shown in purple, prophage genes are shown in blue and the genes associated with macrolides, lincosamides and streptogramines resistance are indicated in orange. The transposase is shown in green and the interrupted gene coding for a 23S rRNA (uracil-C(5))-methyltransferase is shown in black. Genes coding for hypothetical proteins are shown in gray.

TCGAAACTAATATTGT 3′) was used to amplify a 652 bp amplicon of *ermG* and confirmed its presence in the remaining outbreak isolates.

The element containing the *ermG* is located in a region showing evidence of other HGT events (**Figure 3**), such as prophages and putative conjugative transposons (CTn). Overall, PHASTER identified three complete, one questionable and four incomplete prophages (data not shown). All, except for the complete prophage harboring the *ermG*-element, are found in strain M68. One of the incomplete prophages is located 72 kb downstream the homolog of locus CDM68_RS02190. The 72 kb region between this incomplete prophage and the *ermG*-containing element shows a high homology with the 43.5 kb CTn5 element present in *C. difficile* strain 630 (Acc. No. AM180355, genome position 2137789–2181291). This 72 kb region covers 90% of CTn5 with 99% sequence identity but in the isolates of this study it is interrupted by two genetic insertions of 8 and 22 kb (**Figure 3**). This 72 kb region is present in the strain BJ08 (Acc. No. CP003939), but in M68 strain it is shorter, lacking the two aforementioned insertions (42 kb; genome position 407967–449991), and more similar to the CTn5 of *C. difficile* strain 630 (**Figure 3**). The 8 kb insertion shows high homology to a *Campylobacter coli* plasmid (Acc. No. CP017026; 88% coverage, 95% sequence identity), while ∼10 kb of the 22 kb insertion has 99.9% sequence identity with regions of three genomes, namely *Flavonifractor sp.*, *Enterococcus faecium* and *C. difficile* (Acc. Nos. NFHA01000028, LNMU01000054 and MPDX01000112, respectively).

Confirmation of MLS Resistance Mediated by *ermG*

The *ermG*-inducible *C. difficile* 630Δerm strain was subjected to antimicrobial susceptibility testing by diffusion gradient with Etest strips against erythromycin and clindamycin. Confirming that the expression of *ermG* confers resistance to MLS antibiotics, the MICs of erythromycin and clindamycin were both of >256 mg/L in the *C. difficile* 630Δerm conjugant expressing the *ermG*, when comparing with the MICs observed for *C. difficile* 630Δerm *ermG*$^{-}$ strain (0.75 and 1 mg/L, respectively).

DISCUSSION

In the present work, we studied a multidrug resistant TcdA-negative *C. difficile* clone from ribotype 017 implicated in a CDI outbreak and identified several determinants of resistance through WGS data analysis. Two novel mechanisms of resistance were described here, namely, the *ermG* gene, which mediates the resistance to MLS antibiotics and is carried by a putative mobile element exhibiting a mosaic structure, and a mutation in a PBP that is likely associated with imipenem resistance.

Ribotype 017 is the most prevalent TcdA-negative *C. difficile* strain and has been considered a recently emerging type, being associated with outbreaks in some European countries (Van Den Berg et al., 2004; Drudy et al., 2007; Goorhuis et al., 2011; Cairns et al., 2015). In a few countries, such as Poland, China or Korea, ribotype 017 is the most common ribotype overall (Pituch et al., 2011; Collins et al., 2013). As such, the lack of one of *C. difficile* main pathogenicity factors (TcdA) does not seem to affect the spreading or infectious potential of these strains.

The described ribotype 017 clone presented resistance to seven classes of antibiotics (**Table 2**), among which fluoroquinolones, MLS, tetracycline and rifampicin, for which resistance has been described in ribotype 017 in several studies (Barbut et al., 2007; Spigaglia et al., 2011; Dong et al., 2013; Freeman et al., 2015). However, resistance to carbapenems, and its underlying mechanism, is still poorly studied in *C. difficile*. According to a pan-European study, most clinical isolates in Europe are susceptible to imipenem, although ribotype 027 showed elevated MICs compared to other ribotypes (Freeman et al., 2015). Similarly to another clone of ribotype 017 that we described recently (Isidro et al., 2018), the clone characterized in the present study also showed a high-level resistance to imipenem (MIC >32 mg/L).

Resistance to carbapenems in gram-positive bacteria is often associated with single-point mutations in the vicinity of the active site of the PBPs transpeptidase domain, which is carbapenems main target (Davies et al., 2008; Zapun et al., 2008; Papp-Wallace et al., 2011). In this work, we found the mutation Cys721Ser in the transpeptidase domain of PBP3, which is one of the two mutations, along with Ala555Thr in PBP1, that we had previously found in another ribotype 017 imipenem-resistant clone (Isidro et al., 2018). In this previous work, we proposed that these mutations mediate resistance by reducing the binding affinity of imipenem to PBPs. Both the present clone and the one described in the previous study presented a MIC of >32 mg/L but it is possible that their levels of resistance differ at higher concentrations of imipenem, depending on the presence of one or the two mutations, respectively. More studies are therefore needed to fully understand this mechanism of resistance and

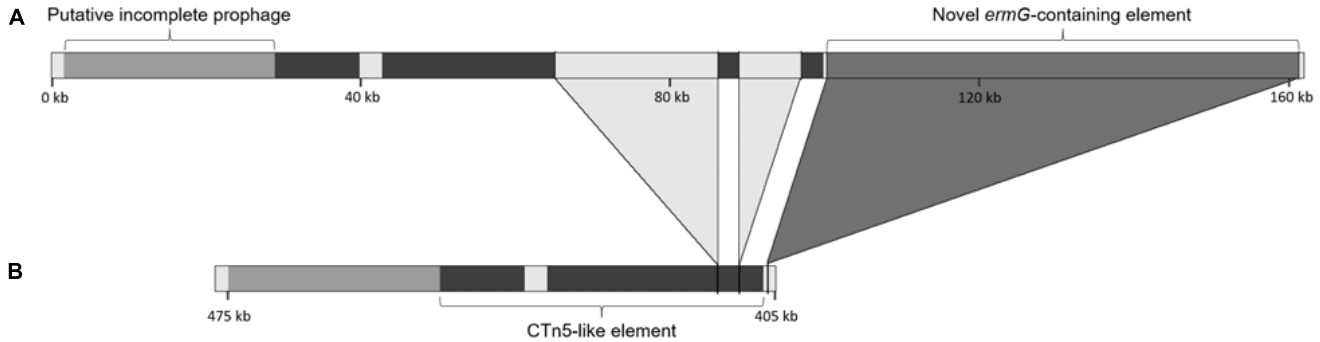

FIGURE 3 | Genetic context of the *ermG*-containing putative mobile element. Genetic context of the novel element containing the *ermG* gene in the strains from the present study **(A)** and comparison of this region with the genome of the reference strain M68 (accession number NC_017175) **(B)**. The darker region represents the homology with the CTn5 element present in strain 630 (accession number AM180355); the light gray regions interrupting this CTn5-like element are not present in strain 630. The two large insertions in the CTn5-like element and the novel *ermG*-containing element differ the strains in this study from strain M68.

determine the contribution of each mutation to the resistance phenotype.

Antibiotic pressure can lead to the selection of resistance and promotes the development and spread of resistant strains (Davies and Davies, 2010). Moreover, CDI shows seasonal variation with a higher incidence in winter months, when there is an increase in both hospital occupancy rates and antibiotic consumption due to respiratory infections (Polgreen et al., 2010; Gilca et al., 2012; Brown et al., 2013). Interestingly, in the present study, carbapenems were the most consumed antibiotics in the outbreak ward, with the hospital also reporting a peak of carbapenems consumption during the last trimester of 2015 (data not shown). Altogether, these conditions might have led to the selection and spread of this imipenem-resistant clone, and subsequently to the outbreak, with the first case occurring in January 2016.

Resistance to MLS antibiotics in *C. difficile* is usually due to ribosomal methylation mediated by the rRNA adenine N-6-methyltransferase encoded by *ermB*, and also, but less frequently, by the chloramphenicol-florfenicol resistance gene, *cfr,* which encodes a 23S rRNA methyltransferase that confers resistance to linezolid (Candela et al., 2017). Both these genes are carried by mobile genetic elements such as conjugative transposons (Spigaglia, 2016). The *C. difficile* isolates in the present study were all highly resistant to clindamycin and erythromycin but neither *ermB* nor *cfr* were found by WGS. Instead, the *ermG* gene was found in the genome of all 11 isolates. Additionally, the genes *mefA*, *msrD* and *vat* were also found immediately upstream of *ermG*. The gene *mefA*, firstly identified in *Streptococcus pyogenes*, mediates macrolides resistance by efflux and is common in *Streptococcus spp.* and amongst Gram-positive bacteria in general. The gene *msrD* is associated with the genetic elements carrying *mefA* in *Streptococcus spp*., and can confer the macrolides efflux phenotype in *S. pneumoniae* (Clancy et al., 1996; Daly et al., 2004; Poole, 2005). However, neither *mefA* nor *msrD* confer resistance to lincosamides or streptogramins. Here, we demonstrated that *ermG* expression alone is sufficient to confer a high level of resistance to clindamycin and to erythromycin upon heterologous expression in the ribotype 012 strain 630Δerm.

The *ermG* was located in a novel putative genetic mobile element with a mosaic structure that is not present in the closest reference strain M68 from ribotype 017. This element contained a RM system, a prophage and a cluster of four MLS resistance genes that showed high sequence identity with elements found in other bacterial genus, which is consistent with transmission to *C. difficile* by HGT. This new element is found in very few *C. difficile* available genomes that, however, have no phenotype data available. Although further investigation is warranted, the fact that one of these genomes is from a non-toxigenic strain from ribotype 009 (Pereira et al., 2016) provides strong evidence for the transmission of this *ermG*-containing element between *C. difficile* strains and highlights the importance of non-toxigenic strains as carriers of resistance determinants.

Several studies have showed evidence of interspecies HGT (Bloemendaal et al., 2010; Goren et al., 2010; Juhas, 2015; von Wintersdorff et al., 2016) and *C. difficile* has also been suggested as a reservoir of resistance genes that might be transferred to other species in the human gut (Johanesen et al., 2015). Consistently, our results show a high degree of sequence identity between determinants of resistance found in *C. difficile* and other relevant human pathogens, As an example, in this work we found two genes encoding aminoglycoside-modifying enzymes, *aadE* and *aac(6′)-Ie-aph(2″)-Ia,* that seem to have a low prevalence in *C. difficile* but are widespread in *Enterococcus* spp., *Campylobacter spp.*, *Staphylococcus spp.* or Streptococcus spp. Anaerobes, such as *C. difficile*, however, are naturally resistant to aminoglycosides (which explains the high MICs generally observed) (Khanafer et al., 2018) and hence the presence of these genes may not directly correlate with the resistance phenotype. Nonetheless, the potential transfer of these genes to other species in which they might contribute to aminoglycoside resistance cannot be disregarded. Overall, these results underline the importance of HGT events in the evolution of *C. difficile* and also point to its potential as a resistance reservoir in the human gut (He et al., 2010; Johanesen et al., 2015). This particular multidrug resistant clone of ribotype 017, harboring such a relevant number of determinants of antimicrobial resistance in mobile elements, may likely trigger the dissemination of these determinants in

clinical settings as well as in the community and the environment, and thus, it should be targeted by an active laboratory and epidemiological surveillance.

In summary, in this study we described a *C. difficile* multidrug resistant clone implicated in a hospital outbreak presenting new resistant determinants that seemingly promoted the spreading success of this clone. Our data show that *C. difficile* is continually evolving through HGT and indicate that antibiotic selective pressure continues to be a major driving force in the development and emergence of new epidemic strains.

AUTHOR CONTRIBUTIONS

All authors contributed to the work described in the paper, as well to the writing and revision of the document.

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Planning a One Health Case Study to Evaluate Methicillin Resistant *Staphylococcus aureus* and Its Economic Burden in Portugal

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important multidrug-resistant nosocomial pathogens worldwide with infections leading to high rates of morbidity and mortality, a significant burden to human and veterinary clinical practices. The ability of *S. aureus* colonies to form biofilms on biotic and abiotic surfaces contributes further to its high antimicrobial resistance (AMR) rates and persistence in both host and non-host environments, adding a major ecological dimension to the problem. While there is a lot of information on MRSA prevalence in humans, data about MRSA in animal populations is scarce, incomplete and dispersed. This project is an attempt to evaluate the current epidemiological status of MRSA in Portugal by making a single case study from a One Health perspective. We aim to determine the prevalence of MRSA in anthropogenic sources liable to contaminate different animal habitats. The results obtained will be compiled with existing data on antibiotic resistant staphylococci from Portugal in a user-friendly database, to generate a geographically detailed epidemiological output for surveillance of AMR in MRSA. To achieve this, we will first characterize AMR and genetic lineages of MRSA circulating in northern Portugal in hospital wastewaters, farms near hospitals, farm animals that contact with humans, and wild animals. This will indicate the extent of the AMR problem in the context of local and regional human-animal-environment interactions. MRSA strains will then be tested for their ability to form biofilms. The proteomes of the strains will be compared to better elucidate their AMR mechanisms. Proteomics data will be integrated with the genomic and transcriptomic data obtained. The vast amount of information expected from this

omics approach will improve our understanding of AMR in MRSA biofilms, and help us identify new vaccine candidates and biomarkers for early diagnosis and innovative therapeutic strategies to tackle MRSA biofilm-associated infections and potentially other AMR superbugs.

Keywords: antimicrobial resistance, surveillance, MRSA, One Health, omics

INTRODUCTION

Staphylococcus aureus is a Gram-positive facultative anaerobe frequently present in the natural human microbiota of the nose and skin that can cause a range of illnesses from minor skin infections and food poisoning to life-threatening diseases such as pneumonia, toxic shock syndrome and sepsis (Sousa et al., 2017). The first methicillin-resistant *S. aureus* (MRSA) was reported only a year after the introduction of methicillin for *S. aureus* treatment (Jevons, 1961). MRSA is resistant to almost all beta-lactams and frequently carries other major classes of antimicrobial resistance (AMR).

Most AMR research has been focused on bacteria growing in planktonic cultures and antimicrobials were originally developed to target individual bacterial cells. However, it is clear that bacteria preferentially develop as complex communities called biofilms (Seneviratne et al., 2012; Penesyan et al., 2015). Recent advances in proteomics techniques have enabled a more in-depth analysis of the possible mechanisms responsible for biofilm AMR and the identification of new anti-biofilm targets (Seneviratne et al., 2012; Azeredo et al., 2017). The use of prefractionation techniques to extract subproteomes significantly enhanced protein identification and coverage of the biofilm proteome (Seneviratne et al., 2012). Also, new shotgun proteomics workflows based on high-resolution tandem mass spectrometry (MS/MS) directly coupled to high performance liquid chromatography (LC) require less protein than conventional two-dimensional gel electrophoresis (2-DE) approaches, allowing a more exhaustive analysis of proteomes or subproteomes and the performance of label-free semi-quantitative comparisons (Azeredo et al., 2017).

Staphylococci have for many decades been recognized as the most frequent cause of biofilm-associated infections (Cihalova et al., 2015; McCarthy et al., 2015). Since the 1990s the epidemiological profile of MRSA has been changing significantly. Its emergence is no longer exclusive to hospitals, as the prevalence of community-acquired infections is increasing (European Centre for Disease Prevention and Control [ECDC], 2017a,b). In fact, several cases of people having had no contact with hospital environments have been diagnosed with MRSA despite having no risk factors for contracting an infection by these organisms (Sousa et al., 2017). In recent years, new genetic lineages of MRSA have been found associated with companion (Leonard and Markey, 2008; Coelho et al., 2011), livestock and food-producing animals, and in various foods (Lee, 2003). However, there is little information on how MRSA spreads and data about the strains recovered from environmental sources, animals and human communities is far from comprehensive. Convergences between habitats can lead to frequent contact between wild animals, other animals and humans, potentially increasing risks to human and animal health. For example, human sources of AMR determinants could contaminate surrounding areas used as food sources for wild animals. More MRSA strains are expected to emerge in the future. The implementation of measures to control zoonotic pathogens and limit the global emergence of resistance traits is required. Integration of human and veterinary systems alone is insufficient as it does not address many structural and environmental issues critical to health.

Biofilm-associated infections are a significant socio-economic burden and have emerged as a major public health concern (Sun et al., 2013; Penesyan et al., 2015). Nearly 80% of all human infections are biofilm-related and one of their most critical features is their considerably higher resistance to environmental stresses, antimicrobials, disinfectants and host immune defenses (Seneviratne et al., 2012; Sun et al., 2013). Despite major advances in biofilm research, knowledge on biofilm formation, propagation and resistance is still very limited and this poor understanding has hampered the development of antimicrobial drugs that specifically target biofilms (Penesyan et al., 2015; Venkatesan et al., 2015).

Antimicrobial resistance acquisition and dissemination rates are outpacing the drug development pipeline (Harbarth et al., 2015; O'Neill, 2016). AMR has the potential to affect anyone of any age in any country (World Health Organization, 2014). If not adequately addressed, AMR could cause 10 million deaths and cost 100 trillion dollars by 2050 (O'Neill, 2014, 2016; European Commission [EC], 2017). Patients with drug-resistant infections or diseases tend to consume more resources and are sick for longer periods, increasing the risk of severe outcomes even if they manage to overcome their main health issue. In addition, the families and entourage of the ill person also end up suffering on personal, practical and economic levels (Ellen et al., 2017). The continuous quantification of the economic burden of these diseases on the individual and on society in general will show the direct consequences of AMR on health system budgets, and other costs that might be associated with losses incurred by different stakeholders (e.g., patients, carers, and governments) (Angelis et al., 2015).

When making such estimates the perspective being taken when considering such scenarios needs to be well defined (Naylor et al., 2018). The payer/provider perspective juxtaposes the patient's perspective, which concerns itself with morbidity, mortality and the clinical outcomes, and the payer's perspective, which focuses on healthcare costs attributable to medical insurance and tax payers (Naylor et al., 2018). The healthcare provider's perspective also needs to be taken into account to estimate the burden on some providers of healthcare like hospitals and primary care practices. Finally, the economic or

societal perspective generally includes the potential impact on the labor force through decreases in productivity, but also the burden on carers and patient out-of-pocket expenses (Naylor et al., 2018). There may be secondary effects of AMR if certain healthcare procedures involving antimicrobial usage are avoided. In a systematic literature review, Naylor and Colleagues (2018) found 187 studies estimated the impact on patient health, 75 studies estimated the payer/provider impact and 11 studies estimated the economic burden. Overall 64% of the studies reviewed were single-center studies. The great majority of studies estimating patient or provider/payer impact used regression analyses. AMR was found to have a significant impact in 48% of the studies that estimated mortality burden. Excess healthcare system costs ranged from non-significant to $1 billion per year, whereas economic burden varied from $21,832 per case to over $3 trillion in GDP loss. Median quality scores (interquartile range) for patient, payer/provider and economic burden studies were 0.67 (0.56–0.67), 0.56 (0.46–0.67), and 0.53 (0.44–0.60), respectively. AMR has therefore become a cause of international concern not only due to the actual and future impact it may have on the population's health, but also on the costs to healthcare systems and gross domestic product (GDP), mainly by the decrease in treatment options.

This project will aim first to provide a better understanding of MRSA prevalence, burden and dissemination in the One Health context of human-animal-environment interactions and then to investigate through proteomics the AMR mechanisms occurring in MRSA biofilms. By characterizing AMR and genetic lineages of MRSA circulating in anthropogenic sources in the North of Portugal, this project will provide epidemiological surveillance data, compiled and easily accessible to the scientific community, public health officials, and the general public. The further proteomic profiling of MRSA biofilms will increase our knowledge of biofilm-specific AMR mechanisms and identify potential vaccine candidates and biomarkers for early rapid diagnosis and new therapeutic strategies.

MATERIALS AND METHODS

Samples

Samples from hospital effluents, and nearby habitats linked to animal farms and wild animal territories will be collected in the Portuguese north province of Trás-os-Montes and Alto Douro annually. Specifically:

(a) Hospital samples will be obtained from the four public hospitals responsible for the public health of the citizens of this province – [Hospital Centre of Trás-os-Montes and Alto Douro (CHTMAD)] – these are located in the cities of Lamego, Peso da Régua, Chaves, and Vila Real. Ethical approval and support has been granted by CHTMAD.

(b) Farm samples will be obtained from soils and farmers, and from animals and their handlers from all the 31 municipalities of the province. The representativeness of the sample of farms will be calculated after obtaining the data referring to the type of farms existing by municipality (e.g., cows, pigs, birds, etc.), and subsequent randomization of the animal samples of each farm.

(c) Wild animal samples will be collected by groups of hunters during the wild rabbit and wild boar hunts and by the Wildlife Recovery Center (Centro de Recuperação de Animais Selvagens, CRAS) at the University of Trás-os-Montes and Alto Douro veterinary hospital.

This data will allow us to map, characterize, and monitor AMR and genetic lineages of MRSA annually, by its presence or absence in the area. It will also indicate the extent of the problem of local and regional human-animal-environment interactions in Trás-os-Montes e Alto Douro (One Health and Eco Health Concepts).

Furthermore, questionnaires regarding the use of antibiotics by the participating entities will be sent to them for annual update – (e.g., hospitals: how many antibiotics have been prescribed by this entity in the last year?; farms: how many of your animals have been administrated antibiotics? How many times has that occurred in the last year?).

MRSA Detection

Staphylococcus aureus and MRSA will be recovered on mannitol salt agar and oxacillin resistance screening agar base (ORSAB), respectively. Presumptive *S. aureus* and MRSA colonies will be identified based on their morphology and re-isolated. Their identity will be confirmed by genotyping using molecular methods and VITEK technology, via PCR amplification of the *nuc* and *mecA* genes. Phenotypic antimicrobial susceptibility will be tested with the EUCAST disk diffusion and broth microdilution methods and the presence of corresponding resistance genes will be investigated by PCR and sequencing. The clonal relationship of isolates will be assessed by pulsed-field gel electrophoresis, *spa*-typing, *agr*-typing and multilocus-sequence-typing (MLST).

Data Collection

All data will be compiled and added to a new web-based application developed so that georeferenced AMR data can be consulted and visualized by medical professionals, the scientific community and others that require it. Existing data on antibiotic resistant staphylococci in Portugal will also be compiled and included in the database. Similar interfaces exist such as the ECDC's Surveillance Atlas of Infectious Diseases and the CDC's Antibiotic Resistance Patient Safety Atlas that allow users to openly interact and manipulate AMR data to customize a variety of maps and tables. However, finer granularity is intended with the possibility to retrieve and filter data at the level of sample collection and isolation details, AMR phenotypic and genotypic profiles, genetic lineages, biofilm-forming ability, among others. Additionally, this information should be traceable to available proteomic, genomic and transcriptomic data of the individual MRSA strains.

Importantly, as AMR prediction and surveillance spans many scientific realms (public health, research, agriculture, drug discovery, etc.), ease-of-use translational tools and data sharing are increasingly needed, requiring a collective dedication to

standardization. Although several global surveillance programs exist that monitor AMR (McArthur and Tsang, 2017), genotypic data is not found in their datasets and accessible databases that combine genotypic and phenotypic AMR data for pathogens in environmental, agricultural, and clinical settings are still not available. Hence, the generation of these informatics resources are of high priority considering their value for epidemiology, antimicrobial stewardship, and drug discovery (McArthur and Tsang, 2017).

Analysis of the Outcomes

All isolated MRSA strains will be tested for their ability to form biofilms. The antimicrobial susceptibility of any biofilm-forming strains will be re-assessed. The proteomes of a number of biofilm-forming MRSA strains will then be characterized. Different subproteomes of MRSA biofilms will be analyzed and compared using both electrophoretic and direct mass spectrophotometric approaches (2-DE-LC-MS/MS and shotgun LC-MS/MS) to identify differentially expressed proteins induced by antimicrobials. The strains selected for proteomic analyses will also be characterized at the genomic and transcriptomic level by whole genome sequencing and RNA sequencing. All omics data will be analyzed and integrated using bioinformatics tools. Several institutions including universities and laboratories will cooperate in this data integration task and all the collaborating international research groups will provide support for data interpretation (**Figure 1**). Biosafety standards will be respected at all stages of the work.

PRELIMINARY AND EXPECTED RESULTS

One Health Focus on Livestock-Associated MRSA in Portugal and Europe

In the past decade, our research group has been surveying AMR in bacteria from a great diversity of environments, collecting over 4,000 samples from more than 75 different sources (humans, wastewaters, food-producing animals, pets, and wild animals), amounting to over 5,000 bacterial isolates. High levels of AMR to critically important drug classes and high rates of clinically relevant multi-resistant strains in non-synanthropic animal species have been found (Marinho et al., 2016). Portugal is one of the countries with highest rates of MRSA and about 44% of the Portuguese hospital *S. aureus* isolates are methicillin-resistant, the second highest rate in Europe (European Centre for Disease Prevention and Control [ECDC], 2017a,b). Our recently published research reveals that MRSA are common in the Portuguese animal communities (Coelho et al., 2011; Marinho et al., 2016) and that the environment and wild animals can be a reservoir or a vehicle of transport for MRSA (Sousa et al., 2017). Currently our research group is one of the few in Portugal that studies antibiotic resistance in wild animals (Oliveira et al., 2010; Clemente et al., 2015; Dias et al., 2015; Jones-Dias et al., 2016; Serrano et al., 2017). For example, we reported the first MRSA isolate of the CC398 (*spa*-type t899) lineage from a wild animal in this country, recovered from a wild boar (*Sus scrofa*). The isolate is *agr*-type I and carries a multi-antibiotic-resistance phenotype, including against beta-lactams (*mec*A gene), tetracycline and ciprofloxacin (Sousa et al., 2017).

We do not know how AMR flows through the environment. This proposal is the next step to investigate the flow of AMR in MRSA and to establish a publicly available, user-friendly database that compiles and integrates the new and existing data on MRSA in Portugal. To illustrate the approach, we can take the spread of the livestock-associated MRSA (LA-MRSA) ST398 as an example. MRSA are indeed becoming frequent in veterinary clinics, in farms, and in livestock animals. In recent years, this particular MRSA clone associated with food production has spread in Europe and is emerging worldwide. Since its discovery, there has been a steady flow of reports of LA-MRSA ST398 among livestock, especially pigs, in numerous European countries (Loeffler et al., 2009). People exposed to livestock are at greater risk of being colonized, and subsequently, infected with LA-MRSA ST398, especially if they are working on farms with a high prevalence. The occupational risk for people exposed to livestock, and those in direct contact with them, has been repeatedly shown. There is not enough data to compare studies in Portugal alone, but if studies from other countries are considered, we can say that LA-MRSA infections may occur outside and independently of hospitals (Pomba et al., 2010). LA-MRSA CC398 is able to cause the same kind of infections that human-adapted MRSA (HA-MRSA) causes in humans. Comparative genome analysis has shown that LA-MRSA has

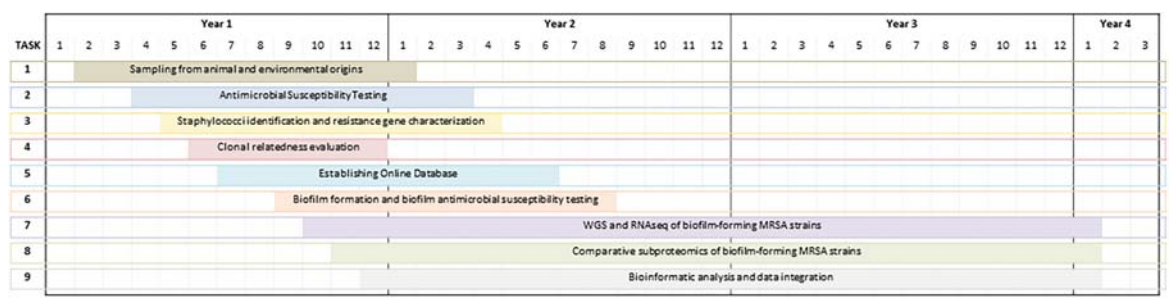

FIGURE 1 | Timeline of case study based on the comparative assessment of antimicrobial resistance of MRSA isolates from Portugal.

evolved from HA-MRSA, and the jump from humans to livestock has been clearly associated with several genetic changes (Price et al., 2012). We will further analyze the proteome and the transcriptome associated to this strain and compare it to the online data on CC398 strains to confirm whether this is an LA-MRSA or a genetically distinguishable strain with zoonotic potential originating from wild animals. Whatever the result, the evolution and re-adaptation of these bacteria to various animal or human populations pose a potential health risk requiring close surveillance.

Beyond surveillance studies, our research group has investigated AMR mechanisms by characterizing a range of resistant strains of interest through proteomic approaches in MRSA (Monteiro et al., 2012, 2015) and other bacterial species (Pinto et al., 2010; Radhouani et al., 2010, 2012; Correia et al., 2014, 2016; Goncalves et al., 2014; Ramos et al., 2015, 2016; Monteiro et al., 2016). However, these proteomic studies, and most AMR research in general, have focused on bacteria growing in planktonic cultures and hence overlooked biofilm-specific AMR mechanisms. These are known to be distinct from the well-characterized intrinsic mechanisms that occur at the cellular level, operating additively to the latter, in a transient and reversible manner, resulting in up to 1000-fold higher resistance levels (Sun et al., 2013; Penesyan et al., 2015; Azeredo et al., 2017). Hence, biofilm-specific mechanisms need to be considered when developing new strategies to combat infectious diseases (Sun et al., 2013; Penesyan et al., 2015).

The objective is to promote collaboration between several public entities as well as different stakeholders from industry and media. We aim to produce information by studying AMR in bacteria from wild animals with zoonotic potential. The potential impacts are both internal, by generating more precise knowledge and collaboration, and external, by improving animal, human, and environmental health in the long term (**Figure 2**). All the MRSA data generated by studying isolates from wild animals will be disseminated according to the principles of One Health information sharing.

Scientific Tasks and Challenges

All *S. aureus* strains should be isolated and MRSA strains identified. Phenotypic and genotypic AMR profiles of all strains should be determined, and molecular typing of strains should be completed. The database should be online and functional. All data on methicillin-sensitive *S. aureus* and MRSA isolates characterized in this project, together with existing data from isolates from Portugal, should be compiled in the database. Testing for biofilm formation and antimicrobial susceptibility should be completed for all MRSA isolates. Strains for further omics approaches should be chosen.

When considering the MRSA issue, we automatically think about outbreaks in the clinical setting. The truth is that this worrisome organism is everywhere, in human clinical isolates, in healthy people who work in clinical or care facilities, in livestock animals and their handlers, in food production and slaughter lines, in wastewater, garbage, and as recently shown, in wild animals. This situation is clearly not new and not wholly unexpected, but in Portugal this environmental MRSA flow is problematic. Our research team has been trying to draw attention to this issue by centering our investigations on the veterinary and environmental aspects. As well as the knowledge and expertise we have accumulated within our team, factors that contributed positively to this work were the collaborations between laboratories and associations, and the individual scholarships from the Portuguese Foundation for Science and Technology (FCT) that made it possible for our students to carry out field work and have access to specialized laboratories. Many factors were challenging at the outset like collecting the samples, often in bad weather conditions. However, with the cooperation of six faculties, stakeholders from industry and media, and Portuguese governmental initiatives we are continuing our surveillance of AMR bacteria recovered from wild animal populations.

FIGURE 2 | Graphical abstract of case study based on the comparative assessment of antimicrobial resistance of MRSA isolates from Portugal.

Strategy for One Health Knowledge Sharing

Two strategic axes have been established to reduce the risk of AMR caused by the use of antibiotics in animals. The aim of the public health protection axis is to reduce the impact of administered veterinary antibiotics on AMR spread. The therapeutic preservation axis is designed to promote the sustainability and efficacy of antimicrobial use. Our plan reflects EU policies because the opinions of a wide range of stakeholders have been taken into account with input from academia and industry, and from practitioners like veterinarians, pharmacists and farmers. One Health learning is expected to involve individual researchers and institutions by the creation of long-term supportive interdisciplinary infrastructures and professional networks.

Effective communication is essential to underpin such a wide-ranging approach. Regular scientific meetings for

consortium updates will complement international congresses to disseminate and discuss findings. Engagement will extend into the community through lectures to high school and university students, and take advantage of social and traditional media outlets. Partners in the countryside will be targeted by providing educational workshops for hunters and cooperating with the League for Nature Protection. Professional guidelines and good practice will be observed, disseminated, promoted and reinforced for all practitioners (distributors, veterinarians, and farmers). For example, active participation in the National Action Plan for Antibiotics Use Reduction in Animals will help to trace and validate veterinary prescription and requisition and to harmonize the register of all medicines administered at farms. With adequate support and training of all professions dealing with animal health and animal production, better selection and use of antibiotics will be promoted, and innovations and alternatives can be explored.

The promotion of investigation, innovation and technological exchanges to incentivize the development of alternative means of treatment, whenever possible and a reinforced monitoring, audits and controls are very important actions to fight against AMR. Research outcomes will be of high quality as we will find out which AMR bacterial variants are associated with each focus of infection and each animal species in a particular habitat. A galvanized network of specialists should more able to prompt the authorities to take action to better regulate antibiotic prescription in hospitals and care facilities (for humans and animals) and on farms, and to take control over how antimicrobials are disposed of, especially when there is a risk of polluting the environment.

Antimicrobial resistance is estimated to cause 25,000 deaths annually and cost over €1.5 billion in healthcare expenses and productivity losses in Europe alone (O'Neill, 2014; European Commission [EC], 2017). In general, higher resistance frequencies are reported by countries in eastern and southern Europe (European Centre for Disease Prevention and Control [ECDC], 2017b). Given the severity of the consequences, MRSA is now a public health priority in Europe and is also one of the highest-priority pathogens in the WHO global priority list to guide research, discovery and development of new antibiotics. The high incidence of MRSA adds to the overall clinical and economic burden in hospitals, causing prolonged hospital stays and higher mortality, mainly due to delayed initiation of appropriate therapy and less effective alternative treatment regimens (O'Neill, 2016; European Centre for Disease Prevention and Control [ECDC], 2017a,b). Given this, there is an impetus to understand the Portuguese situation in more depth, and precision. To facilitate uptake of results and meta-analysis we will base our research on the recommendations of Naylor et al. (2018) by clearly defining data collection and use wherever possible from representative samples of the population studied. Potential

TABLE 1 | Proposed One Health activities, aims and monitoring to implement and integrate knowledge to evaluate the current methicillin resistant *Staphylococcus aureus* situation and estimate its economic burden at the formulation stage of the policy cycle.

One Health initiative specific aims regarding five main activities		Monitoring[1] (transversal activity)	One Health initiative main aim
Thinking	• Stipulate the dimensions that need coverage, and balance different areas of knowledge and multiple perspectives. • Reflect upon the initiative-to-environment match. • Reflect upon the best integrated health approach. • Consider all the system features and targets and, sustainability and socio-ecological-economic impacts. • Think and decide upon relevant performance indicators for each One Health Initiative main activity.	During all the initiative phases the following are of extreme relevance to be under monitoring and assessment for the need for change, development and, innovation: • Research Problem and Design • Team structures • Social and leadership structure • Social and leadership skills • Competence/Skills • Resource allocation • Focus and innovation	Set guidelines that allow for Policy Formulation. (This is possible through the results obtained during the five activities. These will allow evidence which will point out solutions to the AMR problem, and recommendations on how to improve its situation.)
Planning	• Establish common aims that will lead to stakeholder and actor engagement while monitoring, self-assessing and updating each plan when needed.		
Working	• Stimulate the inclusion and collaboration of each stakeholder of the initiative, broadening its impact while balancing its transdisciplinary (cultural, social, and economic) – nature.		
Sharing	• Stimulate systematic general information/awareness sharing, through data and information sharing and development of methods and stimulation of results sharing. These activities intend to develop institutional memory and resilience ability.		
Learning	• Create a general and direct multilevel (individual level, team level and organizational level) learning environment supportive of adaptive and generative learning.		

[1] *Implies monitoring and assessing the processes and results implied in each suggested dimension implied in each initiative activity. This is intended to stimulate the initiatives reflexivity and adaptiveness ability. Adapted from Hitziger et al. (2018).*

confounding factors and biases will be carefully considered when choosing the methodology. All steps of data collection and processing will be clearly recorded. Wider impacts on healthcare systems and economics will be estimated where possible with explanation and justification of any models chosen (**Table 1**).

DISCUSSION

One Health Initiative to Address AMR in Portugal

The One Health approach is the European Commission strategy to tackle AMR, as it recognizes that the health of people, animals and the environment are inextricably linked. This project intends to answer several One Health evaluation questions. (i) How can the spread of AMR be avoided in both human and veterinary medicine? (ii) How can we define the role of wildlife in AMR gene flow? (iii) What steps should we advocate to disseminate our future results? And (iv) How should this issue be addressed in terms of public health?

By involving different universities and stakeholders from industry this project has a One Health attitude from the outset. Different scientific work packages will address the following topics: isolation and identification of strains; genomic and genotypic studies; demographic and socioeconomic characterization; sequencing studies; phenotypic studies; proteomic and transcriptomic analysis; results verification and homologation.

This project will consolidate knowhow in the isolation and identification of MRSA strains from different ecosystems in Portugal. Several collaborations will be maintained and developed between different research groups through this and other projects. This will allow the creation of a bacterial collection with hundreds of strains comprehensively analyzed with genomics and proteomics tools. Knowledge and expertise in using these tools to characterize AMR bacteria will be consolidated, particularly in genotyping techniques by enterobacterial repetitive intergenic consensus PCR and MLST. Purified bacteriocins will be characterized biochemically by MALDI-TOF MS, N-terminal amino acid sequencing by Edman degradation, and sequencing by MALDI TOF/TOF MS. The use of these techniques and the associated equipment will allow us to establish standard protocols for proteomics.

The results of this investigation may add to our knowledge on the occurrence of MRSA strains and the genetic lineages circulating in our surroundings. A more precise local estimate of AMR due to the MRSA burden can inform policy and shape the initiatives to monitor, prevent, treat and limit the spread of resistant infections.

The potential impacts of this case study will lead to better knowledge and collaboration in our interdisciplinary consortium and extended network as well as improvements in animal, human and environmental health.

This project builds on previous efforts of European Commission programs and other programs worldwide and aims to answer priority questions in research and innovation for infectious diseases. First, AMR, genetic lineages and biofilm-forming ability of MRSA strains circulating in anthropogenic sources will be characterized, adding to the emerging picture of the extent of the AMR problem in the context of human-animal-environment interactions. Data will be made available in a free, user-friendly online platform, providing a geoepidemiological output. Further proteomic profiling of MRSA biofilms, integrated with high-throughput genomics and transcriptomics, will provide a large amount of data that will extend the currently limited knowledge on biofilm-specific AMR mechanisms. If this is successful, new molecular candidates for vaccines or biomarkers will be identified that could be developed for early rapid diagnosis and innovative therapeutic strategies to tackle biofilm-associated infections in MRSA and other superbugs with high burden impact. Such an investment in research and innovation taking into consideration the multi-layered burdens of MRSA will improve prevention and treatment and will help us to remain active and vigilant, to develop new, safer and more effective medical treatments, to maintain health and to ensure the viability of health systems. Hopefully this will stimulate more concerted action to reduce the prevalence of MRSA and AMR in Portugal and further afield.

AUTHOR CONTRIBUTIONS

GI, SC, VS, CG, FN, and PP wrote the manuscript. MH, MC, and CT helped design the case study. GI and PP conceived the review. All authors reviewed and contributed to the manuscript.

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Resistance of *Enterococcus* spp. in Dust From Farm Animal Houses: A Retrospective Study

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In a retrospective study, the antimicrobial susceptibility of *Enterococcus* spp. isolated from stored sedimentation dust samples from cattle, pig and poultry barns to 16 antibiotics was determined using a microdilution test. The resistance phenotypes of 70 isolates from different timespans (8 from the 1980s, 15 from the 1990s, 43 from the 2000s and 4 from 2015) were determined. Resistant enterococci were detected in samples from all time periods. Resistances to three or more antibiotics occurred in 69 percent of all isolates. The oldest multidrug resistant isolate was an *Enterococcus faecium* obtained from a 35-year-old pig barn dust sample. No correlations ($\rho = 0.16$, $p = 0.187$) were found between the age of isolates and the number of resistances. Instead, the number of resistances was associated with the origin of the isolates. An exact logistic conditional regression analysis showed significant differences in resistance to ciprofloxacin, erythromycin, penicillin and tylosin between isolates from different animal groups. Interestingly, we isolated ciprofloxacin-resistant *E. faecium* from pig barn dust before fluoroquinolones were introduced into the market for use in animal husbandry. In conclusion, dust from farm animal houses is a reservoir and carrier of multidrug-resistant *Enterococcus* spp. People working in barns are unavoidably exposed to these bacteria. Furthermore, it can be hypothesized that emissions from barns of intensive livestock farming contaminate the environment with multidrug resistant enterococci.

Keywords: *Enterococcus*, survival, dust, livestock, resistance

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INTRODUCTION

Enterococcus spp. can be found in the gut microbiota of mammals and birds and are opportunistic pathogens (Byappanahalli et al., 2012). *Enterococcus* spp. can infect farm animals and cause nosocomial infections in humans (Byappanahalli et al., 2012). Although *Enterococcus* spp. are predominately adapted to their hosts, transmission between animals and humans has been described and is a risk factor for the spread of these organisms (Lu et al., 2002; Kataoka et al., 2014; Lebreton et al., 2014; Milton et al., 2015). Furthermore, the horizontal transfer of resistance genes from animal strains to pathogenic human strains is considered a human hazard (Hammerum, 2012).

One pool of transmissible strains and resistance genes seems to be farm animals (Lu et al., 2002; Donabedian et al., 2006). For instance, in a comprehensive study, Hershberger et al. (2005) showed that farm animals were a reservoir of antibiotic-resistant enterococci and that resistance was more common on farms using antimicrobials. Such strains from animals are potentially able to transfer

resistance genes to pathogenic bacteria. This concern is one of the reasons why trends of resistances in animal isolates are monitored in member states of the European Union and other areas (EFSA, 2015).

Isolates for monitoring programs are commonly obtained from animals, meat, and fecal samples. Furthermore, enterococci are suggested as useful indicator organisms for fecal contaminations of the environment because of their relatively high tenacity outside their hosts (Lukasova and Sustackova, 2003). Since fecal particles are a component of dust in animal housing they could be a source of fecal bacteria (Schulz et al., 2016). This fact explains, for instance, the detection of vancomycin-resistant enterococci (VRE) in dust samples from turkey flocks (Sting et al., 2013). Evidence also suggests that dust from farm animal houses might be a reservoir for multidrug-resistant fecal enterococci, as shown for fecal *Enterobacteriaceae* (Schulz et al., 2016).

Therefore, this retrospective study analyzed the occurrence of enterococci in 125 dust samples from cattle, pig, and poultry barns and the resistance profiles of these bacteria. The dust samples originated from different investigations and studies conducted between 1980 and 2015. During this time span, fluoroquinolones were introduced in the market for use in animal husbandry (Guardabassi et al., 2008). In the same time span, the use of antibiotics as growth promoters was forbidden (Wegener et al., 1999). These events might have had an impact on the resistances of isolates from animal husbandry. Antimicrobial susceptibility testing to determine resistance profiles could be a useful tool in assessing the impacts on the antimicrobial resistances of isolates from farm animal houses (Wiedemann and Heisig, 1999).

MATERIALS AND METHODS

Origins of Dust Samples

From 1980 to 2009, 125 dust samples were collected by sedimentation in Northern Germany. The samples originate from five pig houses, eight poultry barns, and one cattle barn. The samples were taken as parts of various studies. The sedimentation dust samples were collected and stored as described by Schulz et al. (2016). Briefly, collected sedimentation dust samples (between 5 and 50 g) were stored in sterile glass cylinders subsequently sealed with sterile corks and stored in an air-conditioned room at 4°C in the dark.

Additionally, five pooled dust samples collected from a broiler barn in 2015 were included in the study. Dust was transferred by sterile brushes into sterile bags from different dusty surfaces in a barn. After transport to the laboratory, the dust samples were also transferred in sterile glass cylinders. However, a freezer was used to store the samples at 4°C in the dark.

Isolation and Identification of *Enterococcus* spp.

Dust suspensions were prepared as described by Schulz et al. (2016). Subsequently, aliquots (0.1 ml and 0.1 ml of a tenfold dilution and 0.1 ml of a hundred-fold dilution) were plated in triplicate on Bile Aesculin Agar (BAA) (Oxoid Deutschland GmbH, Wesel, Germany) and on BAA supplemented with ciprofloxacin at 4 mg/L (BAACIP) (CIP: Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The detection limit was 1,000 cfu/g of dust. The buffer used to prepare the dust suspensions was plated as a negative control. *Enterococcus faecium* (DSM 2918) and *Enterococcus fecalis* (DSM 20478) were streaked on BAA as growth controls. The plates were incubated at 37°C for 48 h. Presumed enterococci colonies appear with diameters of 1–2 mm and are usually larger than common streptococci, shiny in appearance, and brown with brown or black halos on BAA (Public Health England, 2014; Thermofisher.com, 2017).

At least two putative enterococci colonies of every cultivable sample were randomly selected, streaked on Columbia Agar with sheep blood (COLSB) (Oxoid Deutschland GmbH, Wesel, Germany), and identified as described in the thesis from Liu (2017). Briefly, presumed *Enterococcus* spp. isolates were incubated on API® 20 STREP biochemical test strips in accordance with the manufacturer's protocol (bioMérieux SA, Marcy-l'Étoile, France). After 24 h of incubation, results were analyzed using the apiweb™–API 20 STREP V7.0 software (bioMérieux, Deutschland GmbH, Germany). When the probability of identification was more than 90%, the result was seen as confirmed. However, the differentiation of species from the *E. faecium* group by biochemical tests can fail (Devrise et al., 2002). Therefore, a molecular biological method was used to identify isolates to species level (Stepień-Pyśniak et al., 2017). Stored isolates (at minus 80°C) were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Isolates were incubated on CLOSB at 37°C overnight, afterwards being analyzed by Bruker MALDI Biotyper (Bruker Daltonics, Billerica, USA) in accordance with the manufacturer's protocol. Identified species are summarized in **Table 1**. More detailed results (log(score) values) are shown in the **Supplementary Table 1**.

Antimicrobial Susceptibility

An antimicrobial sensitivity test was performed by the microdilution method for all confirmed enterococci isolates. The code of the commercially prepared microdilution panels is CMV3AGPF (Thermo Fisher Scientific Inc., Waltham, USA). The 15 antibiotics tested were tigecycline (TGC), tetracycline (TET), chloramphenicol (CHL), daptomycin (DAP), streptomycin (STR), tylosin (TYLT), quinupristin/dalfopristin (synercid) (SYN), linezolid (LZD), penicillin (PEN), kanamycin (KAN), erythromycin (ERY), ciprofloxacin (CIP), vancomycin (VAN), lincomycin (LIN), and gentamicin (GEN). The antibiotic concentrations tested are shown in **Tables 2**, **3**.

Due to the absence of trimethoprim-sulfamethoxazole (TMP/SMX) in the prepared panel, sensitivity to these agents was measured separately. Trimethoprim and sulfamethoxazole (Sigma-Aldrich, co., St. Louis, USA) dissolved in methanol were mixed in sterile broth (ratio 1:19). After dilution, the trimethoprim-sulfamethoxazole suspension was added to blank panels. The concentration ranges are also shown in **Tables 2**, **3**.

Fresh Enterococci broth suspension was prepared, and all panels were incubated at 37°C for 24 hours (CLSI, 2016; EUCAST, 2016). *E. faecium* (DSM 2918) was used as a quality

TABLE 1 | Origin and number of isolated species.

Year of sampling	Number of isolates (origin)		
	Enterococcus faecium ($n = 64$)		*Other enterococcus* spp. ($n = 6$)
	From BAA	From BAACIP	From BAA
1981	1 (pig barn)		
1984	2 (pig barn)		
1988	4 (pig barn)		
1989			1 *E. hirae* (pig barn)
1992	1 (pig barn)		
1993	1 (pig barn)		
1994	1 (broiler barn)	6 (broiler barn)	1 *E. hirae* (pig barn)
1995	1 (pig barn)		
1996	1 (pig barn)		
1997			1 *E. hirae* (pig barn)
1998			1 *E. hirae* (pig barn)
1999			1 *E. hirae* (pig barn)
2003	1 (duck barn)		
2004	3 (broiler barn); 1 (turkey barn)	2 (turkey barn)	
2005	2 (pig barn); 3 (cattle barn)	6 (pig barn); 10 (broiler barn); 9 (laying hen house)	
2009	5 (pig barn)	1 (pig barn)	
2015	3 (broiler barn)		1 *E. casseliflavus* (broiler barn)

control. The results were read using the VIZION® system (TREK Diagnostik Systems Ltd., West Sussex, UK). According to guidelines of the Clinical and Laboratory Standards Institute (CLSI), tiny buttons of growth were ignored when reading the minimum inhibitory concentration (MIC) of CHL, ERY, LZD, and TET (CLSI, 2016).

Breakpoints were adopted from CLSI (2016) when available. Three aminoglycosides (gentamicin, kanamycin, and streptomycin) were only tested for high-level resistance, and their breakpoints were obtained from the National Antimicrobial Resistance Monitoring System Animal Isolates (NARMS) of the United States Department of Agriculture (NARMS, 2016). The breakpoints for LIN and TYLT were obtained from NARMS as well (NARMS, 2016). The breakpoints for tigecycline and trimethoprim-sulfamethoxazole were obtained from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016). These figures are also included in **Tables 2**, **3**.

Statistical Analyses

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). For each antibiotic, significant differences between the number of resistant isolates from pigs, fattening poultry (broilers, turkeys, ducks), laying hens, and cattle were analyzed by exact conditional logistic regression using the GENMOD procedure. Exact score tests and odds ratios were calculated to estimate significant differences between the animal groups (Stokes et al., 2012). P-values ≤ 0.05 were interpreted as statistically significant. Isolates were collected from samples between 1980 and 2015. The CORR procedure was used to test if the number of resistances is associated with the age of isolated *Enterococcus* spp. (**Supplementary Table 1**). Pearson's and Spearman's correlation coefficients were calculated and considered as significant when P-values were ≤ 0.05. Associations between total isolates, isolates from BAA, and isolates from BAACIP were tested.

RESULTS

Isolation and Identification of *Enterococcus* spp.

The API® 20 STREP tests identified 70 presumed isolates to *Enterococcus* spp., including 36 from BAA agar and 34 from BAACIP agar. Further identification to species level by MALDI-TOF MS resulted in 64 *E. faecium* isolates, five *E. hirae* isolates and one *E. casseliflavus* isolate. **Table 1** shows the origin of isolates and the year of sampling. *E. faecium* was detected in samples from as early as the early 1980s. *E. hirae* was first cultivated from dust from 1989. Enterococci growing on CIP-supplemented media appeared later in 1994. *E. faecium* was detected in dusts from barns occupied with different animal species, whereas *E. hirae* was isolated from only pig barns.

Frequency of Antimicrobial Resistances in *Enterococcus* spp. Isolates

Figure 1 shows that all isolates were resistant to one or more of the tested antibiotics. Ninety-six percent (67/70) of all isolates were resistant to three or more antimicrobial agents. Overall, isolates from fattening poultry showed higher numbers of resistances, although a single isolate from a pig barn exhibited the highest number of phenotypic resistances ($n = 11$). Isolates from laying hen houses were resistant to fewer antibiotics compared to isolates from pig and fattening poultry barns. Only three isolates from a cattle barn were included. However, the results in **Supplementary Table 1** show that these isolates were resistant to a minimum of three antibiotics from different drug classes.

Seven isolates obtained from dust in fattening poultry barns collected before 2000 were resistant to TYLT. However, for the dust samples collected since 2000, the percentage of resistant isolates was 61.9% (13/21) among fattening poultry. In the isolates from pig farms, the rate of resistant isolates before 2000 was 46.7% (7/15), and then it dropped to 26.7% (4/15) from 2000 onward.

The percentages of SYN-resistant isolates from samples collected before 2000 were 100% (7/7) from fattening poultry barns and 93.3% (14/15) from pig barns. For the younger isolates, the percentages were 76.2% (16/21) and 53.3% (8/15), respectively.

TABLE 2 | MIC distribution for antimicrobial agents of *Enterococcus* spp. from BAA.

Antibiotic[1]		Housed animals	Number of *Enterococcus* spp. with a MIC (μg /ml) of																% Resistant isolates
			0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	
Aminoglycosides[2]	GEN[3]	P												23						0
		FP												10						0
		C												3						0
	KAN[3]	P												11	5	4	1	2		13.0
		FP												4	4	1		1		10.0
		C												1	1			1		33.3
	STR[3]	P														20	1		2	13.0
		FP														7			3	30.0
		C														3				0
Glycopeptides	VAN	P					13	10												0
		FP					3	7												0
		C					1	1	1											0
Lincosamides	LIN[3]	P					3		1		19									82.6
		FP					1				9									90.0
		C							1		2									66.7
Lipopeptides	DAP[5]	P					2	1	3	11	6									NA
		FP						1		3	6									NA
		C								2	1									NA
Macrolides	ERY	P			4	1				6	12									78.3
		FP					1			1	8									90.0
		C				1				1	1									66.7
	TYLT[3]	P						2	1	8	4		8							34.8
		FP							1	1			8							80.0
		C							1		1		1							33.3
Oxazolidinones	LZD	P							22	1										4.3
		FP							10											0
		C							3											0
Penicillins	PEN	P			1			4	3	4	4	7								47.8
		FP							1	2		7								70.0
		C							1		1	1								66.7
Phenicols	CHL	P								4	17	2								8.7
		FP									7	2	1							30.0
		C									3									0

(Continued)

TABLE 2 | Continued

Antibiotic[1]		Housed animals	Number of *Enterococcus* spp. with a MIC (μg /ml) of																% Resistant isolates
			0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	
Quinolones	CIP	P				1	8	5	4	5										39.1
		FP						1	2	7										90.0
		C							1	2										100
Streptogramins	SYN	P					4		8	9	2									82.6
		FP					1	2		5		2								70.0
		C					2			1										33.3
Tetracyclines	TET	P					12	1		1			9							39.1
		FP					2					2	6							80.0
		C								1		2								66.7
	TGC[4]	P	1	11	9	2														0
		FP		3	6	1														0
		C			3															0
Folate pathway inhibitors	TMP/SMX[4]	P			12	6	2			3										13.0
		FP			5	4				1										10.0
		C			1	2														0

P, pigs; FP, fattening poultry; C, cattle.

[1] Breakpoints are adopted from CLSI (2016) when available; [2] Breakpoints for all aminoglycosides are high-level resistance; [3] Breakpoints are established by NARMS (2016); [4] Breakpoints are based on EUCAST (2016); [5] Only a susceptible breakpoint is confirmed; the range of trimethoprim/sulfamethoxazole is shown as the trimethoprim concentration. Unshaded areas show the span of the broth microdilution panels. Single vertical bars indicate a susceptibility breakpoint; double vertical bars are breakpoints for resistance.

NA, not applicable.

TABLE 3 | MIC distribution for antimicrobial agents of *Enterococcus* spp. from BAACIP.

Antibiotic[1]		Housed animals	Number of *Enterococcus* spp. with a MIC (μg /ml) of																% Resistant isolates
			0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024	2,048	4,096	
Aminoglycosides[2]	GEN[3]	P												7						0
		FP												18						0
		L												9						0
	KAN[3]	P												1		3		3		42.9
		FP												2	4	2		10		55.6
		L													8	1				0
	STR[3]	P														2			5	71.4
		FP														11			7	38.9
		L														9				0
Glycopeptides	VAN	P					4	1	2											0
		FP					9	9												0
		L					7	2												0
Lincosamides	LIN[3]	P					1		2		4									57.1
		FP									18									100
		L									9									100
Lipopeptides	DAP[5]	P								4	3									NA
		FP						1	5	8	3	1								NA
		L								4	5									NA
Macrolides	ERY	P			1				1	2	3									71.4
		FP			4					3	11									77.8
		L			5	3					1									11.1
	TYLT[3]	P						2	1	1			3							42.9
		FP							3	2	1		12							66.7
		L						2	3	3			1							11.1
Oxazolidinones	LZD	P							7											0
		FP							18											0
		L							9											0
Penicillins	PEN	P								1		6								85.7
		FP									1	17								100
		L						1	1			7								77.8
Phenicols	CHL	P									4	2	1							42.9
		FP									15	1	2							16.7
		L									9									0

(Continued)

TABLE 3 | Continued

Antibiotic[1]		Housed animals	Number of *Enterococcus* spp. with a MIC (μg /ml) of																% Resistant isolates
			0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024	2,048	4,096	
Quinolones	CIP	P								7										100
		FP								18										100
		L								9										100
Streptogramins	SYN	P					1	3	2		1									42.9
		FP						2	1	10	2		3							88.9
		L					1	3	4	1										55.6
Tetracyclines	TET	P					1						6							85.7
		FP					3		1				14							77.8
		L					4						5							55.6
	TGC[4]	P		3	3	1														0
		FP		4	7	5	2													11.1
		L		3	5	1														0
Folate pathway inhibitors	TMP/SMX[4]	P			3					4										57.1
		FP			18															0
		L			9															0

P, pigs; FP, fattening poultry; L, laying hens.

[1] Breakpoints are adopted from CLSI (2016) when available; [2] Breakpoints for all aminoglycosides are high-level resistance; [3] Breakpoints are established by NARMS (2016); [4] Breakpoints are based on EUCAST (2016); [5] Only a susceptible breakpoint is confirmed; the range of trimethoprim/sulfamethoxazole is shown as the trimethoprim concentration. Unshaded areas show the span of the broth microdilution panels. Single vertical bars indicate a susceptibility breakpoint; double vertical bars are breakpoints for resistance.

NA, not applicable.

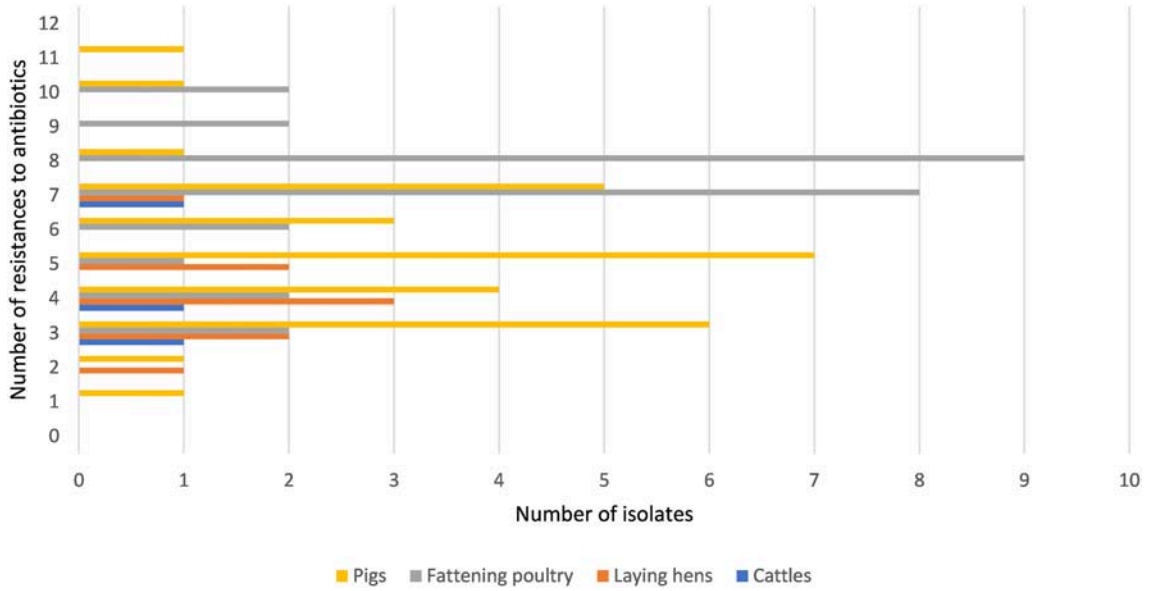

FIGURE 1 | Frequencies of antimicrobial resistances in *Enterococcus* spp. isolates.

Correlations Between Age of Isolates and Number of Resistances

Correlation analyses were carried out to investigate associations between the number of phenotypic resistances (total isolates, isolates from BAA, and isolates from BAACIP) and the age of the isolates. No correlations were found for total isolates (Spearman correlation coefficient, $\rho = 0.16$, $p = 0.187$) and isolates from BAA ($\rho = 0.05$, $p = 0.776$). A moderate monotonic relationship ($\rho = 0.45$, $p = 0.008$) was only obtained when the number of resistances and the age of isolates from BAACIP media were compared.

Antimicrobial Susceptibility

The minimal inhibition concentrations for 16 antimicrobial agents of *Enterococcus* spp. are shown in **Table 2** (isolates from BAA) and **Table 3** (isolates from BAACIP). All enterococci were sensitive to GEN and VAN. Only a few isolates were not susceptible to LZD and TGC. In contrast, relatively high resistance rates of isolates from both media were calculated for LIN, ERY, PEN, TET, SYN, and CIP. All isolates from BAACIP were resistant to CIP (>4 μg/ml, **Table 3**), whereas 15 out of 32 isolates from BAA were susceptible to this antibiotic (**Table 2**). The association between resistant isolates and growing on CIP-containing media was highly significant (Fisher's exact test, $p < 0.0001$). Interestingly, isolates from BAA from the early and late 1980s were already resistant to CIP (**Supplementary Table 1**).

A breakpoint for daptomycin was not available, but the results indicate that most isolates were not susceptible. Obvious differences appear between the resistance rates of different animal groups in **Tables 2**, **3**. However, the numbers of isolates (pigs = 30, fattening poultry = 28, laying hens = 9, cattle = 3) varied between the groups, which hampered the comparison. Therefore, the significance of differences was calculated by a model, and significant outcomes are summarized in **Tables 4**, **5**.

Significant Differences of Antibiotic Resistance Rates Between Animal Groups

An exact conditional logistic regression was conducted to analyze the significant differences between the resistances of isolates from different animal groups. As shown in **Table 4**, the results indicate that the resistances to four antibiotics (ERY, PEN, CIP, and TYLT) were significantly different between isolates from different animal groups. Calculations of the exact odds ratios show significant differences in pairwise comparisons of the animal groups (**Table 5**). The chances of finding resistant isolates showed a general trend. Dust from fattening poultry barns obviously more often contained isolates resistant to ERY, PEN, CIP, and TYLT. Isolates from pig holdings had a higher chance of being resistant to ERY than those from laying hen houses.

DISCUSSION

Enterococcus spp. were isolated from dust samples using BAA. The preparation of dust samples and the subsequent cultivation had a detection limit of 1,000 cfu/g dust. The method enabled the detection of *Enterococcus* spp. in even the oldest sample. Considering that microbial growth in the samples was not possible under storage conditions (Schulz et al., 2016), this means that the oldest isolate (*E. faecium*) survived 35 years in a stored environmental sample. Analyzing more presumed isolates and using an enrichment method would have probably

TABLE 4 | Significant differences ($p \leq 0.05$) of the resistances between isolates from different animal groups.

Antibiotic	CHL	CIP	ERY	KAN	LIN	PEN	SYN	STR	TET	TGC	TYLT
P-Value	0.3997	0.0002	0.0003	0.0515	0.0702	0.0297	0.1725	0.1152	0.1676	0.4720	0.0027

Results of the exact conditional logistic regression.

TABLE 5 | Significant differences in pairwise comparisons of animal groups.

Animal group	Animal group	Antibiotic	Odds ratio	95% confidence limits	*P*-Value
Laying hens	Fattening poultry	ERY	32.045	3.235–>999.999	0.0005
Laying hens	Pigs	ERY	23.729	2.518–>999.999	0.0015
Pigs	Fattening poultry	PEN	6.170	1.402–38.856	0.0113
Pigs	Fattening poultry	CIP	22.467	2.914–>999.999	0.0003
Laying hens	Fattening poultry	TYLT	18.290	1.954–930.221	0.0045
Pigs	Fattening poultry	TYLT	4.201	1.262–15.213	0.0161

enhanced the overall detection rate (Ieven et al., 1999). However, isolating *Enterococcus* spp. from all time periods was possible, and we suggest that *Enterococcus* spp. can be suitable indicator bacteria for retrospective studies with contaminated dry material.

The main species of presumed enterococci was *E. faecium*. This species probably belongs to the typical microbiota in feces from farm animals (Hershberger et al., 2005). Furthermore, *E. faecium* tends to survive longer on dry material than other enterococci (Neely and Maley, 2000). Both of these factors may have influenced the predominant isolation of *E. faecium*. Other species detected were *E. hirae* and *E. casseliflavuss*. *Enterococcus hirae* may be part of the intestinal microbiota of pigs (Larsson et al., 2014) and *E. casseliflavus* was detected also in broiler flocks (Stępień-Pyśniak et al., 2016). The survival for more than two decades in dust also indicates a high tenacity of these species.

The number of phenotypic resistances varied between one and 11. Most of the isolates (98.6%) were multidrug resistant (MDR) according to a definition by Frye and Jackson (2013). The number of antibiotic resistances may vary due to the different treatment regimes in animal husbandry. The treatment status of the sampled barns was unknown. For instance, other studies on isolates of MDR *E. faecium* from food animals revealed 31.7% MDR isolates from cattle, 65.8% MDR isolates from broiler chickens, and 84.6% MDR isolates from pigs (EFSA, 2015; Nowakiewicz et al., 2017). Although the studies are not directly comparable, other sets of antibiotics were tested, so the results of our study and the cited studies indicate that MDR *Enterococcus* spp. is probably widespread in farm animal husbandry.

It is noteworthy that the oldest isolate in the present study (isolated in 1981) was resistant to seven different drug classes. A significant association was not found between the age of the isolates and the number of resistances. It is known that bacteria of animal origin can accumulate antimicrobial drug resistances over time (Tadesse et al., 2012). In the present study, younger isolates showed not more resistances than older isolates. In this context, it must be considered very likely that the heterogeneity of the investigated samples, e.g., different numbers of samples from different time periods and different origins, influenced the results. As an example, isolates from laying hens (all sampled in 2005) showed fewer resistances than older isolates from pigs and fattening poultry.

The susceptibility of isolates to different antimicrobial agents varied greatly (**Table 2**, **3**). Enterococci were completely sensitive to VAN and high-level GEN. Only a few isolates were not susceptible to LZD and TGC. There was a relatively high rate of resistance to LIN, ERY, PEN, TET, SYN, and CIP. BAA supplemented with CIP was used to isolate enterococci from dust samples because fluoroquinolone-resistant enterococci were of special interest. Ciprofloxacin was chosen as a representative of fluoroquinolones because it is a common choice for human bacterial diseases and it is closely related to enrofloxacin, which has been was used extensively in animal husbandry (Guardabassi et al., 2008).

Bacteria show cross-resistance to ciprofloxacin and enrofloxacin (Van den Bogaard et al., 2001). Enrofloxacin was first introduced in German animal husbandry in 1989 (Guardabassi et al., 2008). Thus, the results indicate that the occurrence of CIP-resistant enterococci in the early 1980s was not influenced by the treatment of animals. Resistance to fluoroquinolones in bacteria is multifactorial (Redgrave et al., 2014), and the reason for this early occurrence remains unknown. Isolates from supplemented media were significantly more resistant to ciprofloxacin. However, the resistance among 58% of the isolates from non-selective media and the detection in pig and poultry barns and a cattle barn (**Table 2**) indicate a spread of ciprofloxacin resistance in the farm animal facilities investigated.

High-level resistance breakpoints were used for aminoglycosides because enterococci can prevent aminoglycosides from penetrating the bacterial cell membrane and thus have low-level intrinsic resistance (Zimmermann et al., 1971; EUCAST, 2016). Although high-level resistance against gentamicin was not found, nearly one-third of isolates

had high-level resistance to kanamycin and streptomycin. Resistances to these antibiotics in farm animals might result from the wide and long-term usage of aminoglycosides in Europe (EMA, 2014).

Due to serious nosocomial infections, VRE invariably cause concern among researchers. VRE have been isolated in Germany as early as 1987 (Lütticken and Kunstmann, 1988). Vancomycin resistant enterococci have been isolated from food animals in Sweden, the Netherlands, and Germany (Stobberingh et al., 1999; Nilsson et al., 2009; Sting et al., 2013). However, all enterococci isolated in this study were sensitive to vancomycin.

There was a high percentage of LIN-resistant enterococci, especially in isolates from poultry farm dust. Thirty-six isolates (97.3%) of enterococci from dust in broiler, layer, and turkey houses were resistant to LIN. These findings are consistent with those from another study (Maasjost et al., 2015). Lincosamides and macrolides are important therapeutic agents for the treatment of infections in farm animals (Pyörälä et al., 2014). The resistance to ERY was notable in this study. Except for the isolates from laying hen barns, the percentages of ERY-resistant enterococci were all over 60% (**Tables 2**, **3**). Isolates from pig barns and fattening poultry barns had a higher chance of being resistant to ERY than those from laying hen barns (**Table 5**). It can be assumed that laying hens are generally treated less because of the problem with residues in eggs.

There was a lower percentage of resistance to TYLT, another macrolide, than to ERY (**Tables 2**, **3**). Furthermore, the percentage of TYLT resistant isolates from poultry and pig barns decreased since 2000. It is uncertain whether this observed decrease was influenced by the ban of TYLT as a growth promoter at the end of 1998 in the European Unions (Wegener et al., 1999), but the results show that resistance was still present in isolates from 2015.

Quinupristin/dalfopristin was the first antibiotic for human VRE infections with good clinical effect (Wegener et al., 1999). Virginiamycin and SYN are streptogramins. Due to the "Precautionary Principle," virginiamycin was prohibited as an antibiotic growth promoter at the same time as TYLT (Casewell et al., 2003). A decrease in resistant isolates has been observed for SYN. However, a more comprehensive study would be necessary to confirm this downtrend.

Over 70% of *Enterococcus* spp. were resistant to PEN. Resistance rates of the same magnitude were detected in *E. faecium* isolates from poultry production environments in the United States (Hayes et al., 2004). These high resistance rates may be due to an induced, intrinsic, low-level resistance of *E. faecium* to PEN (Maasjost et al., 2015). A correlation between penicillin and ciprofloxacin resistance has also been observed (Adela et al., 2004).

Although the rate of CHL resistance was <20%, it was obviously higher than in other studies in Germany (Peters et al., 2003; Maasjost et al., 2015). Chloramphenicol was forbidden for use in farm animals in Europe in 1994 (Maasjost et al., 2015). However, in the present study resistant isolates occurred sporadically in poultry and pig barns after the ban.

Linezolid has been allowed for clinical use in humans in Europe since 2001 (Seedat et al., 2006). Although LZD can be used in pets, it should be prescribed only in rare cases (Wijesekara et al., 2017). The first LZD-resistant VRE was found in Germany in 2004 (Halle et al., 2004). In our study, no LZD-resistant *E. faecium* was detected. Only one isolate of *E. hirae* was resistance to LZD. Almost all MIC values for LZD were in the intermediate range (**Tables 2**, **3**). Resistance to TMP/SMX was also scarce. In general, resistance to TMP/SMX seems to be rare in Gram-positive bacteria isolated from German farm animals (Schwarz et al., 2013).

All enterococci in this study were resistant to one or more antimicrobials (**Figure 1**). Approximately 75.0% of isolates from dust from fattening poultry farms were resistant to seven or more antimicrobials compared with only 26.7% from pigs. The resistances to ERY, PEN, CIP, and TYLT were significantly different between isolates from different animal groups (**Table 4**). In a second step, a statistical model revealed that *Enterococcus* spp. isolated from fattening poultry barns were more often resistant to these antibiotics compared to other animal groups (**Table 5**). Furthermore, isolates from fattening poultry barns showed the highest rate resistance to multiple antibiotics (**Figure 1**). These results may be related to the different antibiotic regimes in the environments investigated and suggest that more antibiotics were used in poultry barns.

Metagenomic analyses of environmental samples revealed that antibiotic resistance is an ancient, naturally occurring phenomenon (D'Costa et al., 2011). Although such studies can confirm that genes homologous to resistance genes existed in ancient bacteria, DNA fragments cannot confirm functional resistance against antibiotics (Perron et al., 2015). A study from Perron et al. (2015) revealed functional antibiotic resistance in at last 5,000 years old permafrost. However, whether bacteria survived such a long time or were part of subpopulations remained unknown. This study showed that the long-term survival of enterococci in dust enabled a retrospective view of the phenotypic antimicrobial resistances in isolates from different barns of intensive livestock farming. In comparison to a study from Schulz et al. (2016), the present study detected fluoroquinolone resistant bacteria before these antibiotics were used in farms. The resistance in the absence of fluoroquinolone pressure is likely to be related to the biology of resistance (Redgrave et al., 2014). However, this demonstrates that farm animals can be a reservoir of fluoroquinolone resistant bacteria, although animals came never into contact with these antibiotics. Moreover, it was forbidden to treat laying hens with fluoroquinolones in the European Union (Anonymous, 2002) but all isolates from laying hens in 2005 were resistant to CIP. An eradication of CIP resistant enterococci will not be as simple as prohibiting the use of these agents.

Farmers, animals, and the environment are exposed to dust-bound MDR enterococci shed by carrying animals. Intervention methods such as thoroughly cleaning of all contaminated surfaces in barns are necessary to avoid transmissions. Whether animal strains can be transmitted to humans remains controversial (Donabedian et al., 2006). However, in terms of prevention,

farmers might protect themselves by hygiene measures such as changing clothes, appropriate hand hygiene, and wearing dust masks.

AUTHOR CONTRIBUTIONS

ML isolated and identified the isolates, conducted the antimicrobial susceptibility testing, and carried out the data analysis. NV programmed the statistical model and analyzed the results. JS planned the study and did the correlation analysis. ML, NK, and JS wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03074/full#supplementary-material

Supplementary Table 1 | Origin, identification, and MIC values of isolates.

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frontiers in Microbiology

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Investigation of the Dominant Microbiota in Ready-to-Eat Grasshoppers and Mealworms and Quantification of Carbapenem Resistance Genes by qPCR

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In this study, 30 samples of processed edible mealworms (*Tenebrio molitor* L.) and 30 samples of grasshoppers (*Locusta migratoria migratorioides*) were obtained from producers located in Europe (Belgium and the Netherlands) and Asia (Thailand) and subjected to PCR-DGGE analyses. The PCR-DGGE analyses showed that species in the genus *Staphylococcus* were predominant in the samples of mealworms from Belgium and grasshoppers from the Netherlands; species in the genus *Bacillus* were detected in the samples of mealworms and grasshoppers from Thailand. Moreover, *Weissella cibaria/confusa*/spp. was found in grasshoppers from Belgium. Since data concerning the role of novel foods such as edible insects in the dissemination of carbapenem resistance are currently lacking, the quantification of five carbapenemase encoding genes (bla_{NDM-1}, bla_{VIM}, bla_{GES}, bla_{OXA-48}, and bla_{KPC}) by qPCR was also carried out in all the samples under study. The genes coding for GES and KPC were not detected in the analyzed samples. A very low frequency of bla_{OXA-48} (3%) and bla_{NDM-1} (10%) genes was detected among mealworms. In contrast, grasshoppers were characterized by a high incidence of the genes for OXA-48 and NDM-1, accounting for 57 and 27% of the overall grasshopper samples, respectively. The bla_{VIM} gene was detected exclusively in two grasshopper samples from Thailand, showing only 7% positivity. The analysis of variance showed that all the effects (producers, species, and producers × species) were statistically significant for bla_{NDM-1}, whereas for bla_{OXA-48} and bla_{VIM}, no significant effects were detected for the same source of variation. Further studies are necessary to assess the possible role of edible insects as reservoirs for the resistance to carbapenems and to understand the correlation with the insect microbiota. Furthermore, an intensified surveillance plan examining the occurrence of carbapenemase encoding genes in the food chain and in environmental compartments is needed for a proper risk assessment. In such a context, the appropriate use of antimicrobials represents the main preventive action that should always be applied.

Keywords: edible insects, antibiotic resistance, PCR-DGGE, carbapenemase genes, qPCR

INTRODUCTION

The use of insects for human consumption is a frequent practice worldwide, mainly in Thailand and other Asian countries, Africa, America, and Australia (van Huis et al., 2013; Schlüter et al., 2017). In Europe, edible insects represent an innovative and uncommon protein source, although in some European countries, especially in the Netherlands and Belgium, the rearing and the industrial production of edible insects is gradually increasing (ANSES, 2014; Schlüter et al., 2017). Indeed, several promising aspects are associated with insect consumption since insects (i) are generally characterized by a positive nutrient profile in terms of high-quality proteins and amino acids, good lipids, vitamins, minerals, and fiber, (ii) are easy to breed, and (iii) cause lower emissions of greenhouse gases and ammonia than traditional livestock (Klunder et al., 2012; van Huis et al., 2013; Schlüter et al., 2017). Due to these numerous nutritional, social, and environmental benefits, edible insects are considered the "food of the future" and categorized as novel foods by Regulation (EU) No 2015/2283 of the European Parliament and of the Council.

Among the edible insects, mealworms and grasshoppers are included within those that are already commercialized as food in EU countries (Schlüter et al., 2017) and that have been partially investigated for the presence of relevant pathogens or potentially pathogenic microorganisms (Ali et al., 2010; Klunder et al., 2012; Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2017a, 2018a) as well as for the presence of transferable resistances to antibiotics (Milanović et al., 2016; Osimani et al., 2017b,c, 2018b).

Carbapenems are broad-spectrum β-lactam antibiotics, currently considered the last-line antibiotics for the treatment of severe human infections caused by multidrug-resistant Gram-negative bacteria (EFSA BIOHAZ Panel, 2013; Guerra et al., 2014; Woodford et al., 2014). The production of carbapenemases, which are β-lactamases capable of hydrolyzing carbapenems and almost all β-lactams, represents the main mechanism of resistance to carbapenems (Tzouvelekis et al., 2012; Doi and Paterson, 2015; Fischer et al., 2017). Among the carbapenemases, the plasmid-acquired class A serine-β-lactamases KPC (*Klebsiella pneumoniae* carbapenemase, 17 variants) and GES (Guiana extended spectrum, 9 variants), class B metallo-β-lactamases VIM (Verona integron-encoded metallo-beta-lactamase, 40 variants) and NDM (New Delhi metallo-beta-lactamase, 10 variants), and class D serine-β-lactamases including OXA carbapenemases (Carbapenem-hydrolyzing oxacillinase) such as OXA-48 are among the most common and important from an epidemiological point of view and are thus the main clinical concern (Queenan and Bush, 2007; Grundmann et al., 2010; Miriagou et al., 2010; Walsh, 2010; Cantón et al., 2012; Monteiro et al., 2012; Pfeifer et al., 2012; Guerra et al., 2014; Woodford et al., 2014; Fischer et al., 2017). Carbapenemases are encoded by genes that are easily transferable among bacteria by horizontal gene transfer events since they are located on mobile genetic elements, thus increasing their worldwide spread among bacteria in different reservoirs (Tzouvelekis et al., 2012; Woodford et al., 2014; Doi and Paterson, 2015; Fischer et al., 2017). Indeed, during the last few years, the spread of carbapenem resistance has increased, especially in the Enterobacteriaceae, as well as in non-fermenters such as *Pseudomonas* spp. and *Acinetobacter* spp. and in non-pathogenic bacteria such as *Stenotrophomonas* spp. and *Myroides* spp. (Miriagou et al., 2003; Grundmann et al., 2010; Nordmann et al., 2011, 2012; Cantón et al., 2012; Tzouvelekis et al., 2012; Guerra et al., 2014; Doi and Paterson, 2015; Morrison and Rubin, 2015). In the last decade, the rapid and global dissemination of infections caused by carbapenemase-producing Enterobacteriaceae (CPE) in hospitals and healthcare institutions is of great concern since these outbreaks are often associated with high mortality rates due to the limited and inadequate alternative treatments (Nordmann et al., 2011; Doi and Paterson, 2015; Rossolini, 2015; Grundmann et al., 2017; Zhang et al., 2017). From all of these data, the current opinion is that the acquired carbapenemases are a primary pressing public health threat related to antibiotic resistance (AR) (Tzouvelekis et al., 2012; EFSA BIOHAZ Panel, 2013; Woodford et al., 2014; Doi and Paterson, 2015).

Although the occurrence of carbapenemases was first discovered and mainly investigated at hospitals and healthcare facilities, scientific studies reporting carbapenemase-producers, and carbapenemase-encoding genes (CEG) in animals, the environment and food are increasingly frequent. Specifically, carbapenem resistance has been detected in livestock and in their environments in France, Germany, Switzerland, the USA, and China (Guerra et al., 2014; Webb et al., 2016; Zurfluh et al., 2016; Fischer et al., 2017); companion animals and wildlife (Guerra et al., 2014; Woodford et al., 2014); aquatic environments (Zurfluh et al., 2013; Guerra et al., 2014; Woodford et al., 2014; Fernando et al., 2016); retail chicken meat from Egypt (Abdallah et al., 2015); vegetables and seafood from Asia, India and Brazil (Guerra et al., 2014; Morrison and Rubin, 2015; Zurfluh et al., 2015, 2016). This suggests that non-human sources may be reservoirs of CPE and CEG. Since it is widely recognized that the food chain is one of the main routes for the introduction of antibiotic-resistant bacteria and their genes into the human digestive tract, and for the diffusion and spread of AR in human pathogens (Clementi and Aquilanti, 2011; Rolain, 2013; Milanović et al., 2017), there is a need for intensified surveillance of the occurrence of CEG in the food chain and in different environmental compartments. This is also underscored by the European Food Safety Authority, which has recently recognized the need to improve European legislation to ensure the monitoring of carbapenem resistance in animals and food (EFSA BIOHAZ Panel, 2013).

Based on all these premises, edible insects deserve great attention in terms of safety, including the assessment of the microbiota and of the incidence of AR genes, in particular CEG.

Therefore, in order to obtain an overview of the predominant bacterial species in samples of processed edible mealworms (*Tenebrio molitor* L.) and grasshoppers (*Locusta migratoria migratorioides*) obtained from producers in Europe (Belgium and the Netherlands) and Asia (Thailand), the total microbial DNA was analyzed by culture-independent PCR-DGGE.

Concerning the AR issue, it is worth noting that currently, only a few scientific studies are available on the occurrence of transferable AR genes in edible insects (Milanović et al., 2016;

Osimani et al., 2017b,c, 2018b; Vandeweyer et al., 2018), and to the authors' knowledge, none have been published on the detection of CEG.

To address this gap, the edible insect samples under study were subjected to screening by quantitative PCR (qPCR) of five among the most common carbapenem resistance genes (bla_{NDM-1}, bla_{VIM}, bla_{GES}, bla_{OXA-48}, and bla_{KPC}) (Monteiro et al., 2012). Statistical analyses were performed to determine if edible insect species (mealworms and grasshoppers) or geographical location correlated with the occurrence of carbapenem resistance genes in this study.

MATERIALS AND METHODS

Sampling

Thirty samples of edible mealworms and 30 samples of grasshoppers (boiled, dried, and salted) were purchased via the internet from dealers located in Europe (Belgium and the Netherlands) and Asia (Thailand). Ten mealworm and ten grasshopper samples from each country were collected and marked as follows: TN1-TN10 (mealworms from the Netherlands, Producer 1), TB1-TB10 (mealworms from Belgium, Producer 2), TT1-TT10 (mealworms from Thailand, Producer 3), GN1-GN10 (grasshoppers from the Netherlands, Producer 1), GB1-GB10 (grasshoppers from Belgium, Producer 2), and GT1-GT10 (grasshoppers from Thailand, Producer 3). All the samples were provided in sealed plastic containers and delivered at ambient temperature via international shipping. No information was available on the rearing and hygiene conditions of processing, transport and storage applied to these edible insects before marketing.

Bacterial DNA Extraction

Total microbial DNA was extracted directly from the insect samples using PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) as described by Osimani et al. (2017a). The extracted DNA was quantified and checked for the purity using a NanoDrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and then standardized to 2 ng μL^{-1} for qPCR assays and to 25 ng μL^{-1} for PCR-DGGE analysis. The effective extraction of bacterial DNA was confirmed by conventional PCR amplification of 2 μL (50 ng) of extracted DNA suspensions in a My Cycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using universal prokaryotic primers 27F and 1495R as described by Osimani et al. (2015).

PCR-DGGE Analysis

The equal portions of DNAs extracted from insects were mixed together with the goal of obtaining six pooled samples (TB, TN, TT, GB, GN, and GT), each representing an insect type (mealworms and grasshoppers) and country of origin (Belgium, the Netherlands, and Thailand). The amplification products obtained from the 27F-1495R primer pair as described above were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Subsequently, 2 μL of the purified PCR products was reamplified using the universal prokaryotic primers U968GC (added with GC clamp) and L1401 (Muyzer et al., 1993; Randazzo et al., 2002) following the PCR conditions previously described by Aquilanti et al. (2013). Following amplification, 5 μL of the PCR reaction was loaded on a 1.5% agarose gel together with a 100 bp molecular weight marker (HyperLadder™ 100 bp) to check for the expected product size of 480 bp. Twenty microliters of these PCR amplicons were analyzed by DGGE (30–60% urea-formamide denaturing gradient; 4 h at 130 V) using DCode Universal Mutation Detection System (Bio-Rad Laboratories) as described by Garofalo et al. (2015). All DGGE bands visible under UV light were excised from the gel, and the DNA was eluted overnight at 4°C in 50 μL of molecular biology grade water (Garofalo et al., 2008) and reamplified via PCR as described above, but with the forward primer U968 lacking the GC clamp. The PCR products were sent to Genewiz (Takeley, UK) for purification and sequencing, and the obtained sequences were identified at species level as described above by Osimani et al. (2018c).

Reference Strains

DNA extracted from five reference strains (**Table 1**), each carrying one of the carbapenem resistance genes under study, was used as positive control in the qPCR reactions and for the construction of qPCR standard curves.

qPCR

Absolute quantification of each carbapenemase gene (bla_{NDM-1}, bla_{VIM}, bla_{GES}, bla_{OXA-48}, and bla_{KPC}) in the insect samples was performed by qPCR in a Mastercycler® ep realplex machine (Eppendorf, Hamburg, Germany) using the qPCR primers and cycling conditions described by Monteiro et al. (2012). To check for product specificity, all cycles were followed by a melt curve step analysis with temperature gradually increasing from 65 to 95°C by 0.2°C/s. Each qPCR reaction consisted of 4 μL (8 ng) of the extracted DNA; 5 μL of Type-it 2X HRM PCR Master Mix (Qiagen, Hilden, Germany) containing HotStarTaq Plus DNA Polymerase, EvaGreen Dye, an optimized concentration of Q-solution, dNTPs and $MgCl_2$; 0.2 μM of forward and reverse primers for each gene; and nuclease-free molecular biology grade water to a final reaction volume of 10 μL. The exogenous standards for each gene were prepared by qPCR amplification of the DNA extracted from the reference strains (**Table 1**) as described above but in a final reaction volume of 25 μL. The correct melting temperatures (Tm) and sizes of the obtained PCR products were checked by melting curve analysis and

TABLE 1 | Bacterial reference strains carrying carbapenems resistance genes, used as positive controls in the qPCR reactions.

Bacterial strain	Carbapenems resistance gene
BAA 2146_ *Klebsiella pneumoniae*	bla_{NDM-1}
LEMC_VIM-1_ *Pseudomonas aeruginosa*	bla_{VIM}
LEMC_GES-1_ *Pseudomonas aeruginosa*	bla_{GES}
LEMC_OXA-48_ *Klebsiella pneumoniae*	bla_{OXA-48}
ATCC 1705_ *Klebsiella pneumoniae*	bla_{KPC}

electrophoresis on a 1.5% agarose gel, respectively. The Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) was used for the purification of the amplicons following the manufacturer's instructions. The quantity and the purity of the products were determined using the NanoDrop ND 1000 (Thermo Fisher Scientific). The calculation of each gene copy number was performed using an online calculator (www.idtdna.com) based on the mass and size of the purified qPCR products. The standard curves were created by qPCR amplification of 10-fold serial dilutions of exogenous standards. The amplification efficiencies were estimated from the slopes of the standard curves, and the correlation coefficients (R^2) (Stolovitzky and Cecchi, 1996) were calculated automatically by Mastercycler® ep realplex software. To determine the qPCR detection limit for each gene, the standard curves were generated in the range from ∼1 to 10^7 gene copies per reaction.

For the absolute quantification of CEG, the DNAs extracted from mealworms and grasshoppers were run along with the 10-fold serial dilutions of the standards prepared as described above. The gene copy number of each gene detected in the analyzed insect samples was determined using the slope of the corresponding standard curves. The baseline and threshold calculations were performed automatically by the Mastercycler® ep realplex software. In addition to melting curve analysis, the correct sizes of the amplification products were checked by electrophoresis on 1.5% agarose gels using 100 bp DNA Ladder (HyperLadder™ 100 bp, Bioline, UK) as a molecular weight marker. Moreover, the accuracy of the amplification reactions was validated by the sequencing (Genewiz) of randomly selected positive samples (TB6, GT7, and GN1 for the $bla_{\mathrm{OXA-48}}$ gene; GT2 and TT8 for the $bla_{\mathrm{NDM-1}}$ gene; GT7 and GT8 for the bla_{VIM} gene). The resulting sequences were compared with those from the GenBank database (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). All qPCR reactions were performed in triplicate and the results were expressed as the mean gene copy number per ng of DNA ± standard deviation for each gene.

Statistical Analysis

Descriptive statistics, calculated on 20 samples for each producer and on 30 samples for each insect species were carried out for the $bla_{\mathrm{OXA-48}}$, $bla_{\mathrm{NDM-1}}$, and bla_{VIM} gene copies by computing the means ± standard deviation.

After first checking for conformance to a normal distribution and identification of outliers, analysis of variance (ANOVA) was carried out using JMP statistical software version 11.0.0 (SAS Institute Inc., Cary, NC, USA) to test the following main effects: producers (Belgium, Thailand, the Netherlands), insect species (mealworm, grasshoppers) and producer × species.

Principal component analysis (PCA) was applied to discriminate among mealworms and grasshoppers coming from different producers (Belgium, the Netherlands, and Thailand), and the presence of genes related to resistance to carbapenems ($bla_{\mathrm{NDM-1}}$, bla_{VIM}, bla_{GES}, $bla_{\mathrm{OXA-48}}$, and bla_{KPC}). PCA was carried out using the Unscrambler 7.5 software (CAMO ASA, Oslo, Norway). The mean data were normalized to neutralize any influence of hidden factors. PCA provides a graphical representation of the overall differences in terms of distribution of genes between the insects coming from different producers.

RESULTS AND DISCUSSION

In the present study, the microbiota of commercialized read-to-eat grasshoppers and mealworms from different countries was investigated via PCR-DGGE, as well as the quantification and distribution of five common CEG within the same matrices, in order to have a more complete picture of some safety aspects related to edible insects.

Determination of Microbial Diversity

To have an overview of the predominant bacterial species found in the edible insects considered in this study, the total microbial DNA was extracted from the samples, the DNAs were mixed in order to obtain six pooled samples, each representing an insect type (mealworms, grasshoppers) and country of origin (Belgium, the Netherlands, Thailand) and then analyzed by a culture-independent PCR-DGGE method. The results obtained are reported in **Table 2**.

The dominant species found in mealworms from Belgium and grasshoppers from the Netherlands belonged to the genus *Staphylococcus*, and species in the genus *Bacillus* were found in mealworms and grasshoppers from Thailand. Grasshoppers from Belgium were positive for *Weissella cibaria*/*confusa*/spp., while bacterial species with a percentage identity below 97% were found in mealworms from the Netherlands. It is interesting to note that the predominance of lactic acid bacteria (LAB), and in particular the *Weissella* spp., has been reported for processed and fresh grasshoppers from Belgium and the Netherlands (Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2017a), thus suggesting that the specific rearing conditions may have selected for this microbial group or that this bacterial species is intrinsically associated with this edible insect.

The genera *Staphylococcus* and *Bacillus* identified among the other pooled samples of mealworms and grasshoppers may contain pathogenic species such as *Staphylococcus aureus* and *Bacillus cereus,* and these data are in agreement with other studies on the microbiota in fresh and processed mealworms and grasshoppers (Stoops et al., 2016; Garofalo et al., 2017). The presence of *Staphylococcus* members may be due to an environmental contamination occurring during human handling or processing. Indeed, these insects were boiled and salted, and since *Staphylococcus* spp. is a halophile bacterium usually predominating in environments with low microbial competition, it could have found conditions suitable for growth.

The lack of detection of bacterial DNA belonging to the Enterobacteriaceae has been already reported by Osimani et al. (2018c), although it is generally reported that Enterobacteriaceae represents a predominant bacterial group in the edible insect gut microbiota (Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2017b). These data suggest that good manufacturing practices were applied during rearing and processing of the insects and/or that a degutting step may have been applied. A further explanation is that members

TABLE 2 | Sequencing results of the bands excised from the DGGE gel obtained from the amplified fragments of pooled bacterial DNA extracted directly from mealworms and grasshoppers.

Sample	Closest relative	% Identity[a]	Acc.no[b]
TB	*Staphylococcus warneri*	98	MH211317
	Staphylococcus pasteuri	98	MH158278
	Staphylococcus sp.	98	MH191108
	Staphylococcus kloosii	99	CP027846
	Staphylococcus cohnii	99	KY012323
	Staphylococcus sp.	99	KY865752
TN	*Exiguobacterium* sp.	82	MG859628
	Eikenella corrodens	90	KU663108
	Eikenella sp.	90	KU738863
	Neisseria shayeganii	90	KM462144
TT	*Bacillus* sp.	99	MG757948
	Bacillus sp.	99	LT899995
GB	*Weissella cibaria*	99	CP027427
	Weissella confusa	99	MF327674
	Weissella sp.	99	MG814036
GN	*Staphylococcus haemolyticus*	99	MH179468
	Staphylococcus argenteus	99	LC378381
	Staphylococcus sp.	99	MH021651
	Staphylococcus hominis	99	MF327701
	Staphylococcus aureus	99	MG976640
GT	*Bacillus sonorensis*	99	KY243955
	Bacillus subtilis	99	KU172428
	Bacillus amyloliquefaciens	99	KJ126909
	Bacillus axarquiensis	99	KJ126897
	Bacillus sp.	100	LT899995

TB- pool of 10 (TB1-TB10) mealworm samples from Belgium; TN- pool of 10 (TN1-TN10) mealworm samples from the Netherlands; TT- pool of 10 (TT1-TT10) mealworm samples from Thailand; GB- pool of 10 (GB1-GB10) grasshopper samples from Belgium; GN- pool of 10 (GN1-GN10) grasshopper samples from the Netherlands; GT- pool of 10 (GT1-GT10) grasshopper samples from Thailand.

[a] *Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database.*

[b] *Accession number of the sequence of the closest relative found by a BLAST search.*

of the Enterobacteriaceae family could be present within the processed insect samples but with a lower abundance in respect with other microbial groups. This latter hypotesis is supported by the fact that these microorganisms had previously found below the detection limit of microbial counts (<1 Log cfu g^{-1}) in the same samples (Osimani et al., 2017b,c).

Additionally, the lack of detection of bacterial DNA belonging to Pseudomonadaceae is unusual, but it is possible, as suggested for Enterobacteriaceae, that PCR-DGGE was not able to detect members of this microbial group if they were in the minority relative to the others.

Quantification and Distribution of carbapenem Resistance Genes

This study represents the first report on the screening of five carbapenemase encoding genes (bla_{NDM-1}, bla_{VIM}, bla_{GES}, bla_{OXA-48}, and bla_{KPC}) in processed edible mealworms (*T. molitor* L.) and edible grasshoppers (*L. migratoria migratorioides*) from producers located in Europe (Belgium and the Netherlands) and Asia (Thailand). The identification of the genes coding for these carbapenemases in the samples of edible insects under investigation was conducted by using qPCR. As previously underlined by Monteiro et al. (2012), molecular assays are considered the best solutions for the rapid detection of carbapenem resistance genes and for the identification of the resistance mechanism. The detection limit, defined as the lowest concentration at which 95% of the positive samples are detected was <10 gene copies per reaction for all the genes. The efficiencies of the qPCR reactions were 1.00 for the genes bla_{OXA-48}, bla_{VIM}, and bla_{GES}; 1.01 for the gene bla_{KPC} and 0.96 for the gene bla_{NDM-1}. The R^2 was 1.000 for the genes bla_{OXA-48} and bla_{NDM-1}; 0.999 for the gene bla_{GES}, 0.996 for the gene bla_{KPC} and 0.995 for the gene bla_{VIM}. Moreover, the specificity of the primers used for the amplification of carbapenem resistance genes was confirmed by the results of the sequencing of randomly selected positive samples, which showed >97% similarity with the corresponding gene sequences deposited in the GenBank database. In more detail, the results of the BLAST analysis for the bla_{OXA-48} gene showed 98% of the similarity with the sequences deposited in GenBank such as *K. pneumoniae* (CP031374), *Citrobacter freundii* (MG430338), *Enterobacter ludwigii* (MG436907), *Pantoea agglomerans* (MG436898), *Escherichia coli* (NG_055499), *Shewanella* sp. (NG_055475), and *Proteus mirabilis* (KT175900); for the bla_{NDM-1} gene 99% similarity with the *Enterobacter hormaechei* (AP018835), *K. pneumoniae* (AP018834), *Raoultella planticola* (MH257689), *E. coli* (CP021206), *Pseudomonas aeruginosa* (KT364224), and *Acinetobacter baumannii* (KU180703); and for the bla_{VIM} gene 98% similarity with the *K. pneumoniae* (MH071811), *C. freundii* (NG_061412), *Paenibacillus* sp. (KR822172), *E. coli* (MF169879), *E. hormaechei* (LT991955), *Kluyvera cryocrescens* (MG228427), *Alcaligenes faecalis* (KY623659), *Klebsiella oxytoca* (NG_050362), and *Enterobacter cloacae* (CP030081).

The results of the qPCR quantification of carbapenem resistance genes in samples of ready-to-eat edible mealworms and grasshoppers produced in the Netherlands, Belgium and Thailand are reported in **Tables 3**, **4**.

Regarding mealworms, none of the samples were positive for the genes bla_{GES}, bla_{KPC}, and bla_{VIM}, while only sample TB6 was found positive for bla_{OXA-48} (3% positivity) and samples TN2, TN8, and TT8 were positive for bla_{NDM-1} (10% positivity) (**Table 3**).

Regarding grasshoppers, the genes bla_{GES} and bla_{KPC} were not detected in any of the analyzed samples while only two samples from Thailand (GT7 and GT8) were positive for the bla_{VIM} gene (7% positivity). Interestingly, a high prevalence of bla_{OXA-48} was noted (57% positivity), followed by bla_{NDM-1} (27% positivity) (**Table 4**). Specifically, bla_{OXA-48} was prevalent in 80% of the samples from Belgium, in 50% of the samples from the Netherlands and in 40% of the samples from Thailand. The highest frequency of bla_{NDM-1} was found among samples from Thailand (40%), followed

TABLE 3 | Results of qPCR quantification of carbapenemase genes in samples of ready-to-eat edible mealworms produced in the Netherlands (TN1-TN10), Belgium (TB1-TB10) and Thailand (TT1-TT10).

Producer	Samples	Carbapenemase resistant genes (gene copies ng^{-1} ± standard deviation)				
		bla$_{GES}$	*bla*$_{KPC}$	*bla*$_{OXA-48}$	*bla*$_{NDM-1}$	*bla*$_{VIM}$
1	TN1	n.d.	n.d.	n.d.	n.d.	n.d.
	TN2	n.d.	n.d.	n.d.	2.29 ± 0.31	n.d.
	TN3	n.d.	n.d.	n.d.	n.d.	n.d.
	TN4	n.d.	n.d.	n.d.	n.d.	n.d.
	TN5	n.d.	n.d.	n.d.	n.d.	n.d.
	TN6	n.d.	n.d.	n.d.	n.d.	n.d.
	TN7	n.d.	n.d.	n.d.	n.d.	n.d.
	TN8	n.d.	n.d.	n.d.	0.94 ± 0.13	n.d.
	TN9	n.d.	n.d.	n.d.	n.d.	n.d.
	TN10	n.d.	n.d.	n.d.	n.d.	n.d.
	TN % of positivity for each determinant	n.dr.	n.dr.	n.dr.	20.0	n.dr.
2	TB1	n.d.	n.d.	n.d.	n.d.	n.d.
	TB2	n.d.	n.d.	n.d.	n.d.	n.d.
	TB3	n.d.	n.d.	n.d.	n.d.	n.d.
	TB4	n.d.	n.d.	n.d.	n.d.	n.d.
	TB5	n.d.	n.d.	n.d.	n.d.	n.d.
	TB6	n.d.	n.d.	1.81 ± 0.01	n.d.	n.d.
	TB7	n.d.	n.d.	n.d.	n.d.	n.d.
	TB8	n.d.	n.d.	n.d.	n.d.	n.d.
	TB9	n.d.	n.d.	n.d.	n.d.	n.d.
	TB10	n.d.	n.d.	n.d.	n.d.	n.d.
	TB % of positivity for each determinant	n.dr.	n.dr.	10.0	n.dr.	n.dr.
3	TT1	n.d.	n.d.	n.d.	n.d.	n.d.
	TT2	n.d.	n.d.	n.d.	n.d.	n.d.
	TT3	n.d.	n.d.	n.d.	n.d.	n.d.
	TT4	n.d.	n.d.	n.d.	n.d.	n.d.
	TT5	n.d.	n.d.	n.d.	n.d.	n.d.
	TT6	n.d.	n.d.	n.d.	n.d.	n.d.
	TT7	n.d.	n.d.	n.d.	n.d.	n.d.
	TT8	n.d.	n.d.	n.d.	3.38 ± 0.18	n.d.
	TT9	n.d.	n.d.	n.d.	n.d.	n.d.
	TT10	n.d.	n.d.	n.d.	n.d.	n.d.
	TT % of positivity for each determinant	n.dr.	n.dr.	n.dr.	10.0	n.dr.
	Overall % of positivity for each determinant	n.dr.	n.dr.	3.0	10.0	n.dr.

n.d., not detected. n.dr., not determined.

by samples from Belgium (30%) and the Netherlands (10%) (**Table 4**).

All the insect samples analyzed in this study were previously screened for the presence of 12 selected genes coding for resistance to tetracyclines [*tet*(M), *tet*(O), *tet*(S), and *tet*(K)], macrolide-lincosamide-streptogramin B (MLS_B) [*erm*(A), *erm*(B), *erm*(C)], vancomycin (*vanA* and *vanB*), beta-lactams (*blaZ* and *mecA*) and aminoglycosides [*aac(6′)-Ie aph(2″)-Ia* referred as *aac-aph*] through PCR and nested PCR assays (Osimani et al., 2017b,c). It is interesting to note that the mealworms samples TN2 and TN8 from the Netherlands were also found to be positive for the presence of genes coding for resistance to tetracyclines [*tet*(M), *tet*(K), *tet*(S)] and erythromycin [*erm*(B), *erm*(C)], while sample TB6 was positive for genes coding for resistance to tetracyclines [*tet*(M), *tet*(K)], and sample TT8 from Thailand was positive for genes coding for resistance to tetracyclines [*tet*(K)], erythromycin [*erm*(B)], and aminoglycosides (*aac-aph*) (Osimani et al., 2017b). Moreover, among grasshoppers, almost all of the samples that were found to be positive for *bla*$_{OXA-48}$, *bla*$_{NDM-1}$, and *bla*$_{VIM}$ previously showed positivity for AR genes coding for resistance to tetracyclines [*tet*(M), *tet*(S), *tet*(K)], erythromycin [*erm*(B), *erm*(C)], aminoglycosides (*aac-aph*) and beta-lactams (*blaZ*) (Osimani et al., 2017c).

The average levels of gene copies ng^{-1} in the 60 samples of edible insects were as follows: 0.59 ± 2.39 with a

TABLE 4 | Results of qPCR quantification of carbapenemase genes in samples of ready-to-eat edible grasshoppers produced in the Netherlands (GN1-GN10), Belgium (GB1-GB10) and Thailand (GT1-GT10).

Producer	Samples	Carbapenemase resistant genes (gene copies $ng^{-1} \pm$ standard deviation)				
		bla$_{GES}$	*bla*$_{KPC}$	*bla*$_{OXA-48}$	*bla*$_{NDM-1}$	*bla*$_{VIM}$
1	GN1	n.d.	n.d.	1.17 ± 0.19	n.d.	n.d.
	GN2	n.d.	n.d.	0.24 ± 0.02	n.d.	n.d.
	GN3	n.d.	n.d.	n.d.	n.d.	n.d.
	GN4	n.d.	n.d.	0.86 ± 0.09	n.d.	n.d.
	GN5	n.d.	n.d.	n.d.	n.d.	n.d.
	GN6	n.d.	n.d.	n.d.	n.d.	n.d.
	GN7	n.d.	n.d.	0.89 ± 0.01	n.d.	n.d.
	GN8	n.d.	n.d.	0.30 ± 0.04	n.d.	n.d.
	GN9	n.d.	n.d.	n.d.	2.64 ± 0.19	n.d.
	GN10	n.d.	n.d.	n.d.	n.d.	n.d.
	GN % of positivity for each determinant	n.dr.	n.dr.	50.0	10.0	n.dr.
2	GB1	n.d.	n.d.	0.57 ± 0.02	n.d.	n.d.
	GB2	n.d.	n.d.	0.73 ± 0.01	1.85 ± 0.05	n.d.
	GB3	n.d.	n.d.	0.30 ± 0.08	2.25 ± 0.12	n.d.
	GB4	n.d.	n.d.	0.83 ± 0.01	n.d.	n.d.
	GB5	n.d.	n.d.	0.35 ± 0.11	n.d.	n.d.
	GB6	n.d.	n.d.	0.24 ± 0.01	n.d.	n.d.
	GB7	n.d.	n.d.	n.d.	n.d.	n.d.
	GB8	n.d.	n.d.	n.d.	n.d.	n.d.
	GB9	n.d.	n.d.	0.58 ± 0.03	n.d.	n.d.
	GB10	n.d.	n.d.	0.25 ± 0.05	6.37 ± 0.22	n.d.
	GB % of positivity for each determinant	n.dr.	n.dr.	80.0	30.0	n.dr.
3	GT1	n.d.	n.d.	0.30 ± 0.1	10.31 ± 0.52	n.d.
	GT2	n.d.	n.d.	8.65 ± 1.29	70.38 ± 3.01	n.d.
	GT3	n.d.	n.d.	n.d.	n.d.	n.d.
	GT4	n.d.	n.d.	n.d.	24.88 ± 0.71	n.d.
	GT5	n.d.	n.d.	n.d.	n.d.	n.d.
	GT6	n.d.	n.d.	n.d.	n.d.	n.d.
	GT7	n.d.	n.d.	16.56 ± 0.48	51.13 ± 1.59	392.25 ± 1.77
	GT8	n.d.	n.d.	n.d.	n.d.	42.83 ± 6.82
	GT9	n.d.	n.d.	0.59 ± 0.01	n.d.	n.d.
	GT10	n.d.	n.d.	n.d.	n.d.	n.d.
	GT % of positivity for each determinant	n.dr.	n.dr.	40.0	40.0	20.0
	Overall % of positivity for each determinant	n.dr.	n.dr.	57.0	27.0	7.0

n.d., not detected. n.dr., not determined.

minimum value of 0 and a maximum value of 16.56 for *bla*$_{OXA-48}$, 2.94 ± 11.53 with a minimum value of 0 and a maximum value of 70.37 for *bla*$_{NDM-1}$, 7.25 ± 50.85 with a minimum value of 0 and a maximum value of 392.25 for *bla*$_{VIM}$.

Descriptive statistics on 20 samples from each producer are shown in **Table 5**. In addition, descriptive statistics on 30 samples from each insect species are reported in **Table 6**.

The analysis of variance (**Table 7**) showed that all the variables (producers, species, and producers × species) had significant effects ($P < 0.05$) on the frequency of *bla*$_{NDM-1}$, whereas for *bla*$_{OXA-48}$ and *bla*$_{VIM}$, no significant effects were detected for the same source of variation.

Regarding the distribution of *bla*$_{OXA-48}$ and *bla*$_{VIM}$, multiple comparisons between ACC Least Square Means (LSM) carried out using the Tukey test showed no significant differences among samples from different producers or insect species. Regarding *bla*$_{NDM-1}$, multiple comparisons (Tukey HSD) showed that insect species, but not the origin of the sample, had a significant correlation ($P < 0.05$) with the frequency of the gene.

PCA did not discriminate between the presence of genes encoding resistance to carbapenems among mealworms and grasshoppers coming from different producers. In contrast, in the previous studies on the occurrence of transferable ARs in ready-to-eat edible insects, PCA showed a differentiation among

producers, thus suggesting that different rearing and clinical practices associated with different countries may have played a role in the variability observed (Milanović et al., 2016; Osimani et al., 2017b,c).

As reported by Schlüter et al. (2017), it is presumed that the rearing and processing conditions applied to edible insects will comply with the same food safety regulations as for livestock farming. The use of carbapenems is prohibited in food-producing animals in all countries (OIE, 2015; Webb et al., 2016). Notwithstanding, scientific studies reporting CPE and CEG in livestock and their environment are progressively more frequent (Guerra et al., 2014; Webb et al., 2016; Zurfluh et al., 2016; Fischer et al., 2017). Furthermore, in the last EFSA report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015, the presumptive extended-spectrum beta-lactamase (ESBL)-/AmpC-/carbapenemase-production in *Salmonella* and *E. coli* was monitored in humans, meat (pork and beef), fattening pigs and calves for the first time (EFSA and ECDC, 2017). Varying occurrence/prevalence rates of ESBL-/AmpC-producers were observed between countries, and carbapenemase-producing *E. coli* were detected in single samples of pig meat and from fattening pigs from two Member States (EFSA and ECDC, 2017). These data indicate that other antimicrobial classes could indirectly select CPE outside the hospital setting and that the rapid dissemination of CPE is also promoted by CEG located on plasmids transmissible by horizontal gene transfer events (Tzouvelekis et al., 2012; Woodford et al., 2014). As reviewed by Caniça et al. (2015), AR comprises a dynamic network that involves several environmental niches (e.g., water, soil, and plants) and different reservoirs (e.g., husbandry, hospitals, wild animal, human settings, human hand, food and global trade in foodstuffs) in which the path of dissemination and dynamics of AR genes has to be taken into consideration in order to understand and prevent the AR transmission and spread. Therefore, it is possible to hypothesize that, irrespective of the use of carbapenems in the edible insect rearing, the CEG may derive from the substrates used for feed or from surfaces and hands of operators or from treatments applied for processing, in addition to transport and storage. It is also interesting to note that grasshoppers and mealworms have different dietary habits since grasshoppers are grass-feeders whereas mealworms are usually reared on cereal-based matrices; therefore, the differences

TABLE 5 | Descriptive statistics on 20 samples for each producer.

	Belgium			Thailand			The Netherlands		
	$bla_{\text{OXA-48}}$	$bla_{\text{NDM-1}}$	bla_{VIM}	$bla_{\text{OXA-48}}$	$bla_{\text{NDM-1}}$	bla_{VIM}	$bla_{\text{OXA-48}}$	$bla_{\text{NDM-1}}$	bla_{VIM}
Mean	0.28	0.52	0.00	1.31	8.00	21.75	0.17	0.29	0.00
SD	0.45	1.51	0.00	4.07	19.22	87.73	0.36	0.77	0.00
Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Maximum	1.81	6.37	0.00	16.56	70.37	392.25	1.17	2.64	0.00

SD, standard deviation. Values are expressed as gene copies ng^{-1}.

TABLE 6 | Descriptive statistics on 30 samples for each insect species.

	Mealworm			Grasshoppers		
	$bla_{\text{OXA-48}}$	$bla_{\text{NDM-1}}$	bla_{VIM}	$bla_{\text{OXA-48}}$	$bla_{\text{NDM-1}}$	bla_{VIM}
Mean	0.06	0.22	0	1.11	5.66	14.5
SD	0.33	0.74	0	3.31	15.96	71.77
Minimum	0	0.00	0	0	0	0
Maximum	1.81	3.37	0	16.56	70.37	392.25

SD, standard deviation. Values are expressed as gene copies ng^{-1}.

TABLE 7 | ANOVA results for $bla_{\text{OXA}-48}$, $bla_{\text{NDM}-1}$, bla_{VIM}.

Source of variation	df	$bla_{\text{OXA-48}}$			$bla_{\text{NDM-1}}$			bla_{VIM}		
		SS	MS	*P*	SS	MS	*P*	SS	MS	*P*
Producer	2	15.60	7.80	0.24	769.50	384.75	0.04	6309.68	3154.84	0.30
Species	1	16.64	16.64	0.08	443.85	443.85	0.05	3154.84	3154.84	0.27
Producer × species	2	18.26	9.31	0.19	736.79	368.39	0.04	6309.68	3154.84	0.30

Df, degrees of freedom. Significant at $P < 0.05$. *SS, sum of squares. MS, mean square. P-value.*

in terms of presence and distribution of CEG among these insect species may derive from different rearing practices and substrates.

CONCLUSION

Edible insects such as grasshoppers and mealworms represent a novel food that deserves attention in terms of safety, including the assessment of the incidence of AR genes. The investigation of the microbiota of the mealworm and grasshopper samples in this study revealed the presence of potential pathogenic and non-pathogenic species.

Scientific studies reporting carbapenemase-producing microorganisms and CEG in animals, the environment and food are increasingly frequent. The data presented in this study is the first attempt aimed at determining the incidence of CEG among samples of commercialized ready-to-eat grasshoppers and mealworms from Belgium, the Netherlands and Thailand. Although further studies are necessary to understand the correlation of CEG with the insect microbiota and to assess the possible role of edible insects as reservoirs of resistance to carbapenems, an intensified surveillance plan examining the occurrence of CEG in the food chain and in different environmental compartments, along with a prudent use of carbapenems and antimicrobials in general, are primary measures that should be applied.

AUTHOR CONTRIBUTIONS

VM and AR carried out molecular analyses. MP, ST, LC, and MC performed statistical analyses. CG wrote the manuscript. AO, LA, CV, and FC critically analyzed the results and revised the final manuscript.

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Impact on Public Health of the Spread of High-Level Resistance to Gentamicin and Vancomycin in Enterococci

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Antibiotic resistance has turned into a global public health issue. Enterococci are intrinsically resistant to many antimicrobials groups. These bacteria colonize dairy and meat products and integrate the autochthonous microbiota of mammal's gastrointestinal tract. Over the last decades, detection of *vanA* genotype in *Enterococcus faecium* from animals and from food of animal origin has been reported. Vancomycin-resistant *E. faecium* has become a prevalent nosocomial pathogen. Hospitalized patients are frequently treated with broad-spectrum antimicrobials and this leads to an increase in the presence of VanA or VanB vancomycin-resistant enterococci in patients' gastrointestinal tract and the risk of invasive infections. In humans, *E. faecium* is the main reservoir of VanA and VanB phenotypes. Acquisition of high-level aminoglycoside resistance is a significant therapeutic problem for patients with severe infections because it negates the synergistic effect between aminoglycosides and a cell-wall-active agent. The *aac(6′)-Ie-aph (2″)-Ia* gene is widely spread in *E. faecalis* and has been detected in strains of human origin and in the food of animal origin. Enzyme AAC(6′)-Ie-APH(2″)-Ia confers resistance to available aminoglycosides, except to streptomycin. Due to the fast dissemination of this genetic determinant, the impact of its horizontal transferability among enterococcal species from different origin has been considered. The extensive use of antibiotics in food-producing animals contributes to an increase in drug-resistant animal bacteria that can be transmitted to humans. Innovation is needed for the development of new antibacterial drugs and for the design of combination therapies with conventional antibiotics. Nowadays, semi-purified bacteriocins and probiotics are becoming an attractive alternative to the antibiotic in animal production. Therefore, a better understanding of a complex and relevant issue for Public Health such as high-level vancomycin and gentamicin resistance in enterococci and their impact is needed. Hence, it is necessary to consider the spread of *vanA E. faecium* and high-level gentamicin resistant *E. faecalis* strains of different origin in the environment, and also highlight the potential horizontal transferability of these resistance determinants to other bacteria.

Keywords: enterococci, clinical, foodborne, high-level resistance, gentamicin, vancomycin, clonal, transfer

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INTRODUCTION

Enterococci are resistant to diverse physicochemical conditions and are widespread in nature. They are capable of growing and surviving under harsh environmental conditions and have been found in soil, plants, birds, and insects (Butler, 2006; Ghosh and Zurek, 2015).

In the intestinal tract of humans and other animals, the genus *Enterococcus* can be found among their flora. The microbiological and ecological factors that contribute with intestinal colonization are unknown, even though up to 10^8 CFU/g of enterococci have been found in human feces. In addition, strains from this genus have been isolated from fermented and dairy products. Moreover, some enterococcal strains have been regarded as food biopreservants and probiotics, although their safety remains questioned (Beibei et al., 2015).

Traditionally, enterococci have not been considered as community-acquired pathogens. Usually, these bacteria do not cause infectious diseases in healthy people, except for occasional urinary tract infections; however, *Enterococcus faecium* as well as *E. faecalis*, are prevalent producers of health-care associated opportunistic infections (Woodford and Livermore, 2009). The genomic plasticity of enterococci has contributed with their adaptation to the hospital environment. Their relevance as nosocomial-infections' agents is bolstered by their natural resistance to multiple antimicrobials and an outstanding ability for acquiring and transferring genetic resistance determinants (Werner et al., 2013).

Enterococci express natural (intrinsic) resistance to antibiotics, e.g., clindamycin and trimethoprim-sulfamethoxazole. In addition, enterococci show a naturally low-level resistance to gentamicin. Minimum inhibitory concentration (MIC) values to gentamicin range from 6 to 48 μg/mL (Chow, 2000).

Antimicrobials consumption constitutes an important risk factor for colonization with multi-drug resistant enterococci because of the suppression of the competitive indigenous microbiota in the gastrointestinal tract. The increased number of gut enterococci, due to the decrease of competitive gut indigenous flora, frequently precedes bloodstream infections (Ubeda et al., 2010; Reyes et al., 2017).

Antimicrobials can be used in animal husbandry with therapeutic, prophylactic/metaphylactic and growth promotion purposes. Despite the use of antibiotics as growth promoters has been forbidden in many countries, worldwide, foods supplemented with antimicrobials are freely acquired in several countries with no veterinarian control, including in Argentina. This leads to bacterial exposure to sub-therapeutic concentrations of antibiotics and, hence, it may promote the expression of antibiotic resistance (Andersson and Hughes, 2014). Antimicrobials employed for human therapies and also used in animal production (in decreasing order) are tetracyclines, penicillins, macrolides, sulfonamides, aminoglycosides, lincosamides, and cephalosporins (Love et al., 2011; Kuehn, 2014). Specifically, ceftiofur, sulfamides and tetracyclines are used for prevention and treatment of pneumonia in pigs; gentamicin and neomycin are employed for the therapy of bacterial diarrhea (Dewey et al., 1999; EFSA, 2011).

The addition of antibiotics for growth promotion in animal feed became a common practice without rigorous testing. The mechanism of action in growth promotion induced by antibiotics appears to be related to the reduction of pathogenic bacteria in the intestines. The concentration of antimicrobials used for growth promotion has often been lower than that used for therapy and prophylaxis. These sub-therapeutic doses of antibiotics often create an auspicious condition for selecting antibiotic resistant bacteria (Van Immerseel et al., 2004; Dibner and Richards, 2005). Previously, McDonald et al. (2001) reported antimicrobial resistant enterococci in food produced with animals fed with antibiotics in sub-therapeutic doses.

Extensive use of antimicrobials in animal husbandry has exerted a considerable pressure for the genesis of antimicrobial-resistant bacteria in the environment, such as vancomycin-resistant enterococci (López et al., 2009; Ruzauskas et al., 2009; Marshall and Levy, 2011; Nieto-Arribas et al., 2011; Ribeiro et al., 2011; Sánchez Valenzuela et al., 2013).

Furthermore, enterococci, due to their characteristics of gastrointestinal colonization, environmental persistence, natural and acquired resistance to different antimicrobials and their availability to transfer genes horizontally, can be used as biomarkers of antimicrobial resistance in intensive husbandry.

TRANSFERABLE GENETIC DETERMINANTS OF ANTIMICROBIAL RESISTANCE

Intensive breeding of animals, especially poultry, pigs and cattle, facilitates the selection, spread and resistance determinants transfer of resistant bacteria. Increased antimicrobials resistance in colonizing bacteria from animals and food of this origin was documented (Normanno et al., 2007).

The extended and permanent use of antimicrobials for therapy purposes and growth promotion purposes in husbandry contributed with drug-resistant bacteria selection in humans. When antimicrobials are used in low doses and in prolonged cycles, a selective pressure is exerted that favors the propagation of drug-resistant bacteria (Fey et al., 2000; Graveland et al., 2010).

As a result, antimicrobial-resistant enterococci, as well as other resistant gut bacteria, can be spread in the environment by fecal residues. These bacteria can rapidly transfer their resistance to other strains through genetic determinants carried by mobile elements. Resistant enterococci are able to persist in the animal intestine, contaminate the environment and food of animal origin, and transfer determinants to human gut's isolates (Tasho and Cho, 2017). Moreover, community people can be exposed to antimicrobial resistant enterococci through direct contact.

Use of antimicrobials can enhance gene transfer between bacteria (Malhotra-Kumar et al., 2007). Gene conjugative transfer is frequent in the human gut, as well as in nature. Enterococci acquire antibiotic resistance genes, e.g., for high-level gentamicin

resistance and glycopeptides resistance determinants (Willems et al., 2011; Sparo et al., 2012).

Further, enterococci can horizontally transfer resistance genes to relevant bacteria in clinical settings, such as *Escherichia coli*, *Staphylococcus aureus*, and *Listeria* spp. (Verraes et al., 2013).

Generally, severe infections caused by enterococci are treated with a cell-wall active agent-aminoglycoside (mostly gentamicin) combination. The emergence of β-lactam and glycopeptide resistance and high-level resistance to gentamicin in enterococci has led to the employment of alternative antimicrobials (Arias et al., 2010; Bartash and Nori, 2017).

Figure 1 shows a presumable bidirectional transfer of resistance determinants and/or resistant enterococci between different niches such as human and animal. This transfer can occur through direct contact, foodborne contamination, as well as in health-care settings and the environment (community).

High-Level Vancomycin Resistant Enterococci

In enterococci, vancomycin resistance is associated with different *van* genotypes each corresponding with a typical Van phenotype. These genes are chromosomal or extrachromosomal encoded in transposons and/or plasmids. In human *E. faecalis* and *E. faecium*, VanA and VanB (inducible resistance) are the most relevant types. *vanA* gene cluster is most often found on conjugative or non-conjugative plasmids (Cetinkaya et al., 2000; Top et al., 2008). VanA is encoded by Tn*1546*, or closely related transposons. *vanA* gene is linked with high-level resistance to vancomycin and teicoplanin, while variable-level resistance to vancomycin is associated with a VanB phenotype. The *vanB* operon is found among large conjugative plasmids or in the chromosome (Cetinkaya et al., 2000). The most frequent *vanB* subtype, *vanB2*, is encoded by conjugative transposons Tn*1549*-/Tn*5382*-like. It is interesting to note that Tn*1549*-*vanB* has also been detected in anaerobes that inhabit the human gut (Dahl et al., 2000; Launay et al., 2006).

VanA is the most prevalent glycopeptide resistance phenotype in *Enterococcus* linked with human infections, mainly expressed by *E. faecium* (Freitas et al., 2016). Lester et al. (2006) have proven, in volunteers, the existence of genetic transfer in the human intestine between ingested chicken *vanA*-*E. faecium* and non-resistant to vancomycin human *E. faecium*. It is important to highlight that this research has been performed in a human gut model with its complexity and its diverse microbiota.

Furthermore, there is a global concern regarding plasmid-mediated *vanA* transfer from *E. faecalis* to methicillin-resistant *S. aureus* and their co-colonization, with the likelihood of VanA-*S. aureus* isolation (Flannagan et al., 2003; Weigel et al., 2003).

In the last decades, *vanA*-*E. faecium* were recovered from animals and food of this origin. Initially, the European Union stated that there was a link between Veterinarian use of a glycopeptide (avoparcin) and the emergence of vancomycin resistance (Werner et al., 2008). After avoparcin's ban, glycopeptide-resistance did not disappear. López et al. (2009) reported high-level vancomycin resistant enterococci (4%) from samples of animal origin 10 years after avoparcin was forbidden. Continuous presence of vancomycin-resistant enterococci in farms and in food of animal origin suggests that is possible the co-transfer of resistance genes located in the same conjugative plasmid, such as *vanA* and *ermB*, which encodes for macrolides resistance, widely used in Veterinary medicine. Also, the presence of ABC-type transporter genes and the toxin-antitoxin system may favor the persistence of vancomycin resistance determinants (Aarestrup, 2000). In addition, deficient hygiene conditions in animal husbandry, should not be underestimated (Garcia-Migura et al., 2007). In the same period, a different situation was observed in the United States, since food of animal origin glycopeptide-resistant *E. faecium* were not detected but, nevertheless, they emerged in health-care settings, turning into a pathogen almost as prevalent as *E. faecalis* had been so far (Coque et al., 1996; Ramsey and Zilberberg, 2009). However, in Michigan, United States, vanA-*E.faecium* was detected in farm animals where avoparcin was not used; which supports the existence of alternative ways for spreading of *van* genes, their transfer or carrying isolates from humans to animals (Johnsen et al., 2011; Gordoncillo et al., 2013).

In Argentina, *vanA*-*E. faecium* from artisanal food of animal origin was reported by Delpech et al. (2012). Previously, it was observed that animal-origin vancomycin-resistant *E. faecium* of animal origin were ingested in meats, proving the risk of resistant bacteria colonization when meat products carrying resistant bacteria were consumed (Heuer et al., 2006).

In Argentina, since the late 1990′s vancomycin-resistant *E. faecium* infections have been reported. In several Argentinean hospitals, the prevalence of clonal complex (CC) 17 carrying the *vanA* gene was detected. Most of these enterococci also expressed high-level aminoglycoside resistance (Corso et al., 2007).

Recently, during a year-period (2013), genetic relatedness (PFGE studies) between *vanA* enterococci from humans, food and the hospital environment in the District of Tandil (Argentina) was investigated. *vanA*-*E. faecium* (*n*: 13) were recovered from human, food and hospital environment samples. *van*A enterococci were distributed among seven clonal types; *esp* gene was detected in clinical strains. However, the clonal relationship between *vanA*-*E. faecium* of clinical and food origin was not found. The clonal relationship was observed among isolates from the hospital environment and from patients (Pourcel et al., 2017).

Bacterial conjugation provides an efficient gene transfer pathway and can be considered as the most relevant mechanism for the increase of antimicrobial resistance (Hammerum, 2012). It is possible that bacteria from food can constitute reservoirs of antimicrobial resistance.

The horizontal gene transfer of *vanA*-resistance between food strains and human gut microbiota becomes a possible mechanism

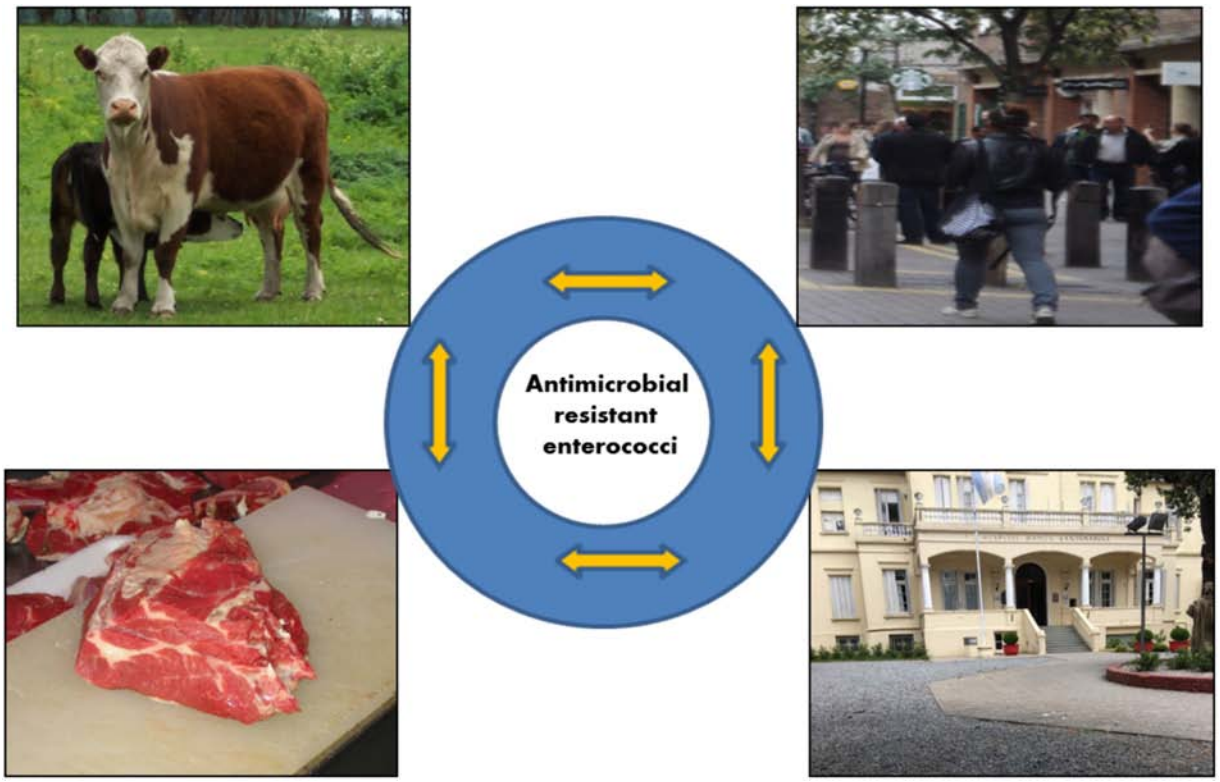

FIGURE 1 | Bidirectional transfer of resistance determinants and/or resistant enterococci between different niches.

of resistance dissemination when enterococci do not fit in the hospital settings (Hammerum et al., 2010).

High-Level Gentamicin Resistant Enterococci

The most prevalent mechanism of high-level aminoglycoside resistance in clinical bacteria is their enzymatic modification. Three families of aminoglycoside modifying enzymes have been recognized: phosphotransferases (APH), acetyltransferases (AAC), and nucleotidyltransferases (ANT). Genes for aminoglycoside modifying enzymes are often plasmidic, with bacteria-bacteria aminoglycoside resistance dissemination (Bassenden et al., 2016).

The following risk factors for the acquisition of infections with high-level gentamicin resistant enterococci have been identified: previous long-term antimicrobial treatment, number of prescribed antimicrobials, previous surgeries, peri-operative antimicrobial prophylaxis, hospitalization term/antimicrobial treatment, urinary catheterization and renal failure. Infections caused by *E. faecalis* with HLGR constitute a severe risk for patients with invasive conditions and long-term hospitalization (Miranda et al., 2001; Wendelbo et al., 2003; Ceci et al., 2015).

The most ubiquitous HLGR gene among human and food enterococci is *aac (6′)-Ie-aph (2″)-Ia* that encodes AAC(6′)-APH(2″)-Ia, with acetyltransferase and phosphotransferase activities. Enterococci with this enzyme express resistance to most of the available aminoglycosides (MIC > 2,000 μg/mL), except for streptomycin (Leclercq et al., 1992). Generally, *aac(6′)-Ie-aph(2″)-Ia* gene is flanked by inverted repeats of IS256, composing transposon Tn*5281* in *E. faecalis* as part of a conjugative plasmid (Rosvoll et al., 2012).

Other monofunctional genes encoding aminoglycoside-modifying enzymes have been described, such as class APH (2″)-subclass I phosphotransferases, chromosomal [e.g., *aph(2″)-Ib* y *aph(2″)-Id*] and plasmidic [e.g., *aph(2″)-Ic*] genes. These resistance determinants were originally found on *Enterococcus* species different than *E. faecalis* and encode enzymes which confer resistance to gentamicin and amikacin. *aph(2″)-Ic* gene is associated with MIC for gentamicin ranging between 128 to 512 μg/mL. Nevertheless, *aph(2″)-Id* gene, initially described in human *E. casseliflavus*, is linked to HLGR. This gene has been detected in clinical vancomycin-resistant *E. faecalis* (Ramirez and Tolmasky, 2010; Economou et al., 2017).

From 2000 to 2002, in Denmark, the proportion of high-level gentamicin resistant *E. faecalis* isolates increased from 2 to 6% in the pig population. Simultaneously, an emergence of HLGR *E. faecalis* isolates among patients with infective endocarditis was detected in the North Denmark Region (DANMAP, 2002). Afterward, Larsen et al. (2010) demonstrated that all of these isolates (human and pig origin) belonged to the same clonal group, suggesting that pigs were a reservoir for high-level gentamicin resistant *E. faecalis* associated with enterococcal infections.

Sparo et al. (2012) proved the spread of enterococci with HLGR from animals to humans through the food chain, and also that enterococci isolated from food of animal origin and humans carried the same aminoglycosides resistant genes, as reported, also, by other authors (Hammerum et al., 2007).

Resistance to ampicillin and vancomycin is infrequent, although *E. faecalis* have been shown to acquire HLGR (Kuch et al., 2012). Recently, over a 1 year period, the presence of cytolysin and HLGR in *E. faecalis* from human (hospital), animal (chicken feces from a farm) and food (minced meat from shops) origin were studied. Clinical samples were obtained from patients with invasive infections in Hospital Ramón Santamarina from Tandil City, Buenos Aires Province (Argentina). In all enterococci with HLGR, *aac (6′) -Ie-aph (2″)-Ia* gene was amplified. *aac (6′)-Ie-aph (2″)-Ia* and *cylA* were detected in human, food and animal *E. faecalis*, proving its environmental spread (Sparo et al., 2013).

In patients presenting risk factors, a high-level intestinal colonization of *E. faecalis* can become a frequent precursor of human invasive infections by bacterial translocation. This event is favored by the enhanced employment of broad-spectrum antimicrobials that exert significant pressure over the intestinal microbiota, hence, resulting in a likely emergency of multi-resistant enterococci. The human gut is a considerable reservoir for microorganisms potentially capable of transfer resistance to conventional antimicrobials. Moreover, the fact that bacteria isolated from food of animal origin can behave as a resistance reservoir needs to be taken into consideration. *In vitro* studies performed to prove genetic exchange between enterococcal strains from humans and food of animal origin, are not conclusive (Sparo et al., 2012). Therefore, *in vivo* models for assessing genetic transfer are needed. Research carried out in animal models with their own microbiota it will not be able to reproduce the conditions of the human intestine. The use of human colon microbiota in germ-free mice is proposed as a model for reproducing the interaction between food strains and human gastrointestinal microbiota (Hirayama, 1999). Recently, HLGR determinants transfer from food to human bacteria was proven in an animal model. Immunocompetent BALB-C mice, colonized with human feces from an infant with no previous antimicrobial treatment, were used. This study showed evidence of the likelihood of high-level gentamicin resistance horizontal transfer from food to human *E. faecalis*. Therefore, a gene transfer model in non-sterile mice colonized with human gastrointestinal microbiota was standardized (Sparo et al., 2012).

It is needed to highlight that the rate of HLGR in vancomycin-resistant enterococci is higher than in vancomycin-susceptible enterococci strains. Mihajlović Ukropina et al. (2011) studied the frequency of antimicrobial resistance in enterococci isolated from blood cultures. HLGR was detected in vancomycin-resistant strains (87.6%) as well as in vancomycin-susceptible strains (9.9%). Hence, according to this study, HLGR in *E. faecium* is higher than in *E. faecalis*.

In an Argentinean study, *E. faecalis* strains with HLRG (*aac (6′)-Ie-aph (2″)-Ia* gene) and without glycopeptide resistance were recovered from human and food samples of animal origin. PFGE patterns showed four clonal types, and also that there was a clonal relationship between *E. faecalis* with HLGR isolated from food and those isolated from humans (Pourcel et al., 2017).

Clonal Complexes of High-Level Vancomycin and Gentamicin Resistant Enterococci

Worldwide, MLST *E. faecium* data established that the majority of the clinical strains belong to the CC17, most of which are resistant to ciprofloxacin and ampicillin, and contain virulence genes. When new algorithms such as the Bayesian analysis of population structure (BAPS) were applied, it showed that CC17 consists of two large groups with different evolutionary origin: BAPS 2-1, containing sequence-type (ST) 78 and BAPS3-3 (ST17 and ST18). Most of the drug-resistant clinical isolates of hospital origin belong to both groups. The majority of community-origin isolates were grouped in the BAPS 2-1 group, genetically and evolutionarily different from hospital isolates and those of hospital origin are evolutionarily closer to those of farm animals. A similar trend was detected among vancomycin-resistant *E. faecium*, investigated in broiler flocks 15 years after the avoparcin ban, diversity was observed as well since they clustered in three BAPS populations (Willems et al., 2012; Bortolaia et al., 2015; Raven et al., 2016).

Several authors have highlighted the predominance of clonal lineages −17, −18 and −78 in human clinical isolates of *E. faecium*. It could be assumed that they have adapted to the intestinal environment and integrate their microbiota (Baquero and Coque, 2011; Faith et al., 2015; Tedim et al., 2015, 2017).

Nowadays, comparison of available genome sequences allowed to support the existence of two clades for *E. faecium*; one of the animal strains and hospital-associated enterococci (clade A) and another one of community strains (clade B), which includes human commensal isolates. Clade A has been subdivided into A1, including most of the clinical isolates (lineages ST17, ST18, and ST78) and A2, containing mainly strains of animal origin. It has also been shown that the genome of the strains included in the clade A1 has a larger size than those ones of strains belonging to A2, which seems to support the recent emergence of this clade and the importance of its recombination (Galloway-Peña et al., 2012; Tedim et al., 2015).

Unlike *E. faecium*, *E. faecalis* lack a clear structure in clades. Some clones are more frequent in hospitalized patients or in the community. Specifically, CC2 and CC9 both present high-level vancomycin resistance and have been described as highly risky due to their adaptation to the hospital environment and global dissemination (Freitas et al., 2009; Kuch et al., 2012; Guzman Prieto et al., 2016).

E. faecalis CC2, a high-risk CC, is frequently found among health-care associated isolates and represents hospital complexes linked with high-level aminoglycoside resistance (Weng et al., 2013). In addition, *E. faecalis* CC87, similar to CC2, expresses multi-drug resistance and can be associated with

invasive infections (Ruiz-Garbajosa et al., 2006; Tedim et al., 2015).

IMPACT IN HUMAN INFECTIONS AND THERAPEUTIC OPTIONS FOR RESISTANT ENTEROCOCCI

Among bloodstream infection (BSI) associated with the healthcare environment, *Enterococci* is the third most common one. Although vancomycin-resistant enterococci have been clinically relevant pathogens for years, the majority of clinical data is retrospective (Wisplinghoff et al., 2004). Nowadays, vancomycin-resistant enterococci are the cause of one-third of all health care associated infections in the United States and one fifth in some European countries (Hidron et al., 2008; European Centre for Disease Prevention and Control [ECDC], 2010). Furthermore, mortality rates in patients with BSIs produced by vancomycin-resistant enterococci range between 20 and 46% (Han et al., 2009; McKinnell et al., 2011; Twilla et al., 2012).

Treatment of vancomycin-resistant enterococci's BSI is particularly challenging. The therapeutic options include linezolid, daptomycin, quinupristin-dalfopristin, tigecycline, and lipoglycopeptides, such as telavancin, dalbavancin and oritavancin.

Due to limited clinical available data of lipoglycopeptides together with resistance issues in VanA enterococci, the role in systemic vancomycin-resistant enterococci infections for telavancin and dalbavancin is irrelevant. Oritavancin (the lipoglycopeptide with the broadest antibacterial coverage) has shown bactericidal activity against VanA and VanB vancomycin-resistant enterococci. This drug was approved for the treatment of acute bacterial skin infections and is currently undergoing clinical trials for the treatment of bacteremia (Zhanel et al., 2010; Messina et al., 2015).

In Europe, Teicoplanin can be used for VanB phenotype infections (Svetitsky et al., 2009).

Tigecycline has not been approved for the treatment of bacteremia because it does not achieve high serum concentrations. This tetracycline can be considered as one of the first-line treatments for polymicrobial intra-abdominal infections associated with vancomycin-resistant enterococci due to its high penetration into the peritoneal space (Arias et al., 2010).

Quinupristin-dalfopristin, effective only against *E. faecium*, has a high molecular weight, which renders it unable to cross the blood-brain barrier. This, added to the facts that it has frequent side effects and that it easily interacts with other drugs, limits its clinical use (Rubinstein et al., 1999).

Since approval, linezolid has been widely employed for vancomycin-resistant enterococci infections. The clinical success rate can vary based on the infection site and generally range between 50 and 80%. Lower success rates are generally seen in patients with bacteremia and infections without known focus (Birmingham et al., 2003; Kraft et al., 2012; Da Silva et al., 2014; Patel et al., 2016).

Linezolid has shown utility for treating infections by vancomycin-resistant enterococci non-susceptible to daptomycin. Surveillance analysis carried out in 2012 showed 99.5% susceptibility for linezolid against enterococci in the United States health systems (Mendes et al., 2014). Prolonged use of linezolid has been associated with resistance emergency (Pogue et al., 2007; McGregor et al., 2012).

Tedizolid is a next-generation parenteral and oral oxazolidinone with a broad spectrum bacteriostatic activity against resistant Gram-positive bacteria including VanA and VanB enterococci. It has been approved for the treatment of acute bacterial skin and soft tissues infections, and, currently, clinical trials for bacteremia and pneumonia treatment are being undergone (Rybak et al., 2014).

Daptomycin has been successful for multidrug-resistant enterococci and vancomycin-resistant enterococci infections' treatment. Multiple analyses of the Cubicin Outcomes and Registry Experience (CORE) have shown a higher clinical success rate when used as first-line therapy for vancomycin-resistant enterococci bacteremia, 87–93% (Sakoulas et al., 2007; Mohr et al., 2009).

β-lactam antibiotics have been evaluated, *in vitro*, combined with daptomycin against vancomycin-resistant enterococci, including ampicillin, ceftaroline, ceftobiprole, and ceftriaxone, all of which produced synergistic effects even when β–lactam resistance was detected (Sakoulas et al., 2012, 2014; Hall Snyder et al., 2014; Werth et al., 2015).

For infectious endocarditis due to ampicillin susceptible and HLGR *E. faecalis*, ampicillin with ceftriaxone should be considered as an alternative treatment option, since it showed a similar efficacy to the observed ones for ampicillin with gentamicin, in susceptible strains, but with less nephrotoxicity. The saturation of several penicillin-binding proteins is the main reason why this combination presents a desirable bactericidal synergy (Mainardi et al., 1995; Murray, 2000; Fernández-Hidalgo et al., 2013; Economou et al., 2017).

Alternatives/Complementary Therapeutic Options

Available evidence about infection control and prevention measures (ICP) to reduce vancomycin-resistant enterococci spread in adult hospitalized patients is insufficient. A systematic review published in 2014 (that included 9 studies with 30,949 participants) emphasized the importance of the implementation of hand hygiene program. A decrease of 47% in the vancomycin-resistant enterococci acquisition rate was observed when this measure is applied. Further studies with appropriate methodological design are urgently needed to define if ICP measures have an impact in reducing the acquisition of vancomycin-resistant enterococci among hospitalized patients (De Angelis et al., 2014).

A proposal for controlling antimicrobial resistance dissemination is to reduce antimicrobials employment in animal husbandry and promoting research of novel therapeutic alternatives. Probiotics are "living microorganisms which when administered in adequate amount confer a health benefit on

the host" (Food and Agriculture Organization/World Health Organzation [FAO/WHO], 2001). These strains improve intestinal microbial balance, provide protection against gut pathogens and modulate the immune system. Probiotics are supplemented into animal feed (cattle, ducks, broilers, and chickens) and have beneficial effects on the food producing animals by enhancing weight gain, increasing egg/milk production, lowering the incidence of disease and mortality rates (Crittenden et al., 2005). Use of probiotics against pathogenic bacteria showed to be effective for reducing food-borne illnesses in consumers, in view of the absence of antibiotics in sub-therapeutic doses (Van Coillie et al., 2007).

A different approach is the use of microbial cell extracts that reduce the risks of bacterial translocation and infection (Sparo et al., 2014; Lemme-Dumit et al., 2018).

Bacteriocins are ribosomally synthesized peptides, with bacteriostatic/bactericidal activity, produced by various bacteria (Gálvez et al., 2007). The use of Gram-positive bacteriocins alone or in combination with antibiotics was proposed as a novel strategy to develop in human and veterinary medicines in order to help conventional antimicrobials against many multi-drug resistant pathogens. These combinations allow decreasing the MIC for achieving a bactericidal effect and, also, reduce undesirable side-effects of antibiotics (Lebel et al., 2013; Naghmouchi et al., 2013; Delpech et al., 2017). Randomized controlled trials are needed for obtaining scientific evidence about the usefulness of these novel compounds against pathogenic enterococci.

CONCLUSION

Worldwide, enterococcal infections are among the most prevalent within those of nosocomial origin. Antimicrobial multi-resistant enterococci and their drug-resistant determinants spread by direct animal-human contact and/or through animal origin food. As mentioned above, the evidence is based on traditional microbiology and molecular tools, such as PFGE and MLST. Therefore, future studies combining phylogeographic methods with whole genomic sequence will provide reliable information for inferring bacteria movement between host populations.

Nowadays more countries are developing antibiotic-limiting policies, and thus arises a need of searching for an alternative or substitute for these drugs for sustainable food production, such as probiotics and bacteriocins.

AUTHOR CONTRIBUTIONS

MS and GD contributed to the writing of the microbiological and resistance aspects of the article, revised it and designed the Figure. NG contributed to the clinical and infectological aspects of the work.

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Advancement of the 5-Amino-1-(Carbamoylmethyl)-1H-1,2,3-Triazole-4-Carboxamide Scaffold to Disarm the Bacterial SOS Response

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Many antibiotics, either directly or indirectly, cause DNA damage thereby activating the bacterial DNA damage (SOS) response. SOS activation results in expression of genes involved in DNA repair and mutagenesis, and the regulation of the SOS response relies on two key proteins, LexA and RecA. Genetic studies have indicated that inactivating the regulatory proteins of this response sensitizes bacteria to antibiotics and slows the appearance of resistance. However, advancement of small molecule inhibitors of the SOS response has lagged, despite their clear promise in addressing the threat of antibiotic resistance. Previously, we had addressed this deficit by performing a high throughput screen of ∼1.8 million compounds that monitored for inhibition of RecA-mediated auto-proteolysis of *Escherichia coli* LexA, the reaction that initiates the SOS response. In this report, the refinement of the 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamide scaffold identified in the screen is detailed. After development of a modular synthesis, a survey of key activity determinants led to the identification of an analog with improved potency and increased breadth, targeting auto-proteolysis of LexA from both *E. coli* and *Pseudomonas aeruginosa*. Comparison of the structure of this compound to those of others in the series suggests structural features that may be required for activity and cross-species breadth. In addition, the feasibility of small molecule modulation of the SOS response was demonstrated *in vivo* by the suppression of the appearance of resistance. These structure activity relationships thus represent an important step toward producing Drugs that Inhibit SOS Activation to Repress Mechanisms Enabling Resistance (DISARMERs).

Keywords: SOS response, antibiotic resistance, structure activity analysis, *Pseudomonas aeruginosa*, DNA damage

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INTRODUCTION

Antibiotic resistant bacteria represent one of the most pressing issues in infectious disease research today (Brown and Wright, 2016). An era is fast approaching when many currently treatable infections may become incurable (Boucher et al., 2009). While important efforts are underway to discover antimicrobials with different mechanisms of action (Clatworthy et al., 2007;

Thaker et al., 2013; Ling et al., 2015), the most conventional approach to overcoming resistance has involved the chemical modification of existing antibiotic scaffolds (Fischbach and Walsh, 2009). Although the resulting "next generation" antibiotics offer a respite, bacteria are likely to rapidly adapt their preexisting resistance mechanisms to counteract these gains. The limitations of conventional approaches highlight the need to pursue alternative strategies.

A promising alternative approach is to target pathways that promote acquired resistance to antibiotics. One such pathway is the bacterial DNA damage response pathway, known as the SOS response (**Figure 1**). Many antibiotics induce the SOS response, either by inducing DNA damage (e.g., fluoroquinolones) or by indirectly promoting DNA damage via targeting essential cellular and metabolic functions (Kohanski et al., 2007; Dwyer et al., 2012; Mo et al., 2016). The SOS response is well conserved across pathogens and involves numerous genes (e.g., >40 in *Escherichia coli*). These proteins include translesion DNA polymerases that promote mutagenesis, recombinases that mobilize antibiotic resistance genes, and proteins that mediate persistence, biofilm formation or directly promote antibiotic evasion (McKenzie et al., 2000; Beaber et al., 2004; Schlacher et al., 2006; Galhardo et al., 2007; Da Re et al., 2009; Dörr et al., 2009, 2010; Gotoh et al., 2010). Thus, suppression of the SOS pathway would be predicted to compromise the response of bacteria to antibiotics.

A means to suppress the SOS pathway is to maintain repression of the SOS response. In the absence of genotoxic stress all genes of the pathway are tightly repressed by the dual-function repressor/protease, LexA (**Figure 1**). In the presence of genotoxic stress the DNA damage sensor protein RecA forms filaments along ssDNA generated by aborted replication. The pathway is triggered when this filamentous RecA (RecA*) promotes a conformational change in LexA that brings one of its protein loops into its own serine protease active site (Luo et al., 2001). Subsequent auto-proteolysis destabilizes LexA, and leads to transcriptional de-repression of SOS pathway genes (Culyba et al., 2018).

Genetic studies targeting either RecA or LexA validate the SOS response as a therapeutic target (**Figure 1**). In a murine thigh infection model an *E. coli* strain harboring a non-cleavable mutant of LexA abrogated resistance both to ciprofloxacin and rifampicin compared to a strain with a cleavable LexA (Cirz et al., 2005). In addition, deletion of RecA, or forced over expression of non-cleavable LexA have been shown to hyper-sensitize bacteria to traditional antibiotics (Lu and Collins, 2009; Thi et al., 2011; Mo et al., 2016). Furthermore, SOS inactivation in resistant bacteria resulted in re-sensitization to a fluoroquinolone (Recacha et al., 2017). Together, these studies suggest that targeting the SOS response could lead to both synergy with DNA damaging antibiotics to lower MIC values and suppression of acquired resistance (Cirz and Romesberg, 2007; Smith and Romesberg, 2007; Culyba et al., 2015).

While specifically targeting RecA has produced some important gains (Wigle et al., 2009; Alam et al., 2016; Bellio et al., 2017), we aimed to inhibit the RecA*-induced cleavage of LexA as this represents the key initiating step in the SOS response. To this end we developed a high throughput screen (HTS) that allowed estimation of RecA*-mediated LexA cleavage. Using this screen some 1.8 million compounds were evaluated for inhibition of RecA*-mediated LexA cleavage (Mo et al., 2018). The result of this screen was the identification of several chemotypes with the potential to modulate the SOS response (Mo et al., 2018). Herein is described the advancement of one of the chemotypes, the 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamide scaffold (**Figure 2**) via a modular synthesis that allowed for evaluation of structure-activity relationships and lead improvement to increase potency and expand the breadth of targetable pathogens. This work underscores the feasibility of developing DISARMERs (Drugs to Inhibit SOS Activation to Repress Mechanisms Enabling Resistance) – molecules that can act as adjuvants in standard antimicrobial therapies to both sensitize bacteria to antibiotics and reduce the rise of acquired resistance.

MATERIALS AND METHODS

Materials

All reagents used in chemical synthesis were purchased from Aldrich Chemical Co., (Milwaukee, WI, United States), Alfa Aesar (Ward Hill, MA, United States), or Thermo Fisher Scientific (Pittsburgh, PA, United States) and were used without further purification. Chemicals used in biochemical assays were obtained from Sigma-Aldrich (St Louis, MO, United States).

Compound Synthesis

Compounds were synthesized using a method that proceeds via a [3+2] cycloaddition, allowing facile, catalytic, non-moisture sensitive, and non-air sensitive syntheses of a variety of 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamides. For the majority of analogs, catalysts employed were either sodium ethoxide (synthesis A, **Table 1**) or cesium carbonate (synthesis B, **Table 1**). The base-mediated cyclization is depicted in **Figure 2**.

For reactions catalyzed by sodium ethoxide (synthesis A), a solution of sodium ethoxide (1.2 mmol) in anhydrous ethanol (10 mL) was maintained under nitrogen and cooled to 0°C with stirring. Once cooled, the cyano component (1.1 mmol) was added to the solution. The resulting solution was stirred for 10 min at 0°C before addition of the azido component (1.0 mmol). The resulting solution was maintained at 0°C for a further 2 min before being allowed to warm to room temperature. Upon reaching room temperature, the solution was lightly sonicated for 20 s before the temperature was raised to 40°C. The solution was maintained at 40°C for 4 h before being allowed to cool to room temperature. Once the solution reached room temperature the reaction was quenched with deionized H_2O (100 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic fractions were washed with deionized H_2O (50 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to yield the crude product. The crude product was dissolved in DMF, filtered through a syringe filter and purified via reverse phase HPLC using acetonitrile in deionized H_2O (with 0.1% TFA in both solvents)

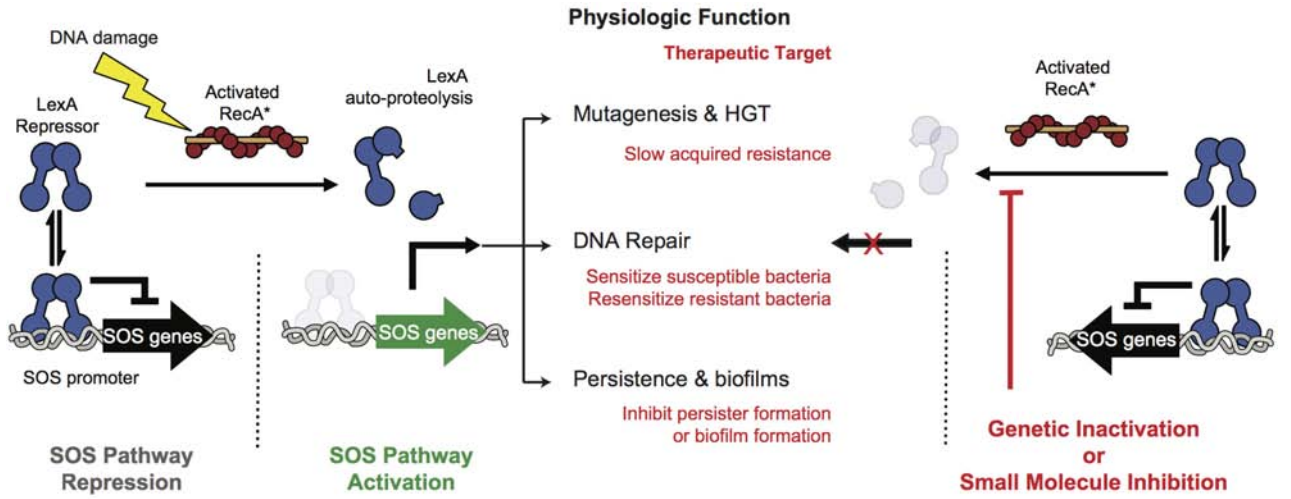


FIGURE 1 | SOS pathway activation and inhibition. At left, under non-stressed conditions the intact LexA dimer binds to the SOS promoter region and tightly represses SOS protein production. Upon DNA damage RecA binds to DNA fragments to form RecA* filaments that stimulate free LexA to undergo auto-proteolysis. Auto-proteolysis of LexA renders it unable to bind to the SOS promoters leading to expression of the effectors of the SOS pathway. These effectors promote DNA repair, accelerate gene transfer and mutagenesis, and contribute to virulence. At right, genetic inhibition of the SOS response has been shown to antagonize these antibiotic-evasion associated phenotypes and small molecule inhibition of the pathway is therefore a viable pathway for improving the efficacy of antibiotics or targeting virulence.

to yield, after evaporation and lyophilization, the desired product.

For reactions catalyzed by cesium carbonate (synthesis B), the azido component (1.1 mmol), the cyano component (1.0 mmol) and cesium carbonate (0.25 mmol) were dissolved in DMSO/deionized H_2O (7:3, 4 mL) with stirring. The reaction vial was capped and stirred for 24 h before being diluted with deionized H_2O, partially concentrated in vacuo, frozen, and lyophilized to remove water and DMSO. The resulting crude material was purified via reverse phase HPLC using acetonitrile in deionized H_2O (with 0.1% TFA in both solvents) to yield, after evaporation and lyophilization, the desired product.

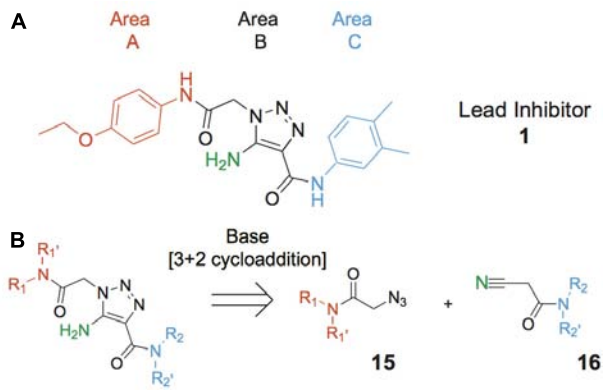


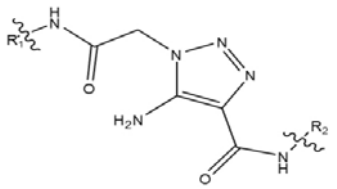
FIGURE 2 | Lead compound and synthetic approach. **(A)** The lead **1** is shown with the Areas A, B and C highlighted. These areas are the focus of diversification in analog synthesis to explore structure-function relationships in the lead series. **(B)** Retrosynthesis of the 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamides is shown, with the core of Area B formed via a cycloaddition of azide **15** and nitrile **16**. In Area B the 5-amino group derived from the nitrile is highlighted to help illustrate the cycloaddition mechanism.

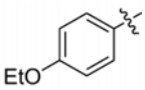
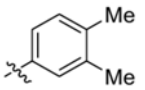
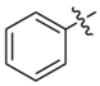
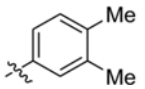
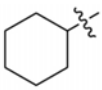
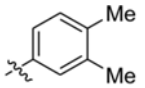
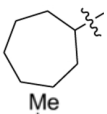
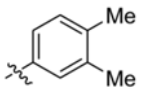
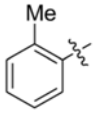
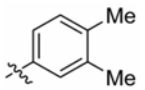
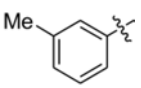
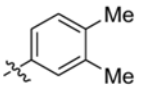
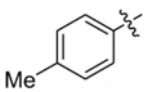
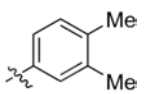
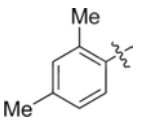
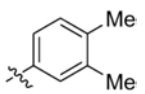
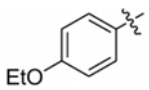
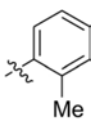
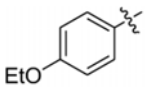
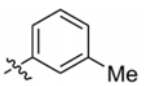
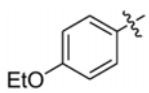
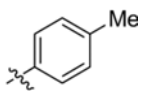
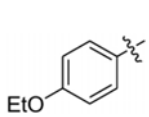
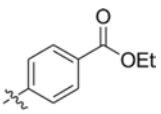
For compound **7** a variation of synthesis A (synthesis C) was employed in which sodium methoxide was used instead of sodium ethoxide. For this synthesis a mixture of the cyano component (0.24 mmol), and sodium methoxide (0.26 mmol) in methanol (1.07 mL) was stirred for 30 min before addition of the azido component (0.21 mmol). The reaction was stirred for 16 h before treatment with methanol (0.25 mL) and stirring for 3 h. Methanol (1 mL) was added, and the reaction was heated at 95°C for 2 h. The mixture was treated with deionized water (25 mL), concentrated HCl (1 drop), and ethyl acetate (10 mL). The aqueous layer was treated with saturated aqueous $NaHCO_3$ (5 mL) and extracted with ethyl acetate (10 mL). The combined organic extracts were washed with saturated aqueous $NaHCO_3$ (10 mL), brine (10 mL), and dried over anhydrous $MgSO_4$ before concentration. The crude material was purified via reverse phase HPLC using acetonitrile in deionized H_2O (with 0.1% TFA in both solvents) to yield, after evaporation and lyophilization, the desired product.

Some compounds in the Supplementary Information were synthesized by alternative methods in which an acetylene component replaced the cyano component. The alternate syntheses are described in the **Supplementary Methods**. Analogs which were not synthesized were obtained from commercial vendors ChemDiv (San Diego, CA, United States) and Vitas-M Laboratory (Champagne, IL).

All compounds were readily soluble in DMSO and were stored as 10 mM frozen (−30°C) stocks when not in use.

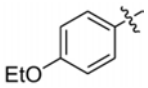
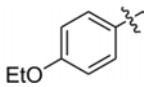
TABLE 1 | Synthesis and inhibition by lead analogs.



Compound	R_1	R_2	*E. coli* $IC_{50}/\mu M$[1]	Synthesis[2]	Yield%
1			32 ± 6	**A/B**	25/25
2			>100	**n/a**	commercial
3			44 ± 4	**A**	27
4			22 ± 3	**B**	34
5			17 ± 2	**n/a**	commercial
6			>100	**n/a**	commercial
7			18 ± 1	**C**	21
8			15 ± 2	**B**	31
9			>100	**n/a**	commercial
10			33 ± 4	**A**	32
11			15 ± 2	**n/a**	commercial
12			19 ± 3	**B**	12

(Continued)

TABLE 1 | Continued

Compound	R_1	R_2	*E. coli* $IC_{50}/\mu M$[1]	Synthesis[2]	Yield%
13			40 ± 3	**B**	33
14			9 ± 1	**B**	39

[1]*IC50 values are the avearge of 4 determinations and the errors are ± 1 SD.* [2]*A-NaOEt conditions. B-CS2CO3 conditions, C-NaOMe conditions.*

FlAsH-LexA Cleavage Assay

IC_{50} values were routinely determined using the *E. coli* FlAsH-LexA cleavage assay previously used to perform HTS (Mo et al., 2018). In this assay RecA-promoted LexA cleavage is monitored using fluorescence polarization. The *E. coli* FlAsH-LexA and RecA were constructed, expressed and purified as previously described (Mo et al., 2018). The conditions were 100 nM *E. coli* FlAsH-LexA, 200 nM RecA, 5 μM ssDNA (SKBT25: GCG TGT GTG GTG GTG TGC) (Tracy and Kowalczykowski, 1996), 5 μM ATPγS in 100 mM Tris-HCl, pH 6.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM TCEP, and 0.01% (w/v) Pluronic-F127. Reactions were performed in 384-well plates and components were added as 10 μL additions of ATPγS, ssDNA and RecA, in buffer and 10 μL of *E. coli* FlAsH-LexA in buffer using a Janus liquid handler (Perkin-Elmer). Compound was added as a DMSO solution using a pin tool, and the final concentration of DMSO in the reaction was 1.2%. Once the reaction components were combined, reactions were centrifuged for 1 min at 500 rpm and incubated for 30 min at room temperature. Reactions were quenched with a 10 μL addition of 50 mM EDTA and plates were read on a Tecan Infinite F200 Pro plate reader (Tecan US, Inc., Morrisville, NC, United States). The final assay conditions resulted in 100–120 mP difference between the uncleaved and cleaved control wells, representing an approximately 60% cleavage of the *E. coli* FlAsH-LexA. On each plate 32 positive (-RecA) and 32 negative controls (+RecA) in which DMSO without compound was added were used to define the range of mP and calculate the fraction inhibited.

IC_{50} values were estimated by non-linear least squares fitting to the data using Equation 1.

$$FI = \frac{[I]^n}{IC_{50}^n + I^n} \quad (1)$$

where FI = Fraction inhibited, [I] = Concentration of compound and n = Hill coefficient. Fitting was performed using Igor Pro (WaveMetrics Inc., Lake Oswego, OR, United States).

In the FlAsH-LexA cleavage assay the highest compound concentration was 111 μM and all of the compounds that demonstrated activity (**1**, **3**, **4**, **5**, **7**, **8**, **10–14**, **22,** and **23**) elicited normal titration curves suggesting that aqueous solubility was maintained up to 111 μM. Representative titrations for the compounds can be found in the **Supplementary Figure 1**.

Orthogonal ^{32}P-LexA Cleavage Assay

Full-length *E. coli* and *P. aeruginosa* LexA were engineered with a RRXS phosphorylation site on the N-terminus of the full-length protein, allowing for ^{32}P labeling by protein kinase A to produce ^{32}P-LexA, as described previously (Mo et al., 2018). Reactions contained 100 nM ^{32}P-LexA, 200 nM RecA and 10 μM ATPγS and the buffer conditions were identical to those in the HTS assay. Compounds were added in DMSO and the final concentration was 2%. Reactions were incubated for 30 min at room temperature after which 2 × Laemmli buffer was added to stop the assay. The stopped reactions were subjected to 15% SDS-PAGE and the gels were visualized via phosphorimaging on a Typhoon imager (GE Healthcare Bio-Sciences, Marlborough, MA, United States). The intact and cleaved bands were quantified using Quantity One (Bio-Rad, Hercules, CA, United States) and the fraction inhibited was calculated. As for the HTS assay, controls contained DMSO and the negative controls contained RecA while the positive controls did not. Plots of fraction inhibited against compound concentration were fitted to Equation 1.

Electrophoretic Mobility Shift Assay

For the electrophoretic mobility shift assay (EMSA) full-length, catalytically inactive LexA-S119A was used (Mo et al., 2014). Increasing concentrations (0–1 μM) of LexA-S119A were mixed with 10 nM SOS operator DNA labeled with Cy5 in EMSA running buffer (70 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 5 mM DTT, 0.1 mg/ml BSA, 10 ng/μL ssDNA, 5% glycerol, 0.04% bromophenol blue) in the presence of 50 μM of compound (or DMSO carrier). After incubation at room temperature for 30 min, 20 μL of each reaction was subjected to 6% native PAGE. Gels were visualized on a Typhoon Imager using default fluorescence filter settings for Cy5. Gel bands were quantified using ImageJ (NIH, Bethesda, MD, United States) to determine the fraction of bound DNA at each LexA concentration. Data were fitted to a variable-slope sigmoidal dose-response curve.

Cell-Based SOS Reporter Assay

An *E. coli* MG1655 strain lacking *sulA* (Δ*sulA*) and the *tolC* transporter (Δ*tolC*) (Mo et al., 2016) was transformed with a reporter plasmid in which *gfp* expression was under the control of the *recA* promoter (pMS201 p*RecA* GFP) (Zaslaver et al., 2006). To perform assays overnight, cultures of the reporter strain were diluted 100-fold in M9 minimal media and grown at 37°C

with agitation to an OD_{595} of ∼0.6. For each reaction sample 100 μL of culture were mixed with 100 μL of M9 minimal media containing ciprofloxacin (256 ng/mL). Pre-diluted compounds were added (5 μL) in DMSO and cultures were incubated at 37°C with agitation for 2 h after which the cells were fixed by adding 200 μL of phosphate buffered saline, pH 7.4 containing 1% paraformaldehyde. After 1 h of fixing, the cells were spun down at 4,000 rpm and re-suspended in phosphate buffered saline, pH 7.4. Fixed cells were analyzed using flow cytometry (BD FACSCalibur, Ex/Em: 488 nm/530 nm) and the mean fluorescence of 20,000 cells in each condition was recorded.

Frequency of Resistance

A starter culture of Δ*tolC E. coli* was cultured overnight at 37°C with shaking in LB broth. The next day the culture was diluted 3×10^7-fold in to LB broth. The dilution was used to produce four sets of twelve cultures, each containing 1 mL. To one set was added 10 μL of deionized H_2O plus 10 μL of DMSO, to the second set was added 10 μL of 125 ng/mL ciprofloxacin in deionized H_2O plus 10 μL of DMSO, to the third set was added 10 μL of deionized H_2O plus 10 μL of a 10 mM solution of **14** in DMSO and to the fourth set was added 10 μL of 125 ng/mL ciprofloxacin in deionized H_2O plus 10 μL of **14** in DMSO. The final concentration of ciprofloxacin (1.25 ng/mL) was below the MIC for ciprofloxacin which was determined to be 5 ng/mL.

The 48 cultures were incubated at 37°C with shaking for 48 h. To determine the population size, spot plating was performed starting with 1 μL of the cultures diluted 10^5-fold. A 100 μL aliquot of each 10^5-fold dilution was transferred to a 96-well plate and serially diluted (10-fold dilutions) into LB broth. The dilutions (5 μL) were spotted on LB agar plates and the plates were incubated overnight at 37°C. To determine the rifampin resistant population, 999 μL of each 1 mL culture was centrifuged at 6000 rpm for 10 min to remove the cells from solution and the cells were suspended in 100 μL of autoclaved 0.15 M NaCl. The 100 μL solutions were plated on the LB plates containing 100 μg/mL rifampin and incubated for 2 days at 37°C. Following counting of the colonies the program bz-rates (Gillet-Markowska et al., 2015) was used to estimate mutation rates.

RESULTS

Among the leads isolated from the HTS performed for inhibitors of RecA*-mediated LexA cleavage, lead **1** was selected for progression (**Figure 2A**). In the initial HTS, the parent 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamide, **1**, had an IC_{50} value of 32 μM (**Table 1**). This chemotype was well behaved in the HTS, producing close to 100% inhibition, and appeared to offer the most chemical tractability to allow for the construction of structure activity relationships (SARs). Furthermore, as LexA cleavage involves formation of a β-turn at the site of self-cleavage (Lee et al., 2005; Whitby et al., 2011), the structural similarity of **1** to β-turn mimetics also suggested that structure-activity relationships could inform on the possible mode of inhibitor action.

In order to better understand SARs, a modular synthesis was devised that would permit generation of informative analogs. While the construction of 5-amino-1,4-disubstituted-1,2,3-triazoles has been extensively investigated (Tome, 2004) no synthetic routes to 5-amino-1-(carbamoylmethyl)-1*H*-1,2,3-triazole-4-carboxamides based on **1** have yet been reported.

Initial synthetic routes that proceeded via the generation of two potential carboxylic acid intermediates followed by peptide couplings to vary the left- and right-hand portions of the final product were unsuccessful. These reactions were low yielding and/or the precursors were prone to decomposition. A more successful strategy proved to be to proceed via the simple structural intermediates, azides (**15**) for the left-hand portion and nitriles (**16**) for the right-hand portion (**Figure 2B**). These intermediates were either synthesized in 1–2 steps (Hering et al., 2005; Ju et al., 2006; Srinivasan et al., 2006; Ng et al., 2008; Xia et al., 2014) or purchased directly and could be combined via known base-mediated conditions to produce the desired aminotriazoles via a [3+2] cycloaddition.

Three sets of reagents that have been previously reported to facilitate such cyclizations were screened: stoichiometric sodium methoxide (Alfred, 1970; L'abbé and and Beenaerts, 1989; Julino and Stevens, 1998), stoichiometric sodium ethoxide (Hoover and Day, 1956; Livi et al., 1979), and catalytic cesium carbonate (Krishna et al., 2015). In most cases the choice of base between stoichiometric sodium ethoxide and catalytic cesium carbonate had little to no impact on the yield (e.g., **1**, **Table 1**). Overall most reactions were successful using the cesium carbonate conditions, however, yields using this route were affected by the time and temperature of the reaction. Using either the sodium ethoxide or catalytic cesium carbonate routes readily permitted modular access to a large variety of analogs, as demonstrated by the fact that aromatic, heteroaromatic, and non-aromatic groups for R_1 and R_2 were tolerated (**Table 1**). This modular approach thus allowed for systematic variation and investigation of structure activity relationships.

Structure Activity Relationships

Initial medicinal chemistry efforts focused on developing an understanding of the necessary features to improve potency. The three areas (A, B, and C) in **Figure 2A** were systematically investigated and the IC_{50} values for selected compounds are listed in **Table 1** with additional data shown in **Supplementary Table 1**. Approaches used to probe the binding of compounds of this class included amino group replacement, linker methylation and N-methylation, methyl probing of the aryl rings, homologated variations, and non-aromatic variations. IC_{50} values were determined using the FlAsH-LexA cleavage assay.

In the linker connecting areas A and B, both mono- and bis-methylated compounds (**17** and **18**) showed no measurable activity, suggesting that substitution was not tolerated at the methylene linker. Similarly, methylation of the amide of the linker, **19**, also abrogated activity. The inability to tolerate substitution in the linker region suggested that it likely lies in a narrow groove and that attempts to modify this area could impact the conformations accessed by the lead. With the linker area appearing not amenable to modification, the

aromatic portion of area A was investigated. Replacement of the para-ethoxy group substituted phenyl ring with an unsubstituted phenyl, **2**, benzyl, **20**, or phenethyl group, **21**, led to loss of activity. However, replacement of the phenyl ring with a cyclohexyl ring, **3**, or a cycloheptyl ring, **4**, returned activity, suggesting that aromaticity was a larger restriction than hydrophobicity. Systematic variation of methyl functionalization on the phenyl ring revealed that substitution at the meta position, **6**, was not tolerated whereas substitution on the ortho, **5**, and para, **7**, positions were preferred. Beyond the single methyl functionalization, mono-substitution at the ortho position on the phenyl showed steric preferences with activities: Me (**5**) > Et (**22**) > OMe (**23**) > OEt (**24**) = H (**2**). Bis-substitution on the aryl ring was additionally investigated with for example, **8**, showing that combination of ortho- and para-substitution was tolerated but not significantly superior to ortho-substitution alone, **5**. In summary, probing of area A revealed interesting substrate preferences but failed to produce a significant increase in potency.

Additional investigations in area B also did not reveal a means to increase potency. Replacement of the amine by hydrogen (**25**), methyl (**26**), or ethyl (**27**) all rendered compounds inactive, suggesting that the amine was making contacts essential for activity. Supporting this conclusion was the finding that mono-substitution on the amine by acetyl (**28**) was tolerated but with reduced potency.

Probing of area C proved more fruitful. As with area A, the amide linker appeared important as methylation was not tolerated (**29**). Probing of the phenyl moiety indicated that its presence and correct positioning are critical. The importance of this ring was indicated by the intolerance to replacement by cyclohexyl (**30**) or methyl (**31**) groups, and the need for correct positioning was indicated by the intolerance to the replacement of the phenyl ring by benzyl (**32**) or phenethyl (**33**). The importance of substitution on the phenyl ring was investigated by systematic methyl substitution around the phenyl ring. Consistent with the meta- and para- substitution pattern on area C of **1**, this analysis indicated that ortho substitution, **9**, was not tolerated whereas individual meta, **10**, and para, **11**, substitutions were allowed. Heteroatom inclusion was also tolerated in Area C, as shown by ester-containing variation **12**, and pyridyl derivative **13**. However, compound **14**, 5-amino-1-{2-[(4-ethoxyphenyl)amino]-2-oxoethyl}-N-phenyl-1H-1,2,3-triazole-4-carboxamide, with no substitution on the phenyl ring proved to be the most potent compound tested in this series with an IC_{50} of 9 μM.

Before proceeding to additional analysis, cytotoxicity testing with HG2 cells was performed for select compounds, including **1** and **14**. Both the initial lead **1** and the most potent analog **14** showed no appreciable toxicity (CC_{50} of 277 μM and > 500 μM, respectively). Due to the increased potency and lack of cytotoxicity, the mechanism and activity of compound **14** was examined in more detail as described below.

Characteristics of Compound 14

A suite of assays was utilized to examine **14** in order to confirm specific inhibition against LexA and demonstrate SOS suppression in cells. To confirm the findings from the

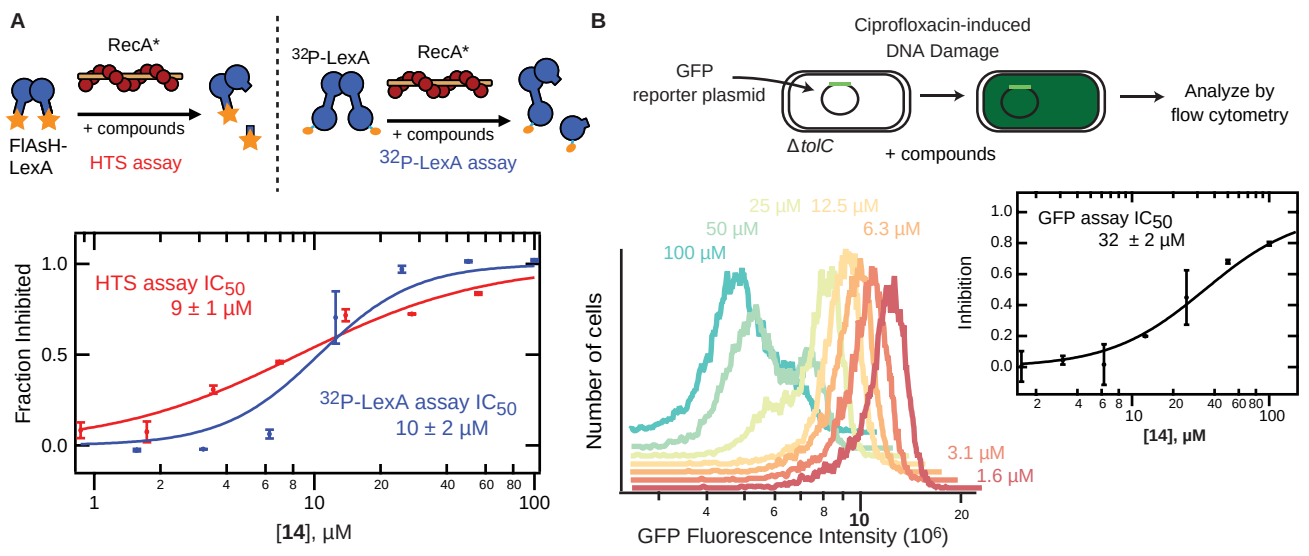


FIGURE 3 | Validation of *in vitro* and *in vivo* activity of **14**. **(A)** The complete dose response curve for **14** performed using two orthogonal *in vitro* assays are shown. The HTS assay employs RecA*-induced changes in fluorescence polarization that can be tracked upon proteolysis of a fluorescent, truncated version of LexA. The ^{32}P-LexA assay uses N-terminal ^{32}P-labeled LexA and tracks formation of the N-terminal fragment upon RecA*-induced cleavage. The calculated IC_{50} values are shown with standard deviation from at least three replicates. **(B)** The SOS reporter assay employs a plasmid with GFP downstream of a *recA* promoter. Expression of GFP can be tracked after initiating DNA damage with ciprofloxacin in a Δ*tolC* MG1655 *E. coli* strain. At left, flow cytometry plots from a representative experiment are shown as a density plot showing the level of GFP expression in the presence of serial dilutions of **14**. At right, the mean GFP fluorescence was used to calculate the level of inhibition relative to a negative control in the absence of **14** and a positive control in the absence of DNA damage.

FlAsH-LexA cleavage assay, a fluorescence-independent assay using a full-length version of *E. coli* LexA was employed. The full-length LexA contained a PKA phosphorylation site at the N-terminus that allowed ^{32}P labeling, such that the extent of auto-proteolysis can be visualized by phosphor-imaging following SDS-PAGE. A plot of the titration curve of **14** obtained using this methodology is shown along with a titration obtained using the FlAsH-LexA assay in **Figure 3A**. The IC_{50} of 10 ± 1 μM indicates that the IC_{50} obtained using the fluorescently labeled truncated *E. coli* LexA in the HTS assay (9 ± 1 μM) was not due to a fluorescence artifact, and that similar potency is observed with full-length and truncated LexA. Interestingly, a similar IC_{50} value was obtained when the slow cleavage in the absence of RecA* was monitored (**Supplementary Figure 2A**). This suggests that **14** binds specifically to LexA, which is further supported by the observation of a thermal shift assay of LexA in the presence of **14** (**Supplementary Figure 2B**).

The dual activities of LexA, DNA binding and protease activity, permit confirmation of specificity. If **14** inhibits RecA*-mediated LexA cleavage in the expected manner, it would be predicted to inhibit the protease function of LexA, but not to alter DNA binding. To examine LexA binding to DNA in the presence of **14** an EMSA was employed. As with **1** (Mo et al., 2018), LexA showed similar DNA binding affinity in the presence or absence of **14** (**Supplementary Figure 3**). This observation confirms that the effects in the HTS and ^{32}P-LexA assays are not due to non-specific aggregation of LexA or other artifacts. Another important consideration is the permeability of **14** in to the bacteria. Permeability was assessed using a Δ*tolC* strain of *E. coli* containing a plasmid that contained the GFP gene under the control of the *recA* promotor (Mo et al., 2018). Compound **14** inhibited the appearance of GFP fluorescence in a dose-dependent manner with an IC_{50} value of 32 ± 2 μM indicating permeability into the Δ*tolC* strain of *E. coli* (**Figure 3B**), without impacting cell size (**Supplementary Figure 4**). The less potent value compared to *in vitro* values suggests that even in the efflux-compromised *E. coli* strain there still remain barriers to entry.

Although the permeability remains in need of further improvement, we also examined whether **14** could suppress the downstream effects of the SOS response *in vivo*. With the knowledge that the IC_{50} for permeability in the Δ*tolC* strain of *E. coli* was 32 ± 2 μM, a concentration of 100 μM **14** was used to assess the ability of **14** to suppress the ciprofloxacin-induced appearance of resistance to rifampicin. As can be seen from **Figure 4**, the lead **14** was effective in reducing the appearance of resistance to rifampicin. In the presence of **14** alone, the mutation rates were comparable to DMSO alone controls. Conversely, exposure to a sub-MIC concentration (1.25 ng/mL) of ciprofloxacin produced an induction of mutagenesis. In the presence of ciprofloxacin and **14** together, an approximately threefold decrease in the per generation mutation rate was observed relative to ciprofloxacin alone.

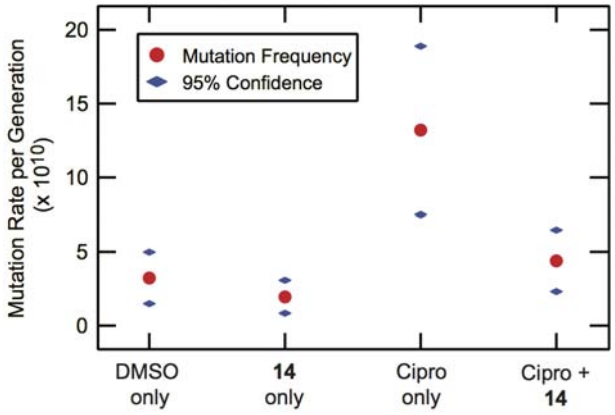


FIGURE 4 | Suppression of ciprofloxacin-induced mutagenesis by **14**. Δ*tolC* MG1655 *E. coli* cultures were grown in the presence or absence of **14** (100 μM) and/or a sub-MIC level of ciprofloxacin (1.25 ng/mL). The cultures were plated without selection to determine total population size and on selective rifampin-containing media (100 μg/mL) to quantify the frequency of rifampin-resistance in the population. The mutational frequency was converted to a per-generation mutation rate, with the rate and 95% confidence interval shown. The rate data were calculated based on at least twelve independent cultures under each condition.

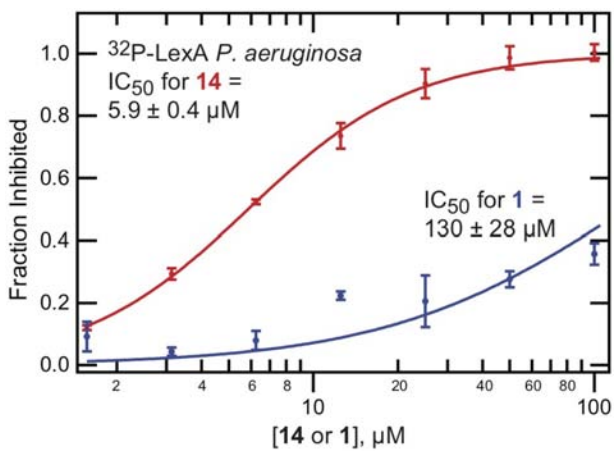


FIGURE 5 | Improved cross-species activity of **14**. The ^{32}P-LexA assay, examining RecA*-mediated cleavage of full length LexA from *P. aeruginosa*, was performed using serial dilutions of either **1** or **14**. The percent inhibition was calculated relative to DMSO controls. The mean value is shown with standard deviation, calculated from at least two replicates.

Cross-Species Reactivity

The HTS and medicinal chemistry efforts were directed at the inhibition of *E. coli* LexA auto-proteolysis. To determine the extent of cross-species reactivity, the effectiveness of **14** in inhibiting the RecA-promoted auto-proteolysis of *Pseudomonas aeruginosa* LexA was examined. As can be seen from **Table 1** and **Figure 5**, compound **14** inhibited the RecA-mediated auto-proteolysis of *P. aeruginosa* LexA with similar potency ($IC_{50} = 5.9 \pm 0.4$ μM) to that demonstrated with full-length *E. coli* LexA ($IC_{50} = 10 \pm 1$ μM). This behavior was not observed with **1** (**Figure 5**) which was a less potent inhibitor of RecA*-induced auto-proteolysis of full-length *P. aeruginosa* LexA ($IC_{50} = 130 \pm 28$ μM). Thus, minor modifications

to **14** compared to **1** had a significant effect on cross-species reactivity and permits potentially expanded species breadth.

DISCUSSION

While the HTS for inhibitors of RecA*-mediated LexA cleavage produced several chemotypes, the 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamide scaffold appeared the most amenable for advancement. The low cytotoxicity and the β-turn mimetic-like structure (see below) were important considerations in the choice to advance this chemotype. A particularly important consideration was the chemical tractability of the lead compound, which permitted the development of a highly modular synthesis that allowed for an initial survey of structure-activity relationships. Our synthetic approach is important because compounds containing the privileged 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamide scaffold have been used to target broad categories of biological activity. Targets have included C3d of the immune response (Morikis and Gorham, 2016), *Mycobacterium tuberculosis* proteasome (Mehra et al., 2015, 2016), microRNA for the treatment of certain cancers (Calin et al., 2002), and a wide range of other diseases (Tili et al., 2007; Huang et al., 2010, 2012, 2013).

Inhibition of the SOS response can now be added to the list of uses for the 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamide scaffold. More specifically, the similar IC_{50} values for **14** in the fluorescence-based LexA cleavage assay and an orthogonal ^{32}P-LexA ± RecA assay suggests on-target activity. This effect appears specific for the self-cleavage activity of LexA, because EMSA testing indicated that **14** does not interfere with the DNA binding ability of LexA. The fact that one LexA function is inhibited while the other is preserved further suggests that **14** is not a Pan-Assay Interference (PAINS) inhibitor (Aldrich et al., 2017). While the data suggest **14** binds specifically, the exact binding site is not clear. We have previously speculated that a β-turn mimetic may prove a useful strategy for targeting the LexA active site given that a β-turn formation plays a role in self-cleavage (Mo et al., 2016). Indeed, speculation that this scaffold could function as a β-turn mimetic was one reason for advancing the 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazole-4-carboxamide. The fact that substitutions that likely perturb the conformational dynamics, such as N-methylation of the amide bonds, is consistent with this hypothesis. Nonetheless, the exact target of lead **1** or analog **14** awaits elucidation through structural or mutational studies and allosteric inhibition may well be the mechanism of action due to the inaccessibility of the active site to all but its natural substrate (Culyba et al., 2015).

One likely driving force for the frequent use of this scaffold in varied therapeutic applications is its low cytotoxicity, as evidenced by the CC_{50} values of 277 μM and > 500 μM. Other properties of **14** also indicate that it is a promising starting point, although ongoing optimization is needed. The properties of the molecule fall within Lipinski's rules for drug-likeness (Lipinski et al., 1997): it has a molecular weight of 380.4 (<500), three hydrogen bond donors (≤5), six hydrogen bond acceptors (≤10) and a cLogP of 1.63 (≤5). In comparison to oral drugs for non-infectious diseases, antibacterial compounds tend to have greater polarity (O'Shea and Moser, 2008; Brown et al., 2014) which provides better solubility (useful for IV drugs) and may enable improved permeability through the outer membrane of Gram-negative bacteria (Nikaido, 2003; Brown et al., 2014). Low lipophilicity is also preferred to avoid off-target activities and cytotoxicity (Livi et al., 1979). Compound **14** has a polar surface area of 124 Å^2 which is below the value of 140 Å^2 above which permeability is typically an issue. These properties define **14** as a drug-like small molecule modulator of the SOS response.

For small molecule SOS modulators to prove useful to address therapeutic challenges, there are two important features of the molecules which will be necessary. First, the molecules must have sufficient breadth to allow for their use against multiple potential pathogens. Although our initial lead **1** showed only limited reactivity against LexA from *P. aeruginosa* (**Figure 5**), our optimization around the scaffold encouragingly revealed **14** as an analog with similar potency against LexA from *E. coli* and *P. aeruginosa*. This development is important because pathogens such as *P. aeruginosa* are associated with chronic infections. Frequent antibiotic exposure in patients with cystic fibrosis or other immunocompromising conditions make the risks of acquired resistance particularly high in these patients. In addition to cross-species reactivity, small molecule modulators must also show sufficient potency *in vivo*. The improved analog **14** shows SOS inhibition activity using the GFP reporter assay in the efflux compromised Δ*tolC E. coli* strain. Encouragingly, at high concentrations, **14** also reduced the rate of ciprofloxacin-induced mutation (**Figure 4**). Although these activities against *E. coli* are promising, these results suggest that the potency of the current leads requires additional improvement, especially because genetic studies not only suggest that potent SOS inhibition is necessary to fully potentiate antibiotic effects but also reveal that mutation rates can be reduced even further (Mo et al., 2016).

The trigger for the activation of the SOS response is genotoxic stress which many antibiotics induce. Molecules that attenuate the activation of the SOS response could therefore reduce the ability of pathogens to adapt and evolve under antimicrobial treatment. Evidence suggests that such a therapeutic would be most effective when used as an adjuvant to an antibiotic whose mechanism of action involves directly damaging DNA, e.g., fluoroquinolones (Mo et al., 2016). The improvements in potency and cross-species activity with **14** suggest that although ongoing work is needed to improve existing leads, discovery of such a therapeutic DISARMER is a feasible pursuit. Combining fluoroquinolones with a potent DISARMER could provide advantages similar to those that β-lactamase inhibitors have provided for β-lactam antibiotic therapy. These possible advantages include extension of the useful lifetime of an antibiotic, increased susceptibility of bacteria to antibiotics, and slowed acquisition of resistance, all of which

offer alternative strategies to address the challenges posed by bacterial pathogens.

AUTHOR CONTRIBUTIONS

TS, RK, AR, and SB designed the experiments. TS, BL, CM, MC, and ZH performed the experiments. TS, RK, and SB analyzed the data. TS, RK, and SB wrote the manuscript. All authors reviewed and edited the manuscript.

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The Prevalence of Colistin Resistant Strains and Antibiotic Resistance Gene Profiles in Funan River, China

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Anthropogenic activities near urban rivers may have significantly increased the acquisition and dissemination of antibiotic resistance. In this study, we investigated the prevalence of colistin resistant strains in the Funan River in Chengdu, China. A total of 18 *mcr-1*-positive isolates (17 *Escherichia coli* and 1 *Enterobacter cloacae*) and 6 *mcr-3*-positive isolates (2 *Aeromonas veronii* and 4 *Aeromonas hydrophila)* were detected, while *mcr-2*, *mcr-4* and *mcr-5* genes were not detected in any isolates. To further explore the overall antibiotic resistance in the Funan River, water samples were assayed for the presence of 15 antibiotic resistance genes (ARGs) and class 1 integrons gene (*intI1*). Nine genes, *sul1*, *sul2*, *intI1*, *aac(6′)-Ib-cr*, bla_{CTX-M}, *tetM*, *ermB*, *qnrS,* and *aph(3′)-IIIa* were found at high frequencies (70–100%) of the water samples. It is worth noting that *mcr-1*, bla_{KPC}, bla_{NDM} and *vanA* genes were also found in water samples, the genes that have been rarely reported in natural river systems. The absolute abundance of selected antibiotic resistance genes *[sul1, aac(6′)-Ib-cr, ermB,* bla_{CTX-M}*, mcr-1,* and *tetM]* ranged from 0 to 6.0 (log_{10} GC/mL) in water samples, as determined by quantitative polymerase chain reaction (qPCR). The *sul1*, *aac(6′)-Ib-cr,* and *ermB* genes exhibited the highest absolute abundances, with 5.8, 5.8, and 6.0 log_{10} GC/mL, respectively. The absolute abundances of six antibiotic resistance genes were highest near a residential sewage outlet. The findings indicated that the discharge of resident sewage might contribute to the dissemination of antibiotic resistant genes in this urban river. The observed high levels of these genes reflect the serious degree of antibiotic resistant pollution in the Funan River, which might present a threat to public health.

Keywords: colistin, antibiotic resistance, *mcr-1*, *mcr-3*, urban river, quantitative polymerase chain reaction

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INTRODUCTION

Multi-drug resistant (MDR) Gram-negative pathogens are resistant to almost all antibiotics, including cephalosporins, quinolones, aminoglycosides and carbapenems, making treatment difficult. Colistin is considered the last line of defense against MDR Gram-negative pathogens, playing an important role in the treatment of severe bacterial infections (Zavascki et al., 2007). Unfortunately, the recent emergence of plasmid-mediated colistin resistance genes in carbapenem-resistant *Enterobacteriaceae* presents a serious new threat to human health. The plasmid-mediated colistin resistance gene *mcr-1* was first discovered Liu et al. (2016). Soon afterward, another mobile

phosphoethanolamine transferase gene, termed *mcr-2*, was discovered in porcine and bovine *Escherichia coli* isolates in Belgium (Xavier et al., 2016). Recently, Yin et al. (2017) discovered a novel *mcr* subtype, *mcr-3*, encoded on an IncI2 plasmid in an *E. coli* isolated from a pig in China. The *mcr-4* and *mcr-5* genes were detected in Europe almost simultaneously (Borowiak et al., 2017; Carattoli et al., 2017). Although there have been numerous reports of colistin resistance genes in animals and humans, fewer studies have focused on *mcr*-bearing isolates from aquatic environments.

Due to the continual release of antibiotic residues and antibiotic resistant bacteria (ARB) into the environment from hospitals, livestock facilities, and sewage treatment plants (STP), antibiotic resistant genes (ARGs) are regarded as environmental contaminants (Pruden et al., 2006; Zurfluh et al., 2017). The occurrence and dissemination of antibiotic resistance in pathogenic and zoonotic bacteria pose a potential threat to human health (Rosenberg Goldstein et al., 2012; Neyra et al., 2014). Moreover, an increasing number of bacteria are resistant to multiple antibiotics, and are able to transfer their resistant determinants among different bacterial species and genera in aquatic environments (Akinbowale et al., 2006). Urban rivers may provide an ideal setting for the acquisition and dissemination of antibiotic resistance because they are frequently impacted by anthropogenic activities. Although antibiotic resistance is a major and developing public health concern, the surveillance of this phenomenon in urban rivers is remarkably limited.

The Funan River, a major urban river in Chengdu used for agricultural activities (e.g., irrigation and cultivation) as well as recreational activities (e.g., swimming and fishing), was used as the model in this study to analyze the magnitude of antibiotic resistance in urban rivers.

The objectives of this study were: (1) to determine the prevalence of colistin resistance strains in the Funan River; (2) to investigate the MDR phenotypes and genotypes of isolated colistin resistant strains; (3) to screen for resistance determinants, including *sul1, sul2,* bla_{CTX-M}, bla_{VIM}, bla_{KPC}, bla_{NDM}, *qnrS*, *aac(6′)-Ib-cr*, *vanA*, *mecA*, *ermB*, *ermF*, *tetM, aph(3′)-IIIa,* and *mcr-1*, and the class 1 integron gene (*intI1*) in water samples from the Funan River.

MATERIALS AND METHODS

Sampling of River Water

To investigate the prevalence of colistin resistant strains, 30 water samples (2 L) were collected from the Funan River near densely populated areas in September 2017. To further explore the antibiotic resistance of bacteria throughout the Funan River, 10 water samples (2 L) were collected from representative locations along the river (**Figure 1**). The representative locations included river intersections, streams near parks, and sewage outlets near residential areas, the hospital, and the municipal wastewater treatment plant (WWTP). The site near the residential sewage outlet is designated RWW and the sample near the municipal wastewater treatment plant is designated WWTP. Sites P1, P2, and P3 are close to various parks and HWW1 and HWW2 are close to the hospital sewage outlet. Site RI is located adjacent to the intersection of a tributary and the mainstream of the river. Sites UWP and DWP are upstream and downstream of Wetland Park, respectively. Water samples were collected from each site, immediately placed on ice, and transported to the laboratory within 4 h. The samples were then maintained at 4°C until investigation.

Bacterial Isolation

A total of 30 water samples were concentrated by vacuum filtration through 0.22 μm filter membranes. The membranes were washed and the collected material was suspended in 10 ml of sterile PBS. A volume of 1 ml thereof was added to 9 ml of Brain Heart Infusion (BHI) broth with polymyxin B at a final concentration of 4 μg/mL. After incubation at 37°C overnight, 100 μl culture samples were streaked onto MacConkey agar plates. Fifty colonies were picked from each MacConkey agar plates and subsequently grown in BHI broth with 4 μg/mL polymyxin B for 18–24 h. Isolates were screened for the presence of *mcr-1, mcr-2, mcr-3, mcr-4,* and *mcr-5* by PCR. Next, *mcr*-positive isolates were purified by subculturing. The *mcr*-positive isolates were identified using 16S rRNA gene sequencing and the BD Phoenix-100 Automated Microbiology System (BD Diagnostic Systems, Sparks, NV, United States).

Antimicrobial Resistance Testing and Detection of *mcr*-Positive Strains Genotype

The minimum inhibitory concentration (MIC) of colistin was determined by broth microdilution. The antimicrobial susceptibility was interpreted according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 6.0 (EUCAST, 2017). Fourteen antimicrobial agents were tested: ampicillin (AMP, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), cefotaxime (CTX, 30 μg), ceftriaxone (CRO, 30 μg), ceftazidime (CAZ, 30 μg), cefoxitin (FOX, 30 μg), imipenem (IPM, 10 μg), ertapenem (ETP, 10 μg), aztreonam (ATM, 30 μg), ciprofloxacin (CIP, 5 μg), fosfomycin (FOS, 50 μg), tetracycline (TE, 30 μg), amikacin (AK, 30 μg) and trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg). Antimicrobial susceptibility was determined by the agar disk diffusion method. Isolates were classified as susceptible, intermediate, or resistant using the breakpoints specified by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2016). *Escherichia coli* ATCC 25922 was used as the quality control strain.

After DNA extraction using the TIANamp bacteria DNA kit (TIANGEN, China), the isolates were screened for the presence of 21 antibiotic resistance genes (bla_{KPC}, bla_{OXA-48}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{SHV}, bla_{TEM}, $bla_{CTX-M-1}$, $bla_{CTX-M-9}$, *fosA3*, *qnrB*, *qnrS*, *floR*, *oqxAB*, *sul1*, *sul2*, *tetM*, *tetA*, *aac(6′)-Ib-cr*, *rmtA,* and *rmtB*) (Berendonk et al., 2015; Zheng et al., 2015; Liu et al., 2016), and the

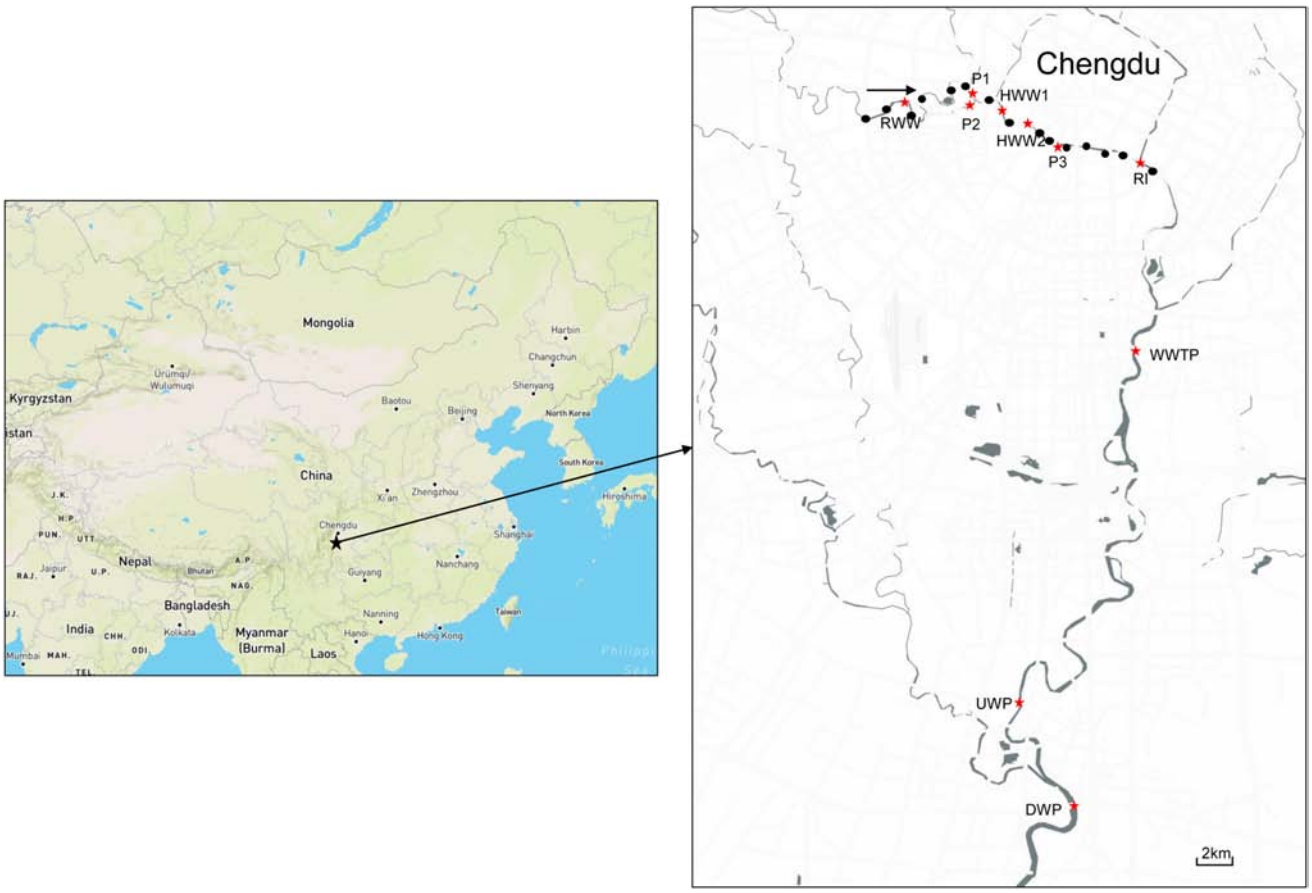

FIGURE 1 | Study area with sampling sites to explore the antibiotic resistance of bacteria throughout the Funan River. Black dots indicate partial sampling sites for the detection of colistin resistant bacteria and red stars indicate sampling sites for the ARG determination in river water. (RWW, Residential Wastewater; WWTP, Municipal Wastewater Treatment Plant; P, Park; HWW, Hospital Wastewater; RI, River Intersection; UWP, Upstream of Wetland Park; DWP, Downstream of Wetland Park).

primers and PCR conditions used are listed in **Table 1**. Negative and positive controls for PCR of each gene were utilized.

Total DNA Extraction and Detection of ARGs

To further explore the extent of antibiotic resistance throughout the Funan River, water samples were collected from 10 locations (**Figure 1**). Total DNA was extracted using the Water DNA kit (OMEGA, United States) from the bacteria sample trapped by 0.22 μm pore filter (2 L samples). Standard PCR performed as listed in **Table 1** was used to detect 15 ARGs (*sul1, sul2,* bla_{CTX-M}, bla_{VIM}, bla_{KPC}, bla_{NDM}, *qnrS*, *aac(6′)-Ib-cr*, *vanA*, *mecA*, *ermB*, *ermF*, *tetM*, *aph(3′)-IIIa* and *mcr-1*) and the class 1 integron gene (*intI1*). Negative and positive controls were used for each set of PCR primers. PCR amplification reactions were conducted in 20 μl volumes containing 1× PCR Master Mix (Tsingke, China), 1.0 μl template DNA, and 0.5 μM of each primer. After amplification, 5 μl samples of the PCR products were loaded on a 1.0% agarose gel containing GoldView, and separated electrophoretically in 1 × TAE buffer at 120 V for 20 min and visualized.

Quantitative Polymerase Chain Reaction

To compare the abundance of ARGs for different sampling sites, the gene copy numbers of the *sul1*, *aac(6′)-Ib-cr*, *ermB*, bla_{CTX-M}, and *tetM* genes were quantified using qPCR assays. These genes confer resistance to five major classes of antibiotics: sulphonamides, aminoglycosides, macrolides, β-lactams, and tetracyclines. The levels of *mcr-1* and 16S rRNA genes were also quantified. To quantitate the amounts of these genes, the levels were compared to the levels in standard samples prepared from plasmids containing these specific genes, as described previously (Chen and Zhang, 2013). The standard samples were diluted to yield a series of 10-fold concentrations and were subsequently used to generate qPCR standard curves. The R^2 values were higher than 0.990 for all standard curves. The 20 μl qPCR mixtures contained 10 μL of SYBR premix Ex Taq^{TM} (TaKaRa, Dalian, China), 0.5 μM of each forward and reverse primer, and 1 μl of template DNA. The final volume was adjusted to

TABLE 1 | Standard primer pairs used in this study.

Target genes	Sequence (5′→3′)	Amplicon size(bp)	Reference
mcr-1	CGGTCAGTCCGTTTGTTC	350	Liu et al., 2016
	CTTGGTCGGTCTGTA GGG		
mcr-2	TGGTACAGCCCCTTTATT	1617	Xavier et al., 2016
	GCTTGAGATTGGGTTATGA		
mcr-3	TTGGCACTGTATTTTGCATTT	542	Yin et al., 2017
	TTAACGAAATTGGCTGGAACA		
mcr-4	ATTGGGATAGTCGCCTTTTT	487	Carattoli et al., 2017
	TTACAGCCAGAATCATTATCA		
mcr-5	ATGCGGTTGTCTGCATTTATC	1644	Borowiak et al., 2017
	TCATTGTGGTTGTCCTTTTCTG		
bla_{KPC}	ATGTCACTGTATCGCCGTC	902	Zheng et al., 2015
	TTACTGCCCGTTGACGCC		
bla_{OXA-48}	TTGGTGGCATCGATTATCGG	744	Zheng et al., 2015
	GAGCACTTCTTTTGTGATGGC		
bla_{NDM}	ATGGAATTGCCCAATATTATGCAC	813	Zheng et al., 2015
	TCAGCGCAGCTTGTCGGC		
bla_{VIM}	TTTGGTCGCATATCGCAACG	500	Zheng et al., 2015
	CCATTCAGCCAGATCGGCAT		
bla_{IMP}	GTTTATGTTCATACWTCG	432	Zheng et al., 2015
	GGTTTAAYAAAACAACCAC		
bla_{SHV}	ATTTGTCGCTTCTTTACTCGC	861	Zheng et al., 2015
	TTTATGGCGTTACCTTTGACC		
bla_{TEM}	ATGAGTATTCAACATTTCCGTG	861	Zheng et al., 2015
	TTACCAATGCTTAATCAGTGAG		
bla_{CTX-M}	TTTGCGATGTGCAGTACCAGTAA	759	Zheng et al., 2015
	CGATATCGTTGGTGGTGCCATA		
$bla_{CTX-M-1}$	AAAAATCACTGCGCCAGTTC	415	Zheng et al., 2015
	AGCTTATTCATCGCCACGTT		
$bla_{CTX-M-9}$	CAAAGAGAGTGCAACGGATG	205	Zheng et al., 2015
	ATTGGAAAGCGTTCATCACC		
fosA3	GCGTCAAGCCTGGCATTT	282	Hou et al., 2012
	GCCGTCAGGGTCGAGAAA		
qnrB	GATCGTGAAAGCCAGAAAGG	469	Wang et al., 2017
	ACGATGCCTGGTAGTTGTCC		
qnrS	ACGACATTCGTCAACTGCAA	540	Wang et al., 2017
	TAAATTGGCACCCTGTAGGC		
oqxAB	CCCTGGACCGCACATAAAG	1140	Wang et al., 2017
	AAAGAACAAGATTCACCGCAAC		
sul1	ATGGTGACGGTGTTCGGCATTCTG	840	Hur et al., 2011
	CTAGGCATGATCTAACCCTCGGTC		
sul2	GAATAAATCGCTCATCATTTTCGG	810	Hur et al., 2011
	CGAATTCTTGCGGTTTCTTTCAGC		
tetM	AGTGGAGCGATTACAGAA	158	Adefisoye and Okoh, 2016
	CATATGTCCTGGCGTGTCTA		
tetA	GCTACATCCTGCTTGCCTTC	210	Adefisoye and Okoh, 2016
	CATAGATCGCCGTGAAGAGG		
aac(6′)-Ib-cr	TTGCGATGCTCTATGAGTGGCTA	482	Eftekhar and Seyedpour, 2015
	CTCGAATGCCTGGCGTGTTT		
rmtA	CTAGCGTCCATCCTTTCCTC	635	Wang et al., 2017
	TTGCTTCCATGCCCTTGCC		
rmtB	GCTTTCTGCGGGCGATGTAA	173	Wang et al., 2017
	ATGCAATGCCGCGCTCGTAT		

(Continued)

TABLE 1 | Continued

Target genes	Sequence (5′→3′)	Amplicon size(bp)	Reference
floR	GTCGAGAAATCCCATGAGTTCA CAGACAGGATACCGACATTCAC	1645	Cloeckaert et al., 2000
intI1	GGGTCAAGGATCTGGATTTCG ACATGCGTGTAAATCATCGTCG	484	Mazel et al., 2000
vanA	AATACTGTTTGGGGGTTGCTC TTTTTCCGGCTCGACTTCCT	734	Kafil and Asgharzadeh, 2014
mecA	TGGTATGTGGAAGTTAGATTGGGAT CTAATCTCATATGTGTTCCTGTATTGGC	155	Paterson et al., 2012
ermB	GATACCGTTTACGAAATTGG GAATCGAGACTTGAGTGTGC	364	Zhang et al., 2016
ermF	CGACACAGCTTTGGTTGAAC GGACCTACCTCATAGACAAG	309	Zhang et al., 2016
aph(3′)-IIIa	GCC GAT GTG GAT TGC GAA AA GCT TGA TCC CCA GTA AGT CA	269	Udo and Dashti, 2000

20 μl by addition of DNase-free water. The IQ^{TM}5 real-time PCR system was employed for amplification and quantification, using the following protocol: 30 s at 95°C, 40 cycles of 5 s at 95°C, 30 s at the annealing temperature, and extension for another 30 s at 72°C. For detection, simultaneous fluorescence signal was scanned at 72°C, followed by a melt curve stage with temperature ramping from 65 to 95°C. Details of the qPCR primers of the target genes and the annealing temperatures are given in **Table 2**. The method design was adopted from prior research (Thornton and Basu, 2011). The copy numbers of the selected ARGs were normalized against the 16S rRNA gene copy number. Therefore, the copy number unit is described as copies/16S.

Statistical Analysis

Statistical analysis was performed using SPSS 17.0 (IBM, United States). One-Way ANOVA was employed to analyze the results and values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The Prevalence of *mcr*-Positive Isolates in the Funan River

The screening of 1500 isolates for *mcr* yielded a total of 24 *mcr*-positive isolates. They included 18 *mcr-1* positive isolates (17 *Escherichia coli* and 1 *Enterobacter cloacae*) and 6 *mcr-3* positive isolates (2 *Aeromonas veronii* and 4 *Aeromonas hydrophila)*. *mcr-2*, *mcr-4*, or *mcr-5* were not observed in any of the isolates.

Many reports have described the presence in *mcr-1* in animal- and human- derived *Enterobacteriaceae* isolates isolated worldwide (Du et al., 2016; Liu et al., 2016; Malhotra-Kumar et al., 2016; Shen et al., 2016), but only two previous studies identified *mcr-1* in waterborne *Enterobacteriaceae*. One study reported

TABLE 2 | Quantitative polymerase chain reaction primer pairs used in this study.

Target genes	Sequence (5′→3′)	Amplicon size(bp)	Annealing temperatures (°C)	Reference
sul1	CACCGGAAACATCGCTGCA AAGTTCCGCCGCAAGGCT	158	60	Luo et al., 2010
aac(6′)-Ib-cr	GTTTCTTCTTCCCACCATCC AGTCCGTCACTCCATACATTG	103	60	Yang et al., 2018
ermB	CACCGAACACTAGGGTTGC TGTGGTATGGCGGGTAAGT	129	55	This study
bla_{CTX-M}	CAGATTCGGTTCGCTTTCAC GCAAATACTTTATCGTGCTGATG	103	55	Yang et al., 2018
mcr-1	CATCGCGGACAATCTCGG AAATCAACACAGGCTTTAGCAC	116	56	Yang et al., 2017
tetM	TTCAGGTTTACTCGGTTCA GAAGTTAAATAGTGTTCTTGGAG	106	55	This study
16S rRNA	CGGTGAATACGTTCYCGG GGWTACCTTGTTACGACTT	128	55	Suzuki et al., 2000

detection of the *mcr-1* gene in 1 out of 74 *Enterobacteriaceae* isolated from 21 rivers and lakes in Switzerland that produced extended spectrum β-lactamases (ESBLs) (Zurfuh et al., 2016). In a separate study, similar to our results, Zhou et al. (2017) isolated 23 *mcr*-1-positive isolates from environmental water sources in Hangzhou, indicating that *mcr-1*-carrying *Enterobacteriaceae* may be common in lakes and rivers in China. Data addressing the prevalence of *mcr-3* is limited. Recently, a novel *mcr* variant, *mcr-3*, was first discovered on an IncI2 plasmid from a strain of *E. coli* isolated from a pig in China (Yin et al., 2017). Since then, *mcr-3*-positive strains have been identified in humans and food (Ling et al., 2017; Liu L. et al., 2017). Worryingly, *mcr-3* has been detected on the chromosome of *Aeromonas veronii*, and these chromosomally encoded *mcr-3* determinants can become plasmid-bound and transferable (Cabello et al., 2017; Ling et al., 2017). Recently, Shen et al. (2018a) presented evidence that *mcr* determinants originated from aquatic environments, including *mcr*-3 harboring *Aeromonas* spp. Because *Aeromonas* species are prevalent in aquatic environments, the occurrence of colistin resistant isolates in urban rivers is of great concern as these strains may contribute to the potential dissemination of *mcr* determinants.

Antimicrobial Resistance Phenotypes and Genotypes of *mcr-1* and *mcr-3*-Positive Strains

As shown in **Table 3**, we next analyzed the antimicrobial resistance phenotypes and genotypes of the isolated *mcr-1* and *mcr-3* positive strains, and found 21 (87.5%) multidrug resistance isolates. The antimicrobial resistance testing showed that all isolates were resistant to colistin (MIC $\geq$ 4 μg/mL). Of the other antimicrobials tested, the most frequent resistance was to CTX (75%, 18 isolates), followed by CAZ (50%, 12 isolates), AMP (50%, 12 isolates), CRO (45.8%, 11 isolates), ATM (45.8%, 11 isolates), SXT (41.7%, 10 isolates), FOS (29.2%, 7 isolates), TE (25%, 6 isolates), AK (20.8%, 5 isolates), CIP (20.8%, 5 isolates), IPM (16.7%, 4 isolates), FOX (12.5%, 3 isolates), AMC (12.5%, 3 isolates), and ETP (4.2%, 1 isolate). The high occurrence of ESBL producers is worrisome, and corresponds to Zurfluh et al. (2013)

TABLE 3 | The antimicrobial resistance genotypes, phenotypes and MIC values of colistin of *mcr-1* and *mcr-3* positive strains.

Isolates	Species	Antibiotic resistant genes	Antimicrobial resistance phenotypes[a]	MIC values of colistin (μg/mL)
E22	*Escherichia coli*	*mcr-1*	CTX, CAZ, AMP, ATM	16
E23	*Escherichia coli*	*mcr-1*	CTX, CAZ	16
E24	*Escherichia coli*	*mcr-1*	CTX, CAZ	16
E25	*Escherichia coli*	*mcr-1*	CTX, CAZ, ATM	16
E26	*Escherichia coli*	*mcr-1*	CTX, CAZ, ATM, AK	16
E27	*Escherichia coli*	*mcr-1, sul2*	CRO, ATM, SXT	16
E28	*Escherichia coli*	*mcr-1*	CTX, CRO, CAZ, ATM, AK	16
E29	*Escherichia coli*	*mcr-1*, $bla_{CTX-M-9}$, *fosA3, qnrS, floR, oqxAB, sul1, sul2, tetA, aac(6′)-Ib-cr*	CTX, CRO, AMP, SXT, CIP	16
E30	*Escherichia coli*	*mcr-1, sul1, tetA*	CRO, FOX, ATM, SXT, FOS, TE	16
E31	*Escherichia coli*	*mcr-1, floR, sul2, tetM*	CTX, CRO, CAZ, SXT	16
E32	*Escherichia coli*	*mcr-1, floR, sul2, tetM*	CTX, CRO, CAZ, SXT	16
E33	*Escherichia coli*	*mcr-1, floR, sul2, tetM*	CTX, CRO, CAZ, SXT	16
E34	*Escherichia coli*	*mcr-1*, bla_{TEM}, $bla_{CTX-M-9}$	CTX, CRO, CAZ, AMP	16
E35	*Escherichia coli*	*mcr-1*, bla_{TEM}, $bla_{CTX-M-9}$, *floR, oqxAB, sul1, sul2, tetM, tetA, aac(6′)-Ib-cr, rmtB*	CTX, CRO, FOX, AMP, ATM, SXT, TE, AK, CIP	16
E36	*Escherichia coli*	*mcr-1*, bla_{TEM}, $bla_{CTX-M-9}$, *fosA3, oqxAB, sul1, sul2*	CTX, CRO, AMP, ATM, SXT, FOS, CIP	16
E38	*Escherichia coli*	*mcr-1, tetM, tetA*	CTX, CAZ, AMP, ATM, TE	16
E39	*Escherichia coli*	*mcr-1, qnrS, tetA*	CTX, ATM, AMC, TE, FOS, CIP	8
E37	*Enterobacter cloacae*	*mcr-1, floR, sul2, rmtA, rmtB*	CTX, FOX, AMP, AMC, SXT, AK, IPM	16
A4	*Aeromonas veronii*	*mcr-3*, bla_{SHV}, *sul1*	CTX, IPM	4
A19	*Aeromonas hydrophila*	*mcr-3*, bla_{TEM}, $bla_{CTX-M-9}$, *qnrB, sul1, sul2, tetA*	CTX, CRO, CAZ, AMP, ATM, AMC, FOS, TE, IPM	16
A48	*Aeromonas hydrophila*	*mcr-3, sul1, rmtA, rmtB*	AMP, FOS	8
A49	*Aeromonas hydrophila*	*mcr-3, sul1, sul2, rmtA, rmtB*	AMP, FOS, AK	8
A52	*Aeromonas hydrophila*	*mcr-3, qnrS, floR, sul1, tetA*	AMP, FOS, TE CIP	4
A54	*Aeromonas veronii*	*mcr-3, sul1*	AMP, SXT, IPM, ETP	4

[a]*CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; FOX, cefoxitin; AMP, ampicillin; ATM, aztreonam; AMC: amoxicillin-clavulanic acid; SXT, trimethoprim-sulfamethoxazole; FOS, fosfomycin; TE, tetracycline; AK, amikacin; CIP, ciprofloxacin; IPM, imipenem; ETP, ertapenem.*

who found 74 ESBL-producing isolates from 21 (36.2%) of 58 rivers and lakes, and all showed the multidrug resistance phenotype. In another study, 70% of fluoroquinolone resistant *E. coli* isolated from an urban river showed resistance to three or more classes of antibiotics (Zurfluh et al., 2014). The widespread distribution of MDR bacteria suggested serious drug-resistant pollution in river water. In this study, cephalosporin resistant strains were found most frequently, which may be related to the extensive use of cephalosporins for clinical and veterinary purposes. Overall, high usage has led to increased occurrence and wide distribution of ESBLs in bacteria (Bradford, 2001; Bonnet, 2004).

The *mcr-1* and *mcr-3* positive isolates were next assayed for the presence of other ARGs. The bla_{SHV}, bla_{TEM} and $bla_{\mathrm{CTX-M-9}}$ genes were detected in 1 (4.2%), 4 (16.7%), and 5 (20.8%) isolates, respectively. None of the isolates were positive for bla_{KPC}, $bla_{\mathrm{OXA-48}}$, bla_{NDM}, bla_{VIM}, bla_{IMP} or $bla_{\mathrm{CTX-M-1}}$. Fifteen (62.5%) of isolates contain sulphonamide resistance genes (*sul1* in 5 isolates, *sul2* in 5 isolates, and *sul1*/*sul2* combined in 5 isolates). Some isolates contained genes encoding tetracycline resistance, with 20.8% and 29.2% positive for *tetM* and *tetA* genes, respectively. Some isolates contained genes encoding fluoroquinolone resistance genes, *qnrB*, *qnrS,* and *oqxAB,* which were detected in 1(4.2%), 3(12.5%), and 3(12.5%) isolates, respectively. Genes associated with aminoglycoside resistance, *aac(6′)-Ib-cr*, *rmtA*, and *rmtB*, were amplified in 2 (8.3%), 3 (12.5%), and 4 (16.7%) isolates, respectively. The *floR* gene was detected in 7 (29.2%) isolates and the *fosA3* gene was identified in 2 (8.3%) isolates. According to a recent report, 77.3% of *mcr-1*-positive *E. coli* (34/44) carried at least 1 ESBL gene, and several isolates carried 3 or more ESBL genes (Wu et al., 2018). Furthermore, $bla_{\mathrm{CTX-M-9}}$ was one of the most prevalent genes among the identified ESBL genes in China (Liu et al., 2015). Consistent with previous reports, sulphonamides and tetracycline resistance genes are the most abundant ARGs in rivers (Yang et al., 2018). We identified two strains (E29 and E36) that carried *mcr-1*, *fosA3*, and $bla_{\mathrm{CTX-M-9}}$ genes from river samples (**Table 3**). The *mcr-1*, *fosA3*, and ESBLs genes were previously identified in *E. coli* isolated from animal and food samples (Liu X. et al., 2017; Lupo et al., 2018), and the presence of these multidrug-resistant strains in urban river may present a serious threat to public health.

Prevalence of Antibiotic Resistance Genes in the Funan River

In this study, the prevalence of ARGs in water samples was investigated by sampling various sites along the Funan River. The *sul1, qnrS, tetM,* and *intI1* genes were detected in samples from all 10 sampling sites (100%). Additionally, *aac(6′)-Ib-cr*, *sul2*, *aph(3′)-IIIa*, *ermB*, and $bla_{\mathrm{CTX-M}}$ were detected at high rates of 90%, 90%, 90%, 80% and 70%, respectively. Many studies have reported the presence of these genes in aquatic environments (Hu et al., 2008; D'Costa et al., 2011; van Hoek et al., 2011; Lin et al., 2015; Makowska et al., 2016). Interestingly, the *aph(3′)-IIIa* gene has rarely been reported in river water microorganisms, but has been reported in clinical specimens (Tuhina et al., 2016). The detection of the *aph(3′)-IIIa* gene was high in this study, suggesting contamination of the Funan River with resistant bacteria carrying the *aph(3′)-IIIa* gene.

Genes conferring resistance to the last line of antibiotics, including *mcr-1*, bla_{NDM}, bla_{KPC} and *vanA* genes, were detected at rates of 30%, 20%, 10%, and 10%, respectively. bla_{VIM} was not detected at any site. The *mcr-1* gene was detected in 30%

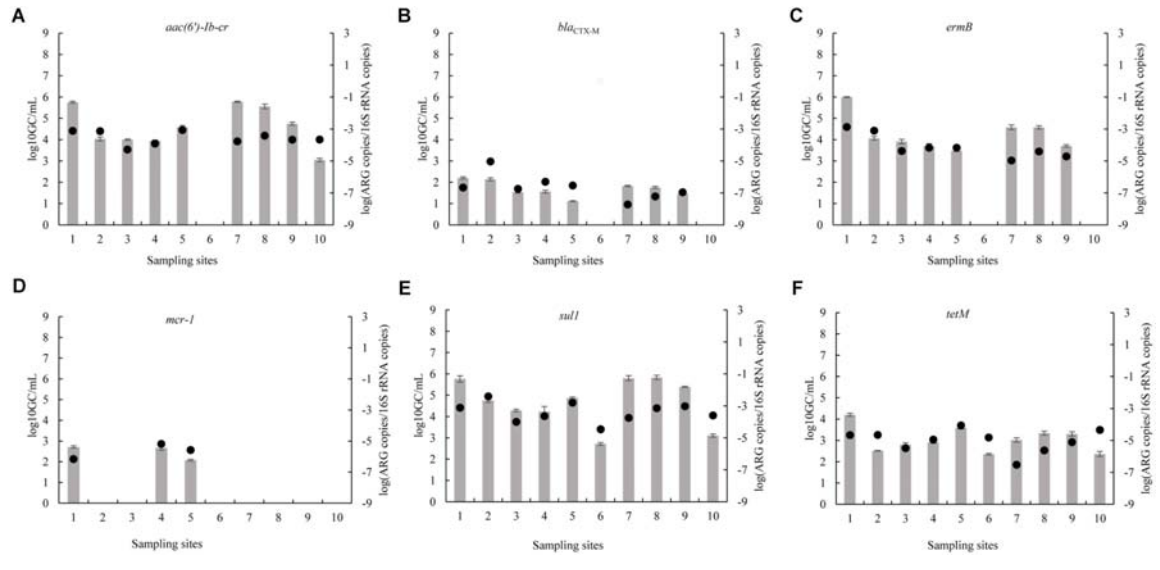

FIGURE 2 | Absolute (bars) and 16S rRNA gene-normalized (symbols) levels of ARGs (**A**: *aac(6′)-Ib-cr*; **B**: $bla_{\mathrm{CTX-M}}$; **C**: *ermB*; **D**: *mcr-1*; **E**: *sul1*; **F**: *tetM*) in water samples collected at various sites (1, RWW, Residential wastewater; 2, P1, Park1; 3, P2, Park2; 4, HWW1, Hospital Wastewater1; 5, HWW2, Hospital Wastewater2; 6, P3, Park3; 7, RI, River Intersection; 8, WWTP, Municipal Wastewater Treatment Plant; 9, UWP, Upstream of Wetland Park; 10, DWP, Downstream of Wetland Park) along the Funan River.

of samples, suggesting the Funan River could act as a reservoir for the *mcr-1* gene. The bla_{NDM}, bla_{KPC} and *vanA* genes were detected near the WWTP (**Figure 1**). Although *mcr-1* is found frequently in human and animal settings, there is only limited data for urban rivers (Marathe et al., 2017; Ovejero et al., 2017; Yang et al., 2017). Similarly, Marathe et al. detected bla_{NDM} and bla_{KPC} genes in the sediments of an Indian river (Marathe et al., 2017). Although a bla_{VIM} positive carbapenem-resistant strain was isolated from a river in Switzerland (Zurfluh et al., 2013), here is a lack of data on bla_{VIM} in the non-clinical environment. The *vanA* gene is associated with vancomycin resistance and has been found in wastewater biofilms and in drinking water biofilms in Mainz (Schwartz et al., 2003). Although these genes have rarely been identified in natural aquatic environments, given the dangerous infections that can arise from ARB (and which subsequently create intractable challenges for clinical treatment), further observation of the prevalence of these genes in aquatic environments is required.

Abundance of ARGs

Concerning the absolute abundance of ARGs in the Funan River, ARGs were detected at levels that ranged from 0 to 6.0 $\log_{10}$ GC/mL (**Figure 2**). The *sul1*, *aac(6′)-Ib-cr,* and *ermB* genes were the dominant ARGs in the Funan River with mean absolute abundances of 4.8, 4.1, and 3.4 $\log_{10}$ GC/mL, respectively. The *sul1* gene exhibited the most prominent average abundance in water samples. Previous studies reported that *sul1* is abundant in numerous water areas, including the Tordera River Basin (Proia et al., 2016) and the Haihe River (Luo et al., 2010). Although the *mcr-1* gene was not detected in water samples at some sites, three sites (RWW, HWW1, and HWW2) displayed 2.0-2.7 $\log_{10}$ GC/mL. Notably, the highest detected level of *mcr-1* (2.7 $\log_{10}$ GC/mL) was higher than that in previous reports about the Haihe river (2.6 $\log_{10}$ GC/mL) (Yang et al., 2017). The absence of *mcr* in some samples may indicate that no *mcr-1* positive strains were present in the water samples or that the levels of *mcr-1* were below the detection limit. Site RWW is located near the residential sewage outlet, suggesting the presence of *mcr-1* was related to human activity. Consistently, *mcr-1* was detected at HWW1 and HWW2, adjacent to the hospital sewage outlets, suggesting the spread of *mcr-1* from hospitals to urban river, although colistin is not used widely in human medicine. The *mcr-1* abundance at RWW (2.7 $\log_{10}$ GC/mL) was slightly higher than that at HWW1 (2.6 $\log_{10}$ GC/mL) and at HWW2 (2.3 $\log_{10}$ GC/mL). Similarly, the prevalence of *mcr-1*-positve *E. coli* from healthy individuals (0.7–6.2%) is higher than the prevalence for inpatients (0.4–2.9%) (Shen et al., 2018b). It is striking that *mcr* is the only gene that was absent from sites other than RWW and HWW. The reasons for high rate of fecal carriage of *mcr* in humans in China may reflect the rapid emergence of plasmid-encoded *mcr-1* within many MDR *E. coli* carried by humans and also be related to the significant diversity and genetic flexibility of MGEs harboring *mcr-1* (Zhong et al., 2018).

At RWW, RI, and WWTP, the absolute abundances of certain ARGs (*sul1*, *aac(6′)-Ib-cr,* and *ermB*) were significantly higher than those at other sampling sites ($P < 0.05$). At P3 and DWP, the absolute abundances of most ARGs were significantly lower than the levels detected at the other sites ($P < 0.05$). RWW was associated with the highest absolute abundance of the six ARGs (*mcr-1*, *sul1*, *aac(6′)-Ib-cr*, *ermB*, bla_{CTX-M}, and *tetM*) (**Figure 2**). Samples near the wastewater treatment plant (WWTP) and densely populated areas exhibited a relatively greater content of resistant genes. Wastewater discharge may contribute to the spread of ARGs into the environment, thereby affecting the bacterial communities of the receiving river (Marti et al., 2013; Xu et al., 2015). Our results indicate that human activities influence the dissemination of resistance genes in the Funan River. Remarkably, the absolute abundances of most ARGs were low at the DWP sampling point, located downstream of the wetland park. This is consistent with a decrease in the ARGs levels of the effluents from a constructed wetland with a free surface flow (Liu et al., 2014).

As shown in **Figure 2**, the relative abundances of each ARG are only partly correlated with their absolute abundance. That is, although the absolute abundances of most ARGs at RWW, RI and WWTP were relatively high, their relative abundances were comparatively low. These differences may be related to the differences in the proportion of resistant bacteria to total bacteria at each site (Tao et al., 2014).

CONCLUSION

This study describes 18 *mcr-1*-positive strains and 6 *mcr-3*-positive strains isolated from the Funan River, of which 87.5% were found to be MDR. The *sul1*, *sul2*, *intI1*, *aac(6′)-Ib-cr*, bla_{CTX-M}, *tetM*, *ermB*, *qnrS* and *aph(3′)-IIIa* genes were abundant in the Funan River. Interestingly, the *mcr-1*, bla_{KPC}, bla_{NDM}, and *vanA* genes were detected, although these four resistance genes have rarely been found in natural river systems. Notably, the *mcr-1* gene was detected at a rate of 30%. Our results suggest urban activities may increase the prevalence of antibiotic resistance genes and demonstrate the current presence of drug-resistance pollution in the Funan River. The processes by which the dissemination of ARGs occurs in urban rivers should be the focus of future studies.

AUTHOR CONTRIBUTIONS

AZ designed the study. HT, DL, XX, and PL carried out the sampling work. HT, YY, XT, and JG performed the experiments. AZ, HT, RX, LK, and CL analyzed the data. AZ, HT, YL, and HW drafted the manuscript. All authors have read and approved the final manuscript.

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Distribution of ExPEC Virulence Factors, *bla*$_{CTX-M}$, *fosA3*, and *mcr*-1 in *Escherichia coli* Isolated From Commercialized Chicken Carcasses

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Pathogenic *Escherichia coli* found in humans and poultry carcasses harbor similar virulence and resistance genes. The present study aimed to analyze the distribution of extraintestinal pathogenic *E. coli* (ExPEC) virulence factors (VF), *bla*$_{CTX-M}$ groups, *fosA3*, and *mcr*-1 genes in *E. coli* isolated from commercialized chicken carcasses in southern Brazil and to evaluate their pathogenic risk. A total of 409 *E. coli* strains were isolated and characterized for genes encoding virulence factors described in ExPEC. Results of antimicrobial susceptibility testing confirmed that the strains were resistant to β-lactams, fosfomycin, colistin, and others resistance groups. The highest prevalence of VFs was observed in isolates belonging to the CTX-M groups, especially the CTX-M-2 group, when compared to those in other susceptible strains or strains with different mechanisms of resistance. Furthermore, ESBL strains were found to be 1.40 times more likely to contain three to five ExPEC virulence genes than non-ESBL strains. Our findings revealed the successful conjugation between ESBL-producing *E. coli* isolated from chicken carcass and the *E. coli* recipient strain J53, which suggested that genetic determinants encoding CTX-M enzymes may have originated from animals and could be transmitted to humans via food chain. In summary, chicken meat is a potential reservoir of MDR *E. coli* strains harboring resistance and virulence genes that could pose serious risks to human public health.

Keywords: ESBL, multidrug-resistance, phylogenetic groups, CTX-M, fosfomycin

INTRODUCTION

Humans and warm-blooded animals naturally harbor bacteria in their intestines, such as *Escherichia coli,* which is usually a non-pathogenic commensal bacterium. However, *E. coli* could cause extraintestinal diseases, including urinary tract infection, septicemia and meningitis in humans or even colibacillosis in poultry, which is attributed to the acquisition of virulence factors (VFs) (Müller et al., 2016).

Extraintestinal pathogenic *E. coli* (ExPEC) strains are characterized by several VF, including adhesins, invasins, protectins, and toxins, as well as several uptake systems for essential nutrients, such as iron (iron-uptake systems) (Johnson et al., 2008b). Commensal and pathogenic *E. coli* can be classified under different phylogenetic groups, since the VF found in each of the varieties are distributed differently (Clermont et al., 2000). Most commensal strains belong to phylogenetic group A or B1, and ExPEC strains, which harbor more VFs than commensal strains, are assigned to phylogenetic group B2 or D (Tenaillon et al., 2010; Cyoia et al., 2015).

In addition to VFs, the spread of resistance elements among human pathogens may be related to the Enterobacteriaceae family, in which *E. coli* belongs. Among the Gram-negative bacteria that are resistant to antibiotics, those that produce CTX-M-type ESBLs represent a serious public health concern worldwide (Xie et al., 2016). In particular, most commonly detected CTX-M groups include CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTXM-25 (Saravanan et al., 2018).

The detection of plasmidial genes that are mainly related to antimicrobial resistance to fosfomycin and colistin represents another major health concern (Sato et al., 2013; McGann et al., 2016). Fosfomycin is used to treat urinary tract infections (UTI) that are mostly caused by Gram-negative and Gram-positive bacteria, which are highly prevalent in North America (Giancola et al., 2017), and have recently received research attention because of the rapid spread of multidrug-resistance. This resistance is related to a novel gene called *fos*A3, which has been reported in *E. coli* and *Klebsiella pneumonia* and is often detected in $bla_{\mathrm{CTX-M}}$ -producing and multidrug-resistant *E. coli* both in animals and in clinical isolates (Ho et al., 2013). Colistin is prescribed for the treatment of UTI and has been associated with many cases of resistance worldwide. Furthermore, renewed attention has been paid to the *mcr*-1 gene because it has been detected not only in clinical isolates but also in animal, food, and environmental samples (Fernandes et al., 2016; McGann et al., 2016; Rapoport et al., 2016; Skov and Monnet, 2016; Zeng et al., 2016).

Pathogenic *E. coli* found in humans and poultry carcass were found to harbor similar virulence and resistance genes in the plasmids (Stromberg et al., 2017). These findings raise the possibility that *E. coli* present in the intestinal tract of healthy individuals could acquire those genes from *E. coli* derived from chicken meat, which could act as a reservoir for bacteria harboring resistance genes (Manges and Johnson, 2012). Therefore, present study aimed to analyze the distribution of ExPEC VFs, $bla_{\mathrm{CTX-M}}$ groups, and the *fos*A3 and *mcr*-1 genes in *E. coli* isolated from chicken carcasses commercialized in southern Brazil (States of Paraná-PR, Santa Catarina-SC, and Rio Grande do Sul-RS).

MATERIALS AND METHODS

Bacterial Isolates

Escherichia coli strains were isolated in the Basic and Applied Bacteriology Laboratory at Londrina State University (Biosafety level 2) from 98 commercial refrigerated chicken carcass (35 chicken carcasses from PR, 23 chicken carcasses from SC, and 40 chicken carcasses from RS), sold in southern Brazil from 2013 to 2014. Each chicken carcass was rinsed into the sterile packaging with 100 mL of Brain Heart Infusion (Himedia Laboratories Pvt. Ltd., Mumbai, India). After homogenization, 0.1 mL of the mixture was smeared onto MacConkey agar (Neogen Corporation Lansing, Michigan) and Violet Red Bile Lactose agar (Oxoid Ltd., Basingstoke, Hants, UK) by the pour plate method. Colonies suspected to be *E. coli* were confirmed by biochemical testing using EPM-MILi and Simmons Citrate agar (PROBAC, Brazil). After biochemical confirmation, one to five strains were collected from each chicken carcass and subsequently analyzed for the genotypic characteristics of ExPEC virulence factors and phenotypic resistance. Only strains that showed difference in those characteristics were selected for further analysis.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was performed using the standard disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2015). The following antimicrobial agents were used in the study: 5 μg of ciprofloxacin; 10 μg of each of ampicillin, gentamicin, norfloxacin, and enrofloxacin; 30 μg of each of cefazolin, cefotaxime, cefoxitin, ceftazidime, tetracycline, nalidixic acid, and chloramphenicol; 300 μg of nitrofurantoin; 1.25/23.75μg of trimethoprim-sulfamethoxazole; 200 μg of fosfomycin; and 20/10 μg of amoxicillin-clavulanic acid (Oxoid Ltd., Basingstoke, Hants, UK). Strains resistant to third-generation cephalosporins were confirmed for ESBL production by double-disk diffusion testing between amoxicillin/clavulanate and cefotaxime or ceftazidime (Jacoby and Han, 1996) or by conducting a combination disc test using cefotaxime, cefotaxime + clavulanic acid (Becton Dickinson, Sparks, MD), ceftazidime, and ceftazidime + clavulanic acid (Becton Dickinson, Sparks, MD), following the CLSI recommendations. The positive strains in the phenotypic tests to ESBL production were screened for ESBL genes, and the strains resistant to fosfomycin were screened for the *fos*A3 gene. The *E. coli* isolate ATCC 25922 was used as a quality control during antimicrobial susceptibility testing. Results were interpreted based on the CLSI criteria.

Detection of Antimicrobial Resistance Genes

ESBL-producing *E. coli* was characterized for ESBL genes encoding CTX-M (groups 1, 2, 8, 9, and 25), TEM, and SHV by Polymerase Chain Reaction (PCR) (Arlet and Philippon, 1991; Bedenić et al., 2001; Woodford et al., 2006). The presence of acquired fosfomycin resistance genes such as *fos*A3 was determined by PCR using specific primer sets (Sato et al., 2013). The strains were additionally tested for the presence of colistin resistance gene *mcr*-1 by PCR (Liu et al., 2016). PCR amplicons were visualized on 2.0% agarose gels stained with GelRed (Biotium, Hayward, CA, USA). After gel electrophoresis, the images were captured using Image Capture Systems (LPixImageHE).

Conjugation Experiments

To verify whether the plasmid harboring bla_{CTX-M} resistance genes could be transferred between *E. coli* strains, the horizontal-transfer efficiencies of the bla_{CTX-M} genes were assessed by performing conjugation experiments between three selected strains harboring bla_{CTX-M} resistance genes. Volumes of cultures of each donor (ESBL-producing *E. coli* isolated from chicken carcass) and azide-resistant *E. coli J53,* recipient strain grown in Luria-Bertani broth (Difco Laboratories, Detroit, Mich) were mixed and incubated for 18–24 h at 37°C. Transconjugants were then selected on MacConkey agar containing 2 μg/mL cefotaxime (Sigma Chemical Co., St. Louis, MO) and 100 μg/mL sodium azide (Sigma Chemical Co., St. Louis, MO) and subsequently used for phylogenetic analysis and testing for the presence of bla_{CTX-M} genes (Xie et al., 2016).

Phylogenetic Classification

E. coli strains were assigned to phylogenetic groups (A, B1, B2, or D) by PCR (Clermont et al., 2000). Each PCR reaction contained 1.25 U of Taq DNA polymerase (Life technologies, Rockville, MD) in 1× PCR buffer (Life Technologies, Rockville, MD), 0.2 mM each dNTP, 2.5 mM $MgCl_2$, and 1 μM each primer. PCR amplicons were visualized on 2.0% agarose gels stained with GelRed (Biotium, Hayward, CA, USA). After gel electrophoresis, the images were captured using Image Capture Systems (LPixImageHE).

Virulence Genes

We surveyed five VF genes that are normally studied in ExPEC strains. The selected genes included: *iut*A (aerobactin siderophore receptor gene), *hly*F (putative avian hemolysin), *iss* (episomal increased serum survival gene), *iro*N (salmochelin siderophore receptor gene), and *omp*T (episomal outer membrane protease gene) (Johnson et al., 2008a). Each PCR reaction contained 1.25 U of Taq DNA polymerase (Life Technologies, Rockville, MD) in 1× PCR buffer (Life Technologies, Rockville, MD), 0.2 mM each dNTP, 2.5 mM $MgCl_2$, and 1 μM each primer. PCR amplicons were visualized on 2.0% agarose gels stained with GelRed (Biotium, Hayward, CA, USA). After gel electrophoresis, the images were captured using Image Capture Systems (LPixImageHE).

Statistical Analysis

Frequencies of ExPEC virulence genes in ESBL-producing and non-ESBL-producing strains were compared by Fisher's exact test and Pearson's Chi-square test. The risk of ESBL-producing *E. coli* harboring more ExPEC genes than non-ESBL-producing *E. coli* at 95% confidence interval (95% CI) was determined by calculating the relative risk (RR). Statistically significant differences were considered at $p < 0.05$. The test was performed using the statistical software R version 3.5.1.

RESULTS

Antimicrobial Resistance of *E. coli* From Poultry Carcasses

A total of 409 *E. coli* isolates from chicken carcasses from southern Brazil were tested. Among these, 121, 135, and 153 were isolated from carcasses from the PR, SC, and RS states. Results of the antimicrobial susceptibility test indicated that strains from chicken carcasses showed a high frequency of antimicrobial resistance, in total 66% of the isolates were resistant to antibiotics. We identified multidrug-resistant *E. coli* strains from chicken carcasses from PR, SC and RS (82, 53, and 80%, respectively). The most common antimicrobial agents for which strains were found to be resistant included tetracycline (68.77%), nalidixic acid (67.61%), and ampicillin (68.77%). The ESBL phenotype was confirmed for 119 isolates (∼32% of PR, 31% of SC, and 35% of RS) of the 409 strains isolated from commercial refrigerated chicken carcasses, which represents 29.1% of all isolates. Furthermore, ESBL-producing *E. coli* were found to be more resistant to a higher number of antimicrobials ($p < 0.05$) compared to non-ESBL-producing *E. coli* (**Figure 1**). Of the 409 *E. coli* strains tested, 99.3% were classified as susceptible to fosfomycin, whereas none showed intermediate resistance and three strains (0.70%) showed resistance to fosfomycin.

Detection of Antimicrobial Resistance Genes

The majority of ESBL-producing *E. coli* isolates (32.23%) were collected from the PR state, while the RS state showed the lowest number of ESBL-producing *E. coli* isolates (27.45%). Out of the 119 ESBL strains, 97 harbored the bla_{CTX-M} gene, six harbored CTX-M-1 group, 61 harbored CTX-M-2 group, and 30 harbored CTX-M-8 group (**Table 1** and **Figure 2**). The CTX-M-9 group and CTX-M-25 group were not detected in the strains (**Figure 2**). The remaining *E. coli* strains harbored the bla_{SHV} (7.56%) and bla_{TEM} (10.08%) genes (**Figure 2**).

Fosfomycin resistance was identified based on phenotypic tests and subsequently confirmed by PCR. The three fosfomycin-resistant strains that harbored the *fos*A3 gene were found to be bla_{CTX-M} positive (3.33%). PCR analysis of the 119 ESBL-producing *E. coli* isolates revealed that 2.50% of the isolates harbored genes encoding resistance to colistin, corresponding to one resistant strain from each state (PR, SC, and RS). Furthermore, these strains were ESBL-producing *E. coli,* and two of these strains harbored five ExPEC virulence genes tested in the present study (*iss*, *iro*N, *iut*A, *hly*F, and *omp*T) (**Table 1**) and were assigned to different phylogenetic groups (A, B2, and B1).

Conjugation Experiments

Among the bla_{CTX-M} positive *E. coli* isolates tested that belonged to phylogenetic group B1, all strains successfully transferred their cefotaxime resistance phenotypes to the *E. coli* recipient strain J53 via conjugation.

Phylogenetic Classification

Phylogenetic analysis revealed that most of the *E. coli* strains belonged to group B1 (36.6%), followed by groups A (31.7%), D

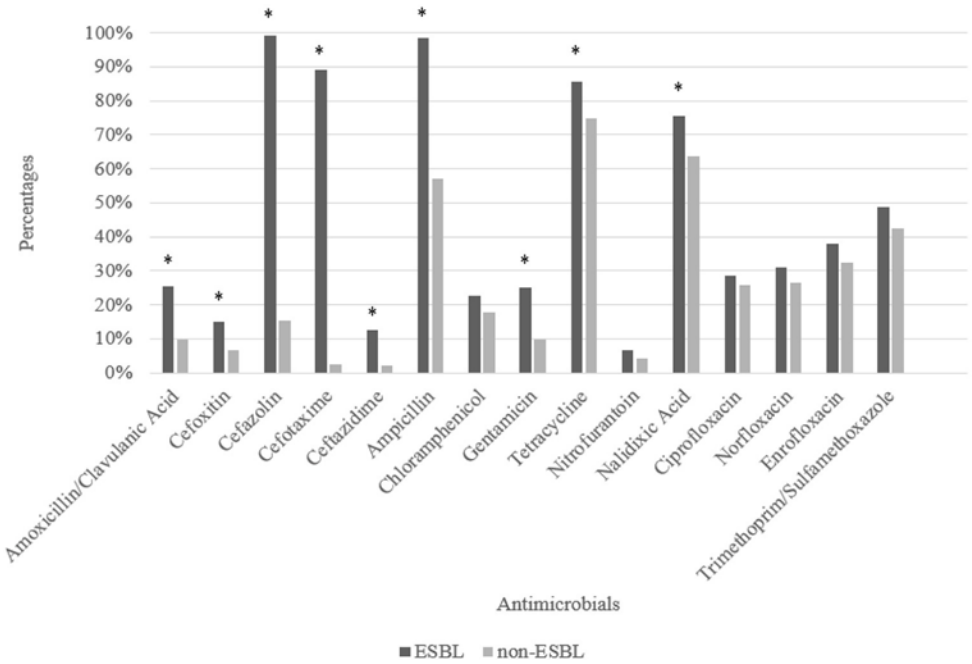

FIGURE 1 | Percentage resistance exhibited by ESBL-producing *E. coli* strains and non-ESBL-producing *E. coli* strains isolated from commercial chicken carcasses in southern Brazil from 2013 to 2014. *$p < 0.05$ by Pearson's Chi-square test.

(28.1%), and B2 (3.40%) (**Table 2**). The determination of *E. coli* phylogenetic groups showed that the majority of the 119 ESBL-producing *E. coli* belonged to phylogenetic group D (36.06%), followed by a nearly even distribution of the remaining three phylogenetic groups, namely, B1 (31.97%), A (27.63%), and B2 (4.22%) (**Table 2**).

Virulence Genes

ExPEC VFs were identified in the various *E. coli* strains. Among the 409 *E. coli* strains analyzed, the prevalence of individual ExPEC VF genes ranged from 33.3% (*iss,* an episomal increased serum survival gene) to 51.6% (*iut*A, an aerobactin siderophore receptor gene). Results indicated that 58% of ESBL-producing *E. coli* harbored three to five ExPEC virulence genes (**Table 1**).

The highest prevalence of ExPEC VFs was observed in strains harboring CTX-M resistance relative to other susceptible strains or even strains with different mechanisms of resistance ($p < 0.01$). The relative risk for ESBL strains that did not contain any ExPEC genes was 0.35 (95 % CI, 0.21–0.57; $p < 0.01$). On the other hand, the RR for ESBL strains harboring three or more ExPEC genes was 1.40 (95 % CI, 1.13–1.73; $p < 0.01$) (**Table 3**). For each non-ESBL strain harboring three or more ExPEC virulence genes (**Supplementary Material**), there are 1.40 ESBL strains harboring three or more ExPEC virulence genes (RR>1). For example, in the PR state, the *iut*A gene was present in 54% of the *E. coli* isolates, and present in 80% of the bla_{CTX-M} producing *E. coli*. Similar results were observed in the other two states for all five virulence genes.

DISCUSSION

In the present study, we analyzed a total of 409 *E. coli* strains from commercial chicken carcasses in Brazil isolated from 2013 to 2014. About 71% of isolates were MDR (Magiorakos et al., 2012), which demonstrate the high antimicrobial resistance. Our current findings are consistent with reports from other countries, which detected MDR in Gram negative bacteria from chicken meat in Italy (66.9% resistant) and India (79.6% resistant) (Ghodousi et al., 2015; Shrestha et al., 2017). In the states of PR and RS, approximately 80% of carcasses were found to be contaminated with *E. coli* that were resistant to three or more antimicrobial groups, whereas the rates of resistance in the state of SC were slightly lower (53%). The higher rates of antimicrobial resistance and MDR in strains could be due to environmental contamination with antibiotic residues in aviculture industries and/or selective pressure caused by the indiscriminate use of antimicrobial compounds as a result of poor monitoring by regulatory bodies (Koga et al., 2015). Importantly, some growth promoters, such as poultry feeds, have been prohibited in animal production in several countries, like in Brazil since 1998 (Brasil Ministério da Agricultura, 2003, 2009).

Almost 30% of the isolates analyzed in the present study were found to be resistant to β-lactams and thus represent a potential health concern. The resistant *E. coli* harbored genes encoding ESBL enzymes that hydrolyze penicillins, cephalosporins, and monobactams and were inhibited by treatment with "classical" β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Bevan et al., 2017; Saravanan et al., 2018). Notably, ESBL-producing *E. coli* showed stronger resistance to

TABLE 1 | Distribution of resistance and virulence genes among 119 ESBL-producing *E. coli* strains isolated from chicken carcasses commercialized in Brazil.

β-lactamases, *fos*A3, *mcr*-1 genes	Virulence genes	Number of isolates
Group 1 CTX-M	*hly*F, *omp*T, *iss, iro*N, *iut*A	1 PR
Group 1 CTX-M	*iut*A	1 RS
Group 1 CTX-M, Group 2 CTX-M	*hly*F, *omp*T, *iss, iro*N, *iut*A	1 PR
Group 1 CTX-M, Group 2 CTX-M	*hly*F, *omp*T, *iss, iut*A	1 SC
Group 1 CTX-M, Group 8 CTX-M, TEM	*iut*A	1 SC
Group 1 CTX-M, TEM	*hly*F, *omp*T, *iss, iro*N, *iut*A	1 SC
Group 2 CTX-M	*hly*F, *omp*T, *iss, iro*N, *iut*A	7 PR 2 SC 3 RS
Group 2 CTX-M	*omp*T, *iss, iro*N, *iut*A	1 RS
Group 2 CTX-M	*hly*F, *omp*T, *iro*N, *iut*A	1 RS
Group 2 CTX-M	*hly*F, *omp*T, *iss, iro*N	2 RS
Group 2 CTX-M	*hly*F, *omp*T, *iut*A	6 PR 2 SC 6 RS
Group 2 CTX-M	*iss, iut*A	1 RS
Group 2 CTX-M	*hly*F, *omp*T	1 RS
Group 2 CTX-M	*iro*N	1 RS
Group 2 CTX-M	*iut*A	8 PR 1 SC 3 RS
Group 2 CTX-M	None	2 SC 6 RS
Group 2 CTX-M, Group 8 CTX-M	None	1 PR
Group 2 CTX-M, Group 8 CTX-M, SHV, *fos*A3	*hly*F, *omp*T, *iss, iro*N, *iut*A	1 PR
Group 2 CTX-M, *mcr*-1	*hly*F, *omp*T, *iss, iro*N, *iut*A	1 PR
Group 2 CTX-M, TEM	*hly*F, *omp*T, *iro*N, *iut*A	1 SC
Group 2 CTX-M, TEM	*hly*F, *omp*T, *iut*A	1 SC
Group 2 CTX-M, TEM	*iut*A	1 SC
Group 8 CTX-M	*hly*F, *omp*T, *iss, iro*N, *iut*A	3 PR 1 SC 3 RS
Group 8 CTX-M	*hly*F, *omp*T, *iss, iro*N	2 SC 3 RS
Group 8 CTX-M	*hly*F, *omp*T, *iro*N, *iut*A	1 RS
Group 8 CTX-M	*hly*F, *omp*T	3 PR
Group 8 CTX-M	*hly*F	1 RS
Group 8 CTX-M	*omp*T	4 PR
Group 8 CTX-M	None	2 RS
Group 8 CTX-M, SHV	*hly*F, *omp*T, *iss, iro*N, *iut*A	2 PR
Group 8 CTX-M, TEM	*hly*F, *omp*T, *iss, iro*N, *iut*A	1 SC
Group 8 CTX-M, TEM	*iut*A	1 SC
SHV	*hly*F, *omp*T, *iss, iro*N, *iut*A	3 RS
SHV	*omp*T, *iss, iro*N, *iut*A	1 RS
SHV, *fos*A3	*hly*F, *omp*T, *iss, iro*N, *iut*A	1 PR
SHV, *mcr*-1	*hly*F, *omp*T, *iss, iro*N, *iut*A	1 RS
TEM	*hly*F, *omp*T, *iss, iut*A	1 SC
TEM	*hly*F, *iut*A	1 SC
TEM	*iro*N, *iut*A	1 SC
TEM	*iut*A	1 SC
TEM	None	1 SC
ND[a], *fos*A3	None	1 SC
ND[a], *mcr*-1	None	1 SC
ND[a]	*hly*F, *omp*T, *iss, iro*N, *iut*A	3 SC
ND[a]	*omp*T, *iss, iro*N, *iut*A	1 SC
ND[a]	*hly*F, *omp*T, *iss, iro*N	1 SC
ND[a]	*iss, iro*N, *iut*A	1 SC
ND[a]	*hly*F, *omp*T, *iut*A	1 RS
ND[a]	*iro*N, *iut*A	1 SC
ND[a]	*iut*A	5 SC
ND[a]	None	2 SC

ND[a], not detected.

others antimicrobials, such as aminoglycosides, quinolones, and tetracyclines, when compared to non-ESBL-producing *E. coli* ($p < 0.05$), further promoting the health risks due to consumption of undercooked meat or the handling or preparation of uncooked poultry products contaminated with resistant strains (Shrestha et al., 2017; Saravanan et al., 2018). CTX-M ß-lactamases are the most widespread type of ESBL and have been identified since the mid-2000s and were specifically detected in clinical isolates of *E. coli* (Bush, 2018). ESBL-producing bacteria have been increasingly detected in meat from food-producing animals such as, poultry (Ghodousi et al., 2015; Shrestha et al., 2017; Poirel et al., 2018). Our findings have raised significant concerns, since the 30% prevalence of ESBL-producing samples in chicken carcasses in southern Brazil was higher than those reported in other regions, as in USA (27%), in India (21%) and in other samples from Brazil (7%) (Freeman et al., 2009; Datta et al., 2014; Gonçalves et al., 2016). Among all ESBL strains, we found 97% classified as $bla_{\text{CTX-M}}$ and the majority belonged to CTX-M-2 group, although the rates varied depending on the region worldwide. Recent studies reported the presence of the CTX-M-1 resistance genes in *E. coli* strains from poultry meat from Sweden (54–58%), Belgium (62%), Canada (66.2%), Italy (8.9%), and Japan (34%) (Smet et al., 2010; Denisuik et al., 2013; Brolund et al., 2014; Ghodousi et al., 2015; Nahar et al., 2018). However, CTX-M-9 represented the most prevalent group in reports of ESBL *E. coli* from Spain (Garrido et al., 2014), Portugal (Fernandes et al., 2014), Japan (Nahar et al., 2018), and Italy (Ghodousi et al., 2015).

One important finding from the current study is the successful conjugation between ESBL-producing *E. coli* isolated from chicken carcass to the *E. coli* recipient strain J53, which suggest that genetic determinants encoding CTX-M enzymes could be conjugative. According to Xie et al. (2016), commensal B1 strains isolated from food-producing animals could act as reservoirs of ESBL genes, which could be disseminated to human bacteria via the food chain, thereby raising a significant public health concern (Leverstein-van Hall et al., 2011; Xie et al., 2016; Poirel et al., 2018). Furthermore, resistance conferred by ESBLs is often associated with resistance to other classes of antibiotics, such as trimethoprim-sulfamethoxazole, aminoglycosides, and fluoroquinolone (Coque et al., 2008; Zeng and Lin, 2017). Therefore, the transfer of CTX-M mobile plasmids are likely to be accompanied by acquisition of other resistance genes. Some

studies reported that plasmid-mediated fosfomycin resistance is frequently detected among CTX-M-producing *E. coli* isolated from food-producing animals (Sato et al., 2013; Xie et al., 2016). During sample collection in 2013, fosfomycin was not commonly used in animal production because of its high cost; nevertheless, 3% of the strains tested positive for the presence of the *fos*A3 gene.

The use of polymyxins (colistin) in food-producing animals, especially in feed additives, represents another health concern. One colistin-resistant *E. coli* strain harboring five ExPEC virulence genes was detected in each of the southern Brazilian states. Several recent studies have also suggested the possibility of transfer of the *mcr*-1 gene to humans via the food chain (Carnevali et al., 2016; Wang et al., 2017). Although the current results indicated a very low presence of the *mcr*-1 gene, other studies indicated that the higher prevalence of colistin resistance could be attributed to the widespread use of colistin in food production in recent years (Huang et al., 2017). Thus, the use of fosfomycin and colistin in food production, such as in poultry, could lead to a public health concern, considering that these antimicrobials are used for the treatment of extraintestinal infections in humans. Therefore, similar to colistin, fosfomycin should also be banned from animal production in many countries.

Current evidence indicates that *E. coli* isolated from chickens and human ExPECs, harbor highly similar virulence genes, thereby suggesting a potential risk to cause diseases in humans (Manges and Johnson, 2012). A higher number of virulence factors present in ExPEC indicates a link to pathogenicity (Pitout, 2012). Furthermore, studies demonstrated an association between ExPEC virulence factors and phylogenetic groups. Intestinal *E. coli* isolates belonging to groups A and B1 harbor fewer ExPEC virulence genes, and ExPECs strains belonging to groups B2 and D contain a higher number of virulence genes (Koga et al., 2015; Müller et al., 2016; Pavlickova et al., 2017). Consistent with previous studies, most *E. coli* strains isolated from chicken carcasses harbor three to five ExPEC virulence genes (33–51%, varying between the five genes) and belonged to phylogenetic group B1 (36%), which represents a group of more multi-resistant commensal strains (Koga et al., 2015; Müller et al., 2016). Among these strains, 58% of ESBL-producing *E. coli* harbored three to five ExPEC virulence genes. Most of these strains were associated with phylogenetic group D, unlike non-ESBL-producing *E. coli,* which were associated with group B1. These rates are high compared to 28% of ExPEC isolated from patients mostly with UTIs in southern Brazil (Cyoia et al., 2015) or very similar to those reported in APEC strains (Mohamed et al., 2018), thereby indicating that some ESBL-producing *E. coli* strains from poultry meat are potentially pathogenic.

Importantly, $bla_{\mathrm{CTX-M}}$ ESBL-producing *E. coli* strains were found to harbor a higher number of ExPEC virulence genes relative to other susceptible strains or even strains that were resistant to other groups of antimicrobials ($p < 0.01$). In addition, ESBL strains are 1.40 times more likely to contain three to five ExPEC virulence genes than non-ESBL strains, which in turn increases their risk for pathogenic potential (RR = 1.40, 95% CI, 1.13–1.73; $p < 0.01$). The above findings suggest that *E. coli*

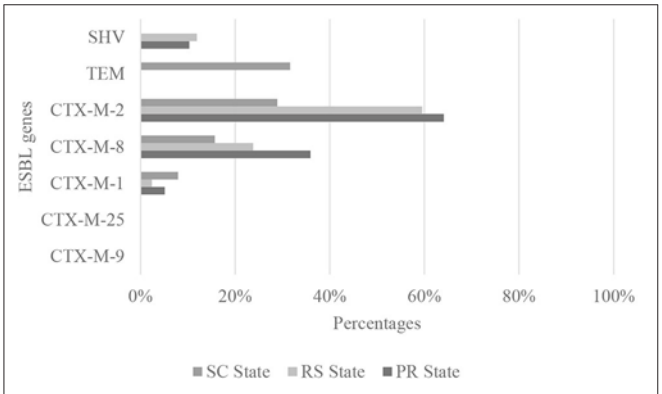

FIGURE 2 | Distribution of ESBL genes encoding CTX-Ms, TEM, and SHV detected in *E. coli* strains isolated from commercial chicken carcasses in southern Brazil from 2013 to 2014.

TABLE 3 | Risk factor analysis indicating that ESBL-producing *E. coli* harbor more virulence genes than non-ESBL-producing *E. coli*.

ExPEC virulence genes	ESBL *n* = 119	Non-ESBL *n* = 290	Relative risk (95% CI)	[a]*p*-value
None	15	105	0.35 (0.21–0.57)	<0.01
1 gene	28	52	1.31 (0.87–1.97)	0.195
2 genes	8	15	1.29 (0.57–2.98)	0.537
3–5 genes	68	118	1.40 (1.13–1.73)	<0.01

[a]*p < 0.05 by Fisher's exact test and Pearson's Chi-square test.*

TABLE 2 | Phylogenetic distribution of 290 non-ESBL-producing *E. coli* strains and 119 ESBL-producing *E. coli* strains isolated from chicken carcasses from different southern Brazilian states.

Phylogenetic groups	Southern Brazilian states—N° of strains (%)								
	PR			SC			RS		
(N° of strains)	Non-ESBL	ESBL	Total	Non-ESBL	ESBL	Total	Non-ESBL	ESBL	Total
A (130)	24 (29.3)	11 (28.2)	35 (28.9)	39 (40.2)	11 (28.9)	50 (37.0)	34 (30.6)	11 (26.2)	45 (29.4)
B1 (150)	33 (40.2)	12 (30.8)	45 (37.2)	33 (34.0)	13 (34.2)	46 (34.1)	46 (41.4)	13 (30.9)	59 (38.6)
B2 (14)	4 (4.9)	1 (2.6)	5 (4.1)	1 (1.0)	2 (5.3)	3 (2.2)	4 (3.6)	2 (4.8)	6 (3.9)
D (115)	21 (25.6)	15 (38.5)	36 (29.8)	24 (24.7)	12 (31.6)	36 (26.7)	27 (24.3)	16 (38.1)	43 (28.1)
	82 (100)	39 (100)	121 (100)	97 (100)	38 (100)	135 (100)	111 (100)	42 (100)	153 (100)

present in chicken meat, which could act as a reservoir for these antimicrobial resistance and virulence genes could be a potential risk for colonization and/or transfer of this resistance to bacteria in the intestinal tracts of humans.

Despite the importance of identifying ESBL-producing *E. coli* belonging to phylogenetic group D, which is commonly associated with strains found in hospitals and ambulatory patients (Pietsch et al., 2017), the detection of commensal strains from group B1 is also notable. Although transferable isolates belonging to phylogenetic group B1 do not comprise the most virulent phylogenetic group (such as B2 or D), these strains still harbor both virulence and resistance genes. Therefore, chicken meat could serve as an important reservoir for resistance genes and could be responsible for the spread of MDR bacteria via the food chain.

CONCLUSION

Our results highlight the high prevalence of ExPEC virulence genes and antimicrobial resistance genes associated with chicken meat. Brazil is the largest exporter of chicken meat and the second largest producer of chicken meat worldwide. These findings further represent a public health concern, considering that chicken meat could serve as a reservoir for the spread of plasmids harboring resistance and virulence genes through the food chain. Future studies should investigate whether both, resistance and virulence genes are transferred together to other bacteria and determine whether they are present in the same plasmid.

AUTHOR CONTRIBUTIONS

PC contributed to the development of experimental research, data analysis, and preparation of the article. VK, BB, and KB contributed to the development of experimental research. EN contributed to the statistical analysis. RK, GN, KB, BB, SH, and CD contributed to and assisted in the design of the work, assisted in critical data interpretation, and in preparation of the article. All authors have participated in this study and commented on the manuscript.

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High Prevalence of Multidrug-Resistant *Klebsiella pneumoniae* Harboring Several Virulence and β-Lactamase Encoding Genes in a Brazilian Intensive Care Unit

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Klebsiella pneumoniae is an important opportunistic pathogen that commonly causes nosocomial infections and contributes to substantial morbidity and mortality. We sought to investigate the antibiotic resistance profile, pathogenic potential and the clonal relationships between *K. pneumoniae* (n = 25) isolated from patients and sources at a tertiary care hospital's intensive care units (ICUs) in the northern region of Brazil. Most of *K. pneumoniae* isolates (n = 21, 84%) were classified as multidrug resistant (MDR) with high-level resistance to β-lactams, aminoglycosides, quinolones, tigecycline, and colistin. All the 25 isolates presented extended-spectrum beta-lactamase-producing (ESBL), including carbapenemase producers, and carried the bla_{KPC} (100%), bla_{TEM} (100%), bla_{SHV} variants (n = 24, 96%), bla_{OXA-1} group (n = 21, 84%) and $bla_{CTX-M-1}$ group (n = 18, 72%) genes. The K2 serotype was found in 4% (n = 1) of the isolates, and the K1 was not detected. The virulence-associated genes found among the 25 isolates were *mrk*D (n = 24, 96%), *fim*H-1 (n = 22, 88%), *ent*B (100%), *iut*A (n = 10, 40%), *ybt*S (n = 15, 60%). The genes related with efflux pumps and outer membrane porins found were *AcrAB* (100%), *tol*C (n = 24, 96%), *mdt*K (n = 22, 88%), *Omp*K35 (n = 15, 60%), and *Omp*K36 (n = 7, 28%). ERIC-PCR was employed to determine the clonal relationship between the different isolated strains. The obtained ERIC-PCR patterns revealed that the similarity between isolates was above 70%. To determine the sequence types (STs) a multilocus sequence typing (MLST) assay was used. The results indicated the presence of high-risk international clones among the isolates. In our study, the wide variety of MDR *K. pneumoniae* harboring β-lactams and virulence genes strongly suggest a necessity for the implementation of effective strategies to prevent and control the spread of antibiotic resistant infections.

Keywords: *Klebsiella pneumoniae*, intensive care units, multi-drug resistance, β-lactams gene, virulence genes

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative opportunistic bacterium that causes infections in hospitalized or otherwise immunocompromised individuals (Gorrie et al., 2017). Currently, *K. pneumoniae* is showing a high resistance to a broad spectrum of drugs including beta-lactam antibiotics, fluoroquinolones, and aminoglycosides (Fair and Tor, 2014; Dsouza et al., 2017). This resistance is resulting in a growing worldwide problem regarding the choice of effective antibiotic treatment for hospital-acquired infections (Davies and Davies, 2010).

Antibiotics of the β-lactam group are commonly prescribed worldwide and include penicillins, cephalosporins, monobactams, and carbapenems (Samaha-Kfoury and Araj, 2003; Ur Rahman et al., 2018). The production of β-lactamase enzymes by the presence of β-lactam-insensitive cell wall transpeptidases, or the active expulsion of β-lactam molecules from Gram-negative bacteria represent the main indications of β-lactam antibiotic resistance (Wilke et al., 2005). Carbapenems are the β-lactams of choice for the treatment of infections caused by extended-spectrum beta-lactamase (ESBL)-producing bacteria (Karuniawati et al., 2013; Okoche et al., 2015), such as *K. pneumoniae*. These antibiotics are also considered the last resort for the management of life-threatening health-care-associated infections (Amjad et al., 2011). Unfortunately, bacterial resistance to carbapenems has been increased and is well documented (Paterson and Bonomo, 2005; World Health Organization [WHO], 2014), and has also been further complicated by the production of β-lactamases, efflux pumps, and mutations that alter the expression and/or function of porins and penicillin-binding proteins (PBPs) (Papp-Wallace et al., 2011).

Antimicrobial resistance is commonly related to the spread of transmissible plasmids and the acquisition of resistance genes that normally occur by horizontal gene transfer, which may also carry virulence determinants (Derakhshan et al., 2016). For pathogen survival, the acquisition of resistance and virulent traits is necessary (Da Silva and Mendonça, 2012), and some reports suggest that such may have an essential role in the pathogenesis of *K. pneumoniae* infections (Vila et al., 2011). Capsule, lipopolysaccharide (LPS), fimbriae (types 1 and 3), and siderophores are virulence factors that contribute to the pathogenicity of *K. pneumoniae*. *K. pneumoniae* strains can synthesize capsules of any of the serotypes *K1* to *K78;* however, K1 and K2 can also be associated with increased pathogenicity (Paczosa and Mecsas, 2016).

Here, we show the antibiotic resistance profile, pathogenic potential, and clonal relationships among *K. pneumoniae* isolated from patients and sources at a tertiary care hospital's intensive care units (ICUs) in the northern region of Brazil.

MATERIALS AND METHODS

Bacterial Strains

Twenty-five *K. pneumoniae* clinical isolates were collected from patients and devices at a tertiary care hospital's ICUs in the state of Tocantins, located in the northern region of Brazil, between January 2014 and May 2015. All *K. pneumoniae* were collected at the bed-side, and then transported to the microbiology laboratory immediately for inoculation on proper culture media and preliminary analysis. Thereafter, the bacterial cultures were sent to the Central Laboratory of Public Health of Tocantins (LACEN/TO), a reference unit from the Brazilian Ministry of Health that receives samples for surveillance of antimicrobial resistance and which is usually located in the capital city of each federal state of Brazil. Strains were isolated from the following sources: tracheal aspirate, rectal swab, surgical drain, wound, catheter tip, cerebrospinal fluid, abscess, urine, and sputum.

Ethics Statement

In this work, all *K. pneumoniae* and the anonymous archival data related patient age, gender, and sample type were obtained from LACEN/TO (data's owner). The study was approved by the Committee of Ethics in Human Research of the Federal University of São Carlos (no. 1.088.936). Permission to conduct the present study was obtained from the Health Department of the State of Tocantins (Secretaria da Sauìde do Estado do Tocantins – SESAU) and LACEN/TO. Patient consent was not required, since the data presented in this study do not relate to any specific person or persons.

Phenotypic Detection of Antibiotic Resistance and Carbapenemase Productions

The identification of *K. pneumoniae* and the evaluation of their susceptibility profiles were performed using the VITEK 2 system (bioMérieux, Inc., Hazelwood, MO, United States) following the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute [CLSI], 2017). All *K. pneumoniae* was tested for their resistance against the following 15 antibiotics: ampicillin/sulbactam (SAM), piperacillin/tazobactam (TZP), cefuroxime (CXM), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), ertapenem (ERP), imipenem (IMP), meropenem (MEM), amikacin (AMK), gentamicin (GEN), ciprofloxacin (CIP), tigecycline (TGC), and colistin (CST). Susceptibility to TGC was interpreted using breakpoints proposed by the European Committee on Antimicrobial Susceptibilities Testing (EUCAST)[1].

Determination of the production of carbapenemase was carried out by modified Hodge test, synergy test, and the ethylenediaminetetraacetic acid (EDTA) test under the CLSI guidelines (Clinical and Laboratory Standards Institute [CLSI], 2017) and as described elsewhere (Miriagou et al., 2010; Nordmann et al., 2011; Okoche et al., 2015).

Multidrug-resistant (MDR) *K. pneumoniae* isolates were defined by non-susceptibility to at least one agent in three or more antibiotic categories (Magiorakos et al., 2012).

[1]http://www.eucast.org/clinical_breakpoints/

Genomic DNA Extraction

Genomic DNA was extracted from an overnight culture using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, United States). The concentration of the DNA extract and purity was determined by measuring absorbance at wavelengths of 260 nm and 280 nm (NanoVue Plus; GE Healthcare Life Sciences, Marlborough, MA, United States). The integrity of genomic DNA was tested by way of electrophoresis.

Detection of Multidrug Resistance Genes

The detection of resistance genes was performed by polymerase chain reaction (PCR) and their identities confirmed by sequencing. Isolates were screened by PCR amplification using specific primers for the detection of ESBL-encoding genes (bla_{TEM}; bla_{SHV}; $bla_{\mathrm{CTX-M}}$; and $bla_{\mathrm{OXA1,4,and30}}$), carbapenemases genes (bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and bla_{OXA48}), a tetracycline resistance gene (*tetB*), and a CST resistance gene (*mcr-1*). Moreover, efflux pump (*AcrAB*, *mdtK*, and *ToIC*), and porin-coding (*Omp*K35 and *Omp*K36) genes were also investigated. The specific primers (Exxtend, São Paulo, Brazil) and the length of expected PCR products are presented in **Table 1**. Amplicons were analyzed by gel electrophoresis in 1.5% agarose and visualized under ultraviolet (UV) light. The forward primers were used for DNA sequencing.

Serotypes and Virulence-Associated Genes Detection

Polymerase chain reaction was used to detect the presence of capsule serotypes (K1 and K2), and virulence-associated genes. These virulence-associated genes included those encoding for regulators of mucoid phenotype A (*rmp*A), type 1 and type 3 adhesins (*fim*H-1 and *mrk*D), enterobactin (*ent*B), yersiniabactin (*Ybt*S), and aerobactin siderophore system (*iut*A). Isolated DNA samples were screened using specific primers (Exxtend, São Paulo, Brazil) for the detection of virulence genes (**Table 2**). The forward primers were used for DNA sequencing.

Sequence Analysis of Resistance and Virulence Genes

The PCR products were extracted from agarose gels, using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, IL, United States), and some of them were randomly selected for DNA Sanger sequencing (Macrogen Inc., Korea). The nucleotide sequences of the corresponding genes of the isolates were submitted to the GenBank database with accession numbers MK106173 to MK106187. The sequences were edited with Ugene v1.18.0 (Okonechnikov et al., 2012). Each sequence was compared using BlastN tools[2] with the *K. pneumoniae* genome as the reference. Access to genetic heritage was approved by the National System for the Management of Genetic Heritage (SisGen) (no. AFF27ED).

Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction

Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) analysis was performed to evaluate the genetic similarity among the bacterial isolates used in this study. ERIC-PCR reactions were executed as previously described by Versalovic et al. (1994), using the primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′). All amplifications were carried out in a total volume of 50 μL, using the enzyme TaKaRa Ex Taq® DNA Polymerase (Takara Bio, Kusatsu, Japan), while standardizing the amount of 100 ng of DNA template for each isolate. The amplified products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide using UV radiation for visualization of the bands. The band profile analysis was performed using the BioNumerics program version 5.1 (Applied Maths, Keistraat, Belgium) for construction of the similarity dendrogram by the unweighted pair group mean method, Dice's similarity coefficient, and 1% band position tolerance. Only bands representing amplicons between 300 bp and 3,000 bp were considered for this analysis. The ERIC-PCR assays were performed in triplicate.

MLST

Ten isolates belonging to the main clusters of the dendrogram obtained by ERIC-PCR were selected for multilocus sequence typing (MLST). Information on the methodology used, including the primers and PCR reaction conditions, is available in the MLST database for *K. pneumoniae*[3]. The alleles and sequence types (STs) of each isolate studied by MLST were determined using the MLST database platform for *K. pneumoniae*.

The determination of the clonal and epidemiological relationships and the formation of clonal complexes (CCs), were completed by analyzing a genetic similarity diagram constructed with the aid of the eBURSTv3 program (eBURSTv3 has been developed and is hosted at The Department of Infectious Disease Epidemiology Imperial College London) (Feil et al., 2004).

Statistical Analysis

The statistical analysis was performed using Fisher's exact test ($p \leq 0.05$).

RESULTS

Antibiotic Resistance Patterns

In the present study, a total of 25 *K. pneumoniae* strains were isolated from samples collected from ICUs patients and devices of a tertiary hospital located in the northern region of Brazil. Most *K. pneumoniae* isolates were obtained from a rectal swab (56%; $n = 14$), followed by tracheal aspirate (16%, $n = 4$), urine (4%, $n = 1$), cerebrospinal fluid (4%, $n = 1$), wound (4%, $n = 1$), sputum (4%, $n = 1$), abscess (4%, $n = 1$), surgical drain (4%, $n = 1$), and catheter tip (4%, $n = 1$). A statistical difference was found

[2]https://blast.ncbi.nlm.nih.gov/

[3]http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html

TABLE 1 | Sequences of primes used for detection of resistance genes and outer membrane porins.

Resistance targeted	Sequence (5'–3'), F/R	T_m (°C)	Amplicon size (bp)	Reference
bla_{KPC}	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	61,3	797	Poirel et al., 2011
bla_{TEM}	TGCGGTATTATCCCGTGTTG TCGTCGTTTGGTATGGCTTC	63	296	Xiong et al., 2007
$bla_{CTX-M-1group}$, (including $bla_{CTX-M-1,3,\ 10,\ 11and12}$)	ACAGCGATAACGTGGCGATG TCGCCCAATGCTTTACCCAG	64	216	Xiong et al., 2004
$bla_{SHVvariants}$	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	55,6	712	Dallenne et al., 2010
$bla_{OXA-1,4and30}$	GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	63	563	Dallenne et al., 2010
bla_{OXA-48}	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	55	438	Poirel et al., 2011
bla_{IMP}	CTACCGCAGCAGAGTCTTTGC ACAACCAGTTTTGCCTTACC	55	587	Martins et al., 2007
bla_{VIM}	AAAGTTATGCCGCACTCACC TGCAACTTCATGTTATGCCG	55	865	Yan et al., 2001
bla_{NDM}	GCAGCTTGTCGGCCATGCGGGC GGTCGCGAAGCTGAGCACCGCAT	60	782	Doyle et al., 2012
*gyr*A	TACCGTCATAGTTATCCACGA GTACTTTACGCCATGAACGT	61,3	387	Wiuff et al., 2000
*tet*B	CAGTGCTGTTGTTGTCATTAA GCTTGGAATACTGAGTGTAA	59,7	571	Call et al., 2003
mcr-1	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	51,6	309	Liu et al., 2015
*Acr*AB	ATCAGCGGCCGGATTGGTAAA CGGGTTCGGGAAAATAGCGCG	58	312	Wasfi et al., 2016
*Tol*C	ATCAGCAACCCCGATCTGCGT CCGGTGACTTGACGCAGTCCT	61	525	Wasfi et al., 2016
*mdt*K	GCGCTTAACTTCAGCTCA GATGATAAATCCACACCAGAA	52	453	Wasfi et al., 2016
*Omp*K35	CTCCAGCTCTAACCGTAGCG GGTCTGTACGTAGCCGATGG	58	241	Wasfi et al., 2016
*Omp*K36	GAAATTTATAACAAAGACGGC GACGTTACGTCGTATACTACG	48	305	Wasfi et al., 2016

TABLE 2 | Sequences of primers used for detection of virulence genes.

Gene	Primer sequence (5'–3'), F/R	Amplicon size (bp)	T_m (°C)	Reference
*rmp*A	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	535	54	Siu et al., 2011
*fim*H-1	TGCTGCTGGGCTGGTCGATG GGGAGGGTGACGGTGACATC	550	61	Schembri et al., 2005
*mrk*D	CCACCAACTATTCCCTCGAA ATGGAACCCACATCGACATT	226	54	El Fertas-Aissani et al., 2013
*iut*A	GGGAAAGGCTTCTCTGCCAT TTATTCGCCACCACGCTCTT	920	56	Compain et al., 2014
*ent*B	CTGCTGGGAAAAGCGATTGTC AAGGCGACTCAGGAGTGGCTT	385	57	Wasfi et al., 2016
*ybt*S	GACGGAAACAGCACGGTAAA GAGCATAATAAGGCGAAAGA	242	52	Compain et al., 2014
K1	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	1283	47	Fang et al., 2007
K2	GGATTATGACAGCCTCTCCT CGACTTGGTCCCAACAGTTT	908	45	Fang et al., 2007

only between the rectal swab and tracheal aspirate for isolates with resistance to the antibiotic TGC (**Supplementary Table S1**). Patients ages ranged from 1 day to 75 years (median age: 39 years old), and no significant differences were found regarding age group or gender and anti-microbial resistance. *K. pneumoniae* strains tested were resistant to all β-lactams (SAM, TZP, CXM-S, CXM, FOX, CAZ, CRO, FEP, ETP, IPM, MEM). These isolates also showed different degrees of resistance to other antibiotics like GEN (80%, n = 20), CIP (64%, n = 16), TGC (52%, n = 13) CST (36%, n = 9), and AMK (4%, n = 1). Demographic characteristics of the patients and antibiotic resistance profiles of the *K. pneumoniae* isolates to the 16 antibiotics tested are shown in **Table 3**.

Detection of Genes Coding for Outer Membrane Porins and Multidrug-Resistant Efflux Pumps and Antimicrobial Susceptibility

The majority of isolates (84%, 21/25) were classified as MDR with high-level resistance to at least one agent in three or more antibiotic categories. Among the MDR *K. pneumoniae*, all (100%, 21/21) isolates contained both *Arc*AB and *Tol*C efflux pumps genes; 86% (18/21) had *AcrAB, mdtK,* and *ToIC* genes, simultaneously; and only 14% (3/21) of isolates did not present with the *mdtK* multidrug efflux gene. PCR results showed that 33% (7/21) of isolates lacked both *Omp*K35 and *Omp*K36 porin genes, while 38% (8/21) of isolates lacked the *Omp*K36 gene.

Of the four isolates (Kp2, Kp67, Kp74, and Kp75) that did not show MDR profiles, three (Kp2, Kp74, and Kp75) had the *Acr*AB, *mdt*K and *ToI*C genes but not the *Omp*K35 and *Omp*K36 porin genes and one isolate (Kp67) carried both the *Acr*AB, and *mdtK* efflux pumps genes and the *Omp*K35 and *Omp*K36 porin genes. The antibiotic resistance profiles of the *K. pneumoniae* isolates are presented in **Table 4**. PCR amplification results for these genes are shown in **Supplementary Figure S1**.

Antibiotic Resistance and Virulence-Associated Genes Detection

The distributions of the antibiotic resistance gene and virulence factors are shown in **Table 5**. All the 25 isolates were positive for the bla_{KPC} gene. In addition, the *K. pneumoniae* isolates carried the bla_{TEM} (100%, n = 25), bla_{SHV} group (96%, n = 24), $bla_{\mathrm{OXA-1}}$ group (84%, n = 21), and $bla_{\mathrm{CTX-M-1}}$ group (72%, n = 18) ESBL-encoding genes. The bla_{IMP}, $bla_{\mathrm{OXA-48}}$, bla_{NDM}, bla_{VIM}, *mcr-1* and *tet*(B) genes were not detected. It was found that a high number of bla_{SHV} in this study that may be associated with the presence of $bla_{\mathrm{SHV-1}}$, which it is reported to be universal in *K. pneumoniae* infection (Babini and Livermore, 2000). Additional PCR amplification results are shown in **Supplementary Figures S2, S3**.

Polymerase chain reaction analysis demonstrated that the *fim*H-1 and *mrk*D genes, encoding type 1 and type 3 fimbrial adhesins, were present in 88% (22/25) and 96% (24/25) of isolates, respectively. Additionally, the enterobactin (*ent*B) gene was found in 100% (25/25), the yersiniabactin (*ybt*S) gene in 60% (15/25) and the aerobactin siderophore system (*iut*A) gene in 40% (10/25) of isolates.

TABLE 3 | Characteristics of the patients and antibiotic resistance profile of the *K. pneumoniae*.

Characteristic	% (*n*)	Antibiotics	% (*n*) profile	
Sex		**Beta lactams**		
Female	44.0 (11)	(SAM, TZP, CXM-S, CXM, FOX, CAZ,	100.0 (25)	R
Male	56.0 (14)	CRO, FEP, ETP, IPM, MEM)		
Age (years)				
0–18	28.0 (7)	**Gentamycin**	80.0 (20)	R
19–59	36.0 (9)	(GEN)	20.0 (5)	S
60 or more	36.0 (9)			
Sample type		**Amikacin**	4.0 (1)	R
Tracheal aspirate	16.0 (4)	(AMK)	96.0 (24)	S
Rectal swab	56.0 (14)			
Drain	4.0 (1)	**Ciprofloxacin**	64.0 (16)	R
Wound	4.0 (1)	(CIP)	36.0 (9)	S
Catheter tip	4.0 (1)			
Cerebrospinal fluid	4.0 (1)		52.0 (13) 48.0 (12)	
Abscess	4.0 (1)	**Tigecycline**		R
Urine	4.0 (1)	(TGC)		S
Sputum	4.0 (1)			
		Colistin	36.0 (9)	R
		(CST)	64.0 (16)	S

Antibiotics: SAM (ampicillin-sulbactam), TZP (piperacillin-tazobactam), CXM-S (cefuroxime sodium), CXM (cefuroxime axetil), FOX (cefoxitin), CAZ (ceftazidime), CRO (ceftriaxone), FEP (cefepime), ETP (ertapenem), IPM (imipenem), MEM (meropenem), GEN (gentamicin), AMK (amikacin), CIP (ciprofloxacin), TGC (tigecycline), CST (colistin). Profile: R, resistance rate; S, sensitivity rate; n, number.

TABLE 4 | Antimicrobial resistance of *Klebsiella pneumoniae* isolates and presence of genes coding for outer membrane porins and efflux pumps.

Isolate no.	Antimicrobial resistance	MDR	Genes coding for porins and efflux pumps				
			OmpK35	*Omp*K36	*TolC*	*AcrA*B	*mdt*K
Kp1	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip, tgc	+	+	+	+	+	+
Kp2*	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen	–	–	–	+	+	+
Kp3	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, amk, gen, cip, tgc, cst	+	+	–	+	+	+
Kp4	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cst	+	–	–	+	+	+
Kp6	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip, tgc	+	+	–	+	+	+
Kp7	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip, tgc	+	+	+	+	+	+
Kp8	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, cip, tgc	+	+	–	+	+	+
Kp16	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip, tgc	+	+	+	+	+	+
Kp17	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cst	+	–	–	+	+	+
Kp21	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip	+	+	+	+	+	–
Kp25	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip, tgc, cst	+	–	–	+	+	+
Kp27	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, cip, tgc, cst	+	+	–	+	+	+
Kp39	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip, tgc	+	+	–	+	+	+
Kp53	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip, tgc, cst	+	–	–	+	+	+
Kp60	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cst	+	–	–	+	+	+
Kp62	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip, tgc	+	–	–	+	+	+
Kp66	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip	+	+	–	+	+	–
Kp67*	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem	–	+	+	–	+	+
Kp68	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, cip, tgc	+	+	+	+	+	+
Kp69	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cst	+	–	–	+	+	+
Kp70	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, cip, tgc	+	+	+	+	+	+
Kp73	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cst	+	+	–	+	+	+
Kp74*	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen	–	–	–	+	+	+
Kp75*	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen	–	–	–	+	+	+
Kp77	sam, tzp, cxm, cxm-s, fox, caz, cro, fep, etp, ipm, mem, gen, cip	+	+	–	+	+	–

*Antibiotics. β-lactams: SAM (ampicillin-sulbactam), TZP (piperacillin-tazobactam), CXM-S (cefuroxime sodium), CXM (cefuroxime axetil), FOX (cefoxitin), CAZ (ceftazidime), CRO (ceftriaxone), FEP (cefepime), ETP (ertapenem), IPM (imipenem), MEM (meropenem); aminoglycosides: GEN (gentamicin) and AMK (amikacin); quinolones: CIP. (ciprofloxacin); glycylcycline: TGC (tigecycline) and polymyxin E: CST (colistin). MDR (multidrug-resistant) = resistance to at least one agent in three or more antibiotic categories. *Isolates that did not susceptible to at least three categories of antimicrobials.*

TABLE 5 | Distribution of serotypes, resistance and virulence genes in *K. pneumoniae* strains.

		Resistance genes											Virulence genes										
		bla$_{KPC}$	*bla*$_{OXA}$	*bla*$_{OXA-48}$	*bla*$_{VIM}$	*bla*$_{IMP}$	*bla*$_{NDM}$	*bla*$_{TEM}$	*bla*$_{SHVvariants}$	*bla*$_{CTX-M1group}$	*mcr-1*	*tetB*	*fimH-1*	*mrkD*	*entB*	*ybtS*	*iutA*	*RmpA*	*TolC*	*AcrAB*	*mdtK*	K1	K2
Isolate	**Sample type**																						
Kp1	Rectal swab	•	•					•	•	•			•	•	•				•	•	•		
Kp2	Rectal swab	•	•					•	•	•				•	•	•	•		•	•	•		
Kp3	Rectal swab	•	•					•	•				•	•	•				•	•	•		
Kp4	Urine	•	•					•	•	•			•		•	•			•	•	•		
Kp6	Rectal swab	•						•	•				•	•	•	•			•	•	•		
Kp7	Rectal swab	•	•					•	•	•			•	•	•				•	•	•		
Kp8	Rectal swab	•	•					•	•				•	•	•				•	•	•		
Kp16	Rectal swab	•	•					•	•	•			•	•	•				•	•	•		
Kp17	Rectal swab	•	•					•	•	•			•	•	•	•	•		•	•	•		
Kp21	Tracheal aspirate	•	•					•	•	•			•	•	•				•	•			
Kp25	Rectal swab	•	•					•	•	•			•	•	•	•	•		•	•	•		
Kp27	Rectal swab	•						•	•				•	•	•				•	•	•		•
Kp39	Rectal swab	•	•					•	•	•			•	•	•	•	•		•	•	•		
Kp53	Rectal swab	•	•					•	•	•			•	•	•	•	•		•	•	•		
Kp60	Cerebrospinal fluid	•	•					•	•	•			•	•	•	•	•		•	•	•		
Kp62	Drain	•	•					•	•	•			•	•	•	•	•		•	•	•		
Kp66	Catheter tip	•	•					•	•				•	•	•				•	•			
Kp67	Tracheal aspirate	•						•					•	•	•					•	•		
Kp68	Rectal swab	•	•					•	•				•	•	•	•	•		•	•	•		
Kp69	Wound	•	•					•	•	•			•	•	•	•	•		•	•	•		
Kp70	Sputum	•						•	•	•				•	•				•	•	•		
Kp73	Abscess	•	•					•	•	•			•	•	•				•	•	•		
Kp74	Rectal swab	•	•					•	•	•				•	•	•	•		•	•	•		
Kp75	Tracheal aspirate	•	•					•	•	•			•	•	•				•	•	•		
Kp77	Tracheal aspirate	•	•					•	•	•			•	•	•				•	•			
Genes present (%)		100	84	0	0	0	0	100	96	72	0	0	88	96	100	60	40	0	96	100	88	0	4

The regulators of the mucoid phenotype A (*rmp*A) gene were not detected. Only one isolate (4%), recovered from swab rectal, presented the capsular serotype K2, and the capsular K1 was not found (**Table 5** and **Supplementary Figure S1**).

Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction

Genetic similarity among isolates was evaluated via ERIC-PCR, and the results indicated the vast majority of the isolates presented a rate of genetic similarity above 70%, separated into two main clusters (A and B) (**Figure 1**). Three isolates (Kp53, Kp60, and Kp62) showed 100% genetic similarity. Only four isolates (Kp4, Kp7, Kp17, and Kp67) were genetically more distant and did not cluster with the other isolates.

MLST

Multilocus sequence typing analysis demonstrated five different STs among 10 selected isolates (**Figure 1**). Four isolates (Kp4, Kp17, Kp60, and Kp65) belonged to ST29, which was the most predominant group. Furthermore, two isolates (Kp7 and Kp66) belonged to ST392, one isolate (Kp27) belonged to ST25, and another one (Kp3) belonged to ST11. The isolate Kp68 presented a novel ST by way of a new allele combination, which was named ST3373. It was not possible to analyze the isolate Kp67 by MLST because it did not show amplification for the *ton*B gene, even after several attempts and adjustments in the reaction.

The eBurst analysis showed that most of the STs (STs 11, 25, 29, and 3373) found were distributed in a more massive clonal complex called CC258 (also called CC258/11). Only the ST392 group, including isolates Kp7 and Kp66, was present into a smaller clonal complex, called CC147 (**Figure 2**).

DISCUSSION

Although *K. pneumoniae* is considered to be an important opportunistic pathogen and a frequent cause of hospital-acquired infections (Struve and Krogfelt, 2004), it is also found in non-clinical habitats, which include the mucosal surfaces of humans and animals, and environmental sources such as water, soil, sewage, and vegetation (Bagley, 1985; Podschun et al., 2001). Previous studies have shown that *K. pneumoniae* strains of environmental origin are similar to those strains of clinical origin in terms of biochemical patterns, virulence, and pathogenicity (Podschun et al., 2001; Struve and Krogfelt, 2004); however, clinical *K. pneumoniae* are significantly more resistant to antibiotics as compared with environmental *K. pneumoniae* (Matsen et al., 1974).

In our study, the vast majority (84%, 21/25) of *K. pneumoniae* isolates showed MDR patterns including a high resistance rate to the common antibiotics used either alone or in association with one another to treat *K. pneumoniae* infections, such as β-lactams (including carbapenems), aminoglycosides, quinolones, glycylcycline, and polymyxin E. Although the high prevalence of MDR *K. pneumoniae* patterns was similar to other results in previous studies (Pereira et al., 2013; Paneru, 2015; Wasfi et al., 2016), this is the first report of a high incidence of MDR *K. pneumoniae* in the state of Tocantins, Brazil. There are many possible contributing factors to the emergence, rise, and spread of antibiotic resistance, including the new acquisition of resistance genes; transfer of antibiotic resistance genes; healthcare exposure; use of indwelling medical devices; limited diagnostic facilities; lack of effective and reliable surveillance systems; immunosuppressed states; travel to areas with a high endemicity of MDR bacteria; lack of new antimicrobial therapeutics; and inappropriate and excessive antibiotic use in health care, food-producing animals, and agriculture (Fletcher, 2015; Vila, 2015; Ayukekbong et al., 2017; Martin and Bachman, 2018; Patolia et al., 2018). Therefore, many of these risk factors may have contributed to the high rates of antibiotic resistance found in our study.

The high rates of resistance to polymyxin E (i.e., CST) and glycylcycline (i.e., TGC) found in our study deserves particular attention because these antibiotic categories have typically been used as the drugs of last resort for the treatment of severe infections caused by *Klebsiella pneumoniae* carbapenemase (KPC)-producing organisms (Pereira et al., 2013). Previous studies have reported that high levels of CST are frequently administered in Brazilian ICUs, mainly after bacteria isolates have become resistant to almost all other available antibiotics (Furtado et al., 2007; Rossi, 2011). Therefore, the overuse and misuse of antibiotics can be associated with an increase of the occurrence of CST resistance found in the current study. The TGC resistance might be due to the presence of the *Acr*AB gene, which encodes the efflux pump *Acr*AB and is considered to be one of the main contributors to a reduced susceptibility to TGC in *K. pneumoniae* clinical isolates (Bialek-Davenet et al., 2015; Wang et al., 2015; Elgendy et al., 2018). In this study, we also found that several TGC-resistant bacteria were isolated from rectal swabs, showing an important association between pathogen-specific and local antibiotic resistance patterns.

K. pneumoniae produces two classics trimeric porins, *Omp*K35 and *Omp*K36, which allow the passage of small hydrophilic molecules such as iron, nutrients, and antibiotics through the outer cell membrane (Tsai et al., 2011). In our study, 28% of all *K. pneumoniae* isolates lacked the *Omp*K36 gene. Our findings are in agreement with those of other authors who reported that the absence of *Omp*K35 or *Omp*K36 can be responsible for resistance to carbapenems in *K. pneumoniae* that produced ESBL (Hernandez-Alles et al., 1999; Wang et al., 2009; Skurnik et al., 2010). The loss of both porins *Omp*K35 and *Omp*K36 produces an increase in carbapenem, CIP, and chloramphenicol resistance (Kaczmarek et al., 2006). However, some of our results are not in complete agreement with the literature, as the presence of *Omp*K35 and *Omp*K36 genes were correlated with both carbapenem and CIP resistance, in 28% of MDR *K. pneumoniae* isolates. In contrast, other studies have suggested that the presence of both porins (*Omp*K35 and *Omp*K36) in MDR isolates can be associated with the presence of point mutations, disruption in the protein coding sequence, or promoter region mutations (Doumith et al., 2009; Wasfi et al., 2016). Further investigations should be performed to evaluate the presence of the mutations in bacteria strains isolated in this study.

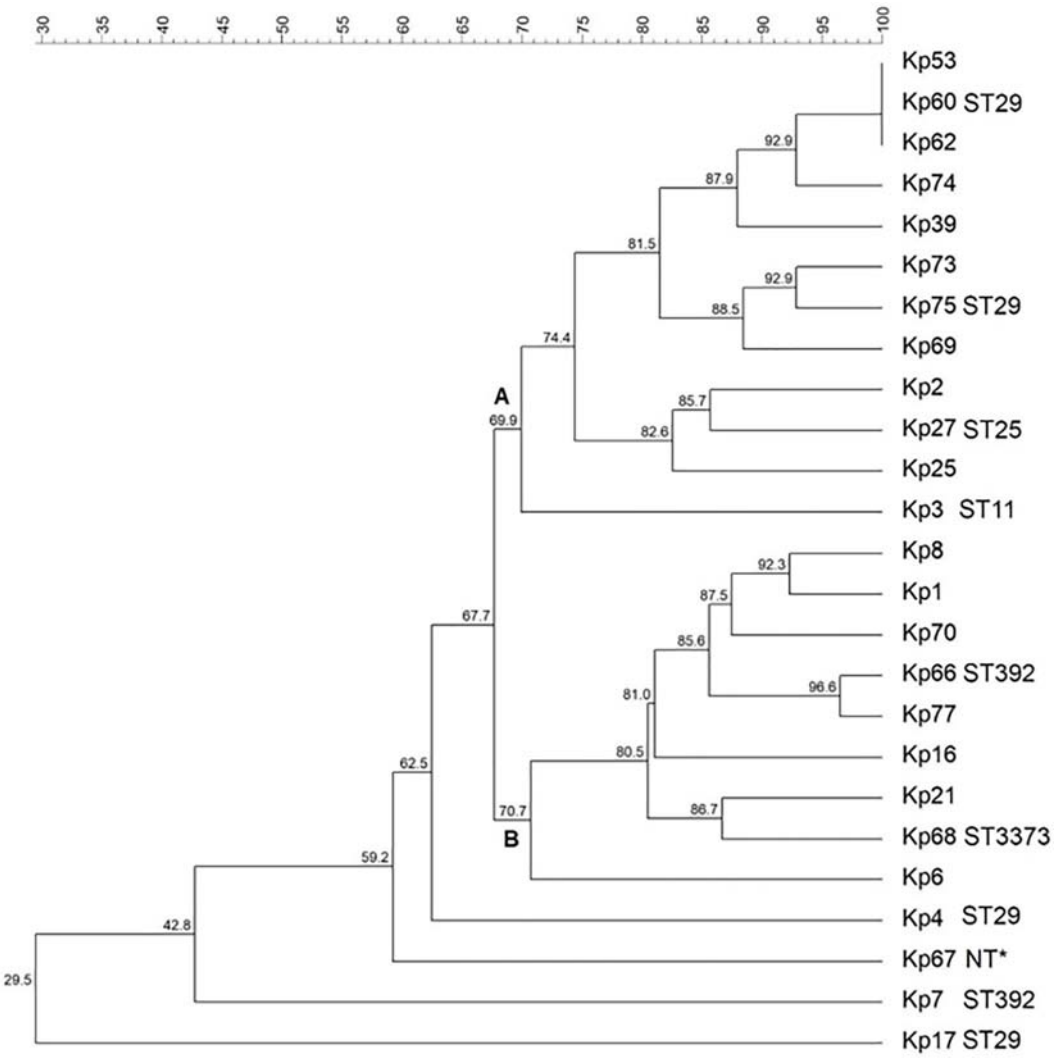

FIGURE 1 | Dendrogram representing the genetic relationship among the 25 *Klebsiella pneumoniae* studied. Clusters were determined using the Unweighted Pair Group Mean (UPGMA) method and the Dice similarity coefficient. Similarity (%) among patterns is represented by the numbers beside the nodes. For each isolate typed by MLST, their respective sequence types (STs) are represented. *NT, not typed by MLST.

Efflux pump systems have been reported as essential mechanisms of resistance and cause of MDR in *K. pneumoniae* (Mahamoud et al., 2007; Meletis et al., 2012). In *K. pneumoniae,* the *Acr*AB and *mdtK* complexes are the best-characterized efflux pumps (Wasfi et al., 2016). Notably, in our research, the presence of *Acr*AB-*Tol*C and *mdt*K genes were strongly associated with MDR *K. pneumoniae* patterns. These results are consistent with other previous studies, that demonstrated that the multidrug efflux pump system (*Acr*AB-*Tol*C) in *K. pneumoniae* was responsible for resistance to quinolones, tetracyclines, TGC, and beta-lactams in various MDR isolates (Padilla et al., 2010; Yuhan et al., 2016).

In *K. pneumoniae*, the genes *fim*H and *mrk*D encode adhesins of type 1 and type 3 fimbriae, which mediate binding to the extracellular matrix; promote biofilm development (Hornick et al., 1992; Struve et al., 2008; Alcántar-Curiel et al., 2013; Fu et al., 2018); and may play a key role in colonization, invasion and pathogenicity (Shah et al., 2017). In the current study, the majority of the MRD *K. pneumoniae* isolates carried both *fimH*-1 and *mrk*D virulence genes. Although studies have reported that many clinical *K. pneumoniae* isolates normally express both type 1 and type 3 fimbrial adhesins (Sahly et al., 2008; Struve et al., 2009; Wasfi et al., 2016), one of the most important steps in the progression to *K. pneumoniae* infection is related to its ability to adhere to host surfaces and demonstrate persistent colonization. *Mrk*D specifically mediates binding to the extracellular matrix, facilitating the adherence of *K. pneumoniae* to damaged tissue and coating indwelling devices (François et al., 1998; Paczosa and Mecsas, 2016), such as urinary catheters (Schroll et al., 2010; Stahlhut et al., 2012) and endotracheal tubes (François et al., 1998). Type 3 fimbriae were found to play an essential role in *K. pneumoniae* biofilm formation (Langstraat et al., 2001;

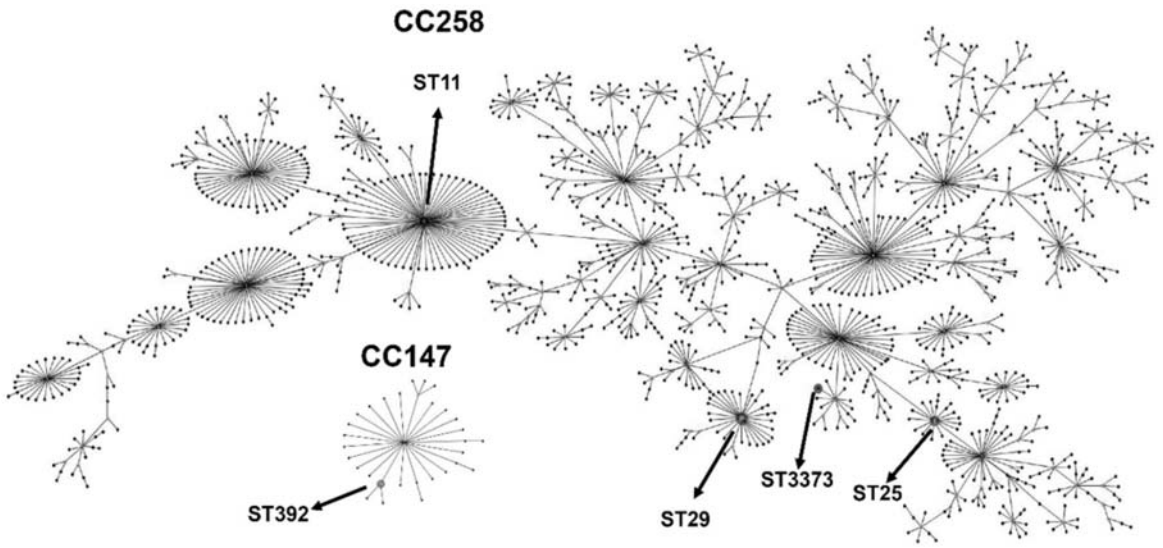

FIGURE 2 | eBURST diagram generated with the MLST data, representing the five different sequence types (STs) obtained in this study (indicated by arrows), distributed in two clonal complexes: CC258, with the STs 11, 29, 25 and the novel ST3373 and CC147, with the ST392. The remaining STs were omitted from the diagram to facilitate visualization. Each dot represents an ST.

Di Martino et al., 2003; Jagnow and Clegg, 2003; Schroll et al., 2010) and they can also mediate the binding of *K. pneumoniae* to endothelial cells and to epithelial cells of the respiratory and urinary tracts (Würker et al., 1990; Hornick et al., 1992; Tarkkanen et al., 1997). Type 1 fimbriae are expressed in 90% of both clinical and environmental *K. pneumoniae* isolates (Stahlhut et al., 2009); however, their precise role in the production of biofilms remains unclear (Paczosa and Mecsas, 2016). Type 1 fimbriae expressed by *K. pneumoniae* in particular cause urinary tract infections (Struve et al., 2008), and may play an important role in colonization of the intestine and in the delivery, entry, and persistence of *K. pneumoniae* in ventilator-associated pneumonia (Kollef, 2004; Struve et al., 2008; Kalanuria et al., 2014). Additionally, the presence of *mrk*D and *fim*H-1 has previously been associated with KPC-positive *K. pneumoniae* (De Cássia et al., 2014), which is in accordance with our findings. Although little is known regarding the potential virulence characteristics of KPC-producing *K. pneumonia* (Andrade et al., 2014; Liu Y. et al., 2014), studies have reported that ESBL-producing isolates of *K. pneumoniae* are able to produce more fimbrial adhesins, are more invasive, and are more resistant to the normal human serum bactericidal effect (Sahly et al., 2004). Therefore, the high frequency of *fim*H-1 (88%) and *mrk*D gene (96%) found in our results, illustrates the importance of evaluating these virulence factors.

The capsule is one of the most important virulence factors (Martin and Bachman, 2018) that protects *K. pneumoniae* from lethal serum factors and phagocytosis (Hsu et al., 2011). In *K pneumoniae*, capsular serotypes K1 and K2 have been considered as predominant virulent strains (Fung et al., 2002; Chuang et al., 2006). Studies using clinical samples have proposed that virulence factors such as K1, K2, K5, *rmp*A and the aerobactin gene, are absent in KPC-producing isolates (Siu et al., 2012). In agreement with these previous studies, our results showed that K1 and *rmp*A were not detected, K2 was present in only one isolate, K5 was not investigated, and all isolates were identified as KPC-producing *K. pneumoniae*. It is important to note that genes encoding *rmp*A, K1, or K2 were highly associated with the hypervirulent (hypermucoviscous) variant of *K. pneumoniae* (hvKP) (Fang et al., 2004; Yeh et al., 2007; Arena et al., 2017; Martin and Bachman, 2018), which causes serious community-acquired infection, and has emerged as a carbapenem-resistant hypervirulent *K. pneumoniae* (CR-HvKP) that can be found in clinical settings (Shon et al., 2013; Liu Y.M. et al., 2014; Zhang et al., 2015; Zhang Y. et al., 2016; Zhang R. et al., 2016). Therefore, this observation suggests that the *K. pneumoniae* in this study did not present molecular characteristics of the hypervirulent (hypermucoviscous) *K. pneumoniae*.

Siderophores are high-affinity, iron-chelating molecules that are critical for bacterial growth, replication, and virulence (Lawlor et al., 2007; Bachman et al., 2015; Holden and Bachman, 2015). The repertoire of siderophores differs among different strains (Behnsen and Raffatellu, 2016); thus, the role of each siderophore in virulence potential can vary (Paczosa and Mecsas, 2016; Lam et al., 2018). Siderophore-associated genes, such as *ent*B, *ybt*S and *iut*A are widely disseminated among *K. pneumonia* strains (Compain et al., 2014). However, *ent*B is only characterized for virulence when it occurs in association with *iut*A, *ybt*S, or *kfu* (Daehre et al., 2018). In agreement with previous studies, all *K. pneumoniae* carried the *ent*B gene (Lavigne et al., 2013; Fu et al., 2018); however, the presence of the genes encoding *ent*B in combination with *iut*A and *ybt*S was found in only 40%, while *ent*B with *ybt*S were found in 60% of all the strains, respectively. Although *K. pneumoniae* secretes a specific combination of siderophores, which can affect tissue localization, systemic spreading, and host survival, the effect of

these molecules on the host during infection is not clear (Holden et al., 2016).

Carbapenems are the antibiotic class of choice for the treatment of severe infections caused by *Enterobacteriaceae*-producing ESBLs (Jacoby and Munoz-Price, 2005). The primary determinant of carbapenem resistance in *K. pneumoniae* is KPC-type carbapenemases (Nordmann et al., 2011), which are encoded by the gene bla_{KPC} and located mainly on a Tn3-based transposon, Tn4401 (Bina et al., 2015), demonstrating exceptional potential to spread throughout the world. In our findings, the presence of bla_{KPC} in all *K. pneumoniae* isolates is in agreement with previous investigations, that suggest the wide dissemination of KPC-producing isolates in various regions of Brazil (Castanheira et al., 2012; Pereira et al., 2013; Biberg et al., 2015; Gonçalves et al., 2017). Besides, PCR analysis demonstrated that most bacteria (84%) coproduced the bla_{KPC} and bla_{OXA-1} group resistance genes. In Brazil, several studies have reported the co-occurrence of bla_{KPC} with the bla_{OXA-1} group in *K. pneumoniae* (Fehlberg et al., 2012; Flores et al., 2016). Furthermore, bla_{IMP}, bla_{VIM}, bla_{OXA48}, and bla_{NDM} are also genes that produce carbapenemases in *K. pneumoniae* (Lascols et al., 2012; Seibert et al., 2014); however, these genes were not found in our study.

Some reports have suggested that TEM (Temoniera), SHV (sulfhydryl variable), and CTX-M (cefotaxime-beta lactamases) are the primary genetic groups of ESBLs among clinically critical Gram-negative bacteria (Bradford, 2001; Paterson and Bonomo, 2005). Additional studies have indicated the presence of bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes in *K. pneumoniae* (Monteiro et al., 2009; Peirano et al., 2009; Seki et al., 2011; Fehlberg et al., 2012), which is in accordance with our results. Globally, the CTX-M type has appeared as the most common type of ESBL, and its incidence is easily surpassing those of SHV and TEM ESBLs in most locales (Jorgensen et al., 2010; Bora et al., 2014). Although our PCR analysis revealed that bla_{TEM} (100%) was the most frequent gene, followed by bla_{SHV} (96%), the presence of the bla_{CTX-M} (72%) group was also high, and can be related to the fluoroquinolone and aminoglycoside resistance (Pitout et al., 2005) found in this study. The co-production of bla_{KPC} with bla_{TEM} was detected in all isolates, while bla_{KPC}, bla_{OXA}, bla_{TEM}, bla_{SHV}, and bla_{CTX-M} were observed in 72% and bla_{KPC}, bla_{TEM}, bla_{SHV}, and bla_{CTX-M} were found in 68% of the *K. pneumoniae* isolates, respectively. Our results suggest that the high antimicrobial resistance found in this study can also be associated with the presence of these β-lactams genes.

Our ERIC-PCR results indicated that, although bacteria were isolated from different patients, the circulating *K. pneumoniae* in this hospital have a high genetic relationship to each other. Ten isolates belonging to the main ERIC-PCR clusters were analyzed by MLST, and four of them (Kp4, Kp17, Kp60, and Kp65) belonged to ST29. ST29 has previously been reported in *K. pneumoniae* strains from various parts of the world, such as Europe, Asia, Oceania, and also in Brazil. Uz Zaman et al. (1994) found ST29 in MDR *K. pneumoniae* carrying the OXA-48 gene that showed variations in outer membrane protein 36, causing an outbreak in a tertiary care hospital in Saudi Arabia. However, the isolates from our study with ST29 were negative for OmpK36 and OXA-48 (**Tables 4**, **5**). The ST25 has been described as being associated with virulent clones, especially belonging to the capsular serotypes K1 and K2 (McCulloh and Opal, 2018). In our study, the only isolate that presented the K2 antigen (Kp27) and various virulence genes also presented the ST25; thus, our findings corroborate with the prior research (**Table 5**). ST11, found in the isolate Kp3, has been described as widespread in Brazil and is considered an international high-risk clone (Gonçalves et al., 2017).

eBURST analysis showed that, except for ST392, all other STs belong to the large clonal complex CC258. Commonly, *K. pneumoniae* isolates grouped into CC258 are associated with the production of carbapenemases and harbor many virulence genes (Gonçalves et al., 2017), which corroborates with our results (**Table 5**). Moreover, the ST392, found in the Kp66 isolate, is part of CC147, which is a small internationally successful clonal complex and has been shown to be an important epidemic clone. Hasan et al. (2014) described a clonal expansion of CC147 by Verone integron-encoded metallo-beta-lactamase (VIM)-producing *K. pneumoniae* strains isolated from Greece. ST392 has been reported worldwide as an emergent clone associated with the spreading KPC-producing *K. pneumoniae* (Yang et al., 2013; Di Mento et al., 2018; Garza-Ramos et al., 2018). In Brazil, ST392 was previously reported in a KPC-2-producing *K. pneumoniae* harboring the *mcr*-1 gene.

CONCLUSION

Our results revealed a worrying situation concerning *K. pneumoniae* that is resistant to the drugs commonly used to treat infections and as well as those used as a last resort for life-threatening infections in patients admitted to the ICU. Additionally, our findings demonstrated the presence of high-risk international clones among isolates. Therefore, our data should be interpreted as an alert for need for prevention and control of the MDR *K. pneumoniae* in hospital settings. A careful and continued surveillance system that provides epidemiological and molecular information is important to limit the risk of infection and the spread of these strains.

AUTHOR CONTRIBUTIONS

RF, BS, and GR performed the experiments. MB kindly provided the strains and aided with the phenotypic detection of antibiotic resistance. ES aided with the writing and edition of the manuscript. EC aided with the sequencing analysis and the sequence submission to the NCBI platform. MCP, AC, AP-S, and CF conceived the idea, wrote the manuscript and analyzed the data. MLST and ERIC-PCR were performed by RN-S.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03198/full#supplementary-material

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Role of Two-Component System Response Regulator *bceR* in the Antimicrobial Resistance, Virulence, Biofilm Formation, and Stress Response of Group B Streptococcus

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Group B Streptococcus (GBS; *Streptococcus agalactiae*) is a leading cause of sepsis in neonates and pregnant mothers worldwide. Whereas the hyper-virulent serogroup III clonal cluster 17 has been associated with neonatal disease and meningitis, serogroup III ST283 was recently implicated in invasive disease among non-pregnant adults in Asia. Here, through comparative genome analyses of invasive and non-invasive ST283 strains, we identified a truncated DNA-binding regulator of a two-component system in a non-invasive strain that was homologous to *Bacillus subtilis bceR*, encoding the *bceRSAB* response regulator, which was conserved among GBS strains. Using isogenic knockout and complementation mutants of the ST283 strain, we demonstrated that resistance to bacitracin and the human antimicrobial peptide cathelicidin LL-37 was reduced in the Δ*bceR* strain with MICs changing from 64 and 256 μg/ml to 0.25 and 64 μg/ml, respectively. Further, the ATP-binding cassette transporter was upregulated by sub-inhibitory concentrations of bacitracin in the wild-type strain. Upregulation of *dltA* in the wild-type strain was also observed and thought to explain the increased resistance to antimicrobial peptides. DltA, an enzyme involved in D-alanylation during the synthesis of wall teichoic acids, which mediates reduced antimicrobial susceptibility, was previously shown to be regulated by the *bceR*-type regulator in *Staphylococcus aureus.* In a murine infection model, we found that the Δ*bceR* mutation significantly reduced the mortality rate compared to that with the wild-type strain ($p < 0.01$). Moreover, this mutant was more susceptible to oxidative stress compared to the wild-type strain ($p < 0.001$) and was associated with reduced biofilm formation ($p < 0.0001$). Based on 2-DGE and mass spectrometry, we showed that downregulation of alkyl hydroperoxide reductase (AhpC), a Gls24 family stress protein, and alcohol dehydrogenase (Adh) in the Δ*bceR* strain might explain the attenuated virulence and compromised stress response. Together, we showed for the first time that the *bceR* regulator in GBS plays an important role in bacitracin and antimicrobial peptide resistance, virulence, survival under oxidative stress, and biofilm formation.

Keywords: Group B Streptococcus, infection, two component system, *bceR*, antimicrobial peptide resistance, virulence, stress response

INTRODUCTION

Group B Streptococcus (GBS) is the leading cause of sepsis in neonates and pregnant mothers worldwide (Russell et al., 2017; Seale et al., 2017). In particular, serogroup III sequence type (ST) 17 has been strongly associated with hyper-virulence as it causes neonatal sepsis and meningitis (D'Urzo et al., 2014; Seale et al., 2016). Further, life-threatening conditions associated with toxic shock syndrome and meningitis due to GBS are being increasingly reported in non-pregnant adults (Ballard et al., 2016). As in other regions, serotypes I, III, and V are predominant in invasive diseases of adults caused by GBS in Hong Kong (Skoff et al., 2009).

Group B Streptococcus serotype III-4/ST283 strains have been implicated in invasive diseases in non-pregnant adults in Asia (Wilder et al., 2000; Chan et al., 2002; Ip et al., 2006, 2016; Kalimuddin et al., 2017). Moreover, this ST283 type has been recently associated with an outbreak of invasive disease in adults in Singapore, which was suspected to be caused by the foodborne ingestion of contaminated freshwater fish as sushi (Kalimuddin et al., 2017). Compared to other serotypes identified in non-pregnant adults, GBS serotype III-4 has a significantly higher propensity to cause meningitis and septicemia, accounting for greater than 50% of all GBS meningitis cases in non-pregnant adults due to serotype III during 1993–2012 in Hong Kong (Ip et al., 2016). In Singapore, an outbreak of this strain type led to invasive diseases associated with spinal infection and septic arthritis in hundreds of young adults (Kalimuddin et al., 2017). Further, over the last 15 years, GBS serotype III-4 strains have remained a single clone of ST283, possessing distinct surface protein genes and mobile genetic elements and exhibiting indistinguishable PFGE fingerprints (Ip et al., 2006), suggesting that GBS III-4 strains might be hyper-virulent and possess special genetic virulent determinants.

Complete GBS genomes available in a public database (Genbank[1]) previously revealed that GBS possesses many pathogenic islands encoding virulence genes and transcriptional regulators, upon comparison with other streptococcal species (Glaser et al., 2002). Moreover, novel regulators involving two component systems (TCSs) associated with GBS pathogenesis have also been identified based on genome analyses (Samen et al., 2006, 2011; Lembo et al., 2010).

Two component systems are key bacterial regulatory systems involved in the detection and response to environmental challenges. Multiple TCSs have been reported in GBS, including covRS (Cumley et al., 2012; Sullivan et al., 2017), CsrRS (Park et al., 2012), RgfA (Al Safadi et al., 2011), and LtdR (Deng et al., 2018). These systems have been shown to play specific roles in colonization, pH tolerance during biofilm formation, and pathogenesis. In Gram-positive bacteria, many *bceR*-like systems have been characterized and comprise part of the antimicrobial peptide detoxification modules (Cui et al., 2005; Dintner et al., 2011). The best studied example of a *bceR*-like system is the bacitracin resistance module (*bceRSAB*) of *Bacillus subtilis* (Ohki et al., 2003; Cui et al., 2005). *In B. subtilis*, this system is linked to the ABC transporter, comprising the BceA ATPase and BceB permease, which serves as a detoxification pump for the removal of antimicrobial peptides (AMPs) (Ohki et al., 2003; Cui et al., 2005; Bernard et al., 2007). AMPs such as cathelicidins have an important role in mammalian innate immune defense and are produced by neutrophils, macrophages, and epithelial cells. However, Gram-positive bacteria have evolved resistance to these AMPs. Specifically, *Staphylococcus aureus* was reported to have two complete TCS/ABC transporter modules termed *graRS-vraFG* and *braRSAB* that either sense the same type of AMP or different AMPs and interact to mediate resistance (Cui et al., 2005; Li et al., 2007a; Meehl et al., 2007). In addition, *bceRS*-like systems such as *apsRS* in *S. epidermidis* and *graRS* in *S. aureus* not only enhance the expression of ABC transporters, but also lower the overall net negative charge of the cell envelope (Li et al., 2007b). This *aps* system decreases the anionic charge of the bacterial surface, which is specifically targeted by cationic AMPs (CAMPs), by upregulating the *dlt* operon and *mprF* (Li et al., 2007b). The *dlt* operon encodes proteins necessary for the D-alanylation of cell wall teichoic acid (TA), which through the repulsion of cations, confers resistance to AMPs (Peschel et al., 1999; Li et al., 2007b). In addition to AMP resistance, *graRS* of *S. aureus* was shown to play an important role in virulence, resistance to oxidative stress, and biofilm formation (Shanks et al., 2008; Falord et al., 2011).

In this work, we identified a key role for the response regulatory gene *bceR* in the determination of pathogenic traits in the clinically invasive GBS ST283 strain, including antimicrobial and oxidative stress resistance, biofilm formation, and virulence using a mouse infection model.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Five GBS III-4 clinical strains were originally obtained from the Prince of Wales Hospital. The GBS strains selected for the current study were based on an archived collection of isolates from the Department of Microbiology, Chinese University of Hong Kong, Prince of Wales Hospital, and were previously characterized by molecular typing. The approval of clinical ethics for the laboratory typing of GBS strains with clinical demographics was obtained as a retrospective study (CRE-2012.054 from the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee) which was published (Ip et al., 2016).

The GBS strains were grown in Todd–Hewitt broth (THB) or THY broth (THB supplemented with 5 g/l yeast extract) or on THY blood agar plates (all from Difco Laboratories, Franklin Lakes, NJ, United States). Recombinant DNA manipulations were performed in *Escherichia coli* strain XL-Blue, grown at 37°C in Luria–Bertani (LB) broth (Difco Laboratories, Franklin Lakes, NJ, United States) or on LB agar plates.

[1]https://www.ncbi.nlm.nih.gov/genome/genomes/186; accessed Mar2018

Whole Genome Sequencing and Comparative Genomics of Five GBS Serotype III-4 Strains

Five GBS strains of serotype III subtype 4 and sequence type ST283 were selected for genome sequencing (CU_GBS_00, CU_GBS_10, CU_GBS_12, CU_GBS_98, and CU_GBS_08). These strains were isolated in Hong Kong between 1998 and 2012, from both invasive and non-invasive sites in adult patients. Genomic DNA from the GBS strains was extracted using the Wizard® Genomic DNA Purification Kit according to the manufacturer's protocol for Gram-positive bacteria (Qiagen, Limburg, Netherlands). Genomes were assembled using the metAMOS pipeline (version 1.5rc3) (Koren et al., 2014). The draft genomes of CU_GBS_00, CU_GBS_10, and CU_GBS_12 were deposited in the NCBI database under GenBank accession numbers JYCT00000000, JYCU00000000, and JYCV00000000, respectively.

The genomes of CU_GBS_98 and CU_GBS_08 were completed (GenBank Accession numbers: CP010875 and CP010874, respectively). Draft genome scaffolds were built using the CONTIGuator software (version 2.7.4) (Galardini et al., 2011), with reference to a GBS complete genome (NEM316, accession number: NC_004368). Gaps between adjacent contigs were defined using Geneious (version R6.1.5[2]) and Mauve software (using progressive Mauve aligner, version 2.3.1; Darling et al., 2010). All gaps were successfully closed by PCR, and the complete genomes of CU_GBS_98 and CU_GBS_08 were deposited in the NCBI database.

We used MUMmer software (version 3.23; Kurtz et al., 2004) to align the GBS genomes to the complete reference genome of CU_GBS_08, to confirm the identified indels and SNPs. We used a cut-off value (breaklen = 500, distance to extend the genome alignment for poor scoring regions) to control for aligned regions considered by MUMmer for SNP and indel identification. The resulting genome alignments were also manually examined to identify gains or losses (and truncations) of genes that differed among GBS strains. Functional effects of the identified indels (in-frame or frame-shift indels) and SNPs (synonymous/non-synonymous/stop-codon mutations) were determined according to gene annotations based on the reference genome.

Generation of Δ*bceR* Strain Using Allelic Replacement

The PCR products containing (a) ∼900 bp of sequence upstream from the *bceR* gene and (b) the last 58 bp of the *bceR* gene to approximately 900 bp downstream of the gene were amplified by PCR (**Supplementary Table S1**). The fragments were digested by the restriction enzyme *EcoRI* and ligated with T4 DNA ligase according to the manufacturer's protocol (NEB, MA, United States). The ligated products were amplified by crossover PCR. The PCR product and the thermosensitive plasmid pJRS233 (Ashbaugh et al., 1998) were digested with restriction enzymes *KpnI* and *BamHI*, ligated, and then transformed into XL1-Blue competent cells (Agilent, CA, United States). The resulting plasmid was extracted with the Plasmid Maxi Kit (Qiagen, Limburg, Netherlands) and transformed by electroporation into CU_GBS_08 (Framson et al., 1997). Transformants were selected at 30°C with 1 μg/ml erythromycin on Todd Hewitt agar with 0.5% yeast extract and 5% defibrinated horse blood. Cells with the plasmid integrated into the chromosome were selected at 37°C under erythromycin pressure, and subsequently passaged at the same temperature in the absence of erythromycin for plasmid excision.

[2]http://www.geneious.com

Construction of Complementation Plasmid to Rescue Δ*bceR* Phenotypes

A plasmid was constructed to express full-length *bceR*, and a 500-bp fragment of the upstream region of this gene was amplified with primers containing *BamHI* and *Xbal* sites and cloned into the *BamH*I and *Xbal* sites of pDL289 (Soualhine et al., 2005) to create the *bceR* expression vector pDL289-bceR. Inserts and reading frames were confirmed by sequencing. pDL289-*bceR* was introduced into the Δ*bceR* strain by electroporation.

Minimum Inhibitory Concentration (MIC) Determination

The MIC of antimicrobial agents was determined by the microbroth dilution method, according to the Clinical and Laboratory Standards Institute (CLSI, 2011).

RNA Extraction and Real Time-PCR

The GBS was plated on blood agar plates and incubated at 35°C in 5% CO_2. Sub-inhibitory bacitracin concentration values were determined by monitoring cell growth in THB with or without a range of bacitracin concentrations in 96-well plates. In brief, overnight cultures of cells were resuspended and adjusted to an OD_{600} of 0.8. A 1% bacterial suspension was prepared to obtain a final inoculum of 1×10^6 to 5×10^6 CFU per well in 200 μl of THB with or without bacitracin at 1/2, 1/4, and 1/8× the MICs. The bacterial cells were then incubated at 37°C, and the OD_{595} was measured every 30 min using a DTX 880 microplate reader (Molecular Devices, San Jose, CA, United States) over 24 h. The minimum concentration that did not alter the bacterial growth curve was considered the sub-inhibitory concentration for the described experiment. Experiments were repeated in triplicate.

Briefly, 2 ml of cultures was harvested at mid-log phase and cells were pelleted by centrifugation at 6000 × *g* at 4°C for 10 min. The pellets were resuspended in TE buffer containing RNA protect (Qiagen, Hilden, Germany) at a ratio of 1:2 TE:RNA protect for RNA stabilization. The bacterial suspension was then incubated with 400 μl of lysozyme (prepared in TE buffer) (Sigma, MO, United States) at 37°C for 30 min. The lysate was treated with 30 μl of 3 M sodium acetate (Sigma, MO, United States), 90 μl of 10% SDS (Merck, Gernsheim, Germany), and 1 ml of Trizol (Life Technologies, Camarillo, CA, United States). This was followed by a 5-min incubation at RT before adding 200 μl of chloroform (Merck, Gernsheim, Germany) for 2 min. All samples were centrifuged at 12,000 × *g* at 4°C for 15 min. The supernatant was transferred to a new tube with 1 ml of isopropanol (Merck, Gernsheim, Germany)

for RNA precipitation. After 2 h of incubation at −20°C, the tubes were centrifuged at 12,000 × *g* at 4°C for 15 min and the supernatant discarded. An equal volume of cold absolute ethanol (Merck, Gernsheim Germany) was then added to the tube, which was centrifuged at 12,000 × *g* at 4°C for 5 min to obtain the RNA pellet. The pellet was resuspended in 100 μl of DNase-free and RNase-free water. Additionally, the sample was treated with 2 U of DNase I (Promega, Fitchburg, WI, United States) followed by a 20-min incubation at 37°C. The RNA quality and quantity were determine using a Nanodrop 1000 (Life Technologies, Camarillo, CA, United States), and the sample was then stored in 20-μl aliquots at −80°C.

Total RNA was extracted with Trizol (Chomczynski and Sacchi, 2006) for three independent experiments. Briefly, 200 ng of total RNA for each sample was subjected to cDNA synthesis using a TURBO DNA-free Kit (Thermo Fisher, MA, United States) according to the manufacturer's protocol. The DNase inactivation reagent was removed by centrifugation at 10,000 × *g* for 1.5 min and the supernatant was aliquoted into fresh tubes for the reverse transcription step using SuperScript III Reverse Transcriptase (Invitrogen, CA, United States) according to the manufacturer's protocol. Real-time PCR was performed using SYBR Green PCR Master Mix (Invitrogen, CA, United States) based on the manufacturer's instructions, with an ABi 7500 Real-Time PCR Detection System (Applied Biosystems, MA, United States). Each sample was run in triplicate with 300 nM of each primer (**Supplementary Table S2**) with the following conditions: 95°C for 10 min, 40 cycles of 95°C for 30 s, and then 60°C for 1 min. Melting curves were generated by a cycle of 95°C for 1 min and 60°C for 1 min. The relative quantitation of mRNA expression was normalized to the constitutive expression of the 16S rRNA housekeeping gene and calculated by the comparative ΔΔCT method (Livak and Schmittgen, 2001; Wang et al., 2014).

Mitogenicity and Cytokine Release in Human Lymphocytes

Bacteria were grown in THB (Oxoid) with 0.2% yeast extract overnight at 37°C. The overnight cultures were then diluted 1:100 in fresh THB, grown to mid-log phase, harvested by centrifugation at 3000 × *g* for 10 min, and then washed three times with phosphate-buffered saline (PBS). Pelleted cells were resuspended in PBS, heat-killed (100°C, 30 min), and subjected to centrifugation at 11,000 × *g* for 20 min at 4°C to remove cell debris. The supernatant (GBS cell extract) was aliquoted and stored at −80°C until required. Protein concentrations were determined using protein assay dye reagent concentrate (Bio-Rad) with bovine serum albumin (Sigma) as a standard.

Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of healthy individuals (obtained from the Hong Kong Red Cross Blood Transfusion Service) by density gradient centrifugation using Ficoll-Paque (GE Healthcare). The human mononuclear cells were washed with PBS, resuspended in medium (RPMI 1640 with 10% FBS), and seeded at 2×10^5 per ml in a 96-well View Plate (Perkin Elmer). Twenty-four hours later, GBS cell extract (prepared as described in the bacterial strains and growth conditions sections) was added at a final concentration of 25 μg/ml. Phytohemagglutinin (PHA, 10 μg/ml) and culture medium alone were included as controls. After incubation for 24 h, the proliferation of lymphocytes was detected using alamarBlue (Life Technologies) according to the manufacturer's protocol. Fluorescence emission was measured using an EnSpire Multimode Plate Reader (Perkin Elmer) at 585 nm with an excitation wavelength of 570 nm. Experiments were performed in triplicate.

Cytokine Measurements

After stimulating PBMCs, the supernatant from cell cultures was collected after incubation for 3, 6, 12, and 24 h to measure cytokine release. Interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, and tumor necrosis factor alpha (TNF-α) were evaluated by ELISA according to the manufacturer's instructions (BD Biosciences). Measurements were performed at an OD of 450 nm (EnSpire Multimode Plate Readers, PerkinElmer).

Mouse Infection Model

Animal experiments were performed with permission of the Animal Experimentation Ethics Committee (AEEC) of the Chinese University of Hong Kong.

The virulence of Δ*bceR* GBS III-4 mutant strains was compared to that of the wild-type strain CU_GBS_08, the CU_GBS_12 strain with a natural truncation of *bceR*, and the ATCC 12403 Type strain as a control using a mouse model. The ATCC strain belongs to serogroup III and originated from a case of fatal septicemia[3]. The GBS inoculum was prepared by diluting overnight cultures 1:100 into THB. Cultures were incubated at 35°C, and then bacteria were harvested by centrifugation at 1200 × *g* for 10 min at 4°C. The pellet was then washed twice and resuspended in 5 ml of PBS. GBS was then prepared by diluting the PBS suspension to 10^7 CFU/ml. Dilutions were confirmed by colony counts on blood agar. Six-week-old CD1 mice were purchased from The Laboratory Animal Services Centre (The Chinese University of Hong Kong, Hong Kong) and infected via intraperitoneal injection with 0.1 ml of the GBS inoculum at 10^7 CFU/ml. The control group was injected with an equivalent volume of sterile PBS. Each group contained 30 mice. The mice were monitored for 10 days and those surviving at this time were sacrificed under anesthesia. The health condition of the mice was monitored daily and animals showing signs of excess weight loss, severe pain, and distress were euthanized before the end of study. The LD_{50} was calculated, and the Kaplan–Meier survival curve for infection and control groups with an endpoint of 10 days was prepared. The study was approved by the University Animal Experimentation Ethics Committee (AEEC; Reference no.:13-063-MIS) and conducted at The Laboratory Animal Services Centre in compliance with International Guiding Principles for Biomedical Research Involving Animals and The Hong Kong Code of Practice for Care and Use of Animals for Experimental Purposes.

[3]https://www.atcc.org/products/all/12403.aspx

H_2O_2 Stress Assay

The GBS strains were plated on blood agar plates and incubated at 35°C in 5% CO_2. Bacterial cells were suspended in pre-warmed THB with shaking at 200 rpm overnight. The overnight cultured bacterial cells were then diluted 1:100 in THB and incubated at 37°C with shaking at 200 rpm to achieve an OD_{600} of 0.8–1.0. The bacteria were resuspended in THB at a concentration of 4×10^7 CFU/ml, and then 40 mM H_2O_2 was added at RT for 15 min. After treatment, fresh THY broth was added to stop the reaction and the bacteria were harvested by centrifugation at $4000 \times g$ for 15 min. Bacterial viability after H_2O_2 treatment was then examined through the culture and enumeration of bacterial colonies. Serial dilutions of medium were used for CFU counting. Each experiment was conducted in triplicate.

Determination of Biofilm Biomass by Crystal Violet Staining and CFU Counting

The GBS strains were plated on blood agar plates and incubated at 35°C in 5% CO_2. Overnight bacterial cultures were then suspended in pre-warmed THB overnight and 24-well flat bottom plates (Costar, Boston, MA, United States) were used to support biofilm growth. Then, the overnight bacterial cultures were diluted 1:100 in THB and incubated at 37°C with shaking at 200 rpm to achieve an OD_{600} of 0.8–1.0. The bacteria were harvested by centrifugation at $4000 \times g$ for 15 min. After washing with PBS, the cells were diluted 1:10 with pre-warmed THB, and 500 μl of cells was added to each well of a 24-well plate and incubated at 37°C with 5% CO_2 overnight without shaking. All samples were run in triplicate.

Biofilm biomass was quantified by measuring the absorbance of crystal violet (Olson et al., 2002). After removing the culture medium, the plates were gently washed with PBS twice to remove the floating cells. Biofilms were stained with 300 μl of 0.5% crystal violet (Sigma, MO, United States) (prepared in 10% ethanol) for 15 min at RT. After staining, the plates were gently washed with PBS three times and dried at RT. Then, 500 μl of 95% ethanol was added to each well and incubated for 15 min to dissolve the biofilms. OD_{595} values were measured using a DTX 880 plate reader (Molecular Devices, San Jose, CA, United States).

Bacterial viability in biofilms was also examined by enumerating bacterial colonies. After removing the culture medium, the plates were gently washed with PBS twice to remove floating cells, which was followed by the addition of 500 μl of fresh THB to each well. The cells were collected by scraping the bottom of each well with a sterile cell scraper. Serial dilutions of the medium were used for CFU enumeration, and each experiment was performed in triplicate.

Two-Dimensional Gel Electrophoresis (2DE) and Mass Spectrometry

The GBS strains were plated on blood agar plates and incubated at 35°C in 5% CO_2. Bacterial cells were suspended in pre-warmed THB with shaking at 200 rpm overnight. Then, the overnight bacterial cultures were diluted1:100 in THB and incubated at 35°C with shaking at 200 rpm to mid-log phase, after which, the bacterial cells were harvested by centrifuging at $4000 \times g$ for 20 min at 4°C. For whole protein extraction, the instructions of the total protein extraction kit (Bio-Rad, United States) were followed, and protein quantitation was performed using RC DC Protein Assay reagent (Bio-Rad, United States). Then, 2DE was conducted following the protocol of a previous study (Jones et al., 2004).

The gel photos were normalized and compared using software PDQuest (Version8.0.1, Bio-Rad, United States). The Boolean method was chosen to compare the intensity of the protein spots to determine both fold-changes and statistically significantly differences between GBS III-4 wild-type and Δ*bceR* strains. From the results, we found that the expression of three proteins was significantly decreased in the Δ*bceR* strain (>2-fold reduction in expression), and these three protein spots were cut from the original 2-DE gel and sent to the proteomic core laboratory of The University of Hong Kong for mass spectrometry-based identification.

Statistical Analysis

Data are expressed as the mean ± SD. Statistical comparisons between different treatment groups were performed using a one-way analysis of variance (ANOVA), followed by a *post hoc* Dunnett's test using GraphPad Prism 6.05 for Windows (GraphPad Software, San Diego CA, United States). Differences were considered as significant at $p < 0.05$, and were denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

Whole Genome Sequencing and Comparative Genomics Analysis of GBS Serotype III-4 Strains

The genomes of three invasive and two non-invasive GBS serotype III-4 strains were sequenced using a Roche 454 and Illumina Solexa Genome Analyzer, according to the manufacturer's instructions, and have been submitted to GenBank as either draft or complete genomes (**Table 1**). The genomes of the meningitis/septicemia strains were compared to those of the non-invasive strains. All single nucleotide polymorphisms (SNPs) from the ORFs were called using Mauve (version 2.3.1) software (Darling et al., 2010). Sequence alignment was performed to compare gene sequence variations among these strains. Genes that encode hypothetical proteins and those related to bacteriophages were not analyzed further. From this, we narrowed down our list to four truncated genes of interest as indicated in **Supplementary Table S3**. These genes showed 100% nucleotide identity to those of other GBS strains in GenBank. SNPs were confirmed by PCR-based Sanger sequencing to filter out false positive SNPs, which can occur with next generation sequencing.

Comparative genome analysis revealed a non-synonymous substitution (truncation) of a DNA binding regulator (Accession no: CU_GBS08_01010) in the non-invasive GBS strain, and the truncation of *bceR* at c.288delG was determined to generate a

TABLE 1 | List of strains in this study.

GBS strain	GenBank accession number	Isolation site	Clinical details	Patient age group	Sequence type	Molecular serotyping group
ATCC12403 (NEM316)	NC_004368	Blood	Septicaemia	Infant	23	III
CU_GBS_00	JYCT00000000	Wound	Non-invasive	Non-pregnant adult	283	III-4
CU_GBS_08	CP010874	Blood	Toxic shock syndrome	Non-pregnant adult	283	III-4
CU_GBS_10	JYCU00000000	Blood	Septic arthritis	Non-pregnant adult	283	III-4
CU_GBS_12	JYCV00000000	Vaginal-rectal swab	Non-invasive	Pregnant adult	283	III-4
CU_GBS_98	CP010875	Cerebrospinal fluid	Meningitis	Non-pregnant adult	283	III-4
CU_GBS_08_Δ*bceR*	*bceR* deletion mutant of CU_GBS_08					
CU_GBS_08_Δ*bceR*+*pbceR*	*bceR complementation* of CU_GBS_08_Δ*bceR*					

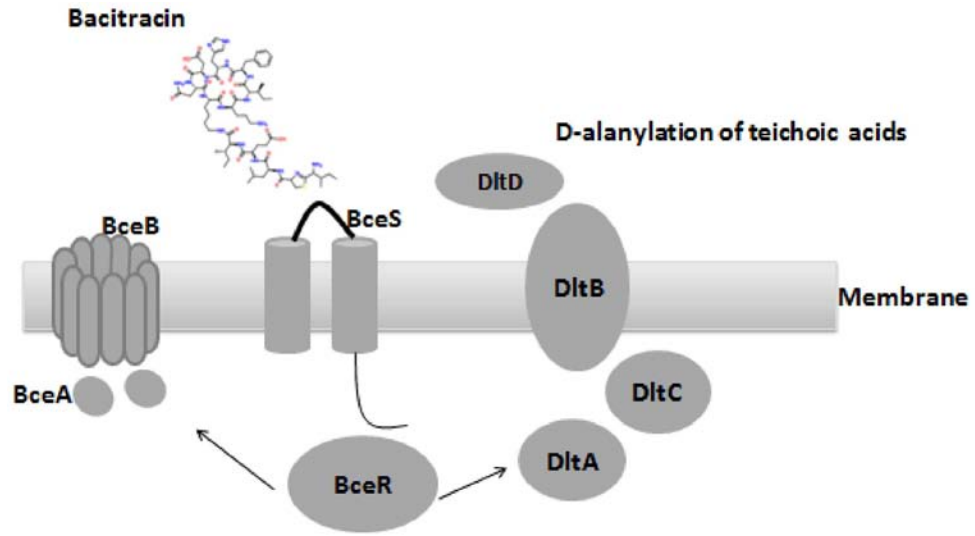

FIGURE 1 | Proposed organization structure of BceRS system and working model in GBS. AMPs that usually function by pore formation in the membrane (bacitracin was used in this figure), bacitracin bind to the anionic loop of BceS and cause the phosphorylation of BceR, which result in the activation of BceR regulated AMPs resistance in GBS. (1) up-regulated the expression of the transporter BceAB to facilitate the resistance by expelling the bacitracin **(left)**. (2) D-alanylation of teichoic acids by the *dlt* system, which in turn decreased negative charge of the bacterial membrane and ensure the resistance **(right)**.

stop codon, abrogating expression of a region of the mRNA encoding the last 20 aa of the receiver domain and the DNA-binding domain. BLAST analyses revealed that this regulator was most closely related to the TCS response regulator protein BceR of *S. gallolyticus*, with 69% protein sequence homology (GenBank no: CDO17747.1). Although this gene was present in all GBS strains examined, the sequences harbored ∼30% differences compared to the *bceR* genes of other bacteria, suggesting that this gene might have specific functions in GBS. Based on the location of the truncation of the response gene, we predicted that the *bceR*-like response would be aborted in the non-invasive strain. The present study therefore focused on the role of this response regulator gene in this TCS of GBS. We thus knocked down this gene in the wild-type invasive strain CU_GBS_08 to elucidate its role in antimicrobial peptide resistance, stress response, and virulence in this invasive GBS strain. Our working model is depicted in **Figure 1**. Together with evidence that the transporter-encoding *bceAB* gene is activated by bacitracin, we have re-named this regulator *bceR* of the two-component system *bceRS* in this complete genome (GenBank genome: CP010874).

The Δ*bceR* Strain Is More Sensitive to Bacitracin and Antimicrobial Peptides

It is known that *bceR*-like systems comprise components of antimicrobial peptide detoxification modules, such as the *graRS* system of *S. aureus*, as the MIC values of some AMPs were decreased in strains with mutations in this system (Cui et al., 2005; Meehl et al., 2007). Here, the MICs of selected AMPs and antibiotics were measured for the Δ*bceR*, complementation, and wild-type strains (**Table 2**). MICs for the mutant strain were 256- and 4-fold lower for bacitracin and LL-37, respectively, compared to those for the wild-type strain. However, Δ*bceR* complementation with the pDL289-*bceR* plasmid restored resistance to both bacitracin and LL-37 (**Table 2**). No difference in resistance was observed between the wild-type strain and the isogenic Δ*bceR* strain for other antibiotics.

TABLE 2 | Minimal inhibitory concentrations (MIC) of antimicrobial peptides and other antibiotics in GBS strains.

	MICs (μg/ml)[a]				
Antibiotics	**CU_GBS_08**	**CU_GBS_08_Δ*bceR***	**CU_GBS_08_Δ*bceR*+*pbceR***	**CU_GBS_12**	**ATCC49619**
Bacitracin	64	0.25	64	0.25	8
LL-37	256	64	256	128	2
Polymyxin B	128	128	128	64	128
Ampicillin	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
Cefotaxime	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
Penicillin	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
Vancomycin	0.5	0.5	0.5	0.5	0.5
Erythromycin	≤0.06	≤0.06	≤ 0.06	≤0.06	≤0.06
Ciprofloxacin	0.5	0.5	0.5	0.5	0.5

[a]*Minimum inhibitory concentration, obtained according to CLSI protocol (CLSI, 2011).*

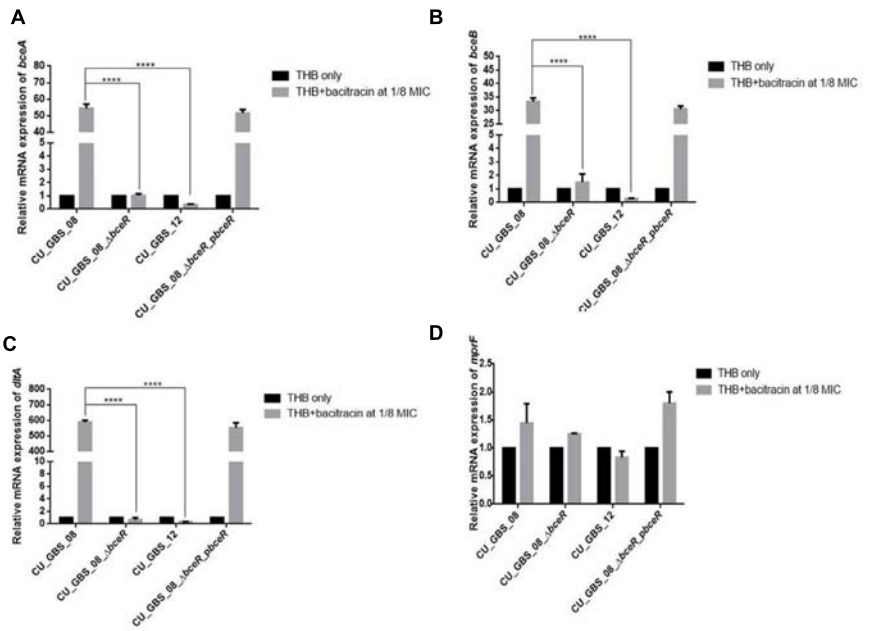

FIGURE 2 | Relative expression of *bceA*, *bceB*, *dltA,* and *mprF* **(A–D)** in GBS strains with addition of bacitracin. Genes expression of *bceA*, *bceB*, *dltA,* and *mprF* in wild type GBS III-4 strain, CU_GBS_08 (bacitracin MIC 64 μg/ml), isogenic mutant (CU_GBS_08_Δ*bceR*, bacitracin MIC 0.25 μg/ml), and non-invasive GBS III-4 strain CU_GBS_12 (bacitracin MIC 0.25 μg/ml) were shown. GBS strains without treatment were normalized to 1. Error bars represent the standard deviation of the mean values from three independent experiments. Significance was determined by one-way ANOVA (*$p < 0.05$, ****$p < 0.0001$).

Expression of *bceA*, *bceB*, and *dltA* Is Reduced in the Δ*bceR* GBS Strain

graRS, a *bceRS*-like system of *S. aureus*, was reported to induce AMP resistance not only by pumping AMPs out via an ABC transporter, but also by lowering the overall negative net charge of the cell envelope by upregulating expression of the *dlt* operon and *mprF* (Li et al., 2007; Meehl et al., 2007). Thus, the expression of *bceA, bceB*, *dltA*, and *mprF* was evaluated in the presence of a sub-inhibitory concentration of bacitracin in wild-type and Δ*bceR* strains and normalized to 16s rRNA expression. Expression levels in GBS strains grown in THB only were used as controls and adjusted to 1. As shown in **Figure 2**, levels of *bceA*, *bceB*, and *dltA* were higher when respective strains were grown in THB containing bacitracin at 1/8 the MIC value for CU_GBS_08 (bacitracin: MIC, 64 μg/ml) compared to those when bacteria were grown in the presence of bacitracin at 1/8 the MIC value for CU_GBS_Δ*bceR* (bacitracin: MIC, 0.25 μg/ml; $p < 0.0001$) and for CU_GBS_12 (bacitracin: MIC, 0.25 μg/ml; $p < 0.0001$; **Figures 2A–C**). However, no significant difference of *mprF* expression was found between the wild-type strain and Δ*bceR* strain (**Figure 2D**).

Mitogenicity and Pro-inflammatory Response Induced by GBS in Human PBMCs

The proliferation of PBMCs was evaluated after 24 h of stimulation with GBS or 10 μg/ml PHA to evaluate mitogenicity and the ability of GBS to induce the proliferation of these

cells. As shown in **Figure 3**, although all bacteria induced the proliferation of PBMCs, the Δ*bceR* strain demonstrated a significantly reduced immunogenicity ($p < 0.0001$). Similarly, levels of the cytokines TNF-α, IL-6, IL-8, IL-1β, IL-10, and IL-12 were determined, as shown in **Figures 4A–F**. The isogenic mutant strain Δ*bceR* induced a significant decrease in the expression of pro-inflammatory cytokines when compared to that with the wild-type strain. The decreased release of TNF-α was the most obvious ($p < 0.0001$) and was approximately fourfold decreased compared to that with the wild-type strain. This was followed by IL-6, IL-1β, and IL-10, which were decreased by approximately twofold with the Δ*bceR* strain ($p < 0.001$ for IL-6 and IL-10 and $p < 0.01$ for IL-1β). Peak IL-6 expression was delayed to 24 h with the Δ*bceR* strain, and the release of IL-8 was approximately 1.4-fold lower for this strain ($p < 0.0001$). The release of IL-12 could not be detected in the presence of both wild-type and mutant strains. Further, the complementation of Δ*bceR* using pDL289 reversed the change in cytokine release.

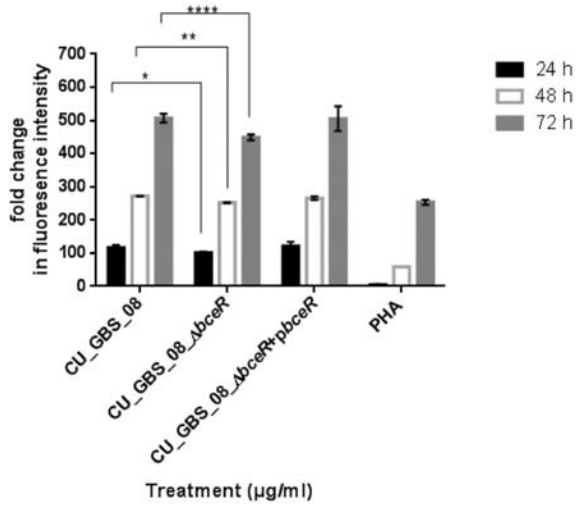

FIGURE 3 | Effect of *bceR* deletion on proliferation of PBMCs. PBMCs were cultured in 25 μg/ml heat-killed GBS strains for 24 h, with phytohaemagglutinin (PHA) (10 μg/ml) only. Cell proliferation was determined by fluorescence intensity. Three independent experiments were performed and the mean ± SD was illustrated with the error bar. Statistical significance at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ were reached when GBS wild type strain was compared to the mutant (CU_GBS_08_Δ*bceR*). The mitogenicity effect between the wild type and Δ*bceR* strain was most significant at 72 h ($p < 0.001$).

The Deletion of *bceR* Attenuates Virulence in a Mouse Infection Model

The virulence of the wild-type and Δ*bceR* strains was studied using a mouse infection model via intraperitoneal inoculation. The lethal concentration (LD_{50}) at which 50% of the mice died in the tested group at the specified time point was then calculated. The LD_{50} values of the Δ*bceR* and wild-type strains were 1×10^7 and 3×10^6 CFU, respectively (**Supplementary Table S4**). Moreover, the survival rates of mice infected intraperitoneally with GBS at 10^7 CFU after 10 days of inoculation are shown in **Figure 5**. As observed, the virulence of the Δ*bceR* strain was attenuated compared to that of the wild-type strain, as revealed by the increased survival rate of 23.3% versus 0% with the wild-type strain ($p < 0.01$).

Bacterial Survival in Response to H_2O_2 Stress Is Decreased in the Δ*bceR* Strain

Next, the response of the Δ*bceR*, wild-type, and complementation strains to H_2O_2 stress was assessed (**Figure 6**).

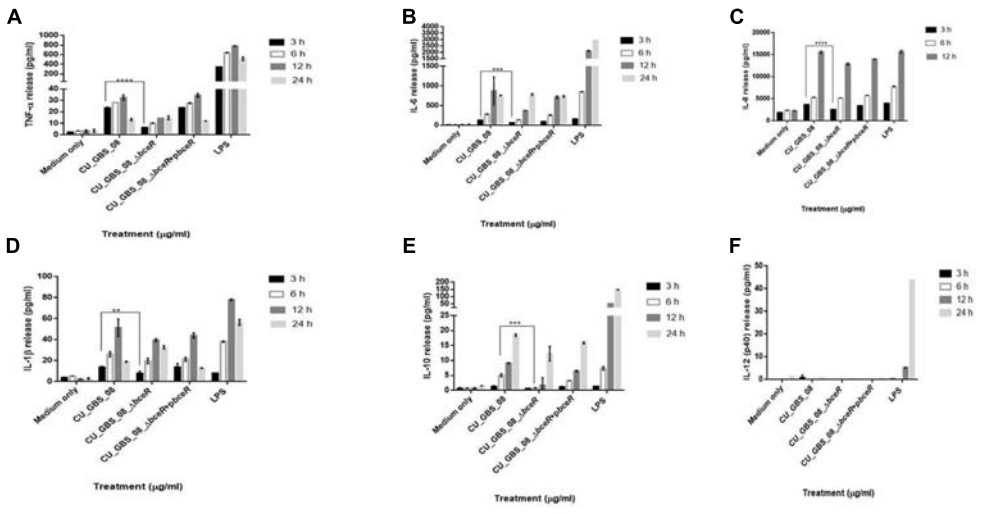

FIGURE 4 | Release of TNF-α, IL-6, IL-8, IL-1β, IL-10, and IL-12 **(A–F)** from human lymphocytes as induced by wild type GBS and Δ*bceR* strain. Heat-killed GBS extract (5 μg/ml) (invasive strain CU_GBS_08; CU_GBS_08_Δ*bceR*; CU_GBS_08_Δ*bceR*+*pbceR*, *bceR* complementation strain) was incubated with human lymphocytes. Cytokine release was measured at 3, 6, 12, and 24 h. LPS represents the lipopolysaccharide control. Data are expressed as mean ± SD ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

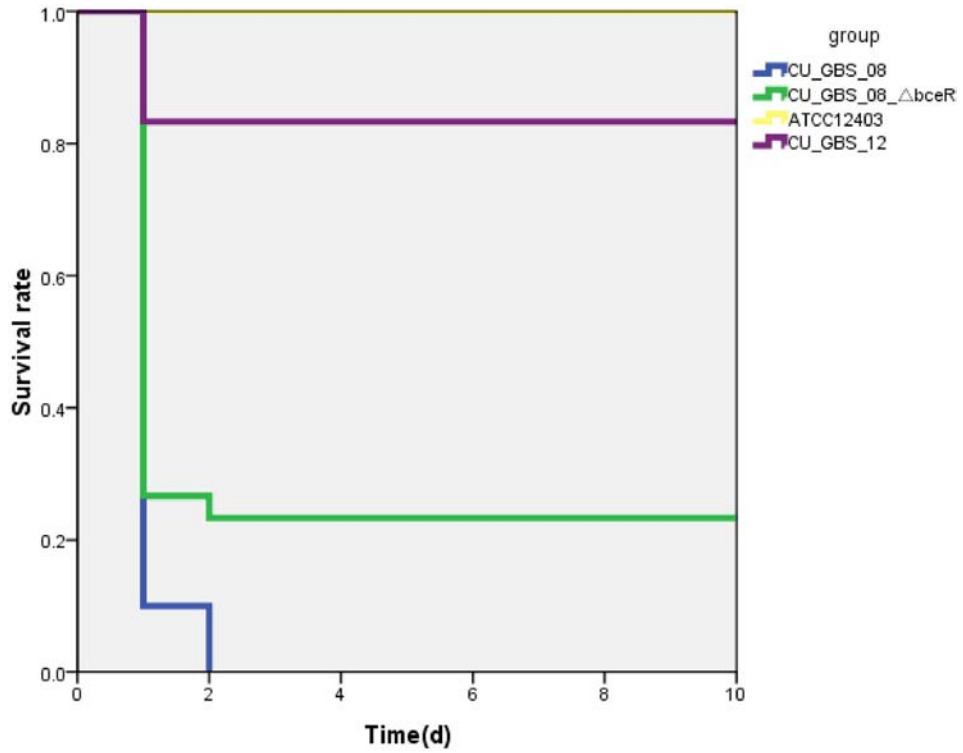

FIGURE 5 | Kaplan–Meier survival curve. Survival rate was calculated at 10 days post-intraperitoneal injection. The difference in survival rate between CU_GBS_08 strain (wild type) and CU_GBS_08_ΔbceR strain is statistically significant with P < 0.01 by Fisher's exact test.

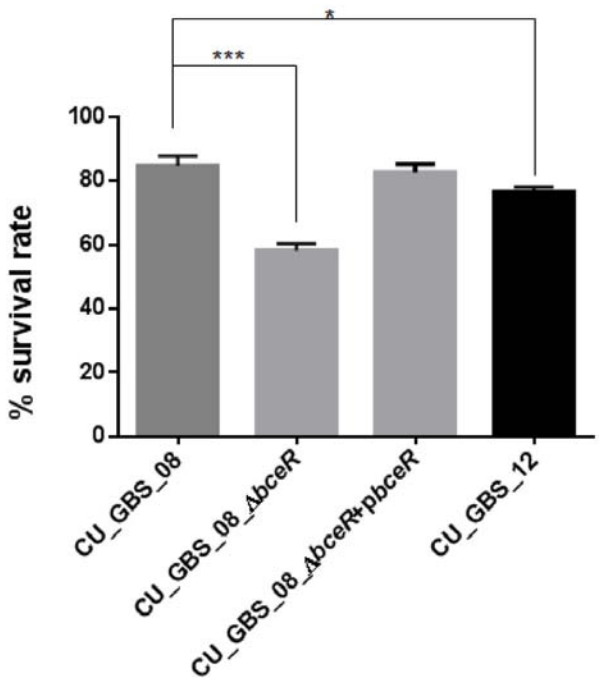

FIGURE 6 | Effect of H_2O_2 stress on GBS. The Δ*bceR* was significantly more susceptibility to H_2O_2 (40 mM) exposure than wild type (*$p < 0.05$, ***$p < 0.001$).

The mutant strain was significantly more susceptible to H_2O_2 than the wild-type strain. Specifically, the survival rate of the mutant strain was reduced by 20% compared to that of the wild-type strain ($p < 0.001$); however, no significant difference in susceptibility was observed between wild-type and non-invasive CU_GBS_12 strains.

Biofilm Formation Is Impaired in the Δ*bceR* Strain

The ability of the wild-type, Δ*bceR*, Δ*bceR* complementation, and CU_GBS_12 (non-invasive) strains to form biofilms was assessed by crystal violet staining and CFU enumeration (**Figures 7A,B**). One-way ANOVA analysis showed that biofilm formation was impaired significantly in the Δ*bceR* strain when compared to that in the wild-type strain ($p < 0.05$ and $p < 0.0001$, for crystal violet staining and CFU numbers, respectively), which was reversed by complementation. The biofilms were also evaluated by confocal microscopy (CLSM), wherein the cell density (xy images) and thickness (xz images) of biofilms were assessed. As shown in **Supplementary Figures S1A–C**, most cells in the biofilms were stained green, indicating that more live cells were present. However, a decreased signal was detected, based on the xy and xz images, for the *bceR* strain when compared to that with the wild-type strain, which indicated that fewer living or dead cells were present with the Δ*bceR* strain. Thus, CLSM images revealed that loss of the *bceR*-like regulator inhibited

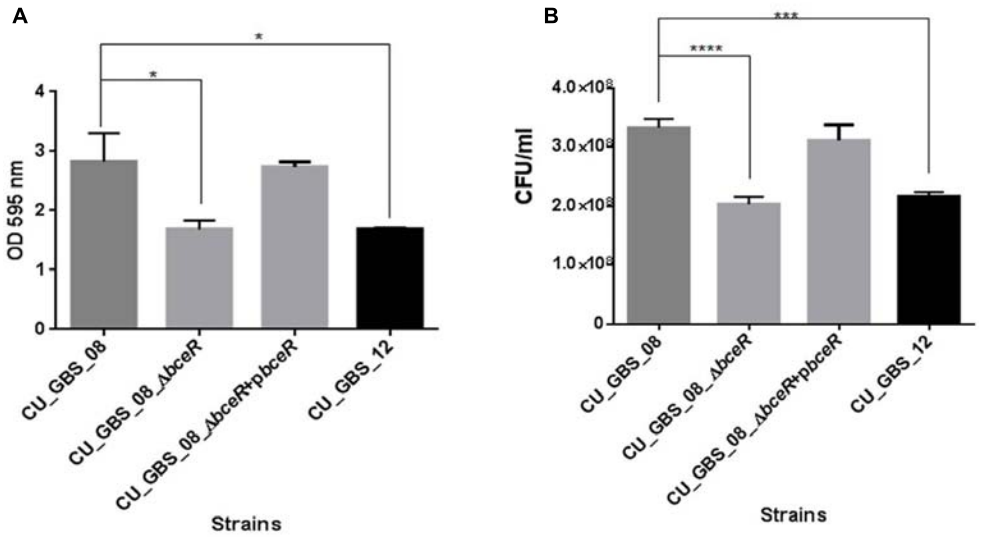

FIGURE 7 | Evaluation the role of two-component regulator *bceR* on biofilm formation in GBS using crystal violet staining **(A)** and bacterial counting **(B)**. Bacteria stained with crystal violet were measured at OD_{595}. Significance was determined by one-way ANOVA (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$).

TABLE 3 | Results of proteins identification by mass spectrometry.

Spot	Protein name	Protein score	Database	Accession number	Reference
1	Alkyl hydroperoxide reductase (AhpC)	391	NCBInr	gi| 403643060	Cosgrove et al., 2007
2	Gls24 family stress protein (Gls24)	291	NCBInr	gi| 446353446	Teng et al., 2005
3	Zinc-dependent alcohol dehydrogenase (Adh)	409	NCBInr	gi| 446571849	Suvarna et al., 2000; Mukherjee et al., 2006

biofilm formation, resulting in a lower cell density and reduced thickness.

Deletion of *bceR* Alters Protein Expression in the GBS Strain

Proteomic analysis of bacteria harvested at mid-log phase was performed using 2-DE and mass spectrometry. This revealed three proteins that were reduced by greater than twofold in the Δ*bceR* strain; the Boolean operation of the PDQuest software (version 8.0.1, Bio-Rad, United States) was then used to compare the intensities of the protein spots (**Table 3** and **Figure 8**). This analysis indicated that alkyl hydroperoxide reductase (AhpC), the Gls24 family stress protein (Gls24), and alcohol dehydrogenase (Adh) were decreased by 2.72-, 2.79-, and 2.59-fold, respectively. Real-time PCR was conducted to confirm the results of 2DE-mass spectrometry at the RNA level, and these three markers were reduced by 6.73-, 3.56-, and 6.7-fold, respectively, in the Δ*bceR* strain (**Figure 9**).

DISCUSSION

In this study, the *bceR*-like gene, belonging to the *bceRS*-like TCS family was described in GBS, and was found to mediate AMP and environmental stress resistance. The *bceR*-like system is associated with resistance to cell wall-targeting antimicrobial peptides in *B. subtilis* (Bernard et al., 2007; Dintner et al., 2014). Moreover, the *bceR*-like system (*graR*) of *S. aureus* was previously found to respond to vancomycin and polymyxin B, and the homologous proteins encoded by these genes were determined to mediate resistance to bacitracin and nisin in *S. mutans* and *Lactococcus lactis*, respectively (Tsuda et al., 2002; Kramer et al., 2006). In GBS, we found that the deletion of *bceR* resulted in an increased sensitivity to bacitracin and human cathelicidin LL-37. The regulatory effect of *bceR* on the ABC transporter *bceAB*, which encodes a protein that can pump out AMPs from the bacterial cells, is possibly the major mechanism of AMP resistance conferred by the *bceR*-like system of GBS. However, the loss of *bceR* in GBS did not alter sensitivity to erythromycin and beta-lactam antibiotics; these results demonstrate that the structurally homologous *bceRS* system might play a specific role in GBS, which highlights the importance of determining the individual roles of *bceR*-like systems in the pathogenesis of different Gram-positive species.

In *S. epidermidis*, the TA alanylation system, *dltAB*, and *mprF*, which encodes a lipid modification enzyme, were also found to be controlled by the *bceR*-like system (Li et al., 2007b; Sass et al., 2008). In GBS, the D-alanylation of TA was found to confer resistance to cationic peptides, and the lack of DltA was related to increased sensitivity to phagocytic cells and attenuated bacterial virulence (Poyart et al., 2003; Saar et al., 2012). DltA is a cytoplasmic carrier protein ligase that catalyzes the D-alanylation of the D-alanyl carrier protein DltC. DltB is a transmembrane protein that was reported to be involved in the efflux of activated

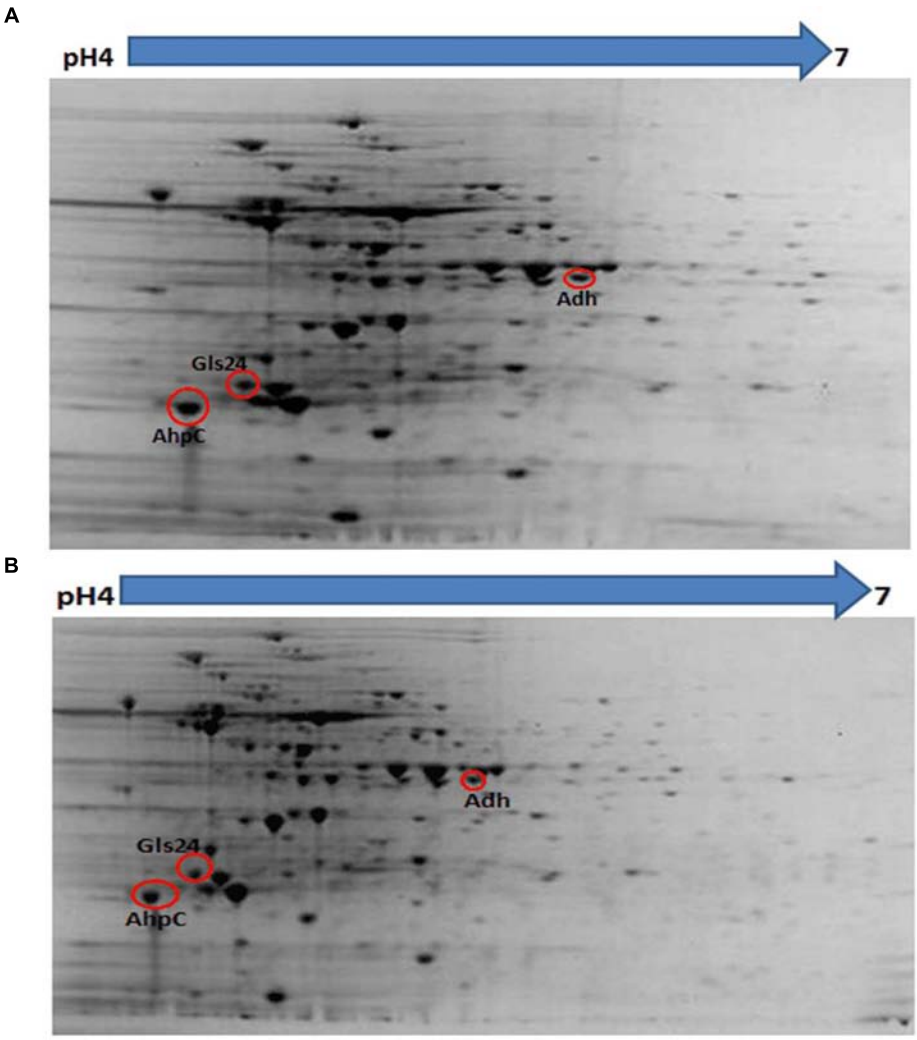

FIGURE 8 | Photographs of 2-DE gel in wild type **(A)** and Δ*bceR* strain **(B)** GBS strain. Red circles showed the proteins that found to have significantly decreased expression in Δ*bceR* strain. They were identified as alkyl hydroperoxide reductase (AhpC), The Gls24 family stress protein (Gls24), and alcohol dehydrogenase (Adh), respectively, by mass spectrometry. The gel photos were normalized and compared using software PDQuest (Version8.0.1, Bio-Rad, United States), Boolean method was chosen for detecting the proteins with statistic significantly difference in expression between GBS III-4 wild type and mutant strains.

D-alanine to the site of acylation (Joseph et al., 2004; Mandin et al., 2005). In GBS, we found that the expression of *dltA* was downregulated in the Δ*bceR* strain in the presence of bacitracin, suggesting that it might be regulated by the *bceR*-like system. Suppressing the D-alanylation of lipoteichoic acids through the repression of *dltA* would increase the negative charge of the GBS envelope, resulting in susceptibility of the Δ*bceR* strain to AMPs. In addition to the negative charge of bacteria, the density of the cell wall was shown to be altered in *dltA* mutants of *Streptococcus pyogenes* and the deletion of this gene was found to suppress the production of virulence-related proteins (Cox et al., 2009; Grubaugh et al., 2018; Luo et al., 2018). Moreover, the *bceR*-like system (*virRS*) was identified to regulate bacterial adhesion and entry into eukaryotic cells in *Listeria monocytogenes*, and the *dlt* operon, *mprF*, and *bceAB* were all found to be controlled by the regulator *virR* (Abachin et al., 2002; Camejo et al., 2009), suggesting that *dltA* might contribute to virulence in GBS, which requires further investigation. *mprF* was not differentially expressed in the presence or absence of bacitracin, indicating that this gene might respond to other inducers.

In addition to resistance to AMPs, GBS *bceR* was found to mediate environmental stress resistance and biofilm formation. Accordingly, the Δ*bceR* strain displayed increased sensitivity to H_2O_2 stress when compared to the invasive CU_GBS_08 strain, which was similar to results reported for the TCS *graRS* of *S. aureus*, which was found to be involved in resistance to superoxide radicals (Falord et al., 2011). The underlying mechanism is still unclear, but we found that the Δ*bceR* strain exhibited reduced expression of the alkyl hydroperoxide reductase AhpC, the zinc-dependent alcohol dehydrogenase Adh, and a Gls24 family protein. These proteins have been reported to be involved in oxidative stress resistance and biofilm formation

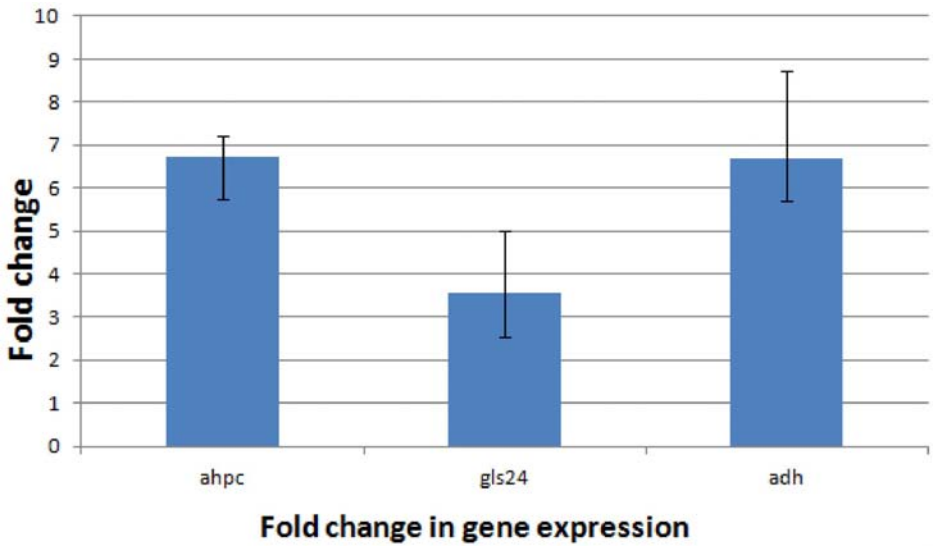

FIGURE 9 | Application of the $2^{-\Delta\Delta C_T}$ method. The experiment was conducted to validate the effect of *bceR* gene knockout on the expression of candidate genes. *ahpC,* alkyl hydroperoxide reductase; *gls24*, gls24 family general stress protein; *adh*, zinc-dependent alcohol dehydrogenase. Error bars represent the standard deviation of the mean values from at least three replicate.

(Becker et al., 2001; Teng et al., 2005; Cosgrove et al., 2007), implying the contribution of the *bceR*-like regulator to these processes in GBS. Experiments demonstrating the effects of other environmental factors on the survival of the wild-type/mutant strains, such as different pH, temperature, and osmotic pressure, were also consistent with results from previous studies on GBS (Yang et al., 2012). However, significant differences in pH tolerance, temperature tolerance, and osmotic stress resistance between wild-type and Δ*bceR* strains were not detected (data not shown). Bacterial cells within biofilms are difficult to eradicate, as they are highly resistant to antibiotics and the host immune system. The difference in biofilm-forming ability between GBS isolates from asymptomatic pregnant women (carriers) and those isolated from clinical infections was previously found to be statistically significant (Olson et al., 2002). The protein Adh was previously reported to catalyze the reversible conversion of acetaldehyde to ethanol, which is known to enhance the production of *Staphylococcus* biofilms; moreover, Adh expression was found to be upregulated in *Staphylococcus* biofilms (Becker et al., 2001; Finelli et al., 2003). In our study, all strains were able to form biofilms, but the biofilm biomass of the wild-type strain was significantly greater than that of the Δ*bceR* strain. This is consistent with a previous report suggesting that the TCS *graRS* is involved in biofilm formation in *S. aureus* (Shanks et al., 2008).

The invasive CU_GBS_08 strain used in this study was isolated from a non-pregnant adult with toxic shock syndrome, indicating the virulence of this invasive clinical strain. Therefore, the role of the *bceR*-like system in virulence was assessed by using both *in vitro* cytokine release assays and an *in vivo* mouse infection model. Our results demonstrated the mitogenic nature of this regulator and its ability to induce a significant pro-inflammatory cytokine response, which is a characteristic of the development of sepsis and septic shock. Cytokines are soluble proteins that play a significant role in inflammation and the regulation of immune responses (von Hunolstein et al., 1997). Significantly increased production of TNF-α, IL-6, and IL-1β was detected after infection with the wild-type strain compared to that with the Δ*bceR* strain. These three cytokines were reported to be positively related to disease severity (De Bont et al., 1993; Cusumano et al., 1996; von Hunolstein et al., 1997). It was previously reported that *S. epidermidis* and *S. aureus* mutant strains devoid of the *bceR*-like system are more susceptible to neutrophil-mediated killing (Cheung et al., 2010). Moreover, the expression of IL-8, a major activator of neutrophils and lymphocytes (Cusumano et al., 1996; Vallejo et al., 1996; von Hunolstein et al., 1997) was found to be reduced in Δ*bceR* strains. However, the deletion of *bceR* did not completely abrogate the proliferation of mononuclear cells and cytokine release, suggesting that other factors are also involved in the virulence and pathogenicity of this strain.

In our mouse infection model, ATCC12403, which originated from a case of fatal septicemia, was used as a control. Our wild-type invasive strain resulted in lethality that was decreased by two orders of magnitude compared to that with the ATCC strain, thus indicating its hyper-virulence. Further the attenuation of virulence in the Δ*bceR* strain was demonstrated; moreover, the Gls24 family protein was previously found to be related to bacterial virulence (Teng et al., 2005). The *bceR*-like system was previously found regulate numerous virulence factors in *S. aureus* and *L. monocytogenes* (Joseph et al., 2004; Falord et al., 2011), which in turn indicates that *bceR* might be involved in cross-talk with other regulator(s) in GBS. The non-invasive GBS strain was the least virulent among the stains tested, and harbors mutations in addition to the *bceR* truncation; this indicates that other gene(s) involved in bacterial virulence need

to be characterized. TCSs are widely used as signal transduction systems by bacteria to respond to changing growth conditions. The ability of GBS to efficiently adapt to different host niches during the infectious cycle is important for the pathogenicity of these strains. *bceRS*-like TCSs are widespread in Gram-positive bacteria and are associated with a range of bacterial activities. Further, their contributions to these activities in GBS have not been sufficiently recognized. Our results indicated that *bceR* is involved in environmental stress resistance, antimicrobial peptide resistance, and virulence, processes that are crucial for the survival of GBS in response to different microenvironments that are encountered during infection. Thus, *bceR* could be a potential target to modulate and attenuate virulence.

AUTHOR CONTRIBUTIONS

YY, ML, HZ, CL, and AL performed the experimental work. YY analyzed the data with supervision of MI and prepared first draft of the manuscript. MI and KF contributed to the GBS strains collection and design of the project. MI and GZ contributed essential ideas and discussion. All authors contributed to the drafts of the manuscript, revision and approved the manuscript submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00010/full#supplementary-material

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In vitro Effects of Antimicrobial Agents on Planktonic and Biofilm Forms of *Staphylococcus saprophyticus* Isolated From Patients With Urinary Tract Infections

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Bacterial biofilms play an important role in urinary tract infections (UTIs), being responsible for persistent infections that lead to recurrences and relapses. *Staphylococcus saprophyticus* is one of the main etiological agents of UTIs, however, little is known about biofilm production in this species and especially about its response to the antimicrobial agents used to treat UTIs when a biofilm is present. For this reason, the aim of this work was to evaluate the response of *S. saprophyticus* biofilms to five antimicrobial agents. *Staphylococcus saprophyticus* was evaluated for antimicrobial susceptibility in its planktonic form by means of minimum inhibitory concentration (MIC) and in biofilms by means of minimum inhibitory concentration in biofilm (MICB) against the following antimicrobial agents by the microdilution technique: vancomycin, oxacillin, trimethoprim/sulfamethoxazole, ciprofloxacin, and norfloxacin. Of the 169 *S. saprophyticus* studied, 119 produced a biofilm as demonstrated by the polystyrene plate adherence method. Biofilm cells of *S. saprophyticus* exhibited a considerable increase in MICB when compared to the planktonic forms, with an increase of more than 32 times in the MICB of some drugs. Some isolates switched from the category of susceptible in the planktonic condition to resistant in the biofilm state. Statistical analysis of the results showed a significant increase in MICB ($p < 0.0001$) for all five drugs tested in the biofilm state compared to the planktonic form. Regarding determination of the minimum bactericidal concentration in biofilm (MBCB), there were isolates for which the minimum bactericidal concentration of all drugs was equal to or higher than the highest concentration tested.

Keywords: *Staphylococcus saprophyticus*, biofilm, antimicrobial resistance, MICB, MBCB

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INTRODUCTION

In order to survive in hostile environments such as in host tissues (antibodies, phagocytes, etc.,) or on an inert surface where they are exposed to inhospitable conditions (UV light, desiccation, heat, cold), bacteria adapt by forming adherent populations (sessile bacteria) organized in a structure called biofilm (Mah and O'Toole, 2001).

Li et al. (2005) demonstrated that biofilm formation in *Staphylococcus* spp. depends on Polysaccharide Intercellular Adhesin (PIA), whose biosynthesis is mediated by the *ica* operon. This operon contains the *ica*ADBC genes and the regulatory *ica*R gene, which is transcribed in the direction opposite to the *ica* operon. In the case of the *ica*R gene, some studies have suggested that its product is a transcription repressor that plays an adaptive role in the regulation of the expression of the *ica* operon according to environmental conditions. Some factors such as anaerobic growth, the presence of antibiotics at subinhibitory concentrations, and environmental stress such as high osmolarity may increase expression of the *ica* operon. In addition to PIA, the existence of *ica*-independent mechanisms for biofilm formation in *Staphylococcus* spp., such as proteins and DNA, has been highlighted (Mendoza-Olazarán et al., 2015).

Once formed, these biofilms render the cells less accessible to the defense system of the organism, impairing the action of antibiotics. Biofilms thus represent basic survival strategies of these microorganisms, a fact that explains why biofilms are considered to be of major public health importance. Furthermore, the proximity of cells inside microcolonies or between microcolonies provides an excellent environment for the exchange of genetic material. The mechanism of conjugation, i.e., the transfer of plasmids between bacteria, occurs at a higher proportion between bacterial cells in biofilms than between planktonic cells (Águila-Arcos et al., 2017).

In the laboratory, the effectiveness of an antibiotic is evaluated with the microorganism in its planktonic form (free cells). However, these assays only reveal the concentration of the chemotherapeutic agent that is necessary to inhibit growth or kill planktonic bacteria (Jorgensen and Ferraro, 2009). Maximum resistance to antibiotics is achieved once microorganisms complete the formation of the mature biofilm (Høiby et al., 2010). For some antibiotics, the concentration required to kill sessile bacteria can be up to a thousand times greater than the concentration required to kill exactly the same strain in its planktonic form (Nickel et al., 1985; Aslam, 2008). Therefore, in some circumstances, the use of planktonic bacteria for the selection of chemotherapeutic agents may be inappropriate.

Biofilm formation can be considered a virulence determinant that is responsible for the long-term persistence of bacteria in the genitourinary tract (Costerton et al., 1999). Urinary catheters and other prosthetic devices predispose to urinary tract infections (UTIs) by destroying natural barriers (urethral sphincter) and providing a nidus for infection that serves as a substrate for biofilm formation. Bacterial biofilms play an important role in UTIs, being responsible for persistent infections that lead to recurrences and relapses (Delcaru et al., 2016).

The most commonly prescribed antibiotics for the treatment of UTIs are trimethoprim/sulfamethoxazole, fluoroquinolones, first- and second-generation cephalosporins, amoxicillin + clavulanate, and nitrofurantoin (Lee et al., 2008). According to the CLSI M100-S26 document (2016), routine susceptibility testing of urinary *S. saprophyticus* isolates is not recommended since this microorganism is normally susceptible to the antimicrobial agents used to treat acute uncomplicated UTIs (nitrofurantoin, sulfamethoxazole/trimethoprim, or a fluoroquinolone). However, 17.6% of the *S. saprophyticus* isolated from UTIs tested by Ferreira et al. (2012) were resistant to sulfamethoxazole/trimethoprim, a fact that may lead to therapeutic failure when UTIs are treated empirically. Antibiotic resistance seems to have emerged also among *S. saprophyticus* strains and antimicrobial susceptibility testing of these strains is therefore necessary.

Staphylococcus saprophyticus is one of the main etiological agents of UTIs, however, little is known about biofilm production in this species and especially about its response to the antimicrobial agents used to treat UTIs when a biofilm is present. For this reason, the aim of this work was to evaluate the response of *S. saprophyticus* biofilms to five antimicrobial agents.

MATERIALS AND METHODS

Samples

Staphylococcus saprophyticus isolated from the urine of different patients were used in the study. The strains were obtained in a prospective study through isolation in the Laboratory of Microbiology, University Hospital of the Botucatu School of Medicine (HC-FMB), SP, Brazil, in 2013 and 2014 or were obtained from a culture collection established in 2008. The samples were collected from patients originating from wards, outpatient clinics, emergency rooms, and basic health units of Botucatu and region. The present study was approved by the institutional Ethics Committee (Protocol 16269813.1.0000.5411) and was exempt from the requirement of free informed consent of the participants in this study since we did not use clinical data of the patients and had no contact with the patients. Bacteria had previously been isolated from the patients and were stored at the Laboratory of Microbiology (HC-FMB).

Individuals of both genders and all ages with *S. saprophyticus*-positive urine cultures compatible with UTI, with a colony count equal to or greater than 100,000 colony forming units per milliliter of urine ($\geq 10^5$ CFU/mL) according to the criteria of Kass (1956), were included. Samples were collected according to the urine collection protocol of the service.

The isolates were seeded on blood agar with 5% sheep blood (secondary isolation) and stained by the Gram staining method for the assessment of purity and observation of their specific morphology and staining. After confirmation of these characteristics, the strains were submitted to the catalase, DNAse, and tube coagulase (gold standard) tests to distinguish *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) as recommended by Koneman et al. (1997).

DNA Extraction and Identification of *S. saprophyticus*

DNA was extracted from isolates identified as CoNS with the Illustra®Kit (GE Healthcare) according to manufacturer's instructions.

Isolates identified as CoNS were genotyped using primers targeting conserved sequences adjacent to the 16S and 23S genes by the internal transcribed spacer-PCR (ITS-PCR) technique described by Couto et al. (2001). The G1 "GAAGTCGTAACAAGG" 16S and L1 "CAAGGCATCCA CCGT" 23S primers were used. The efficiency of the amplifications was monitored by electrophoresis on 3% MetaPhor agarose prepared in 1X TBE buffer and stained with SYBR Safe. The following international reference strains were used as controls: *S. epidermidis* (ATCC 12228), *S. epidermidis* (ATCC 35983), *S. haemolyticus* (ATCC 29970), *S. hominis* (ATCC 27844), *S. hominis* subsp. *novobiosepticus* (ATCC 700237), *S. lugdunensis* (ATCC 700328), *S. saprophyticus* (ATCC 15305), and *S. warneri* (ATCC 10209).

Detection of *mecA* Gene for Oxacillin Resistance

For detection of the *mecA* gene, PCR was performed using the *mecA1* (AAA ATC GAT GGT AAA GGT TGG) and *mecA2* (AGT TCT GCA GTA CCG GAT TTG) – 533 (bp) primers according to the parameters described by Murakami et al. (1991). International reference strains were included in all reactions: *S. aureus* ATCC 33591 (positive) and *S. aureus* ATCC 25923 (negative).

Agarose gels were prepared at a concentration of 2% in 1X TBE, stained with SYBR Safe DNA Gel Stain® (Invitrogen), and visualized under a UV transilluminator.

Detection of Biofilm Production by the Polystyrene Plate Adherence Method (Christensen et al., 1985) Modified by Oliveira and Cunha (2010)

The method of detecting biofilm production in culture plates proposed by Christensen et al. (1985) was used, with modifications proposed by Oliveira and Cunha (2010). This method is based on the spectrophotometric reading of optical density of the adherent material produced by the bacteria. International reference strains used as positive (*S. aureus* ATCC 29213, *S. epidermidis* ATCC 35983) and negative controls (*S. aureus* ATCC 33591, *S. epidermidis* ATCC12228) and sterile TSB were included in all tests. Optical density reading was carried out in an ELISA reader (Labsystems, model Multiskan EX) using a 540-nm filter. Samples were classified as negative when the cut-off value corresponded to the classification of non-adherent ($\leq$ 0.111) and as positive when the cut-off value corresponded to the classification of weakly adherent (>0.111 or $\leq$0.222) or strongly adherent (>0.222). These cut-offs values were established by Oliveira and Cunha (2010).

Evaluation of Biofilm Formation With Visualization by Scanning Electron Microscopy (SEM) in an Isolate of Biofilm-Producing *S. saprophyticus*

A biofilm-producing *S. saprophyticus* isolate in the polystyrene plate adherence test was selected for confirmation of biofilm production by SEM. The biofilm-producing strain was first isolated in BHI broth and 10^8 CFU of bacteria were transferred to a conical tube (Falcon-CORNING) containing 2 mL TSB culture medium prepared with 2% glucose and a 0.5-cm segment of VYGON umbilical catheter (reference 1270.04, 0.8 mm × 1.5 mm diameter). The tube was incubated under constant stirring for 48 h at 100 rpm/37°C for bacterial growth and biofilm formation. After this period, the catheter segment was removed, washed with PBS, immersed in 2.5% glutaraldehyde solution, fixed in an increasing alcohol series (15, 30, 50, 70, 90, and 100%) for 15 min each, dried in a vacuum centrifuge for 5 min, metallized with gold, and visualized under a scanning electron microscope to evidence biofilm production.

Determination of MIC of Vancomycin, Oxacillin, Norfloxacin, Ciprofloxacin, and Trimethoprim/Sulfamethoxazole for Planktonic Cells of *S. saprophyticus* by the Broth Microdilution Method

The broth microdilution method was used for determination of the (MIC) for planktonic cells of *S. saprophyticus*. Sterile microtiterplates with Müller-Hinton broth adjusted with cations (Oxoid, United Kingdom) as recommended by the CLSI (2016) were used. A stock solution of each drug was prepared in 3,200 μg/mL distilled water. Serial dilutions were made in a microtiter plate containing Müller-Hinton broth at concentrations on a logarithmic scale of two, comprising the breakpoints (CLSI, 2016), in a final volume of 100 μL. For preparation of the inoculum, the isolates were first seeded on blood agar. After incubation for 24 h, isolated colonies were seeded in BHI broth and the bacterial suspensions were adjusted to a turbidity of 0.5 McFarland standard (1×10^8 CFU/mL), diluted at 1:1000, and added to the wells in a final volume of 200 μL and final bacterial concentration of 5×10^4 CFU/well. The plates were incubated in an oven at 35°C and the MIC was read after 24 and 48 h of incubation. A positive control containing the broth and bacterial suspension and a negative control containing only the Müller-Hinton broth were used. In addition, *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 29213 (susceptible to vancomycin) were used as negative controls, and *E. faecalis* ATCC 51299 (resistant to vancomycin) and *S. aureus* ATCC 33591 (resistant to oxacillin) were used as positive controls. The MIC was defined as the lowest concentration of antimicrobial that completely inhibited the growth of the microorganism as detected by the naked eye. Wells with turbidity and/or the presence of bacteria at the bottom of the well were classified as positive growth. The susceptibility and resistance cut-offs recommended by the CLSI (2016) were

used to determine the MIC for planktonic cells. The same cut-offs were used to evaluate the biofilm antimicrobial susceptibility of the isolates since no standards exist for biofilm tests.

Determination of (MICB) and (MBCB) of Vancomycin, Oxacillin, Norfloxacin, Ciprofloxacin, and Trimethoprim/Sulfamethoxazole for *S. saprophyticus* Biofilm by the Broth Microdilution Method

Bactericidal concentrations for biofilms (MBCB) were determined by adapting the test method described by Frank et al. (2007). The isolates cultured for 22 h in TSB with 2% glucose were adjusted to a turbidity of 1.0 McFarland standard (corresponding to 1×10^8 to 2×10^8 CFU/mL) and diluted at 1:50 in TSB with 2% glucose. Aliquots (200 μL) were plated in 96-well flat bottom plates (Nuclon Delta, Nunc, Denmark), covered with a 96-pin cap (Nunc-TSP; Nunc), and incubated for 24 h to allow biofilm formation on the pins. To remove non-adherent cells, the biofilms formed on the pins were washed by immersion in a series of three 96-well plates filled with 200 μL of sterile saline phosphate-buffered saline (PBS). The cap with the pins was placed on a flat bottom plate prepared for broth microdilution susceptibility testing. The wells contained 200 μL of antimicrobial agent diluted in CAMHB (Müller-Hinton broth supplemented with cations, 100 mg/mL calcium, and 50 mg/mL magnesium) or 200 μL of CAMHB without drugs as positive growth control. The biofilms were exposed to the antimicrobials for 24 h. The cap with the pins was removed, washed three times in PBS as described above, and transferred to 96-well plates containing 200 μL TSB plus 2% glucose. On that occasion, prior to discarding the plate with the antibiotics, a "naked eye" reading was performed to determine the MIC of the antibiotics for biofilm cells (MICB). Subsequently, the biofilm cells formed on the cap pins were dislodged by sonication for 5 min at 40 kHz (Hielscher, Ultrasonic Technology, UIP250MTP) in 96-well plates containing fresh culture medium for cell recovery. The cap with the pins was discarded and replaced with a normal cap and optical density was measured in a plate reader equipped with a 600-nm filter. Wells containing TSB plus 2% pure glucose (without inoculation) were used as spectrophotometric sterility controls. The plate was incubated for 24 h and a second optical density measurement at 600 nm was taken. The MBCB was defined as the lowest concentration of the drug that exhibited a change in optical density at 600 nm of 10% of the reading obtained for the positive growth control between the readings performed before incubation and after 24 h. For better control of the efficacy of the test, we used the biofilm-producing strain *S. epidermidis* ATCC 35983 and the non-producing strain *S. epidermidis* ATCC 12228 as controls.

Statistical Analysis

Correlation analysis between antimicrobial susceptibility and the inhibitory concentration of the drugs for planktonic and biofilm bacteria was performed using the Chi-squared test or Fisher's exact test (SPSS® 13.0 software), adopting a level of significance <0.05.

RESULTS

Detection of Biofilm Production by the Polystyrene Plate Adherence Method

A total of 169 samples of *S. saprophyticus* isolated from patients with UTI were used. Of these, 119 (70.4%) produced a biofilm and 88 (52.1%) were classified as strongly adherent and 31 (18.3%) as weakly adherent.

Evaluation of Biofilm Formation With Visualization by SEM

An *S. saprophyticus* isolate classified as strongly adherent in the evaluation of biofilm production on polystyrene plates was selected for SEM analysis of biofilm production. **Figure 1** shows the biofilm structure produced by *S. saprophyticus* isolated from a case of UTI.

Evaluation of Antimicrobial Susceptibility of Planktonic and Biofilm Cells of *S. saprophyticus*

Biofilm antimicrobial susceptibility was evaluated in the 119 isolates producing biofilms on polystyrene plates. The same drugs as those employed to evaluate antimicrobial susceptibility in planktonic isolates for determination of MIC were used to test the biofilm antimicrobial susceptibility by establishing the (MICB; **Table 1**).

The determination of MIC in planktonic cells against the five antimicrobials revealed that 117 (98.3%) isolates were resistant to oxacillin, with MIC_{50} of 1 μg/mL and MIC_{90} of 2 μg/mL, but only three isolates (2.5%) were positive for the *mecA* gene. These three isolates exhibited the highest MIC (256 μg/mL), while the other 116 showed MIC ranging from ≤ 0.25 to 2 μg/mL. In addition, 21 (17.7%) isolates were resistant to trimethoprim/sulfamethoxazole, with MIC_{50} of 0.25/2.38 μg/mL and MIC_{90} of 4/76 μg/mL. All isolates were susceptible to vancomycin with MIC_{50} of 1 μg/mL and MIC_{90} of 2 μg/mL, to norfloxacin with MIC_{50} of 2 μg/mL and MIC_{90} of 4 μg/mL, and to ciprofloxacin with MIC_{50} and MIC_{90} of 0.25 μg/mL (**Figure 2**).

Using the criteria for interpretation of susceptibility tests recommended by the CLSI (2016) for determination of MIC in planktonic CoNS as a guideline to evaluate the antimicrobial susceptibility of the biofilm isolates, none of the drugs was found to be totally effective against the biofilm isolates. Statistical analysis of the results showed a significant increase in MICB ($p < 0.0001$) for all five drugs tested in the biofilm state compared to the planktonic forms (**Figure 2**).

There was a considerable increase in susceptible planktonic isolates that became resistant in the biofilm state (**Table 1**). Of the 119 biofilm isolates analyzed, 28 (23.5%) exhibited intermediate resistance or resistance to vancomycin (MICB 1 to 64 μg/mL). All isolates were resistant to oxacillin (MICB 0.5 to 2048),

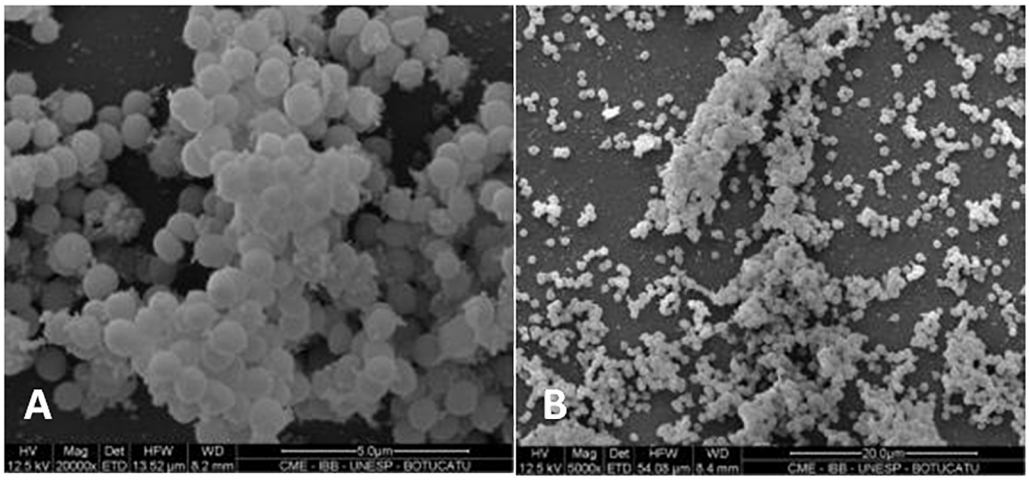

FIGURE 1 | Scanning electron micrograph showing the biofilm structure in *Staphylococcus saprophyticus*. Magnification: **(A)** 20,000×; **(B)** 5,000×.

41 (34.4%) exhibited intermediate resistance or resistance to norfloxacin (MICB 2 to 64 μg/mL), 30 (25.2%) demonstrated intermediate resistance or resistance to ciprofloxacin (MICB 0.125 to 64 μg/mL), and 58 (48.7%) were resistant to trimethoprim/sulfamethoxazole (MICB 0.06/1.18 μg/mL to 64/1,216 μg/mL), considering the CLSI (2016) cut-off point for resistance in planktonic cells (**Table 1** and **Figure 2**). Regarding resistance to trimethoprim/sulfamethoxazole, it is important to note that 21 (17.7%) of the 58 (48.7%) isolates resistant to MICB were already resistant in the MIC evaluation of this drug; thus, 37 (31.1%) of the isolates changed from susceptible to resistant in the biofilm state.

The biofilm isolates exhibited a considerable increase in MICB when compared to the planktonic forms, with an increase of more than 32 times in the values of some drugs. Some isolates switched from the category of susceptible in the planktonic condition to resistant in the biofilm state (**Figure 2** and **Table 2**).

Regarding determination of the MBCB, there were isolates for which the minimum bactericidal concentration of all drugs was equal to or higher than the highest concentration tested (**Figure 3**), with emphasis on norfloxacin with 33 (27.7%) samples with MBCB > 128 μg/mL and trimethoprim/sulfamethoxazole with 36 (30.2%) samples with MBCB > 128/2,432 μg/mL.

TABLE 1 | Comparison of drug resistance profile between planktonic and biofilm cells of *Staphylococcus saprophyticus*.

	Planktonic bacteria		Bacteria in biofilm	
Drug	**R (%)**	**IR (%)**	**R (%)**	**IR (%)**
Vancomycin	0 (0)	0 (0)	9 (7.6)	19 (16.0)
Oxacillin	117 (98.3)	•	119 (100.0)	•
Norfloxacin	0 (0)	0 (0)	26 (21.8)	15 (12.6)
Ciprofloxacin	0 (0)	0 (0)	24 (20.2)	6 (5.0)
Trim/Sut	21 (17.7)	•	58 (48.7)	•

•, *Drugs without intermediate resistance MIC; R, resistant; IR, intermediate resistance; Trim/Sut, trimethoprim/sulfamethoxazole.*

DISCUSSION

The formation of bacterial biofilms is the basis of many persistent infectious diseases. This persistence is attributed mainly to the increased antibiotic resistance of biofilm cells (Mah, 2012).

The MIC has been used as a gold standard to determine the antimicrobial susceptibility of pathogenic bacteria (Costerton et al., 1995). When MIC determination reveals that the drug is not effective in inhibiting the growth of a given organism, the drug in question will not be used for the treatment of infection because it will be clinically ineffective (Potera, 1999). However, if a microorganism is considered susceptible *in vitro*, it does not necessarily mean that the drug will have the same effect *in vivo* (Pratt and Kolter, 1999; Mendoza-Olazarán et al., 2015; Algburi et al., 2017). In routine clinical laboratories, antimicrobial susceptibility testing for antibiotic selection continues to be performed using planktonic cells, a fact that impairs evaluation of the efficacy of the antimicrobial tested since these bacteria are protected by the biofilm in the patient and the response will not be the same as obtained in the tests.

The determination of MIC to evaluate the susceptibility of planktonic *S. saprophyticus* cells revealed that most samples were susceptible to the antibiotics tested. Regarding oxacillin resistance, 98.3% of the planktonic cells were resistant in the microdilution test, but only three isolates were positive for the *mecA* gene. The three samples that were positive for the *mecA* gene showed the highest MIC (256 μg/mL) and the remaining 116 had MIC ranging from ≤ 0.25 to 2 μg/mL. Similar results have been reported in other studies and might be due to the fact that the breakpoint recommended by the CLSI overestimates resistance in this species (Ferreira et al., 2012).

In general, the antibiotics tested proved to be ineffective in *S. saprophyticus* biofilms as resistant isolates were found for all

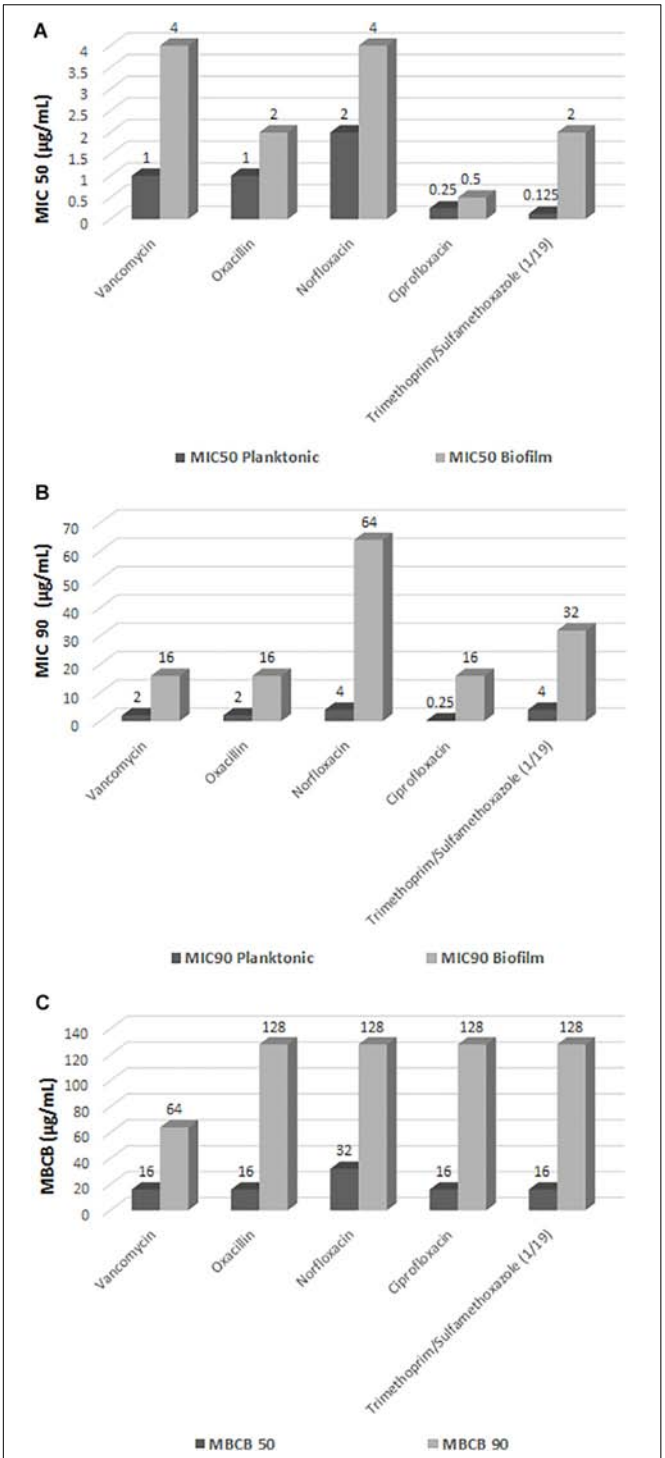

FIGURE 2 | Minimum inhibitory concentration (MIC) in planktonic cells of *Staphylococcus saprophyticus*, minimum inhibitory concentration in biofilm (MICB), and minimum bactericidal concentration in biofilm (MBCB). **(A)** MIC 50 (μg/mL); **(B)** MIC 90 (μg/mL); **(C)** MBCB.

drugs tested. This is a matter of concern because high doses of antibiotics would be necessary to eliminate these microorganisms organized in biofilms, which is clinically impractical. Biofilm cells may be more resistant to antibiotics because the bacteria are protected against the action of the drugs, with the biofilm impairing the entry of molecules by acting as a physical barrier for diffusion. In addition, biofilm cells have reduced metabolic and growth rates and the biofilm matrix can adsorb or react with the antibiotics, thereby reducing the amount of antibiotics available to interact with cells in the biofilm. Another possibility is that the biofilm cells are tolerant to antibiotics. Hence, treatment may lead to the eradication of most part of the biofilm population, but a fraction of persistent cells is not affected and thus acts as a nucleus for reinfection after therapy discontinuation (Lewis, 2012).

The microorganisms inside a biofilm express different phenotypic characteristics when compared to their free-living homologs. In a study investigating whether the antibiotic resistance genes *aac6-aph2a*, *ermC*, and *tetK*, which confer resistance to gentamicin, erythromycin and tetracycline, are likely to be disseminated via conjugative transfer, Águila-Arcos et al. (2017) searched for horizontal transfer genes from two common staphylococcal plasmids, (i) conjugative pSK41 and (ii) mobilizable pT181, in 25 staphylococcal biofilm-forming clinical isolates belonging to the species *S. aureus*, *S. epidermidis, S. hominis,* and *S. capitis*. Both horizontal transfer and antibiotic resistance genes were detected in these staphylococcal isolates. Therefore, biofilms represent a hot spot for horizontal gene transfer by bacterial conjugation. This horizontal gene transfer is important for the genetic diversity of microbial communities and favors the exchange of genes that can contribute to the chronic nature of infections (Vuotto et al., 2014). The detection of horizontal transfer and antibiotic resistance genes in these clinical staphylococcal strains isolated from biofilms points to the potential risk of the development and dissemination of multidrug-resistant bacteria.

The most commonly prescribed antibiotics for the treatment of UTIs are trimethoprim/sulfamethoxazole, fluoroquinolones (ciprofloxacin or norfloxacin), first and second generations of cephalosporins, amoxicillin + clavulanate, and nitrofurantoin (Lee et al., 2008). In the present study, 17.7% of the samples were already resistant to trimethoprim/sulfamethoxazole in the evaluation of planktonic MIC, while 48.7% of the biofilm samples were resistant. In addition, 31.1% of the samples changed from susceptible to resistant in the biofilm state, an alarming finding considering that the trimethoprim/sulfamethoxazole combination is considered the first-line drug for the treatment of uncomplicated UTIs (Drekonja and Johnson, 2008). Thus, the frequent use of the drug in empirical therapy is associated with an increase in the clinical failure rate, especially if the microorganism grows in biofilms, as observed in the present study.

The administration of fluoroquinolones is recommended for uncomplicated UTIs in areas where the incidence of trimethoprim/sulfamethoxazole resistance is higher than 10%, as well as for the treatment of complicated UTIs and acute pyelonephritis (Blondeau, 2004). Fluoroquinolones have been successfully used to treat a wide range of community-acquired and hospital-acquired infections, and rates of resistance to fluoroquinolones remain low (Oliveira et al., 2016). In fact, in the present study, all planktonic samples were susceptible

TABLE 2 | Variation of the increase in MIC and change of the category from susceptible to resistant in relation to planktonic cells and in biofilm.

	2X (%)	4X(%)	8X(%)	16X(%)	32X(%)	64X(%)	128X(%)	256X (%)	S-I(%)	S-R(%)
Vancomycin	59 (49.5)	32 (26.9)	10 (8.4)	9 (7.6)	7 (5.9)	2 (1.7)	–	–	19 (16.0)	9 (7.6)
Oxacillin	71 (59.7)	21 (17.6)	9 (7.6)	8 (6.7)	7 (5.9)	2 (1.7)	1 (0.8)	–	•	2 (1.7)
Norfloxacin	83 (69.7)	10 (8.4)	7 (5.9)	10 (8.4)	9 (7.6)	–	–	–	15 (12.6)	26 (21.8)
Ciprofloxacin	57 (47.9)	27 (22.7)	11 (9.3)	3 (2.5)	5 (4.2)	6 (5.0)	5 (4.2)	5 (4.2)	6 (5.0)	24 (20.2)
Trim/Sut	28 (23.5)	28 (23.5)	14 (11.8)	14 (11.8)	13 (10.9)	7 (5.9)	7 (5.9)	8 (6.7)	•	37 (31.1)

•, *Drugs without intermediate resistance MIC; X, Number of times MIC increased in biofilm samples; S-I, susceptible-intermediate: percentage of isolates with intermediate resistance only in the presence of the biofilm; S-R, susceptible-resistant: percentage of isolates that were resistant only in the presence of the biofilm; Trim/Sut, trimethoprim/sulfamethoxazole.*

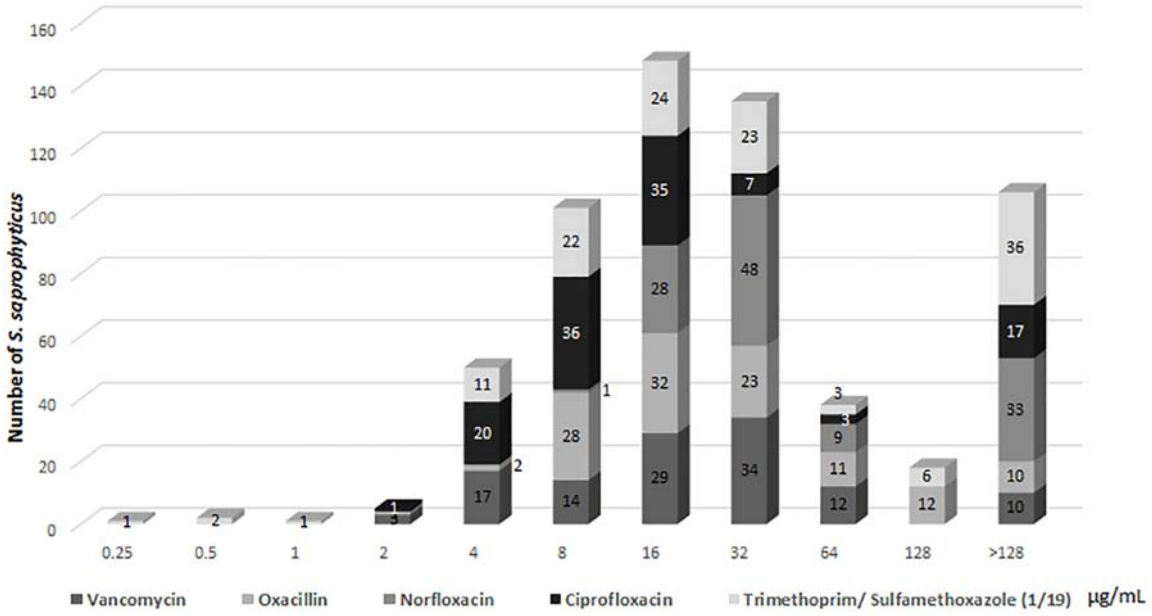

FIGURE 3 | Profile of (MBCB) of *Staphylococcus saprophyticus.*

to norfloxacin and ciprofloxacin, however, the same was not observed for the biofilm samples, with 34.4% of the isolates exhibiting intermediate resistance or resistance to norfloxacin and 25.2% exhibiting intermediate resistance or resistance to ciprofloxacin. The presence of the biofilm increased the MIC by two, four, eight and up to 32 times the values obtained for some drugs, with some samples switching from the category of susceptible in the planktonic condition to resistant in the biofilm state. This phenomenon was more frequently observed for norfloxacin, ciprofloxacin, and trimethoprim/sulfamethoxazole.

Oliveira et al. (2016) evaluated the MIC for planktonic and biofilm cells of *Staphylococcus* spp. comparing six drugs, and observed a two-, four-, eight-, and up to 16-fold increase of MIC in the presence of the biofilm compared to planktonic cells, mainly for the drugs vancomycin and erythromycin. In that study, among the 20 *S. saprophyticus* isolates studied, no planktonic samples were resistant to vancomycin and linezolid. However, regarding the MICB, the percentage of samples that moved from susceptible to resistant or intermediate resistant was 53.8% for vancomycin and 30.8% for erythromycin. The authors also observed that *S. haemolyticus*, *S. hominis*, *S. warneri*, and *S. lugdunensis* isolates did not exhibit much variation of MIC in the presence of the biofilm, probably because these species are poor biofilm producers.

Regarding determination of MBCB in the present study, there were isolates for which the MBCM of all drugs was equal to or higher than the highest concentration tested. The results corroborate the observation that microorganisms susceptible to certain antimicrobials in conventional laboratory tests may be highly resistant to the same antimicrobials when grown in biofilms. Consequently, infectious diseases involving biofilms are generally difficult to treat. Bacterial biofilms play an important role in UTIs, being responsible for persistent infections that lead to recurrences and relapses (Delcaru et al., 2016).

Studies have demonstrated the importance of bacterial biofilm formation in UTIs, particularly chronic cystitis and catheter-associated infections (Hancock et al., 2007). Urinary catheters and other prosthetic devices predispose to UTIs by serving as a substrate for biofilm formation, carrying a higher bacterial burden and increasing the risk of epithelial adhesion.

The finding that *S. saprophyticus* isolates can produce biofilms, in addition to the observation of resistance to the antimicrobial

agents when these microorganisms were grown in biofilms, suggests that biofilm formation is a very important virulence factor for *S. saprophyticus*, which permits this species to establish persistent UTIs. This study demonstrated that the severity of UTIs depends not only on the susceptibility of the microorganism to the antibiotics commonly used for treatment, but also on the virulence of the bacteria. Biofilm production by *S. saprophyticus* and its role in UTIs remain poorly studied. Treatment of this infection is usually simple and rapid, however, if not treated correctly with efficient antimicrobials, progression to much more severe infection of the kidneys (pyelonephritis) may occur that can lead to generalized infection, renal abscesses, and loss of kidney function. No data are available correlating the inefficacy of antibiotics in the treatment of UTIs with the biofilm formation by *S. saprophyticus* or any other species. However, the results of the present study show that more attention should be given to this virulence factor in *S. saprophyticus* and to the antimicrobial treatments used since *in vitro* biofilm formation decreases the susceptibility of the microorganisms to the antibiotics tested. The results of conventional antimicrobial susceptibility tests (MIC) cannot be applied to microorganisms grown in biofilms as the antimicrobials tested were unable to eradicate biofilm-bound bacteria. This was clearly demonstrated in the present study.

CONCLUSION

The present study shows that biofilm production is a successful strategy for the microbial survival of *S. saprophyticus* and should be taken into account in the treatment of UTIs that do not consistently respond to therapeutic concentrations, as the response to antimicrobials may be impaired in bacterial biofilms. This virulence factor may increase the survival capacity of the pathogen during the treatment of infection with antimicrobial agents.

AUTHOR CONTRIBUTIONS

KM participated in the conception and design of the study, carried out the microbiological tests, and wrote the paper. AF provided the clinical material and helped with the conception and design of the study. VP, LP, and AO helped with the microbiological tests. MC was responsible for the conception and design of the study, coordination of laboratory work, data analysis, and manuscript writing.

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Signal Transduction Proteins in *Acinetobacter baumannii*: Role in Antibiotic Resistance, Virulence, and Potential as Drug Targets

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Acinetobacter baumannii is a notorious pathogen in health care settings around the world, primarily due to high resistance to antibiotics. *A. baumannii* also shows an impressive capability to adapt to harsh conditions in clinical settings, which contributes to its persistence in such conditions. Following their traditional role, the **T**wo **C**omponent **S**ystems (TCSs) present in *A. baumannii* play a crucial role in sensing and adapting to the changing environmental conditions. This provides *A. baumannii* with a greater chance of survival even in unfavorable conditions. Since all the TCSs characterized to date in *A. baumannii* play a role in its antibiotic resistance and virulence, understanding the underlying molecular mechanisms behind TCSs can help with a better understanding of the pathways that regulate these phenotypes. This can also guide efforts to target TCSs as novel drug targets. In this review, we discuss the roles of TCSs in *A. baumannii*, their molecular mechanisms, and most importantly, the potential of using small molecule inhibitors of TCSs as potential novel drug targets.

Keywords: two-component systems, PmrAB, AdeRS, BfmRS, stress

INTRODUCTION

Acinetobacter baumannii is a Gram-negative coccobacillus, which is an important opportunistic human pathogen that causes hospital-acquired infections (Peleg et al., 2008a, 2012; Visca et al., 2011; Wong et al., 2017). Clinical importance of *A. baumannii* is emphasized by the fact that it is listed by the WHO as the "top priority" pathogen that urgently need novel and effective therapeutic options (http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf). The success of *A. baumannii* in hospital environments can be mainly attributed to its ability to display multi-drug resistant phenotypes due to the rather robust acquisition of antibiotic resistance mechanisms (Dijkshoorn et al., 2007; Antunes et al., 2014). These include antibiotic modifying enzymes, decreased permeability to antibiotic molecules, and efflux pumps that extrude the antibiotic molecules out to the periplasm and beyond (Gordon and Wareham, 2010; Lee et al., 2017).

Multi- and pan-drug resistance in *A. baumannii* is an alarming development for healthcare facilities around the world (Rodriguez-Bano et al., 2004; Agodi et al., 2010; Sievert et al., 2013; Labarca et al., 2016). As a result, some infections caused by multi-drug resistant *A. baumannii* have become virtually untreatable with our current arsenal of antibiotics (Maragakis and Perl, 2008). Further, without any new antibiotics for Gram-negative bacteria, such as *A. baumannii* in the developmental pipeline, we are on the verge of a post-antibiotic era where even a minor infection could have lethal consequences for the patient (Xie et al., 2018).

Apart from its multidrug resistance, the success of *A. baumannii* can also be attributed to its ability to survive and persist in the harsh conditions found within hospital environmental niches (Jawad et al., 1998; Rajamohan et al., 2010). Constant and prolonged exposure to antiseptics and desiccating agents, endurance of less than optimal temperatures, and sudden changes of the environmental and nutritional conditions when transferred into the human body from an abiotic surface are some of the challenges that *A. baumannii* faces in its role as an opportunistic human pathogen. Therefore, in order to be a successful pathogen, *A. baumannii* needs to sense and adapt to these changes in an efficient and timely manner.

Signal transduction mechanisms in bacteria play a crucial role in adapting to environmental changes. TCSs are one of the most ubiquitous signal transduction systems present in bacteria that help them sense and adapt to the environmental conditions (Alm et al., 2006; Wood et al., 2018). TCSs therefore play a role in bacterial adaptive responses which can lead to the modulation of their antibiotic susceptibility and virulence. Consequently, these systems are vital to study in order to understand the mechanisms of antibiotic resistance and virulence in bacteria (Poole, 2012; Kroger et al., 2016; Schaefers et al., 2017; Kenney, 2018; Lingzhi et al., 2018). Further, TCSs can also serve as an attractive target when developing anti-virulence therapeutics (Gotoh et al., 2010b). In this review, we describe the roles of TCSs in the resistance and virulence of *A. baumannii* and their potential to be used as novel therapeutic targets.

TWO COMPONENT SYSTEMS (TCSs)

TCSs are the most widespread signal transduction system present in bacteria and archaea (Stock et al., 2000). Typically, a TCS consists of two components, a histidine kinase (HK) and a response regulator (RR) (**Figure 1**). A high level of specificity with the HK and the RR is observed within the TCSs of a bacterial cell (Szurmant et al., 2007). However, there are instances where a single HK protein can have multiple cognate RR proteins (Lopez-Redondo et al., 2010) or when a single RR protein can be activated by multiple HK proteins (Laub and Goulian, 2007). Since their first description in 1986 (Nixon et al., 1986), an enormous amount of both HK and RR proteins have been discovered and characterized in a wide variety of bacteria (Whitworth and Cock, 2009). It is estimated that an average bacterial genome can contain up to 50–60 TCS-encoding genes (Whitworth, 2008; Whitworth and Cock, 2008; Wuichet et al., 2010). Given the advancement in bioinformatics and next generation sequencing techniques, specific databases dedicated to TCSs have become available that provide valuable information about these proteins (Ulrich and Zhulin, 2007; Barakat et al., 2011).

The TCSs in bacterial systems have implications for a wide variety of regulatory functions relating to sensing and adapting to their environment. In pathogenic bacteria, these functions often include but are not limited to antibiotic susceptibility modulation and virulence-related phenotypes, such as biofilm formation and motility (Tiwari et al., 2017).

TCSs IN *Acinetobacter baumannii*

An overview of various genomes of well-characterized *A. baumannii* clinical isolates show the presence of close to 20 different genes/operons that encode for TCSs (**Table 1**). Most of these genes and operons have a high degree of conservation at nucleotide level, indicating that they may be involved in the important functions. However, as mentioned above, the effector domains of *A. baumannii* RR proteins can be quite diverse which is shown in **Figure 2**. Below we describe the TCSs in *A. baumannii* that have been characterized to date.

AdeRS

AdeRS is the first characterized and also the most studied TCS in *A. baumannii*. It was first described in a clinical strain *A. baumannii* BM4454, when the inactivation of *adeS* resulted in an increased susceptibility to aminoglycosides due to the downregulation of the RND efflux pump AdeABC (Marchand et al., 2004) (**Figure 3**). Since it was first identified, a number of mutations in either *adeR*, *adeS,* or both have been shown to be directly responsible for the overexpression of the AdeABC pump (Ruzin et al., 2007; Yoon et al., 2013; Sun et al., 2016). Considering AdeRS system's role in the expression of AdeABC, it can be said that it plays a role in the susceptibility of *A. baumannii* to antibiotics that are substrates of the AdeABC pump. Further, the overexpression of AdeABC efflux pump has been associated with the decreased susceptibility to tigecycline observed in some clinical isolates of *A. baumannii* (Sun et al., 2014; Yuhan et al., 2016) thus implicating an indirect role of AdeRS in the susceptibility toward tigecycline. This is important since tigecycline is one of the last resort antibiotics for the treatment of multidrug resistant *A. baumannii* infections (Ni et al., 2016). However, there needs to be further investigations into this due to the possibility of involvement of other factors for the observed tigecycline susceptibility (Yoon et al., 2013).

Recent transcriptomics data suggest that the role of AdeRS extends well-beyond the expression of AdeABC efflux pump. A study in *A. baumannii* AYE showed that AdeRS controls the expression of almost 600 different genes (Richmond et al., 2016). Products of a number of these genes are believed to play a role in virulence, biofilm formation and multi drug efflux activity. However, deletion of *adeB* in the same strain resulted in similar phenotypes as deletion of *adeRS*. This suggests that at least some phenotypic changes observed upon the *adeRS* deletion may be a result of the decreased expression of the AdeABC efflux pump (Richmond et al., 2016).

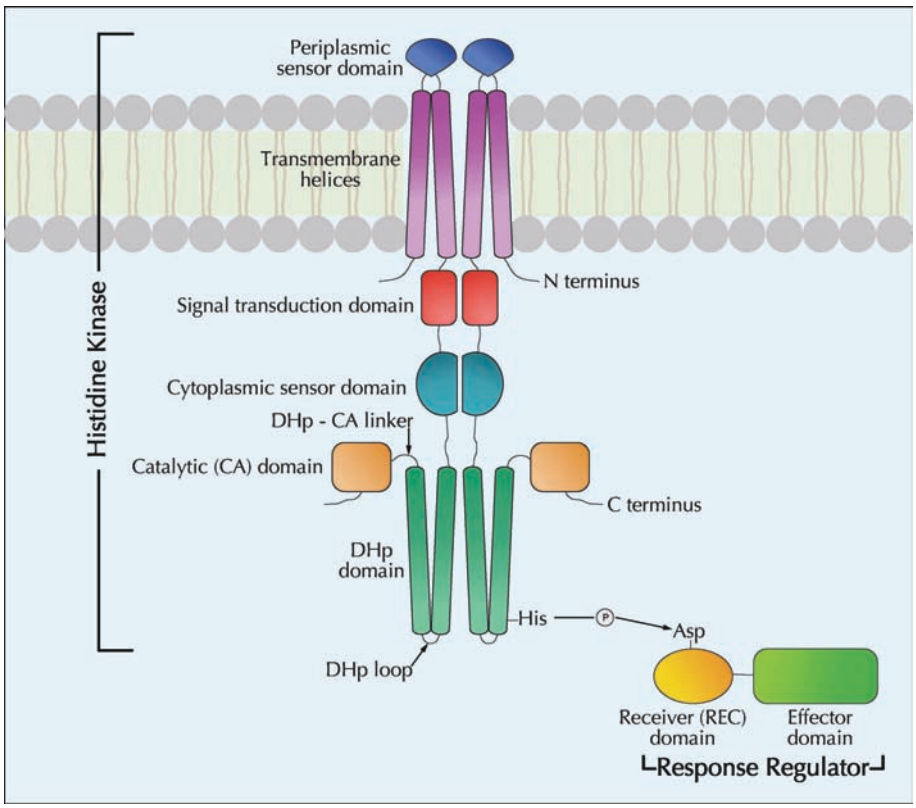

FIGURE 1 | Schematic diagram showing the cellular architecture of a typical two-component regulatory system as well the mechanism of phosphotransfer between two components (modified with permission from Springer Nature Du et al., 2018. A prototypical TCS, comprised of a membrane-bound sensory **h**istidine **k**inase (HK) and a cytosolic **r**esponse **r**egulator (RR) protein, is shown. The basic mechanism of a TCS involves the HK sensing the environmental changes and relaying the message to the RR effectively through phosphorelays to initiate the necessary response. HK proteins, usually dimers, possess several conserved domains that are essential for their function, such as **d**imerization and **h**istidine **p**hosphotransfer (DHp) domain and **c**atalytic **A**TP binding (CA) domain which make up the catalytic core of the HK (Bhate et al., 2015). The H-box containing the conserved histidine residue, that gets phosphorylated, is located in the DHp domain (Casino et al., 2009, 2010). The CA domain binds ATP and phosphorylates the histidine residue, thus initiating the HK autophosphorylation (Zschiedrich et al., 2016). The DHp and CA domains are conserved among all HK proteins and the sensory domains are variable conferring specificity of signal recognition. The phosphoryl group from the H-box of the HK is ultimately transferred to a conserved aspartate residue of the receiver (REC) domain of the cognate RR thus activating the RR (Yamamoto et al., 2005). While the REC domain is highly conserved, the effector domains of RR display variability conferring specificity to the protein (Zschiedrich et al., 2016). Following its activation, dephosphorylation of the RR is critical to maintain the efficient regulatory capacity of the TCSs (Kenney, 2010). This is achieved through the phosphatase activity of the HK (Hsing and Silhavy, 1997).

The multifaceted regulon of AdeRS remains to be explored further, especially in clinically relevant phenotypes of *A. baumannii*. Further, environmental signals that activate the sensor kinase, AdeS, remain mostly unknown. However, we recently uncovered evidence that AdeRS system maybe responding to the NaCl concentrations in the growth medium (De Silva and Kumar, 2017). This work links adaption to environmental conditions, such as NaCl concentration to antibiotic susceptibility (as a result of expression of the AdeABC pump) as well as virulence factors, such as biofilm formation and surface-associated motility. It is therefore obvious that AdeRS plays a role in the antibiotic susceptibility of *A. baumannii* but also possibly in its virulence. However, it's role in antibiotic susceptibility and virulence is likely to be more strain-specific, as it is not uncommon to find disrupted copies of *adeRS* genes in clinical isolates of *A. baumannii*, such as LAC-4 and AB031 (**Table 1**).

BaeSR

BaeSR, named such because of its homology with an *E. coli* TCS (Leblanc et al., 2011), mediates a possible "cross–talk" with other TCSs. It has been shown to regulate overlapping regulons with other TCSs in *A. baumannii*. BaeSR was initially thought to be associated with the regulation of AdeABC RND efflux pump expression (Lin et al., 2014) (**Figure 3**). This is indicative of a possible cross–talk between BaeSR and AdeRS. Further investigations into the BaeSR revealed that it may also modulate the expression of AdeIJK and MacAB-TolC efflux pumps (Henry et al., 2012). However, efforts to determine the DNA binding sites in the promoters corresponding to the observed target genes remain unsuccessful, leaving room for further explorations (Lin et al., 2015). A phenotypic microarray screen revealed that the deletion of *baeR* resulted in reduced tolerance of *A. baumannii* to tannic acid (Lin et al., 2015), a diverse group of natural antibacterial compound (Henis et al., 1964). Tannic acids

TABLE 1 | Conservation of the TCSs in *A. baumannii* in select sequenced and publicly available clinical strains.

Gene	17978mff CP012004.1		AB030 CP009257.1		Ab04-mff CP012006.1		LAC4 CP007712.1		AB0057 CP001182.2		AB5075-UW CP008706.1		AB031 CP009256.1		AYE CU459141.1	
	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation
A1S_0234	99%	446/447	98%	438/447	97%	434/447	97%	434/447	98%	437/447	98%	437/447	93%	417/447	98%	437/447
A1S_0235	100%	1,524/1,524	98%	1,497/1,524	99%	1,505/1,524	99%	1,506/1,524	98%	1,501/1,524	98%	1,501/1,524	98%	1,500/1,524	98%	1,501/1,524
A1S_0236 gacA	100%	636/636	99%	634/636	99%	633/636	99%	632/636	99%	631/636	99%	631/636	99%	631/636	99%	631/636
A1S_0260	100%	1,140/1,140	99%	1,134/1,140	99%	1,132/1,140	99%	1,132/1,140	99%	1,128/1,140	99%	1,128/1,140	99%	1,131/1,140	99%	1,128/1,140
A1S_0261	100%	741/741	99%	737/741	99%	736/741	99%	735/741	99%	733/741	99%	733/741	99%	736/741	99%	733/741
A1S_0574 gacS	100%	2,808/2,808	99%	2,780/2,808	99%	2,769/2,808	99%	2,769/2,808	99%	2,773/2,808	99%	2,773/2,808	99%	2,773/2,808	99%	2,773/2,808
A1S_0748 bfmR	100%	717/717	99%	713/717	99%	708/717	99%	708/717	99%	713/717	99%	713/717	100%	717/717	99%	713/717
A1S_0749 bfmS	100%	1,593/1,593	98%	1,564/1,593	97%	1,550/1,593	97%	1,550/1,593	98%	1,566/1,593	98%	1,566/1,593	99%	1,587/1,593	98%	1,566/1,593
A1S_1393	100%	3,465/3,465	94%	3,261/3,465	94%	3,258/3,465	94%	3,257/3,465	95%	3,299/3,465	95%	3,299/3,465	95%	3,303/3,465	95%	3,298/3,465
A1S_1394	100%	960/960	96%	920/960	99%	952/960	99%	951/960	97%	928/960	97%	928/960	96%	922/960	97%	928/960
A1S_1753 adeR	100%	744/744	98%	731/744	–	–	–	–	99%	735/744	99%	735/744	97%	721/744	99%	735/744
A1S_1754 adeS	100%	1,086/1,086	98%	1,041/1,065	–	–	–	–	97%	1,052/1,086	97%	1,052/1,086	Disrupted	Disrupted	97%	1,052/1,086
A1S_1977	100%	1,110/1,110	99%	1,094/1,110	99%	1,094/1,110	99%	1,094/1,110	98%	1,093/1,110	98%	1,093/1,110	99%	1,096/1,110	98%	1,093/1,110
A1S_1978	100%	1,494/1,494	97%	1,454/1,494	97%	1,452/1,494	97%	1,451/1,494	97%	1,448/1,494	97%	1,447/1,494	97%	1,449/1,494	97%	1,448/1,494
A1S_2006	100%	591/591	Disrupted		100%	591/591	100%	591/591	99%	588/591	99%	588/591	99%	588/591	99%	588/591
A1S_2137	100%	717/717	93%	669/717	95%	674/711	95%	673/711	95%	681/717	95%	681/717	98%	703/717	95%	680/717
A1S_2138	100%	2,655/2,655	95%	2,526/2,656	94%	2,509/2,656	95%	2,510/2,656	96%	2,537/2,656	96%	2,537/2,656	94%	2,502/2,656	96%	2,538/2,656
A1S_2287	99%	1,358/1,360	99%	1,342/1,360	99%	1,356/1,360	99%	1,355/1,360	98%	1,336/1,360	98%	1,336/1,360	99%	1,344/1,360	98%	1,336/1,360
A1S_2750 pmrB	100%	1,335/1,335	99%	1,318/1,335	99%	1,330/1,335	99%	1,330/1,335	99%	1,316/1,335	99%	1,316/1,335	99%	1,318/1,335	99%	1,316/1,335
A1S_2751 pmrA	100%	675/675	99%	670/675	99%	665/675	99%	665/675	99%	668/675	99%	668/675	99%	671/675	99%	668/675
A1S_2811	100%	4,521/4,521	98%	4,426/4,521	98%	4,441/4,521	98%	4,442/4,521	98%	4,433/4,521	98%	4,433/4,521	98%	4,442/4,521	98%	4,433/4,521
A1S_2814	100%	363/363	99%	361/363	99%	362/363	99%	362/363	99%	362/363	99%	362/363	99%	362/363	99%	362/363
A1S_2815	100%	384/384	99%	383/384	99%	381/384	99%	381/384	99%	383/384	99%	383/384	99%	383/384	99%	383/384
A1S_2883 baeR	100%	687/687	99%	680/687	99%	684/687	99%	684/687	99%	678/687	99%	678/687	99%	679/687	99%	678/687
A1S_2884 baeS	99%	1,463/1,464	99%	1,458/1,464	99%	1,457/1,464	99%	1,456/1,464	99%	1,454/1,464	99%	1,454/1,464	99%	1,449/1,464	99%	1,454/1,464
A1S_2906	100%	1,269/1,269	99%	1,250/1,269	99%	1,256/1,269	99%	1,256/1,269	99%	1,255/1,269	99%	1,255/1,269	99%	1,255/1,269	99%	1,255/1,269
A1S_2937	100%	684/684	–	–	85%	579/684	100%	684/684	87%	592/684	87%	592/684	–	–	87%	592/684
A1S_2938	100%	1,374/1,374	–	–	–	–	99%	1,372/1,374	91%	1,256/1,377	91%	1,256/1,377	–	–	91%	1,256/1,377

(Continued)

TABLE 1 | Continued

Gene	17978mff CP012004.1		AB030 CP009257.1		Ab04-mff CP012006.1		LAC4 CP007712.1		AB0057 CP001182.2		AB5075-UW CP008706.1		AB031 CP009256.1		AYE CU459141.1	
	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation	Identity	Nucleotide conservation
A1S_3229	100%	765/765	99%	760/765	99%	757/765	99%	757/765	99%	756/765	99%	756/765	99%	761/765	99%	756/765
A1S_3230	100%	1,458/1,458	99%	1,444/1,458	99%	1,445/1,458	99%	1,445/1,458	99%	1,440/1,458	99%	1,440/1,458	99%	1,452/1,458	99%	1,440/1,458
A1S_3302	100%	3,498/3,498	98%	3,441/3,498	99%	3,450/3,498	99%	3,450/3,498	98%	3,434/3,498	98%	3,434/3,498	98%	3,432/3,498	98%	3,434/3,498
A1S_3304	100%	651/651	99%	644/651	99%	645/651	99%	645/651	99%	643/651	99%	643/651	99%	645/651	99%	643/651
A1S_3374	100%	164/164	100%	164/164	99%	163/164	99%	163/164	100%	164/164	100%	164/164	100%	164/164	100%	164/164
A1S_3376	100%	1,359/1,359	99%	1,339/1,359	98%	1,329/1,359	98%	1,329/1,359	98%	1,331/1,359	98%	1,331/1,359	99%	1,341/1,359	98%	1,331/1,359

The list of putative TCSs were extracted from the P2CS database (www.p2cs.org) from the A. baumannii ATCC17978 strain (Accession number: CP000521.1).

has been used as a topical agent in burn patients (Hupkens et al., 1995) as they display effective antibacterial activity against various bacteria, including *E. coli, Staphylococcus aureus, Staphylococcus epidermidis, Salmonella* spp. etc (Kim et al., 2016). Tannic acid has also been shown to inhibit biofilm formation in *Staphylococcus aureus* (Payne et al., 2013). In *A. baumannii,* Tannic acids are being explored as adjuvants for antimicrobial therapy. They were shown to synergize the activity of novobiocin, rifampicin, and fusidic acid against MDR *A. baumannii* (Chusri et al., 2009). However, the role of BaeSR TCS in tannic acid as well as the expression of efflux pumps controlled by BaeSR will have to be considered to in order to explore the clinical usage of tannic acid as an adjuvant therapy options against *A. baumannii*.

Studies on the environmental signals that BaeSR responds to remain limited. However, expression of *baeR* and *baeS* in *A. baumannii* is induced by sucrose (20% w/v) (Lin et al., 2014), suggesting that BaeSR may be involved in *A. baumannii*'s response to osmotic stress.

PmrAB

A. baumannii's resistance to commonly used antibiotics has led to an increased use of "last resort" antibiotics, such as colistin (Karaiskos et al., 2017; Jiménez-Guerra et al., 2018). As a result, emergence of colistin resistance is becoming more common in *A. baumannii* (Cai et al., 2012; Lean et al., 2015). Investigations into the mechanisms of resistance to colistin in *A. baumannii* have revealed the involvement of PmrAB resistance (Park et al., 2011; Rolain et al., 2013), named so for its role in polymixin (**Figure 3**). PmrAB has been described in various Gram-negative pathogens including *E. coli* (Quesada et al., 2015), *Salmonella enterica* (Gunn, 2008), *Klebsiella pneumoniae* (Cheng et al., 2010), and *Pseudomonas aeruginosa* (Lee and Ko, 2014) and has been shown to have a similar function colistin resistance. Observations of mutations in both *pmrA* (RR) and *pmrB* (HK) leading to decreased susceptibility to colistin presented preliminary evidence of the connection between PmrAB and colistin susceptibility in *A. baumannii* (Adams et al., 2009). Further, both colistin-resistant clinical isolates as well as laboratory generated spontaneous mutants showed phosphoethanolamine modification of lipid A of lipopolysaccharide (LPS) within the outer membrane (Arroyo et al., 2011; Beceiro et al., 2011). The modification of lipid A is mediated by PmrC which is generally part of the same operon as *pmrAB* (Raetz et al., 2007). PmrC can add phosphoethanolamine to either 4′ or 1′ phosphate of lipid A (Da Silva and Domingues, 2017). This modification of LPS results in a positively charged phosphate groups and prevents the binding of the cationic colistin (Tamayo et al., 2005a,b; Arroyo et al., 2011). Mutations in both *pmrA* and *pmrB* cause the overexpression of the *pmrCAB* operon.

Observations that low pH or supplementation of Fe^{3+} in the growth medium (Adams et al., 2009) lead to colistin resistance may suggest that PmrB could be responding to those signals (Gunn, 2008). However, growth of *A. baumannii* under low pH or in iron supplemented growth media failed to alter the expression of *pmrA* (Adams et al., 2009). Therefore, the environmental signals to which PmrAB responds to in *A. baumannii* remain elusive.

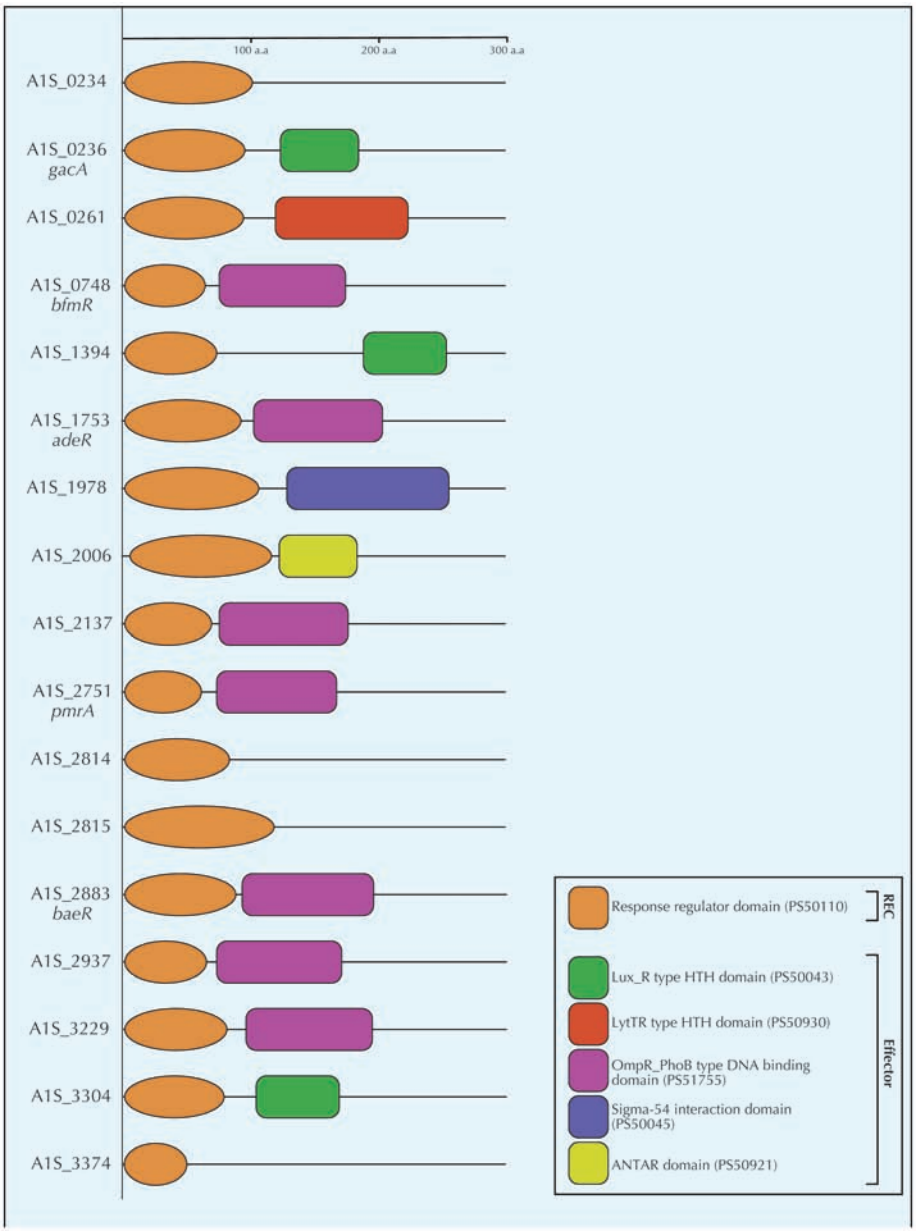

FIGURE 2 | Schematic diagram of the conserved domains of all the response regulators of *A. baumannii* ATCC17978 as determined by a ScanProsite (de Castro et al., 2006). The figure depicts the receiver domain (orange) and the different effector domains identified by ScanProsite (Lux_R type HTH domain in green, LytTR type HTH domain in red, OmpR_PhoB type DNA binding domain in violet, Sigma-54 interaction domain in purple, and ANTAR domain in yellow). Most abundant effector domain was the OmpR_PhoB type DNA binding domain which was present in seven response regulators followed by the Lux_R type HTH domain which was present in three response regulators. The other three types of effector domains were exclusive to single response regulators. The numbers in parenthesis refer to the PROSITE accession numbers of the respective domains. The hybrid sensor kinase A1S_2811 was not included in the figure due to the lack of a distinct response regulator protein.

GacSA

GacSA is a TCS that is well-characterized in *Pseudomonas sp*. (Gooderham and Hancock, 2009). GacSA in *A. baumannii* ATCC19606 was identified when the transposon insertions in the *gacS* sensor kinase gene rendered the mutants incapable of utilizing citrate as the sole carbon source (Dorsey et al., 2002). This suggests that GacSA is involved in citrate metabolism. Since the initial characterization of GacSA in *A. baumannii* ATCC19606, a number of subsequent studies have carried out the functional characterization of GacSA TCS in *A. baumannii* ATCC17978. Interestingly, in *A. baumannii* ATCC17978, the *gacS* gene is not linked to the response regulator-encoding gene. Rather, it has both a HisKA domain and a REC domain suggesting that it could function as a hybrid sensor kinase.

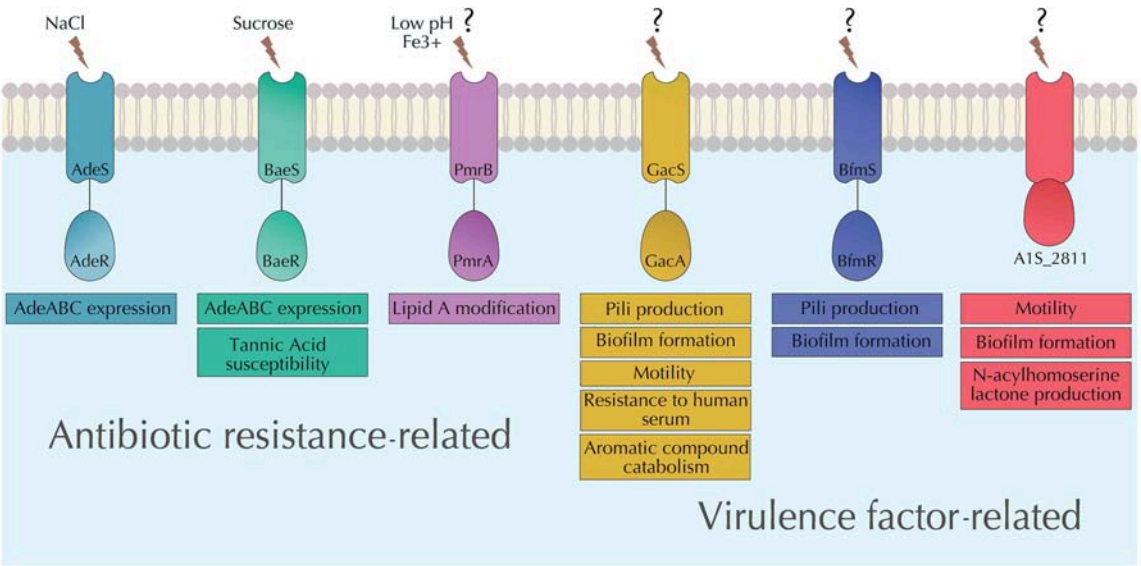

FIGURE 3 | Summary of the characterized TCSs in *A. baumannii*. Functions of each of the characterized TCSs in *A. baumannii* (AdeRS, BaeSR, PmrAB, GacSA, BfmRS, and A1S_2811) as well as their known stimuli are depicted.

Although, there is also a possibility that in *A. baumannii* ATCC17978, the response regulator for GacS is encoded elsewhere in the genome. This indicates that the organization of the *gacSA* genes may vary from strain to strain in *A. baumannii*.

In addition to the initially observed metabolic role of *gacS*, a transposon mutant with a disrupted *gacS* gene displayed significantly reduced *A. baumannii*'s ability to inhibit *Candida albicans* (Peleg et al., 2008b). *gacS* deletion mutant also displayed attenuated virulence in a mouse infection model (Cerqueira et al., 2014). Deletion of *gacS* also led to the revelation of its involvement in a number of other virulence related functions. These include control of pili synthesis, motility, and biofilm formation, resistance against human serum, and metabolism of aromatic compounds (Cerqueira et al., 2014) (**Figure 3**). GacSA is involved in the regulation of the aromatic compound catabolism through the *paa* operon that encodes the components of the phenylacetic acid catabolic pathway. The *paa* gene cluster is significantly downregulated in the *gacSA* deletion mutants which may explain their attenuated virulence in a mouse septicaemia model (Cerqueira et al., 2014). The attenuated virulence of *gacSA* deletion mutants was observed in a later study involving a zebra fish virulence model as well (Bhuiyan et al., 2016) adding to the repertoire of studies that suggest that GacSA may function as a global virulence regulator in *A. baumannii*.

BfmRS

Biofilm formation is an important virulence factor of pathogens, such as *A. baumannii* that helps them survive harsh conditions present in hospital environments. The ability of *A. baumannii* to form biofilms starts with its attachment to surfaces that is mediated by the expression of pili. The expression of pili is mediated by the *csu* operon in *A. baumannii* and is under the regulatory control of BfmRS (Tomaras et al., 2008). Deletion of the response regulator *bfmR* in *A. baumannii* ATCC19606 resulted in the complete abolishment of biofilm formation (Tomaras et al., 2008) (**Figure 3**). While of the role of *csu* operon in the attachment of *A. baumannii* on abiotic surfaces is well-established (Tomaras et al., 2003; Moon et al., 2017; Pakharukova et al., 2018), its role in the adherence of *A. baumannii* to human epithelial cells remains ambiguous. It was observed that *A. baumannii* ATCC19606 strain lacking *csuE* in fact adhered to bronchial epithelial cells better than the wild-type parent making the role pili in adherence to epithelial cells unclear (de Breij et al., 2009). It is possible that this was a strain specific outcome and further investigations are required to draw definitive conclusions.

In addition to regulating biofilm formation, BfmRS also plays a role in regulating the exopolysaccharide production (Geisinger et al., 2018). Exopolysaccharides play an important role in virulence of *A. baumannii* as they are a component of the capsule, which protects *A. baumannii* against serum killing and increasing the virulence in animal models. Further, antibiotic exposure leads to an increase in capsule production in *A. baumannii* mediated by increased expression of genes in K-locus, which in turn is regulated by the BfmRS system (Geisinger and Isberg, 2015).

Crystal structure of BfmR shows that it binds to its own promoter with higher affinity in an inactive (dephosphorylated) state compared to the active (phosphorylated) state (Draughn et al., 2018). This is unusual behavior highlights a unique self-regulation strategy of BfmRS system Therefore, BfmRS system is an excellent candidate to study not only the mechanisms that regulate virulence factors in *A. baumannii* but also the functioning of the TCSs systems in general.

A1S_2811

A1S_2811 is a recently characterized hybrid sensor kinase possessing four histidine–containing phosphotransfer domains as well as a regulatory CheA-like domain and a CheY-like receiver domain (Chen et al., 2017). CheA and CheY homologs in *E. coli* and *P. aeruginosa* are associated with regulatory roles in controlling motility via regulating either pili or flagella (Li et al., 1995; Alon et al., 1998; Bertrand et al., 2010). Interestingly, in *A. baumannii,* this hybrid sensor kinase is expressed in an operon composed of five genes where the four other genes upstream are *pilJ, pilI, pilH,* and *pilG.* Phenotypic analysis of the deletion mutant of *A1S_2811* revealed a significant reduction in surface motility and biofilm formation at the gas-liquid interface. More intriguingly, *abaI,* which encodes a N-acylhomoserine lactone involved in quorum sensing, was also significantly downregulated. Supplementation with synthetic homoserine lactone complemented the biofilm and motility phenotypes (**Figure 3**). This suggests that A1S_2811 regulates biofilm formation and surface motility through an AbaI-associated quorum sensing pathway rather than the conventional pili associated pathway (Chen et al., 2017). This is in contrast to the BfmRS mediated regulon controlling biofilm formation in *A. baumannii*. Association of both BfmRS and A1S_2811 with biofilm formation is also an example of one phenotype being under the control of multiple regulatory networks formed by different TCSs.

TCSs AS POTENTIAL NOVEL DRUG TARGETS IN BACTERIAL PATHOGENS

Given the important role that TCSs play in regulating the clinically-relevant phenotypes (virulence and/or antibiotic resistance) of bacterial pathogens, it has been proposed that targeting them therapeutically can offer an alternate treatment strategy against multidrug resistant pathogens (Barrett and Hoch, 1998; Stephenson and Hoch, 2002a,b, 2004; Gotoh et al., 2010b; Cardona et al., 2018). TCSs in *A. baumannii* as well as other organisms offer promise as novel drug targets because of a number of reasons; (i) their conserved nature among bacteria, (ii) their involvement in modulating antibiotic resistance and virulence phenotypes, (iii) their absence in mammalian cells thus reducing off–target toxicity, (iv) lesser potential of resistance development, as the focus of the approach is to supress virulence and/or antibiotic susceptibility rather than killing the cells. It is therefore not all that surprising that TCSs from different organisms have been studied as potential therapeutic targets. **Table 2** summarizes a few examples of the use of TCSs inhibitors used in bacterial pathogens other than *A. baumannii.*

POTENTIAL OF TCSs AS NOVEL DRUG TARGETS IN *A. baumannii*

In *A. baumannii*, small molecule inhibitors, such as 2-aminoimidazole compounds have shown great promise in inhibiting the action of both PmrA and BfmR. The 2-aminoimidazole-based adjuvants used in combination with colistin were able to reverse colistin resistance in *A. baumannii* clinical isolates through inhibiting PmrAB and thereby abolishing the lipid A modification (Brackett et al., 2016). A promising feature of this strategy was that no resistance toward the PmrAB inhibitor was observed during the testing period of 7-days (Harris et al., 2014). Yet another example is the use of small molecule 2-aminoimidazole derivatives to inhibit the functions of BfmR (Thompson et al., 2012), such as biofilm formation (Milton et al., 2018). However, as with any other small molecule inhibitor, the cytotoxicity of the compounds used against PmrAB and BfmRS remains to be determined before the inhibitors could be deployed in a clinical setting.

Preliminary findings on the inhibition of BfmRS and PmrAB system are encouraging. In addition, AdeRS, A1S_2811, or GacSA can potentially be explored as therapeutic targets because of the important role they have been shown to play in the antibiotic resistance and virulence of *A. baumannii*.

CHALLENGES IN TARGETING TCSs FOR THERAPUTICS

Despite the fact that the investigations of the TCSs show an increasing amount of information being uncovered during the

TABLE 2 | A brief summary of the examples of using TCS inhibitors as a therapeutic option in bacterial pathogens other than *A. baumannii*.

TCS	Organism(s)	Inhibitor	Inhibitory action	References
AlgR1/AlgR2	*Pseudomonas aeruginosa*	Thiazole derivatives	Inhibition of AlgR1 phosphorylation and AlgR2 kinase activity	Roychoudhury et al., 1993
WalKR	*Staphylococcus aureus* *Bacillus subtilis*	Walkmycin B Waldiomycin	Inhibition of autophosphorylation of WalK	Okada et al., 2010; Igarashi et al., 2013
		Walrycin A Walrycin B	Inhibition of phosphotransfer from WalR	Gotoh et al., 2010a
QseCB	Enterohemorrhagic *E. coli* (EHEC)	LED209	Inhibition of autophosphorylation of QseC	Rasko et al., 2008
PhoPQ	*Salmonella sp.*	Radicicol	Activity against PhoQ	Guarnieri et al., 2008
VanSR	*Enterococcus faecium*	Thiazole derivatives	Inhibition of phosphotransfer to VanR	Ulijasz and Weisblum, 1999

recent years, the majority of these efforts have focused on the cellular functions carried out by TCSs. This has left a void of information about the environmental signals that act as a trigger for the histidine kinase stimulation. The proposed stimuli for the already characterized TCSs are limited to osmotic stress for BaeSR (Lin et al., 2014), monovalent cations for AdeRS (De Silva and Kumar, 2017); and possibly low pH and Fe^{3+} for PmrAB (Gunn, 2008; Adams et al., 2009). Uncovering the environmental stimuli that activate a TCS response is critical in understanding the molecular pathways that are used for gene regulation by a particular TCS. These pathways can then be better exploited to render *A. baumannii* non-virulent and/or antibiotic susceptible. However, it is often difficult to determine these signals due to an array of practical reasons including, but not limited to, the potential ability of sensor kinases to detect multiple stimuli and difficulty in expressing, purifying, and experimenting with histidine kinase proteins *in vitro* in their natural conformations.

CONCLUSIONS AND FUTURE PERSPECTIVES

It is evident that the characterized TCSs present in *A. baumannii* are responsible for controlling a number of antibiotic resistance and virulence associated phenotypes, which contribute to the success of this organism as a human pathogen. Research on TCSs in *A. baumannii* has extended our knowledge on virulence and resistance mechanisms in this organism over the last few years. However, there is still a considerable knowledge gap in comprehensive understanding of the complete TCS regulatory networks. Nonetheless, TCSs present themselves as potential targets for drug design and the use of 2-aminoimidazole compounds is are encouraging. A better characterization of these systems both genetically and functionally is key for the potential use of TCS as therapeutic targets.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Variation in Mutant Prevention Concentrations

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Objectives: Understanding how phenotypic traits vary has been a longstanding goal of evolutionary biologists. When examining antibiotic-resistance in bacteria, it is generally understood that the minimum inhibitory concentration (MIC) has minimal variation specific to each bacterial strain-antibiotic combination. However, there is a less studied resistance trait, the mutant prevention concentration (MPC), which measures the MIC of the most resistant sub-population. Whether and how MPC varies has been poorly understood. Here, we ask a simple, yet important question: How much does the MPC vary, within a single strain-antibiotic association? Using a *Staphylococcus* species and five antibiotics from five different antibiotic classes—ciprofloxacin, doxycycline, gentamicin, nitrofurantoin, and oxacillin—we examined the frequency of resistance for a wide range of concentrations per antibiotic, and measured the repeatability of the MPC, the lowest amount of antibiotic that would ensure no surviving cells in a 10^{10} population of bacteria.

Results: We found a wide variation within the MPC and distributions that were rarely normal. When antibiotic resistance evolved, the distribution of the MPC changed, with all distributions becoming wider and some multi-modal.

Conclusion: Unlike the MIC, there is high variability in the MPC for a given bacterial strain-antibiotic combination.

Keywords: antibiotic resistance, selection, *Staphylococcus epidermidis*, repeatability, replication

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INTRODUCTION

The increase in antibiotic-resistant bacteria is globally an urgent public health issue (Dijkshoorn et al., 2007; Nordmann et al., 2007; Davies and Davies, 2010; Brusselaers et al., 2011; Bush et al., 2011; Morehead and Scarbrough, 2018). The minimum inhibitory concentration (MIC), defined as the lowest concentration of an antimicrobial agent that inhibits growth of the wild type population, assuming no mutations, by 99% (Haight and Finland, 1952; Sanders et al., 1984; Sanders, 2001; Obolski et al., 2015) has been used extensively to classify bacteria as resistant to an antibiotic (Dong et al., 1999; Drlica, 2003; Epstein et al., 2004). Yet the MIC is a single measurement of resistance; it captures one parameter of resistance, but not all.

As antibiotic concentrations increase, the first steep decline in colony numbers, representing an ∼1% recovery, corresponds to the MIC. After exposing cells to antibiotics at MIC levels, there

will often still exist a population of resistant mutants due to spontaneous mutations, considered to be single-step resistant mutants. As concentrations increase beyond the MIC, these single step mutants will remain until a concentration that reduces colony recovery to 0% is achieved. Above this concentration, no single-step mutants can exist. This concentration is the second metric of resistance, the mutant prevention concentration (MPC). The MPC is defined as the MIC of the least-susceptible, single-step mutant (Dong et al., 1999; Firsov et al., 2003; Allen et al., 2004; Drlica et al., 2006; Hansen et al., 2006; Drlica and Zhao, 2007; Firsov et al., 2008). This is experimentally measured by determining the lowest antibiotic concentration that can kill all single-step resistant mutants within a population size of 10^{10} cells (Feldman, 1976; König et al., 1998; Zhao and Drlica, 2001; Gould and MacKenzie, 2002). This concentration of cells is similar to the numbers of cells found in some infectious cases in clinical situations (Zhao and Drlica, 2001; Gould and MacKenzie, 2002). The concentrations between MIC and MPC, defined as the mutant selection window (MSW), signify the antibiotic concentration range for which evolution of resistance can occur by selecting for the non-susceptible portion of the population (**Figure 1**; Drlica, 2003; Drlica and Zhao, 2007).

While the MIC for each bacterial antibiotic-strain pair is typically considered a single value with high repeatability (Dong et al., 1999; Zhao and Drlica, 2001; Li et al., 2002; Firsov et al., 2003; Zinner et al., 2003; Allen et al., 2004; Li et al., 2004; Metzler et al., 2004a,b; Marcusson et al., 2005; Hansen et al., 2006; Olofsson et al., 2006; Drlica and Zhao, 2007; Firsov et al., 2008; Liu et al., 2013; Oshima et al., 2017; Zhang et al., 2017), it is unclear if this is true for the MPC. Because the MPC is dependent on the probability and timing of mutations that confer resistance, it seems likely that the MPC would have a greater variance than MICs, but the variation in the MPC has not been well studied.

Previous work typically has examined MPCs using fluoroquinolone antibiotics. Studies using *Staphylococcus aureus* (Dong et al., 1999; Drlica, 2003; Firsov et al., 2003; Allen et al., 2004; Metzler et al., 2004a; Firsov et al., 2008), *Mycobacterium tuberculosis* (Rodriguez et al., 2004; Drlica and Zhao, 2007), and the poultry pathogen *Mycoplasma gallisepcticum* (Zhang et al., 2017) have obtained values for the MPC, and the MSW, by examining the presence of resistant mutants at sub-MPC and MPC antibiotic concentrations *in vitro*. Their results confirm that resistant mutants are enriched when bacteria were exposed to concentrations that fall within the MSW. While the MPC and MSW have been widely described in *M. tuberculosis* in adults as defined values (Rodriguez et al., 2004), in one review of the antibiotic dosing used in child tuberculosis, it was found that the heterogeneity of MICs could result in a range of MPCs (Jaganath et al., 2017). Multiple studies using *Streptococcus pneumonia* (Li et al., 2002; Drlica, 2003; Zinner et al., 2003) and *Haemophilus influenzae* (Li et al., 2004; Metzler et al., 2004b) emphasize the variability in mutation accumulation and observe increasing MSWs with successive mutations. Many studies on the MPC also consider the pharmacokinetics/pharmacodynamics of the antibiotics (Drlica, 2003; Marcusson et al., 2005; Olofsson et al., 2006). Interestingly, one such study found the MIC to be weakly correlated to the MPC using *E. coli* (Marcusson et al., 2005), also suggesting that the MPC may be a more unpredictable resistance parameter. In all of the studies mentioned, it is important to note that there were less than five replicates of the MPC obtained.

Our study focuses on a strain of *Staphylococcus epidermidis*, a gram positive bacterium that colonizes the skin and mucus membranes of the human body, and represents a large part of the normal microflora (Widerström et al., 2012). An opportunistic pathogen, *S. epidermidis* is also the leading cause of infections due to intravenous medical devices, resulting in significant healthcare costs (Uckay et al., 2009). There has been little work done to determine MPC variation using *S. epidermidis*, with one study showing stability in MPC values using two replicate experiments (Liu et al., 2013). Our study uses 20 replicate experiments per bacteria-antibiotic strain to investigate the variability of MPCs. Specifically, we address the following questions: Are the MPCs replicable in highly controlled laboratory conditions? What is the variation in MPCs? Does the variation differ between antibiotics and/or strains? Here we show that the MPC can vary significantly, and the ranges differ between antibiotics and through the evolution of resistance. Our results indicate a large role for stochasticity in determining the MPC of a bacterial strain with a specific antibiotic.

MATERIALS AND METHODS

Culture Conditions

A master tube of *S. epidermidis* (ATCC 14990), was our ancestral strain and grown overnight in Luria Broth (LB) media (10 g tryptone, 5 g yeast extract, and 10 g NaCl), and then frozen with 25% glycerol at −80°C. Several hundred aliquots were made from the master tube and also kept frozen with 25% glycerol at −80°C. *S. epidermidis* (ATCC 14990) was evolved to each of five antibiotics: ciprofloxacin, doxycycline, gentamicin, nitrofurantoin, and oxacillin. We obtained and purified one independent spontaneously resistant mutant for each antibiotic, resulting in five resistant strains. For all resistant strains collected, we confirmed resistance by streak-purifying colonies onto agar plates containing antibiotic concentrations above the known MIC. For all experiments described here, we used freshly thawed aliquots of the ancestral strain and the resistant strains. Each replicate experiment required one aliquot. Strains were grown (aerated) in LB media for approximately 8 h at 37°C to a density of roughly 10^9 cells per ml and serially diluted to approximately 10^5 cells per mL for MIC determination on agar plates.

Antibiotics

We used five antibiotics: Ciprofloxacin hydrochloride (CPR) (MP Biochemicals 199020), Doxycycline hyclate (DOX) (Sigma-Aldrich D9891), Gentamycin sulfate salt (GEN) (Sigma-Aldrich G1264), Nitrofurantoin (NTR) (Sigma-Aldrich N7878), and Oxacillin sodium salt (OX) (Sigma-Aldrich 28221). Ciprofloxacin, a synthetic second-generation fluoroquinolone, inhibits DNA synthesis by inhibiting bacterial enzymes DNA gyrase and topoisomerase, which are involved in the unwinding

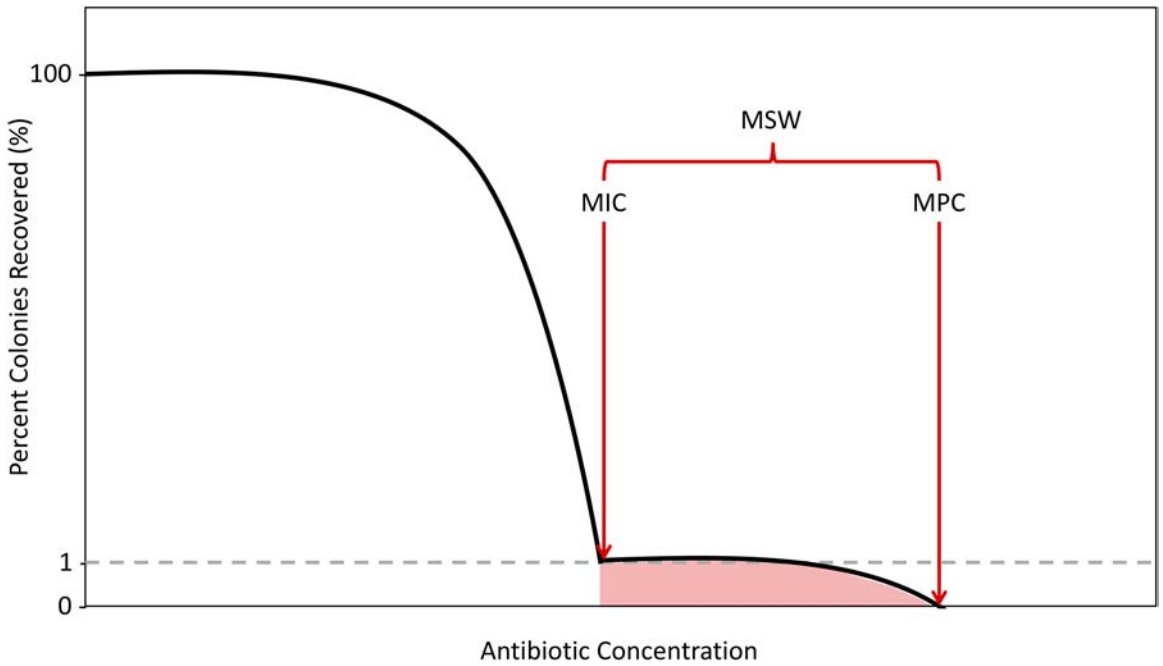

FIGURE 1 | Schematic of the Mutant Selection Window. As antibiotic concentrations increase, the percentage of colonies recovered decreases with two sharp declines demarcating the boundaries of the mutant selection window (MSW), in red. The shaded region selects for single-step resistant mutants. The first decline, which results in a 99% decrease in colonies recovered is determined to be the minimum inhibitory concentration (MIC). The second decline, where there is a 100% decrease in colonies recovered is determined to be the mutation prevention concentration (MPC).

and supercoiling of DNA during DNA replication (Hooper et al., 1987). Doxycycline, a broad-spectrum tetracycline, inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit and preventing aminoacyl tRNA from binding (Roberts, 1996; Chopra and Roberts, 2001). Gentamicin, an aminoglycoside, inhibits bacterial protein synthesis by targeting the ribosomal A site (Hahn and Sarre, 1969; Yoshizawa et al., 1998). Nitrofurantoin, a multiple-mechanism nitrofuran, inhibits a variety of bacterial enzymes, including those involved in DNA and RNA synthesis as well as carbohydrate synthesis (Shah and Wade, 1989; McOsker and Fitzpatrick, 1994). Oxacillin, a beta-lactam penicillin, inhibits bacterial cell wall synthesis (Park and Strominger, 1957). These antibiotics were chosen because of their clinical importance, widespread use, and different mechanisms of action.

Determination of Liquid Minimum Inhibitory Concentration (MIC) Estimates

MIC estimates in liquid culture were determined using microtiter plates with serial and equidistant dilutions of antibiotics. Approximately 10^3–10^4 cells were inoculated in each well with 100 μl LB and allowed to grow for 22 h, shaken at 220 revolutions per minute (rpm) and incubated at 37°C (Tecan Infinite M200 PRO Multimode Microplate Reader). The liquid MIC estimate was determined by the lowest antibiotic concentration observed to inhibit growth by at least 95%, compared to the positive control. We also included negative controls on each 96 well-plate to validate no contamination of media.

Determination of Agar MIC

Liquid MIC levels were used as a starting point to determine agar MIC levels. Agar tests tend to yield very similar MIC levels, but on occasion there may be minor differences. We plated two 100 mm agar plates for antibiotic concentrations ranging from 0.2 × liquid MIC and ending at 1.7 × liquid MIC estimate in increments of 0.1 × liquid MIC. Viable cells were quantified as colony forming units (CFUs). We inoculated each plate using 10^5 cells, resulting in a CFU population that has a limited probability of spontaneous mutation (Martinez and Baquero, 2000; O'neill et al., 2001). These cells were spread via the Copacabana method (Worthington et al., 2001; Mills et al., 2005), which involves the equal distribution of bacteria via sterile glass beads. We conducted the agar MIC assays in duplicate and recorded the median and range for each MIC for each bacterial strain. We prepared agar plates using 1000 mL of MilliQ water, 15 g agar powder, and one 25 g LB tablet (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1.5 g/L Tris/Tris HCl).

Determination of Mutant Prevention Concentration (MPC)

MPC was determined as the antibiotic concentration that prevents the growth of any resistant mutants following an inoculum of 10^{10} cells on LB plates containing dilutions of antibiotic (Dong et al., 1999; Drlica, 2003). A population of 10^{10}, allows for the consideration of single-step mutants, which is imperative in defining the MPC (Martinez and Baquero, 2000; O'neill et al., 2001). From a frozen aliquot, we grew a

bacterial culture overnight for 18 h at 37°C and then inoculated this culture in LB until the inoculum reached an OD_{600} between 0.45 and 0.7. We then centrifuged the bacterial culture (4000 rpm × 4 min, 4°C). We resuspended and combined all bacterial pellets in 7.5 mL of the original supernatant to give 10^{10} cells. We used liquid MIC estimates to plan the incremental concentrations used in MPC experiments. We performed two preliminary MPC experiments with concentrations ranging from 1 × liquid MIC estimate to 64 × liquid MIC estimate, increasing by a factor of two. We repeated MPC experiments 20 times, with three replicates per antibiotic concentration. To measure MPC, we plated at least 10^{10} bacterial cells on agar plates and spread the inoculum via the Copacabana method (Worthington et al., 2001; Mills et al., 2005). Plates were then incubated at 37°C for 72 h. We determined MPC to be the lowest concentration of antibiotic where all three agar plates for a single concentration showed zero colonies. We prepared agar plates using 1000 mL of MilliQ water, 15 g agar powder, and one 25 g LB tablet (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1.5 g/L Tris/Tris HCl).

Mutant Selection Window (MSW)

Using MICs and MPCs, we determined the MSWs of ancestral and resistant strains in terms of the MIC of the ancestral strain. Using the MIC of the ancestral strain allowed us to directly compare the MSWs between the two strains.

RESULTS

We found that MPC estimates varied widely within a single antibiotic, indicating low repeatability of MPC. This was true of most antibiotics tested (**Table 1** and **Figure 2**). The inter-quartile range (IQR) varied among the antibiotics used and whether the strain was the resistant or ancestral strain. The ancestral strain had a more robust signal for a single MPC value where the resistant strain was much more variable (**Figure 2**).

The distribution of most MPCs do not appear normal (**Figure 2**). All of the resistant strains did not meet the requirements of a normal distribution (Shapiro-Wilk test ($p < 0.05$) and Kolmogorov-Smirnov test ($p < 0.001$)). The ancestral strains did have a mix of distributions; doxycycline and nitrofurantoin both failed to reject the null hypothesis of a Shapiro-Wilk test ($p > 0.05$). We also demonstrate using a two-sample Kolmogorov–Smirnov test, that the MPC distributions change as resistance evolves. In all direct comparisons of ancestral and resistant strains (with the same antibiotic) the distributions of the MPC values are different ($p < 0.001$).

We also found that the MSW changed when resistance is evolved (**Figure 3**). There is less variation in the MIC values than there is in the MPC values. The MSW not only shifts but also widens as resistance evolves.

TABLE 1 | Mean, standard deviation, median, and IQR of MPCs for both strains of *Staphylococcus epidermidis* (ancestral and resistant) for all antibiotics tested. All values reported in micrograms per milliliter.

Antibiotic	Strain	Mean	Standard Deviation	Median	IQR
Ciprofloxacin	Ancestral	1.2	0.22	1.2	0.25
	Resistant	4.8	0.68	4.6	0.65
Doxycycline	Ancestral	12.2	1.27	12	2
	Resistant	20.8	3.59	20	3.56
Gentamycin	Ancestral	11	1.54	11.7	2.34
	Resistant	107.3	15.91	110	22
Nitrofurantoin	Ancestral	1	0.25	1.1	0.19
	Resistant	3.2	0.57	3.3	0.35
Oxacillin	Ancestral	24.8	3.81	24	5
	Resistant	47	5.72	46.2	8.4

DISCUSSION

Our results show a range of MPCs in replicate experiments, indicating a large role for stochasticity and limited repeatability for this trait. In this study, the MPC trait is not easily predictable. This variation in MPCs is in contrast to MICs, which are generally predictable for each bacterial strain-antibiotic combination within a particular laboratory setting. For example, although variation in the MIC among different labs has been shown as a result of variations in strains as well as assay variations, individual studies within labs show consistency in the determination of the MIC (Mouton et al., 2017). Thus, while one trait (MIC) is more predictable and repeatable given a certain selection pressure, another (MPC) varies greatly due to stochastic processes. While previous studies indicate that MPCs can be fairly stable (Blondeau et al., 2001; Li et al., 2004; Marcusson et al., 2005; Olofsson et al., 2006), the number of replicates in these studies (two or three), would be insufficient to examine effects of stochasticity on the appearance of mutants.

The change in the MPC is large enough to account for the change of distribution and variation within the resistant strain as there is little to no overlap in the inter-quartile range (IQR). This supports the idea that although the MPC distribution is large and somewhat unpredictable, we can be confident that the MPC of a resistant strain is higher than an ancestral strain.

Our results here suggest two potentially relevant clinical notes. First, it has been proposed that if clinicians target MPCs, there can be no resistant bacteria left in a population within an individual patient (Dong et al., 1999). While this has not proven practical in most cases given the high concentrations of antibiotics needed, there has been work towards determining antibiotic combinations that lower the MPC (Michel et al., 2008). If used clinically (which is entirely hypothetical, since it is not currently used in the clinic), there should be care to understand that MPCs can vary with each bacteria and antibiotic combination and that failure to recognize variation in the MPC could result in inaccurate dosing. Therefore, this study suggests that MPCs should be understood as a range with confidence intervals, rather than as a single number. This study also reveals a significant change in the distribution of the MPC between ancestral and resistant strains, emphasizing the unpredictability of this trait when a bacterial strain acquires a spontaneous mutation conferring antibiotic resistance. Not only do distributions of the MPC in resistant strains increase, but

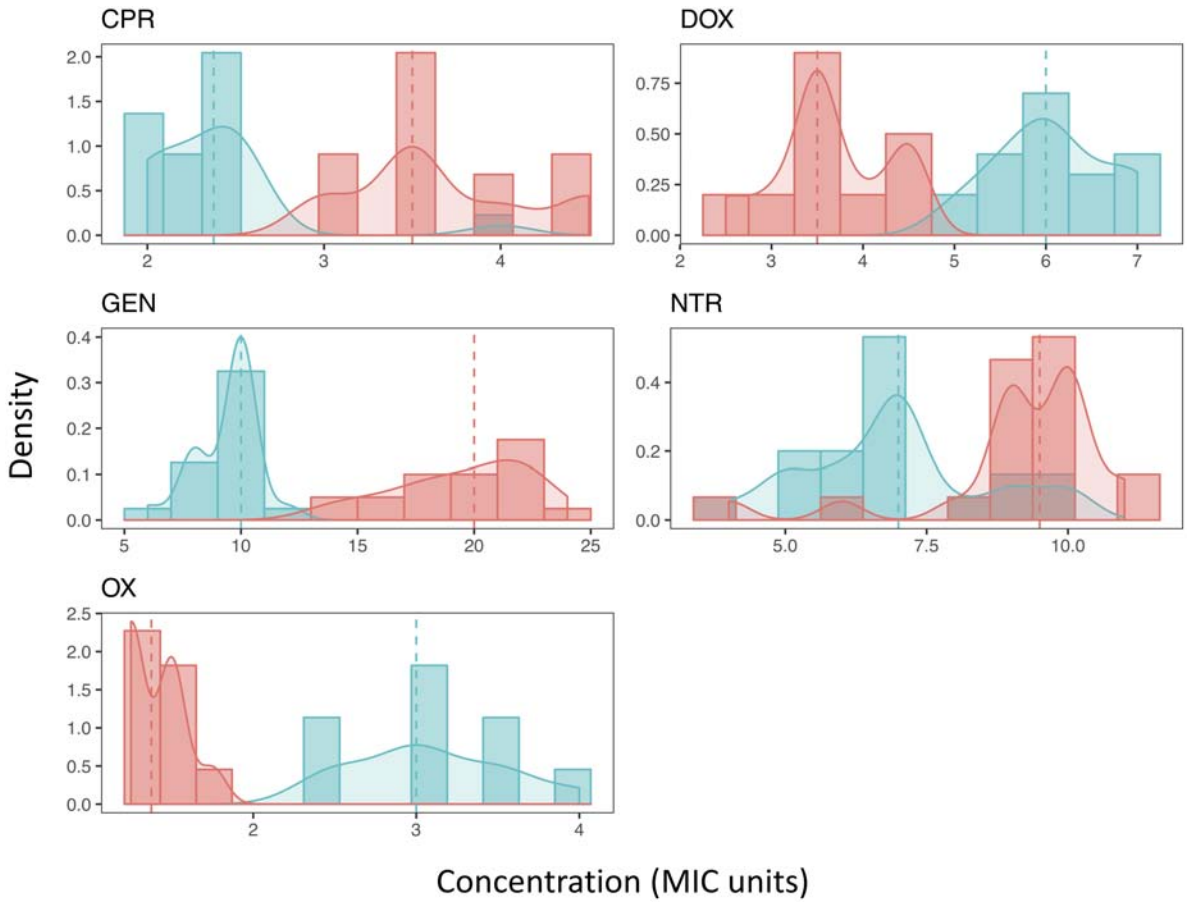

FIGURE 2 | The distribution of Mutant Prevention Concentrations. The MPC distribution of both the ancestral strain (blue) and antibiotic resistant strain (red) for each antibiotic tested. Both the histograms of the data along with the kernel-density estimation is shown. The dashed line represents the median of each sample, respectively. A Shapiro-Wilk test ($p < 0.05$) and Kolmogorov-Smirnov test ($p < 0.001$) both show that all resistant strains distributions cannot be considered normal. Most ancestral strains are also not considered to be a normal distribution ($p < 0.05$), the distribution of the MPCs of the ancestral strain when exposed to either DOX or NTR fail to reject the null hypothesis of a Shapiro-Wilk test ($p > 0.05$). Furthermore, when comparing the MPC distributions between the ancestral strain and the resistant strain for each antibiotic the distributions are not the same (2-sample Kolmogorov-Smirnov test, $p < 0.001$).

the shapes of the distributions also change considerably. With nitrofurantoin and oxacillin, the distribution of the MPC changes from unimodal distributions in the ancestral strains to bimodal distributions in the resistant strains (See **Figure 2**). In either case, any intermediate steps taken to move a population off its trajectory towards maximal resistance—for example, using a different antibiotic against a population of bacteria—needs to consider the fact that there may not be a deterministic response of the pathogen population to the new stressor.

There has been some contention as to the utility of MPCs when the resistance mechanisms evaluated *in vitro* do not match the resistance mechanisms that would be found in a clinical setting (Smith et al., 2003). In this study, the acquisition of spontaneous chromosomal mutations was the primary mechanism of resistance when isolating and purifying resistant strains. However, horizontal transfer is typically required for resistance to aminoglycosides like oxacillin, β-lactams like gentamicin, and tetracyclines like doxycycline (Roberts, 1996; Smith et al., 2003). The distributions found in this study offer a first look at the unpredictability of MPC variation in resistant strains. Moreover, ciprofloxacin is a fluoroquinolone in which the mechanism of resistance is largely spontaneous chromosomal mutations (Pantosti et al., 2007).

It is known that the MIC fluctuates with inoculum size, with smaller inocula leading to lower MIC estimates (Granier et al., 2002; Egervärn et al., 2007; Wiegand et al., 2008). Even when testing the MIC values between liquid and agar media, slight differences are found. It would be worthwhile to investigate whether similar fluctuations exist for MPC testing. To elucidate evolutionary potentials in variation, this study used 10^{10} cells, an inoculum size similar to the number of bacterial cells found in naturally-occurring bacterial infections (Feldman, 1976; König et al., 1998; Zhao and Drlica, 2001; Gould and MacKenzie, 2002). Testing a range of large inoculum concentrations may provide further information about how MPCs depend upon cell concentrations present at the time of antibiotic administration. Our findings are particularly relevant to understanding variation in bacterial responses to antibiotics at high cell densities.

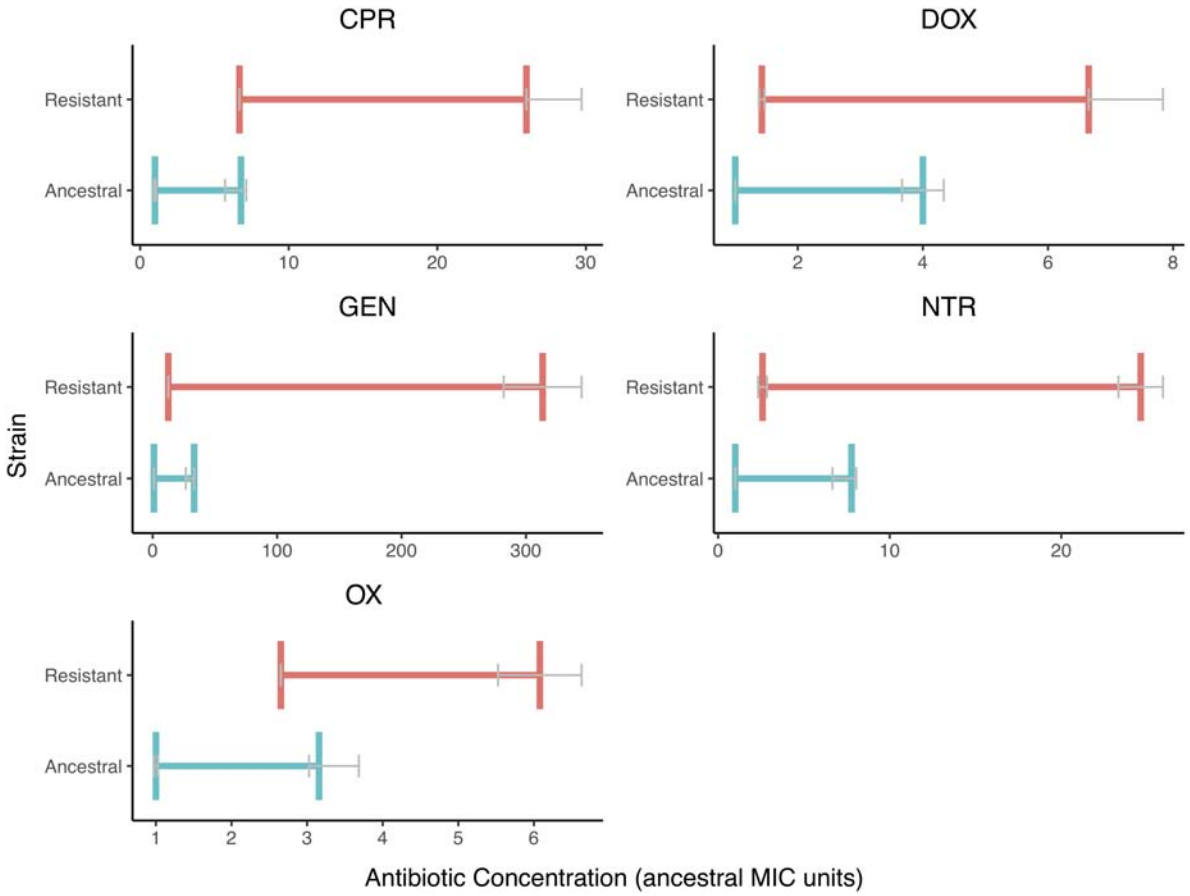

FIGURE 3 | The Mutant Selection Windows of the Ancestral and Resistant Strains. The mutant selection window windows are shown for both resistant (red) and ancestral (blue) strains for each of the five drugs tested. The MIC and MPC shown here are the median value with error bars (IQR) represented in gray. The mutant selection windows for the resist stain are all shifted to higher values and are wider in size. The MPC appears to vary more in the resistant strain than in the ancestral strain.

Toprak et al. (2012) showed that resistance to different antibiotics involved different types of pathways: some antibiotics had a very stereotyped pathway with similar mutations evolved in the same order, whereas other antibiotics had much more variation in timing and type of mutation (Toprak et al., 2012). With regards to the MPC, it could be illuminating to quantify and examine the specific genetic mutations underlying resistant strains of bacteria at similar and dissimilar MPCs. This would give more information regarding which specific mutations are needed, and how many unique mutations or combinations of mutations exist, to yield high antibiotic resistance. A better understanding of the amount of variation by bacteria and antibiotic could provide a more complete story regarding the variation underlying MPCs. This current study provides a first step, which shows high variability in this important resistance trait.

Luria-Delbruck fluctuations, defined as fluctuations in the frequency of spontaneous mutations in microbial populations (Luria and Delbrück, 1943), may affect the evolutionary trajectory of populations. If a mutation occurs early on in the growth of the population there would be more cells with mutations because of the exponential characteristic of cell division in bacteria (Sarkar, 1991). Conversely, if a mutation arises later, there will be fewer cells exhibiting that mutation. Thus, a low probability event, which occurs early on, may have drastic and amplified results (Skipper, 1983; Rosche and Foster, 2000). Luria-Delbruck fluctuations can, but do not necessarily, have a large impact on the number of resistant mutants in a given population of bacteria (Ford et al., 2013). If a spontaneous mutant arises early in the population growth phase and happens to confer resistance to a given antibiotic, then in the presence of the antibiotic, the ending population will be comprised largely of this resistant mutant and daughter cells. Depending on the exact timing of the appearance of the mutation, a population may exhibit many resistant cells, or very few. Understanding, therefore, the mutations and patterns below the MPC would also be a very useful future study in elucidating fluctuations in the MPC and MSW.

In summary, we find that even in highly controlled laboratory environments, MPCs vary widely, not only from differences in strain and antibiotic, but from replicates with the same strain and same antibiotic. Several other factors may also affect MPC variation, such as CFU concentrations, mutation type, and

inocula size and in the future, these factors should be investigated. Understanding how and why the MPC varies can allow us to lay the foundations for more comprehensive dosing strategies that take into consideration the presence and elimination of single-step resistant mutants. From a clinical perspective, caution should be taken when determining how reliable certain therapeutic treatments will be in terms of completely eliminating resistant mutants. From an evolutionary perspective, we show the significant role of stochasticity in bacteria evolving antibiotic resistance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

PY conceived of the presented idea and planned the experiments. CG, NL, CH, PK, AB, AV, LS, GB, MW, EC, LF, and JN carried out the experiments. NL analyzed the data. CG, NL, and PY discussed and contributed to the interpretation of results. CG, NL, CH, MW, and PY contributed to the final version of the manuscript. PY supervised the project.

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Multidrug-Resistant *Enterobacter cloacae* Complex Emerging as a Global, Diversifying Threat

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The *Enterobacter cloacae* complex (ECC) includes common nosocomial pathogens capable of producing a wide variety of infections. Broad-spectrum antibiotic resistance, including the recent emergence of resistance to last-resort carbapenems, has led to increased interest in this group of organisms and carbapenem-resistant *E. cloacae* complex (CREC) in particular. Molecular typing methods based on heat-shock protein sequence, pulsed-field gel electrophoresis, comparative genomic hybridization, and, most recently, multilocus sequence typing have led to the identification of over 1069 ECC sequence types in 18 phylogenetic clusters across the globe. Whole-genome sequencing and comparative genomics, moreover, have facilitated global analyses of clonal composition of ECC and specifically of CREC. Epidemiological and genomic studies have revealed diverse multidrug-resistant ECC clones including several potential epidemic lineages. Together with intrinsic β-lactam resistance, members of the ECC exhibit a unique ability to acquire genes encoding resistance to multiple classes of antibiotics, including a variety of carbapenemase genes. In this review, we address recent advances in the molecular epidemiology of multidrug-resistant *E. cloacae* complex, focusing on the global expansion of CREC.

Keywords: carbapenem-resistant Enterobacteriaceae, carbapenem-resistant *Enterobacter cloacae* complex, carbapenemase, multidrug-resistance, bacterial genomics

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INTRODUCTION

Enterobacter spp., the second most common carbapenem-resistant Enterobacteriaceae (CRE) in the United States, increasingly contribute to the spread of carbapenem-resistant infections (Wilson et al., 2017). In particular, *Enterobacter cloacae* complex (ECC) are common nosocomial pathogens capable of producing a wide variety of infections, such as pneumonia, urinary tract infections, and septicemia (Sanders et al., 1997; Wisplinghoff et al., 2004). The emergence of multidrug resistance (MDR), including resistance to the last-resort carbapenems meropenem, imipenem, and ertapenem, has led to an increased interest in these organisms.

Molecular analyses based on multilocus sequence typing (MLST) and heat-shock protein (*hsp*) typing have led to the re-definition of members within this complex (Hoffmann and Roggenkamp, 2003; Paauw et al., 2008; Miyoshi-Akiyama et al., 2013). Whole-genome sequencing (WGS), moreover, has allowed for reproducible population-level analyses to determine clonal structure and diversity in ECC and CREC collections ranging from localized, regional outbreaks to global studies

(Chavda et al., 2016; Gomez-Simmonds et al., 2018). These methods have facilitated analyses of phylogenetic structure and evolutionary history on a global scale.

Importantly, clinical and genomic studies have revealed a striking facility for ECC to acquire genes encoding broad-spectrum antibiotic resistance, including a variety of carbapenemase genes, superimposed on intrinsic β-lactam resistance conferred by chromosomal *ampC* genes. Here, we address recent advances in the molecular epidemiology, resistance mechanisms, global spread, and genomics of MDR ECC, focusing on CREC.

MOLECULAR EPIDEMIOLOGY OF *E. cloacae* COMPLEX

The *E. cloacae* complex is polyphyletic based on the traditionally employed 16S rRNA gene typing (Mezzatesta et al., 2012). Phenotypic methods and antibiotic susceptibility patterns were insufficient to resolve this genetically diverse species cluster. Molecular and genomic advances have enabled more refined species designations of ECC based on single amplicon (*hsp*60 or *rpoB*) genotyping, multilocus sequence analysis (MLSA), comparative genomic hybridization (CGH), pulsed-field gel electrophoresis (PFGE), and more recently, MLST and WGS. Based on *hsp*60 allelic variation, ECC was previously classified into thirteen genovars (clusters I-XIII). These encompass *Enterobacter asburiae* (cluster I), *Enterobacter kobei* (cluster II), *Enterobacter ludwigii* (cluster V), *Enterobacter hormaechei* subsp. *oharae* (cluster VI), subsp. *hormaechei* (cluster VII), and subsp. *steigerwaltii* (cluster VIII), *Enterobacter nimipressuralis* (cluster X), *E. cloacae* subsp. *cloacae* (cluster XI) and subsp. *dissolvens* (cluster XII), unnamed *E. cloacae* Hoffmann clusters III, IV, and IX, and an unstable *E. cloacae* sequence crowd (cluster XIII) (Brenner et al., 1986; Kosako et al., 1996; Hoffmann and Roggenkamp, 2003; Hoffmann et al., 2005a,b,c). However, using *hsp*60 or *rpoB* alone led to significant discrepancies in identification of subspecies (Paauw et al., 2008).

Multilocus sequence analysis based on 6 housekeeping genes (*rpoB*, *fusA*, *gyrB*, *leuS*, *pyrG*, and *rplB*) suggested the emergence of two distinct ECC clades: a recent clade including the three *E. hormaechei* subspecies and a heterogeneous older clade including multiple ECC clusters. The observed recombination:mutation ratio of 1.04 (95% confidence interval 0.72–1.45) across ancestral clades also indicates potential recombination events in the early evolution of ECC, likely accounting for discrepancies between single amplicon methods (Paauw et al., 2008). Based on MLSA, *Enterobacter mori* (Zhu et al., 2011), *Enterobacter xiangfangensis* (cluster VI), and *Enterobacter cancerogenus* were recently classified (Schonheyder et al., 1994). The remarkable genomic heterogeneity within ECC has even been used to suggest broad re-classification of the complex into five distinct genera based on MLSA (Brady et al., 2013). Despite ongoing debate regarding nomenclature within ECC, *E. cloacae* and *E. hormaechei* and related subspecies remain the most clinically relevant. In 2013, *dnaA* was added to the six genes of MLSA to develop an MLST scheme, which has emerged as a more robust tool for identifying closely related ECC isolates (Miyoshi-Akiyama et al., 2013). To date, 1069 sequence types (STs) have been reported.[1]

Comparison of the entire genome through WGS provides the opportunity to explore the genetic relationships between genomes at even higher resolution (Kluytmans-van den Bergh et al., 2016), and has further refined ECC classification into 18 clusters (A-R). These encompass the 12 Hoffmann clusters, *E. mori*, and five novel clusters (K, L, N, O, and P) (Chavda et al., 2016 and **Supplementary Figure S1**). Thus, the advent of WGS has greatly improved the ability to identify, investigate, and compare the emergence of ECC in diverse settings with high resolution, despite its polyphyletic and genomic diversity.

MULTIDRUG- AND CARBAPENEM-RESISTANCE IN ECC

A variety of intrinsic and acquired antimicrobial resistance mechanisms have diminished the arsenal of effective therapeutics for treatment of ECC infections. ECC is intrinsically resistant to penicillins and first- and second-generation cephalosporins due to low-level expression of chromosomal *ampC* genes encoding an inducible AmpC-type Bush group 1 (class C) cephalosporinase. Resistance to third-generation cephalosporins and aztreonam can result from mutations, usually in *ampD,* leading to constitutive hyperproduction (derepression) of AmpC (Seeberg et al., 1983; Kaneko et al., 2005; Cheng et al., 2017).

Extended-spectrum β-lactamase (ESBL) genes confer resistance to most β-lactam antibiotics, including extended spectrum (i.e., second and third-generation) cephalosporins (ESCs) and monobactams (i.e., aztreonam). These genes are typically plasmid-encoded and were first identified in ECC in 1989 (De Champs et al., 1989). Since then, ESBL-encoding ECC have increased in prevalence, particularly in nosocomial settings and among patients with previous antibiotic exposure (Kluytmans-van den Bergh et al., 2016; Jean and Hsueh, 2017; Peirano et al., 2018). ESBL- and AmpC-mediated resistance now commonly coincide, leading to near-pan-resistance to β-lactams (Pitout et al., 1997).

Carbapenem-resistance in ECC is conferred through either constitutive overexpression of AmpC combined with disrupted membrane permeability, or more commonly through the acquisition of plasmid-encoded carbapenemase genes. Two major categories of carbapenemases have been identified in CREC, carbapenem-hydrolyzing serine β-lactamases (Ambler class A and D) and metallo-β-lactamases (MBLs; Ambler class B) (**Supplementary Table S1**). The *Klebsiella pneumoniae* carbapenemase (KPC), a class A β-lactamase which predominates in the United States, and the New Delhi metallo-β-lactamase-1 (NDM-1) have been most frequently described in ECC (Chavda et al., 2016), although substantial regional variation has been reported (Peirano et al., 2018). Rarely, ECC may also harbor chromosomally encoded carbapenemase genes (Boyd et al., 2017).

[1] http://pubmlst.org/ecloacae/

In addition to β-lactam resistance, ECC harbor a variety of multi-class antibiotic resistance genes. This includes aminoglycoside resistance primarily due to the acquisition of plasmids or mobile genetic cassettes encoding aminoglycoside 6'-N-acetyltransferase type I [AAC(6')-I] (Neonakis et al., 2003). Mutations in DNA gyrase, DNA topoisomerase, or efflux pump genes have been associated with resistance to fluoroquinolones (Ruiz, 2003; Baucheron et al., 2004). Notably, ESBL and carbapenemase genes are often collocated with aminoglycoside-resistance genes on plasmids, engendering multi-class antibiotic resistance phenotypes (Chen et al., 2014; Chavda et al., 2016; Gomez-Simmonds et al., 2018).

An AAC(6′)-I variant produced by *aac(6′)-Ib-cr,* or the presence of plasmid-borne *qnr* or *qep* genes, can confer low-level quinolone resistance in ECC (Park et al., 2007; Périchon et al., 2007; Xiong et al., 2008; Cano et al., 2009; Kim et al., 2009). In addition, specific substitutions in chromosomal fluoroquinolone resistance-determining regions (QRDRs), such as the previously characterized double-serine/threonine substitutions in *gyrA* and *parC* (Hiramatsu et al., 2012), have been associated with improved fitness in major STs of other Enterobacteriaceae, including ESBL-producing *Escherichia coli* (Johnson et al., 2015) and *K. pneumoniae* (Tóth et al., 2014). This fitness advantage has been hypothesized to contribute to the spread of high-risk international STs while selecting against minor STs (Fuzi et al., 2017). QRDR mutations have been detected in ECC and appear to be widespread in CREC (Cano et al., 2009; Gomez-Simmonds et al., 2018; Guillard et al., 2015). However, their contribution to the spread of specific ECC and CREC clones has yet to be determined.

GLOBAL EMERGENCE OF CREC

E. cloacae complex was one of the first KPC-producing organisms identified (Bratu et al., 2005), and has recently demonstrated an increase in prevalence and regional distribution (Park et al., 2016; Wilson et al., 2017). Current literature indicates that the emergence and spread of CREC is due to high diversity of clonal lineages and carbapenemases. A recent study leveraging two global surveillance programs demonstrated the remarkable dissemination and variety of carbapenemase genes in ECC (Peirano et al., 2018).

We found 61 publicly available English-language publications identifying carbapenemase alleles in ECC with a corresponding geographic location (**Supplementary Table S2**). These encompassed 36 carbapenemase alleles (IMP-1,4,8,11,13,14,26,34; IMI-1,2,3,4,5,6,7,9; KPC-2,3,4,5,18; NDM-1,5,6,7; NMC-A; OXA-48; VIM-1,2,4,5,11,23,31; FRI-1,2; GES-7) in ECC from 44 countries, including single isolates and single or multi-institutional outbreak collections (**Figure 1** and **Supplementary Table S2**). In the United States and Canada, bla_{KPC}-positive ECC have been mostly encountered, with rare reports of IMI- and NMC-A-encoding organisms. Isolates harboring bla_{KPC} have also been detected in Europe and South America. While $bla_{\mathrm{NDM-1}}$ is endemic in the Indian subcontinent, multiple bla_{NDM} alleles were detected in hospitals throughout Eastern China (Jin et al., 2018; Wang et al., 2018). IMP-encoding genes have been reported widely in Southeast Asia, including China, Japan, Korea, the Philippines, Taiwan, and Australia, and are thought to be endemic to this area. On the other hand, VIM variants are more prevalent across Europe with rare reports from South America and Southeast Asia. OXA-48-like carbapenemases, thought to originate in Turkey, have spread into the Middle East, North Africa, and Europe (Poirel et al., 2011).

Previous multinational surveillance studies employing MLST found substantial clonal diversity of both ESBL-producing ECC and CREC, with evidence for several potential high-risk clones. The most widespread ESBL-producing ECC were ST66, ST78, ST108, and ST114, each having at least 10 isolates from three to five countries (Izdebski et al., 2015). Several epidemic clonal complexes (CC), such as CC74 (including ST78) or CC114 (including ST66) were identified, including specific ST66, ST78, and ST114 pulsotypes associated with carriage of CTX-M-15 β-lactamase. Likewise, ST114, (*E. xiangfangensis*), ST93 and ST90 (*E. hormaechei* subsp. *steigerwaltii*), and ST78 (*E. cloacae* cluster III) were widespread among global CREC isolates from 37 countries (Peirano et al., 2018), while ST105 (*E. xiangfangensis*) and ST108 were also identified in multiple countries.

GENOMIC INSIGHTS INTO THE SPREAD OF CREC WITHIN THE UNITED STATES

While carbapenem-resistant *K. pneumoniae* (CRKP) appears to be declining in high-prevalence areas such as the Northeastern United States, multiple sites across the United States have reported increasing prevalence of CREC (Frieden et al., 2018). By 2015, over 4% of ECC clinical isolates collected in the United States Veteran's Health Administration (VHA) nationwide were carbapenem non-susceptible, with especially high rates along the West Coast and Southwestern United States (Wilson et al., 2017). Most recently, New York City, Boston, Western Pennsylvania, North Carolina, and Minnesota/North Dakota have reported significant increases in CREC infections (Ahn et al., 2014; Hargreaves et al., 2015; Pecora et al., 2015; Gomez-Simmonds et al., 2016; Kanamori et al., 2017).

Limited information is available regarding specific genomic features of ECC potentiating its transmission and recent epidemiological success. However, the few available genomic studies suggest that establishment of successful clones as well as acquisition of MDR phenotypes by diverse lineages may have been substantial contributors.

ST171 has been identified as a major CREC clone with epidemic potential in the United States (Hargreaves et al., 2015; Chavda et al., 2016; Gomez-Simmonds et al., 2018). We previously found phylogenomic evidence that all ST171 with publicly available sequences formed two major clades which diverged and spread in parallel from the Northeastern to the Mid-Atlantic and Midwestern United States (Gomez-Simmonds et al., 2018). Our analysis estimated that these clades diverged prior to 1962, roughly two decades before the widespread

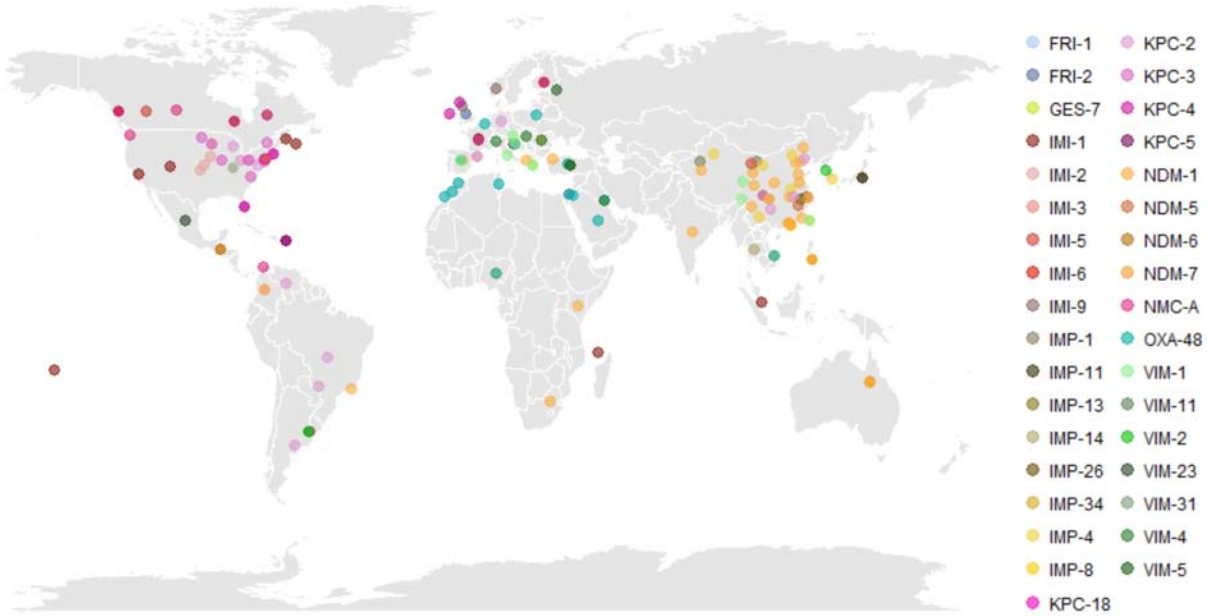

FIGURE 1 | Global distribution of carbapenem-resistant *Enterobacter cloacae* complex (CREC). Literature review identified 61 English-language publications identifying carbapenemase subtypes in CREC with a specified geographic location of isolation. The regional emergence of carbapenemases is evident, with KPC and IMI predominant in North America, OXA-48 and VIM predominant in Europe and the Middle East, and NDM and IMP predominant in China and Southeast Asia. Underlying data and referenced publications can be found in **Supplementary Table S2**. Abbreviations: FRI, French imipenemase; GES, Guiana extended-spectrum β-lactamase; IMI, imipenem-hydrolyzing carbapenemase; IMP, active-on-imipenem carbapenemase; KPC, *K. pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; NMC, non-metallo carbapenemase; OXA, oxacillinase; VIM, Verona integron-encoded metallo-β-lactamase.

use of carbapenems and fluoroquinolones, suggesting antibiotic pressure as a key factor in the proliferation of ST171.

ST171 is primarily associated with bla_{KPC-3}, although a handful of bla_{KPC-2}- and bla_{KPC-4}-containing isolates have been identified. In the Northeast, CREC ST171 primarily contained a bla_{KPC-3} gene located on IncFIA plasmids (e.g., p34978, pNR3024) (Gomez-Simmonds et al., 2018). These plasmids were nearly identical to pBK30683, a ∼70 kb IncFIA plasmid which was widespread among bla_{KPC}-producing *K. pneumoniae* in New York and New Jersey hospitals (Chen et al., 2014). Interestingly, a different study reported ST171 isolates from Minnesota and North Dakota which contained bla_{KPC-3} on a truncated (∼120 kb) IncFIA plasmid pMNCRE44 (Hargreaves et al., 2015). The truncated pMNCRE44 shared key regions with other ST171 IncFIA plasmids, but lacked genes encoding conjugation machinery. A small cluster of ST171 isolates from Boston instead contained bla_{KPC-4} on an unrelated IncHI2 plasmid (Pecora et al., 2015). A duodenoscope-mediated outbreak of CREC in a Michigan hospital also found likely patient-to-patient transmission of bla_{KPC}-positive ST171 (KPC allele unreported) (Hawken et al., 2018). However, the hospital collection included diverse clones in which carbapenem-resistance was driven primarily by chromosomal mutations rather than carbapenemase genes. ST171 was rare in global surveys of both primarily carbapenem-susceptible (Girlich et al., 2015; Izdebski et al., 2015) and carbapenemase-producing ECC (Peirano et al., 2018), harboring three different carbapenemase genes presumably on different plasmid backbones. This suggests that stable uptake of the IncFIA plasmid by ST171 largely enabled its successful proliferation throughout the Northeastern United States, while isolates lacking this plasmid remain uncommon.

In contrast, ST78 was identified as a high-risk clone among both ESBL-producing ECC and CREC. CREC ST78 has largely been isolated in the Northeastern United States, with multiple sporadic uptake events of bla_{KPC}-containing plasmids (Gomez-Simmonds et al., 2018), and has not exhibited the same rapid clonal proliferation as ST171. ST78 has been associated with various KPC-types on IncN plasmids, even within the New York City area (Gomez-Simmonds et al., 2018). Global carbapenemase-producing ST78 isolates have also been associated with a variety of plasmid backbones, highlighting its unique ability to acquire MDR plasmids. Peirano et al. (2018) demonstrated 4 different carbapenemases (bla_{VIM-1}, bla_{IMP-4}, bla_{IMP-8}, bla_{OXA-48}) on multiple different genetic backbones in ST78, although the carbapenemase-harboring plasmid could not be determined using short-read sequencing. In Japan, ST78 isolates harbored bla_{IMP-1} on class 1 integrons encoded on multiple different plasmids including IncHI2, IncW, and IncFIB (Aoki et al., 2018).

Other CREC STs have been associated with diverse KPC subtypes on IncN, IncX7, IncL/M, IncA/C, pKpQIL, and pKPC_UVA01-like plasmids, and plasmids with unknown replicon types (Chavda et al., 2016). However, few molecular studies include complete plasmid analyses, particularly for non-bla_{KPC} carbapenemases. Notably, although region-specific

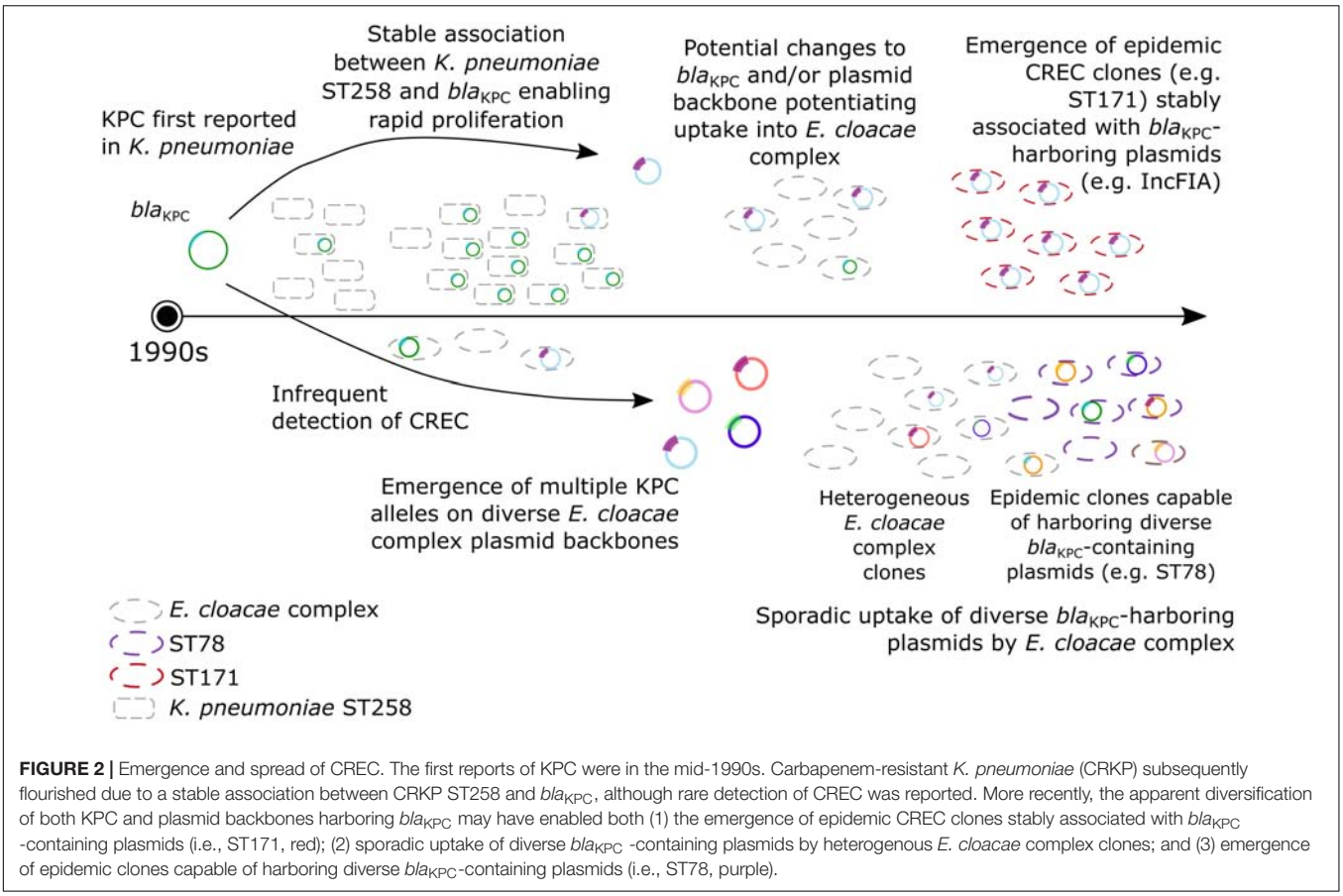

FIGURE 2 | Emergence and spread of CREC. The first reports of KPC were in the mid-1990s. Carbapenem-resistant *K. pneumoniae* (CRKP) subsequently flourished due to a stable association between CRKP ST258 and *bla*$_{KPC}$, although rare detection of CREC was reported. More recently, the apparent diversification of both KPC and plasmid backbones harboring *bla*$_{KPC}$ may have enabled both (1) the emergence of epidemic CREC clones stably associated with *bla*$_{KPC}$ -containing plasmids (i.e., ST171, red); (2) sporadic uptake of diverse *bla*$_{KPC}$ -containing plasmids by heterogenous *E. cloacae* complex clones; and (3) emergence of epidemic clones capable of harboring diverse *bla*$_{KPC}$-containing plasmids (i.e., ST78, purple).

associations between carbapenemase genes and specific genetic backbones have been reported, shuffling of these genetic structures among different ECC clones appears to occur commonly including in geographically diverse areas (Peirano et al., 2018).

Since the mid-1990s, when KPC was first described, the spread of CRKP has largely been attributed to the stable association between *bla*$_{KPC}$ and the successful CRKP clone ST258 (Kitchel et al., 2009; **Figure 2**). Although isolated instances of CREC were reported around the same time, the diversification of both *bla*$_{KPC}$ and plasmid backbones harboring these genes may have enabled uptake into diverse ECC. In contrast to CRKP, the spread of CREC can be attributed to not only stable *bla*$_{KPC}$-clone associations, as in the case of ST171 and *bla*$_{KPC-3}$-encoding IncFIA plasmids, but also the sporadic uptake of diverse plasmids by heterogeneous clones. This includes clones with epidemic potential capable of harboring diverse *bla*$_{KPC}$-containing plasmid backbones, such as ST78.

OTHER GENOMIC AND VIRULENCE FACTORS POTENTIATING THE SPREAD OF CREC

In addition to the presence of *bla*$_{KPC}$ genes, other genomic factors linked to carbapenem- or other MDR may have aided in the rapid proliferation of CREC. Several lineage-specific genomic islands in both ST171 and ST78, encode for toxin-antitoxin and cell stress response systems (Gomez-Simmonds et al., 2018). Genes for toxin-antitoxin systems and heavy metal resistance have been found on MDR plasmids in CREC isolates (Aoki et al., 2018). These factors may further contribute to the success of this organism, particularly in nosocomial settings, although their specific impact on virulence and fitness has yet to be determined.

Virulence of CREC compared to carbapenem-susceptible ECC has not been extensively assessed. However, murine macrophage cytotoxicity assays did reveal significantly reduced cell killing of CREC vs. ESBL isolates and, more specifically, reduced toxicity of CREC ST171 vs. ESBL ST78 isolates (Gomez-Simmonds et al., 2018). Of note, no significant differences in cytotoxicity by site of collection or KPC-subtype were observed. Although previous studies reported detection of Shiga-like toxins in ECC (Paton et al., 1996; Probert et al., 2014), candidate genes were not detected in genomic analysis of these clones (Gomez-Simmonds et al., 2018). Thus, CREC may be a low-virulence pathogen with specific adaptations that enable success in nosocomial environments. In particular, cross-class antibiotic resistance and the acquisition of carbapenem- and fluoroquinolone-resistance determinants prior to the widespread use of these drugs point to the role of antibiotic pressure in hospital settings, rather

than increased virulence, in the spread of CREC ST171 in the United States (Gomez-Simmonds et al., 2018). However, as previously suggested, potential fitness advantages conferred by QRDR mutations may play a role in the spread of major CREC STs, including ST171 and ST78, and should be evaluated further.

Analogous to the pan-genome, the concept of a "pan-metabolome" has also been applied to ECC (Rees et al., 2018). Several metabolite targets were identified, which discriminated between CREC and carbapenem-susceptible ECC, indicating a distinct metabolomic signature for each phenotype, beyond the presence of a single carbapenemase gene. The use of metabolomics and transcriptomics in future studies will be important to fully understand the complex relationships between genomic background, acquired carbapenemase resistance and virulence factors, and variable resistance phenotypes.

FUTURE DIRECTIONS

Several gaps remain in our understanding of CREC. The notable diversity of CREC clones, carbapenemase genes, and plasmid backbones harboring MDR genes have thus far led to uncertainty regarding a clear timeline and evolutionary history of these organisms. Virulence, fitness, or other genomic factors potentiating the spread of CREC have not been completely defined or assessed *in vitro*. Moreover, despite recent advancements potentiated by WGS and comparative genomics, transcriptomics and/or metabolomics approaches may be useful in future studies to define the metabolic activity of CREC under different conditions. Lastly, the underreporting of CREC remains a possibility, and may influence findings regarding both population-level diversity and genomic mechanisms of resistance.

Regardless, the unique diversity of CREC, even compared to other CRE such as CRKP, necessitates a tailored approach to preventing its transmission and further diversification. The establishment of high-risk global CREC clones, coupled with the apparent high frequency of plasmid uptake into diverse ECC, suggests that vigilant tracking of both localized outbreaks and the potential for horizontal plasmid transfer is required.

AUTHOR CONTRIBUTIONS

A-CU initiated the review. AG-S performed a literature search. MKA wrote the first draft. All authors edited and reviewed the manuscript draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00044/full#supplementary-material

FIGURE S1 | Phylogenetic tree of representative *E. cloacae* complex (ECC) isolates showing relationships between Hoffmann clusters I-XII, genomic groups A-R, and selected sequence types (STs). At least one isolate with publicly available short-read sequences was selected from each ST previously reported in two recent genomic studies of CREC (Chavda et al., 2016; Gomez-Simmonds et al., 2018). NCBI Sequencing Read Archive (SRA) accession numbers are shown for each isolate in **Supplementary Table S3**. A public ST171 genome (GenBank CP012165) was used as the reference sequence for calling of concatenated core genome SNPs with snippy (https://github.com/tseemann/snippy) after removing mobile genetic elements and phage regions. The maximum likelihood tree was generated using RaxML with 100 bootstraps and visualized in iTOL (https://itol.embl.de/).

TABLE S1 | Carbapenemase classes identified in carbapenem-resistant *Enterobacter cloacae* complex.

TABLE S2 | Carbapenemase alleles by reported location.

TABLE S3 | Metadata for selected isolates with publicly available whole-genome short-read data for phylogenetic analysis (**Supplementary Figure S1**).

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frontiers in Microbiology

ORIGINAL RESEARCH
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Multiple Benefits of Plasmid-Mediated Quinolone Resistance Determinants in *Klebsiella pneumoniae* ST11 High-Risk Clone and Recently Emerging ST307 Clone

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International high-risk clones of *Klebsiella pneumoniae* are among the most common nosocomial pathogens. Increased diversity of plasmid-encoded antimicrobial resistance genes facilitates spread of these clones causing significant therapeutic difficulties. The purpose of our study was to investigate fluoroquinolone resistance in extended-spectrum beta-lactamase (ESBL)-producing strains, including four *K. pneumoniae* and a single *K. oxytoca*, isolated from blood cultures in Hungary. Whole-genome sequencing and molecular typing including multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were performed in selected strains. Gene expression of plasmid-mediated quinolone resistance determinants (PMQR) was investigated by quantitative-PCR. MLST revealed that three *K. pneumoniae* strains belonged to ST11 and one to ST307 whereas *K. oxytoca* belonged to ST52. The isolates harbored different β-lactamase genes, however, all *K. pneumoniae* uniformly carried $bla_{CTX-M-15}$. The *K. pneumoniae* isolates exhibited resistance to fluoroquinolones and carried various PMQR genes namely, two ST11 strains harbored *qnrB4*, the ST307 strain harbored *qnrB1* and all *K. pneumoniae* harbored *oqxAB* efflux pump. Levofloxacin and moxifloxacin MIC values of *K. pneumoniae* ST11 and ST307 clones correlated with *qnr* and *oqxAB* expression levels. The *qnrA1* carrying *K. oxytoca* ST52 exhibited reduced susceptibility to fluoroquinolones. The maintained expression of *qnr* genes in parallel with chromosomal mutations indicate an additional protective role of Qnr proteins that can support dissemination of high-risk clones. During development of high-level fluoroquinolone resistance, high-risk clones retain fitness thus, enabling them for dissemination in hospital environment. Based on our knowledge this is the first report of ST307 clone in Hungary, that is emerging as a potential high-risk clone worldwide. High-level fluoroquinolone resistance in parallel with upregulated PMQR gene expression are linked to high-risk *K. pneumoniae* clones.

Keywords: international clones, multi-drug resistance, whole genome sequence analysis, gene expression, plasmid-mediated quinolone resistance

INTRODUCTION

International high-risk clones of *Klebsiella pneumoniae* are among the most common Gram- negative pathogens. In addition to community-acquired infections, it has been known for decades that due to their ability to spread rapidly in hospital environment, these bacteria can cause several outbreaks. Multi-drug resistant (MDR) *K. pneumoniae* emerged and dramatically increased prevalence of nosocomial infections while *K. oxytoca* has been isolated in hospital infections with less frequency (Podschun and Ullmann, 1998; Kang et al., 2006; Zhou et al., 2016).

Multi-drug resistant *K. pneumoniae* acquires various resistance mechanisms that confer antibiotic resistance to commonly used antibiotics. Among the most frequent resistance mechanisms are extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC enzyme (pAmpCs), carbapenemases, plasmid-mediated quinolone resistance (PMQR) genes, aminoglycoside-modifying enzymes (AMEs), as well as exogenously acquired 16S rRNA methyltransferase that have been detected in clinical isolates (Yan et al., 2002; Ko et al., 2010; Cao et al., 2014; Bi et al., 2017). Presence of PMQR genes including *qnr* determinants, *aac(6′)-Ib-cr, qepA* and *oqxAB* efflux pumps confer reduced susceptibility to fluoroquinolones and facilitate selection of fluoroquinolone resistance in Enterobacterales (Rodríguez-Martínez et al., 2011; Carattoli, 2013). High-risk *K. pneumoniae* clones have acquired these antibiotic resistance determinants, that enabled them to increase their pathogenicity and survival skills. These clones have tenacity and flexibility to accumulate resistance determinants and they have contributed to disseminate global multi-drug resistance (Woodford et al., 2011). Consequently, increased diversity of plasmid-encoded antimicrobial resistance genes facilitates spread of these clones, causing significant therapeutic difficulties.

Multi-drug resistant *K. pneumoniae* strains mainly belong to certain sequence types (ST) namely, ST11, ST14, ST15, ST37, ST101, ST147, ST258, ST336, ST340, and ST874. These represent high-risk international clones that played major role in dissemination in hospital settings and increased frequency in nosocomial infections (Damjanova et al., 2008; Hrabák et al., 2009; Baquero et al., 2013; Munoz-Price et al., 2013; Rodrigues et al., 2014; Gonçalves et al., 2017). Among these clones ST258 has been reported as a hybrid clone that was created by a large recombination event between ST11 and ST442 (Mathers et al., 2015).

International high-risk *K. pneumoniae* ST11 has been frequently detected worldwide as a successful pathogen being associated with important co-resistance and virulence factors (Damjanova et al., 2008; Andrade et al., 2014). However, in recent years, new drug-resistant lineages have emerged internationally and among them, KPC-producing *K. pneumoniae* ST307 has been recognized in the United States which was initially associated with production of CTX-M-15 (Castanheira et al., 2013). Later on, this clone has been reported in several countries including Italy, United Kingdom, Columbia, Pakistan, Morocco, Korea, Tunisia, China, Serbia (Habeeb et al., 2013; Girlich et al., 2014; Gona et al., 2014; Park et al., 2015; Ocampo et al., 2016; Mansour et al., 2017; Novović, 2017; Villa et al., 2017; Xie et al., 2017).

Recent studies related to dissemination and antibiotic resistance of *K. pneumoniae* clones clearly showed that "fitness cost advantage" associated with high-level resistance to fluoroquinolones contributed to emergence of international high-risk *K. pneumoniae* clones. In hospital settings where fluoroquinolones are extensively used, international clones are selected out, allowing dominance over other clones (Tóth et al., 2014; Fuzi, 2016; Fuzi et al., 2017). This capacity will provide these clones increased opportunities to spread as well as allow time to acquire antimicrobial drug resistance determinants from other bacteria (Mathers et al., 2015). Whole-genome sequence analysis contributes to detect markers of pathogens, therefore in our study the aim was to investigate high-level fluoroquinolone resistance in *K. pneumoniae* high-risk clone ST11 and currently emerging ST307.

MATERIALS AND METHODS

Bacterial Strains

In our preliminary examination, a total of 54 *Klebsiella* strains (53 *K. pneumoniae* and a single *K. oxytoca*) isolated from bloodstream infections of patients treated at intensive care units of Semmelweis University between 2010 and 2014 were collected. Species identification was done by MALDI-TOF/MS (Bruker Daltonics, Bremen, Germany). Minimum inhibitory concentration determination was performed by microdilution method based on EUCAST recommendation.[1] All *Klebsiella* strains were resistant to third-generation cephalosporins and showed reduced susceptibility or resistance to fluoroquinolones. All strains were tested for presence of PMQR genes and all of them were ESBL producers by phenotypic test. In this study, selection of strains was done based on the following criteria: (1) presence of *qnr* gene and non-wild type fluoroquinolone MIC values: Kox37 (isolated in 2010); (2) presence of *qnr* gene and high fluoroquinolone MIC values: Kpn33 (isolated in 2010), Kpn47 (isolated in 2014), Kpn125 (isolated in 2013); (3) multiple PMQR gene carriage together with high fluoroquinolone MIC values: Kpn115 (isolated in 2013) (Domokos et al., 2016).

Multilocus Sequence Typing (MLST)

Genotype of each strain was determined by MLST. The sequences of seven housekeeping genes namely, *gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB* were amplified and directly sequenced. Alleles and sequence types were assigned by using the MLST database[2] (Diancourt et al., 2005). The distance based relationship between the strains was investigated by BacWGST (Ruan and Feng, 2016) using both the whole-genome MLST and SNP (sequenced based) strategies. Multiple genome analysis was carried out using all the draft genomes of this study and the *HS11286_CP003200_ST11* as a reference genome (**Figure 1**).

[1] www.eucast.org

[2] http://www.pasteur.fr/mlst/Kpneumoniae.html

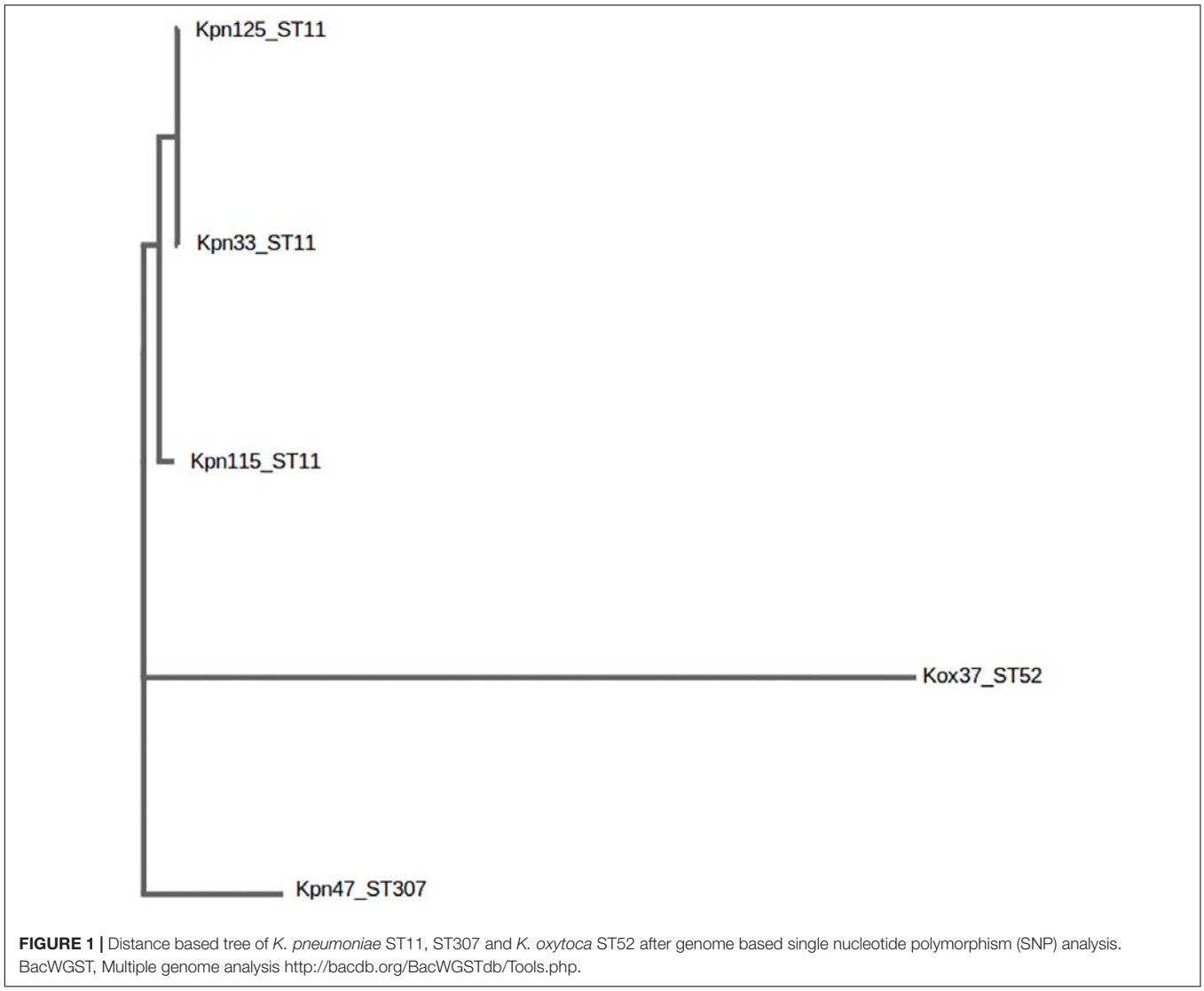


FIGURE 1 | Distance based tree of *K. pneumoniae* ST11, ST307 and *K. oxytoca* ST52 after genome based single nucleotide polymorphism (SNP) analysis. BacWGST, Multiple genome analysis http://bacdb.org/BacWGSTdb/Tools.php.

Pulsed Field Gel Electrophoresis (PFGE) Typing

Clonal relatedness of the four *K. pneumoniae* strains was analyzed by PFGE according to CDC (2000) protocol. Prepared genomic DNA of each strain was digested by *Xba*I restriction endonuclease (Fermentas, ABI, Germany), and DNA fragments were separated in a PFGE CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA, United States). Banding patterns were analyzed by Fingerprinting II Informatix Software (Bio-Rad). *Salmonella enterica* serotype Braenderup H9812 was used as a size marker (Hunter et al., 2005).

Whole-Genome Sequencing (WGS)

DNA of each strain was extracted by UltraClean Microbial DNA Isolation Kit (Qiagen GmbH, Hilden, Germany). Libraries were prepared using SureSelect QXT Library Prep Kit (Agilent Technologies, Santa Clara, United States). Sequencing was performed on an Illumina MiSeq system using the MiSeq reagent kit v2 generating 250-bp paired-end reads. Trimmomatic (Bolger et al., 2014) was used for preprocessing the WGS data. If the average quality score was below 20 in a sliding window of 4 the adapter sequences and the leading and trailing bases were removed as well as the first 18 bases. Only the reads longer than 50 nucleotides were used for subsequent analysis. *De novo* genome assembly was performed with SPAdes Genome Assembler 3.13.0 (Bankevich et al., 2012). Each assembled genome was accepted for further analysis if it met all of the following quality criteria: (i) average coverage > 30 times, (ii) N50 > 15,000 bases, (iii) maximum contig length > 50,000 bases, and (iv) assembled genome size between 5,000,000 and 6,500,000 bases. Assembled genomes were uploaded to the online bioinformatics tools ResFinder (Zankari et al., 2012), PlasmidFinder (Carattoli et al., 2014) (Center for Genomic Epidemiology, Technical University of Denmark, Lyngby, Denmark) to analyse resistome and plasmid replicon types of the isolates.

Quantitative PCR (qPCR)

Total RNA of tested strains was isolated by RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The qPCR was carried out in a Step One Real-Time PCR System (Applied BioSystems, Thermo Fisher Scientific). Separate expression of *qnrA1, qnrB1 qnrB4*, *oqxA*, and *oqxB* genes were investigated whereas chromosomal *rpoB* was chosen as housekeeping gene. Set of primers and 6-FAM or VIC labeled probes were designed by Primer Express 3.0 software. All oligonucleotide primers and probes for qPCR are listed in **Table 1**. Each RNA sample was tested in triplicate. The qPCR was applied in default setting 60°C 30 s; 50°C 5 min; 95°C 10 min; 40 cycles of [95°C 15 s and 60°C 1 min] 60°C 30 s. The C_T values of genes of interest were normalized (ΔC_T) to the C_T values of housekeeping gene *rpoB* and the relative expression of each gene of interest was calculated as $2^{-\Delta C}{}_T = C_{T\ (geneofinterest)} - C_{T\ (rpoB)}$.

RESULTS

In our study, four *K. pneumoniae* and a single *K. oxytoca* were investigated by MLST and PFGE. Three different STs were identified, including ST11 (Kpn33, Kpn115, Kpn125), ST307 (Kpn47), and ST52 (Kox37).

Pulsed-field gel electrophoresis analysis detected three pulsotypes (PT) among *K. pneumoniae* strains, namely, KP053, S and KP197. Two isolates belonged to KP053 (Kpn33 and Kpn125) and one was detected as S PT (Kpn115). These strains belonged to the ST11 international high-risk clone. By contrast, Kpn47 was classified as KP197 PT (**Figure 2**).

The initial assembled draft genome sequences were 5611026 bp (Kpn33); 6370417 bp (Kox37); 5451744 bp, (Kpn47); 5450412 bp (Kpn115), and 5593358 bp (Kpn125). Seventeen antibiotic resistance genes were found in two ST11 *K. pneumoniae* strains (Kpn33 and Kpn125), twelve were in the third ST11 strain (Kpn115), sixteen resistant genes were in ST307 strain (Kpn47) and ten resistance genes were detected in Kox37. Sequence analysis revealed that the isolates harbored different β-lactamase genes, including bla_{DHA-1}, bla_{OXA-1}, bla_{OXA-2}, bla_{OXA-9}, bla_{HV-11}, bla_{HV-28}, and bla_{TEM-1A}, bla_{TEM-1B}, $bla_{OXY-1-3}$, bla_{TLA-1}; and all *K. pneumoniae* strains carried $bla_{CTX-M-15}$. Among aminoglycoside resistance genes all isolates were positive for *aac(3)-IIa*. Only Kpn47 carried a tetracycline resistance (*tetA*) gene. Except for Kox37, all strains were identified positive for *fosA* gene nevertheless, *sul1* or *sul2* and trimethoprim resistance (*dfrA12, dfrA14, dfrA29*) genes were detected in four strains. PMQR genes were found in each tested strain namely, in Kpn33 *qnrB4,* in Kox37 *qnrA1,* in Kpn47 *qnrB1,* in Kpn125 *qnrB4.* All *K. pneumoniae* strains harbored *oqxAB* efflux pump and *aac(6′)-Ib-cr*, but one of the ST11 strains (Kpn115) carried no *qnr* gene. Presence of phenicol resistance gene (*catA1* or *catB3*) was observed in all strains. Chromosomal mutations conferring fluoroquinolone resistance in *K. pneumoniae* strains were also detected, Ser83Phe and Asp87Ala substitutions were in DNA gyrase subunit A of Kpn115 (ST11), but all other *K. pneumoniae* strains had only Ser83Ile in gyrase while on the other hand all *K. pneumoniae* had a Ser80Ile substitution in DNA topoisomerase IV. Based on the sequencing data, IncFIB, IncFII, and IncR replicons were uniformly present in all ST11 strains. In the case of ST307 IncFIB, IncL/M, IncHI1B were detected. The detected resistance genes and plasmid replicons are listed in **Table 2** and **Figure 3**.

Among *qnr* genes, *qnrB4* of two ST11 strains (Kpn33 and Kpn125) showed 9.74 and 3.55 fold expression, respectively. Interestingly, Kpn33 (ST11) was characterized approximately 3-fold higher expression, compared to the genetically similar Kpn125 (ST11). The lowest expression level (1.64) among *qnr* genes was detected in *K. oxytoca*, that exhibited reduced susceptibility to ciprofloxacin. In the case of *qnrB1* in Kpn47 (ST307), it showed 2.39 fold expression.

Expression of *oqxA* ranged between 1.47 and 3.92 and that of *oqxB* from 3.09 to 8.53. The highest *oqxA* and *oqxB* expressions were observed in Kpn33 (ST11) and Kpn47 (ST307). These were followed by Kpn125 (ST11) and Kpn115 (ST11). Interestingly, Kpn115 a strain of ST11 high-risk clone carried no *qnr* gene moreover, it showed the lowest *oqxAB* expression. It is conspicuous that in every *K. pneumoniae* strain the *oqxB* is expressed 2–3 fold higher than *oqxA*.

TABLE 1 | Primers used for qPCR (F, forward; R, reverse; P, probe).

Gene	Primer sequence
qnrA1-F	5′-TTGAGTGACAGCCGTTTTCG-3′
qnrA1-R	5′-GCAGCTGACAGTGGCTGAAG-3′
qnrA1-P	6-FAM-CTGCCGCTTTTATC-MGB
qnrB1-F	5′-GTGCGCTGGGCATTGAA-3′
qnrB1-R	5′-CGGAAATCTGCGCCTTGT-3′
qnrB1-P	6-FAM-TTCGCCACTGCCGC-MGB
qnrB4-F	5′-TGCGCTGGGAATCGAAA-3′
qnrB4-R	5′-CGCGAAAATCTGACCCTTGT-3′
qnrB4-P	6-FAM-TCGCCACTGCCGGG-MGB
oqxA-F	5′-GTCGACGGCTTACAAAAAGTGTT-3′
oqxA-R	5′-GCAACGGTTTTGGCGTTAA-3′
oqxA-P	6-FAM-ATGCCGGGTATGCC-MGB
oqxB-F	5′-CTGGATTTTCCGTCCGTTTAAC-3′
oqxB-R	5′-TTGCCTACCAGTCCCTGATAGC-3′
oqxB-P	6-FAM-CTGCGCAGCTCGAA-MGB
rpoB-F	5′-GTCGCGGCTGAACAAGCT-3′
rpoB-R	5′-AACGGCCACTTCGTAGAAGATC-3′
rpoB-P	VIC-CTACGGCAGGTAACC-MGB

DISCUSSION

International high-risk *K. pneumoniae* ST11 clone has been frequently detected worldwide as a successful pathogen being associated with important virulence (Damjanova et al., 2008; Andrade et al., 2014), and resistance determinants including VIM, NDM and KPC-production (Yan et al., 2002; Kristóf et al., 2010; Qi et al., 2011; Yu et al., 2016; Campana et al., 2017). In our study, all strains of ST11 international high-risk clone carried $bla_{SCTX-M-15}$ ESBL that correlates well with earlier studies as the most common global ESBLs are the

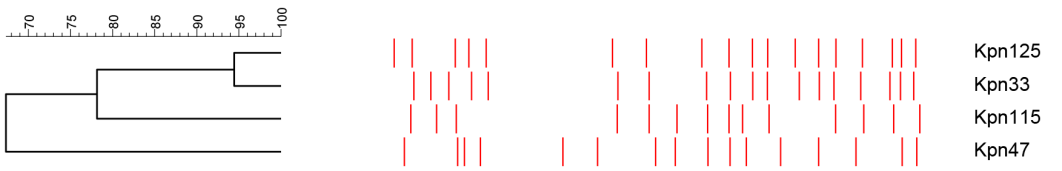


FIGURE 2 | PFGE of *K. pneumoniae* ST11 and ST307.

TABLE 2 | Distribution of the different resistance genes and plasmid replicons of tested strains.

ST11 Kpn33	ST52 Kox37	ST307 Kpn47	ST11 Kpn115	ST11 Kpn125	Genes
■				■	*aadA1*
■	■	■	■	■	*aac(3)-IIa*
■				■	*aac(6')-Ib*
	■				*aph(3')-Ic*
■				■	*aadA2*
		■	■		*strA*
		■	■		*strB*
■	■			■	*sul1*
		■			*sul2*
■		■	■	■	*fosA*
				■	*dfrA12*
		■			*dfrA14*
	■				*dfrA29*
■		■	■	■	***oqxA***
■		■	■	■	***oqxB***
■		■	■	■	***aac(6')-Ib-cr***
	■				***qnrA1***
		■			***qnrB1***
■				■	***qnrB4***
		■			*tet(A)*
	■				***blaOXY-1-3***
					blaTLA-1
				■	***blaTEM-1A***
	■	■	■		***blaTEM-1B***
■				■	***blaDHA-1***
		■	■		***blaOXA-1***
	■				***blaOXA-2***
■				■	***blaOXA-9***
			■		***blaSHV-11***
		■			***blaSHV-28***
■			■	■	***blaCTX-M-15***
■	■			■	*catA1*
		■	■		*catB3*

ST11 Kpn33	ST52 Kox37	ST307 Kpn47	ST11 Kpn115	ST11 Kpn125	Plasmid replicons
▒			▒	▒	*IncFII(K)*
					IncFIA(HI1)
			▒		*IncR*
	▒	▒	▒		*IncFIB(K)*
					IncL/M (pmu407)
		▒			*IncFIB(Mar)*
					IncHI1B
	▒				*ColRNA1*

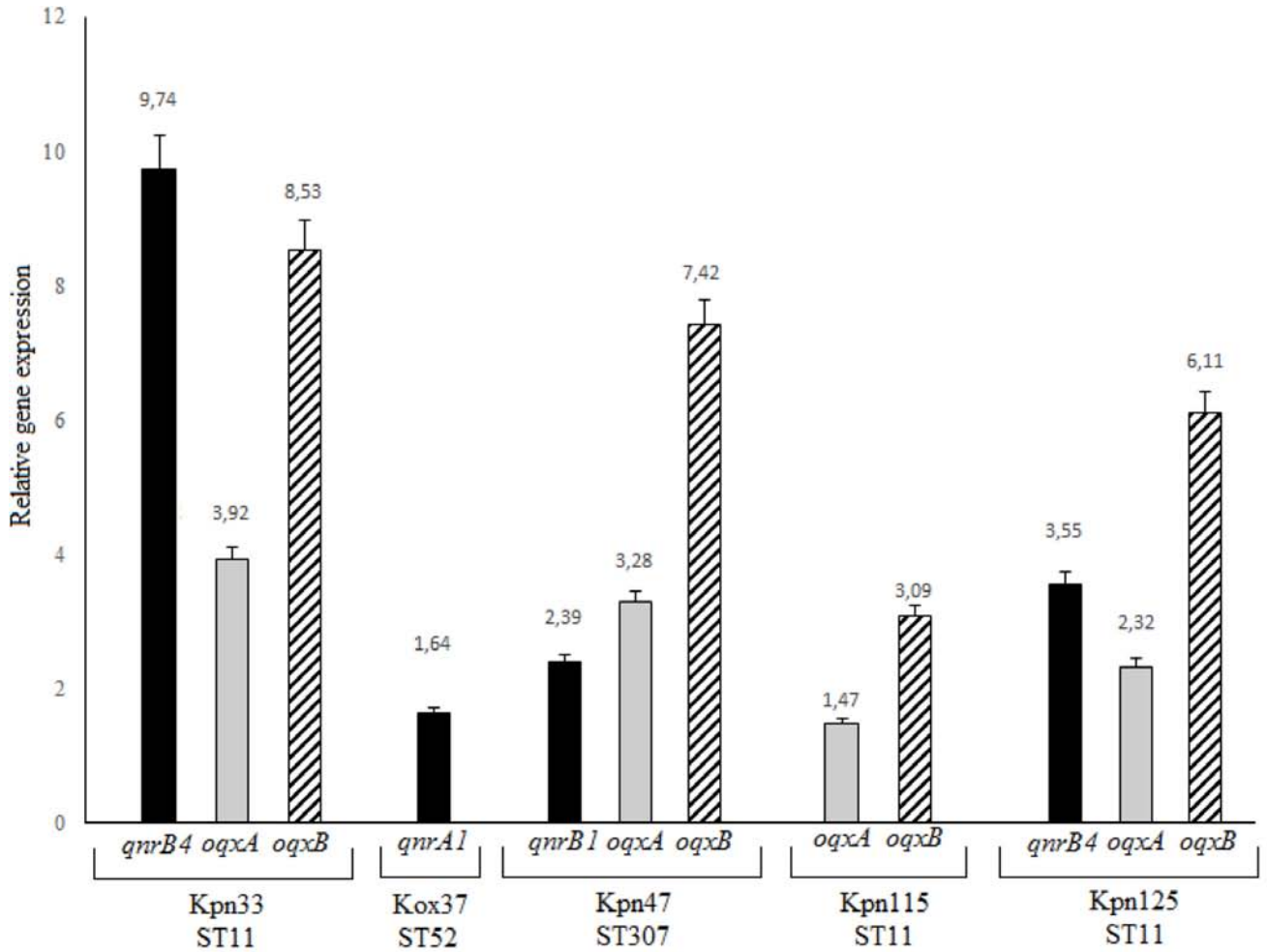
CTX-M type beta-lactamases in Enterobacterales (Nordmann and Poirel, 2014). Recently, in a Bulgarian study among 82 ESBL-producing *K. pneumoniae* and four *K. oxytoca* CTX-M-15 (87%) was predominant (Markovska et al., 2017). *K. pneumoniae* ST11 has been already reported in Hungary, as a widely disseminated clone in all over the country (Damjanova et al., 2008). In Poland, an inter-regional outbreak was reported that was dominated by NDM-1 and CTX-M-15 coproducing *K. pneumoniae* ST11 clone (Baraniak et al., 2016). A high prevalence (30.2%) of CTX-M-15-producing *K. pneumoniae* was detected in raw bovine milk too. This finding highlights the spread of CTX-M-15-producing *K. pneumoniae* also in the food chain (Diab et al., 2017).

In recent years, new drug-resistant international lineages have emerged, among them, KPC-producing *K. pneumoniae* ST307 has been recognized in several countries (Castanheira et al., 2013; Villa et al., 2017). To the best of our knowledge, our study is the first description of ST307 in Hungary that is has been reported as a potential high-risk clone. High similarity has been found in our ST307 isolate compared to that of detected by Villa et al. (2017).

Three pulsotypes were identified among the investigated *K. pneumoniae* strains: KP053, S PT, and KP197. Two ST11 isolates belonged to KP053 (Kpn33 and Kpn125) and the third ST11 was detected as S PT (Kpn115) that was earlier reported in Hungary (Damjanova et al., 2008). In a Hungarian study, PFGE typing revealed 12 pulsotypes; of these, KP053 (262/312) and KP070 (38/312) belonged to sequence type ST11 (Kis et al., 2016); these data also prove the spread of KP053/ST11 clone in our country. *K. pneumoniae* ST307 (Kpn47) was classified as KP197 pulsotype, however, this type was not registered until 2014 by the National Public Health Institute. Since 2015, altogether 30 strains have been identified with this pulsotype in Hungary (unpublished data).

In this study, mutations in gyrase and topoisomerase coding genes and various PMQRs were detected in *K. pneumoniae* and *K. oxytoca*. Of the detected PMQRs in this study *oqxAB* was present in all *K. pneumoniae* clinical isolates but not in *K. oxytoca*. This result can be explained by the fact that the *oqxAB* is a chromosomally-encoded gene in *K. pneumoniae* (Yuan et al., 2012). The *qnrB* genes were observed in *K. pneumoniae* ST11 correlating with the international data (Hidalgo et al., 2013; Jaidane et al., 2018). However, this is the first report of the *qnr* gene in *K. oxytoca* ST52. Regarding plasmid replicon types, the most common replicon was IncFIB, that was present in all ST11, ST52, and ST307, which confirms earlier studies (Anes et al., 2017).

Acquisition of *qnr* determinants can have multiple advantages. In the case of *K. oxytoca,* the presence and expression of *qnrA1* caused reduced susceptibility to quinolones. Levofloxacin and moxifloxacin MIC values of *K. pneumoniae* ST11 and ST307 clones correlated with *qnr* and *oqxAB* expression levels (**Figure 3**).



	Kpn33	Kox37	Kpn47	Kpn115	Kpn125
MIC values					
NAL	>256	16	>256	>256	>256
CIP	128	0.25	128	128	128
LEV	128	0.25	32	8	64
MOX	128	0.5	128	8	64
QRDRs					
gyrA	Ser83Ile	-	Ser83Ile	Ser83Phe, Asp87Ala	Ser83Ile
parC	Ser80Ile	-	Ser80Ile, Asn304Ser	Ser80Ile	Ser80Ile

FIGURE 3 | Level of *qnrB4* (Kpn33 and Kpn125), *qnrA1* (Kox37), and *qnrB1, oqxA*, and *oqxB* relative gene expression. QRDRs: quinolone resistance determining regions. All MIC values are in mg/L.

Further beneficial effect of Qnr proteins can be explained by the toxin-antitoxin effect. Qnr proteins are considered antitoxins, that protect gyrase and topoisomerase IV enzymes from naturally occuring toxins. This theory was described by Ellington and Woodford (2006) and it can be valid also in internationally disseminated high-risk clones (Ellington and Woodford, 2006). During development of fluoroquinolone resistance PMQR determinants play a role in reduced susceptibility, and they maintain low-level fluoroquinolone resistance (Garoff et al., 2018). Later, by chromosomal mutations in QRDRs high-level fluoroquinolone resistance develops, but PMQR expression is maintained thus, indicating further role of PMQRs such as protection of gyrase and topoisomerase IV enzymes (Tran et al., 2005a,b; Redgrave et al., 2014).

It has been also established that the development of fluoroquinolone resistance is diverse among different clones and in the case of international high-risk *K. pneumoniae* clones the fluoroquinolone resistant strains retain fitness that facilitates their dissemination in hospital environment (Fuzi, 2016). Moreover, Redgrave et al. indicated that fluoroquinolone resistance played a key role in evolutionary success of *K. pneumoniae* clones (Redgrave et al., 2014).

Emergence and possible dissemination of *K. pneumoniae* ST307 in hospital settings raises also public health concerns, therefore continous monitoring of high-risk and potential high-risk clones is necessary.

REPOSITORY DATA

Assembled genomes of all investigated strains were deposited in NCBI Genbank under the following accession numbers. Raw sequence data of each strain in this study was submitted to Sequence Read Archive (SRA)

Kpn 33: Bioproject: PRJNA511518, Biosample SAMN10639440
Kox37: Bioproject PRJNA511522, Biosample: SAMN10639457
Kpn47: Bioproject: PRJNA511523, Biosample: SAMN10639726
Kpn115 Bioproject: PRJNA511524, BioSamples SAMN10639736
Kpn125: Bioproject: PRJNA511525, BioSamples SAMN10639737.

AUTHOR CONTRIBUTIONS

JD performed pulsed-field gel electrophoresis, multilocus sequence typing, and handled the manuscript. ID performed pulsed-field gel electrophoresis and whole-genome sequencing. KK identified and handled strains from clinical specimen. BL performed whole-genome sequence analysis. BK performed qPCR, analyzed the data, and handled the manuscript. DS was laboratory chief, contributed to study design, and handled the manuscript.

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Emergence of Colistin Resistance Gene *mcr-8* and Its Variant in *Raoultella ornithinolytica*

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Recently, a novel mobile colistin resistance gene, *mcr-8*, was identified in *Klebsiella pneumoniae*. Here, we report the identification of *mcr-8* and its variant, *mcr-8.4*, in *Raoultella ornithinolytica* isolates which also belong to Enterobacteriaceae family. The *mcr-8* gene was located on transferrable plasmids with difference sizes. Notably, the transferability of *mcr-8*-carrying plasmids was enhanced once they entered into *Escherichia coli* hosts and multiple β-lactamase genes could co-transfer with *mcr-8*. These findings expand our knowledge of *mcr-8*-carrying bacterial species.

Keywords: colistin resistance, *mcr-8*, *mcr-8.4*, β-lactamase, *Raoultella ornithinolytica*

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INTRODUCTION

Colistin (polymyxin E), a polypeptide antibiotic, was originally isolated from the soil bacterium *Paenibacillus polymyxa* subsp. *colistin* (Poirel et al., 2017). Colistin is effective against most Gram-negative bacteria and was considered as one of the last-resort antibiotics for the treatment of human infections caused by multidrug resistant Gram-negative bacteria, especially, carbapenem-resistant Enterobacteriaceae (CRE) (Li et al., 2006). However, in 2016, the first plasmid mediated colistin resistance gene *mcr-1* was identified in *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Liu et al., 2016). To date, the *mcr-1* gene has been detected in Enterobacteriaceae isolated from food, animals, human and environment in over 50 countries across five different continents (Hembach et al., 2017; Huang et al., 2017). Subsequently, plasmid-mediated colistin resistance genes *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, and *mcr-7* have been identified in various bacterial species from humans and animals (Partridge et al., 2018). Recently, we reported the identification of *mcr-8* located on an InFII-type conjugative plasmid in *Klebsiella pneumoniae* isolated from chickens and pigs in China (Wang et al., 2018).

Raoultella ornithinolytica is closely related to *Klebsiella* and belongs to Enterobacteriaceae family (Beye et al., 2018; Hajjar et al., 2018). *R. ornithinolytica* is usually found in animals, soil, and botanical environment. This organism caused human infections, initially rare, are increasing according to several reports (Sun et al., 2015; Ponce-Alonso et al., 2016; Beye et al., 2018). So far, multi-drug resistance has been detected in *R. ornithinolytica* (Walckenaer et al., 2004; Castanheira et al., 2009; Khajuria et al., 2013), including *mcr-1* positive isolates (Luo et al., 2017). Here, we report the emergence of *mcr-8* in *R. ornithinolytica*.

MATERIALS AND METHODS

Bacterial Isolation and Identification

A total of 300 cloaca samples were collected from chicken in commercial poultry farms of Shandong Province, China, in 2016. All the samples were screened on the CHROMAgar Orientation agar plate (bioMérieux, Lyon, France) containing 2 μg/ml colistin. The identification of bacterial species was performed using MALDI-TOF MS (BruKer Daltonik, Bremen, Germany), and then confirmed by 16S rDNA sequence analysis as described previously (Zhang et al., 2015; Luo et al., 2017). The presence of *mcr* (*mcr-1* to *mcr-8*) in *R. ornithinolytica* was determined by PCR amplification and followed by Sanger sequencing as described previously (Wang et al., 2018).

Before collection the study samples, we have drafted an application "Detection of plasmid mediated colistin resistance genes of Enterobacteriaceae in Shandong, China," within which chicken are designed to be used as research object in this antimicrobial resistance study. Those experiments are guaranteed to conduct in accordance with the principles of the Beijing Municipality Review of Welfare and Ethics of Laboratory Animals, as well as rules and regulations from China Agricultural University's committee on animal welfare and ethics. Finally, this application was approved by committee on Animal Welfare and Ethics in China Agricultural University.

S1-PFGE and Southern Blotting

S1 nuclease-PFGE and Southern blotting were performed to locate the *mcr-8* gene in both donor and recipient strains as described previously (Zheng et al., 2017). Briefly, agarose gel plugs embedded strains were digested with S1 nuclease (TakaRa, Dalian, China), and Southern blotting was performed using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). The genomic DNA of the *Salmonella enterica* serovar Braenderup H9812 strain restricted with *Xba*I was used as the DNA marker. The *mcr-8* probe was the one, which we previously reported (Wang et al., 2018).

Conjugation Assay

The horizontal transferability of *mcr-8* was examined using conjugation assay with *E. coli* J53 (azide-resistant) or *E. coli* EC600 (rifampicin-resistant) as the recipient strain. Considering colistin resistance spontaneous mutants might be confused with colistin transconjugants, the conjugation assay with *E. coli* J53 were performed twice, first was selected on LB agar plates containing 4 μg/ml colistin and 100 μg/ml azide, second was selected on 16 μg/ml amoxicillin and 100 μg/ml azide LB agar plates. In parallel, QDRO1 and QDRO2, and recipient strains J53 were plated on conjugation plates as control. Transconjugants were confirmed by PCR targeting the *mcr-8* and β-lactamase genes, $bla_{\mathrm{TEM-1B}}$ and $bla_{\mathrm{OXA-1}}$ in QDRO1 and QDRO2 transconjugants, respectively, as well as *Xba*I enzyme digested pulsed field gel electrophoresis (PFGE). For analysis of the transfer ability of *mcr-8* in the same genus, we further performed conjugation assay using the above identified QDRO1 and QDRO2 transconjugants (T-QDRO1 and T-QDRO2) as donor strains and *E. coli* EC600 as recipient strain. The transfer frequency was calculated as the number of transconjugants per recipient as previous reported (Zhao et al., 2017).

Antimicrobial Susceptibility Test

The MICs of wild strains and transconjugants to antimicrobial agents (listed in **Table 1**) were determined by broth microdilution method, and the results were interpreted according to CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST). The *E. coli* ATCC 25922 was used as a quality control strain.

Genome Sequencing and Analysis of Antibiotic Resistance Genes

Genomic DNA of the isolates were extracted using the Wizard Genomic DNA Purification kit (Promega), then subjected to WGS on the Illumina HiSeq 2500 platform according to the manufacturer's protocols, which produced 150-bp paired-end reads. For each isolate analyzed by WGS, at least 100-fold coverage of raw reads was collected. The draft genomes were assembled using CLC Genomics Workbench 9.0 (CLC Bio, Aarhus, Denmark). Reference sequences of antibiotic resistance genes were from database ARG-ANNOT (de Man and Limbago, 2016).

RESULTS AND DISCUSSION

Presence and Location of *mcr-8* in *Raoultella spp*

A total of 15 *Raoultella spp* strains obtained from 300 chicken cloaca samples, among which 12 *R. ornithinolytica*, 2 *R. planticola*, and 1 *R. terrigena*. PCR assays showed that two *R. ornithinolytica* strains, named QDRO1 and QDRO2, were positive for *mcr-8*, but no other *mcr* genes were identified in this 15 *Raoultella spp* strains. S1-PFGE and Southern blotting assay indicated that *mcr-8* were located on ∼90-kb and ∼200-kb plasmids in QDRO1 and QDRO2, respectively (**Figure 1**). These two *mcr-8*-carrying plasmids were named as pQDRO1 and pQDRO2, respectively.

Transferability of *mcr-8* Gene

Conjugation assays showed that the pQDRO1 and pQDRO2 plasmids were transferable from *R. ornithinolytica* to recipient *E. coli* strains. The transfer frequencies of the pQDRO1 and pQDRO2 plasmids to *E. coli* J53 were $2.28 \pm 1.64 \times 10^{-8}$ and $1.71 \pm 1.01 \times 10^{-8}$, respectively. Meanwhile, transconjugants from amoxicillin and azide plates were resistant to colistin and *mcr-8* positive. These suggested that *mcr-8* was co-transferred with β-lactamase genes. As expected, donor strains QDRO1 and QDRO2, and recipient J53 did not grow on colistin and azide plates, or amoxicillin and azide plates. To determine whether the adaptation of *mcr-8*-carrying plasmids in *E. coli* could affect their transfer frequencies, we further performed the conjugation assays using the transconjugants as donor strains and *E. coli* EC600 as recipient strain. We found that the transfer frequencies

TABLE 1 | The minimum inhibitory concentrations of tested antimicrobial agents for the studied bacterial isolates.

Bacterial isolate[1]	MICs (μg/ml)[2]									
	CST	PB	AMC	AZT	CAZ	GEN	TET	FFC	CHL	CIP
R. ornithinolytica QDRO1	**16**	**8**	**128/64**	**2**	**64**	**>512**	**>256**	**>256**	**>256**	**128**
T-QDRO1	**16**	**4**	**32/16**	**2**	**16**	0.25	0.5	4	4	0.004
R. ornithinolytica QDRO2	**8**	**4**	**128/64**	**8**	**32**	**>512**	**>256**	**>256**	**>256**	**128**
T-QDRO2	**8**	**4**	**32/16**	**4**	**16**	0.25	0.5	4	4	0.004
R. ornithinolytica QDRO3	**4**	**4**	**128/64**	**2**	**64**	**>512**	**>256**	**>256**	**>256**	**16**
R. ornithinolytica QDRO4	**8**	**8**	**64/32**	**2**	**32**	**>512**	**>256**	**>256**	**>256**	**128**
R. ornithinolytica QDRO5	**16**	**8**	**128/64**	**4**	**32**	**>512**	**>256**	**>256**	**>256**	**64**
R. ornithinolytica QDRO6	**8**	**8**	**64/32**	**4**	**32**	**>512**	**>256**	**>256**	**>256**	**32**
R. ornithinolytica QDRO7	**8**	**8**	**64/32**	**4**	**16**	**>512**	**>256**	**>256**	**>256**	0.008
R. ornithinolytica QDRO8	**16**	**16**	**128/64**	**4**	**32**	**>512**	**>256**	**>256**	**>256**	**64**
R. ornithinolytica QDRO9	**32**	**16**	**128/64**	**4**	**32**	**>512**	**>256**	**>256**	**>256**	**64**
R. ornithinolytica QDRO10	**64**	**32**	**128/64**	**4**	**64**	**>512**	**>256**	**>256**	**>256**	**128**
R. ornithinolytica QDRO11	**4**	**4**	**64/32**	**2**	**32**	**>512**	**>256**	**>256**	**>256**	**64**
R. ornithinolytica QDRO12	**8**	**8**	**128/64**	**4**	**32**	**>512**	**>256**	**>256**	**>256**	**128**
R. planticola QDRP1	**8**	**8**	**64/32**	**4**	**8**	**>512**	**>256**	**>256**	**>256**	**16**
R. planticola QDRP2	**4**	**4**	**64/32**	**4**	**16**	**>512**	**>256**	**>256**	**>256**	**8**
R. terrigena QDRT1	**2**	**2**	**32/16**	**4**	**8**	**>512**	**>256**	**>256**	**>256**	0.016

[1] T-QDRO1 and T-QDRO2 represent the transconjugations of R.ornithinolytica QDRO1 and R.ornithinolytica QDRO2. [2] Antimicrobial agents are abbreviated as follows: CST, colistin; PB, polymyxin B; AMC, amoxicillin-clavulanate; AZT, aztreonam; CAZ, ceftazidime; GEN, gentamycin; TET, tetracycline; FFC, florfenicol; CHL, chloramphenicol; CIP, ciprofloxacin. The bold numbers mean the isolates are resistant to the tested antimicrobial agent.

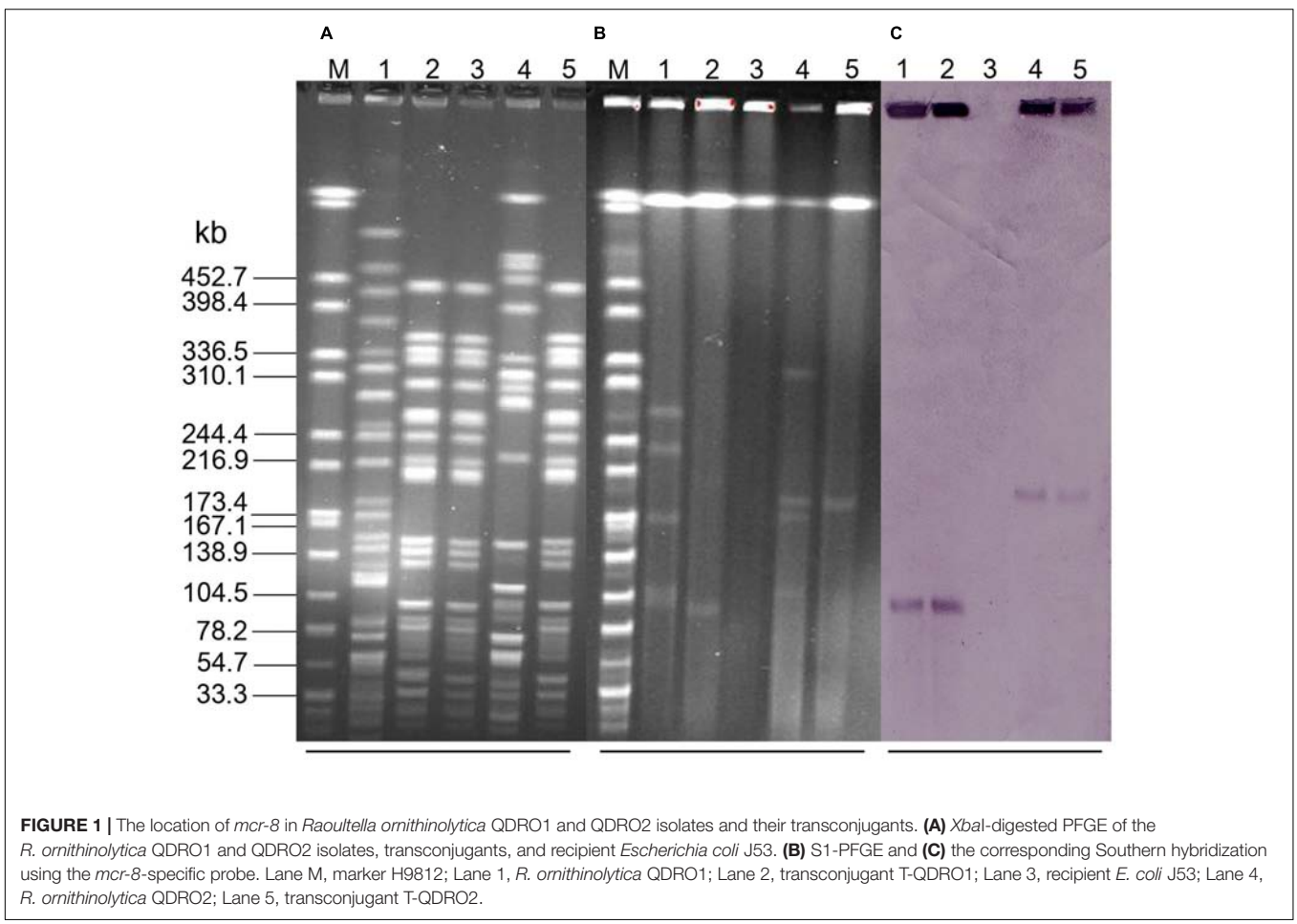

FIGURE 1 | The location of *mcr-8* in *Raoultella ornithinolytica* QDRO1 and QDRO2 isolates and their transconjugants. **(A)** *Xba*I-digested PFGE of the *R. ornithinolytica* QDRO1 and QDRO2 isolates, transconjugants, and recipient *Escherichia coli* J53. **(B)** S1-PFGE and **(C)** the corresponding Southern hybridization using the *mcr-8*-specific probe. Lane M, marker H9812; Lane 1, *R. ornithinolytica* QDRO1; Lane 2, transconjugant T-QDRO1; Lane 3, recipient *E. coli* J53; Lane 4, *R. ornithinolytica* QDRO2; Lane 5, transconjugant T-QDRO2.

of the pQDRO1 and pQDRO2 plasmids increased 10^3 and 10^4 folds, respectively, compared with the transfer frequencies of plasmids from *R. ornithinolytica* QDRO1 and QDRO2 to *E. coli*, respectively. To determine if the transfer frequencies of plasmids could be affected by the recipient bacteria, we performed the conjugation assays using the parental strains *R. ornithinolytica* QDRO1 and QDRO2 as donors and *E. coli* EC600 as recipients. The transfer frequencies of the pQDRO1 and pQDRO2 plasmids from *R. ornithinolytica* to *E. coli* EC600 were $4.17 \pm 1.35 \times 10^{-7}$ and $3.09 \pm 1.29 \times 10^{-7}$, respectively. We further performed the conjugation assays using the obtained transconjugants as donor strains and *E. coli* J53 as recipient strain. The transfer frequencies of pQDRO1 and pQDRO2 were $2.74 \pm 1.31 \times 10^{-4}$ and $3.71 \pm 1.98 \times 10^{-4}$, respectively. Similar to the previous results, increased transfer frequencies were observed for the pQDRO1 and pQDRO2 plasmids once they adapted to the *E. coli* host. These findings demonstrated that *mcr-8* gene is able to transfer between different bacterial species, which may further promote the dissemination of drug resistance.

Antimicrobial Susceptibility

Antimicrobial susceptibility test showed that this 15 *Raoultella spp* strains were all resistant to colistin, polymyxin B, amoxicillin-clavulanate, aztreonam, ceftazidime, tetracycline, florfenicol, chloramphenicol, and only *R. ornithinolytica* QDRO7 and *R. terrigena* QDRT1 were sensitivity to ciprofloxacin (**Table 1**). Both transconjugants were not only resistant to colistin and polymyxin B, but also resistant to β-lactam antibiotics, such as amoxicillin-clavulanate, aztreonam and ceftazidime, which implied that β-lactamase producing genes might be co-transferred with *mcr-8*.

Whole Genome Sequencing Analysis

WGS analysis showed that a 16.5-kb contig (GenBank: QWIX00000000) of *R. ornithinolytica* QDRO1 carrying *mcr-8* showed 100% query coverage and 99% identity to the corresponding segment of the *mcr-8*-carrying plasmid pKP91 from *K. pneumoniae* (Genbank number: MG736312) by Blastin in the NCBI database. A *mcr-8* variant, termed *mcr-8.4* (Genbank number: MH791448), was found in this 16.5-kb contig. Compared with *mcr-8*, *mcr-8.4* gene carried an A1209C transversion, which resulted in Serine to Arginine substitution. Similarly, the 25.5-kb *mcr-8*-carrying contig (Genbank number: MK097469) of *R. ornithinolytica* QDRO2 showed 83% query coverage and 99% identity to the corresponding segment of the *mcr-8*-carrying plasmid pKP91 from *K. pneumoniae*. Genetic structure analysis of the two *mcr-8*-carrying contigs showed that two copies of ΔIS*903B* located upstream and downstream of *mcr-8.4* in *R. ornithinolytica* QDRO1, while, only one copy of ΔIS*903B* located upstream of *mcr-8* in *R. ornithinolytica* QDRO2 (**Supplementary Figure S1**). Plasmid replicon type was carried out using the Center for Genomic Epidemiology[1], and showed that *R. ornithinolytica* QDRO1 contained IncHI2, IncA/C2, IncX3, and IncFII-type plasmids, and *R. ornithinolytica* QDRO2 contained IncHI2, IncFIB, IncHI1B, and IncFII-type plasmids. To further identify the replicon type of plasmids pQDRO1 and pQDRO2, we detected the replicon genes, which found in wild strains, in transconjugants of *R. ornithinolytica* QDRO1 and QDRO2 using primers listed in **Supplementary Table S1**. Results showed that the plasmids pQDRO1 and pQDRO2 both belong to IncFII-type, which is same with plasmid pKP91.

Analysis of the whole genome sequences of QDRO1 and QDRO2 isolates showed that these two strains contained multiple resistance genes (**Table 1**). As shown, except *mcr-8.4*, *R. ornithinolytica* QDRO1 also contained *aadA1*, *aph(3′)-Ia*, *strA*, *strB*, *aac(6′)-Ib*, and *armA*, *fosA*, *mph(E)*, *floR*, *cml*, *qnrB4*, *sul*, *tet(B)*, *tet(34)*, $bla_{\mathrm{TEM-1B}}$, $bla_{\mathrm{OXA-1}}$, $bla_{\mathrm{DHA-1}}$. Similarly, except *mcr-8*, *R. ornithinolytica* QDRO2 contained *aac(3)-IVa*, *aph(4)-Ia*, *aadA2*, *fosA, mph*(A), *mph*(E), *cat*, *floR*, *cml*, *QnrS4*, *oqxAB, QnrB52*, *sul1*, *sul2* and *sul3, tet*(A), *tet*(34), *tet*(O), *tet*(B), $bla_{\mathrm{TEM-1B}}$, $bla_{\mathrm{OXA-1}}$, $bla_{\mathrm{SHV-73}}$.

Our above antimicrobial susceptibility assay suggests that β-lactamase genes might be co-transferred with *mcr-8*. In order to determine the co-transfer of these genes, PCR amplification was performed to detect the presence of β-lactamase genes in transconjugants using primers listed in **Supplementary Table S1**. $bla_{\mathrm{TEM-1B}}$ and $bla_{\mathrm{DHA-1}}$ were detected in QDRO1 transconjugant, while $bla_{\mathrm{OXA-1}}$ and $bla_{\mathrm{SHV-73}}$ were present in QDRO2 transconjugant. These findings indicated that $bla_{\mathrm{TEM-1B}}$, $bla_{\mathrm{DHA-1}}$, $bla_{\mathrm{OXA-1}}$, and $bla_{\mathrm{SHV-73}}$ could co-transfer with *mcr-8*.

CONCLUSION

This study identified colistin resistance genes *mcr-8* and its variant, *mcr-8.4*, in *R. ornithinolytica*. The two *mcr-8*-carrying IncFII-type plasmids could be transferred to *E. coli* by conjugation. In addition, the transferability of the two plasmids were enhanced once they entered into *E. coli* hosts, which might further accelerate the dissemination of *mcr-8* among *Enterobacteriaceae*. It is worth noting that the co-transferability of *mcr-8* with several β-lactamase genes may further facilitate the dissemination of *mcr-8* among Enterobacteriaceae.

AUTHOR CONTRIBUTIONS

ZS, SZ, and YaoW conceived and designed the experiments. XW, YaoW, YZ, and ZW performed the experiments. ZS and XW analyzed the data. XW, YanW, and ZS wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00228/full#supplementary-material

[1] http://genomicepidemiology.org/

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ORIGINAL RESEARCH
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Extended Spectrum Beta-Lactamase-Producing Gram-Negative Bacteria Recovered From an Amazonian Lake Near the City of Belém, Brazil

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Aquatic systems have been described as antibiotic resistance reservoirs, where water may act as a vehicle for the spread of resistant bacteria and resistance genes. We evaluated the occurrence and diversity of third generation cephalosporin-resistant gram-negative bacteria in a lake in the Amazonia region. This water is used for human activities, including consumption after appropriate treatment. Eighteen samples were obtained from six sites in October 2014. Water quality parameters were generally within the legislation limits. Thirty-three bacterial isolates were identified as *Escherichia* (n = 7 isolates), *Acinetobacter*, *Enterobacter,* and *Klebsiella* (n = 5 each), *Pseudomonas* (n = 4), *Shigella* (n = 3), and *Chromobacterium*, *Citrobacter*, *Leclercia*, *Phytobacter* (1 isolate each). Twenty nine out of 33 isolates (88%) were resistant to most beta-lactams, except carbapenems, and 88% (n = 29) were resistant to antibiotics included in at least three different classes. Among the beta-lactamase genes inspected, the bla_{CTX-M} was the most prevalent (n = 12 positive isolates), followed by bla_{TEM} (n = 5) and bla_{SHV} (n = 4). $bla_{CTX-M-15}$ (n = 5), $bla_{CTX-M-14}$ (n = 1) and $bla_{CTX-M-2}$ (n = 1) variants were detected in conserved genomic contexts: $bla_{CTX-M-15}$ flanked by IS*Ecp1* and Orf477; $bla_{CTX-M-14}$ flanked by IS*Ecp1* and IS*903*; and $bla_{CTX-M-2}$ associated to an ISCR element. For 4 strains the transfer of bla_{CTX-M} was confirmed by conjugation assays. Compared with the recipient, the transconjugants showed more than 500-fold increases in the MICs of cefotaxime and 16 to 32-fold increases in the MICs of ceftazidime. Two isolates (*Escherichia coli* APC43A and *Acinetobacter baumannii* APC25) were selected for whole genome analysis. APC43A was predicted as a *E. coli* pathogen of the high-risk clone ST471 and serotype O154:H18. $bla_{CTX-M-15}$ as well as determinants related to efflux of antibiotics, were noted in APC43A genome. *A. baumannii* APC25 was susceptible to carbapenems and antibiotic resistance genes detected in its genome were intrinsic determinants (e.g., $bla_{OXA-208}$ and $bla_{ADC-like}$). The strain was not predicted as a human pathogen and belongs to a new sequence type. Operons related

to metal resistance were predicted in both genomes as well as pathogenicity and resistance islands. Results suggest a high dissemination of ESBL-producing bacteria in Lake Água Preta which, although not presenting characteristics of a strongly impacted environment, contains multi-drug resistant pathogenic strains.

Keywords: antibiotic resistance, *Escherichia coli*, *Acinetobacter baumannii*, bla_{CTX-M}, whole genome analysis

INTRODUCTION

Bacterial resistance to antibiotics is currently one of the most serious public health concerns. The environment and particularly aquatic systems have been pointed as important reservoirs of resistance (Baquero et al., 2008; Taylor et al., 2011; Marti et al., 2014). These settings bring together indigenous bacterial communities and bacteria resulting from anthropogenic contamination, creating a milieu that may promote horizontal gene transfer (Pei and Gunsch, 2009; Jiao et al., 2017). Furthermore, significant quantities of contaminants accumulate in polluted aquatic systems and some of these contaminants were implicated in the selection of resistant bacteria (e.g., antibiotics, metals, disinfectants) (Henriques et al., 2016; Jiao et al., 2017). The environment was also confirmed as the origin of some of the most successfully widespread antibiotic resistance genes (e.g., bla_{CTX-M} and bla_{OXA-48}; Poirel et al., 2002; Tacão et al., 2018). These evidences urgently ask to better understand the ecology of antibiotic resistance and the factors involved in resistance selection in aquatic systems. Dissemination of antibiotic resistance in these systems is particularly relevant when water is used for purposes that facilitate the transmission of bacteria to humans, namely for consumption, irrigation, recreational activities and fishing. Increasing our understanding of antibiotic resistance in specific aquatic systems is essential to suggest and implement mitigation strategies.

Nowadays, the spread of resistance to third generation cephalosporins in gram-negative bacteria is one of the major concerns in terms of antibiotic resistance. These antibiotics have great human health importance being often the first choice for the treatment of infectious diseases caused by gram-negative bacteria. Nevertheless, the levels of resistance to third generation cephalosporins have been increasing, and in several countries have reached levels that threaten their usefulness (WHO, 2014; ECDC, 2017). The most common and successful mechanism of resistance is the production of extended-spectrum beta-lactamases. According to a recent World Health Organization report, ESBL-producing Enterobacteriaceae are a critical human health concern (WHO, 2014). ESBLs can be classified into Ambler's classes A (e.g., TEM, SHV, CTX-M, PER, VEB, GES) and D (OXA) (Ambler, 1980). Among these, enzymes of the CTX-M family are currently globally disseminated, often found in pathogenic bacteria of the family Enterobacteriaceae, and associated with mobile genetic elements (Bevan et al., 2017). In Brazil, CTX-M-producing bacteria have been frequently reported in hospital settings, with the most common variants being CTX-M-15 and CTX-M-2 (Rocha et al., 2016).

The problematic summarized above demands from the authorities measures to contain the spread of resistance to antibiotics. Aquatic environments may be one of the most important intervention areas. The occurrence of ESBL genes, including bla_{CTX-M}, in different aquatic systems has been reported in several countries (Tacão et al., 2012; Zurfluh et al., 2013; Alves et al., 2014; Nascimento et al., 2017). In Brazilian aquatic systems, clinically relevant bacteria producing CTX-M enzymes have been recently described, e.g., in lakes (Nascimento et al., 2017), rivers (de Oliveira et al., 2017), wastewater (Dropa et al., 2016) and coastal water (Sellera et al., 2017). For the measures to be effective further studies are required to reveal which bacteria and which resistance and transfer mechanisms are present in these settings. There is a need to address different geographic areas, particularly ecologically relevant aquatic systems whose water is used for human activities.

In this work, we collected samples in an Amazonian lake. Water from this lake is used for water supply, irrigation and recreational activities (Santos et al., 2015). Gram-negative bacteria resistant to antibiotics were selected and mechanisms of resistance were characterized. The occurrence of genetic platforms that may contribute to multi-drug resistance in these bacteria (i.e., integrons) was also assessed. Two isolates belonging to species of public health interest (i.e., *Escherichia coli* and *Acinetobacter baumanii*) were selected for whole genome sequencing and analysis.

MATERIALS AND METHODS

Sampling and Sample Analysis

Lake Água Preta (1°25′7.849″S, 48°26′19.02″W) is an Amazonian mesotrophic lake located in the Utinga State Park, Pará, Brazil. It is located near a densely populated area that includes the city of Belém (population of approximately 1.5 million). This lake was chosen considering its importance in water supply, irrigation and recreational activities. It has great ecological relevance in the Amazonian area (Santos et al., 2015). The lake has a surface area of approximately 7 km^2 and a maximum depth of 8.5 m. There are no relevant agricultural or livestock activities on the banks of the lake. There is, however, a record of untreated wastewater discharges resulting from a large number of illegal homes in the vicinity of the lake. Six sampling points were selected (**Figure 1**). One liter of water was collected in triplicate at each sampling point in October 2014. Samples were collected in 1 L polypropylene flasks, packed in an isothermal box with ice, and sent to the Faculty of Sanitary and Environmental Engineering laboratory, Federal University of Pará, Brazil. Water samples collected for microbiological analysis were stored in previously sterilized polypropylene flasks

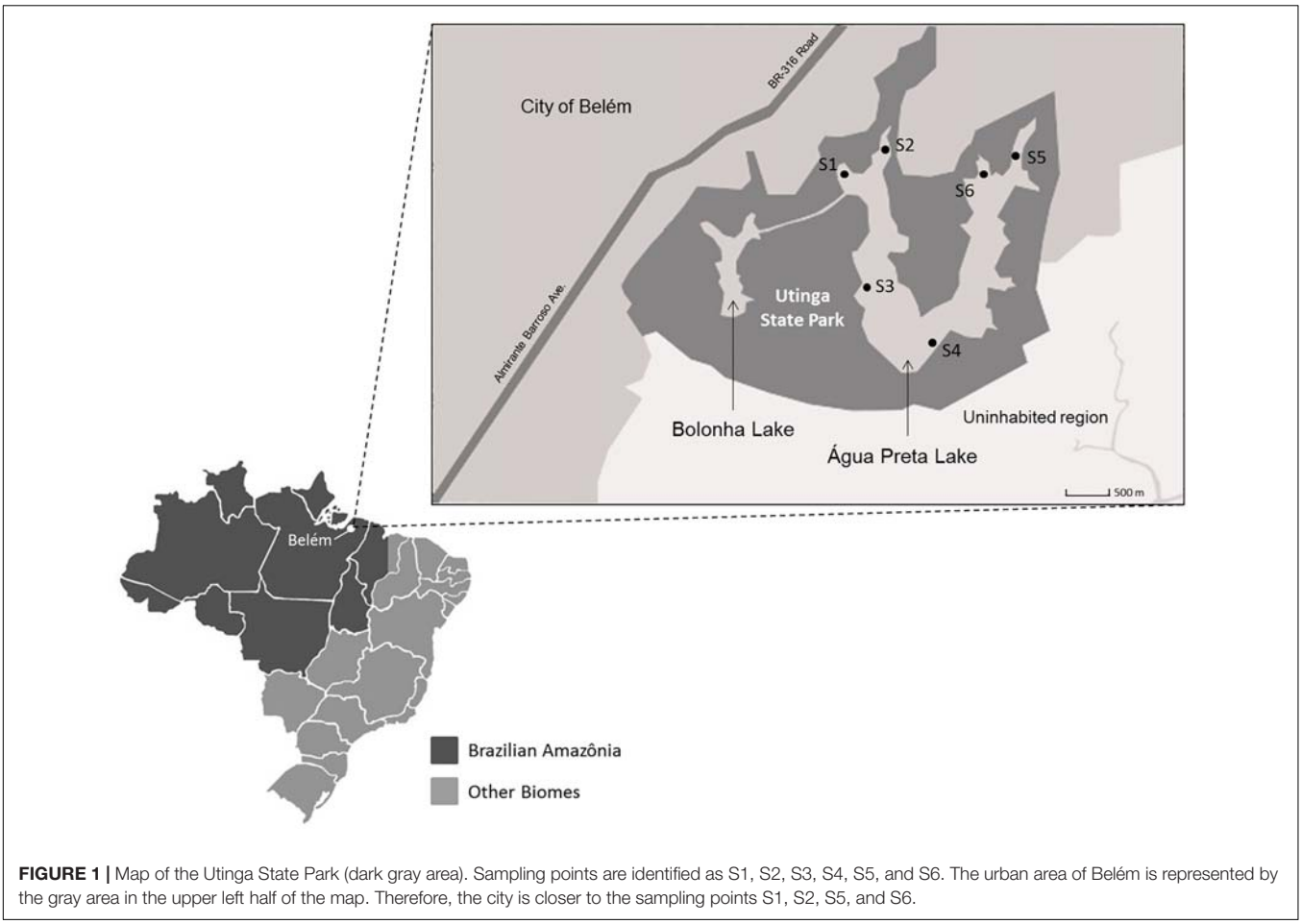

FIGURE 1 | Map of the Utinga State Park (dark gray area). Sampling points are identified as S1, S2, S3, S4, S5, and S6. The urban area of Belém is represented by the gray area in the upper left half of the map. Therefore, the city is closer to the sampling points S1, S2, S5, and S6.

of 250 mL. Sampling and analytical methods were performed according to the procedures and recommendations described in Standards Methods for the Examination of Water and Wastewater (Rice et al., 2012). Physico-chemical parameters such as pH, conductivity, temperature, dissolved oxygen and salinity were analyzed at the sampling points by potentiometry using a multi-parametric probe (556 MPS; YSI, United States). The following parameters were determined by UV spectrophotometry (UV DR 2800; HACH, Germany): turbidity, total solids, true color, apparent color, total phosphorous, total nitrogen, total iron, chemical oxygen demand (COD), and the concentration of the ions nitrite, nitrate, ammonia, chloride, aluminum, manganese, nickel, cadmium, copper, zinc and sulfate. Biochemical oxygen demand (BOD) was determined using a manometric respirometric test in the equipment BODTrack II (HACH, United States). The Most Probable Number (MPN) of total coliforms and *E. coli* was determined using the chromogenic substrate Colilert 18/QUANTI-TRAY (IDEXX Laboratories, United States) according to the manufacturers' protocol. Odor intensity was measured using sensorial panel, while alkalinity and acidity were determined by titrimetry.

Results were evaluated according to the resolution no. 357/2005 of the Environment National Council of Brazil (CONAMA, 2005).

Bacteria Growth Conditions and Isolation

Water samples (1, 10, and 50 mL) were filtered through 0.45-μm-pore-size cellulose ester filters (Millipore). Membranes were placed onto MacConkey agar medium supplemented with cefotaxime (8 μg mL^{-1}) (Sigma-Aldrich) and incubated at 37°C for 16 h. Individual colonies were purified in the same medium and stored in 20% glycerol at –80°C.

DNA Extraction and Identification of the Isolates

For DNA extraction, the bacterial isolates were inoculated in Tryptic Soy Broth medium (Himedia) supplemented with cefotaxime (8 μg mL^{-1}) and cultivated at 37°C overnight with aeration. An aliquot of 5 ml of the culture was centrifuged at 6,000 *g* at 4°C for 10 min. The cell pellet was subjected to DNA extraction using the DNeasy Blood and Tissue kit (Qiagen), according to the manufacturer's protocol. The integrity of the DNA was visualized on 1% agarose gel. DNA was stored in TE buffer (Tris 10 mM, EDTA 1mM, pH 8.0) at –20°C.

To determine the phylogenetic affiliation of the isolates, the 16S rRNA gene was amplified using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR was carried

out in 50 μL reaction mixtures containing buffer 1×, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.2 pmol of each primer, 1 U of Taq DNA polymerase (Invitrogen) and 50–100 ng of DNA. Cycling conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension step of 72°C for 10 min. Amplicons were sequenced using the ABI 3730 DNA Analyzer platform (Thermo Fisher Scientific). Reverse and forward sequences were assembled with BioEdit v. 7.2.6.1 (Hall, 1999) and the consensus sequences (∼1.5 kb) were compared to the GenBank database using BLASTn[1].

Antibiotic Susceptibility Testing

To estimate the level of resistance of the isolates, the disk-diffusion method was used (Bauer et al., 1966). *E. coli* ATCC 25922 was used as quality control strain. Sixteen antibiotics were tested including amoxicillin (10 μg), amoxicillin + clavulanic acid (20–10 μg), ampicillin (10 μg), cephalotin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), imipenem (10 μg), aztreonam (30 μg), kanamycin (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), tetracycline (30 μg) and the combination of sulfamethoxazole + trimethoprim (25 μg). CLSI (2017) breakpoints were used to classify strains as susceptible, intermediate or resistant. Antibiotics were selected based on the CLSI guidelines, which specify the antibiotics that should be considered when characterizing Gram-negative non-fastidious organisms (e.g., Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas aeruginosa*). Minimal inhibitory concentrations (MIC) were determined for cefotaxime and ceftazidime, following CLSI guidelines.

PCR Amplification of Resistance Genes and Mobile Genetic Elements

Isolates were screened by PCR to determine the presence of genes conferring resistance to beta-lactams (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{IMP}, bla_{VIM}, bla_{KPC}). We also analyzed the isolates for the presence of genes encoding integrases of class 1 (*intI1*) and 2 (*intI2*). The PCR reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystem) using DNA purified as described above. PCR was carried out using buffer 1×, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.2 pmol of each primer and 1 U of Taq DNA polymerase (Invitrogen) with sufficient water for 25 μl of reaction. Primers used and PCR conditions were as previously described (Dallenne et al., 2010; Alves et al., 2014). The genomic context of bla_{CTX-M} was characterized by PCR-targeting IS*Ecp1*, IS*26*, orf477 and IS*903*, as previously described (Tacão et al., 2012). A negative and a positive control were included in each PCR experiment. The negative control differed from the reaction mixture by substituting DNA for the same volume of sterile dH_2O. The amplicons were visualized on 1% agarose gels using the 1 kb Plus DNA ladder (Invitrogen) to assist in the identification of the PCR products.

[1]http://www.ncbi.nlm.nih.gov/

Mating Assays

Mating assays were performed for bla_{CTX-M}-positive strains, as previously described (Moura et al., 2012). In short, donor strains and the rifampicin-resistant *E. coli* CV601 (recipient strain) were grown overnight in Luria–Bertani broth (LB) at 37°C, 180 rpm. Donor and recipient strains were mixed at a 1:1 ratio and centrifuged (5 min, 7,000 *g*) to precipitate cells. After discarding the supernatant, 1 mL of fresh LB was added and left overnight at 37°C, without shaking. Then, cells were centrifuged (5 min, 7,000 *g*) and resuspended in a 0.9% NaCl solution. Putative transconjugants were selected by plating 100 μL of this suspension in plate count agar (PCA) supplemented with rifampicin (100 μg/mL), and cefotaxime (8 μg/mL). To confirm the identity of the transconjugants we used BOX-PCR typing (Versalovic et al., 1994) and bla_{CTX-M} PCR amplification as described above.

Genome Sequencing, Assembly and Analysis

Two multi-drug resistant isolates were selected randomly to represent phylogenetic groups with high clinical relevance (i.e., *Acinetobacter baumanii* and *E. coli*) and their genome was sequenced. Genomic DNA, extracted as described in Section "DNA Extraction and Identification of the Isolates," and sequenced in the Ion Torrent Personal Genome Machine (Thermo Fisher Scientific) using chip 318 v.2 according to the manufacturer's protocol. The quality of the reads was visualized using FastQC[2]. The reads were trimmed, discarding bases with Phred values below 20, and filtered, discarding reads with less than 100 nucleotides. The reads were assembled in contigs using the software MIRA 4 (Chevreux et al., 2004). Redundant contigs were removed using the SeqMan Pro tool of the Lasergene software (DNASTAR). The sequenced genomes were submitted to the GenBank database under the accession numbers PKCA01000000 (*E. coli* APC43A) and PYSX01000000 (*A. baumannii* APC25).

The contigs were ordered in scaffolds with MAUVE (Darling et al., 2004). Automatic genome annotation was performed in RAST (Rapid Annotation using System Technology) (Aziz et al., 2008). The RAST SEED subsystems (Overbeek et al., 2014), CARD (Comprehensive Antibiotic Resistance Database) (McArthur et al., 2013) and Resfinder v.2.1 (Zankari et al., 2012) were used to search for resistance genes in the sequenced genomes.

An *in silico* analysis of Plasmid Multilocus Sequence Typing (MLST) was performed using the web tool pMLST v.1.8 (Larsen et al., 2012) available at the site of the Center for Genomic Epidemiology[3]. PlasmidFinder v.1.3 (Carattoli et al., 2014) was used for detection of plasmid sequences, PathogenFinder v.1.1 (Cosentino et al., 2013) was used to determine the strains' pathogenicity level, SerotypeFinder v.1.1 (Joensen et al., 2015) was used for serotyping, and VirulenceFinder v.1.5 (Joensen et al., 2014) was used to detect virulence determinants.

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[3]http://www.genomicepidemiology.org/

Pathogenicity Islands (PAIs) and Resistance Islands (RIs) were predicted using the software GIPSy v.1.1.2 (Soares et al., 2016). *E. coli* K-12 substr. MG1655 (NC_000913.3) and *Acinetobacter calcoaceticus* CA16 (NZ_CP020000.1) were used as reference strains. The nucleotide sequence of each PAI and RI were recovered using the genome browser Artemis v.14.0.0 (Rutherford et al., 2000). In order to determine the location of PAIs and RIs, we designed a circular map using BLASTn in the software BRIG (Blast Ring Image Generator) (Alikhan et al., 2011).

A phylogenomic approach was used to determine the isolates species affiliation. Genomes used for comparison were obtained from GenBank. Four phylogenetic markers: 16S rRNA, *rpoB*, *gyrB*, and *dnaJ* were used to calculate a distance matrix based on a BLASTn comparison all-against-all in the software Gegenees v.2.2.1 (Ågren et al., 2012).

RESULTS AND DISCUSSION

Water Quality and Characterization of Cultivable Antibiotic-Resistant Bacteria

The majority of physical, chemical and microbiological parameters were within the limits established by the Brazilian law for freshwater environments intended for human consumption after appropriate treatment (**Supplementary Table S1**). However, BOD in sampling points 1, 4, 5, and 6 was above the recommended values. Additionally, dissolved oxygen (DO) concentration was below the limit in all sampling points analyzed. These two results suggest high oxygen consumption by the microbial community in Lake Água Preta during the sampling period.

For this study, sampling was performed only in October, and seasonal variation was not assessed. The temperature in this geographic region, immediately below the equator, is high throughout the year, though there are significant differences in terms of rainfall. The decision to sample in the dry season (July to November) was due to logistics issues related to lake access. However, in future studies it would be interesting to evaluate seasonal factors that may affect water quality and antibiotic resistance in Lake Água Preta.

Thirty-three isolates were obtained in this study (**Table 1**). Isolates affiliated mostly to genus *Escherichia* (7 isolates), followed by genera *Acinetobacter*, *Enterobacter,* and *Klebsiella* (5 isolates each), *Pseudomonas* (4 isolates), *Shigella* (3 isolates), and *Chromobacterium*, *Citrobacter*, *Leclercia* and *Phytobacter* (1 isolate each).

Most isolates were classified as multi-drug resistant (29/33–88%), meaning resistant to at least three classes of antibiotics. All isolates showed resistance to penicillins such as amoxicillin, ampicillin or both (**Table 1**), and 79% were also resistant when the penicillin (amoxicillin) was combined with a beta-lactamase inhibitor (clavulanic acid). Twenty-one of the 33 isolates showed resistance to cefotaxime (63.6%) and six showed intermediate resistance (18.2%). Resistance to carbapenems was detected only in the *Chromobacterium* isolate (**Table 1**). This genus has been commonly isolated from aquatic ecosystems and presents intrinsic resistance to these last-resort antibiotics (Lima-Bittencourt et al., 2011). The importance of *Chromobacterium* as progenitor of KPC carbapenemases has been recently discussed (Gudeta et al., 2016). For non-beta-lactam antibiotics, high levels of resistance or intermediate resistance were observed against aminoglycosides (76% of resistant isolates), tetracycline (64%), ciprofloxacin (58%) and the combination trimethoprim/sulfamethoxazole (55%). These results are in accordance with previous studies, which reported high levels of multi-drug resistance among strains resistant to third generation cephalosporins (Tacão et al., 2014). The presence of multi-drug resistant bacteria in natural aquatic systems may result from several anthropogenic pressures (Taylor et al., 2011; Tacão et al., 2012). The values of BOD and DO within Lake Água Preta are consistent with an impacted environment. An important cause may be the disposal of untreated sewage, resulting from an increasing number of illegal houses constructed along the margins. As in other geographic locations (e.g., Alves et al., 2014), wild life may also contribute to antibiotic resistance spread in this region. Finally, the presence of sub-lethal concentrations of antibiotics in aquatic systems has been reported to select for antibiotic resistant bacteria. In Brazil, until recently, antibiotics were among the most consumed medical drugs, and sold without medical prescription (Mattos et al., 2017). This situation might have contributed to the contamination of aquatic systems. These systems have been reported to act as reservoirs and to promote the transfer of antibiotic resistance genes among bacteria, thus contributing to multi-drug resistance spread.

The most frequently detected beta-lactamase gene was $bla_{\mathrm{CTX-M}}$ (n = 12 positive isolates), followed by bla_{TEM} (n = 5) and bla_{SHV} (n = 4) (**Table 1**). As in our study, CTX-M is the most frequently reported ESBL worldwide (Tacão et al., 2012; Bevan et al., 2017). Carbapenemase genes bla_{IMP}, bla_{VIM}, and bla_{KPC} were not detected among the isolates. Of the 22 isolates resistant to third generation cephalosporins, the gene $bla_{\mathrm{CTX-M}}$ was not detected in 10. These isolates affiliated to the genera *Acinetobacter* (n = 3), *Pseudomonas* (n = 2), *Citrobacter* (n = 1), *Enterobacter* n = 1), *Phytobacter* (n = 1), *Chromobacterium* (n = 1) and *Klebsiella* (n = 1). The bla_{SHV} is known to be intrinsic to *Klebsiella pneumoniae* (Babini and Livermore, 2000). Although we have used two sets of primers targeting this gene, under the conditions tested it was not detected in two of the isolates that affiliated with this species, including isolate API34 which showed resistance to cefotaxime. This result may be related to primer-template mismatches or to the affiliation of these isolates to a different *Klebsiella* species. Resistance to cefotaxime in *Klebsiella* spp. may be related with overproduction of other chromosomal beta-lactamases (e.g., bla_{OXY}, bla_{LEN}, bla_{OKP}) due to mutations in the gene promoter regions (Hæggman et al., 2004). Overexpression of chromosomal beta-lactamases may also be the mechanism responsible for resistance to third-generation cephalosporins in isolates affiliated to other bacterial genera such as *Enterobacter*, *Citrobacter*, *Chromobacterium*, and *Pseudomonas* (intrinsic bla_{AmpC}; Jacoby, 2009), or *Acinetobacter* (e.g., bla_{ADC} genes; Zhong et al., 2008). The $bla_{\mathrm{CTX-M-15}}$

TABLE 1 | Characteristics of isolates obtained from Lake Água Preta.

Isolate	16S rRNA gene Affiliation	Resistance (and intermediate) phenotype	Resistance genotype	Integrase genes
APC43A	*Escherichia coli* NBRC 102203 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, NAL (KAN)[b]	$bla_{CTX-M-15}$[a]	–
APC25	*Acinetobacter baumannii* DSM 30007 (97%)	AML, AMC, AMP, CEF, CAZ, CTX, ATM, CIP, NAL (KAN)	–[a]	–
APC4	*Citrobacter werkmanii* CDC 0876-58 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, ATM, CIP, NAL, SXT, TET (KAN)	–	–
APC6	*Enterobacter sp.* A2 (99%)	AML, AMC, AMP, CEF, CAZ, ATM, CTX, CIP, GEN, KAN, NAL, TET	bla_{TEM}–bla_{SHV}	–
APC11	*Pseudomonas putida* F1 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, ATM, CHL, NAL, SXT	bla_{CTX-M}	–
APC13	*Acinetobacter baumannii* DSM 30007 (97%)	AML, AMP, CEF, CAZ, FEP, ATM (CTX, NAL)	–	–
APC14	*Pseudomonas mosselii* CFML 90-83 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, TET (NAL)	–	–
APC15	*Chromobacterium haemolyticum* MDA0585 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, IPM, ATM, KAN, NAL	–	–
APC19	*Escherichia coli* O157:H7 Sakai (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, GEN, KAN, NAL, SXT, TET	bla_{CTX-M}	*intI*2
APC20	*Pseudomonas mosselii* CFML 90-83 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM (NAL)	–	–
APC22	*Shigella sonnei* Ss046 (99%)	AML, AMP, CEF, CTX, FEP, ATM, CIP, GEN, KAN, NAL, SXT (AMC)	$bla_{CTX-M-2}$	*intI*2
APC24B	*Escherichia fergusonii* ATCC 35469 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, KAN, NAL, SXT, TET	bla_{CTX-M}	*IntI*1
APC28	*Klebsiella pneumoniae* DSM 30104 (96%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, KAN, NAL, SXT, TET (C)	bla_{TEM-} bla_{SHV}-$bla_{CTX-M-15}$	*intI*1
APC32	*Escherichia fergusonii* ATCC 35469 (97%)	AML, AMP, CEF, CAZ, CTX, FEP, ATM, SXT (AMC, CIP, KAN)	bla_{TEM}-$bla_{CTX-M-15}$	–
APC33	*Shigella sonnei* Ss046 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, NAL	$bla_{CTX-M-15}$	–
APC34	*Escherichia coli* NBRC 102203 (98%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, KAN, NAL, SXT, TET	bla_{CTX-M}	*IntI*2
APC38	*Escherichia fergusonii* ATCC 35469 (98%)	AML, AMC, AMP, CEF, CTX, FEP, ATM, CIP, GEN, CHL, KAN, NAL, SXT, TET	bla_{CTX-M}	*IntI*2
APC39	*Acinetobacter nosocomialis* RUH2376 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, ATM, CIP, KAN, NAL, SXT, TET	–	–
APC40A	*Escherichia fergusonii* ATCC 35469 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, GEN, CHL, NAL, SXT (KAN)	$bla_{CTX-M-14}$	*intI*1-*intI*2
APC42	*Acinetobacter baumanii* DSM 30007 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, KAN, NAL, SXT, TET (CHL)		–
APC43B	*Shigella sonnei* Ss046 (99%)	AML, AMC, AMP, CEF, CAZ, CTX, FEP, ATM, CIP, GEN, KAN, NAL, SXT, TET (CHL)	$bla_{CTX-M-15}$	–
API2	*Klebsiella pneumoniae* R-70 (99%)	AML, ATM, C, KAN, SXT, TET (AMP, CIP)	–	*IntI*1
API3	*Enterobacter asburiae* JCM6051 (94%)	AML, AMC, AMP, CEF, CAZ, ATM, CHL, KAN, NAL, TET	–	–
API4	*Klebsiella pneumoniae* 07A044 (99%)	AML, AMC, AMP, CEF, ATM, SXT, TET (CTX, NAL)	bla_{SHV}	–
API6	*Leclercia adecarboxylata* CIP 82.92 (99%)	AML, AMC, AMP, CEF, ATM, CHL, KAN, NAL, TET (CTX)	–	–
API7	*Enterobacter cloacae* LMG 2683 (99%)	AML, AMC, AMP, CEF (CTX, ATM)	–	–
API10	*Enterobacter tabaci* YIM Hb-3 (99%)	AML, AMC, AMP, CEF, ATM, CHL, KAN, NAL (SXT, TET)	–	–
API12	*Pseudomonas otitidis* MCC10330 (99%)	AML, AMC, AMP, CEF, ATM, KAN, TET (NAL)	–	–
API16	*Enterobacter tabaci* YIM Hb-3 (100%)	AML, AMC, AMP, CEF, ATM, GEN, CHL, NAL, SXT, TET (CTX)	–	–
API20	*Acinetobacter seifertii* LUH 1472 (99%)	AML, AMC, AMP, CEF, ATM, CIP, KAN, TET (CAZ, CTX, GEN, CHL)	–	–
API24	*Phytobacter diazotrophicus* Ls8 (98%)	AMP, CAZ, CTX, ATM, KAN, NAL (AML, FEP, TET)	bla_{TEM}	–
API29	*Klebsiella pneumoniae* DSM 30104 (99%)	AML, AMP, CEF	bla_{TEM-} bla_{SHV}	–
API34	*Klebsiella pneumoniae* DSM 30104 (98%)	AML, AMP, CEF, CTX, FEP, ATM, CIP, GEN, CHL, NAL, SXT, TET	–	–

The BLASTn identity result for each isolate is presented within parentheses after the 16S rRNA affiliation. The abbreviation of antibiotics is as follows: amoxicillin (AML); amoxicillin + clavulanic acid (AMC); ampicillin (AMP); cephalotin (CEF); ceftazidime (CAZ); cefotaxime (CTX); aztreonam (ATM); cefepime (FEP); imipenem (IPM); kanamycin (KAN); gentamicin (GEN); nalidixic acid (NAL); ciprofloxacin (CIP); chloramphenicol (CHL); tetracycline (TET); sulfamethoxazole + trimethoprim (SXT). [a]*Isolates selected for whole genome analysis. The complete analysis of its resistance genotype is presented in main text and in* ***Table 2****.* [b]*Parentheses indicate intermediate susceptibility to the antibiotic.*

gene was found in 5 isolates (affiliated with genera *Klebsiella*, *Escherichia* and *Shigella*), the $bla_{CTX-M-14}$ gene was found in 1 isolate (affiliated with *Escherichia*), and the $bla_{CTX-M-2}$ gene was detected in only 1 isolate (affiliated with *Shigella*). These variants have previously been reported in Brazil in both clinics and environmental settings (Dropa et al., 2016; Rocha et al., 2016; Nascimento et al., 2017; Sellera et al., 2017). For the remaining bla_{CTX-M}-positive isolates, it was only possible to sequence a portion of the gene, insufficient to accurately determine its variant. For these isolates, PCR products were not obtained with the primers used to characterize the genomic context of bla_{CTX-M}. IS*Ecp1* was found in the upstream region of all $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ genes. Downstream, all $bla_{CTX-M-15}$ genes presented Orf477 and $bla_{CTX-M-14}$ presented the insertion sequence IS*903*. The same contexts were previously reported for these genes in clinical and environmental isolates worldwide (Eckert et al., 2006; Tacão et al., 2012). Particularly, the association of IS*Ecp1* element with ESBL genes seems to be one of the reasons for the successful spread of these genes, being a major concern in clinical settings (Tian et al., 2011). The genetic context of $bla_{CTX-M-2}$ carried by *Shigella* sp. APC22 was identical to that previously described (Eckert et al., 2006): an upstream region with a *sul1* gene (encoding resistance to sulfonamides) followed by an IS*CR1* element; and downstream an open reading frame designated Orf3, followed by *qacEdelta1* (encoding for a quaternary ammonium compound resistance protein) and a *sul1* gene. These CR-like elements are usually associated to complex class 1 integrons, usually identified between duplications of 3'conserved sequence (CS) regions, along with antibiotic resistance genes like $bla_{CTX-M-2}$ (Toleman et al., 2006).

Conjugations assays were performed for nine out of twelve bla_{CTX-M}-positive isolates. Three isolates were able to grow on rifampicin and were excluded from these experiments. Under the used conjugation conditions, 4 out of 9 donor strains generated transconjugants carrying bla_{CTX-M}. In contrast with the recipient strain *E. coli* CV601, all transconjugants showed MIC for cefotaxime from 32 to >256 μg/mL, while for ceftazidime MICs varied from 2 to 8 μg/mL (**Supplementary Table S2**). Overall, the association of bla_{CTX-M} genes to conjugative plasmids in these isolates was confirmed indicating that their mobilization to different hosts may be facilitated.

Previous studies highlighted the important contribution of integrons to multi-drug resistance profiles among ESBL-producers (Tacão et al., 2014). In this study, the integrase genes *intI*1 and *intI*2 were detected in 4 and 5 isolates, respectively (**Table 1**). All but one of these isolates were positive for the bla_{CTX-M} gene.

TABLE 2 | Major genomic features of two isolates from Lake Água Preta and resistance genes annotated by CARD and/or ResFinder.

***E. coli* APC43A**	**%GC**	**CDS**	**contigs**	**N50**	**RIs**	**PAIs**	**Size (bp)**	**MLST**	**Serotype**	**Plasmids**
	50.5	5923	195	3283348	5	25	5,035,455	ST471	O154:H18	IncX4 e IncFIA
Resistance Genes	**ARO category**							**Genes**	**Contig localization**	
	Antibiotic inactivation enzyme; beta-lactam resistance proteins							$bla_{CTX-M-15}$	22_18911	
								intrinsic bla_{AmpC}		
	Efflux pump conferring antibiotic resistance							*acrE*	15_8461	
								emrB	10_26638	
								mdtB	5_4183	
								mdtL	8_54031	
								msbA	11_125192	
								tolC	7_57761	
	Efflux pump conferring antibiotic resistance; antibiotic resistance gene cluster, cassette, or operon							*mdtE*	37_5848	
	Efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux							*acrS*	15_10016	
								cpxR	19_63902	
								emrR	10_24793	
								H-NS	20_22613	
***A. baumanii* APC25**	**%GC**	**CDS**	**contigs**	**N50**	**RIs**	**PAIs**	**Size (bp)**	**MLST**	**Serotype**	**Plasmids**
	39.0	5063	121	244684	10	11	4,860,843	–[a]	–[a]	–[b]
Resistance Genes	**ARO category**							**Genes**	**Contig localization**	
	Efflux pump complex or subunit conferring antibiotic resistance							*adeK*	11_73107	
	Antibiotic inactivation enzyme; beta-lactam resistance protein							$bla_{OXA-208}$	23_160179	
								$bla_{ADC-like}$	6_85724	

[a] *pMLST and SeroTypeFinder do not have support for this taxon.* [b] *PlasmidFinder did not detected any plasmid sequence.*

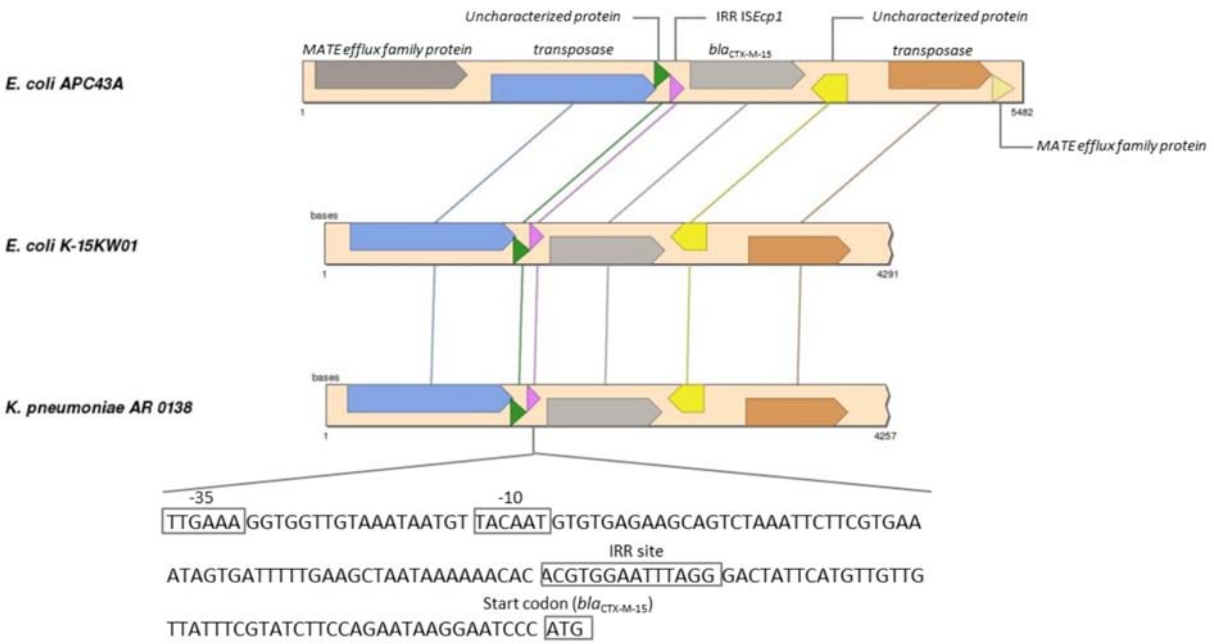

FIGURE 2 | Synteny analysis of IS*Ecp1* element carrying $bla_{\text{CTX-M-15}}$ gene in *Escherichia coli* APC43A. Conserved genes are connected by lines. The pink arrow represents the IS*Ecp1* element that is described in detail at the bottom of the figure. *E. coli* APC43A contains two CDSs identified as MATE efflux family proteins flanking the IS element. These CDSs are complementary and probably are part of the same gene that was fragmented during the insertion of the IS element.

Genomic Analysis of Two Multi-Drug Resistant Isolates

To obtain an in-depth characterization of the resistome of selected isolates, as well as insights into their mobilome and virulence potential, two isolates (i.e., *E. coli* APC43A and *Acinetobacter baumannii* APC25) were selected for whole genome sequencing. These isolates were randomly selected among isolates that: (1) belong to bacterial groups of public health concern, (2) presented multi-drug resistance profiles.

Identification at species level was confirmed using a phylogenomic approach as described in Material and Methods. Both strains were resistant to all beta-lactams except to imipenem (APC43A) or to imipenem and cefepime (APC25). Additionally, strains showed resistance to ciprofloxacin, nalidixic acid, and an intermediate susceptibility to kanamycin. Summary of both strains genomic features is presented in **Table 2**.

Escherichia coli APC43A Genomic Analysis

For *E. coli* APC43A the RAST server classified 162 CDSs in the subsystem of Virulence, Disease and Defense (3.2% of total genes) (**Supplementary Table S3**). Among them, 122 were genes related to antibiotic resistance and toxic compounds. Two beta-lactamase genes were predicted in the genome. As described above, $bla_{\text{CTX-M-15}}$ gene was located between IS*Ecp1* and orf477. Genomic analysis revealed that a transposase gene followed orf477 and that two fragments of a truncated gene encoding a MATE efflux family protein flanked this entire region (**Figure 2**). This region showed identity values higher than 99% and coverage higher than 93% with the genomes of *K. pneumoniae* AR 0138 (CP021757.1) and *E. coli* K-15KW01 (CP016358.1) (Zurfluh et al., 2016). In *E. coli* K-15KW01 the $bla_{\text{CTX-M-15}}$ gene was embedded at the right-hand extremity of an IS*Ecp1* element (**Figure 2**). In our strain APC43A, the inverted repeat sequence (IRR) (ACGTGGAATTTAGG), and the –35 (TTGAAA) and –10 (TACAAT) sites of the IS*Ecp1* element were conserved 48 base pairs upstream of the ATG start codon of $bla_{\text{CTX-M-15}}$ (**Figure 2**). The annotation of the other identified beta-lactamase gene was evaluated by comparing its nucleotide sequence to the Uniprot database through BLASTn. The gene showed high identity with an intrinsic AmpC beta-lactamase encoding gene (above 99%), emphasizing its correct annotation. Mutations previously related to enzyme overexpression (Jacoby, 2009) were not detected in the bla_{AmpC} gene promoter. Besides beta-lactamase genes, genes encoding resistance to other classes of antibiotics were detected in the genome of strain APC43A, mostly related with efflux pumps (**Table 2**).

Sequences representing two plasmids, assigned to the incompatibility groups IncX4 and IncFIA, were detected in the genome of *E. coli* APC43A (**Table 2**). The contig corresponding to replicon IncX4 has a size of 30,306 bp, which is very similar to the size of *E. coli* IncX4 plasmids in the GenBank database (e.g., accession number JX981514.1). This plasmid was detected in the porcine enterotoxigenic strain *E. coli* UMNF18 carrying genes for type II secretion system (Shepard et al., 2012). IncFIA is a fertility plasmid of *E. coli* and part of this plasmid was detected in a 9,933 bp contig. No resistance genes were found within plasmids.

PathogenFinder analysis showed that *E. coli* APC43A is a human pathogen and the SerotypeFinder tool classified this strain in the O154:H18 serotype. Six virulence factors (*gad*,

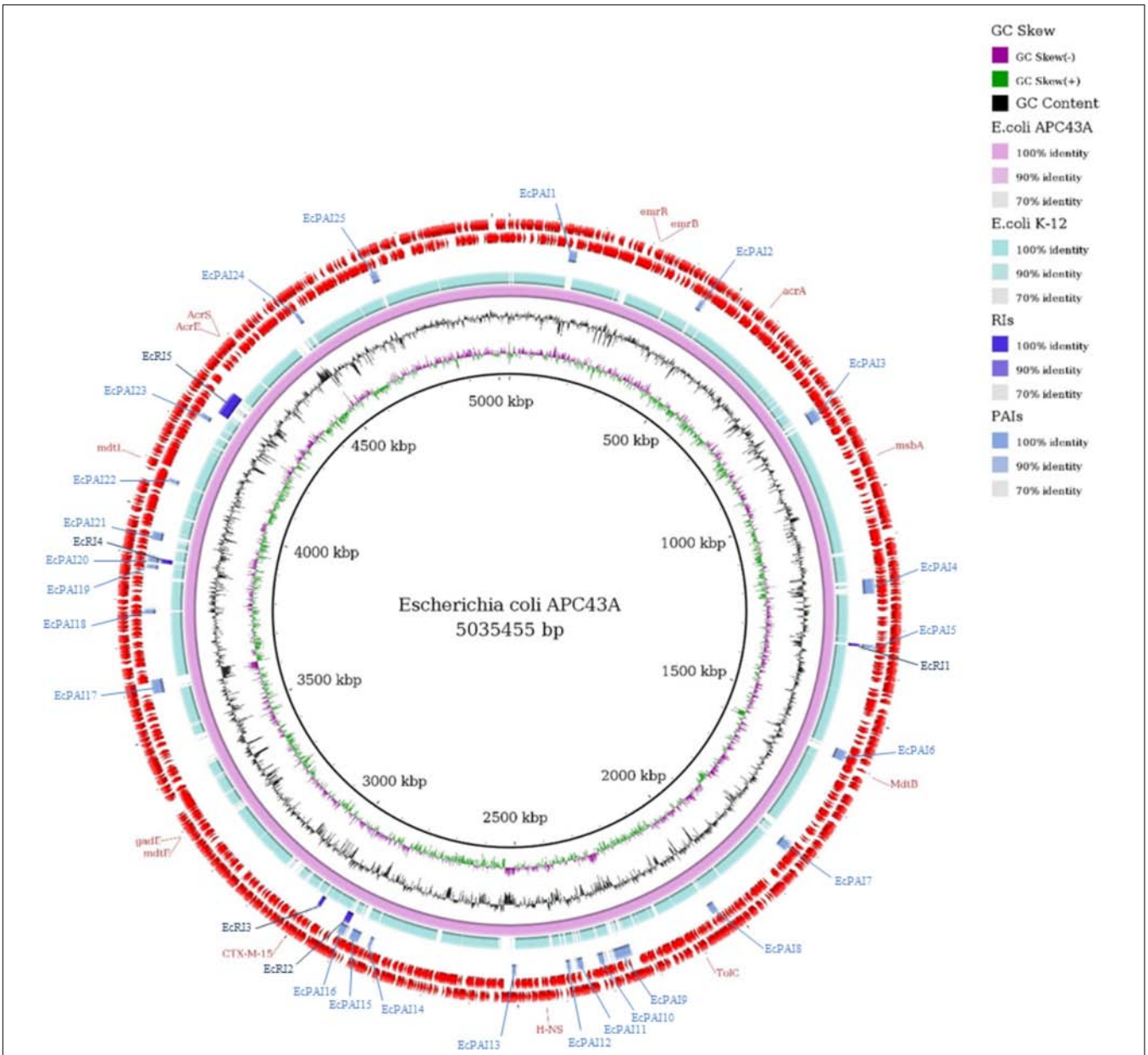

FIGURE 3 | Comparative genomic ring designed in Gegenees program. The innermost ring to the outermost is presented in this figure, as follows: GC skew and the GC content of *E. coli* APC43A; the genome sequence of *E. coli* APC43A and *E. coli* K-12; the Resistance Islands (RIs) detected by GIPSy; the Pathogenicity Islands (PAIs) detected by GIPSy; CDSs identified in the genome of *E. coli* APC43A. The location of the resistance genes detected by CARD and ResFinder are shown and identified by their respective names in red.

lpfA, *ltcA*, *astA*, *cba* e *cma*) normally found in pathogenic *E. coli* were detected in the genome of *E. coli* APC43A. These virulence genes are involved in host-pathogen interaction during gastrointestinal infections caused by ingestion of contaminated food or water (Joensen et al., 2014). The strain was assigned to ST471, a high-risk clone previously reported in clinical settings and commonly associated with ESBL genes and genes encoding carbapenemases (Kapmaz et al., 2016; Yi et al., 2017). In Brazil, this sequence type was described in clinical isolates from Rio de Janeiro (Peirano et al., 2011).

Acinetobacter baumannii APC25 Genomic Analysis

High levels of intrinsic resistance to a number of antibiotics have been reported for *A. baumanii*, seriously compromising the treatment of patients infected with this pathogen. Intrinsic resistance mechanisms in members of this species include the production of chromosomal beta-lactamases and aminoglycoside-modifying enzymes, expression of efflux pumps and permeability defects. Nevertheless, *A. baumanii* is also known for its ability to acquire genes encoding resistance determinants.

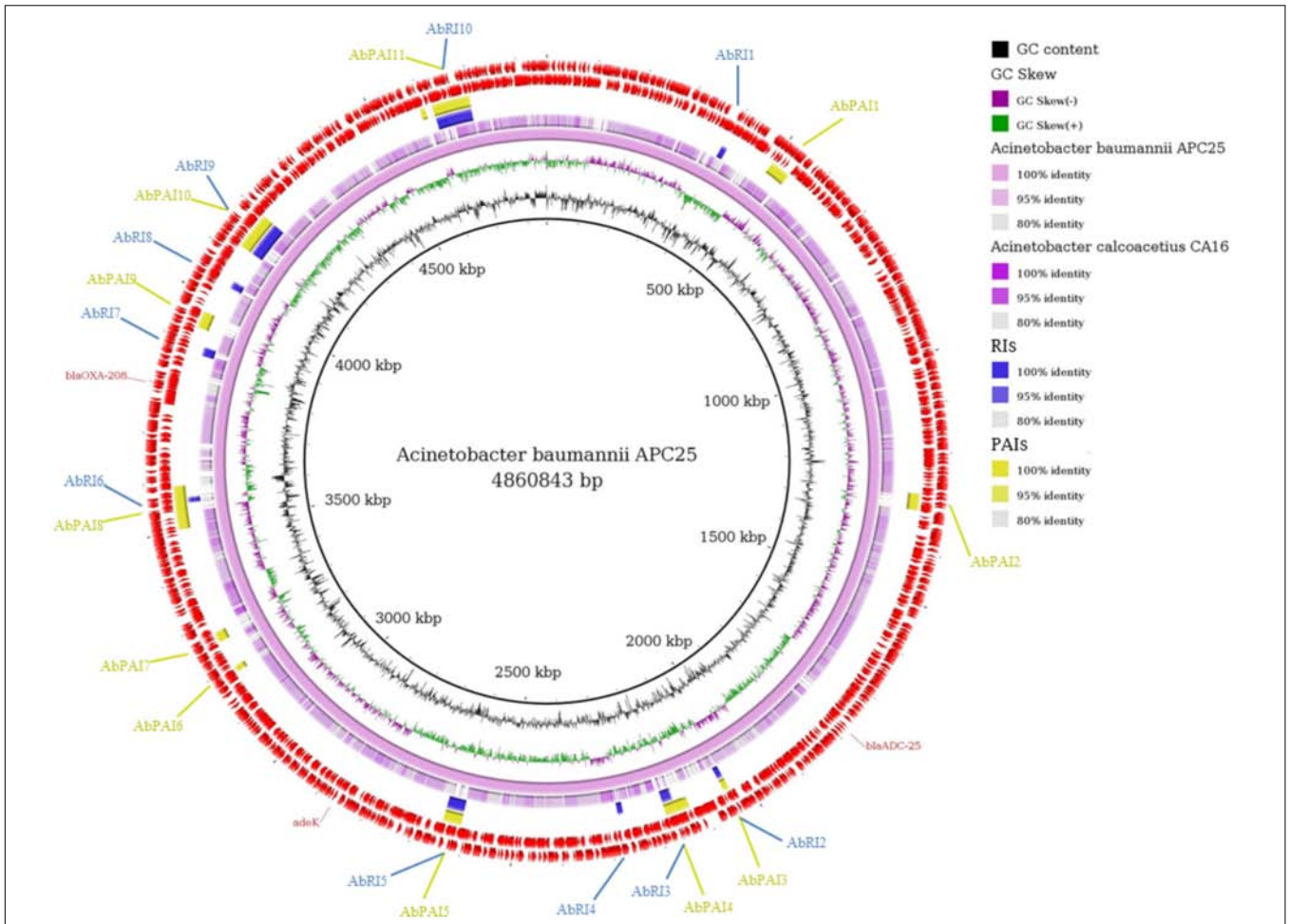

FIGURE 4 | Comparative genomic ring designed in Gegenees program. The innermost ring to the outermost is presented in this figure, as follows: GC skew and the GC content of *Acinetobacter baumannii* APC25; the genome sequence of *A. baumannii* APC25 and *A. calcoacetius* CA16; the Resistance Islands (RIs) detected by GIPSy; the Pathogenicity Islands (PAIs) detected by GIPSy; CDSs identified in the genome of *A. baumannii* APC25. The location of the resistance genes detected by CARD and ResFinder are shown and identified by their respective names in red.

For the genome of *A. baumannii* APC25 the RAST server classified 109 CDSs in the subsystem of Virulence, Disease and Defense (2.6% of total genes) (**Supplementary Table S4**). Eighty-three of these 109 CDSs are related to resistance to antibiotics and toxic compounds. The beta-lactamase genes $bla_{\mathrm{OXA-208}}$ and $bla_{\mathrm{ADC-like}}$ (98% similar to $bla_{\mathrm{ADC-25}}$) were detected (**Table 2**). Both genes were previously reported as intrinsic genetic determinants in the chromosome of *A. baumannii* (Zhao and Hu, 2012). $bla_{\mathrm{OXA-208}}$ encodes an OXA-51-like chromosomally encoded beta-lactamase (Evans and Amyes, 2014). Clinically relevant oxacillinases have been reported in clinical isolates from sixteen states in Brazil, mostly OXA-23 and OXA-143 (Medeiros and Lincopan, 2013). The $bla_{\mathrm{ADC-25}}$ encodes a cephalosporinase recently described to confer resistance to second and third generation cephalosporins (Zhong et al., 2008; Lee et al., 2012), a result that is in line with the antibiotic susceptibility profile of strain APC25.

Plasmids were not detected in *A. baumannii* APC25 and the isolate was not predicted as a human pathogen by the PathogenFinder tool (**Supplementary Table S5**). MLST sequences were uploaded to the *Acinetobacter*-MLST Pasteur database and since an unreported allele combination was observed, a new sequence type (ST1278) was assigned.

Resistance to Metals and Genomic Islands Prediction

Operons related to resistance to metals were determined in the sequenced strains. *E. coli* APC43A possesses incomplete mercury resistance operons (**Supplementary Figure S1**). In addition, the two-component system *cusR-cusS* and the efflux pump *cusCFBA*, described as responsible for copper and silver resistance in other strains of *E. coli* (Gudipaty and McEvoy, 2014), were annotated in the genome. In *A. baumannii* APC25, the zinc, cadmium, and cobalt resistance may be mediated by the operon *czcABC*, which was found duplicated in its genome (**Supplementary Figure S1**). Both genomes showed operons for resistance to arsenic. *A. baumannii* APC25 has an operon composed by an arsenical resistance-3 (ACR3) family protein, while *E. coli* APC43A has an *arsRBC* type operon

(**Supplementary Figure S1**). Several studies have showed that some pollutants such as metals could co-select for antibiotic resistance (Wright et al., 2008; Rosewarne et al., 2010; Henriques et al., 2016). However, the level of aluminum, manganese, nickel, cadmium, copper and zinc in Lake Água Preta was in accordance to the standard values for mesotrophic lakes (**Supplementary Table S1**).

Twenty-five PAIs and five RIs were identified in the genome of *E. coli* APC43A (**Figure 3**). The location of the islands is shown in the comparative ring of **Figure 4**. It is worth noting that these islands are almost completely absent in the genome of the non-pathogenic *E. coli* K-12 (**Figure 3**). Interestingly, among the detected resistance genes only the gene *mdtB* was within a GEI (EcPAI16), suggesting that these resistance islands may encode resistance to other classes of compounds. In some cases, the program identified PAIs and RIs in the same genome region, e.g., EcPAI5 and EcRI1, which means that these regions may encode both resistance and virulence factors.

The genome of *A. baumannii* APC25 has 11 PAIs and 10 RIs (**Figure 4**). The low number of PAIs is in accordance with the prediction of PathogenFinder that classified the isolate as a non-pathogenic strain. The majority of PAIs and RIs were found in the same location of the genome similar to that observed for *E. coli* (**Figure 4**). No resistance genes predicted by CARD or ResFinder were located within GEI.

CONCLUSION

Lake Água Preta is an Amazonian mesotrophic lake located near a densely populated area that presented physical, chemical and microbiological parameters in accordance to the Brazilian environmental laws, with some exceptions. The majority of bacterial strains (29 out of 31; 88%) isolated from the lake, in media supplemented with cefotaxime, were multi-drug resistant, classified in the Enterobacteriaceae family, and carried ESBL genes, primarily $bla_{\mathrm{CTX-M}}$. In some cases the transfer potential of these genes were confirmed in conjugation assays. These results suggest a high dissemination of ESBL genes in Gram-negative bacteria of Lake Água Preta, which although not presenting characteristics of a highly impacted environment, contains multi-drug resistant pathogenic strains such as *E. coli* APC43A (ST471).

AUTHOR CONTRIBUTIONS

AS, IH, AF, and RB conceived and designed the experiments. DF, SA, JA, and MT performed the experiments. DF, RR, and RB were involved in genome analysis. DF, SA, MT, RB, and IH prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00364/full#supplementary-material

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Evolution of Penicillin Non-susceptibility Among *Streptococcus pneumoniae* Isolates Recovered From Asymptomatic Carriage and Invasive Disease Over 25 years in Brazil, 1990–2014

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Streptococcus pneumoniae is a major cause of community-acquired pneumonia and meningitis, and it is also found as a commensal, colonizing the human upper respiratory tract of a portion of the human population. Its polysaccharide capsule allows the recognition of more than 90 capsular types and represents the target of the currently available pneumococcal conjugate vaccines (PCVs), such as the 10-valent (PCV10) and the 13-valent (PCV13). Penicillin non-susceptible pneumococci (PNSP) have been listed as one of the current major antimicrobial-resistant pathogen threats. In Brazil, the emergence of PNSP was initially detected in the mid 1990s and PCV10 has been part of the National Immunization Program since 2010. Here, we investigated the distribution of capsular types and penicillin susceptibility profiles of 783 pneumococcal strains isolated in Brazil between 1990 and 2014 to assess the evolution of penicillin non-susceptibility among pneumococci associated with asymptomatic carriage and invasive pneumococcal disease (IPD). The most common serotypes among carriage isolates were 19F, 6B, 6C, 23F, and 14. Among IPD isolates, the most frequent types were 14, 3, 6B, 5, 19F, and 4. We detected 21 types exclusively associated with IPD isolates, whereas non-typeable (NT) isolates were only detected in carriage. Nearly half of the isolates belonged to PCV10 serotypes, which remarkably decreased in occurrence (by nearly 50%) after PCV10 introduction (2011–2014), while non-PCV10 serotypes increased. PNSP frequency and levels were much higher among carriage isolates, but PNSP belonging to PCV10 serotypes were more common in IPD. While the occurrence of PNSP has decreased significantly among IPD isolates since 2011, it kept increasing

among carriage strains. Such a difference can be attributed to the serotypes that emerged in each clinical source after PCV10 usage. PNSP with multidrug resistance profiles that emerged within carriage isolates comprised mostly serotypes 6C and 35B, as well as NT isolates. In turn, penicillin-susceptible capsular types 3, 20, and 8 have risen among IPD. Overall, our results reinforce the relevance of PNSP surveillance over a long period of time to better understand the dynamics of antimicrobial resistance in response to PCV introduction and may also contribute to improve control measures toward drug-resistant pneumococci.

Keywords: *Streptococcus pneumoniae*, penicillin non-susceptibility, asymptomatic carriage, invasive pneumococcal disease, capsular type

INTRODUCTION

Streptococcus pneumoniae, or pneumococcus, is a leading cause of infections, such as pneumonia and meningitis, among children > 5 years old. In addition, this microorganism is also commonly found colonizing the human upper respiratory tract, a niche considered as its major reservoir and the main entry for the establishment of invasive pneumococcal disease (IPD) (Lynch and Zhanel, 2009; Weiser, 2010; Tan, 2012; Donkor, 2013).

This pathogen presents a polysaccharide capsule as the most important virulence factor (Bogaert et al., 2004; Kadioglu et al., 2008; Hyams et al., 2010). The pneumococcal capsule is antigenically diverse allowing the recognition of more than 90 serotypes (Bentley et al., 2006; Mostowy et al., 2017). In addition, the polysaccharide capsule is the basis of licensed vaccine formulations against pneumococcal disease, including the 7-valent pneumococcal conjugate vaccine (PCV7), the 10-valent PCV (PCV10), and the 13-valent PCV (PCV13) (WHO, 2012).

Penicillin non-susceptible pneumococci (PNSP) were recently listed as one of the most important antimicrobial-resistant threats worldwide (CDC, 2013; WHO, 2017). Increasing occurrence of PNSP has been detected since the first report in 1967 in Australia (Hansmann and Bullen, 1967; Castañeda et al., 1998; Appelbaum, 2002; Sadowy et al., 2010; Hackel et al., 2013; Kim et al., 2016). This characteristic seems to be more commonly associated with certain serotypes, such as serotype 14 and those included in serogroups 6, 19, and 23 (McGee et al., 2001; Lee et al., 2014). In Brazil, the emergence of PNSP was initially documented in the mid 1990s and it was initially attributed to the introduction of an internationally disseminated clone (namely ST156) expressing the capsular type 14 (Brandileone et al., 2006; Pinto et al., 2016).

Different measures can affect the epidemiology and evolution of PNSP isolates, including antibiotic therapy policies and the implementation of vaccines. However, such interventions may vary according to the geographical region (Guillemot et al., 1998; McCormick et al., 2003; Kim et al., 2016). Brazil is one of the 32 countries that have introduced PCV10 into the national immunization program, starting in 2010 (Brazil Ministry of Health, 2010). In turn, PCV13 has simultaneously replaced PCV7 in private immunization clinics. Thus, the aim of the present study was to investigate the distribution of capsular types and penicillin susceptibility profiles among pneumococcal isolates recovered from asymptomatic carriage and IPD over a period of 25 years in Brazil, comprising the periods before and after PCV introduction.

MATERIALS AND METHODS

Bacterial Strains

A total of 783 peumococcal isolates were included in the study, comprising 355 isolates recovered from asymptomatic carriers (nasopharynx or oropharynx specimens) and 428 strains derived from IPD (blood or cerebrospinal fluid specimens). They were isolated from children and adults between 1990 and 2014 in five different cities (Campos dos Goytacazes, Niterói, Ribeirão Preto, Rio de Janeiro, and São Paulo) of Southeastern Brazil.

Isolates were recovered during surveillance studies or received from health institutions. Isolates obtained from cases of infection were recovered from clinical specimens taken as part of the standard patient care procedures and did not require ethical approval for their use. Carriage isolates were recovered from specimens collected during surveillance studies approved by ethics committees.

The isolates were previously subjected to phenotypic identification tests according to standard procedures (Spellerberg and Brandt, 2011), including observation of colony morphology and hemolysis on blood agar plates, cellular characteristics as observed after Gram stain, and catalase production, optochin susceptibility and bile-solubility testing.

Determination of Capsular Types

The capsular types were determined by either multiplex PCR (Dias et al., 2007) or the standard Quellung reaction (Sørensen, 1993) with antisera provided by the *Streptococcus* Laboratory at the Centers for Disease Control and Prevention (CDC, Atlanta, GA, United States).

Evaluation of Penicillin Susceptibility Profiles

Susceptibility to penicillin was evaluated according to the CLSI recommendations and interpretative criteria (CLSI, 2016). Minimal inhibitory concentrations (MICs) of penicillin were determined by either using the broth microdilution method or E-test® strips (Oxoid, bioMérieux). All isolates showing

penicillin MICs ≥ 0.12 μg/ml were classified as PNSP. In addition, isolates showing penicillin MICs ≥ 0.12 < 2 μg/ml were classified as pneumococci with reduced susceptibility to penicillin (PRSP), those with MICs ≥ 2 < 4 μg/ml were classified as penicillin-resistant pneumococci (PRP) and those with MICs ≥ 4 μg/ml were classified as high-level penicillin resistant pneumococci (HLPRP).

Statistical Analyses

Distribution of pneumococcal capsular types and penicillin resistance rates and levels were analyzed by the Chi-square or Fisher's exact tests using the software GraphPad Prism v5.0. *p*-Values < 0.05 were considered significant.

RESULTS

Distribution of Capsular Types

Sixty capsular types, as well as 13 non-typeable (NT) isolates, were detected among 783 pneumococcal isolates. Thirty-nine serotypes and NT isolates were identified among the 355 carriage isolates, and 59 serotypes were detected among the 428 IPD isolates. Twenty-one capsular types were exclusively observed in IPD derived strains, while only one serotype (7B) as well as NT isolates were exclusively identified in carriage strains. **Supplementary Table S1** shows the distribution of capsular types among all 783 pneumococcal strains according to the clinical source.

Overall, the most common serotypes were 14 (n = 86; 11%), 6B (n = 63; 8%), 19F (n = 62; 7.9%), 23F (n = 51; 6.5%), 3 and 6C (n = 40; 5.1% each), 6A (n = 26; 3.3%), and 5 (n = 25; 3.2%). These eight capsular types accounted for nearly half of the 783 strains. The most frequent serotypes among carriage strains were 19F (11.8%), 6B (9.6%), 6C (9%), 23F (8.7%), and 14 (8.2%); accounting for 47.3% of the isolates. In turn, the most common serotypes among IPD were 14 (13.3%), 3 (7.2%), 6B (6.5%), 5 (5.1%), 19F (5.1%) and 4 (4.7%), making up 41.9%. Distribution of serotypes fluctuated over time and a higher diversity of capsular types was detected in the late study period (**Figure 1**).

Nearly half of the 783 pneumococcal isolates belonged to PCV serotypes (**Table 1** and **Supplementary Table S1**). Occurrence of PCV10 serotypes remarkably decreased during 2011–2014, while non-PCV10 serotypes, including non-vaccine (NV) serotypes and those exclusively covered by PCV13, increased in this same period (**Figure 2**). This trend was noted regardless of clinical source ($p < 0.01$). Of note, although detected in low numbers until 2010, all newly emerging non-PCV10 serotypes in the period 2011–2014 have been circulating in our setting since the early period of isolation included in the present study (1990s).

Penicillin Susceptibility Profiles

Around 20% (176) of the 783 isolates were PNSP, showing penicillin MICs ranging from 0.12 to 8 μg/ml. Differences were noted regarding distribution of PRSP, PRP, and HLPRP between carriage and IPD, with significantly higher numbers and levels of penicillin resistance among carriage strains (**Table 2**; $p < 0.05$).

Overall, PNSP were associated with 24 serotypes and NT isolates (**Supplementary Table S1**); eight serotypes (6A, 6B, 6C, 14, 19A, 19F, 23F, and 35B) and NT isolates were mostly associated with penicillin resistance (**Table 3**). These serotypes included six (6A, 6B, 6C, 14, 19F, and 23F) of the most frequently

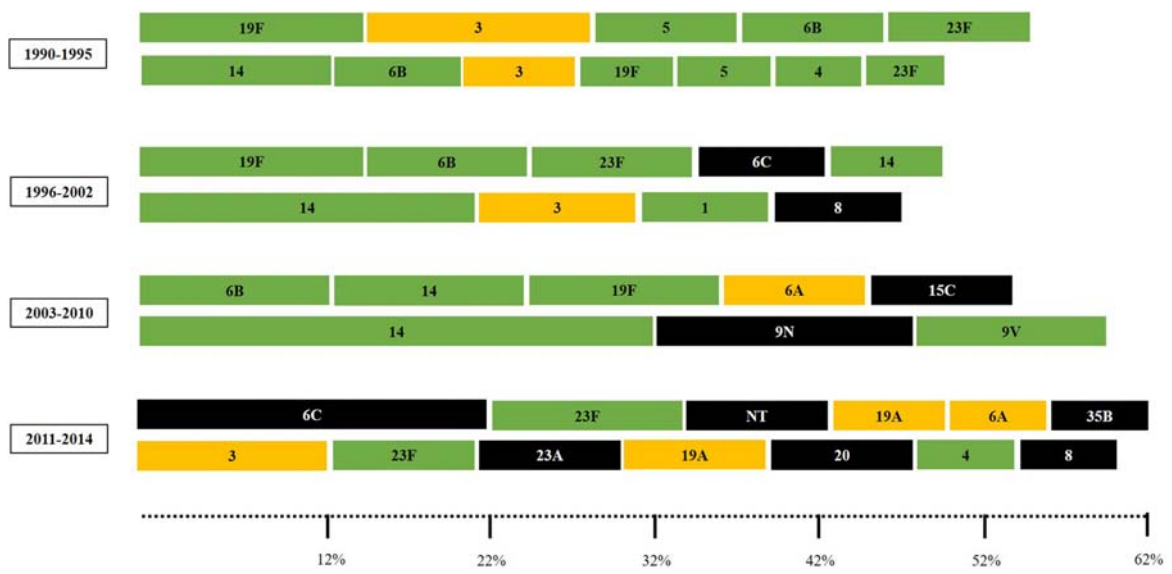

FIGURE 1 | The most common pneumococcal capsular types (comprising 50 to 60% of the pneumococcal isolates) according to the clinical source and period of time. Carriage isolates (represented in the upper lines) included strains recovered from nasopharynx or oropharynx specimens while IPD (invasive pneumococcal disease) isolates (represented in the bottom lines) included strains recovered from blood or cerebrospinal fluid. Serotypes colored in green are included in the 10-valent pneumococcal conjugate vaccine (PCV); those colored in yellow are only included in the 13-valent PCV; and those colored in black are not included in any PCV currently available.

TABLE 1 | Distribution of capsular types included in pneumococcal conjugate vaccines currently available among 783 *Streptococcus pneumoniae* isolates according to the clinical source.

Clinical source[a] (n)	% (n) strains belonging to capsular types included in[b]	
	10-Valent PCV	13-Valent PCV
Carriage (355)	44.5 (158)	55.5 (197)
IPD (428)	50.5 (216)	61.9 (265)
All (783)	45.1 (353)	56.3 (441)

[a]*Carriage isolates included strains recovered from nasopharynx or oropharynx specimens while IPD (invasive pneumococcal disease) isolates included those recovered from blood or cerebrospinal fluid.*
[b]*PCV, pneumococcal conjugate vaccine; 10-valent PCV includes serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F; 13-valent PCV also includes 3, 6A, and 19A.*

found among the 783 isolates investigated. In addition, four (6B, 14, 19F, and 23F) of them were PCV10 serotypes. Nevertheless, the most common PNSP serotypes varied according to the clinical source (**Table 3**). Of note, a much higher proportion of PNSP strains belonging to PCV10 serotypes was isolated from IPD (**Table 4**; $p < 0.01$).

PRSP, PRP and HLPRP showed an increasing trend during the study period among carriage strains (**Figure 3A** and **Table 5**; $p < 0.01$). Regarding IPD, this increasing trend was observed only until 2010; between 2011 and 2014, PNSP numbers and levels significantly decreased (**Figure 3B** and **Table 5**; $p < 0.01$).

Distribution of PNSP serotypes also varied according to the study period. Overall, PNSP belonging to PCV10 serotypes showed a decreasing trend, while PNSP associated with non-PCV10 serotypes showed an increasing trend (**Figure 4**; $p < 0.01$). However, the most frequent serotypes in each period varied according to the clinical source. In addition, a higher diversity of serotypes was associated with PNSP isolated in the late period (**Figure 5**).

DISCUSSION

Differences in the distribution of pneumococcal serotypes between carriage and IPD isolates were observed. Some serotypes, including 3, 4, and 5, were exclusively detected among IPD cases. Previous studies have shown that certain capsular types are more prone to cause IPD while others are well-adapted to nasopharynx colonization (Bender et al., 2008; Weiser, 2010; Weinberger et al.,

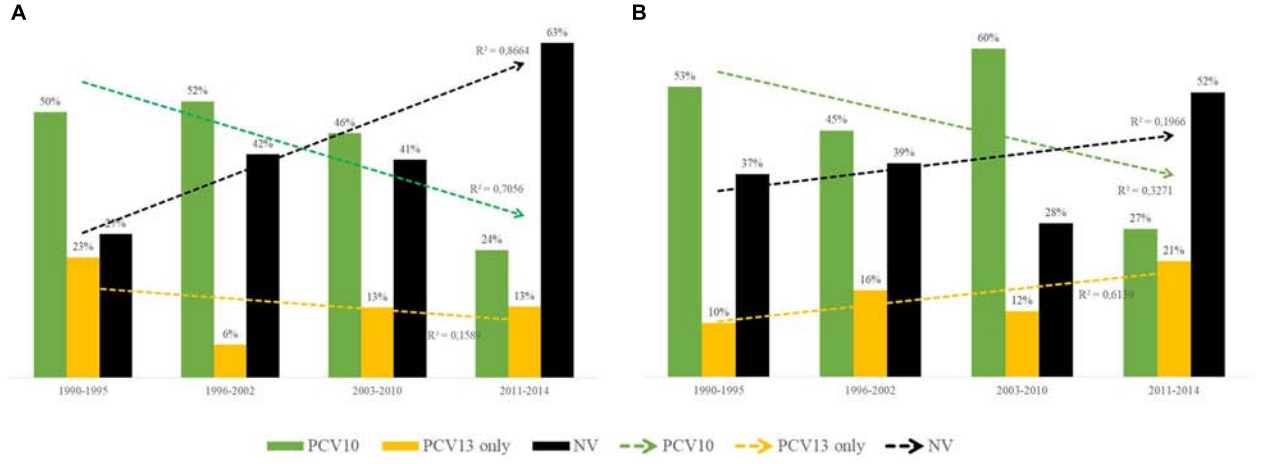

FIGURE 2 | Distribution over time of capsular types included in the 10-valent pneumococcal conjugate vaccine (PCV10; in green), of those included only in the 13-valent pneumococcal conjugate vaccine (PCV13; in yellow) and of those not included in any PCV currently available [non-vaccine (NV), in black]. **(A)** Distribution among 355 *Streptococcus pneumoniae* isolates recovered from asymptomatic carriers. **(B)** Distribution among 428 *S. pneumoniae* isolates recovered from patients with IPD. 10-valent PCV includes serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F; 13-valent PCV also includes 3, 6A, and 19A.

TABLE 2 | Distribution of *Streptococcus pneumoniae* isolates with reduced susceptibility to penicillin (PRSP), resistant to penicillin (PRP), and high-level resistant to penicillin (HLPRP) according to the clinical source.

Clinical source[a]	PRSP% (N)	PRP% (N)	HLPRP% (N)	MIC50 (μg/ml)	MIC90 (μg/ml)
Carriage	23.9 (85)	3.7 (13)	5.6 (20)	0.06	1.5
IPD	11.4 (49)	0.5 (2)	1.6 (7)	0.03	0.12
All	17.1 (134)	1.9 (15)	3.4 (27)	0.03	0.5

[a]*Carriage isolates included those recovered from nasopharynx or oropharynx specimens while IPD (invasive pneumococcal disease) isolates included those recovered from blood or cerebrospinal fluid. Isolates showing penicillin MICs ≥ 0.12 < 2 μg/ml were classified as pneumococci with reduced susceptibility to penicillin (PRSP), those with MICs ≥ 2 < 4 μg/ml were classified as penicillin-resistant pneumococci (PRP) and those with MICs ≥ 4 μg/ml were classified as high-level penicillin resistant pneumococci (HLPRP).*

TABLE 3 | Distribution of *Streptococcus pneumoniae* isolates non-susceptible to penicillin (PNSP) among nine capsular types mostly associated with penicillin resistance, according to the clinical source.

Capsular type (n)	% PNSP (n)	Carriage[a]			IPD[a]		
		% PNSP	MIC50[b]	MIC90[b]	% PNSP	MIC50[b]	MIC90[b]
6A (26)	26.9 (7)	36.8	0.06	2	0	0.01	0.03
6B (63)	38.1 (24)	31.4	0.06	0.25	46.4	0.06	0.32
6C (40)	40 (16)	50	0.06	0.75	0	0.01	0.06
14 (86)	52.3 (45)	82.7	2	4	36.8	0.06	1.5
19A (22)	36.4 (8)	45.4	0.06	8	27.3	0.05	8
19F (62)	17.7 (11)	17.5	0.06	0.12	18.2	0.06	0.25
23F (51)	49 (25)	61.3	0.12	0.25	30	0.06	0.25
35B (7)	57.1 (4)	66.7	1	4	0	0.01	0.01
NT (13)	61.5 (8)	61.5	0.19	4	0	NA	NA

[a]Carriage isolates included strains recovered from nasopharynx or oropharynx specimens while IPD (invasive pneumococcal disease) isolates included those recovered from blood or cerebrospinal fluid.
[b] μg/ml. NA, not applicable since no strain belonging to such serotype was detected. Strains showing penicillin MICs ≥ 0.12 μg/ml were classified as penicillin non-susceptible pneumococci (PNSP). Serotypes comprised by PCV10 are highlighted in green, those included only in PCV13 are highlighted in yellow, those not included in any PCV currently available are not colored.

TABLE 4 | Distribution of capsular types included in pneumococcal conjugate vaccines currently available among 176 *Streptococcus pneumoniae* isolates non-susceptible to penicillin (PNSP) according to the clinical source.

Clinical source[a] (n)	% (n) of isolates belonging to capsular types included in[b]	
	10-Valent PCV	13-Valent PCV
Carriage (118)	53.4 (63)	63.5 (75)
IPD (58)	87.9 (51)	93.1 (54)
All (176)	45.1 (353)	56.3 (441)

[a]Carriage isolates included strains recovered from nasopharynx or oropharynx specimens while IPD (invasive pneumococcal disease) isolates included those recovered from blood or cerebrospinal fluid.
[b]PCV, pneumococcal conjugate vaccine; 10-valent PCV includes serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F; 13-valent PCV also includes 3, 6A, and 19A.

2011). Pneumococcal strains lacking the polysaccharide capsule (NT isolates), for example, are believed to be less virulent (Sharma et al., 2013). Accordingly, NT isolates were only identified among pneumococcal isolates recovered from asymptomatic carriage. On the other hand, a group of serotypes seems to be highly versatile, being frequently found in both carriage and IPD. In this study, three capsular types were frequently found regardless of clinical source, including 6B, 14, and 19F. Indeed, these serotypes are known to be common among carriage and IPD worldwide before PCV introduction (Hausdorff, 2007; Weinberger et al., 2011; Song et al., 2013).

Nearly 20% of all the isolates were PNSP, which is in accordance with previous data from Brazilian studies (Neves et al., 2013; Mott et al., 2014; dos Santos et al., 2015). However, differences on the distribution of penicillin resistance were also noted when carriage and IPD isolates were compared. PNSP occurrence, as well as penicillin MIC levels, were higher among carriage isolates. Indeed, certain serotypes almost exclusively found in IPD, such as serotype 3, were fully susceptible to penicillin. Several studies have shown that pneumococcal serotypes commonly found in carriage are more frequently associated with antimicrobial resistance than those exclusively found in IPD isolates (Weiser, 2010; Song et al., 2013; Zhou et al., 2015; Kim et al., 2016; Neves et al., 2017). This observation may be due, at least in part, to the fact that the human nasopharynx, in contrast to blood or cerebrospinal fluid, is a highly populated niche where genetic exchange among bacteria occurs and, thus, emergence of antimicrobial resistance traits can be favored (Andam and Hanage, 2015; Kim et al., 2016).

Although fluctuations on the occurrence of serotypes over time can happen naturally and should be carefully evaluated, our results suggest that the introduction of PCV7 and PCV13 in 2001 and 2010, respectively, did not seem to have affected pneumococcal epidemiology regarding serotype and PNSP distribution in our setting. This might be due, at least in part, to the fact that these PCVs were made available only in private clinics in Brazil. Indeed, usage of PCV7 and PCV13 in Brazil is very low due to their high cost (Brazil Ministry of Health, 2006; Medeiros et al., 2017; Neves et al., 2017). On the other hand, according to previous studies conducted in Brazil (dos Santos et al., 2013; Medeiros et al., 2017; Neves et al., 2018), our results suggest an important impact on serotype replacement after the implementation of PCV10. PCV10 serotypes showed a decreasing trend over time, especially in the late study period (2011–2014). In parallel, occurrence of non-PCV10 serotypes increased over time, surpassing the numbers of PCV10 serotypes in both carriage and IPD between 2011 and 2014. Similar observations have been made in other countries where PCV10 was routinely adopted, such as the Netherlands, Mozambique and Finland (Knol et al., 2015; Nhantumbo et al., 2017; Sihvonen et al., 2017).

Among the non-PCV10 serotypes emerging after PCV10 introduction, serotype 19A was an important serotype associated with both carriage and IPD. Although emergence of this serotype after PCV7 introduction in certain high-income countries is a well-established fact (Isaacman et al., 2010; Isturiz et al., 2017), serotype 19A emergence after PCV10 introduction in Brazil

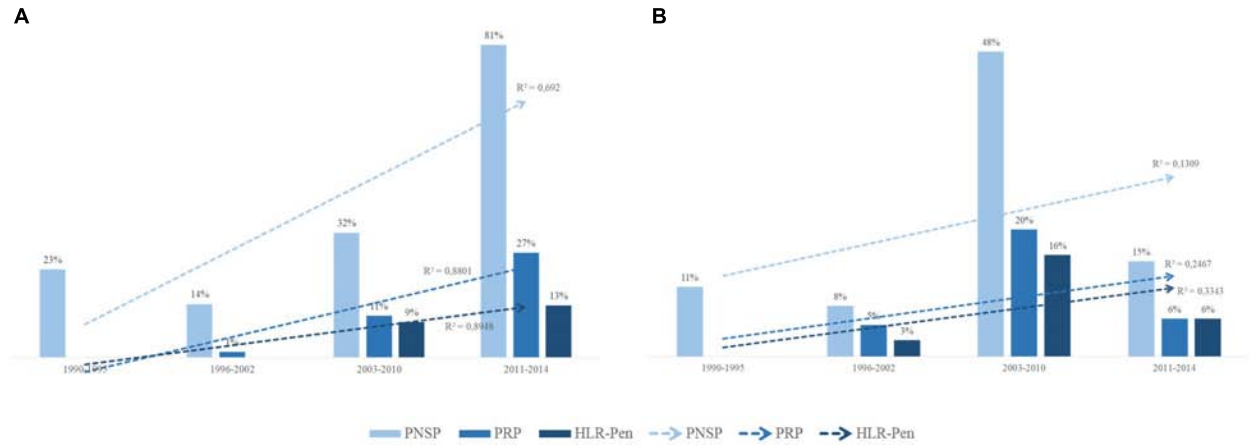

FIGURE 3 | Distribution over time of pneumococci with reduced susceptibility to penicillin (PRSP), penicillin-resistant pneumococci (PRP), and high-level penicillin resistant pneumococci (HLPRP). **(A)** Distribution among 355 *S. pneumoniae* isolates recovered from asymptomatic carriers. **(B)** Distribution among 428 *S. pneumoniae* isolates recovered from patients with IPD. Isolates showing penicillin MICs ≥ 0.12 < 2 μg/ml were classified as PRSP, those with MICs ≥ 2 < 4 μg/ml were classified as PRP and those with MIC ≥ 4 μg/ml were classified as HLPRP.

still seems to be a contradictory issue. While certain studies reveal that occurrence of this serotype has not significantly changed (Medeiros et al., 2017; Neves et al., 2018), others report an increasing rate (Cassiolato et al., 2018; Christophe et al., 2018). We also observed that serotypes 3, 8, 20, and 23A emerged among isolates from IPD cases, whereas serotypes 6C, 35B, and NT isolates were more commonly associated with asymptomatic carriage. Emergence of serotype 6C in carriage and of serotypes 3 and 8 in IPD after PCV10 implementation in Brazil has been recently described (Medeiros et al., 2017; Christophe et al., 2018; Neves et al., 2018). Of note, all these emerging non-PCV10 serotypes have been circulating in our setting since the 1990s, reinforcing the possibility of serotype replacement phenomenon.

Moreover, while PNSP numbers and levels decreased significantly in the late period of the present study (2011–2014) among IPD isolates, they kept increasing among isolates from carriage. Accordingly, many studies have reported lower frequencies and levels of PNSP among IPD isolates after PCV10 introduction in Brazil (dos Santos et al., 2013; Medeiros et al., 2017). In turn, antimicrobial resistance levels among pneumococcal isolates from asymptomatic carriage have been increasing despite of vaccination. Recently, Neves et al. (2018) have suggested that this is probably due to the emergence of multidrug resistant lineages belonging to non-PCV10 serotypes, such as the serotype 6C-CC386, among carriage isolates. On the other hand, serotypes emerging among IPD isolates after PCV10 introduction, such as 3, 8 and 20, were shown to be fully susceptible to penicillin. These observations suggest that the PCV10 impact on the reduction of PNSP occurrence and level might be more relevant for IPD than for carriage. This suggestion can also be supported by the observation that, before PCV10 introduction, PNSP isolates recovered from IPD were almost completely represented by PCV10 serotypes (nearly 90%), while only half of PNSP strains recovered from asymptomatic carriage comprised PCV10 serotypes.

Penicillin non-susceptible pneumococci have been listed as one of the major antimicrobial resistance threats among bacterial pathogens (CDC, 2013; WHO, 2017). Although they represent a global public health threat, occurrence and epidemiology of PNSP vary according to the geographic region. Taken our results into consideration, from the mid 1990s until 2010, serotype 14 played a major role in the dispersion of penicillin non-susceptibility, especially among IPD isolates. Indeed, it was previously shown that an internationally disseminated clone belonging to this serotype (namely ST156), which was also frequently associated with IPD worldwide, was the main reason for PNSP emergence in Brazil in the pre-vaccination era (Barroso

TABLE 5 | Distribution of penicillin minimum inhibitory concentration (MIC) levels among *Streptococcus pneumoniae*, according to the period of time and clinical source.

Clinical source[a]	MIC50 (μg/ml)				MIC90 (μg/ml)			
	1990–1995	1996–2002	2003–2010	2011–2014	1990–1995	1996–2002	2003–2010	2011–2014
Carriage	0.03	0.06	0.03	0.50	0.25	0.12	2	4
IPD	0.03	0.06	0.09	0.01	0.12	0.12	4	0.12

[a]*Carriage isolates included strains recovered from nasopharynx or oropharynx specimens while IPD (invasive pneumococcal disease) isolates included those recovered from blood or cerebrospinal fluid.*

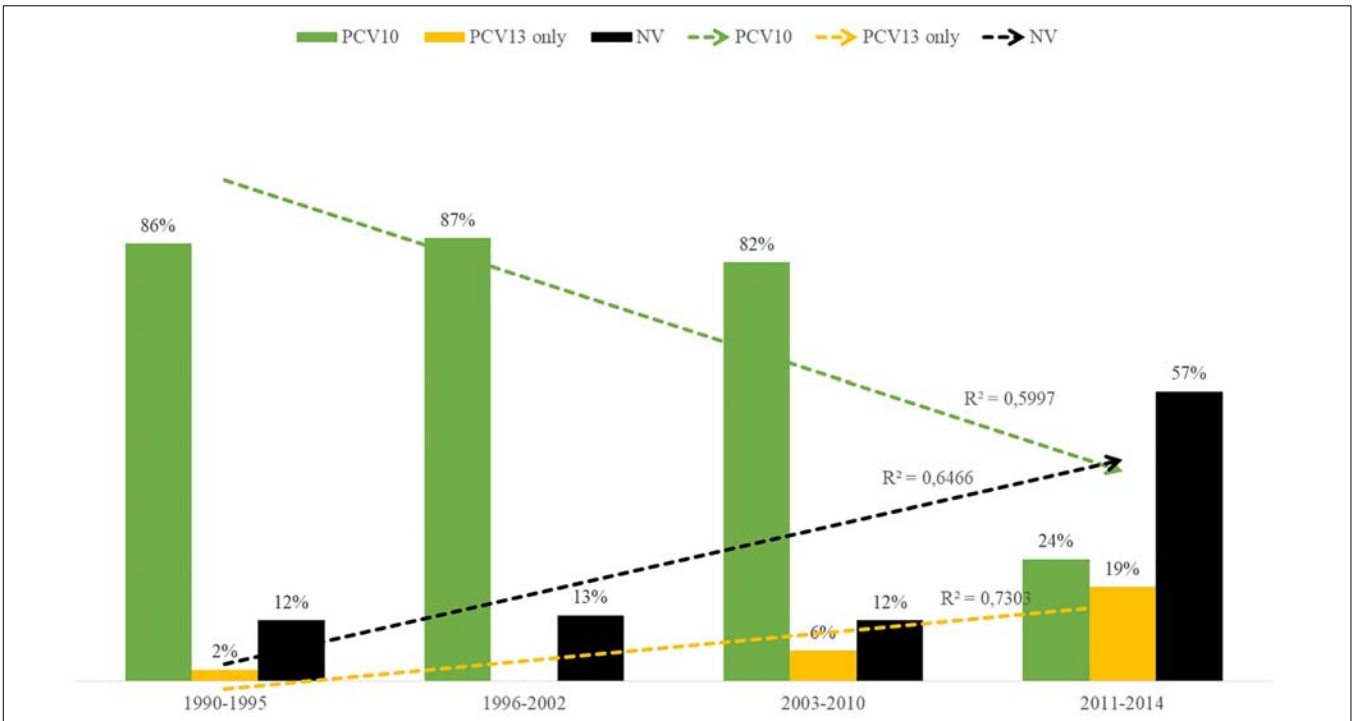

FIGURE 4 | Distribution over time of capsular types included in the 10-valent pneumococcal conjugate vaccine (PCV10; in green), of those included only in the 13-valent pneumococcal conjugate vaccine (PCV13; in yellow) and of those not included in any PCV currently available (in black) among penicillin non-susceptible pneumococci (PNSP).

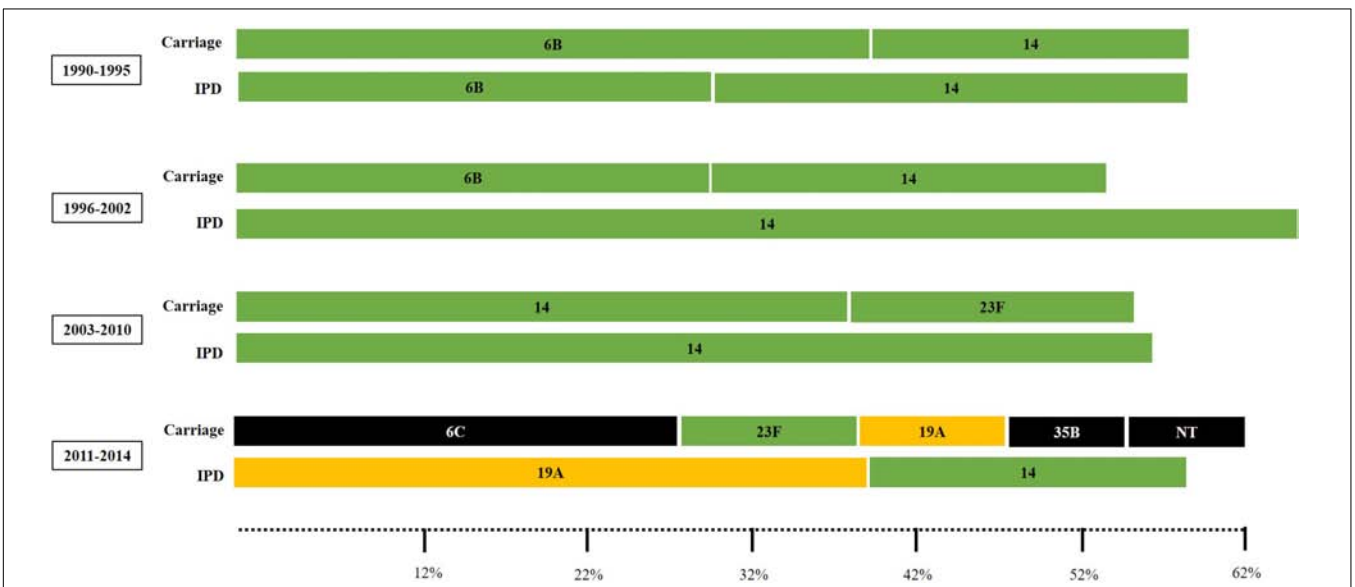

FIGURE 5 | Sets of the most common pneumococcal capsular types associated with penicillin non-susceptibility (comprising 50 to 60% of the pneumococcal isolates) according to the clinical source and period of time. Carriage isolates included those recovered from nasopharynx or oropharynx specimens while IPD isolates included those recovered from blood or cerebrospinal fluid. Serotypes colored in green are included in the 10-valent pneumococcal conjugate vaccine (PCV); those colored in yellow are only included in the 13-valent PCV; and those colored in black are not included in any PCV currently available.

et al., 2012; Pinto et al., 2016). After 2010, however, this scenario has changed and a more diversified panel of serotypes has been associated with penicillin non-susceptibility, regardless of clinical source. Among IPD isolates specifically, serotype 19A PNSP emerged significantly, surpassing the previous number of serotype 14 PNSP isolates.

Major limitations of this study are related to the characteristics of the population included. It is known that age of individuals is an important feature and may have an influence on serotype distribution. However, we were not able to assess this issue in detail since information was not available for a large proportion of the isolates analyzed, although we estimate from available data that most of strains were recovered from children. In addition, although Brazil is a country with continental dimensions and, thus, might present discrepancies between regions, the Southeastern region, represented here by five different cities, is the most populated one. According to the last official demographic survey conducted in Brazil (Instituto Brasileiro de Geografia e Estatística [IBGE], 2010), population living in the Southeastern region accounted for nearly half of the whole Brazilian population. Moreover, this region can be considered as representative of the ethnic, social, and economic diversity of the Brazilian population due to the historic high flow of domestic in-migration.

Penicillin non-susceptible pneumococci evolution can be driven by different interventions such as antibiotic therapy policies and vaccine implementation (Guillemot et al., 1998; McCormick et al., 2003; Kim et al., 2016). These aspects usually differ by country; for example, Brazil is one of the 32 countries that have adopted PCV10 in the national immunization program instead of PCV7/PCV13, adopted by other 98 countries (Brazil Ministry of Health, 2010). Therefore, gathering information on PNSP epidemiology over a long period of time can contribute to a better understanding of their evolution and the impact of different vaccination strategies. Overall, our results show the emergence of non-PCV10 serotypes after 2010 in Brazil and the emergence and spread of PNSP associated with carriage. On the other hand, PCV10 has been effective in decreasing PNSP rates and levels among IPD isolates, but it has not avoided serotype replacement. These results reinforce the need of continuous surveillance of PNSP in the post-vaccine introduction era and may contribute to the development of more effective measures to control the spread of drug-resistant pneumococci.

AUTHOR CONTRIBUTIONS

JP and LT coordinated the study. TP, LT, and JP contributed to the conception and design of the work. TP, FN, AS, LO, NC, CM-S, and LC performed the experiments and analyzed the data. LO performed the statistical analyses. TP, LO, JP, and LT wrote the manuscript. All authors revised and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00486/full#supplementary-material

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IncX4 Plasmid Carrying the New *mcr-1.9* Gene Variant in a CTX-M-8-Producing *Escherichia coli* Isolate Recovered From Swine

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We studied a commensal colistin-resistant *Escherichia coli* isolated from a swine cecum sample collected at a slaughter, in Portugal. Antimicrobial susceptibility phenotype of *E. coli* LV23529 showed resistance to colistin at a minimum inhibitory concentration of 4 mg/L. Whole genome of *E. coli* LV23529 was sequenced using a MiSeq system and the assembled contigs were analyzed for the presence of antibiotic resistance and plasmid replicon types using bioinformatics tools. We report a novel *mcr-1* gene variant (*mcr-1.9*), carried by an IncX4 plasmid, where one-point mutation at nucleotide T1238C leads to Val413Ala substitution. The *mcr-1.9* genetic context was characterized by an IS*26* element upstream of the *mcr-pap2* element and by the absence of IS*Apl1*. Bioinformatic analysis also revealed genes conferring resistance to β-lactams, sulphamethoxazole, trimethoprim, chloramphenicol and colistin, corresponding to the phenotype noticed. Moreover, we highlight the presence of *mcr-1.9* plus $bla_{\text{CTX-M-8}}$, a bla_{ESBL} gene rarely detected in Europe in isolates of animal origin; these two genes were located on different plasmids with 33,303 and 89,458 bp, respectively. MCR-1.9-harboring plasmid showed high identity to other X4-type *mcr-1*-harboring plasmids characterized worldwide, which strongly suggests that the presence of PMCR-encoding genes in food-producing animals, such as MCR-1.9, represent a potential threat to humans, as it is located in mobile genetic elements that have the potential to spread horizontally.

Keywords: MCR-1.9, plasmid-mediated colistin resistance, IncX4, CTX-M-8, Portugal

INTRODUCTION

Since the report of a plasmid-mediated colistin resistance (PMCR) mechanism, designated MCR-1, in *Escherichia coli* and *Klebsiella pneumoniae* isolated from animals, food and humans in China, further reports exposed the global dissemination of *mcr*-type gene in various bacterial species isolated from a wide range of different sources (Caniaux et al., 2017). In Portugal, PMCR has also

been detected in a wide range of different sources and species, including humans, food-producing animals and meat, and in the environment (Campos et al., 2016; Figueiredo et al., 2016; Jones-Dias et al., 2016; Kieffer et al., 2017; Manageiro et al., 2017; Tacão et al., 2017; Mendes et al., 2018). Noteworthy, are the recent report of two cases presumably associated with the travel of patients from Portugal, one involving animals: a patient repatriated to France after hospitalization for 2 months in Portugal, in 2015 (Beyrouthy et al., 2017), and a New York state patient returning from Portugal in 2016 after staying on a farm with chickens and pigs (Gilrane et al., 2017).

More worrisome is the presence of *mcr* genes in *Enterobacteriaceae* carrying other resistance determinants namely, extended-spectrum β-lactamases (ESBL)- and/or carbapenemase-encoding genes. Since the first report of co-localization of *mcr-1* and ESBL- in 2016 in bovines in France, an increase encoding genes in the proportion of *mcr-1* genes among ESBL-producing *E. coli* in animals has been noticed, suggesting that the use of extended-spectrum cephalosporins may have simultaneously favored the spread of *mcr-1* (Haenni et al., 2016). Here we describe the first detection of a novel *mcr* variant, hereafter-named *mcr-1.9*, identified in a commensal *E. coli* LV23529 isolated from a swine cecum sample collected at a slaughter, in Portugal.

MATERIALS AND METHODS

Bacterial Isolate

Escherichia coli LV23529 was isolated in 2015 from a swine cecum sample collected at a Portuguese slaughter, during an evaluation study of commensal *E. coli* recovered from swine samples for antimicrobial susceptibility testing.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined by microdilution method as previously described (Manageiro et al., 2017). In order to assess decreased susceptibility of the strain, interpretation of the results was done according to the epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST[1]).

Screening and Characterization of PMCR- and ESBL-Resistance Mechanisms

Molecular Detection of *mcr-1* and bla_{ESBL}-Encoding Genes

Following phenotypic characteristics, PMCR- and ESBL-resistance mechanisms were searched and identified by molecular methods, as previously described (Manageiro et al., 2017).

Transfer Experiments

Conjugation experiments were performed using sodium azide-resistant *E. coli* J53 as a recipient strain. Transconjugants were selected on McConkey agar supplemented with sodium azide (150 mg/L) and either cefotaxime (2 mg/L) or colistin (2 mg/L). Plasmid DNA was extracted from *E. coli* LV23529 using a NucleoBond Xtra Plus kit (Macherey-Nagel), and transformed into *E. coli* TOP10 OneShot chemically competent cells (Invitrogen), accordingly to manufacture's protocol. *E. coli* transformants were selected on MacConkey agar supplemented with 2 mg/L of colistin. PCR for $bla_{CTX\text{-}M\text{-}8}$ or *mcr-1*-type and MICs of recipients and transformants were determined as mentioned above.

Genetic Context of *mcr-1.9* Gene

Colistin-resistant *E. coli* LV23529 was genotypically characterized by whole-genome sequencing (WGS), as previously described (Manageiro et al., 2017). Sequence reads were trimmed and filtered according to quality criteria, and de novo assembled into contigs by means of CLC Genomics Workbench 10.0 (Qiagen). The assembled contigs were analyzed and studied for the presence of antibiotic resistance, virulence genes and plasmid replicon types, serotype, multi-locus sequence type (ST) and *fim*-type, using bioinformatics tools[2]. The NCBI prokaryotic genome automatic annotation pipeline (PGAAP) was used for annotation.

Plasmid sequencing was also performed on a MiSeq Illumina platform using 150 bp paired-end reads, after plasmid DNA extraction from TLV23529 (*mcr-1.9*) using a NucleoBond Xtra Plus kit (Macherey-Nagel), and quantification using Qubit 1.0 Fluorometer (Invitrogen), as previously described (Manageiro et al., 2017). Sequence reads were trimmed and filtered according to quality criteria, and mapped against *E. coli* ATCC 25922 genome (NZ_CP009073). Unmapped reads (80.2%/total reads) were then used for plasmids structure construction by mapping assembly based on the genetic organization of the closest plasmid sequences obtained by BLASTn; this was followed by contig neighbor's prediction from assembly information using CLC Genomics Workbench 10.0 (Qiagen). NCBI Microbial genomes BLAST analysis tool[3] was used to search for plasmid sequences. Plasmid alignments and ORF representations were also done using EasyFig v. 2.2.3 (Sullivan et al., 2011).

Genomic Epidemiological Analysis

BacWGSTdb database was used for genotyping and source tracking bacterial pathogen (Ruan and Feng, 2016).

Nucleotide Sequence Accession Number

The pLV23529-MCR-1.9 and pLV23529-CTX-M-8 nucleotide sequences from this study were submitted to the NCBI GenBank Database with accession numbers KY964067 and KY964068, respectively. The new *mcr-1.9* nucleotide sequence was submitted with accession number KY780959.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession SBIH00000000. The version described in this paper is version SBIH01000000.

[1]http://mic.eucast.org/Eucast2/

[2]https://cge.cbs.dtu.dk/services/

[3]https://www.ncbi.nlm.nih.gov/genome/microbes/

RESULTS AND DISCUSSION

MIC results showed that LV23529 was non-wild-type to third- and fourth-generation cephalosporins (ceftazidime 2 mg/L, cefotaxime 32 mg/L, cefepime 8 mg/L) with synergy with clavulanic acid; this isolate was also non-wild-type to chloramphenicol (>128 mg/L), sulphamethoxazole (>1024 mg/L), trimethoprim (>32 mg/L), tetracycline (>64 mg/L), and colistin (4 mg/L). LV23529 remained wild-type to carbapenems, fluoroquinolones, aminoglycosides and tigecycline (**Table 1**).

Molecular characterization of the *E. coli* LV23529 isolate allowed the detection of $bla_{\text{CTX-M-8}}$ and *mcr-1*-type genes.

Only the transferability of the $bla_{\text{CTX-M-8}}$ gene was achieved by conjugation, with TcLV23529 ($bla_{\text{CTX-M-8}}$) exhibiting the ESBL phenotype from LV23529 isolate (cefotaxime 2 mg/L, cefepime 2 mg/L) with synergy with clavulanic acid, and wild-type to colistin (≤1 mg/L) (**Table 1**). Although conjugation assays for *mcr-1*-type were negative, the colistin resistance determinant could be transferred to *E. coli* TOP10 competent cells; transformant TLV23529 (*mcr-1*-type) showed the respective resistance to colistin (4 mg/L) (**Table 1**).

The WGS assembly of *E. coli* LV23529 yielded 193 contigs (average 143.7-fold coverage), which together comprised 5,122,415bp, showing a GC content of 50.7%. The largest contig was 320,931 bp long; the N50 statistic, which stands for the minimum contig length of at least 50% of the contigs, was 113,197 bp. The average length of the obtained contigs was 26,541 bp. Overall, the genome sequence comprised 5,124 putative genes, among which 5,037 consisted of protein encoding sequences.

The WGS analysis showed that *E. coli* LV23529 belongs to serotype O8:H19, usually associated with porcine stx_{2e}-producing *E. coli* (Zweifel et al., 2006; Bai et al., 2015), and to MLST (Achtman scheme) ST201 [clonal complex 469 (CC469)] and to the FimH-type determinant *fimH32*. This ST201 was encountered worldwide mainly in isolates collected from livestock samples (*Escherichia*/*Shigella* Enterobase database, Alikhan et al., 2018). Three virulence factors were detected: *astA* (heat-stable enterotoxin 1), *lpfA* (long polar fimbriae), and *gad*-type (glutamate decarboxylase).

Further bioinformatics analysis of *E. coli* LV23529 isolate revealed acquired-genes conferring resistance to β-lactams ($bla_{\text{CTX-M-8}}$ and $bla_{\text{TEM-1}}$), aminoglycosides (*aadA1* and *aadA2*),

TABLE 1 | Phenotypic and genotypic context of CTX-M-8 and MCR-1.9 producing *E. coli* clinical isolate, transformant, transconjugant, and the respective recipient strains.

Antibiotic	LV23529[b]	Transformation		Conjugation	
	($bla_{\text{CTX-M-8}}$, *mcr-1.9*, *cmlA1*, *sul3*, *tetA*, *tetM*, *dfrA12*, *aadA1*, *aadA2*)	*E. coli* TOP10[c]	TLV23529[d] (*mcr-1.9*)	ECJ53AZNa[e]	TcLV2352[f] ($bla_{\text{CTX-M-8}}$)
Ampicillin	>64	4	8	2	>64
Cefoxitine	4	4	4	4	8
Ceftazidime	2	0.5	0.5	≤0.5	1
Ceftazidime plus clavulanate[a]	≤0.125/4	0.5	0.5	≤0.125/4	≤0.25/4
Cefotaxime	32	≤0.25	≤0.25	≤0.25	2
Cefotaxime plus clavulanate[a]	≤0.06/4	0.125	0.125	≤0.06/4	≤0.06/4
Cefepime	8	0.125	0.125	≤0.06	2
Imipenem	0.25	0.5	0.5	0.25	0.25
Meropenem	≤0.03	0.06	0.06	≤0.03	≤0.03
Ertapenem	≤0.015	≤0.015	≤0.015	≤0.015	≤ 0.015
Nalidixic acid	≤4	≤4	≤4	≤4	≤4
Ciprofloxacin	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015
Chloramphenicol	>128	≤8	≤8	≤8	≤8
Sulphamethoxazole	>1024	≤8	≤8	≤8	≤8
Tetracycline	>64	≤2	≤2	≤2	≤2
Trimethoprim	>32	≤0.25	≤0.25	≤0.25	≤0.25
Gentamicin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Colistin	4	≤1	2	≤1	≤1
Tigecycline	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25

MICs in mg/L.

[a] *Clavulanate 4 mg/L.*

[b] *E. coli LV23529 was the clinical isolate harboring the acquired antibiotic resistance genes $bla_{\text{CTX-M-8}}$, mcr-1.9, cmlA1, sul3, tetA, tetM, dfrA12, aadA1, and aadA2.*

[c] *E. coli TOP10 was the recipient strain in the transformation experiment.*

[d] *TLV23529 is a transformant of LV23529 (harboring mcr-1.9).*

[e] *E. coli J53AZNa was the recipient strain in the conjugation experiment.*

[f] *TcLV23529 is a transconjugant of LV23529 (harboring $bla_{\text{CTX-M-8}}$).*

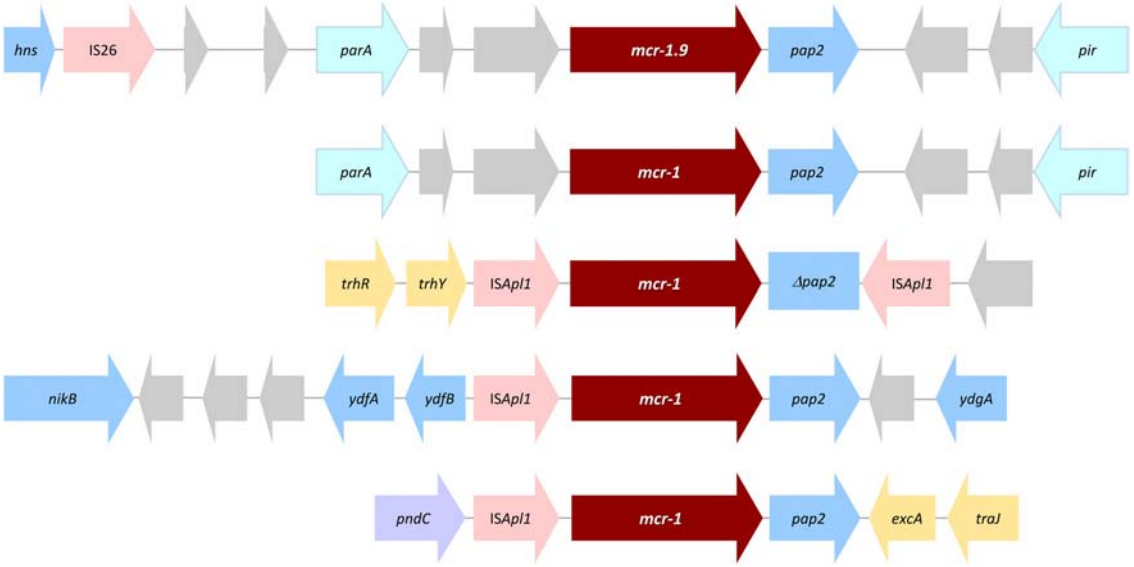

FIGURE 1 | Linear comparison of IncX4-pLV23529-MCR-1.9 with the top six *mcr-1*-harboring plasmids showing the highest identities (>99.9%, E-value 0.0), in different *E. coli* isolates. Boxed arrows represent the position and transcriptional direction of ORFs. Gray vertical blocks indicate the shared similarity regions according to TBLASTX identity. Genes associated with pilus and plasmid transfer are colored yellow, antibiotic resistance genes are colored red, mobile genetic elements are colored pink, and other genes are colored gray (hypothetical proteins) or blue (other).

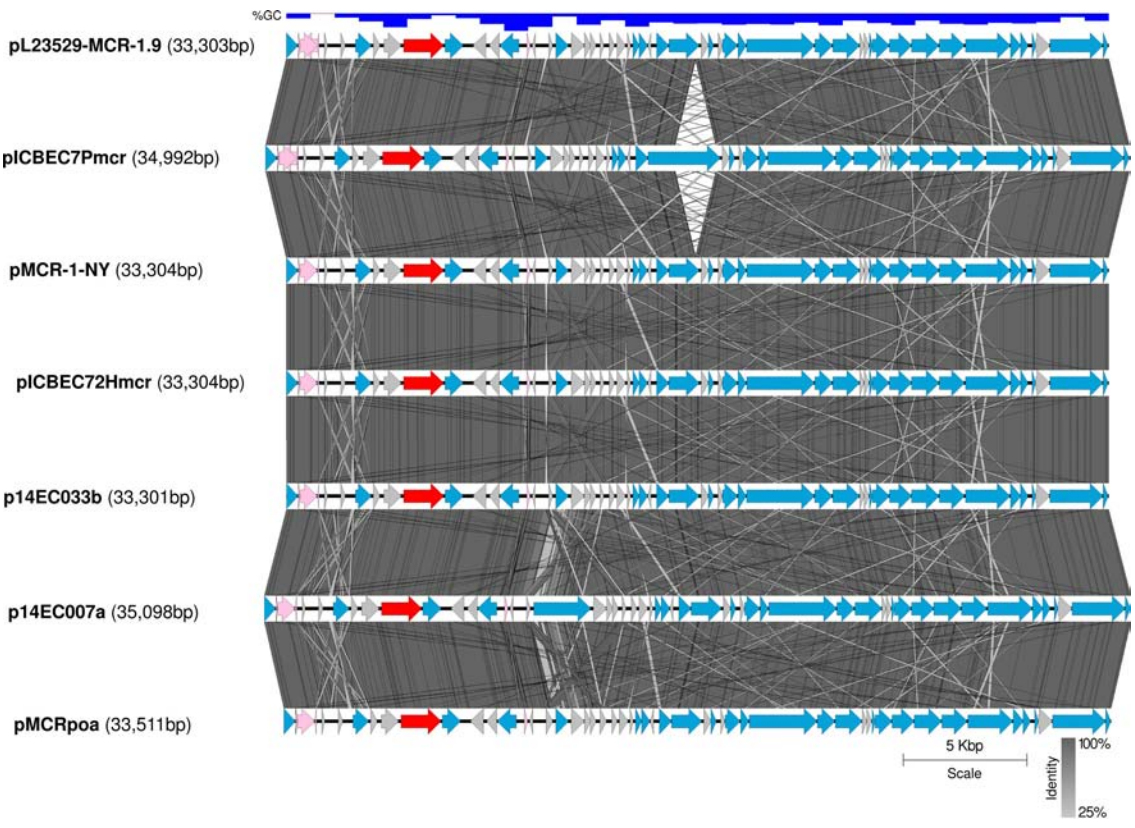

FIGURE 2 | Schematic representation of the genetic environment of *mcr-1.9* in comparison with other *mcr-1*-type representative environments. Boxed arrows represent the position and transcriptional direction of ORFs. Genes are not drawn to scale. Genes associated with pilus and plasmid transfer are colored yellow, antibiotic resistance genes are in red, mobile genetic elements in pink, plasmid maintenance and stability genes in violet, plasmid replication associated genes are in light blue, and other genes are colored gray (hypothetical proteins) or blue (other).

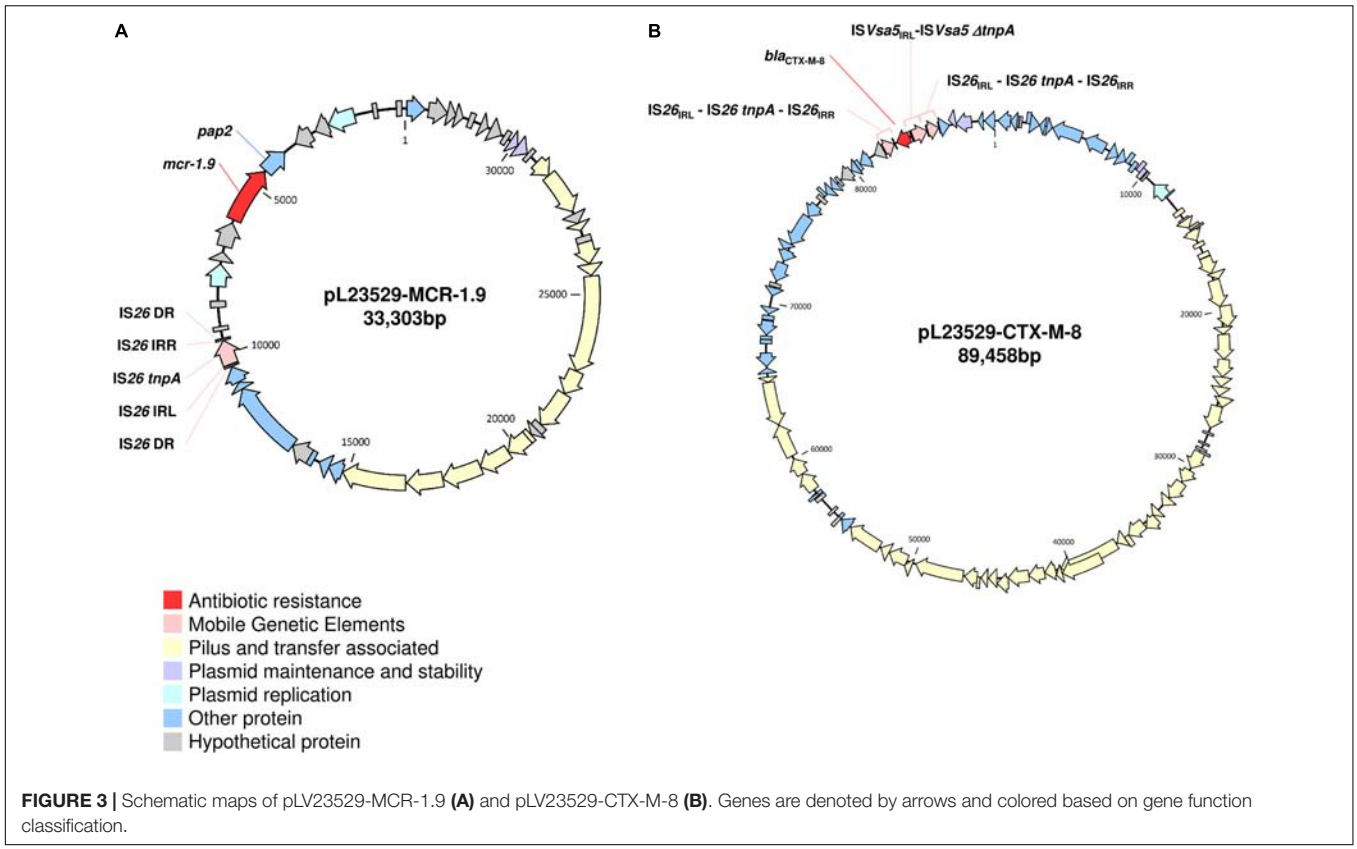

FIGURE 3 | Schematic maps of pLV23529-MCR-1.9 **(A)** and pLV23529-CTX-M-8 **(B)**. Genes are denoted by arrows and colored based on gene function classification.

TABLE 2 | Comparison of IncX4-pLV23529-MCR-1.9 with the top six *mcr-1*-harboring plasmids showing the highest identities (>99.9%, E-value 0.0), in different *E. coli* isolates.

IncX4-type Plasmid (bp)	*E. coli* strain (MLST[a])	Source/Country/Year	Identity (%)	Mismatches/ gap opens (No. of nucleotides)	Query alignment overlap (%)	pLV23529-MCR-1.9 Alignment overlap (%)	Plasmid GenBank Acc. No.
pMCRpoa (33,511)	3431F (ST744)	Human patient/Brazil/2014	99.98	**15/1**	99.4	100.0	CM007714
pI4EC007a (35,098)	14Ec007 (ST301)	Human patient/China/2014	99.98	**15/1**	94.9	100.0	CP024132
pMCR-1-NY (33,304)	MDR56 (ST117)	Human patient /United States/2015	99.97	**19/1**	100.0	100.0	CP019908
pICBEC72Hmcr (33,304)	ICBEC72H (ST101)	Human patient/Brazil/2016	99.95	**17/0**	100.0	100.0	CP015977
pI4EC033b (33,301)	14EC033 (ST2064)	Human patient/China/2014	99.92	**16/3**	100.0	100.0	CP024149
pICBEC7Pmcr (34,992)	ICBEC7P (ST10)	Magellanic penguins/Brazil/2013	99.92	**16/3**	95.2	100.0	CPO17246

[a]*MLST accordingly with Warwick scheme (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).*

phenicol (*cmlA1-type* and *floR*-type), sulphamethoxazole (*sul3*), tetracycline [*tet(A)*-type and *tet(M)*-type], trimethoprim (*dfrA12*), and colistin (*mcr-1-type*), justifying the phenotype noticed. Additionally, several unknown mutations in the *ampC* (promoter region), *parC*, 16S *rrsB*, 16S *rrsC*, 23S and *pmrB* chromosomal genes were detected, the last gene being described as the primary mechanism for the development of chromosomally encoded resistance to polymyxins (Phan et al., 2017).

The named *mcr-1.9*, differed from *mcr-1* by one-point mutation (T1238C), leading to Val413Ala substitution. The MCR-1 protein contains a transmembrane domain and a phosphoethanolamine (PEA) transferase domain with 8α, 12β, and 12η units (Gao et al., 2016). The amino acid substitution of MCR-1.9 occurred in the region between η7 e η8 of the PEA transferase domain, which have been found not to influence the function of MCR-1 (Gao et al., 2016).

The *mcr-1.9* genetic context was characterized by an IS*26* element upstream of the *mcr-pap2* element and by the absence of IS*Apl1* (**Figure 1**), which is in accordance with other studies about *mcr-1* gene (Veldman et al., 2016; Sun et al., 2017). The *mcr-1.9* gene can be mobilized within an IS*Apl1*-flanked

composite transposon (Tn*6330*), although many sequences have been identified without IS*Apl1* or with just a single copy (Snesrud et al., 2018). Indeed, it has been described that initially IS*Apl1* was presumably involved in the transposition of the *mcr-1* cassette and then was lost, contributing for the stability of *mcr* gene on IncX4 plasmids (Sun et al., 2017; Snesrud et al., 2018).

The PMCR-encoding gene was found in an IncX4 plasmid (pLV23529-MCR-1.9), showing highest identities (>99.9%) with six IncX4-type *mcr-1*-harboring plasmids identified worldwide, in unrelated *E. coli* isolates, mainly collected from human patients (**Figure 2** and **Table 2**). Indeed, all belonged to different MLST, which might suggest a resistance plasmid dissemination across strains (plasmid outbreak) rather than clonal transmission of MCR-1-type-producing strains. Furthermore, no *E. coli* LV23529 closely related isolates were detected among those currently deposited in the public database BacWGSTdb (Ruan and Feng, 2016), which reinforce the importance of the horizontal gene transfer in this study.

Like pLV23529-MCR-1.9, the six plasmids (**Table 2**) doesn't have the IS*Apl1* element. Hence, similarities may suggest that the one-point mutation (T1238C) in *mcr-1.9* occurred on the X4 plasmid, since mobilization of *mcr-1* occurs as part of a composite transposon (Tn*6330*) and that structures lacking the downstream IS*Apl1* are not capable of mobilization (Snesrud et al., 2018). The IS*26* upstream of the *mcr-pap2* element is flanked by an 8bp direct repeat (**Figure 3A**), indicating that its insertion wouldn't seems to be related to the *mcr-1.9* context, justifying the differences found with other IncX4 *mcr-1*-harboring plasmids. IncX4 plasmid has been widely implicated in the spread of MCR-1 gene in Europe (Caniaux et al., 2017). In Portugal, this plasmid type is circulating among diverse hosts (humans, pigs, poultry), being responsible for hospital-based outbreak caused by MCR-1 plus KPC-3-producing *K. pneumoniae* (Mendes et al., 2018), as well as for the diffusion of this PMCR at the farm level (Kieffer et al., 2017). Indeed, IncX4 plasmids seem to be efficiently transferred at different temperatures and different lack-of-fitness burdens among bacterial hosts, which may facilitate the transfer of *mcr-type* among *Enterobacteriaceae* (Lo et al., 2014; Wu R. et al., 2018). The pLV23529-MCR-1.9 plasmid backbone contains all the core genes common to IncX plasmids involved in segregation, stability, replication, and conjugative transfer of the plasmid (**Figure 3A**), namely the IncX-type pilus synthesis operon (*pilX1-pilX11*). However, pLV23529-MCR-1.9 was mobilizable, but not self-transmissible. Of note, we found a one-point mutation (G64T), leading to Asp22Tyr substitution, in the PilX1, a peptidoglycan hydrolase involved in T-DNA plasmid transfer. This mutation might explain why the attempts to conjugate *mcr-1.9* from *E. coli* LV23529 were unsuccessful (Chen et al., 2009).

Further plasmid analysis revealed the presence of two other plasmids: IncF [F2:A-:B-], IncR and the colicinogenic IncI1-ST113-carrying the $bla_{\text{CTX-M-8}}$ (pLV23529-CTX-M-8, **Figure 3B**). Of note, the *mcr-1.9*-positive isolate, co-harboring $bla_{\text{CTX-M-8}}$ and $bla_{\text{TEM-1}}$ genes, is here reported for the first time in an *E. coli* isolate of animal origin. In fact, $bla_{\text{CTX-M-8}}$ gene is rarely detected in Europe in isolates of animal origin (Börjesson et al., 2016), but in humans seems to be emerging (Eller et al., 2014). Indeed, a recent phylogenetic study suggested an increasing trend of co-existence and transmission of $bla_{\text{CTX-M}}$ and *mcr-1* in both clinical medicine and veterinary medicine (Wu C. et al., 2018).

In conclusion, the presence of PMCR-encoding genes, such as MCR-1.9, in food-producing animals represents a potential threat to humans, as it is located in mobile genetic elements that have the potential to spread horizontally. As mentioned, in Portugal, PMCR is an emerging problem and its international spread is a worrying reality (Beyrouthy et al., 2017; Gilrane et al., 2017).

AUTHOR CONTRIBUTIONS

VM designed the study, performed the molecular experiments and bioinformatics analysis, interpreted the data, and wrote the manuscript. LC, RR, and EF performed the microbiological and molecular experiments. CS and LV performed the Illumina genome sequencing experiments. MC designed the study, wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Biofilm Forming Antibiotic Resistant Gram-Positive Pathogens Isolated From Surfaces on the International Space Station

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The International Space Station (ISS) is a closed habitat in a uniquely extreme and hostile environment. Due to these special conditions, the human microflora can undergo unusual changes and may represent health risks for the crew. To address this problem, we investigated the antimicrobial activity of AGXX®, a novel surface coating consisting of micro-galvanic elements of silver and ruthenium along with examining the activity of a conventional silver coating. The antimicrobial materials were exposed on the ISS for 6, 12, and 19 months each at a place frequently visited by the crew. Bacteria that survived on the antimicrobial coatings [AGXX® and silver (Ag)] or the uncoated stainless steel carrier (V2A, control material) were recovered, phylogenetically affiliated and characterized in terms of antibiotic resistance (phenotype and genotype), plasmid content, biofilm formation capacity and antibiotic resistance transferability. On all three materials, surviving bacteria were dominated by Gram-positive bacteria and among those by *Staphylococcus*, *Bacillus* and *Enterococcus* spp. The novel antimicrobial surface coating proved to be highly effective. The conventional Ag coating showed only little antimicrobial activity. Microbial diversity increased with increasing exposure time on all three materials. The number of recovered bacteria decreased significantly from V2A to V2A-Ag to AGXX®. After 6 months exposure on the ISS no bacteria were recovered from AGXX®, after 12 months nine and after 19 months three isolates were obtained. Most Gram-positive pathogenic isolates were multidrug resistant (resistant to more than three antibiotics). Sulfamethoxazole, erythromycin and ampicillin resistance were most prevalent. An *Enterococcus faecalis* strain recovered from V2A steel after 12 months exposure exhibited the highest number of resistances ($n = 9$). The most prevalent resistance genes were *ermC* (erythromycin resistance) and *tetK* (tetracycline resistance). Average transfer frequency of erythromycin, tetracycline and gentamicin resistance from selected ISS isolates was 10^{-5} transconjugants/recipient. Most importantly, no serious human pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA) or

vancomycin-resistant Enterococci (VRE) were found on any surface. Thus, the infection risk for the crew is low, especially when antimicrobial surfaces such as AGXX® are applied to surfaces prone to microbial contamination.

Keywords: antimicrobial surface, gram-positive human-pathogenic bacteria, antibiotic resistance, biofilm, conjugative transfer, International Space Station, hostile environment

INTRODUCTION

The International Space Station is an isolated habitat in a hostile environment. Microgravity, solar and cosmic radiation alter the immune-regulatory responses of the crew rendering them more susceptible to bacterial infections (Sonnenfeld, 2005; Crucian et al., 2008; Guéguinou et al., 2009). The microorganisms in the spaceship are human-derived; they originate from the crew and helpers who prepare the mission. The spaceship provides a special environmental niche for microorganisms, which directly or indirectly affect the health, safety or performance of the crew (Taylor, 2015). Microgravity can affect the virulence (Nickerson et al., 2004; Wilson et al., 2007; Rosenzweig et al., 2010; Crabbé et al., 2011), growth kinetics (Klaus et al., 1997; Kacena et al., 1999; Nickerson et al., 2004) and biofilm formation of microorganisms (Mauclaire and Egli, 2010). To assess the risk microorganisms pose to astronauts, the composition and properties of microbial communities in spaceships were analyzed. Two hundred and thirty-four bacterial and fungal species were found on the MIR space station, among those strong biofilm formers. *Staphylococcus* spp., followed by *Bacillus* spp. and *Corynebacterium* spp. were abundant in air as well as in surface samples (Novikova, 2004; Novikova et al., 2006). Schiwon et al. (2013) analyzed ISS samples from air and crewmembers in-flight and post-flight. *Bacillus* spp., *Staphylococcus* spp. and *Enterococcus* spp. were the most prevalent. 75.8% of the isolates exhibited resistance to one or more antibiotics. Corresponding resistance genes were found in 86% of the antibiotic-resistant bacteria. In 86.2% of the isolates horizontal transfer genes were detected. Eighty-three percent of the isolates were able to form biofilms (Schiwon et al., 2013).

Under spaceflight conditions, bacteria were shown to exhibit enhanced secondary metabolite and extracellular polysaccharide production as well as enhanced biofilm formation (Mauclaire and Egli, 2010; Vukanti et al., 2012). In space, the cell wall of *S. aureus* was significantly thicker than in the same strain grown on Earth (Novikova et al., 2006; Taylor, 2015). Various bacteria exhibited enhanced virulence, increased antibiotic resistance and differential gene expression under space conditions (Horneck et al., 2010; Yamaguchi et al., 2014; Taylor, 2015). Thus, these bacteria could spread their virulence and/or antibiotic resistance genes through horizontal gene transfer (HGT) and turn harmless bacteria into potential pathogens.

HGT is mediated by mobile genetic elements (MGEs), such as conjugative plasmids, conjugative transposons, integron-specific gene cassettes, or phages that are able to facilitate their own transfer. Plasmid-mediated HGT plays a primordial role in the emergence of new pathogens (Frost et al., 2005; Garbisu et al., 2018). Schiwon et al. (2013) found conjugative plasmids in bacterial isolates from the ISS and could demonstrate that some of these strains were able to transfer their antibiotic resistance genes to other bacteria. The HGT rate was shown to be higher in microbial biofilms than in planktonic cultures (Holmes et al., 2015). Biofilms represent a protected mode of microbial growth and confer significant survival advantages in hostile environments (Li et al., 2007; Thallinger et al., 2013). Thus, biofilm forming organisms show increased resistance to antibiotics, either due to decreased penetration of the antibiotic through the biofilm matrix or due to expression of more complex biofilm-specific resistance mechanisms.

Multiple antibiotic resistant and strong biofilm forming *Staphylococcus* and *Enterococcus* isolates detected on the ISS could pose an increased health risk on the crew (Schiwon et al., 2013). Several studies report, that bacteria from astronauts in-flight were more resistant to antibiotics due to enhanced biofilm formation or changes in cell morphology, e.g., thicker cell walls than isolates obtained from the same individuals either pre- or post-flight. As medical aid on the ISS is restricted, there is an urgent need for new antimicrobial materials, which can be used there to prevent infections by multi-resistant biofilm forming bacteria.

Heavy metals, e.g., copper and silver, have been known for a long time to possess antimicrobial activity. Silver was officially approved as an antimicrobial agent in the twentieth century (Chopra, 2007; Schäberle and Hack, 2014; Guridi et al., 2015; Vaishampayan et al., 2018). However, after the discovery of antibiotics the use of metals to combat bacterial infections has declined (Chopra, 2007; Grass et al., 2011). Later on, due to the increased occurrence of antibiotic resistant pathogens, silver and copper have again found widespread use, both in medicine and in everyday life (Maillard and Hartemann, 2012; Warnes and Keevil, 2013; Schäberle and Hack, 2014). These metals are easy to use as coatings on a variety of substrates and have a lethal effect on bacteria and fungi via the so-called contact killing (Grass et al., 2011). Silver is one of the best-studied bactericidal agents in water supplies (Russell and Hugo, 1994; Rohr et al., 1999; Vonberg et al., 2008; Vaishampayan et al., 2018). However, as occurred with antibiotics, bacteria have also developed resistance mechanisms against silver (Gupta et al., 1999). Like the excessive use of antibiotics, the extended use of silver is questioned due to its toxicity to the environment as well as to the human body (Landsdown, 2010). Plain ruthenium is not applied as antibacterial agent, but antibacterial activity has been demonstrated for ruthenium(II) polypyridyl complexes (Bolhuis et al., 2011; Li et al., 2011, 2015).

Due to the increasing resistance of bacteria to both antibiotics and commonly used antimicrobial metals, there is an urgent need to develop new approaches to combat bacterial infections. A new

antimicrobial surface coating is AGXX® consisting of micro-galvanic elements of the two noble metals, silver and ruthenium, surface-conditioned with ascorbic acid (Vaishampayan et al., 2018). Both metals can be galvanically applied to diverse surfaces such as stainless steel, plastics, or cellulose fibers. The coating proved to be active against both Gram-positive and Gram-negative bacteria, but also against filamentous fungi, yeasts and some viruses (Guridi et al., 2015; Landau et al., 2017a,b; Vaishampayan et al., 2018). Recently, we demonstrated that it efficiently inhibits the growth of MRSA (Vaishampayan et al., 2018). The postulated mode of action is based on the formation of reactive oxygen species, particularly superoxide anions (Meyer, C., personal communication), which affect biomolecules, such as nucleic acids, proteins, and lipids. AGXX® has self-regenerating activity based on two coupled redox reactions taking place on the micro-galvanic silver and ruthenium elements on the surface of the material. They result in effective regeneration of the coating (Clauss-Lendzian et al., 2018).

In this study, we investigated the long-term antimicrobial effect of two different antimicrobial coatings. Three sets of V2A steel samples (uncoated, silver-coated, AGXX®-coated) were exposed and analyzed after six, 12, and 19 months on the ISS. Seventy-eight human pathogenic bacteria, which survived on the antimicrobial coatings or on the uncoated steel carrier (control) were phylogenetically affiliated and further characterized. The number of human pathogenic isolates decreased from V2A steel ($n = 39$) to V2A-Ag ($n = 31$) to V2A-AGXX® ($n = 8$). After 6 months of exposure, no bacteria survived on AGXX®, whereas six human pathogens were obtained after 12 and two after 19 months. From all materials, predominantly staphylococci and bacilli were isolated. Multi-antibiotic resistant, plasmid harboring staphylococcal and enterococcal ISS isolates transferred erythromycin, gentamicin and tetracycline resistance with average transfer frequencies of 10^{-5} transconjugants/recipient.

MATERIALS AND METHODS

Preparation of Antimicrobial Metal Sheets

The material was provided by Largentec GmbH, Berlin, Germany. V2A (DIN ISO 1.4301) stainless steel sheets were used as reference material and as base material for Ag and AGXX® coatings. The coatings were prepared as described in detail in Clauss-Lendzian et al. (2018). Prior to use in the experiments, the metal sheets (coated and uncoated) were autoclaved at 121°C for 20 min. The metal sheets had a size of 4 cm^2 each and were placed on the door to the bathroom of the ISS. Three sets of test sheets, one for each time point, - time points 12 and 19 months thus representing a cumulative bacterial load—were exposed on the ISS.

Reference Strains

Bacterial strains used as reference in biofilm formation assays and PCRs or as recipients in mating experiments are listed in **Table 1**. *Staphylococcus* and *Enterococcus* strains were grown in Tryptic Soy Broth (TSB, Sigma-Aldrich Chemie GmbH, Munich, Germany) or Brain Heart Infusion broth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) at 37°C with shaking. *Bacillus* strains were grown in Lysogeny Broth (LB, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) at 30°C with shaking.

Bacteria Isolation and Phylogenetic Affiliation

Bacteria were isolated from V2A steel surfaces (uncoated, Ag-coated, AGXX®-coated) exposed on the ISS for 6, 12 and 19 months, respectively. The bacteria were detached from the surfaces by rinsing with Phosphate Buffered Saline (PBS) followed by cultivation in Reasoner's 2A broth (R2A, Lab M Limited, Heywood, England) at 25° and 37°C under shaking. Appropriate dilutions of the cultures were passaged several times onto R2A agar until pure isolates were obtained. Isolates were phylogenetically affiliated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics MALDI Biotyper system) according to the manufacturer's instructions (Bruker Daltonics). Mass spectra were compared with the MALDI-BDAL Database (Version 3.1, 7311rntries). If identification with MALDI-TOF MS failed, the isolate was sent for 16S rRNA gene sequencing (SMB Ruedersdorf, Germany). Analysis of the 16S rDNA sequences was performed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and ChromasPro (Version 2.1.8). The isolates are denominated according to following scheme (i) the material they were isolated from, (ii) the exposure time on the ISS in months, and (iii) the order of isolation, e.g., *E. faecalis* V2A-12-03 was isolated from uncoated V2A steel after 12 months exposure, and it is the third isolate obtained from this material at this time-point.

Biofilm Screening Assay

Biofilm formation test was carried out according to Vaishampayan et al. (2018). *E. faecalis* T9 and *S. aureus* 04-02981, both strong biofilm formers, were used as positive controls (Schiwon et al., 2013; Vaishampayan et al., 2018). For *Staphylococcus* spp., TSB, for *E. faecalis,* BHI medium was used as negative control (Schiwon et al., 2013). Biofilm formation was measured in EnSpire Multimode Plate Reader 2300-0000 (Perkin Elmer, Turku, Finland) at 570 nm (OD_{570}). The assays were performed in triplicates. Normalized biofilm formation was calculated by dividing the biofilm measure at OD_{570} by the bacterial growth at OD_{600}. Biofilm classification criteria were applied according to Nyenje et al. (2013).

Antibiotic Disc Diffusion Method

Antibiotic resistance of the isolates toward 15 different antibiotics was analyzed with the disc diffusion method (discs from Oxoid, Wesel, Germany) on Mueller Hinton agar (Sifin diagnostic GmbH, Berlin, Germany) according to the guidelines of the Clinical and Laboratory Standards Institute, (CLSI, 2013). Details are given in **Table 2**. Each test was performed in triplicates. For sulfamethoxazole (RL25), no comparable data were found for Staphylococci, Enterococci and Bacilli. Thus, isolates lacking an inhibition zone were classified as resistant, those without inhibition zone were classified as susceptible.

TABLE 1 | Bacterial species used as references for AB-R-screening, biofilm formation, plasmid isolation, and in biparental mating.

Species	Genotype/Characteristics	References
Bacillus subtilis		
BD662	pBD90 [*ermD*]; *trpC2, thr-5*	Schiwon et al., 2013
BD1156	pBD370 [*ermG*]; *leu, mer, hisH*	Schiwon et al., 2013
Enterobacter cloacae DSM46348	*ampC*	Schiwon et al., 2013
Enterococcus casseliflavus UC73	*aph(2)-Id, vanC*	Schiwon et al., 2013
Enterococcus gallinarum SF9117	*aph(2)-Ic, vanC, ermB*	Schiwon et al., 2013
Enterococcus faecalis		
DS16	*tetM*; pAD1, pAD2	Schiwon et al., 2013
RE25	pRE25 [*ermB*, cat_{pIP501}, *aph(3)-III, sat4, ant(6)-Ia, tra*$^{+}$], *tetM*	Schiwon et al., 2013
JH2-2	pIP501 [cat_{pIP501}, *ermB, tra*$^{+}$]	Schiwon et al., 2013
T9	clinical isolate, Tet^{R}, strong biofilm former	Creti et al., 2006
OG1X	$Strep^{R}$, $Gent^{R}$, protease-negative	Schiwon et al., 2013
MISSEX 78	pre_{pSK41}, *aph3-III, ermB, ermD, tetM*, cat_{pIP501}	
TU-79	*aph3-III, ermB, tetM, tetO*, cat_{pIP501}, pre_{pSK41}	Schiwon et al., 2013
Enterococcus faecium		
SF11770	*aac(6)-Im, aph(2)-Ib, aac(6)-Ii, ant(4)-Ia, ant(6)-Ia, aph(3)-III, ermB, sat4, tetL, tetM, vanA, vanZ*	Schiwon et al., 2013
Escherichia coli		
PS84	*qnrS, sul2*	Broszat et al., 2014
Hm06-20	*qnrA, sul1*	Broszat et al., 2014
Lactococcus lactis K214	pK214 [*tetS, catLM, mdt(A), str, mob*$^{+}$]	DSMZ
Klebsiella pneumoniae		
K2-78	*qnrB*	Broszat et al., 2014
DSM 16609	*blaSHV-5*	Broszat et al., 2014
Staphylococcus aureus		
SK5428	pSK41 [*ant(4)-Ia (synonym: aadD), aac(6)-Ie-aph(2)-Ia, ble, qacC, tra*$^{+}$]	Schiwon et al., 2013
DSM13661	*mecA*	Schiwon et al., 2013
04-02981	strong biofilm former, methicillin resistant, *ermA*	Nuebel et al., 2010
Staphylococcus epidermidis MISSEX 66	pre_{pSK41}, *aph3-III*	Schiwon et al., 2013
Staphylococcus haemolyticus VPS617	*tetK, mph(C), ermC, msr, blaZ, mecA, dfrA, aph(3)-III, aph(2)-Ia, aac(6)-Ie, ant(6)-IalnorA, sat4*	Schiwon et al., 2013

DSMZ, German collection of microorganisms and cell cultures, Braunschweig. AB-R, antibiotic resistance; $Strep^{R}$*,* Tet^{R}*, tetracycline resistance;* $Gent^{R}$*, gentamicin resistance; AB-R genes for resistance against ampicillin, ampC (E. cloacae DSM46348), ciprofloxacin, qnrA (E. coli Hm06-20), qnrB (K. pneumoniae K2-78), qnrS (E. coli PS84); erythromycin, ermA (S. aureus 04-02981), ermB (E. faecium SF11770), ermC (S. haemolyticus VPS617), ermD (B. subtilis BD662), ermG (B. subtilis BD1156); gentamicin, aac(6′)-Ie-aph(2″)-Ia (E. faecium SF11770), aph(2″)-Ib (E. faecium SF11770), aph(2″)-Ic (E. gallinarum SF9117), aph(2″)-Id (E. casseliflavus UC73); kanamycin, aadD [S. aureus SK5428 (pSK41)], aph(3′)-III (E. faecalis RE25) against oxacillin, mecA (S. aureus DSM13661); ß-lactams, blaSHV-5 (K. pneumoniae DSM13661), blaZ (S. haemolyticus VPS617); sulfamethoxazole, sul1 (E. coli Hm06-20), sul2 (E. coli PS84), and tetracycline, tetK (S. haemolyticus VPS617), tetL (E. faecium SF11770), tetM (E. faecium SF11770), tetO (E. faecalis TU-79), tetS (L. lactis K214).*

PCR Assays

For the PCR assays, cell lysates prepared from 100 μL overnight cultures were used. Cell pellets were re-suspended in 20 μL lysis buffer (50 mM NaOH, 0.25% sodium dodecyl sulfate) and incubated at 95°C for 20 min. Prior to use in PCR, they were diluted 1:10 with distilled water. Twenty-five microliter PCR reactions contained 0.125 μL Taq-Polymerase (5 U/μL), 2.5 μL 1x PCR buffer, 0.2 μM of each primer (**Table 3**), 0.5 μL of deoxynucleoside triphosphates (200 μM) and 1 μL template DNA (lysate). DNA amplifications were carried out in a Biometra T3 Thermocycler (Analytik Jena AG, Jena, Germany). The temperature profiles are given in **Supplementary Table 1**.

Plasmid DNA Isolation

Plasmid DNA from Staphylococci was extracted as described in Schiwon et al. (2013) with some minor modifications. After washing the plasmid DNA with 70% ethanol, 1 μL of RNase A (10 μg/mL; Merck KGaA, Darmstadt) and 3 μL of Proteinase K (20 mg/mL; Merck KGaA, Darmstadt) were added, followed by 1 h incubation at room temperature. Plasmid DNA extraction from Enterococci was performed as described in (Schiwon et al., 2013).

Mating Assays

On basis of multiple antibiotic resistance and occurrence of plasmids >20 kbp, ISS isolates were selected as donors for biparental matings. As recipients, the methicillin resistant clinical isolate, *S. aureus* 04-02981 and the *E. faecalis* lab strain OG1X were selected. Details on all of the matings are given in **Table 4**. Overnight cultures of Staphylococci were diluted 1:5 in TSB medium, overnight cultures of Enterococci 1:5 in BHI medium containing the appropriate antibiotics (**Table 4**) and grown until OD_{600} = 0.5. Donors and recipients were washed with PBS prior to mixing in 1:10 ratio, spotted onto a TSA plate

TABLE 2 | Antibiotic disc diffusion method.

Antibiotic	Abbreviation	Concentration μg/mL	References		
			***Staphylococcus* spp.**	***Enterococcus* spp.**	***Bacillus* spp.**
Ampicillin	AMP	10	CLSI, 2013	EUCAST, 2013	Mohammadou et al., 2014
Chloramphenicol	C	30	EUCAST, 2013	Liofilchem®, 2017[a]	Mohammadou et al., 2014
Ciprofloxacin	CIP	5	EUCAST, 2013	EUCAST, 2013	Banerjee et al., 2011
Gentamicin	CN	10	EUCAST, 2013	Oliveira et al., 2010	Banerjee et al., 2011
Clindamycin	DA	10	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]
Doxycycline	DO	30	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]
Erythromycin	E	15	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]	Mohammadou et al., 2014
Kanamycin	K	4	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]
Cephalothin	KF	30	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]
Meropenem	MEM	10	Liofilchem®, 2017[a]	Andrews, 2007	Liofilchem®, 2017[a]
Oxacillin	OX	5	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]	Liofilchem®, 2017[a]
Tigecycline	TCG	5	EUCAST, 2013	EUCAST, 2013	EUCAST, 2013
Tetracycline	TE	10	EUCAST, 2013	Andrews, 2007	EUCAST, 2013
Vancomycin	VA	30	EUCAST, 2013	Tamanna et al., 2014	EUCAST, 2013

For Enterococcus spp., inhibition zones from DA10, E5, K5, KF30, OX5 and for Bacillus spp., inhibition zones from DA10, DO30, K5, KF30, MEM10, OX5, TCG15, TE10 were evaluated as for Staphylococcus spp.

[a] *http://www.liofilchem.net/antibioticdisc/.*

for *Staphylococcus* recipients, on a BHI plate for *Enterococcus* recipients and incubated for 16 h at 37°C. Cells were recovered in 1 mL PBS, serial dilutions were incubated at 37°C on TSA/BHI plates for 16 h to enumerate transconjugants. The number of recipients was also determined after 16 h at 37°C. Transfer frequencies are given as number of transconjugants/recipient.

RESULTS

Bacterial Isolates From V2A, V2A-Ag and V2A-AGXX® Surfaces

A total number of 112 bacterial isolates were recovered from the different materials after the three time intervals (6, 12, and 19 months). 73.6% of the isolates are human pathogens. All isolates were identified to species level by MALDI-TOF biotyping or 16S rRNA gene sequencing. In total, 49 isolates were obtained after 6 months, 51 after 12 months and 22 after 19 months exposure of the antimicrobial materials on the ISS. The non-human pathogenic bacteria include *Bacillus* spp. ($n = 20$; *B. astrophaeus, B. infantis, B. korlensis, B. licheniformis, B. megaterium, B. niacini, B. pumilus, B. tequilensis,* and *B. thuringiensis*), *Enhydrobacter aerosaccus* ($n = 2$), *Micrococcus yunannensis* ($n = 1$), *Paenibacillus polymyxa* ($n = 1$), *Pseudomonas psychrotolerans* ($n = 1$), and *Staphylococcus capitis* ($n = 9$). To assess the infection risk for the crew, only the human-pathogenic bacteria ($n = 78$) were characterized in terms of biofilm formation and antibiotic resistance profile. Three *Moraxella osloensis* strains obtained from V2A ($n = 1$) and V2A-Ag ($n = 2$) after 19 months were the only Gram-negative human-pathogenic bacteria. Seventy-five Gram-positive human pathogenic bacteria were selected for the study: 32 from 6 months, 21 from 12 months, and 22 isolates from 19 months exposure.

The longer the exposure time of the three materials, the higher was the bacterial diversity on the materials (**Figure 1** and **Table 5**). All pathogenic isolates recovered from V2A and V2A-Ag after six months belonged to the genus *Staphylococcus*. No bacteria were recovered from AGXX® after 6 months. In total, 17 Staphylococci and three *E. faecalis* were detected after 12 months: Seven Staphylococci and one *E. faecalis* strain from V2A, six Staphylococci from V2A-Ag and four Staphylococci and two *E. faecalis* strains from AGXX®. After 19 months, seven Staphylococci and seven *B. cereus* strains were recovered from V2A and three Staphylococci and three *B. cereus* strains from V2A-Ag. Only one *B. cereus* and one *S. epidermidis* strain were isolated from AGXX® after 19 months exposure. In summary, a considerably lower bacterial number survived on AGXX® than on the other two surfaces. Nevertheless, the silver coating also showed a slight antimicrobial effect.

Biofilm Formation of Pathogenic ISS-Isolates

Biofilm formation of pathogenic isolates was determined by crystal violet staining, biofilms were classified according to Nyenje et al. (2013). The data are summarized in **Table 5**. Twenty-six V2A-isolates showed strong (66.7% of all pathogenic isolates from V2A-steel), ten moderate (25.5%) and three weak (7.8%) biofilm formation. Twenty-one isolates from V2A-Ag were strong biofilm formers (91.3% of all pathogenic isolates from V2-Ag), one isolate showed moderate (4.3%), one isolate weak (4.3%) biofilm formation. Of the eight AGXX®-isolates, six had strong (75.0% of all pathogenic isolates from V2A-AGXX®) and two (25%) weak biofilm formation ability. Interestingly, 43 Staphylococci (52 pathogenic Staphylococci in total) formed strong biofilms (82.7%), eight Staphylococci (15.4%) were moderate biofilm formers and one *Staphylococcus* isolate (1.9%)

TABLE 3 | Oligonucleotides used for the detection of antibiotic resistance genes.

Primer	Antibiotic	Sequence (5′ → 3′)	GenBank Acc. No.	Amplicon size [bp]	Annealing temperature [°C]	References
aac6-aph2a fw	gentamicin	GCCAGAACATGAATTACACGAG	NC_005024	610	56	Schiwon et al., 2013
aac6-aph2a rev		CTGTTGTTGCATTTAGTCTTTCC				
aadD_pSK41 fw	kanamycin	TGTCGTTCTGTCCACTCCTG	AF051917	525	62	Schiwon et al., 2013
aadD_pSK41 rev		ATGAATGGACAACCGGTGAG				
ampC fw	ampicillin	GTGACCAGATACTGGCCACA	AJ005633	821	55	Schiwon et al., 2013
ampC rev		TTACTGTAGCGCCTCGAGGA				
aph(2)-Ib fw	gentamicin	AGGATGCCCTTGCATATGATGAAGCGACGT	AF207840	449	56	Schiwon et al., 2013
aph(2)-Ib rev		ATCAGCATAAGGCGCCGGAAGTAGCAGAAA				
aph(2)-Ic fw	gentamicin	AGCATACAATCCGTCGAGTCGCTTGGTGAG	U51479	641	56	Schiwon et al., 2013
aph(2)-Ic rev		CTGGCGCTGCAACTTGCTGAGTTCATGAAT				
aph(2)-Id fw	gentamicin	GTGGTTTTTACAGGAATGCCATC	AF016483	134	56	Schiwon et al., 2013
aph(2)-Id rev		CCCTCTTCATACCAATCCATATAACC				
aph3-III fw	kanamycin	CCGCTGCGTAAAAGATAC	X92945	592	56	Schiwon et al., 2013
aph3-III rev		GTCATACCACTTGTCCGC				
blaSHV-5 fw	ß-lactams	TGTTAGCCACCCTGCCGCT		825	60	Schiwon et al., 2013
blaSHV-5 rev		GTTGCCAGTGCTCGATCAG				
blaZ fw	ß-lactams	TTAAAGTCTTAC CGAAAGCAG	AB245468	777	60	Sidhu et al., 2002
blaZ rev		TAAGAGATTTGC CTATGCTT				
ermA fw	erythromycin	ACGATATTCACGGTTTACCCACTTA	WP_001072201	584	58	Khan et al., 1999
ermA rev		AACCAGAAAAACCCTAAAGACACG				
ermB fw	erythromycin	GCATTTAACGACGAAACTGGCT	U00453	572	56	Schiwon et al., 2013
ermB rev		GACAATACTTGCTCATAAGTAATGGT				
ermC fw	erythromycin	CGTAACTGCCATTGAAATAGACC	V01278	519	58	Schiwon et al., 2013
ermC rev		TCCTGCATGTTTTAAGGAATTG				
ermD fw	erythromycin	CGGGCAAATATTAGCATAGACG	M29832	463	56	Schiwon et al., 2013
ermD rev		ATTCTGACCATTGCCGAGTC				
ermG fw	erythromycin	TGCAGGGAAAGGTCATTTTAC	M15332	483	56	Schiwon et al., 2013
ermG rev		AACCCATTTCATTACAAAAGTTTC				
mecA fw	methicillin	TAATAGTTGTAGTTGTCGGGTTTG	X52593	707	60	Schiwon et al., 2013
mecA rev		TAACCTAATAGATGTGAAGTCGCT				
qnrB (B1, B7) fw	fluoroquinolone	AGCGGCACTGAATTTAT		497	56	Broszat, 2014
qnrB (B1, B7) rev		GTTTGCTGCTCGCCAGTC				
qnrS1 fw	fluoroquinolone	GGAAACCTACAATCATACATA		600	56	Broszat, 2014
qnrS1 rev		GTCAGGATAAACAATACC				
sul1 fw	sulfamethoxazole	CACCGGAAACATCGCTGCA		158	60	Broszat, 2014
sul1 rev		AAGTTCCGCCGCAAGGCT				

(Continued)

TABLE 3 | Continued

Primer	Antibiotic	Sequence (5′ → 3′)	GenBank Acc. No.	Amplicon size [bp]	Annealing temperature [°C]	Reference
sul2 fw	sulfamethoxazole	CTCCGATGGAGGCCGGTAT		190	60	Broszat, 2014
sul2 rev		GGGAATGCCATCTGCCTTGA				
tetK_pT181 fw	tetracycline	TTTGAGCTGTCTTGGTTCATTG	CP000045	539	55	Schiwon et al., 2013
tetK_pT181 rev		AGCCCACCAGAAAACAAACC				
tetL	tetracycline	CATTTGGTCTTATTGGATCG	AY081910	475	55	Schiwon et al., 2013
tetL		ATTACACTTCCGATTTCGG				
tetM fw	tetracycline	GAACTCGAACAAGAGGAAAGC	M85225	729	55	Schiwon et al., 2013
tetM rev		ATGGAAGCCCAGAAAGGAT				
tetO fw	tetracycline	GGATGGCATACAGGCACAGA	M18896	737	55	Schiwon et al., 2013
tetO rev		GTTTGGATCATAGGGAGAGGAT				
tetS fw	tetracycline	TGGTCAACGGCTTGTCTATG	X92946	546	55	Schiwon et al., 2013
tetS rev		AGCCCAGAAAGGATTTGGAG				

Reference strain for bla-SHV-5, K. pneumoniae DSM13661; sul1, E. coli Hm06-20; sul2, E. coli PS84.

formed only weak biofilms. Of the *B. cereus* isolates (11 in total), one showed strong (9.1%), three moderate (27.3%), and seven (63.6%) showed only weak biofilm formation capacity. In contrast, all three *E. faecalis* isolates were classified as strong biofilm formers.

Prevalence of Antibiotic Resistances in the Pathogenic Isolates

Antibiotic sensitivity testing of the isolates showed that 32.0% of the pathogenic isolates were resistant to <3 of the tested antibiotics (15 antibiotics in total were tested), 68.0% were resistant to three or more antibiotics. Eighteen isolates had three antibiotic resistances (24.0% of the isolates), 23 isolates were resistant to four antibiotics (30.7% of the isolates), six isolates were resistant to five antibiotics (8.0%) and three isolates had six different antibiotic resistances (4.0%). *E. faecalis* V2A-12-03 (from V2A steel after 12 months) had the highest number of resistances. It was resistant to nine different antibiotics, chloramphenicol, gentamicin, clindamycin, doxycycline, erythromycin, kanamycin, meropenem, sulfamethoxazole, and tetracycline.

In total, 97.3% of the pathogenic Gram-positive isolates were resistant to 25 μg sulfamethoxazole, 74.7% were resistant to 15 μg erythromycin and 61.3% were resistant to 10 μg ampicillin. Interestingly, these resistances were found with similar prevalence on all three surfaces, irrespective of the exposure time. No oxacillin resistant *Staphylococcus* was detected, whereas all *B. cereus* isolates (all of the 11 isolates after 19 months) were resistant to oxacillin. One *B. cereus* (V2A-AG-19-10) isolate showed resistances against six different antibiotics (AMP10, C30, E15, K5, OX5, RL25).

None of the isolates was resistant to vancomycin or cephalothin. Two *E. faecalis* (V2A-AGXX-12-02,-03) isolates were resistant to six antibiotics (CN10, DA10, E15, K5, RL25, TE10) and one *E. faecalis* isolate (V2A-12-03) was resistant to nine antibiotics (C30, CN10, DA10, DO30, E15, K5, MEM10, RL25, TE10). Meropenem resistance was detected in three strains, *E. faecalis* V2A-12-03, *S. hominis* V2A-12-04, and *S. hominis* V2A-AG-12-05.

To identify the resistance genes in the isolates resistant to three or more antibiotics (68.0% of the pathogenic isolates), gene-specific PCRs were performed. Gentamicin [*aac6-aph2a* ($n = 1$), *aph(2)-ic* ($n = 2$)], kanamycin [*aadD* ($n = 4$), *aph3-III* ($n = 5$)], erythromycin [*ermC* ($n = 19$), *ermB* ($n = 1$)], and tetracycline [*tetK* ($n = 9$), *tetL* ($n = 1$), t*etM* ($n = 1$)] resistance genes were detected in the number of isolates indicated in parentheses (**Table 5**). *ermC* and *tetK* were the most prevalent resistance genes. No sulfamethoxazole (*sul1, sul2*) resistance gene was found in any of the isolates.

Plasmid Profiles of ISS-Isolates

Exemplarily, plasmid DNA profiles of 20 out of total 45 staphylococcal isolates resistant to three or more antibiotics forming moderate or strong biofilms were obtained (**Table 5**). All isolates contained plasmids <20 kbp, the number of plasmid bands varied from one to seven. Interestingly, 17 isolates harbored plasmids >20 kbp likely able to self-transfer. Plasmid

TABLE 4 | Efficiency of gentamicin, erythromycin, and tetracycline resistance transfer from ISS-isolates to *S. aureus* 04-02981 and *E. faecalis* OG1X.

Donor	Selection	Recipient	Selection	Transconjugant selection	Transfer efficiency per recipient
E. faecalis V2A-12-03	CN10	*S. aureus* 04-02981	CIP5	CN10, CIP5	8.3×10^{-4}
E. faecalis V2A-AGXX-12-02	CN10		CIP5	CN10, CIP5	-
E. faecalis V2A-AGXX-12-03	CN10		CIP5	CN10, CIP5	9.2×10^{-7}
S. hominis V2A-6-05	TE10		CIP5	TE10, CIP5	2.7×10^{-5}
S. hominis V2A-6-06	TE10		CIP5	TE10, CIP5	3.3×10^{-8}
S. haemolyticus V2A-12-08	TE10		CIP5	TE10, CIP5	6.6×10^{-7}
S. hominis V2A-AG-12-06	TE10		CIP5	TE10, CIP5	4.2×10^{-4}
S. hominis V2A-AGXX-12-01	TE10		CIP5	TE10, CIP5	6.8×10^{-4}
S. hominis V2A-AGXX-12-03	TE10		CIP5	TE10, CIP5	-
S. haemolyticus V2A-AGXX-12-05	TE10		CIP5	TE10, CIP5	1.2×10^{-7}
S. hominis V2A-6-03	E15	*E. faecalis* OG1X	SM1000	E15, SM1000	1.6×10^{-4}
S. hominis V2A-6-11	E15		SM1000	E15, SM1000	4.2×10^{-4}
S. aureus V2A-6-13	E15		SM1000	E15, SM1000	9.1×10^{-5}
S. aureus V2A-6-14	E15		SM1000	E15, SM1000	2.5×10^{-4}
E. faecalis V2A-12-03	E15		SM1000	E15, SM1000	-
S. haemolyticus V2A-AGXX-12-05	E15		SM1000	E15, SM1000	-
S. hominis V2A-6-05	E15		CN10	E15, CN10	-
S. hominis V2A-6-06	E15		CN10	E15, CN10	7.9×10^{-6}
S. hominis V2A-6-09	E15		CN10	E15, CN10	-
S. hominis V2A-6-10	E15		CN10	E15, CN10	-
S. hominis V2A-6-12	E15		CN10	E15, CN10	-
S. hominis V2A-12-04	E15		CN10	E15, CN10	1.6×10^{-6}
S. haemolyticus V2A-12-08	E15		CN10	E15, CN10	1.1×10^{-6}
S. hominis V2A-AG-12-05	E15		CN10	E15, CN10	-
S. hominis V2A-AG-12-06	E15		CN10	E15, CN10	5.1×10^{-6}
S. hominis V2A-AG-12-08	E15		CN10	E15, CN10	-
E. faecalis V2A-AGXX-12-03	E15		CN10	E15, CN10	-
S. hominis V2A-AGXX-12-06	E15		CN10	E15, CN10	1.1×10^{-5}

CIP, Ciprofloxacin; CN, gentamicin; E, erythromycin; SM, streptomycin; TE, tetracycline. (-) no transconjugants were obtained. The concentrations of the antibiotics are given in μg/mL.

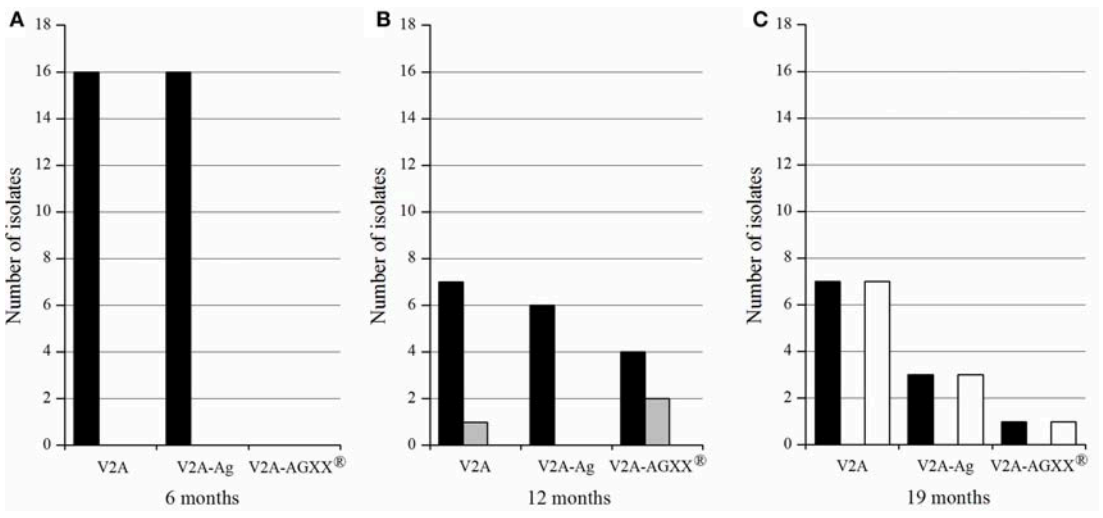

FIGURE 1 | Number of Gram-positive pathogenic bacteria recovered from the different materials (V2A, V2A-Ag, V2A-AGXX®), after 6 months **(A)**, 12 months **(B)**, and 19 months **(C)** exposure on the ISS. In black, *Staphylococcus* spp.; gray, *E. faecalis*; white, *B. cereus*.

TABLE 5 | Characteristics of all isolates from V2A, V2A-Ag, V2A-AGXX® after 6, 12, and 19 months.

No.	Name	Species	Biofilm formation	AB-R		Number of plasmid-bands >20 kbp
				Phenotype	Genotype	
1	V2A-6-01	*S. hominis*	+++	AMP10, DA10, E15, RL25	n.d.	n.d.
2	V2A-6-02	*S. hominis*	++	DA10, E15, RL25	*ermC*	n.d.
3	V2A-6-03	*S. hominis*	+++	AMP10, DA10, E15, RL25	*ermC*	1
4	V2A-6-04	*S. hominis*	+++	DA10, E15, RL25	n.d.	n.d.
5	V2A-6-05	*S. hominis*	+++	AMP10, E15, RL25, TE10	*tetK, ermC*	2
6	V2A-6-06	*S. hominis*	++	AMP10, E15, RL25, TE10	*tetK, tetO, ermC*	2
7	V2A-6-07	*S. hominis*	++	AMP10, E15, RL25, TE10	*tetK, ermC*	1
8	V2A-6-08	*S. hominis*	++	DA10, E15, RL25	n.d.	n.d.
9	V2A-6-09	*S. hominis*	+++	AMP10, DA10, E15, RL25	*tetK*	2
10	V2A-6-10	*S. hominis*	+++	AMP10, E15, RL25, TE10	n.d.	0
11	V2A-6-11	*S. hominis*	+++	AMP10, E15, RL25, TE10	*ermC*	1
12	V2A-6-12	*S. hominis*	+++	AMP10, E15, RL25, TE10	*ermC*	1
13	V2A-6-13	*S. aureus*	+++	AMP10, E15, K5, RL25	*ermC*	1
14	V2A-6-14	*S. aureus*	+++	AMP10, DA10, E15, K5, RL25	*tetK*	0
15	V2A-6-15	*S. hominis*	+++	AMP10, E15, RL25, TE10	*ermC*	1
16	V2A-6-16	*S. aureus*	++	AMP10, E15, K5, RL25, TE10	*ermC*	n.d.
17	V2A-AG-6-01	*S. epidermidis*	+++	E15, RL25	*ermC*	n.d.
18	V2A-AG-6-02	*S. epidermidis*	+++	E15, RL25	n.d.	n.d.
19	V2A-AG-6-03	*S. aureus*	+++	AMP10, E15, RL25	*ermC*	n.d.
20	V2A-AG-6-04	*S. aureus*	+++	AMP10, E15, RL25	*ermC*	n.d.
21	V2A-AG-6-05	*S. aureus*	+++	AMP10, E15, K5, RL25	n.d.	n.d.
22	V2A-AG-6-06	*S. epidermidis*	+++	E15, RL25	n.d.	n.d.
23	V2A-AG-6-07	*S. aureus*	++	AMP10, E15, RL25	n.d.	n.d.
24	V2A-AG-6-09	*S. epidermidis*	+++	E15, RL25	n.d.	n.d.
25	V2A-AG-6-11	*S. aureus*	+++	AMP10, E15, RL25	n.d.	n.d.
26	V2A-AG-6-14	*S. aureus*	+++	AMP10, E15, RL25	n.d.	n.d.
27	V2A-AG-6-15	*S. epidermidis*	+++	AMP10, E15, RL25	n.d.	n.d.
28	V2A-AG-6-16	*S. epidermidis*	+++	E15, RL25	*ermC*	n.d.
29	V2A-AG-6-21	*S. epidermidis*	+++	E15, RL25	n.d.	n.d.
30	V2A-AG-6-22	*S. epidermidis*	+++	E15, RL25	n.d.	n.d.
31	V2A-AG-6-23	*S. epidermidis*	+++	AMP10, E15, RL25	n.d.	n.d.
32	V2A-AG-6-24	*S. epidermidis*	+++	E15, RL25	n.d.	n.d.
33	V2A-12-02	*S. hominis*	++	AMP10, E15, MEM10, RL25	n.d.	n.d.
34	V2A-12-03	*E. faecalis*	+++	C30, CN10, DA10, DO30, E15, K5, MEM10, RL25, TE10	*aac6-aph2a, aph3-III, ermC*	1
35	V2A-12-04	*S. hominis*	+++	AMP10, E15, K5, RL25, TE10	*aph3-III, tetO*	1
36	V2A-AGXX-12-01	*S. hominis*	+++	AMP10, E15, RL25, TE10	*tetK, tetL*	1
37	V2A-AG-12-03	*S. hominis*	+++	AMP10, K5, RL25	*aadD, aph3-III*	1
38	V2A-AG-12-04	*S. hominis*	+++	AMP10	n.d.	n.d.
39	V2A-AG-12-05	*S. hominis*	+++	AMP10, E15, K5, MEM10, RL25	*aph3-III, aadD, ermC*	0
40	V2A-AGXX-12-02	*E. faecalis*	+++	CN10, DO30, K5, RL25	*aph(2)-ic, aph3-III, aadD*	1
41	V2A-AGXX-12-03	*E. faecalis*	+++	CN10, DA10, E15, K5, RL25, TE10	*aph(2)-ic, aadD, aph3-III, ermB, tetK, tetM, tetO*	1
42	V2A-AG-12-06	*S. hominis*	+++	AMP10, DO30, E15, RL25, TCG15, TE10	*tetK*	1
43	V2A-12-07	*S. epidermidis*	+++	AMP10, DO30, RL25	n.d.	n.d.
44	V2A-AGXX-12-05	*S. haemolyticus*	+++	C30, DO30, E15, RL25, TE10	*tetO*	3

(Continued)

TABLE 5 | Continued

No.	Name	Species	Biofilm formation	AB-R		Number of plasmid-bands >20 kbp
				Phenotype	Genotype	
45	V2A-AG-12-08	*S. hominis*	+++	AMP10, DA10, E15, RL25	*ermC*	2
46	V2A-12-08	*S. haemolyticus*	+++	C30, DO30, E15, RL25, TE10	*tetK*	1
47	V2A-AG-12-10	*S. hominis*	+++	RL25	n.d.	n.d.
48	V2A-12-09	*S. lugdunensis*	+++	RL25	n.d.	n.d.
49	V2A-AGXX-12-06	*S. hominis*	+++	AMP10, DA10, E15, RL25	*ermC*	1
50	V2A-AGXX-12-09	*S. hominis*	+++	AMP10, RL25	n.d.	n.d.
51	V2A-12-10	*S. caprae*	+++	AMP10, C30, E15, RL25	*ermC*	n.d.
52	V2A-12-12	*S. lugdunensis*	+++	RL25	0	0
53	V2A-12-13	*S. hominis*	+++	AMP10, DA10, RL25	n.d.	n.d.
54	V2A-19-02	*S. hominis*	++	AMP10, E15, RL25	n.d.	n.d.
55	V2A-AGXX-19-01	*S. epidermidis*	+	E15, RL25	n.d.	n.d.
56	V2A-19-03	*S. hominis*	+++	E15, RL25	n.d.	n.d.
60	V2A-19-05	*S. hominis*	+++	E15, RL25	n.d.	n.d.
61	V2A-19-06	*S. hominis*	+++	E15, RL25	n.d.	n.d.
62	V2A-19-07	*S. hominis*	+++	E15, RL25	n.d.	n.d.
63	V2A-AG-19-06	*S. hominis*	+++	RL25	n.d.	n.d.
64	V2A-19-09	*S. hominis*	+++	E15, RL25	n.d.	n.d.
65	V2A-19-10	*S. hominis*	+++	E15, RL25	n.d.	n.d.
66	V2A-AG-19-07	*S. hominis*	+++	E15, RL25	n.d.	n.d.
67	V2A-AG-19-08	*S. hominis*	+++	E15, RL25	n.d.	n.d.
68	V2A-19-14	*B. cereus*	+++	AMP10, K5, OX5, RL25	n.d.	n.d.
69	V2A-19-15	*B. cereus*	+	AMP10, K5, OX5, RL25	n.d.	n.d.
70	V2A-AG-19-10	*B. cereus*	+	AMP10, C30, E15, K5, OX5, RL25	n.d.	n.d.
71	V2A-19-16	*B. cereus*	+	AMP10, OX5, RL25, TCG15	n.d.	n.d.
72	V2A-19-17	*B. cereus*	++	AMP10, K5, OX5, RL25	n.d.	n.d.
73	V2A-19-18	*B. cereus*	+	AMP10, OX5	n.d.	n.d.
74	V2A-AG-19-12	*B. cereus*	+	AMP10, OX5, RL25	n.d.	n.d.
75	V2A-19-19	*B. cereus*	++	AMP10, OX5, RL25	n.d.	n.d.
76	V2A-19-20	*B. cereus*	++	AMP10, OX5, RL25	n.d.	n.d.
77	V2A-AG-19-14	*B. cereus*	+	AMP10, E15, OX5, RL25	n.d.	n.d.
78	V2A-AGXX-19-03	*B. cereus*	+	AMP10, OX5, RL25	n.d.	n.d.

AB-R, antibiotic resistance; +++, strong biofilm formation; ++, moderate biofilm formation; +, weak biofilm formation; AMP, ampicillin; C, chloramphenicol; CN, gentamicin; DA, clindamycin; DO, doxycycline; E, erythromycin; K, kanamycin; MEM, meropenem; OX, oxacillin; RL, sulfamethoxazole; TCG, tigecycline; TE, tetracycline. The concentration of the antibiotics is given in μg/mL. ermC, ermB, erythromycin resistance genes; aac6-aph2a, aph(2)-ic, gentamicin resistance genes; blaSHV-5, ß-lactam antibiotic resistance gene; aadD, aph3-III, kanamycin resistance genes; tetK, tetM, tetL, tetO, tetracycline resistance genes. n.d., not determined.

DNA profiles were also obtained from the three *E. faecalis* isolates; all of them were multi-drug resistant and strong biofilm formers. All, *E. faecalis* V2A 12-03, *E. faecalis* V2A-AGXX-12-02 and *E. faecalis* V2A-AGXX-12-03 harbored putative conjugative plasmids >20 kbp. Interestingly, *E. faecalis* V2A 12-03 showed additionally three small plasmid bands in the size range between 3 and 1.5 kbp.

Mating Experiments

Antibiotic resistance transfer of selected ISS-isolates was studied in biparental matings (Laverde et al., 2017). Isolates resistant to tetracycline, gentamicin or erythromycin and harboring a plasmid >20 kbp were selected as donors, plasmid-free *S. aureus* 04-02981 and *E. faecalis* OG1X were used as recipients. The results of all of the matings are summarized in **Table 4**. Gentamicin resistance transfer to *S. aureus* 04-02981 was successful from *E. faecalis* V2A-12-03 (*aac6-aph2a*-encoded gentamicin resistance) with a transfer frequency of 8.3×10^{-4} transconjugants/recipient and from *E. faecalis* V2A-AGXX-12-03 (*aph(2)-ic*-encoded gentamicin resistance) with a transfer frequency of 9.2×10^{-7} transconjugants/recipient.

Erythromycin resistance transfer of six *Staphylococcus* donors harboring the *ermC* resistance gene and of three *Staphylococcus* donors harboring an unknown erythromycin resistance gene to *E. faecalis* OG1X was successful with transfer frequencies in the range of 1.1×10^{-6} to 4.2×10^{-4} transconjugants/recipient. Tetracycline resistance transfer from four *S. hominis* strains and two *S. haemolyticus* strains to *S. aureus* 04-02981 was successful.

Three of the staphylococci harbored only the *tetK* resistance gene, one only *tetO*. One *S. hominis* strain harbored *tetK* and *tetO*, while another harbored the resistance genes *tetK* and *tetL*. Tetracycline resistance transfer frequencies varied considerably ranging from 3.3×10^{-8} to 6.8×10^{-4} transconjugants/recipient.

Ten out of the 17 successful matings were randomly chosen for plasmid DNA isolation of the transconjugants. In nine of the ten matings large plasmid bands comparable in size to those of the donors were detected in the transconjugants (data not shown).

DISCUSSION

We proved that the novel antimicrobial coating AGXX® strongly reduced the bacterial load on surfaces on the ISS particularly prone to microbial contamination. However, over time—with exposure times >6 months—some nosocomial pathogens survived even on the novel antimicrobial coating. Moreover, an interesting shift in the composition of the microbial communities was observed over time.

Bacterial Survivors Isolated From V2A, V2A-Ag and V2A-AGXX® Surfaces

The bacterial community isolated from the surfaces was always dominated by *Staphylococcus* spp. (63.4% of 112 isolates) and *Bacillus* spp. (24.1%) irrespective of the exposure time. 46.4% of the Staphylococci are affiliated to the coagulase-negative Staphylococci, including pathogens such as *S. epidermidis*, *S. lugdunensis*, *S. haemolyticus*, *S. hominis*, and *S. caprae*. Coagulase-positive Staphylococci such as *S. aureus* (8.9% of all isolates) were only found on V2A and V2A-Ag surfaces after 6 months exposure. *B. cereus* (9.8% of all isolates) was the only pathogenic *Bacillus*. Only three *E. faecalis* (2.7% of all isolates) were recovered from V2A and V2A-AGXX® surfaces after 12 months. Schiwon et al. reported that predominantly *S. hominis*, *S. aureus,* and *S. epidermidis* were detected on crew-members and in air-filters on the ISS (Schiwon et al., 2013). *S. hominis* and *S. epidermidis* were the most prevalent Staphylococci associated with debris collected from the crew's quarters on the ISS (Venkateswaran et al. (2014). In addition, 13 *E. faecalis* and eight *B. cereus* strains were isolated from the crew and air-filters on the ISS (Schiwon et al., 2013). Taking the data of this study and others together (Van Houdt et al., 2012; Schiwon et al., 2013; Venkateswaran et al., 2014; Mayer et al., 2016) it can be concluded that the bacteria that survived on the different surfaces were predominantly human-associated.

Microbial diversity on the test materials increased over time. After 6 months only Staphylococci and Bacilli were found, after 12 months Staphylococci, Bacilli, *E. faecalis* and one *P. polymyxa* strain were isolated while after 19 months, Staphylococci, Bacilli, *E. aerosaccus*, *M. osloensis, M. yunnanensis*, and *P. psychrotolerans* were recovered. Novikova (2004) reported a similar diversity on surfaces on the MIR station including Staphylococci, Bacilli, *Micrococcus*, *Moraxella*, and *Pseudomonas*.

A decline of the number of Gram-positive human-pathogens recovered from V2A ($n = 39$) to V2A-Ag ($n = 28$) to V2A-AGXX® ($n = 8$) was observed. In total, only 12 bacteria were recovered from AGXX®-coated surfaces after 12 and 19 months exposure. AGXX® showed a pronounced antimicrobial effect, it reduced the microbial load by 79.5%. Silver also had a slight antimicrobial effect, it reduced the microbial load by 28.2%.

The antimicrobial test-materials are static surfaces, where dead cells, dust particles and cell debris can deposit. These deposits might interfere with the direct contact between the antimicrobial surface and the bacteria, which is required for effective antimicrobial activity of contact catalysts, such as Ag and AGXX®. Over time the deposits might have grown in size and thickness resulting in increasing interference with the antimicrobial activity. Possibly, this effect could explain that after 6 months no bacteria were recovered from AGXX®, whereas with prolonged exposure time a few bacteria escaped the antimicrobial action.

Strong Biofilm Forming ISS Isolates

Biofilms provide microbes shelter from antimicrobials and the host immune system (Foulquié Moreno et al., 2006; Chen and Wen, 2011; Rafii, 2015; Qi et al., 2016; Hall and Mah, 2017). Bacterial biofilms have been associated with diseases such as cystic fibrosis, periodontitis, and nosocomial infections on catheters and prosthetic heart valves (Storti et al., 2005; Delle Bovi et al., 2011). Eradication of biofilms is difficult due to impaired penetration of antibiotics and the decreased host immune response. Thus, they can pose a health risk to immunosuppressed people, such as the crew on the ISS.

Most *Staphylococcus* and all *Enterococcus* isolates from this study formed strong biofilms. *B. cereus* isolates were more diverse in terms of biofilm formation: Seven isolates produced a weak, three a moderate and only one produced a strong biofilm. The fact that all bacterial isolates were able to form biofilms could be due to the long exposure to adverse space conditions.

Prevalence of Antibiotic Resistances in Human Pathogenic Isolates

Astronauts have a suppressed immune response in-flight and as a consequence they are more susceptible to bacterial infections (Van Houdt et al., 2012; Taylor, 2015). The potential infection by pathogenic Staphylococci and Enterococci increases with duration of the mission (Schiwon et al., 2013). Therefore, treatability of bacterial infections on the ISS and on even longer space missions with limited amounts of antimicrobial drugs available is a health concern which has to be tackled.

In this study, all Gram-positive pathogenic isolates were resistant to at least one antibiotic. 68.0%, mostly Staphylococci, were multidrug resistant (resistant to more than three antibiotics). After 12 months exposure, also multi-resistant Enterococci occurred, one *E. faecalis* strain from V2A steel and two *E. faecalis* strains from V2A-AGXX®. *E. faecalis* V2A-12-03 had with nine resistances the largest number of resistances.

In total, the isolates were tested against 15 different antibiotics. Seven different antibiotic resistances were found after 6 months, 13 after 12 months and after 19 months, the number of resistances equalled the number after 6 months. This could be partly due to the fact, that the number of resistances in the Staphylococci declined after 19 months (most isolates had only

one or two resistances), while *Bacillus* strains with more than three resistances came up.

All Staphylococci had similar antibiotic resistance profiles. The *B. cereus* isolates after 19 months exposure also showed similar resistance profiles. Most Bacilli and Staphylococci were resistant to ampicillin and erythromycin. Gentamicin resistance only occurred in *E. faecalis* isolates. Interestingly, all of them were also resistant to kanamycin. *E. faecalis* strains are known to be intrinsically resistant to low-level aminoglycosides (gentamicin, kanamycin) or have acquired high-level aminoglycoside resistance e.g., by uptake of *aac6-aph2a* or *aph(2)-ic* (Chow, 2000; Wendelbo et al., 2003; Dadfarma et al., 2013). As *E. faecalis* V2A-12-03 encodes *aac6-aph2a* and *E. faecalis* V2A-AGXX-12-02 and−03 encode *aph(2)-ic*, they are likely high level gentamicin resistant. *aac6-aph2a* was found on plasmids pSK41, pGO1, pLW1043, pSK1, pTEF1, on Tn*4001*-like transposons and on the chromosome (Schiwon, 2011). *aph(2)-ic* was found on conjugative plasmid pYN134 (Hollenbeck and Rice, 2012) in *E. gallinarum* but was shown to readily transfer to *E. faecalis* (Chow et al., 1997). Therefore, it is likely that gentamicin resistance spreads via these conjugative plasmids (Chow et al., 1997).

Most ISS-isolates were resistant to sulfamethoxazole, which interferes with bacterial synthesis of folic acid. It could be speculated that changes in the thickness of the cell wall due to exposure to space conditions might be involved in resistance to sulfamethoxazole by inhibiting the uptake of the antibiotic.

Most abundant resistance genes in the ISS-isolates were *ermC* and *tetK* coding for erythromycin and tetracycline resistance, respectively. Both genes are plasmid-borne and have been detected in Staphylococci of human origin (Schiwon et al., 2013). *ermC* was found on pSK41-like conjugative plasmid pUSA03 isolated from the community-acquired MRSA strain USA300 (Grohmann et al., 2003; Smillie et al., 2010; Schiwon et al., 2013). A pSK41-like plasmid could have spread *ermC* among the *S. aureus* strains V2A-6-13 and V2A-6-16, and between V2A-AG-6-03 and V2A-AG-6-04 isolated from the same material. Indeed, from *S. aureus* V2A-6-16 a plasmid >20 kbp was isolated. *ermB* is another plasmid-encoded erythromycin resistance gene. It is one of the 33 erythromycin resistance genes found in Staphylococci (Schiwon et al., 2013). However, *ermB* is not abundant in Staphylococci. No ISS-isolate from crew and air-filters harbored *ermB* (Zmantar et al., 2011; Schiwon et al., 2013). Also in this study, only *E. faecalis* V2A-AGXX-12-03 encoded *ermB*. De Leener et al. (2005) reported that *ermB* is present on Tn*1545*-like elements and that is likely associated with the occurrence of the tetracycline-resistance gene *tetM*. Interestingly, *E. faecalis* V2A-AGXX-12-03 harbored *tetM* along with *ermB*.

tetK is found on small mobilizable plasmids, which can be integrated into the *Staphylococcus* chromosome or into larger staphylococcal plasmids (Gillespie et al., 1987; Needham et al., 1994; Roberts, 2005). *tetO* and *tetK* can be found on pT181-like small mobilizable plasmids (Khan and Novick, 1983; Chopra and Roberts, 2001). *S. hominis* V2A-AGXX-12-01 (*tetK, tetO*) and *S. haemolyticus* V2A-AGXX-12-05 (*tetK*) likely carry pT181-like plasmids as small plasmid bands in the range of 2000–6000 bp were observed on the gel (data not shown). Both strains were isolated from the same material after the same time-period. Thus, the resistance genes might have spread via HGT among them. Along with *tetK,* pT181-like plasmids can carry *tetL* as well (Chopra and Roberts, 2001). Both genes were found in *S. hominis* V2A-AGXX-12-01.

Kanamycin resistance occurred both in Staphylococci and Enterococci. The kanamycin-resistance gene *aph3-III* was found in *S. hominis* V2A-12-04, *S. hominis* V2A-AG-12-05 and *E. faecalis* V2A-12-03, *E. faecalis* V2A-AGXX-12-02, and *E. faecalis* V2A-AGXX-12-03, all isolated from V2A and the two antimicrobial surfaces after 12 months. *aph3-III* is located on transposons of the Tn*916*-Tn*1545* type encoding a broad spectrum of resistances, toward tetracycline, macrolides, lincosamides, streptogramins, and kanamycin (Fons et al., 1997; Soge et al., 2008; Roberts and Mullany, 2011). The kanamycin-resistance gene *aadD* was detected in *S. hominis* V2A-AG-12-03, V2A-AG-12-05 and in *E. faecalis* V2A-AGXX-12-02 and V2A-AGXX-12-03. *aadD* is encoded on *S. aureus* plasmid pUB110 (4548 bp) (McKenzie et al., 1986; Allignet et al., 1998). As the two *S. hominis* and two *E. faecalis* strains were isolated from the same materials, V2A-Ag and V2A-AGXX®, respectively, transfer of the *aadD* gene might have taken place. *S. hominis* V2A-AG-12-05 showed plasmid-bands in the range of 2000-3000 bp and around 7000 bp likely indicating the presence of pUB110-like plasmids (data not shown). Occurrence of *aph3-III* and *aadD* genes in *Staphylococcus* and *Enterococcus* isolates from the ISS has already been reported (Schiwon et al., 2013).

Antibiotic Resistance Transfer of the ISS-Isolates

Plasmids are the key players in HGT of antibiotic resistances (Kohler et al., 2018). Twenty multidrug-resistant, biofilm forming human-pathogenic staphylococcal isolates obtained from the three different materials after 6, 12, and 19 months were applied to plasmid DNA isolation. All isolates harbored plasmids <20 kbp and 17 of them also harbored plasmids >20 kbp. Commonly, *S. aureus* strains contain one or more plasmids ranging in size from <2000 bp to >60 kbp (Kwong et al., 2008).

Fourteen of the 17 *Staphylococcus* isolates with large plasmids were applied as donors to biparental matings to test the transferability of tetracycline and erythromycin resistance. In total, six out of seven tetracycline resistance transfer experiments (*S. hominis* V2A-6-05, *S. hominis* V2A-6-06, *S. haemolyticus* V2A-12-08, *S. hominis* V2A-AG-12-06, *S. hominis* V2A-AGXX-12-01, and *S. haemolyticus* V2A-AGXX-12-05) were successful whereas nine out of 18 erythromycin resistance transfer experiments (*S. hominis* V2A-6-03, *S. hominis* V2A-6-06, *S. hominis* V2A-6-11, *S. aure*us V2A-6-13, *S. aureus* V2A-6-14, *S. hominis* V2A-12-04, *S. haemolyticus* V2A-12-08, *S. hominis* V2A-AG-12-06, and *S. hominis* V2A-AGXX-12-06) were successful. Thus, these nine isolates likely harbor conjugative elements encoding erythromycin resistance. Indeed, in *E. faecalis* OG1X transconjugants of four of these matings large plasmids similar in size to those of the donors were found. pSK41 (46.4 kbp) and pUSA03 (37 kbp) are well known staphylococcal conjugative plasmids. Both carry *ermC* (Berg et al., 1998; Kennedy et al.,

2010; Smillie et al., 2010) which was also detected in five of the successful donors.

Tetracycline resistance transfer frequencies from *S. hominis* V2A-6-05 (*tetK*), *S. hominis* V2A-6-06 (*tetK, tetO*), *S. haemolyticus* V2A-12-08 (*tetK*), *S. hominis* V2A-AG-12-06 (*tetK*), *S. hominis* V2A-AGXX-12-01 (*tetK, tetL*), *S. haemolyticus* V2A-AGXX-12-05 (*tetO*) to *S. aureus* 04-02891 ranged from 1.2×10^{-7} to 6.8×10^{-4} transconjugants/recipient. *tetK* is only rarely found on large staphylococcal plasmids. It is rather encoded on small mobilizable staphylococcal plasmids in the size range of 4.4 to 4.7 kbp, such as pT181 (Chopra and Roberts, 2001). Thus, in the successful matings with donors harboring *tetO* or *tetK* mobilizable pT181-like plasmids might have played a role in the transmission of the resistance to *S. aureus* 04-02981. As pT181 is non self-transmissible another conjugative element has participated in the transfer of the tetracycline resistance. All donors that were successful in the tetracycline resistance matings contained in addition to plasmid-bands <20 kbp at least one plasmid-band >20 kbp, which could represent the conjugative plasmid. Thus, it is likely that the successful donors harbor a pT181-like plasmid which was transferred by the help of a conjugative plasmid. Indeed, in *S. aureus* 04-02981 transconjugants from all of those matings large plasmids similar in size to those of the donors were detected. In addition, small plasmids in the size range of pT181-like plasmids were found in transconjugants of three of these matings.

Transfer frequency of gentamicin resistance (8.3×10^{-4} transconjugants/recipient) from *E. faecalis* V2A-12-03 (*aac6-aph2a*) to *S. aureus* 04-02891 was higher than from *E. faecalis* V2A-AGXX-12-03 (*aph(2)-ic*) to the same recipient (9.2×10^{-7} transconjugants/recipient). *aac6-aph2a* can be found on conjugative plasmids, such as pSK41, pGO1, pLW1043, pSK1, pTEF1, Tn*4001*-like transposons but also on the chromosome (Schiwon, 2011). The uptake of *aac6-aph2a* by *S. aureus* 04-02891 indicates that *E. faecalis* V2A-12-03 likely harbors one of these conjugative elements. Indeed, this observation was corroborated by isolation of a plasmid >20 kbp from *E. faecalis* V2A-12-03. *aph(2)-ic* was found on the 34-kbp conjugative plasmid pYN134 (Chow et al., 1997; Hollenbeck and Rice, 2012). The uptake of *aph(2)-ic* by *S. aureus* 04-02891 suggests that *E. faecalis* V2A-AGXX-12-03 likely harbors a pYN134-like plasmid. This argument was corroborated by the observation of a plasmid band >20 kbp for *E. faecalis* V2A-AGXX-12-03.

The data of this study confirm erythromycin and tetracycline resistance transfer in ISS-isolates from air-filters and the crew as reported by Schiwon et al. (2013). Further transfer studies between ISS-isolates could deepen our knowledge in the transmissibility of antibiotic resistances. However, no methicillin resistant Staphylococci and no vancomycin resistant enterococci were found. Thus, the generation of serious multi-resistant pathogens by horizontal transfer is unlikely.

Further Applications of the Antimicrobial Surface

AGXX® proved to be a long-term efficient antimicrobial, even under the harsh conditions on the ISS. The antimicrobial coating has been also successfully applied against other Gram-positive and Gram-negative pathogens. It also strongly reduced the bacterial load of *Legionella* and the highly pathogenic Shiga toxin-producing *E. coli* O104:H4 strain (Guridi et al., 2015). It is available in diverse application forms, such as powders, thin sheets, as coating on diverse plastic materials and on cellulose fleece and will be recently tested in the 4 months SIRIUS isolation study for future lunar flights.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

EG designed the project and supervised all the experiments. L-YS, KR, JF, WS, PO, and AV performed the experiments. L-YS, KR, and EG wrote the manuscript and designed the figures and tables. NN provided us access to the BIORISK experiment on the ISS and contributed with insightful discussions on the experimental design. All authors read and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00543/full#supplementary-material

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Clonally Diverse Methicillin and Multidrug Resistant Coagulase Negative Staphylococci Are Ubiquitous and Pose Transfer Ability Between Pets and Their Owners

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Sixty-eight owners and 66 pets, from 43 unrelated pet-owning households were screened for methicillin-resistant coagulase negative staphylococci (MRCoNS), potential cases of MRCoNS interspecies transmission (IT), and persistence. MRCoNS isolates were identified by microbiological and molecular tests. MLST-based phylogenetic analysis was performed in *Staphylococcus epidermidis* isolates. Antimicrobial susceptibility was evaluated using phenotypic and molecular methods. SCC*mec* type and the presence of biofilm-related *ica* locus was PCR-tested. Isolates suspected for MRCoNS IT cases were subjected to *Sma*I-PFGE analysis and individuals from positive households were followed-up for 1 year for carriage dynamics (every 3 months, T0–T4). Nineteen MRCoNS isolates from owners (27.9%) and 12 from pets (16.7%) were detected, coming from 20 households (46.5%). *S. epidermidis* was predominant (90 and 67% of human and animal strains, respectively), showing high phylogenetic diversity (16 STs among 24 strains). Methicillin-resistant *S. epidermidis* (MRSE) strains belonged to CC5 (75%), CC11 (12.5%), singleton S556 (8.3%), and S560 (4.17%). Significant host-associated differences were observed for resistance to aminoglycosides, co-trimoxazole, chloramphenicol (higher in animal isolates) and tetracycline (higher among human strains). Multidrug resistance (MDR) was common (68.4%) and associated with human strains. Great diversity of *ccr* and *mec* complexes were detected, most strains being non-typeable, followed by SCC*mec*IV and V. Over one third of isolates (most from owners), carried the *ica* locus, all MRSE CC5. Two sporadic IT cases (T0) were identified in owners and dogs from two households (4.7%), with diverse interspecies-exchanged clones detected along the sampling year, especially in dogs. A comparative analysis of all MRCoNS, with all nasal coagulase positive staphylococci (CoPS) recovered from the same individuals at T0, revealed that CoPS alone was predominant in owners and pets, followed by co-carriage of CoPS and MRCoNS in owners but single MRCoNS in pets. Statistical analyses revealed that owners are more prone to co-carriage and that co-existence of IT cases and co-carriage are positively interrelated. MRCoNS from

healthy owners and their pets are genetically heterogeneous MDR strains that are spread in the community. Therefore, pets also contribute to the dissemination of successful human clones. Owner-pet inhabitancy increases the risk for staphylococcal temporal concomitance with its subsequent risk for bacterial infection and genetic exchange.

Keywords: methicillin-resistant coagulase negative staphylococci, *Staphylococcus epidermidis*, multidrug resistance, interspecies transmission, carriage dynamics, co-carriage, owner, pet

INTRODUCTION

Staphylococci are normal commensal bacteria of the skin and mucous membranes of humans and other animals. They can be differentiated by their ability to produce coagulase. Coagulase positive staphylococci (CoPS), with *Staphylococcus aureus* as major representative in humans and *Staphylococcus pseudintermedius* in dogs, pose, in general, higher pathogenic potential than coagulase negative staphylococcal (CoNS) species (Becker et al., 2014). CoNS are less often involved in community-associated diseases, but represent one of the major nosocomial pathogens, and have a substantial impact on human life and health (Becker et al., 2014; May et al., 2014). In humans, *Staphylococcus epidermidis* is the most common species among CoNS infections (24–80%), and the most frequent cause of medical device-associated infections (Miragaia et al., 2009; Becker et al., 2014). Regardless of the sparse data available, CoNS have occasionally been confirmed as causative agents for different site infections in dogs (Malik et al., 2006; Kern and Perreten, 2013; LoPinto et al., 2015; Couto et al., 2016). Yet, their zoonotic potential and importance in veterinary medicine is unclear.

Staphylococci, especially CoNS, are notorious for their ability to accrue antimicrobial resistance (AMR) determinants and to produce a biofilm, which makes associated infections particularly difficult to treat (Miragaia et al., 2009; Becker et al., 2014). Further, methicillin resistance is normally associated with additional resistances, which may pose a risk for the AMR gene transfer between staphylococci with higher pathogenic properties, such as *S. aureus* (Bloemendaal et al., 2010). On top of this, multidrug resistant (MDR) strains drastically limit the therapeutic options available and represent a human and animal health problem.

Nasal *S. aureus* and *S. pseudintermedius* can be exchanged between owners and cohabitant pets, and such acquisition can persist over time (Gomez-Sanz et al., 2013a,b). However, no data are available on the incidence and diversity of MRCoNS in healthy owners and their companion animals at the household, on potential cases of interspecies transmission (IT) and on its persistence over time.

The potential association between owner-pet companionship and the concomitant carriage of more than one staphylococcal type (CoPS and MRCoNS), as well as the potential host tropism for these subpopulations is unknown, but is essential to appraise potential owner-pet cohabitation as a risk factor for staphylococcal acquisition, infection and transmission. In addition, simultaneous carriage of CoPS and MRCoNS represents a potential risk for AMR transfer, which is barely considered in AMR surveillance studies.

The **goal** of this study is to determine the nasal occurrence, diversity, clonal distribution, and molecular characterization of MRCoNS in healthy owners and their pets, residing in common households, as well as to address potential IT cases and their carriage dynamics. We subsequently analyzed the MRCoNS and concomitant CoPS nasal patterns to determine whether there was any bacterial species- and/or host-associated tropism.

MATERIALS AND METHODS

Study Population and Sampling Criteria

Individuals from 43 unrelated pet-owning households were sampled in La Rioja region (Northern Spain) for the nasal carriage of MRCoNS and for IT potential cases. IT was defined as the presence of the same MRCoNS clone in owner and cohabitant pet. Samples were taken from March 2009 to February 2011. Individuals tested were, in parallel, sampled for the nasal occurrence of CoPS (Gomez-Sanz et al., 2013b). Only MRCoNS were further characterized in this study. Inclusion criteria for households tested included healthy humans whose profession did not involve any direct animal contact. None of the individuals tested had received antimicrobial treatment within the 4 months prior sampling. Household recruitment was on a voluntary basis. Sixty-eight humans and 66 animals (54 dogs, 12 cats) were included (Gomez-Sanz et al., 2013a,b). All individuals gave written informed consent to participate in this study, as well as for the sampling of their animals. This study was included in a project approved by the Ethical Committee of Clinical Research of La Rioja (reference: METC 09-399/C). One to five owners and one to five pets were tested from each household, showing 10 different combinations. In most cases (19, 44.2%), only one person and one animal were sampled per household. Nine and 11 of the 43 household units included more than one pet (20.9%) and more than one owner (25.6%), respectively. Four households included both more than one animal and more than one owner (9.3%). In total, 36 of 66 pets lived with other sampled animals (dog/cat) (54.5%), while 40 of 68 owners lived with other sampled humans (58.8%). Of note, all cohabitant pets within a sampled household were included in the study whereas owners were not always all sampled. Swabs were transported to the lab within 5 h after sampling and were either immediately analyzed or stored at -20°C until further analysis.

Isolation and Identification of MRCoNS

Sampled nasal swabs were inoculated into Brain-Heart-Infusion broth (BHI, Difco) supplemented with 6.5% NaCl and incubated at 37°C for 24 h. One-hundred microliters were inoculated

on Oxacillin-Resistant-Staphylococcal-Agar-Base (ORSAB; OXOID) plates supplemented with 2 mg/L of oxacillin. Plates were incubated at 35°C for 24–48 h. All blueish to white (potential MRCoNS) colonies with different morphologies were sub-cultured on BHI agar and further studied. Preliminary identification of MRCoNS isolates was based on colony morphology, Gram staining, and catalase and DNase activities. Presence of the *mecA* gene was investigated by PCR in all isolates (Gomez-Sanz et al., 2013a). Identification of MRCoNS was performed by amplification and sequencing of the *sodA* gene in all *mecA* positive CoNS isolates (Poyart et al., 2001). In addition, isolates that were difficult to type by Multi Locus Sequence Typing (MLST) were also identified by amplification and sequencing of the 16S rRNA (Hogg and Lehane, 1999), and by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). When different isolates from the same individual were recovered, which belonged to the same bacterial species and shared the same AMR phenotype, only one isolate was further characterized. Individual nomenclature was as follows: household number (1–43) – isolate host [Human (H); dog (D); cat (C)]. – number of individuals when more than one (1–5).

Multi-locus Sequence Typing (MLST) of Methicillin Resistant *S. epidermidis* (MRSE) Isolates

All 25 MRSE isolates were subjected to MLST as recommended by Thomas et al. (2007). Two novel sets of primers for *aroE* (aroE-fw2: 5′-TTCATTATCGCATTGATGC-3′, aroE-rv2: 5′-TCAGCACCTTGATGAACGAA-3′) and *tpi* (tpi-fw2: 5′-TAGCCGGAAACTGGAAAATG-3′, tpi-rv2: 5′-GCACCTTCTAACAATTGTACG-3′) alleles were employed for isolates that could not be amplified with the standard primers. Allele and ST identification was used following the *S. epidermidis* MLST database[1]. The MLST data were analyzed using the goeBURST algorithm[2] for ST clustering within clonal complexes (CC) (as of November 2017). For this, Phyloviz2 grouping was generated by Hierarchical Clustering (Hamming Method, UPGMA) using allelic profiles (Nascimento et al., 2017). In addition, a phylogenetic relationship of concatenated sequences was investigated by the construction of a distance tree including metadata on isolates characteristics for each of the different MLST profiles obtained (CLC Genomics Workbench 10.0.1, Qiagen Bioinformatics).

Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Classification

The SCC*mec* type was determined based on the chromosomal cassette recombinase *ccr* gene/s and on the type of *mec* complex as described by Kondo et al. (2007), while confirmation of SCC*mec* type was tested using SCC*mec* primers described by Zhang et al. (2005). In addition, allele *ccrAB4*, present in SCC*mec* types VI and VIII (Oliveira et al., 2006) was included. Following this approach, cassettes I–IX could be identified.

Typeability of the SCC*mec* cassettes was defined as follows: (i) Typeable (T) SCC*mec* cassettes were considered those for which *ccr*, type of *mec* complex (Kondo et al., 2007) and/or SCC*mec* (Zhang et al., 2005) were identified; (ii) Non-Ascribed (NA) SCC*mec* types were those with a novel combination of *ccr*, *mec* complex, and/or SCC*mec*, and (iii) Non-Typeable (NT) were considered those that did not yield positive results with the primer sets used, per scheme. New SCC*mec* were defined as those enclosed within NA and NT categories.

Characterization of Antimicrobial Resistance Profile

Susceptibility to 17 antimicrobial agents was performed using an agar disk-diffusion method (CLSI, 2013). Antimicrobial agents tested were as follows (class of agent/s): penicillin, oxacillin [+ 2% NaCl], cefoxitin (β-lactams); gentamicin, kanamycin, tobramycin, streptomycin (aminoglycosides); co-trimoxazole (aminopyrimidine/sulfonamide); erythromycin (macrolides); clindamycin (lincosamides); tetracycline (tetracyclines); chloramphenicol (amphenicols); vancomycin (glycopeptides); ciprofloxacin (fluoroquinolones); mupirocin (pseudomonic acid); fusidic acid (steroids); and linezolid (oxazolidinones). Procedures and breakpoints were those proposed for CoNS in CLSI document M100-S23 (CLSI, 2013). For streptomycin and fusidic acid, the methods and breakpoints employed were those recommended by the Société Française de Microbiologie[3]. The double-disk diffusion test (D-test) was performed on all isolates to detect inducible clindamycin resistance (CLSI, 2013). Multidrug resistance (MDR) was considered when a resistance to > 3 antimicrobial classes was observed.

The presence of 33 AMR genes, in addition to the *mecA* gene, was investigated by PCR: *blaZ,* *tet*(K), *tet*(M), *tet*(L), *erm*(A), *erm*(B), *erm*(C), *erm*(T), *erm*(F), *mph*(C), *msr*(A)/*msr*(B), *lnu*(A), *vga*(A), *vga*(C), *aacA-aphD, aphA3, aadE*, *aadD*, *aadA, str*, *dfr*(A), *dfr*(D), *dfr*(G), *dfr*(K), *mupA*, *fexA, cfr,* cat_{pC194}, cat_{pC221}, cat_{pC223}, *fusB*, and *fusC* (Gomez-Sanz et al., 2013a,b). Positive controls from the collection of the University of La Rioja were included in each reaction.

Mutations within the quinolone resistance determining region (QRDR) of *gyrA* and *gyrB* genes (DNA gyrase subunits), and within *parC* and *parE* genes (DNA topoisomerase IV subunits) were investigated in ciprofloxacin resistant isolates (Yamada et al., 2008). The corresponding genes of the quinolone susceptible *S. epidermidis* strain ATCC 12228 (GenBank ac. no NZ_CP022247.1) were used as a reference for mutation detection and positioning within the gene.

Presence of Virulence Genes Involved in Biofilm Formation

PCR based determination of several genes involved in biofilm formation was implemented. Genes tested were the *S. aureus* biofilm matrix protein *bap* (Cucarella et al., 2001); the Staphylococcal intercellular adhesin (*icaADBC*) operon-containing genes *icaA*, *icaB*, *icaC,* and *icaD*, responsible for

[1]https://pubmlst.org/sepidermidis/

[2]http://goeBURST.phyloviz.net

[3]http://www.sfm-microbiologie.org/

the synthesis of the biofilm matrix polysaccharide intercellular adhesion (PIA) (Ziebuhr et al., 1999; Arciola et al., 2006); the transcriptional repressor of the *ica* locus, the *icaR* gene (Conlon et al., 2002); as well as the insertion sequence IS*256*, which has been observed to play a role in phase variation of virulence by *ica* locus in *S. epidermidis* (Ziebuhr et al., 1999).

Determination of Cases of Interspecies Transmission (IT)

The genetic relatedness of MRCoNS isolates suspected for cases of direct IT – i.e., those isolates of the same species recovered from cohabiting individuals that exhibited identical AMR profile, MLST for *S. epidermidis*, and SCC*mec* type – was addressed by Pulsed Field Gel Electrophoresis (PFGE) of the total DNA digested with a *SmaI* macro-restriction enzyme following the HARMONY protocol (Murchan et al., 2003).

Longitudinal Approach: Carriage Status Definition and IT Dynamics

All individuals from households with cases of direct IT were followed-up with for a year. For this, nasal samples from the anterior nares of owners and pets were studied once every 3 months (five sampling times in total, T0–T4) with a total of 24 additional samples analyzed (T0–T4). Studied subjects positive for MRCoNS in at least four of the five samplings (including T0) were considered persistent carriers; those positive in two or three samplings were defined as intermittent carriers; individuals positive in a single sampling were reported sporadic carriers; and those negative throughout the study were defined as non-carriers. Dynamics of the IT cases over time was defined likewise (persistent, intermittent, and sporadic).

MRCoNS and Coagulase Positive Staphylococci (CoPS) Individual and Household Concomitance

In a former study Gomez-Sanz et al. (2013a,b), all coagulase positive staphylococcal (CoPS) isolates recovered from the same individuals at the same sampling (T0) were characterized (36 *S. aureus* and 18 *S. pseudintermedius*). At this stage, we aimed at making a summative and comparative analysis of the MRCoNS and CoPS concomitant carriage of individuals tested in T0 and, subsequently, of respective households. Such concomitance was also analyzed along the longitudinal study with the individuals from households with cases of IT (**Supplementary File S1**). Potential association of concomitant carriage, host, and/or being involved in an IT case was evaluated.

Statistical Analysis

The characteristics of the owner and pet isolates were compared for consistent differences. Statistical analysis tests were performed in R (R Development Core Team, 2018). SCC*mec*, AMR, and *ica* locus profiles between owners and dogs were compared using the Fisher's Exact test. Potential significant differences in MRCoNS carriage and MRCoNS/CoPS co-carriage between owners and pets at individual and household level were likewise evaluated. Correlations between presence of *ica* locus and (i) bacterial species, (ii) CC, (iii) host, and (iv) household of origin were analyzed by dependence measure of variables using multivariable Logistic Regression test. Correlations between owner and pet cohabitation and bacterial nasal carriage, as well as between involvement in **IT** cases and bacterial simultaneous carriage (MRCoNS; CoPS), at individual and household level, were likewise evaluated [variables: (i) host, (ii) presence of more than one pet per household, (iii) involvement in IT case, (iv) bacterial concomitance]. Correlation analyses were performed using the Corrplot R package. All analyses were performed at a 95% confidence interval (CIs). The degree of genetic diversity for ST and SCC*mec* types was assessed by Simpon's Index of Diversity (SID). SID represents the probability (0 = low diversity, 1 = high diversity) that any two randomly selected species from the sample will be different. In this analysis, each ST or SCC*mec* element (*ccr*, *mec* complex combination) was considered a "type" or "species."

RESULTS

Occurrence of MRCoNS in Individuals and Households

Thirty-one MRCoNS isolates, 19 isolates from 19 owners (27.9%) and 12 isolates from 11 pets (16.7%) (14.81% dogs, 25% cats) were detected. MRCoNS species distribution in owners and pets is shown in **Figure 1**. *S. epidermidis* and *Staphylococcus lentus* were detected in both owners and pets. *Staphylococcus haemolyticus* was detected in one owner only and *Staphylococcus cohnii* and *Staphylococcus vitulinus* in dogs (the latter in two cohabitant dogs). One dog (1-D1, from household no. 1) carried one *S. lentus* and one *S. epidermidis* isolate (**Table 1**). For both, owners and pets, *S. epidermidis* was the predominant species, accounting for 89.5 and 66.0% of strains, respectively. In total, 25% of owners and 12.1% of pets (9.3% among dogs, 25% in cats) carried MRSE.

In 20 of the 43 (46.5%) tested households there was at least one individual (either owner and/or pet) positive for MRCoNS. In two households (4.6%) (numbered 1–2) there was concurrent MRCoNS carriage of at least one owner-pet pair (**Table 1**). Instead, in 11 residences (25.6%) (no. 3–13) only owners were positive for MRCoNS whereas in seven households (16.3%) (no. 14–20) only pets carried MRCoNS (**Table 1**).

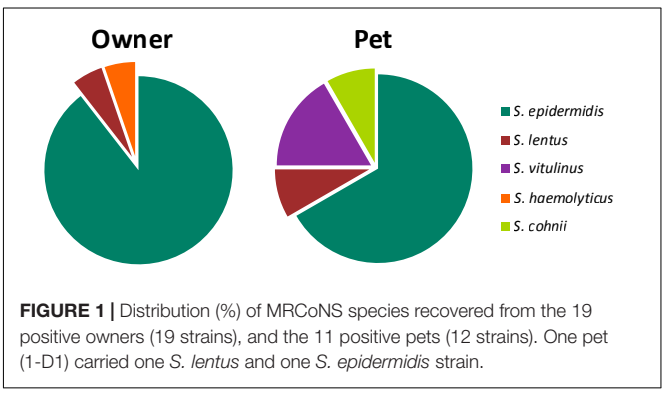

FIGURE 1 | Distribution (%) of MRCoNS species recovered from the 19 positive owners (19 strains), and the 11 positive pets (12 strains). One pet (1-D1) carried one *S. lentus* and one *S. epidermidis* strain.

TABLE 1 | Molecular characterization of the 31 MRCoNS strains recovered from healthy owners and their pets from 20 households.

HH[a]	Individual ID	Strain ID	MRCoNS species (%)[b]	MLST (*S. epidermidis*)	SCC*mec*		Antimicrobial resistance profile to non β-lactams[c]		Determinants involved or influencing biofilm formation		
					Kondo et al., 2007	Zhang et al., 2005	Phenotype[d]	Resistance genes detected	*icaADBC*	*icaR*	IS*256*
Households where at least one owner and one pet carried MRCoNS											
1	**1-H1, 1-D1**	**C3031/C3030**	***S. lentus* (99%)**	–	–	**NA**	**NT**	**E-Cc-G-To-K-Xi**	***erm*(A), *mph*(C), *aacA-aphD***	–	**–**	**+**
	1-H2	C5116	*S. haemolyticus* (99%)	–	–	NA	V	E-T	*msrA/B*, *mph*(C), *tet*(K)	-	-	-
	1-D1	C3029	*S. epidermidis* (99%)	ST555	CC5	NA	V	E	*msrA/B*, *mph*(C)	+	+	–
2	**2-H1, 2-D1**	**C3041/C3033**	***S. epidermidis* (96%)**	**ST130**	CC5	**IV**	**NT**	**To**	***aadD***	**–**	**–**	**-**
	2-H2	C3040	*S. epidermidis* (96%)	ST2	CC5	NT	NT	–	–	+	+	-
Households where only owners carried MRCoNS											
3	3-H1	C3910	*S. epidermidis* (98%)	ST22	CC5	NT	NT	M-Cp	*mupA*	+	+	–
4	4-H1	C3034	*S. epidermidis* (94%)	ST290[e]	CC11	IV	IV	E-M	*msrA/B*, *mupA*	–	–	–
5	5-H1	C5110	*S. epidermidis* (99%)	ST5	CC5	IV	NA	E-M-Cp	*msrA/B*, *mupA*	–	–	–
6	6-H1	C5112	*S. epidermidis* (99%)	ST556	S556	IV	NT	Che-F	–	–	–	–
7	7-H1	C3043	*S. epidermidis* (98%)	ST553	CC5	NT	NT	E-Ci-T	*erm*(C), *tet*(K)	+	+	–
8	8-H1	C3914	*S. epidermidis* (99%)	ST559[e]	CC11	IV	IV	S-T	*str*, *tet*(K)	–	–	–
9	9-H1	C5114	*S. epidermidis* (99%)	ST556	S556	IV	NT	X	*dfr*(A)	–	–	–
10	10-H1	C3922	*S. epidermidis* (99%)	ST5	CC5	NT	NA	E-M-Cp	*msrA/B*, *mupA*	–	–	–
11	11-H1	C3926	*S. epidermidis* (99%)	ST554	CC5	V	V	–	–	+	+	–
	11-H2	C3928	*S. epidermidis* (99%)	ST35	CC5	NA	V	E-T	*msrA/B*, *tet*(K)	+	+	–
12	12-H1	C3932	*S. epidermidis* (99%)	ST555	CC5	NA	V	E-M	*msrA/B*, *mph*(C), *mupA*	+	+	-
	12-H2	C3933	*S. epidermidis* (99%)	ST20	CC5	NA	NT	–	–	+	+	-
	12-H3	C3934	*S. epidermidis* (99%)	ST20	CC5	NA	NT	E	*msrA/B*, *mph*(C)	+	+	–
13	13-H1	C3937	*S. epidermidis* (99%)	ST83	CC5	NT	IV	Cc-G-K-To-M	*vga*(A), *aacA-aphD*, *mupA*	+	+	+
	13-H2	C3938	*S. epidermidis* (99%)	NT[f]	–	NA	V	E-Cc	*msrA/B*, *lnu*(A)	–	–	–
Households where only pets carried MRCoNS											
14	14-D1	C3911	*S. vitulinus* (100%)	–	–	NA	NT	–	–	–	–	–
15	15-D1	C3913	*S. vitulinus* (99%)	–	–	NA	NT	–	–	–	–	–
16	16-D1	PA84	*S. epidermidis* (99%)	ST130	CC5	IV	NT	–	–	–	–	–
17	17-D1	C3044	*S. epidermidis* (99%)	ST5	CC5	IV	NA	E-Ci-G-To-K-X-M-Cp	*erm*(C), *aacA-aphD*, *dfr*(A), *mupA*	–	–	+
	17-D2	C3045	*S. cohnii* (98%)	–	–	NA	III	E	*msrA/B*, *mph*(C)	–	–	–
	17-D3	C3046	*S. epidermidis* (97%)	ST5	CC5	NT	NA	E-Ci-G-To-K-X-M-Cp	*erm*(C), *aacA-aphD*, *dfr*(A), *mupA*	–	–	+
18	18-C1	C3035	*S. epidermidis* (98%)	ST558[e]	CC11	NA	NA	–	–	–	–	–
19	19-C1	C3036	*S. epidermidis* (99%)	ST60	CC5	NT	NT	Cc	*vga*(A)	–	–	–
20	20-C1	C3921	*S. epidermidis* (98%)	ST560[e]	S560	NA	V	E-Ch-M	*msrA/B*, cat_{pC223}, *mupA*	–	–	–

Individuals involved in cases of Interspecies Transmission and strain characteristics are marked in bold. [a]HH, household no. Households 1–2 compile data from household individuals where at least one owner and one pet carried MRCoNS Households 3–13 cluster those where only owners were positive for MRCoNS; and Households 14–20 include those where only pets carried MRCoNS. [b]MRCoNS, Methicillin-Resistant Coagulase Negative Staphylococci. (%) refers to the percentage of identity to the best NCBI BLASTN hit. [c]Mutations in the quinolone resistance determining regions (QRDR) of GyrA and GyrB subunits of DNA Gyrase as well as of ParC and ParE subunits of DNA topoisomerase IV are not displayed. [d]E, erythromycin; Cc, constitutive clindamycin resistance; Ci, inducible clindamycin resistance; Che, clindamycin hetero resistance; T, tetracycline; G, gentamicin; To, tobramycin; K, kanamycin; S, streptomycin; M, mupirocin; X, trimethoprim/sulfamethoxazole; Xi, intermediate resistance trimethoprim/sulfamethoxazole; to Ch, chloramphenicol; Cp, ciprofloxacin; F, fusidic acid. [e]Sequence type for which aroE and tpi alleles could not be amplified with standard primers.[f]NT, non-typeable. Cells with gray background highlight the concordant results between both SCCmec classification schemes.

Not significant differences were observed in MRCoNS carriage among owners or pets where more than one pet was in the house ($p = 0.7946$ versus 0.4321, respectively).

Clonal Lineages of MRCoNS Isolates

Molecular characterization of the 31 MRCoNS isolates recovered is displayed in **Table 1**. In total, 24 of the 25 MRSE isolates were typed by MLST, with 16 different STs detected. One human MRSE isolate (C3938) could not be typed due to reiterate lack of amplification of several of the MLST-schemed alleles (*gtr*, *pyr*, *yqil,* and *mutS*), regardless MALDI-TOF confirmed that it was *S. epidermidis*. In addition, four isolates were not typeable using the standard *aroE* and *tpi* primers[4,5], but did amplify with in-house designed primers (ST290 and the novel ST558, ST559, and ST560).

Nine of 24 (37.5%) MRSE strains revealed novel STs (seven different ones), with either novel allele (ST553, ST554, ST555, ST556) or novel allele combination (ST558, ST559, ST560) (**Supplementary Table S1**). Fifteen MRSE strains (62.5%) belonged to already known STs (nine different STs). ST5 (primary ST founder of CC5) was predominant, being present in two MRSE from unrelated owners and two from related dogs. Most MRSE belonged to CC5 (75%), which is the major group within the *S. epidermidis* MLST scheme, three strains belonged to CC11 (12.5%) and three strains were singletons [S556 (8.3%) and S560 (4.2%)] (**Figure 2A**). All previously known STs (ST2, ST35, ST22, ST60, ST20, ST130, ST83, and ST290) represented subgroup founders (by default settings, i.e., an ST with at least three links to other STs, including the link to its assumed progenitor), with ST2 as the biggest subgroup founder within CC5 (formerly compiling CC2) (**Figure 2A**).

The distance tree of the 16 concatenate ST sequences detected among the 24 MRSE strains revealed high profile diversity. All cases were concordant with the CC and STs subgroup clusters (based on allelic profile) represented by Phyloviz2 clustering using the goeBURST algorithm, except for ST35, which formed an independent branch from the closest variants (ST2, ST22, and ST553). Remarkably, all four STs that could not be amplified using the standard primers (all 3 CC11 and S560) clustered together in a distant branch from the rest of STs (**Figure 2B**).

All canine MRSE strains exhibiting STs were also detected in owners (1 ST155, 2 ST5, 2 ST130, all CC5), while all feline (ST60-CC5, ST560-S560, ST558-CC11) and some human MRSE strains were unique (**Figure 2B**).

The Simpon's Index of Diversity (SID) was remarkably high (0.96), reflecting a 95.6% chance of randomly picking two strains from the sample cohort that are different.

ccr and *mec* Complex Diversity Among MRCoNS Isolates (SCC*mec* Profile)

Based on a scheme by Kondo et al. (2007), high diversity of *ccr* types, *mec* complexes and *ccr-mec* complex combinations were detected among the 31 studied isolates (**Table 1**). *ccrAB2* ($n = 21$) and *mec* complex A ($n = 13$) were predominant within their respective category (see **Supplementary Table S2**). All eight SCC*mec* cassettes carrying *ccrC* presented additional *ccr* genes (*ccrAB2*, $n = 5$; *ccrAB1*, $n = 2$; or *ccrAB1*+*ccrAB2*, $n = 1$) (**Supplementary Table S2**). More than one *ccr* type was detected in 11 isolates (35.5%). A total of 21 SCC*mec* cassettes were either NT or NA (67.7%), nine were SCC*mec* IV (29%), and one was SCC*mec* V (3.2%).

According to scheme by Zhang et al. (2005), 20 strains were either SCC*mec* NT or NA (64.5%), seven were SCC*mec* V (22.6%), three were SCC*mec* IV (9.7%), and one was SCC*mec* III (3.2%). Four strains were positive for more than one SCC*mec* cassette.

Eight of 31 strains (25.8%) were concordantly typed with both typing schemes (**Table 1** and **Supplementary Table S2**). Among them, SCC*mec* NT was predominant ($n = 4$), followed by SCC*mec* IV ($n = 2$) and SCC*mec* V and SCC*mec* NA (one each), respectively. Both schemes categorized seven additional cassettes in different strains, with a SID of 0.89 by Kondo et al. (2007) and SID 0.71 by Zhang et al. (2005). In total, as a consensus of both schemes, 18 strains of SCC*mec* were NT (58.1%), 10 NA (32.3%), two SCC*mec* IV (6.5%), and one SCC*mec* V (3.2%) (**Supplementary Table S2**).

Comparing owner versus pet MRCoNS isolates by Kondo et al. (2007), SCC*mec* NT or NA were predominant among both human and animal strains (combined 63.2% for owners versus 91.7% for pets) ($p = 0.02203$). SCC*mec* IV was the most commonly known SCC*mec* cassette among both host isolates (six from humans, 31.6%; three from dogs, 25%), while SCC*mec* V was only detected in two owners (10.5%).

According to both schemes performed, one MRSH from an owner and one MRSE from her pet (1-H2 and 1-D1, household 1) shared the same SCC*mec* cassette (**Table 1** and **Supplementary Table S2**).

Antimicrobial Resistance (AMR) Pattern

Prevalence of resistance to non β-lactams among human and animal isolates, as well as the detected resistance genes, is shown in **Figure 3**. Erythromycin resistance [*erm*(A), *erm*(C)] was the most common pattern (51.6% of isolates), followed by mupirocin (*mupA*) (29%) and clindamycin [*vga*(A), *lnu*(A)] (29%) resistance. Subsequently, MLS was the antimicrobial class to which most strains exhibited resistance. Mupirocin resistance was only present in MRSE strains (36% of MRSE). Inducible clindamycin resistance was only observed in the three isolates carrying the *erm*(C) gene (see **Table 1**).

Mutations identified in the QRDR of the *gyrA*, *parC* and *parE* genes of the five MRSE ciprofloxacin resistant strains are summarized in **Table 2**. All detected substitutions are displayed in **Supplementary Table S3**. No mutation was observed in any strain within the *gyrB* gene sequence region. The most common mutation was Ser84Phe (5/5) and Ser84Tyr (3/5) in GyrA and ParC, respectively.

Resistance to aminoglycosides ($p = 0.008$–0.016), co-trimoxazole ($p = 0.016$) and chloramphenicol ($p = 0.007$) was significantly higher in animal isolates (with the latter being exclusively detected in pets), whereas resistance to tetracycline was only present and abundant in owner isolates ($p = 2.95E-07$).

[4]https://pubmlst.org/sepidermidis/

[5]http://sepidermidis.mlst.net

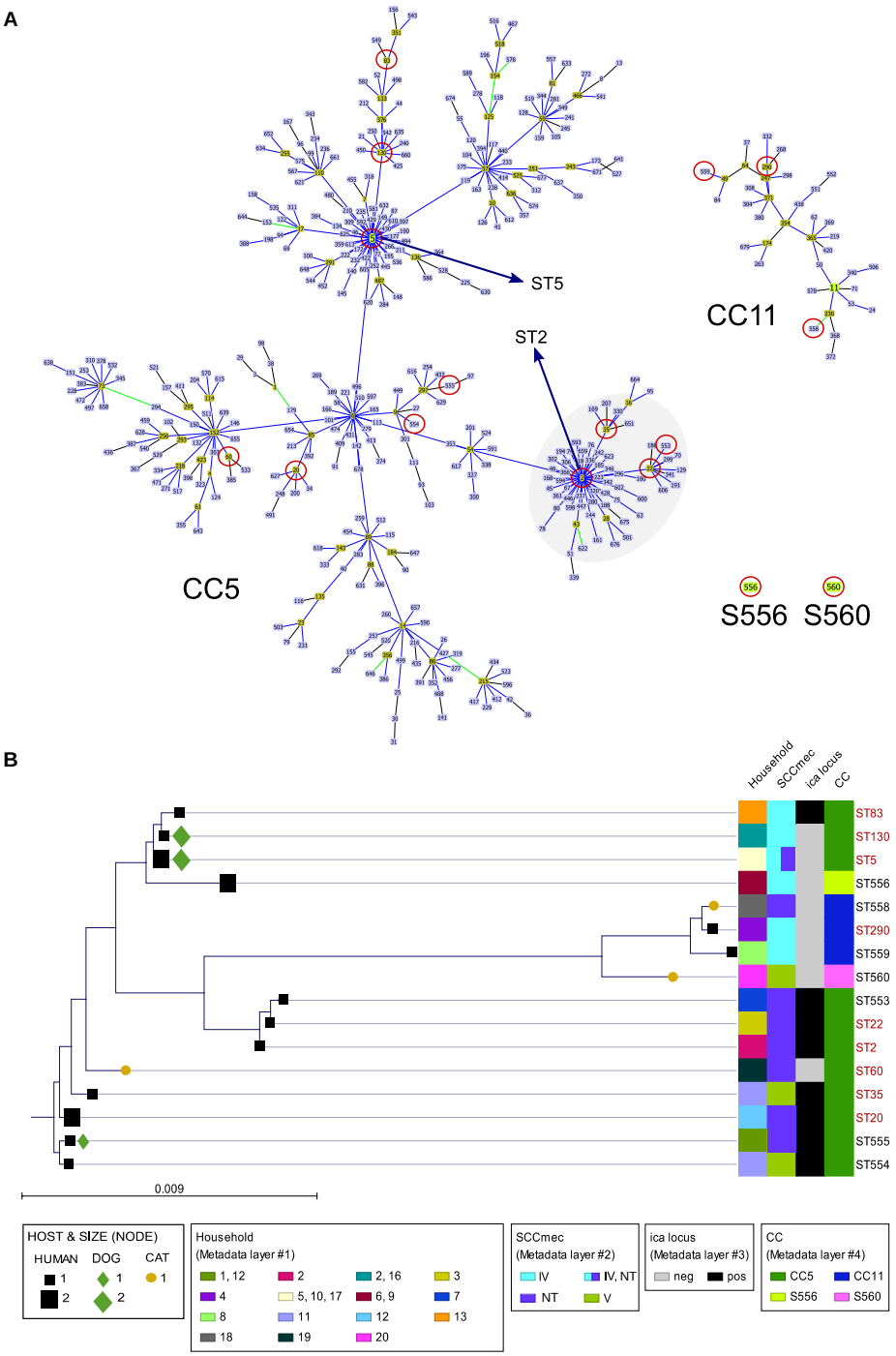

FIGURE 2 | (A) Clustering analysis of the *S. epidermidis* STs detected in this study by goeBURST algorithm using Phyloviz 2 software (Nascimento et al., 2017). The most restricted level [level 1 – Single Locus Variant (SLV)] was used, requiring six of seven alleles shared to the linked ST. Cyan STs indicate probable ancestors (group founders) and green STs constitute subgroup founders. Blue STs correspond to STs that share the same background (CC). Circles in red indicate the STs detected in this study. Specific location of ST5 (CC5 ST primary founder) and ST2 (major subgroup founder of the cluster) within CC5 are indicated. **(B)** Distance tree of the 16 concatenate ST sequences detected among the 24 *S. epidermidis* isolates constructed using CLC Genomics Workbench 10.0.1 (https://www.qiagenbioinformatics.com/). Sequences were aligned using internal parameters, and the tree was built with a Neighbor Joining method using Jukes-Cantor as Nucleotide Distance measure, with a bootstrap analysis of 500 replicates. The bar length indicates the number of substitutions per site. STs in black color are those with new ST, either by the presence of a new allele or new allele combination.

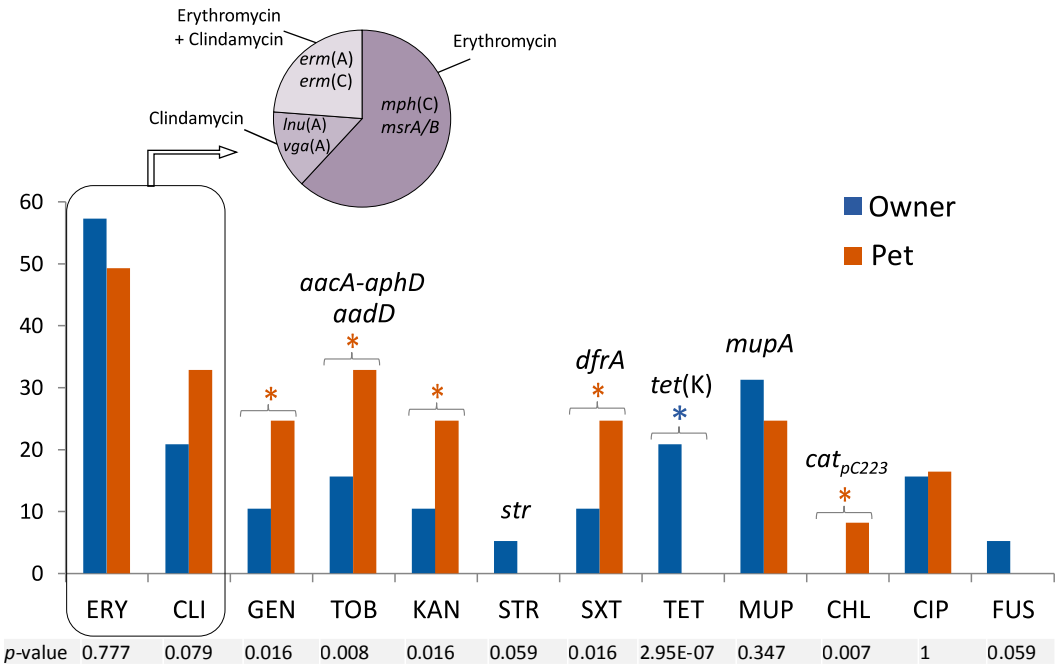

FIGURE 3 | Percentage of resistance to non β-lactams and antimicrobial resistance genes detected among the 31 MRCoNS isolates investigated in T0. FUS, fusidic acid; CHL, chloramphenicol; TET, tetracycline; CIP, ciprofloxacin; SXT, co-trimoxazole; GEN/TOB/KAN/STR, gentamicin/tobramycin/kanamycin/streptomycin; MUP, mupirocin; ERY/CLI, erythromycin/clindamycin. All isolates were susceptible to vancomycin and linezolid. Individual *P*-value (Fisher's Exact Test for count data) to account for significant difference at 95% confidence interval is indicated at the bottom of the histogram. Asterisks (blue or red) above the bars represent those agents for which statistical differences were detected, with the asterisk color remaking the host (owner or pet, respectively) of the bacteria involved in the significance.

TABLE 2 | Mutations in the quinolone resistance determining regions (QRDR) of GyrA (DNA Gyrase), ParC, and ParE (DNA topoisomerase IV) of the quinolone resistant strains.

Strain	GyrA		ParC		ParE	
	Synonymous substitution	Non-synonymous substitution	Synonymous substitution	Non-synonymous substitution	Synonymous substitution	Non-synonymous substitution
C3044, C3046	**S84F**	P34P, V74V	**S80F, D84G**	V70V, Q73Q, G104G	–	L411L, L430L, L442L, V458V
C3910	**S84F, E88K**	P34P, V74V	**S80Y, D84Y**	V70V, Q73Q, G104G	–	L411L, L430L, L442L, V458V
C3922	**S84F, E88K**	P34P, V74V	**S80Y, D84Y**	V70V, Q73Q, G104G	–	L411L, L430L, L442L, V458V
C5110	**S84F**	P34P, V74V	**S80Y**	V70V, Q73Q, G104G	–	L411L, L430L, L442L, V458V

GyrB is not represented given that no mutations were observed. Synonymous substitutions are marked in bold.

Resistance to fusidic acid and streptomycin were only detected in human isolates at low rates, but no significant differences were observed with the Fisher's Exact test.

Remarkably, one methicillin-resistant *S. lentus* (MRSL) clone (isolates C3030 and C3031, from owner 1-H1 and cohabitant dog 1-D1) showed intermediate resistance to trimethoprim and co-trimoxazole but did not harbor any of the trimethoprim resistance genes so far described in staphylococci. The human MRSE-S556 strain (C5112) also showed hetero-resistance to clindamycin but was negative for the corresponding genes tested. This strain was also resistant to fusidic acid and lacked the acquired *fusB* and *fusC* genes.

Significant differences were observed between the rate of owners and pets carrying MDR MRCoNS isolates (68% versus 33%) (p = 1.205E-06). In total, 54.84% of isolates were MDR.

Presence of Determinants for Biofilm Formation

A total of 32.3% of isolates were positive for the genes enclosed within the *ica* locus (*icaADBC*) as well as for the *icaADBC* transcriptional regulator *icaR* (**Table 1**); all of which were MRSE of the CC5 lineage (see **Figure 2B**). If divided by the bacterial host, 47.4% of human isolates and a single MRSE canine strain (C3029) (8.3%) were positive (p = 4.49e-10).

Subsequently, the presence of the *ica* locus gene cluster in human MRSA-CC5 isolates was strongly positively correlated. Through logistic regression analysis, positive association was observed between presence of the *ica* locus and owners, only when the variable household of origin was not considered in the equation (association was observed at 0.1 significance code otherwise).

The IS*256* was detected in four *icaADBC*-negative isolates (12.9%). These isolates also contained the bifunctional aminoglycoside resistance *aacA-aphD* gene, which is normally enclosed within Tn*4001* (IS*256*_*aacA-aphD*_IS*256*).

Owner/Pet MRCoNS IT Cases and Longitudinal Overview

Based on all molecular techniques performed, two cases of IT were identified in the owner and cohabitant dog in two unrelated households (4.7% of tested residences; 10% of households with MRCoNS-carrying individuals): (i) a MDR MRSL clone (1-H1 and 1-D1), resistant to erythromycin/clindamycin and gentamicin/tobramycin/kanamycin; and (ii) a MRSE-ST130-CC5 clone (2-H1 and 2-D1) resistant to tobramycin/kanamycin (**Table 1** and **Figure 4**).

According to the 1 year longitudinal study, in case 1, sporadic carriage by the involved MRSL clone was observed in both individuals (1-H1, 1-D1). Instead, the involved dog (1-D1) also carried a MRSE ST155-CC5-SCC*mec*V strain (resistant to erythromycin) in T0 which was also present when sampling T3 in the same animal (intermittent carrier of such clone) as well as in the other cohabitant owner (1-H2), representing an additional *S. epidermidis* sporadic IT case (**Figure 4**). In total, three different MRCoNS species (*S. lentus, S. epidermidis, S. haemolyticus*) and one clone of each were detected along the sampling year. Dog 1-D1 carried two of these clones while the owners carried one clone each.

In case 2, sporadic carriage by the involved MRSE ST130-CC5 clone was also observed. Notably, the same owner and dog (2-H1, 2-D1) carried an identical non-concurrent MRSE clone (only resistant to β-lactams) in different samplings: T2 for the owner and T3 for the dog (**Figure 4**), indicating transient carriage and suggesting that such a clone might be circulating within the household. Along the sampling year, these two subjects revealed to be intermittent carriers of different *S. epidermidis* clones with different resistance patterns (**Figure 4**). In total, a single MRCoNS species (*S. epidermidis*) was detected throughout the sampling year, however, five different MRSE clones were observed, three of them found in dog 2-D1, three in owner 2-H1 and a single clone in owner 2-H2.

None of the individuals, from both cases, were persistent carriers by any of the recovered MRCoNS strains. None of the IT-involved isolates in T0 exhibited any of the genes of the *ica* locus. However, the MRSE C3029 clone (from case 1), which carried the *ica*-locus, was detected again in this animal and one owner in T3 (IT case).

The dynamics of all CoPS staphylococci detected in the same samplings (T0–T4) are described in the **Supplementary File S1** as well as in **Supplementary Figure S1**.

Individual and Household MRCoNS and/or CoPS Concomitance

Eighty-five staphylococcal strains [MRCoNS (n = 31) and CoPS (n = 54)] (Gomez-Sanz et al., 2013b) from the 68 positive individuals recovered at the same sampling point were compared here (**Supplementary Table S4**). This comprehensive picture revealed a total of nine cases of IT (two MRCoNS, 7 CoPS) at sampling T0 (11.9% of subjects coming from 18.6% of tested households) (Gomez-Sanz et al., 2013a,b). Altogether, 55.9% of owners and 45.5% of pets were positive for MRCoNS and/or CoPS (**Supplementary Table S5**).

Single presence of CoPS was the most common pattern, with owners and pets predominantly carrying only *S. aureus* (26.5%) or *S. pseudintermedius* (22.7%), respectively (**Figure 5A**). The carriage rate of MRCoNS as the single species recovered was similar in owners and pets tested (ca. 10.5%) (**Figure 5A**). Alternatively, 17.7% of owners and 6.1% of pets simultaneous carried both bacterial types (p = 0.015) (**Table 3**). Concomitant carriage of MRCoNS and *S. aureus* was significantly higher among owners than pets (14.7% versus 1.5%), while no significant differences were detected for co-carriage of MRCoNS and *S. pseudintermedius* (2.9% versus 4.6%) (**Figure 5A** and **Supplementary Table S5**).

Eleven of the 17 MRCoNS (64.7%) strains involved in the simultaneous carriage were MDR and six of 17 (35.3%) contained the *ica*-locus genes, involved in biofilm formation (**Table 3**). IT cases were more common among individuals with concomitant carriage (6/16, 37.5%) (p = 0.004).

At the household level, based on the strains recovered from individuals tested, 32 households were positive for any of the tested bacterial species (74.4%; 34.9% positive for one bacterial type, 39.5% positive for both MRCoNS and CoPS) (**Figure 5B**). Co-presence of *S. aureus* and MRCoNS was the most predominant pattern (18.6%), followed by *S. aureus* alone (16.3%), and co-presence of *S. aureus*, *S. pseudintermedius* and MRCoNS (14%). Considering the 32 positive residences, *S. aureus* was the predominant species among households with a single bacterial type (21.9%), and half (50%) presented both MRCoNS and CoPS bacterial types (**Figure 5B**).

In total, 23.3% of households contained individuals simultaneously harboring both bacterial types (**Table 3**). Half (5/10) of these households enclosed subjects directly involved in IT cases (p = 0.011). Further, all four pets and seven of the 12 owners who tested positive for concomitant MRCoNS and CoPS (11/16, 68.8%) originated from households where IT cases occurred, even if they were not the individuals directly involved in the case (**Table 3**).

Association Between MRCoNS and CoPS Concomitance, IT Cases, and Host

Logistic regression analysis confirmed a strong positive correlation between individual staphylococcal concomitance and involvement in IT case (0.001 significance code). A positive association (0.05 significance code) was observed between concomitance and owners, only when the household of origin

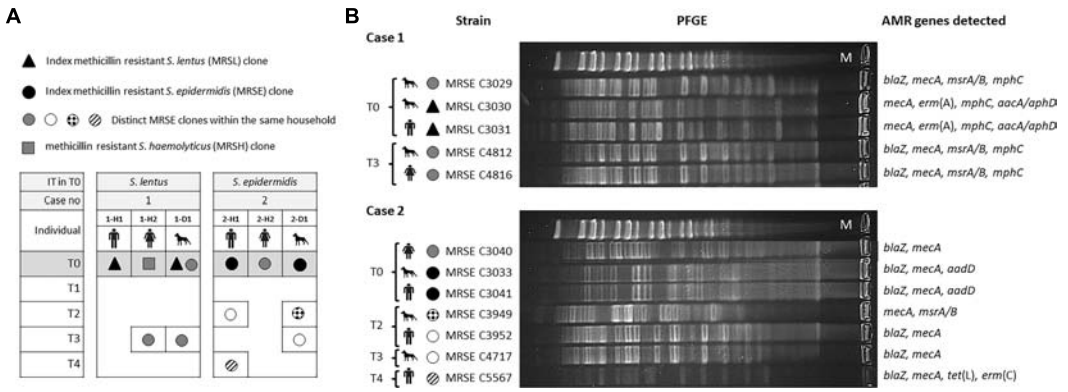

FIGURE 4 | (A) Schematic representation of the methicillin resistant coagulase negative staphylococcal carriage dynamics of both households investigated along 1 year. IT, bacterial species responsible for interspecies transmission. T0–T4 indicate the different sampling times along the sampling year. Individuals are named H (for human) or D (dog) followed by the case number (1 or 2) and a lower-case letter to differentiate subjects per household. **(B)** Pulsed-field Gel Electrophoresis (PFGE) profile of genomic DNA digested with SmaI restriction enzyme of isolates recovered from individuals involved in both cases of possible direct interspecies transmission. Upper lane in PFGE per case corresponds to MidRange PFGE Marker (New England Biolabs). Antimicrobial resistance (AMR) genes detected in each strain are also indicated.

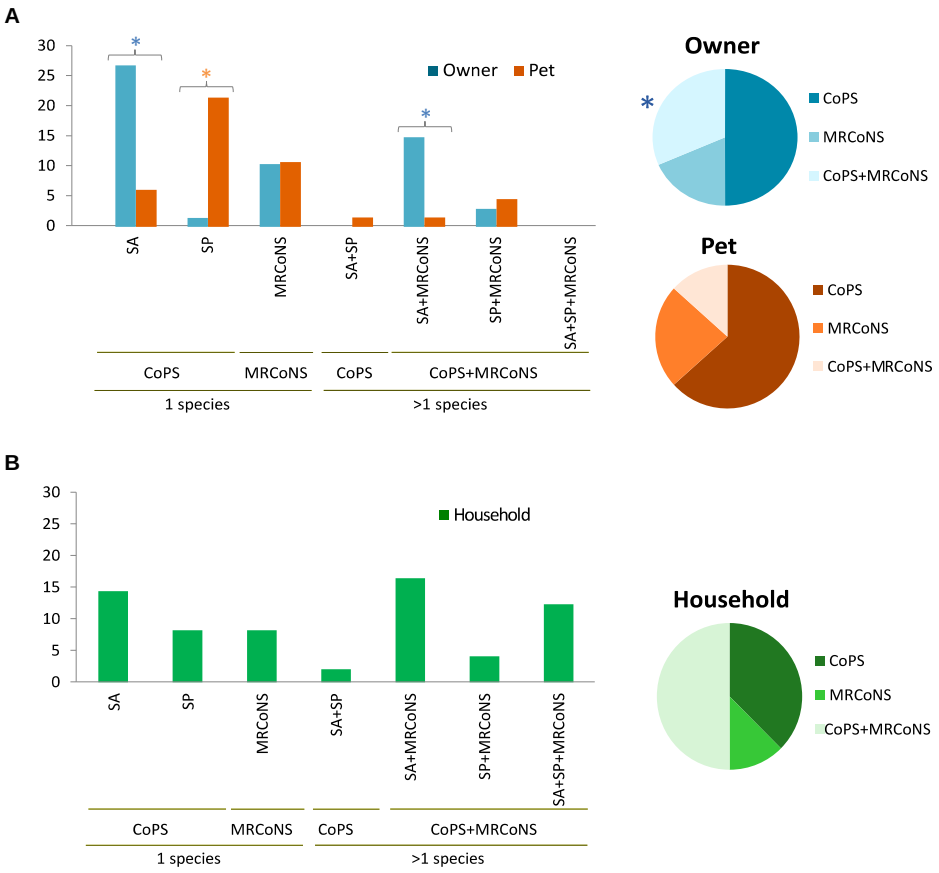

FIGURE 5 | (A) Left panel, bar chart showing the percentage of owners and pets that carried Coagulase Positive Staphylococci (CoPS), i.e., *S. aureus* (SA) and/or *S. pseudintermedius* (SP); MRCoNS; or CoPS + MRCoNS in sampling T0 (Gomez-Sanz et al., 2013b). Right panel, graphical view of the distribution of CoPS and/or MRCoNS detected among the individuals positive for such bacterial species. **(B)** Left panel, bar chart displaying the percentage of households with individuals positive for CoPS (SA, SP), MRCoNS or CoPS + MRCoNS in sampling T0. Right panel, graphical representation of the distribution of CoPS and/or MRCoNS detected among the households with individuals positive for such bacterial species. Colored stars indicate values with significant differences between human and animal strains.

was not considered in the equation (association was observed at 0.1 significance code otherwise).

No significant differences were observed between the presence of more than one animal in the house (animal cohabitation) and (i) staphylococcal carriage ($p = 0.3145$ for pets, $p = 0.1644$ for owners), or (ii) MRCoNS and CoPS individual co-carriage ($p = 1$ for pets, $p = 0.7781$ for owners).

DISCUSSION

The present study provides novel information on frequency, population structure, genetic diversity, AMR and virulence potential among MRCoNS from companion animals and their owners within the household, as well as on staphylococcal human-pet interaction and persistence. The MRCoNS carriage rate detected among healthy owners (28%) is remarkably higher than those detected in former studies among healthy individuals in non-healthcare settings, with rates ranging between 7 and 17% (Barbier et al., 2010; Rolo et al., 2012; Du et al., 2013; Abadi et al., 2015; Xu et al., 2018). Higher nasal MRCoNS rates (30, 47–51%) were detected in Japanese children in day-care centers and kindergartens (Jamaluddin et al., 2008) and among a remote population in French Guiana (Lebeaux et al., 2012). On the other hand, a recent international study on nasal staphylococcal colonization among healthcare workers from 75 different countries revealed a nasal MRCoNS carriage rate of 21.4% (Morgenstern et al., 2016). All these data reflect that nasal distribution of MRCoNS markedly depend on the cohort studied. Remarkably, scarce data are available on the nasal MRCoNS colonization rate among pet owners, and or the animal-owner contact as a possible contributor in increased MRCoNS carriage. Only a couple of recent studies analyzed the risk factors of MRS carriage among individuals in contact with companion animals (Han et al., 2016; Rodrigues et al., 2018). Rodrigues et al. (2018) reported an overall prevalence of MRCoNS of 54.2% among healthy humans in professional daily contact with companion animals in Portugal. In this report, being a veterinary professional was identified as a risk factor for methicillin-resistant staphylococcal carriage (both CoNS and CoPS) colonization (Rodrigues et al., 2018). The relatively high MRCoNS rate detected here might therefore be due, at least partially, to direct pet-human contact, and might be considered as a risk factor for colonization. However, the lack of a "control" population in the current study forces us to interpret these data with caution. Among pets, very sparse data are available on the specific nasal MRCoNS rates. Lower rates (1–15%) than those detected here (17%) have been observed among healthy dogs from several body sites (nasal, rectal, oral, anal, belly) (Vengust et al., 2006; Bagcigil et al., 2007; Aslantas et al., 2013; Gandolfi-Decristophoris et al., 2013; Garbacz et al., 2013; Chah et al., 2014; Davis et al., 2014; Wedley et al., 2014; Siugzdaite and Gabinaitiene, 2017). Interestingly, MRCoNS was isolated from 42% of healthy non-vet visiting and non-antimicrobial treated Labrador retrievers in the United Kingdom (Schmidt et al., 2014). In the latter study, both nasal and perineal samples were collected, suggesting that different sampling methodologies may affect observed prevalence.

In humans, *S. epidermidis* is the most frequently recovered staphylococcal species, colonizing the body surface (Becker et al., 2014; Schmidt et al., 2014). Moreover, the *S. epidermidis* group (predominantly *S. epidermidis* and *S. haemolyticus*) is the most significant species within CoNS representing one of the major nosocomial pathogens (Becker et al., 2014). As such, MRSE was the MRCoNS predominant species detected (25%, 99% of human strains). *S. epidermidis* was also the predominant species among tested animals, with an overall prevalence of 12.1% (9.3% among dogs versus 25% in cats), corresponding to 66% of strains. A diverse range of MRCoNS species have been detected among dogs, such as *Staphylococcus sciuri*, *Staphylococcus warneri, S. lentus*, *S. vitulinus,* or *Staphylococcus fleurettii* (Bagcigil et al., 2007; Becker et al., 2014; Chah et al., 2014; Davis et al., 2014; Schmidt et al., 2014; Siugzdaite and Gabinaitiene, 2017). Regardless *S. epidermidis* has a more defined role in humans; it may also form part of the normal microbiota of animals and, although at lower rates, has been detected as the predominant CoNS species among healthy dogs (Aslantas et al., 2013; Schmidt et al., 2014; Han et al., 2016). Nevertheless, *S. epidermidis* is a predominantly human associated bacterium and the observed distribution here may be influenced by the human-pet direct or indirect contact within the household.

S. epidermidis is the most studied species within CoNS and it is characterized by pronounced genomic diversity (Becker et al., 2014). This agrees with the high diversity of MRSE STs detected (SID of 0.96). In spite of the scattered data available on MRSE lineages from healthy individuals, former reports have also reported high rates of novel STs among *S. epidermidis* isolates (Xu et al., 2018), evidencing high intra-species diversity. MRSE CC5 was predominant, clustering 75% of MRSE isolates from owners. This clonal lineage (with ST5 as primary founder) represents the biggest group within the MLST scheme for this species. MRSE ST2 and ST22, among others, currently enclosed within CC5 but traditionally constituting its own CC (CC2), have been shown to be predominant among hospital environments (Miragaia et al., 2007; Rolo et al., 2012; Cherifi et al., 2013; Becker et al., 2014; Widerstrom et al., 2016; Gordeev et al., 2017). In the community, a high diversity of STs have been identified among healthy individuals (Miragaia et al., 2007; Rolo et al., 2012; Cherifi et al., 2013; Becker et al., 2014; Widerstrom et al., 2016; Gordeev et al., 2017). In contrast, recent studies have revealed a high diversity of lineages among MRSE from both clinical and healthy individuals, with either no increased abundance of CC5 strains among clinical isolates (Jena et al., 2017) or with CC5 predominance in both settings (Rolo et al., 2012; Du et al., 2013). This may be due to the fact that most STs already cluster into CC5 by eBURST/goeBURST analyses, which may hamper attempts to identify lineages that might be associated with different regimes (Thomas et al., 2014). For this reason, a couple of recent studies implemented a Bayesian clustering approach to appraise the real species-wide population structure and ecology of *S. epidermidis*, detecting six genetic cluster (GCs) based on their adaptation to nosocomial or commensal lifestyles (Thomas et al., 2014; Tolo et al., 2016). Following this classification for the already known

TABLE 3 | Individuals concomitantly carrying at least one MRCoNS and one CoPS isolate ranked by household type (based on carriage and IT) and major strain characteristics.

HH[a]	Origin[b]	Individual ID	Strain ID	Staphylococcal bacterial species[c]			MLST	*ica* locus	SCC*mec*[d]	Resistance genes detected
				CoPS		MRCoNS				
Households with individuals involved in cases of interspecies transmission (IT)										
1	H	1-H1	C3494	MSSA			$ST1654_{[new]}$	ND[e]		*blaZ*
		1-H1	C3031			MRSL	-	-	NT	*mecA, erm*(A), *mphC, aacA-aphD*
	D	1-D1	C2729	MSSA			$ST1654_{[new]}$	ND	NT	*blaZ*
		1-D1	C3030			MRSL	-	-		*mecA, erm*(A), *mphC, aacA-aphD*
		1-D1	C3029			MRSE	$ST555_{[new]}$	+	V	*blaZ, mecA, msrA/B, mphC*
2	H	2-H1	C3495	MSSA			$ST1733_{[new]}$	ND		*blaZ*
		2-H1	C3041			MRSE	ST130	-	NT	*blaZ, mecA, aadD*
	H	2-H2	C2730	MSSA			$ST1733_{[new]}$	ND		*blaZ*
		2-H2	C3040			MRSE	ST2	+	NT	*blaZ, mecA*
5	H	5-H1	C2919	MSSA			ST45	ND		*blaZ*
		5-H1	C5110			MRSE	ST5	-	NA	*blaZ, mecA, msrA/B, mupA*
6	H	6-H1	C2915		MSSP		$ST142_{[new]}$	ND		*blaZ-tet*(K)-*tet*(M)-*erm*(B)-[*aadE-sat4-aphA3*]-*dfr*(G)-cat_{pC221}
		6-H1	C5112			MRSE	$ST556_{[new]}$	-	NT	*blaZ, mecA*
12	H	12-H1	C3931	MSSA			ST121	ND		*blaZ-tet*(K)
		12-H1	C3932			MRSE	$ST555_{[new]}$	+	V	*blaZ, mecA, msrA/B, mphC, mupA*
	H	12-H3	C5120	MSSA			ST5	ND		*blaZ*
		12-H3	C3934			MRSE	ST20	+	NT	*blaZ, mecA, msrA/B, mphC*
17	D	17-D1	C3007		MSSP		ST42	ND		*blaZ*
		17-D1	C3044			MRSE	ST5	-	NA	*blaZ, mecA, erm*(C), *aacA-aphD, dfrA, mupA*
	D	17-D2	C3008		MSSP		ST141	ND		*blaZ-tet*(M)
		17-D2	C3045			MRSCo	-	-	NA	*mecA, msrA/B, mphC*
	D	17-D3	C3009		MSSP		$ST154_{[new]}$	ND		*blaZ*-[*erm*(B)-*aadE-sat4-aphA3*]
		17-D3	C3046			MRSE	ST5	-	NT	*blaZ, mecA, erm*(C), *aacA-aphD, dfrA, mupA*
Households lacking IT cases										
4	H	4-H1	C2732	MSSA			ST30	ND		*blaZ-str*
		4-H1	C3034			MRSE	ST290	-	NT	*blaZ, mecA, erm*(B), *msrA/B, mupA*
9	H	9-H1	C3917		MSSP		ST21	ND		*blaZ*
		9-H1	C5114			MRSE	$ST556_{[new]}$	-	NT	*blaZ, mecA, dfrA*
11	H	11-H1	C3925	MSSA			$ST2177_{[new]}$	ND		*blaZ*
		11-H1	C3926			MRSE	$ST554_{[new]}$	+	V	*mecA*
	H	11-H2	C3927	MSSA			ST95	ND		*blaZ-erm*(C)
		11-H2	C3928			MRSE	ST35	+	NA	*blaZ, mecA, msrA/B, tetK*
13	H	13-H2	C4897	MSSA			ST121	ND		*blaZ*
		13-H2	C3938			MRSE	NT[f]	-	NA	*mecA, msrA/B, lnu*(A)

Cells highlighted in faint gray indicate individuals directly involved in cases of interspecies transmission by any of tested staphylococcal type (from this study and Gomez-Sanz et al., 2013b). [a]HH, household no. Nomenclature based on the current study (different nomenclature was used in Gomez-Sanz et al., 2013a,b). Households 1, 2, 5, 6, 12, and 17 include individuals involved in cases of Interspecies Transmission (including by CoPS, Gomez-Sanz et al., 2013b). Households 4, 9, 11, and 13 group individuals where only owners co-carried MRCoNS and CoPS. [b]H, Human; D, dog. [c]CoPS, coagulase positive staphylococci; MSSA, methicillin-susceptible S. aureus, MSSP, methicillin-susceptible S. pseudintermedius; MRSP, methicillin-resistant S. pseudintermedius; MRCoNS, methicillin-resistant coagulase negative staphylococci; MRSL, methicillin-resistant Staphylococcus lentus; MRSE, methicillin-resistant Staphylococcus epidermidis; MRSCo, methicillin-resistant Staphylococcus cohnii. [d]Consensus SCCmec cassette. [e]ND, non-determined. [f]NT, non-typeable.

STs, (i) ST2 and ST22 were more suited to a nosocomial lifestyle (GC5); (ii) ST290 to a more commensal lifestyle (GC4), (iii) ST5, ST83, and ST130 were adapted to a more generalist-to-non-hospital sources (GC1); and (iv) ST20, ST35, and ST60 were better suited for generalist-to-infection-associated lifestyles (GC6).

Very scarce data are available on MRSE lineages among pets. A few studies among clinical samples detected ST5 and/or ST2 (both CC5) as predominant, in line with data from humans (Kern and Perreten, 2013; Weiss et al., 2013; Couto et al., 2016). However, data on the circulating MRSE lineages in the community and whether they reflect the human circulating lineages within a target system, are lacking. Here, clear clustering of human and canine strains was observed, as all STs detected among dogs were also detected among different owners from different households. This lack of host tropism of specific lineages suggests the adaptability potential of MRSE to different hosts within a shared habitat and/or the easiness of host sporadic acquisition of circulating lineages. In contrast, the three STs detected among MRSE from feline isolates were unique. This might indicate that, while dogs tend to share the same clonal lineages as owners, cats might pose feline-associated lineages. Further studies with a bigger sample size are needed on the ecology of MRCoNS and MRSE among different inhabitant species, and how cohabitation may influence host staphylococcal profiles.

High diversity of SCC*mec* types was detected, most being either NT or NA (90.3%, 28/31). These values are notably higher than those detected among both clinical and community MRCoNS human isolates (Barbier et al., 2010; Lebeaux et al., 2012; Aslantas et al., 2013; Abadi et al., 2015; McManus et al., 2015). This high rate may be partially due to the higher discriminatory power of using two schemes. Remarkably, slightly similar values (83%) were recently detected among MRSE from the nares of neonates at hospital admission (Salgueiro et al., 2017). It is challenging to define whether the NTs cassettes identified here are identical to those previously described as NTs, due to variances in typing methods and the lack of full analysis of the genetic organization and composition of these elements. For this, further in-depth analyses, such as whole genome sequencing (WGS), are definitively needed.

Lack of robust concordance was observed between results obtained by both schemes, with guidelines from Kondo et al. (2007) showing a remarkable high diversity index (SID 0.89 versus 0.71), and reflecting the high intergenic diversity within MRCoNS cassettes. SCC*mec* IV was the predominant typeable cassette for both owner and pets, and despite, additional cassettes have been sporadically detected, it is also the most prevalent cassette among humans and companion animals (Ruppe et al., 2009; Barbier et al., 2010; Lebeaux et al., 2012; Aslantas et al., 2013; Kern and Perreten, 2013; Park et al., 2013; Weiss et al., 2013; Becker et al., 2014; Abadi et al., 2015; McManus et al., 2015; Couto et al., 2016).

Several *ccr* genes were detected in 35.5% of strains, showing a high variety of site-specific recombinases among MRCoNS. The possibility that primers are not specific enough for potential new *ccr* cannot be discarded. Further, *ccr2* and *ccrC* were co-present in all but one detected cases, suggesting that clustering of both *ccr* genes might imply and adaptive advantage. Further analyses should be performed to unveil the real presence and functionality of redundant *ccr* genes, and whether this implies an adaptive advantage under specific conditions. The high SCC*mec* variability, lack of typeability and presence of novel *ccr* and *mec* complex combinations reflect an ever-increasing complexity among SCC*mec* cassettes among CoNS from healthy individuals. Such mobile elements may represent a source for the potential transfer to concurrent staphylococci sharing the same niche. In this study, however, transmission of β-lactams resistance between MRCoNS and CoPS appears negligible among the population tested.

Macrolides-Lincosamides-Streptogramins (MLS), especially erythromycin, was the antimicrobial class for which most strains exhibited resistance among owners and pets (64.5%). MLS are important antibiotics for treatment of staphylococcal infections in both humans and animals (Guardabassi et al., 2004; Bagcigil et al., 2007). Subsequently, it is not surprising that MLS resistance is common among staphylococci in the community (Aslantas et al., 2013; Gandolfi-Decristophoris et al., 2013; Garbacz et al., 2013; Wedley et al., 2014; Couto et al., 2016; Han et al., 2016). Of note, combined resistance to erythromycin and clindamycin is the most common MLS pattern among CoPS isolates (Gomez-Sanz et al., 2013a,b), however, most MRCoNS isolates here were either resistant only to erythromycin or to clindamycin. This pattern reflects the potential differential ability to acquire different resistance genes between CoPS and MRCoNS populations.

Resistance to Aminoglycosides, co-trimoxazole and chloramphenicol was significantly higher among pet isolates. Resistance to these agents, especially to aminoglycosides and trimethoprim, has been reported as common among staphylococci of healthy dogs, and these agents are used extensively in hospital and veterinary settings (Guardabassi et al., 2004; Penna et al., 2010; Chah et al., 2014; Wedley et al., 2014; McManus et al., 2015; Han et al., 2016; Conner et al., 2018). Interestingly, Tetracycline was only detected among human strains, while this antibiotic is widely used in both human and animal medicine (Guardabassi et al., 2004). The lack of resistance among animal strains differs from former studies among both healthy and clinical canine isolates, with rates ranging between 40 and 81% (Aslantas et al., 2013; Kern and Perreten, 2013; Chah et al., 2014; Wedley et al., 2014; Couto et al., 2016; Siugzdaite and Gabinaitiene, 2017). Such differences are most likely due to the groups studied and the geographical area of the sampling. Further research is therefore needed to ponder these profiles as common trends among MRCoNS from healthy pets in Spain.

Interestingly, mupirocin and ciprofloxacin resistance were associated to MRSE and only detected in this species (36 and 20%, respectively). This association is relevant and may reflect a higher exposure of MRSE strains to these agents, which might be partially due to the higher pathogenic potential of this CoNS species. Little is known about the real prevalence of mupirocin resistance (MR) among the CoNS population (Becker et al., 2014), and even less among staphylococci from pets. A couple of studies have detected lower resistance levels, even among clinical samples (8-20%) (Aslantas et al., 2013; Kern and Perreten,

2013; Wedley et al., 2014; Couto et al., 2016). The high rate of mupirocin resistance detected among MRSE (both in owners and pets) is alarming as it is the key antibiotic used for decolonization of methicillin-resistant *S. aureus* (Becker et al., 2014).

MDR was high (54.8%) and significantly higher among human isolates (68.4% versus 33.3%). This difference may again reflect higher exposure of humans to antimicrobial therapy or the clinical settings, or to the coexistence of resistance strains within the same ecological niche, which may favor the horizontal transfer of their mobile elements. Diverse MDR values have been observed among staphylococci from healthy dog owners and pets (17–93%), with most studies reporting very high MDR values (Gandolfi-Decristophoris et al., 2013; Garbacz et al., 2013; Wedley et al., 2014; Han et al., 2016; Siugzdaite and Gabinaitiene, 2017; Conner et al., 2018). Therefore, MRCoNS from healthy owners and pets represent a reservoir for AMR gene transfer in the community and may hamper successful treatment of staphylococcal infections in both animals and humans.

A relatively high rate of isolates (32%) was positive for *ica* locus, which is one of the key elements involved in the early stages of biofilm formation (intercellular adherence and cell agglutination) (Becker et al., 2014). Several studies have shown that *S. epidermidis* from healthy individuals or community environments less frequently carry *ica*ADBC-cluster genes, in comparison to clinical samples or hospital-associated environments (Fey and Olson, 2010; Becker et al., 2014; Szczuka et al., 2016; Seng et al., 2017). The rates detected here are therefore outstanding and reflect that MRCoNS strains spread in the community pose notable virulence properties. Interestingly, in the current study, *ica*ADBC was positively correlated with human MRSE CC5 isolates (47.7%). Harris et al. (2016) recently identified *S. epidermidis* of this lineage as *ica*ADBC-containing biofilm producers. However, they could not establish lineage-biofilm formation associations, as the genes involved were present in divergent lineages, showing evidence for horizontal gene transfer. Alternatively, although most cases of biofilm-forming CoNS isolates and biofilm-associated infections containing the *ica*-locus are from *S. epidermidis*, other CoNS species have occasionally been detected to form biofilms and to contain this operon (Szczuka et al., 2016; Seng et al., 2017).

To the best of our knowledge, this is the first study addressing the occurrence and persistence of MRCoNS transmission between owners and their pets. Two cases of IT were detected in T0 (4.7%). Diverse sequential MRCoNS clones were observed on the longitudinal approach among tested individuals, revealing a MRCoNS existent flow in the household setting and the vector-role of dogs for human staphylococcal acquisition, and vice verse. In addition, dog 1-D1, involved in the MRSL IT case in T0, was also positive for a MRSE *ica*-locus positive strain (C3029 MRSE-ST155-CC5-SCC*mec*V), which was responsible for an additional case of IT in T3 (9 months after first sampling). *S. epidermidis* is a human related species, whereas *S. lentus* is considered animal-associated (Becker et al., 2014). Subsequently, the MRSE-involved IT cases here are suggested to have an anthropozoonotic origin, whereas the MRSL case may be regarded as of zoonotic origin. These data provide evidence that MDR and virulent MRCoNS strains can be exchanged and at least temporarily persist between owners and in-contact pets, contributing to the dissemination of resistant staphylococci, with the subsequent risk of infection. To this end, the household environment could also play a role as source for MRCoNS and persistence in the sampled population, as recently reported from community environments (20.5%) (Seng et al., 2017).

To our knowledge, this is also the first study addressing simultaneous nasal carriage of CoPS and MRCoNS in owners and their pets. A single study, focused on the occurrence of CoPS and MRCoNS in dogs, observed slightly higher carriage rates to the ones detected here (45.5%), with 55% of animals positive for CoPS and/or MRCoNS (Wedley et al., 2014). However, CoPS and MRCoNS co-carriage was as low as 2.2%, in comparison to the 6.1 and 16.2% detected among our animal and human population, respectively. Alternatively, although owners and pets differed in the CoPS predominant species when occurring alone or in concomitance with MRCoNS, no significant differences were observed when addressing the single presence of MRCoNS. Again, this might indicate a less prone host-tropism among MRCoNS than among *S. aureus* or *S. pseudintermedius*, or the capacity to adapt or temporarily coexists in different hosts. In addition, owners tend to simultaneously carry both bacterial types. Based on the strong association between involvement in an IT case and CoPS-MRCoNS simultaneous carriage, we reveal that owner-pet inhabitance favors the coincident coexistence of the staphylococcal species with high virulence potential and/or MDR pattern. This scenario does not only disclose an exchange of relevant bacteria between owners and pets, but also paves the way for the exchange of AMR and virulence factors between concomitant strains. Whether these owner-pet exchanged microbes have a true niche on these pairs, versus transient detection after direct or indirect contact, is unknown. However, these results suggest that owner-pet inhabitance may significantly shape the staphylococcal population composition of one another.

CONCLUSION

MRCoNS, especially MRSE, are common colonizers of healthy owners and pets. They show high clonal diversity, represent a reservoir of AMR genes and pose IT potential. The detection of MRSE clonal lineages that circulate in human hospitals and the community suggests that companion animals can contribute to the dissemination of highly successful human clones. Due to the sequential MRCoNS clones detected in owners and pets over time, more longitudinal studies are required to distinguish between persistent colonization, transient carriage or mere contamination, as well the implication of what the different statuses can imply for public health. Individuals involved in cases of IT revealed to be prone to simultaneous CoPS-MRCoNS co-carriage. These data highlight the importance of companion animals as reservoirs of important MDR opportunistic pathogens, which can be transferred to in-contact individuals. Further epidemiological studies including samples from environmental sites are needed to elucidate the conditions by which MRCoNS

are propagated within household settings, as well as to confirm owner and pet cohabitation as a risk factor for the acquisition and subsequent infection by MDR staphylococci.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

EG-S, CT, and MZ conceived and designed the experiments. EG-S, SC, and LR-R performed the experiments. EG-S analyzed the data and wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00485/full#supplementary-material

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Methicillin-Resistant *Staphylococcus aureus* Blood Isolates Harboring a Novel Pseudo-staphylococcal Cassette Chromosome *mec* Element

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The aim of this work was to assess a novel pseudo-staphylococcal cassette chromosome *mec* (ΨSCC*mec*) element in methicillin-resistant *Staphylococcus aureus* (MRSA) blood isolates. Community-associated MRSA E16SA093 and healthcare-associated MRSA F17SA003 isolates were recovered from the blood specimens of patients with *S. aureus* bacteremia in 2016 and in 2017, respectively. Antimicrobial susceptibility was determined *via* the disk diffusion method, and SCC*mec* typing was conducted by multiplex polymerase chain reaction. Whole genome sequencing was carried out by single molecule real-time long-read sequencing. Both isolates belonged to sequence type 72 and *agr*-type I, and they were negative for Panton-Valentine leukocidin and toxic shock syndrome toxin. The *spa*-types of E16SA093 and F17SA003 were t324 and t2460, respectively. They had a SCC*mec* IV-like element devoid of the cassette chromosome recombinase (*ccr*) gene complex, designated as ΨSCC*mec*$_{E16SA093}$. The element was manufactured from SCC*mec* type IV and the deletion of the *ccr* gene complex and a 7.0- and 31.9-kb portion of each chromosome. The deficiency of the *ccr* gene complex in the SCC*mec* unit is likely resulting in mobility loss, which would be an adaptive evolutionary mechanism. The dissemination of this clone should be monitored closely.

Keywords: methicillin-resistant *Staphylococcus aureus*, sequence type 72, pseudo-SCC*mec*, *ccr* gene, blood isolates

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates were first identified in the early 1960s, immediately after the introduction of penicillinase-stable penicillins in the clinical setting (Jevons et al., 1963). Now, the spread of MRSA strains represents a global concern with a recognizable healthcare burden. The *mecA* and *mecC* genes encoding penicillin-binding protein (PBP) 2a of low beta-lactam binding affinity confer beta-lactam resistance to the bacterial host by composing a mobile genetic element, namely, the staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito et al., 1999).

The SCC*mec* element harbors two fundamental components: a *mec* gene complex and a cassette chromosome recombinase (*ccr*) gene complex. A unique combination of the *mec* gene complex class and the *ccr* gene complex allotype determines the type of the SCC*mec* element, and its variation within the joining- (J-) regions determine the subtypes of each SCC*mec* type. To date, 13 SCC*mec* types have been deposited together with numerous subtypes (International Working Group on the Classification of Staphylococcal Cassette Chromosome, 2009; Baig et al., 2018). The *mec* gene complex includes the *mecA* or *mecC* gene, along with the regulatory *mecR1* and *mecI* genes. The *ccr* gene complex comprises one or more *ccr* genes (Deurenberg and Stobberingh, 2008) accounting for the integration/excision of the SCC*mec* element into and out of the *orfX* gene in the staphylococcal chromosome (Katayama et al., 2000). The SCC*mec*-like elements devoid of the *mecA* gene are denominated as an SCC as long as they share the following characteristics with SCC*mec*: carriage of the *ccr* gene(s), integration at integrated site sequences in the chromosome, and the presence of flanking direct repeat sequences. And those without the *ccr* genes are termed as the pseudo-(Ψ) SCC*mec* element.

Through a nationwide antimicrobial resistance surveillance in South Korea (Lee et al., 2018), two *mecA*-positive MRSA blood isolates were identified as those carrying a non-typeable SCC*mec* element. To assess the non-typeable SCC*mec*, both genomes were entirely sequenced, and a novel ΨSCC*mec*$_{E16SA093}$ element was identified.

MATERIALS AND METHODS

Bacterial Isolates

A total of 586 *S. aureus* blood isolates collected between May 2016 and April 2017 from six general hospitals in South Korea (Lee et al., 2018) were screened. Among the 319 cefoxitin-resistant isolates, E16SA093 and F17SA003, whose SCC*mec* elements could not be typified, were selected for further study.

Antimicrobial Susceptibility Testing and the Determination of SCC*mec* Types

Antimicrobial susceptibility to antimicrobials used for staphylococci infection was determined by disk diffusion tests on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA), following the CLSI guidelines (CLSI, 2018). *S. aureus* ATCC 25923 was simultaneously tested in each batch for quality control. MRSA isolates were subjected to polymerase chain reaction (PCR) for *mecA* gene and SCC*mec* typing, as previously described (Oliveira and de Lencastre, 2002).

Multilocus Sequence Typing, *agr* Typing, and *spa* Typing

Multilocus sequence typing (MLST) was carried out by PCR and sequencing of the seven housekeeping genes, *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*. Allelic numbers and sequence types (STs) were determined by comparing the obtained sequences to the database for *S. aureus*[1]. The *agr* type was determined by multiplex PCR (Gilot et al., 2002), and *spa* typing was conducted by comparing the PCR-amplified nucleotide sequence of the variable repeat region of the *spa* gene against the Ridom SpaServer[2].

Whole Genome Sequencing

Bacterial whole genomes were sequenced with single-molecule real-time (SMRT) sequencing on a PacBio RSII instrument (Pacific Biosciences, Menlo Park, CA, USA) using genomic DNA from the *S. aureus* isolates extracted by a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). SMRTbell template libraries were prepared, and adapter ligation was performed. Acquired sequencing data were *de novo* assembled by PacBio SMRT, read with the PacBio SMRT analysis software suite (version 2.3.0). Coding sequences (CDS), including tRNA and rRNA, were annotated using the NCBI Prokaryotic Genome Annotation Pipeline[3]. Nucleic acid sequences were compared using Basic Local Alignment Search Tool,[4] and resistance and virulence determinants were searched for using ResFinder[5] and VirulenceFinder[6], respectively. Prophages were searched for using the PHAge Search Tool Enhanced Release[7]. For any putative *ccr* gene, the site-specific serine recombinase motif (Wang and Archer, 2010) and a modified motif by the consensus pattern (Perreten et al., 2013) were searched for against the coding sequences of both genomes.

Nucleotide Accession Numbers

The nucleotide sequences of the entire genomes of *S. aureus* E16SA093 and F17SA003 were deposited in GenBank under accession numbers CP031130 and CP031131 for F17SA003 and E16SA093, respectively.

RESULTS AND DISCUSSION

Epidemiological Features of MRSA ST72

Following the one-year collection of the 586 *S. aureus* blood isolates, a total of 319 isolates (54.4%) were MRSA conferred by the *mecA* gene. A total of 176 (30.0%) isolates belonged to ST72; 112 of those isolates (63.6%) were MRSA, 65 were healthcare-associated (HA) MRSA, and 47 were community-associated MRSA (CA-MRSA). All but three MRSA ST72 isolates (97.3%, 109/112) carried SCC*mec* type IV, while one possessed SCC*mec* type II and the remaining two isolates (E16SA093 and F17SA003) had non-typeable elements.

[1]http://pubmlst.org/saureus
[2]http://www.spaserver.ridom.de
[3]http://www.ncbi.nlm.nih.gov/books/NBK174280
[4]http://blast.ncbi.nlm.nih.gov
[5]https://cge.cbs.dtu.dk/services/ResFinder
[6]https://cge.cbs.dtu.dk/services/VirulenceFinder
[7]http://phaster.ca

The MRSA ST72 harboring SCC*mec* IV (ST72-MRSA-IV) was one of the top three CA-MRSA clones in the USA until 2002 as a pulse-field type USA700; however, the clone was suddenly eliminated from the country in 2004 (Tenover et al., 2008). In South Korea, the ST72-MRSA-IV acceded a major CA-MRSA clone by 2005 (Kim et al., 2007), and the ST72-MRSA-IV subsequently grew to be a major HA-MRSA clone. This finding supports the idea that the ST72-MRSA-IV clone was disseminating from communities to hospitals (Song et al., 2011).

Two *mecA*-Positive MRSA ST72 Blood Isolates Carrying a Non-typeable SCC*mec* Element

The CA-MRSA E16SA093 was recovered in September 2016 from an 86-year-old female patient with acute infective endocarditis and infective spondylopathy. The patient was transferred from an acute care hospital to a general hospital located in Gwangju city, and blood cultures were carried straightaway. The bacteremia originated from a bone infection, and empirical treatment was started with cefazoline. Definitive treatment was followed with teicoplanin within 72 h after the initial blood culture, and the patient was cured. The HA-MRSA 17SA003 was recovered in January 2017 from a 63-year-old male patient with diabetes mellitus and stage 4 chronic kidney disease hospitalized in a general hospital in Busan city. An initial blood culture was performed on the 13th day of hospitalization, and the origin of *S. aureus* bacteremia was unidentified. Empirical treatment with cefazoline was replaced to vancomycin within 72 h, and the patient was cured.

Both MRSA isolates belonged to ST72 and *agr*-type I. They were negative for both Panton-Valentine leukocidin and toxic shock syndrome toxin (**Table 1**). The *spa*-types of E16SA093 and F17SA003 were t324 and t2460, respectively. Among the 10 antimicrobials tested, the E16SA093 isolate was resistant only to cefoxitin, while F17SA003 was resistant not only to cefoxitin but also to erythromycin and clindamycin.

Genome Sequencing and Identification of the Novel ΨSCC*mec*$_{E16SA093}$

The *de novo* assembly of the genome resulted in a 2,767,631,390-bp circularized chromosome, including 2,564 assigned CDSs, 60 tRNAs, and 19 rRNAs for E16SA093, and a 2,849,947,596-bp circularized chromosome, including 2,546 CDSs, 60 tRNAs, and 19 rRNAs for F17SA003. The overall GC contents were 32.9% for both. No plasmid was identified. Acquired genetic elements by both chromosomes were alike, including two intact *Staphylococcus* prophages (44.1-kb ϕNM3 and 41.2-kb Sap26), 17 virulence factors, and three antimicrobial resistance genes, with an extra copy of *blaZ* for F17SA003. No known heavy metal resistance genes were identified for either.

For the SCC*mec* element, a class B *mec* gene complex lacking the Ψ*IS1272* upstream from the *mecA* gene was identified, and neither the *ccr* gene nor any putative site-specific serine recombinase gene was identified elsewhere in the chromosome (**Figure 1**). The ΨSCC*mec*, designated as ΨSCC*mec*$_{E16SA093}$, resembled a SCC*mec* type IV, which is common in MRSA ST72. When compared with the genome of HL1 that is a CA ST72-MRSA-IV recovered in South Korea before 2010 (Chen et al., 2013), a 12.6-kb region was deleted from the SCC*mec* type IV element, and 7.0- and 31.9-kb chromosomal DNA region was deleted in the E16SA093 and F17SA003, respectively. The Ccr recombinase involves the site-specific integration/excision of SCC*mec* elements (International Working Group on the Classification of Staphylococcal Cassette Chromosome, 2009), and the CcrA2/CcrB2 in the SCC*mec* IV is targeting the *attB* site at the *orfX* gene (Wang and Archer, 2010). The ΨSCC*mec*$_{E16SA093}$ was indeed integrated exactly at *attB*, suggesting the subsequent elimination of the *ccrA2*/*ccrB2* genes from the SCC*mec* IV element after the integration event. As the Ψ*IS1272* was absent, IS-associated recombination was suspected. However, no palindromic sequences were observed in either end of the deleted 19.7- and 44.5-kb DNA fragments targetable by IS*1272*, with an insertion sequence involved in the structure-dependent transposition or stem-loop replacement (Wan et al., 2017).

TABLE 1 | ST72 MRSA isolates harboring the ΨSCC*mec*$_{E16SA093}$ element.

Isolate	Year of isolation	Infection type	Clinical details/sex	Antimicrobial resistance pattern[a]	Antimicrobial resistance gene[b]	Virulence-associated gene[c]	*spa*[d]	*pvl*[e]	TSST-1[e]	*agr*
E16SA093	2016	CA	Bloodstream infection originated from bone infection/female	FOX	*mecA, aadD, blaZ*	*seo, sem, sei, seu, sen, seg, lukE, lukD, aur, splA, splB, hlgB, hlgC, hlgA, hlb, sak, scn*	t324	ND	ND	I
F17SA003	2017	HA	Bloodstream infection of unspecified origin of infection/male	FOX, EM, CLN	*mecA, aadD blaZ*[f]	*seo, sem, sei, seu, sen, seg, lukE, lukD, aur, splA, splB, hlgB, hlgC, hlgA, hlb, sak, scn*	t2460	ND	ND	I

CA, community-associated; HA, healthcare-associated; spa, staphylococcal protein A; pvl, Panton-Valentine leukocidin; TSST-1, toxic shock syndrome toxin; agr, accessory gene regulator. [a]*The antimicrobial resistance was determined against a panel of 10 antistaphylococcal agents, including cefoxitin (FOX), erythromycin (EM), clindamycin (CLN), quinupristin/dalfopristin, cotrimoxazole, mupirocin, vancomycin, teicoplanin, linezolid, and tigecycline.*

[b]*The acquired antimicrobial resistance gene was searched for against the database of ResFinder.*

[c]*The virulence-associated gene was searched for against the database of VirulenceFinder.*

[d]*The spa type t324 was 07-23-12-12-17-20-17-12-12-17, and the spa type t2460 was 26-17-34-34-17-20-17-17-17-16.*

[e]*ND, Not detected.*

[f]*Two copies of the blaZ gene were identified in the F17SA003 chromosome.*

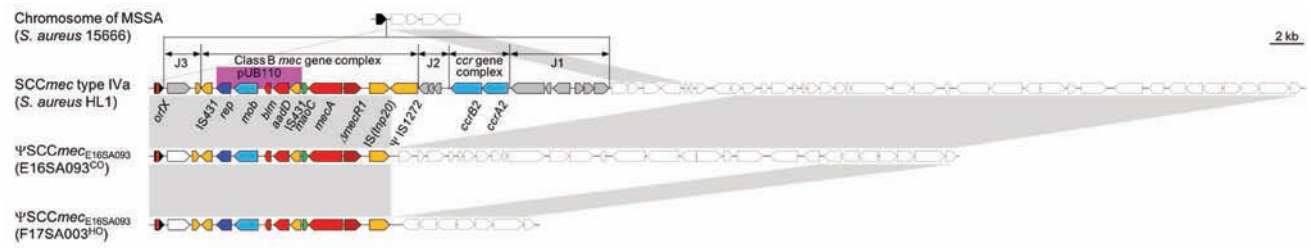

FIGURE 1 | Genomic structures and environments of the ΨSCC*mec*$_{E16SA093}$ element in comparison with the SCC*mec* type IVa in MRSA HL1 (Chen et al., 2013) (GenBank accession, CP003979) and *orfX* in MSSA 15666 (GenBank accession, EU272085). The conserved region in the gray box showed a resemblance of >99% nt identity. Filled arrows represented the components consisting of the SCC*mec* element. The open arrows indicated the presence of chromosomal genes in *S. aureus*. Arrows in black, *orfX*; red, resistance genes; yellow, transposase; blue, replication origin; sky blue, recombinase; gray, hypothetical coding sequence.

Epidemiology of ΨSCC*mec*

The fitness benefit of the *ccr*-gene-loss from SCC*mec*, resulting in an inherent *mecA* in the chromosome, has never been assessed, while spontaneous *mecA*-gene-loss in the absence of selective pressure, driven by the huge biological cost of gene expression, has been demonstrated (Noto et al., 2008). The furnished *mecA* gene could provide advantages to MRSA in the beta-lactam-abundant habitat, such as the clinical settings, suggesting a course of adaptive evolution for MRSA. While ΨSCC*mec* is occasionally identified in MRSA (Chen et al., 2010), methicillin-resistant coagulase-negative staphylococci (MRCNS) carrying the element is much more common (Perreten et al., 2013; Shore and Coleman, 2013). The speculation of MRCNS to be a reservoir of SCC*mec* (Archer et al., 1994), in the MRSA is inspiring.

In this study, we evaluated MRSA ST72 isolates carrying ΨSCC*mec*$_{E16SA093}$, which was likely being fabricated from the SCC*mec* type IV. Excised portions of the chromosomes differed in size, and the event likely occurred independently, indicating that the clonal dissemination of ST72-MRSA-ΨSCC*mec*$_{E16SA093}$ has not yet been occurred. The immobile *mecA* gene could make the MRSA fit the antimicrobial-abundant habitat, even though the *mecA* gene expression is known to be costly. Further study of the molecular mechanisms driving *ccr* gene loss is needed, and dissemination of the clone should be surveilled.

ETHICS STATEMENT

The research, which has no involvement of human subject but the clinical isolates, does meet the exempt category without approval from Ethics Committee on Human Research of the Health Ministry in South Korea and the study design has not been reviewed by the committee.

AUTHOR CONTRIBUTIONS

HL and SJ conceived the study. E-JY, HL, and SJ analyzed the data. E-JY and SJ wrote the manuscript. DK, JoS, and JeS collected the strains.

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frontiers in Microbiology

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Fecal Colonization With Multidrug-Resistant *E. coli* Among Healthy Infants in Rural Bangladesh

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Third generation cephalosporins (3GC) are one of the main choices for treatment of infections caused by multidrug-resistant (MDR) Gram-negative bacteria. Due to their overuse, an increasing trend of resistance to 3GC has been observed in developing countries. Here, we describe fecal colonization of 3GC-resistant (3GCr) *Escherichia coli* in healthy infants (1–12 months old) living in rural areas of Bangladesh. We found that stool samples of 82% of infants (n = 100) were positive for 3GCr *E. coli* with a mean ± standard deviation of 6.21 ± 1.32 $\log_{10}$ CFU/g wet weight of stool. 3GCr *E. coli* encompasses an average one third (33%) of the total *E. coli* of stool. Almost 77% (n = 63) of these 3GCr *E. coli* were MDR (or resistant to ≥3 classes of antibiotics). Around 90% (n = 74) of 3GCr *E. coli* were extended spectrum beta-lactamase (ESBL)-producing in which $bla_{\text{CTX-M-group-1}}$ was the predominant (96%, n = 71) ESBL-gene followed by bla_{TEM} (41%, n = 30) and $bla_{\text{OXA-1}}$ (11%, n = 8). A significant proportion (26.5%, n = 22) of 3GCr *E. coli* was pathogenic, comprising two types, enteroaggregative (EAEC, n = 19) and enteropathogenic (EPEC, n = 3). Colonization of 3GCr *E. coli* in infant guts was not associated with demographic characteristics such as age, sex, mode of delivery, maternal and infant antibiotic use, disease morbidity, and feeding practices. The high rate of colonization of 3GCr *E. coli* in infants' guts is a serious public health concern which needs immediate attention and warrants further studies to explore the cause.

Keywords: colonization, multidrug-resistant, *E. coli*, ESBL, third generation cephalosporins

INTRODUCTION

The rapid rise of multidrug-resistant (MDR) bacterial infections is a major public health concern and a growing threat to the global health security. Unregulated use of broad spectrum antibiotics and widespread reservoirs of these pathogens are main contributors to this problem (Hilty et al., 2012). Broad spectrum antibiotics, in particular third generation cephalosporins (3GC), are among

the most frequently prescribed drugs for the treatment of infections caused by Enterobacteriaceae (Pereira et al., 2004; World Health Organization [WHO], 2017). Failure of treatment with these antibiotics has increasingly been reported due to the emergence of extended spectrum beta-lactamases (ESBLs) during the last two decades (Pitout and Laupland, 2008). Several studies have suggested that children are more likely to be exposed to antibiotics both directly (Alexander et al., 2011; Saari et al., 2015) and indirectly, through exposure to antibiotics taken by their mothers (Verani et al., 2010; Macones et al., 2012; Ledger and Blaser, 2013; de Tejada, 2014). This direct and/or indirect consumption of antibiotics might thus affect infants' intestinal microflora, including *Escherichia coli*, which is one of the first bacterial species that colonizes the infant gut (Hewitt and Rigby, 1976).

Antimicrobial resistance in commensal bacteria is worrisome due to its ability to spread to pathogens (Munk et al., 2018). Recent studies have showed that school children and children up to 2 years of age were colonized by *E. coli* resistant to broad spectrum antibiotics and ciprofloxacin, respectively (Gurnee et al., 2015; Ferjani et al., 2017). However, there is no information on the carriage rate and abundance of this antibiotic-resistant *E. coli* in relation to the total number of *E. coli* present in the gut. In addition, there is no data available on the carriage rate of MDR *E. coli*, including ESBL-producing *E. coli*, among infants under 1 year old. Therefore, this study evaluated the prevalence and rate of colonization of this organism during the early life of infants. We determined the prevalence, abundance, and carriage rate of 3GC-resistant (3GCr) *E. coli*, including pathogenic *E. coli*, by analyzing culturable *E. coli* in infant's stool samples.

MATERIALS AND METHODS

Ethics Statement

The research and the ethical review committees of icddr,b approved and monitored the progress of the study. Informed written consent was obtained from mothers of all infants either by signature or, for those who were not literate, by thumbprint after verbal communication. Samples were identified with codes to preserve anonymity. A witness also signed each informed-consent form. All authors vouch for the completeness and accuracy of the data and analyses presented.

Study Design, Site, and Enrollment of Participants

We conducted a cross-sectional study of children <1 year of age living in five rural villages of Matlab and Hajiganj, sub-districts of Chandpur district of Bangladesh, between March and October 2017. Hajigonj has a total area of 189 km^2 with 327,367 people living in 64,257 households at 11 unions, whereas Matlab Uttar has a total area of 279 km^2 with 382,195 people in 62,418 households at 15 unions (DGHS Health Bulletin, 2014). According to the The World Bank (2016) the crude birth rate for Bangladesh is 18.95 per 1000 people as of 2016, so an approximation for the number of infants in Hajigonj and Matlab Uttar is 6,200 and 7,250, respectively, or less than 13,500 total infants (2016). For study sites we included one union from each sub-district. A total of 100 households (50 from each union) containing one infant (age ≤1 year) in each household were enrolled in the study after obtaining written informed consent from the mothers of enrolled infants. A pre-tested survey questionnaire was used to collect information on age, sex, mode of delivery, maternal and child antibiotic consumption, disease morbidity, and feeding practices.

Sample Collection

A total of 100 stool samples were collected from 100 infants located in the selected households using sterile stool containers provided earlier to all the mothers on the date of the interview. Assuming an infant population in Hajigong and Matlab Uttar of 13,500, the sample size of 100 stool samples would imply a margin of error of approximately 10% with 95% confidence interval for prevalence rates of 3GCr *E. coli* (Dhand and Khatkar, 2014). The field staff collected the samples on the following day and transported it to icddr,b laboratory (Dhaka, Bangladesh) on ice for microbiological analyses within 4 h.

Enumeration and Isolation of *E. coli*

Both total and 3GCr *E. coli* were enumerated using the drop plate method as described previously (Herigstad et al., 2001). In brief, MacConkey agar plates (Becton Dickson, MD) with and without fixed concentrations of cefotaxime (1.0 μg/mL) were used to enumerate 3GCr *E. coli* and total *E. coli*, respectively. A total of four 10-fold serial dilutions (10^{-1} to 10^{-4}) of each stool sample were made and 50 μl from each dilution was inoculated onto MacConkey agar plates with and without antibiotic added to the culture media. All plates were incubated at 37°C for 18 h and the number of colony forming units (CFUs) per gram wet weight of stool sample were counted from the dilution of readable range. Proportion of 3GC-sensitive (3GCs) *E. coli* CFUs per gram feces (CFU/g) count was calculated by subtracting 3GCr CFU/g count from corresponding total *E. coli* CFU/g. Further, proportion of 3GCr *E. coli* count was measured in respect to the total *E. coli* count obtained on MacConkey agar plate. At least three colonies from each sample were confirmed as *E. coli* by API-20E (bioMerieux, France) and stored at −80°C for further characterization.

Antibiotics Susceptibility Test

Antibiotic susceptibility of *E. coli* (one isolate per sample) was determined by standard disk diffusion technique following the Clinical and Laboratory Standards Institute (CLSI) guidelines (Patel, 2017). The antibiotics used in this study were ampicillin (10 μg), gentamycin (10 μg), tetracycline (30 μg), meropenem (10 μg), imipenem (10 μg), ceftriaxone (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), colistin (10 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), azithromycin (15 μg), trimethoprim/sulfamethoxazole (25 μg), nitrofurantoin (30 μg), and chloramphenicol (30 μg) (Oxoid, United Kingdom). The zone of inhibition was measured and the isolates were classified as resistant, intermediate, or sensitive according to the interpretation guideline provided by the CLSI (Patel, 2017).

An isolate was considered MDR if resistant to three or more classes of antibiotics.

Test for Extended Spectrum Beta-Lactamase (ESBL)

Extended spectrum beta-lactamase was tested by combination disk test (CDT) as described by CLSI (Patel, 2017). Disks containing a 3GC, including cefotaxime, CTX (30 μg) or ceftazidime, CAZ (30 μg) alone and in combination with clavulanic acid (CLA, 10 μg) were applied (Oxoid, United Kingdom). The zone of inhibition around the CTX or CAZ disk combined with CLA was compared with the inhibition zone around CTX or CAZ disks alone. The test was considered positive for ESBL-production if the inhibition zone diameter was ≥5 mm larger with CLA than without (Patel, 2017).

PCR for ESBL Genes and *E. coli* Pathotypes

All 3GCr *E. coli* were tested for ESBL genes considered priorities due to their clinical relevance, specifically: $bla_{\text{CTX-M-group-1}}$, bla_{SHV}, bla_{TEM}, and $bla_{\text{OXA-1}}$. These genes were tested by PCR using primer sequences and PCR conditions as described previously (Islam et al., 2017). The pathotypes of *E. coli* (EPEC, ETEC, EAEC, EIEC, and STEC) were identified by multiplex PCR of different pathogenic genes according to the procedure described earlier (Talukdar et al., 2013).

Statistical Data Analysis

Data were entered in SPSS 20.0 (IBM Inc., Chicago, IL, United States). Data cleaning, statistical analysis and graphical presentation were done in Stata 13.0 (College Station, TX, United States) and R-3.4.2 (R Core Team, 2014). *E. coli* concentrations were $\log_{10}$ transformed in order to assess the association between demographic variables with 3GCr *E. coli* carriage using chi-square test and non-parametric Mann–Whitney *U*-test. Population counts of the susceptible and resistant isolates from the same infant were compared using Wilcoxon Rank-Sum test on the paired data. Statistical significance was determined using alpha = 0.05 for all tests except for the Wilcoxon Rank-Sum test which stratified analyses by age (1–3, 4–6, 7–9, and 10–12 months). For this, the conservative Bonferroni correction was applied to adjust alpha to 0.0125 (0.5/4) to correct for multiple comparisons.

RESULTS

Carriage of 3GCr *E. coli* in Infant's Fecal Sample

Of the 100 stool samples from infants, 82% were positive for 3GCr *E. coli*. Mean count ± standard deviation for total *E. coli* was 6.86 ± 1.56 $\log_{10}$ *E. coli* CFU/g of stool while the mean count of 3GCr *E. coli* was 6.21 ± 1.32 $\log_{10}$ CFU/g. On average, 3GCr *E. coli* encompasses approximately one third of (33%) of the total *E. coli* present/g wet weight of stool.

Antibiotic Susceptibility of 3GCr *E. coli*

All the 3GCr (n = 82) *E. coli* isolates were tested for susceptibility against a panel of 13 antibiotics. Resistance to multiple antibiotics other than 3GCr was very common, with 77% (n = 63) of isolates classified as MDR. None of the isolates were resistant to colistin or carbapenem (**Figure 1**).

Prevalence of ESBLs Among 3GCr *E. coli* Isolates

CDT of all 3GCr *E. coli* (n = 82) isolates revealed that more than 90% (n = 74) were ESBL-producing. Among ESBL-producing isolates, 96% (n = 71) were positive for $bla_{\text{CTX-M-group-1}}$, 41% ($n$ = 30) for bla_{TEM}, and 11% (n = 8) for $bla_{\text{OXA-1}}$. Of the 8 CDT negative isolates, 4 were positive for $bla_{\text{CTX-M-group-1}}$ indicating that these isolates might have co-produced ESBL and AmpC enzymes. Given the high rates of positivity for detection of $bla_{\text{CTX-M-group-1}}$, further molecular characterization for resistance genes was not conducted; carriage of multiple mechanisms of resistance is possible but was not explored.

Prevalence of *E. coli* Pathotypes Among 3GCr *E. coli* Isolates

Analysis of virulence genes among 3GCr isolates revealed that 23% (n = 19) of the isolates were positive for genes specific for EAEC (*aat*A, *aai*C) and 3.6% (n = 3) of the isolates were positive for genes specific for EPEC (*bfp*, *eae*). No other virulence genes were detected (*lt*, *st*, *ipa*H, and *ial*).

Determinants of 3GCr *E. coli* Carriage Among Infants

Statistical analysis of results did not reveal any significant association between the presence of 3GCr *E. coli* in infant stool and characteristics of the infant, including gender, religion, age,

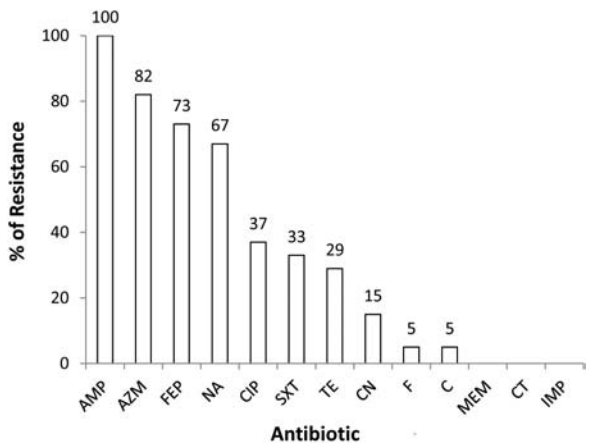

FIGURE 1 | Prevalence of 3GCr *E. coli* resistant to different classes of antibiotics in infants aged from 1 to 12 months (n = 82). AMP, ampicillin; AZM, azithromycin; FEP, cefepime; NA, nalidixic acid; CIP, ciprofloxacin; SXT, sulfamethoxazole; TE, tetracycline; CN, gentamycin; F, nitrofurantoin; C, chloramphenicol; MEM, meropenem; CT, colistin; IMP, imipenem; 3GCr, third generation cephalosporins resistant.

mode of delivery, feeding practice of child, diarrheal history, and maternal and child antibiotic consumption (**Table 1**). Moreover, association between the rate of colonization of 3GCr *E. coli* in the child's gut and demographic characteristics of infant (age, $p = 0.5$; delivery mode, $p = 0.8$; infant and mother antibiotic consumption, $p = 0.2$, $p = 0.4$; and infant diarrhea, $p = 0.2$) were not statistically significant in non-parametric Mann–Whitney *U*-test (**Table 2**). Therefore, the presence or abundance of 3GCr *E. coli* in infant stools in this study could not be explained by some of the most common risk factors, including prior exposures to antibiotics.

Age-Wise Distribution of Fecal Carriage of 3GCr *E. coli* in Respect to 3GCs *E. coli*

We calculated the mean count of 3GCr *E. coli* among infants of the same age groups and plotted the distribution with 1 month intervals in order to determine if the CFU count of resistant microflora changes with infant age. Analysis of mean count of 3GCr up to 12 months showed that $\log_{10}$ CFU mean count of 3GCr even at 3 months of age was high (6.43 $\log_{10}$) and consistent in subsequent months (**Figure 2**), indicating early appearance of 3GCr *E. coli* in infants. However, the percentage of infants infected with resistant *E. coli* population (3GCr and MDR) was progressively increased as infant age grown by months (**Figure 3**).

We compared the differences between 3GCr and 3GCs *E. coli* counts among infants of the same age groups at 1–3, 4–6, 7–9, and 10–12 months of age to examine whether 3GCr *E. coli* co-exist with the 3GCs favorably without selective pressure of antibiotics. There was no significant difference in the population of 3GCr and 3GCs *E. coli* using Wilcoxon Rank-Sum test at any age group except for the oldest one, 10–12 months ($p = 0.011$) (**Figure 4**), suggesting that 3GCr *E. coli* can stably persist like 3GCs *E. coli* from early months of life. Notably, statistical significance in the difference in 3GCr and 3GCs *E. coli* amongst infants 10–12 months old is borderline significant compared to the Bonferroni adjusted significance level of alpha = 0.0125 for the age-stratified Wilcoxon Rank-Sum test.

TABLE 1 | Demographic characteristics of infants with and without fecal carriage of third generation cephalosporins resistant (3GCr) *E. coli*.

Characteristics	3GCr *E. coli*		*p*-Value
	Positive, *n* = 82 (%)	Negative, *n* = 8 (%)	
Sex (Male)	44 (54)	3 (38)	0.472
Religion (Muslim/Hindu)	72 (88)	5 (63)	0.087
Age ≤6 months	33 (40)	5 (63)	0.275
Mode of delivery (CS)*	27 (32)	3 (38)	1.000
Mode of delivery (NVD)*	27 (32)	3 (38)	1.000
Exclusively breast feeding	1 (1)	0	1.000
Exclusively formula feeding	1 (1)	0	1.000
Complementary feeding	80 (98)	7 (88)	1.000
Diarrhea (Yes)	9 (11)	0	0.593
Antibiotic consumption (6 months before)	53 (65)	3 (38)	0.149
Other complication (Cold)	47 (57)	2 (25)	0.135
Maternal antibiotic consumption	12 (15)	1 (13)	1.000

**NVD, normal vaginal delivery; CS, cesarean section. *Mode of Delivery (CS and NVD) was obtained for 64 cases, and information from the remaining 36 cases was not available. Number in the parentheses indicates percentage.*

TABLE 2 | Association between demographic variables and third generation cephalosporins resistant (3GCr) *E. coli* colonization in infants' gut.

Factors	Characteristics	Frequency (*n*)	3GCr *E. coli* mean count (CFU/g wet weight of stool)	*p*-Value
Infant age	Age <= 6	33	3.2×10^6	0.630
	Age > 6	49	2.1×10^6	
Mode of delivery	NVD	27	3.3×10^6	0.665
	CS	27	2.4×10^6	
Child antibiotics	Yes	53	3.2×10^6	0.259
	No	29	1.6×10^6	
Child diarrhea	Yes	9	1.3×10^6	0.632
	No	70	2.2×10^6	
Maternal antibiotics	Yes	12	4.2×10^6	0.758
	No	70	2.3×10^6	

NVD, normal vaginal delivery; CS, cesarean section.

DISCUSSION

We found an extremely high prevalence of both 3GCr *E. coli* (82%) and ESBL-producing *E. coli* (74%) in stool samples of healthy infants living in rural areas of Bangladesh. Despite the relatively small sample size ($n = 100$) chosen based on logistic constraints, the high prevalence rates are likely representative of Bangladeshi infants under 1 year old in the study area within a margin of error of 10% (with 95% confidence interval). Even with the associated uncertainty, this is the highest prevalence of ESBL-producing *E. coli* in healthy human guts observed to date. For example, in a study in Tunisia, the prevalence of MDR *E. coli* was 6.6% in children aged between 6 and 12 years (Ferjani et al., 2017). The rates of 3GCr *E. coli* in healthy children at various ages was reported as 2.9% in Sweden, 2.7% in Portugal, and 10% in Senegal (Guimaraes et al., 2009; Kaarme et al., 2013). The fecal carriage rate of ESBL-producing Enterobacteriaceae in healthy children (0–59 months) was much higher (59%) in central Africa (Farra et al., 2016). None of these studies have reported the data on culturable 3GCr *E. coli* as a proportion of total culturable *E. coli* in stool samples. In our study we found that around one third of the total *E. coli* colonies obtained in culture were 3GCr, which is alarming.

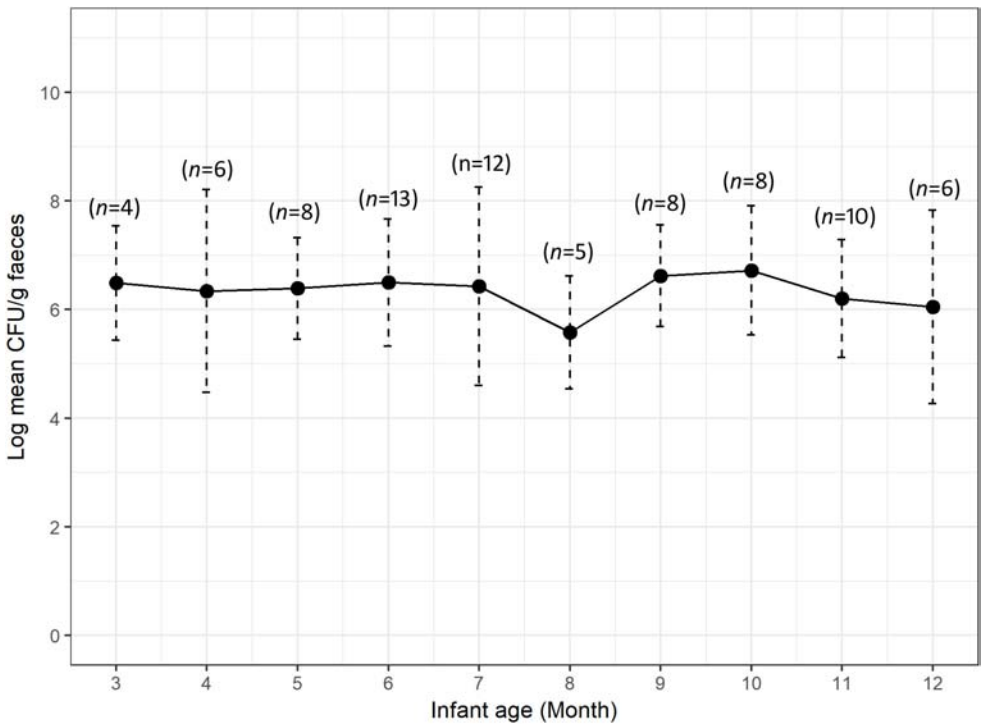

FIGURE 2 | Variations in carriage rates of 3GCr *E. coli* in infants at different ages from 3 to 12 months. The $\log_{10}$ mean count of 3GCr CFU from infant at 1 and 2 month of age was excluded because only one infant of each month was obtained for these time periods. Error bars are the standard errors of the results for each age group. 3GCr, third generation cephalosporins resistant.

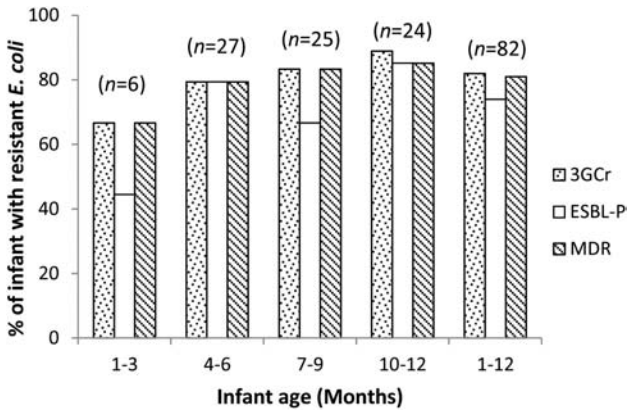

FIGURE 3 | Age wise distribution of prevalence of 3GCr, MDR, and ESBL-P *E. coli* in infant stool samples. 3GCr, third generation cephalosporins resistant; ESBL-P, ESBL-producing; MDR, multidrug-resistant.

The difference between 3GCr and 3GCs *E. coli* gives an indication of fitness costs for the maintenance of resistance within the gut microbial community. The proportion of 3GCr amongst the total *E. coli* (sum of 3GCr and 3GCs *E. coli*) was not significant in infants at different age groups, indicating that the competitiveness of resistant bacteria with normal residential flora within the gut is not influenced by age. There may be a low fitness cost associated with persistence and dissemination of resistance. Indeed, Cottell et al. (2012) suggest a low fitness cost associated with plasmid (pCT) carrying the resistance gene $bla_{\mathrm{CTX-M-14}}$ for *E. coli*, as the *E. coli* were able to persist and disseminate readily even in the absence of selective pressure from antibiotics (Cottell et al., 2012). Our findings also displayed congruence with previous reports demonstrating that tetracycline- and ampicillin-resistant isolates persist continuously without any selective pressure of antibiotics in the gut of different age groups of children (Karami et al., 2006, 2008).

The implication of this high load of 3GCr *E. coli* is substantial in the context of child health safety. Antibiotic resistant *E. coli* and other common commensals of the gut including *Klebsiella* spp. and *Acinetobacter* spp. are amongst the leading causes of community-acquired serious infection in Southeast Asia. In a cross sectional study at five sites across Southeast Asia, Saha et al. (2018) found that only 17% of possible serious bacterial infections (pSBI) identified in young children were resistant to first line antibiotics. Resistant infections, as compared to susceptible infections, are linked with worse outcomes. For example, in Tanzania, children with septicemia caused by bacteria producing extended-spectrum beta-lactamases were almost twice as likely to die compared to non-ESBL infections (71% mortality rate vs. 39%) (Blomberg et al., 2007). In the present study, the observed high carriage rate and high relative proportion of culturable 3GCr *E. coli* may harbinger higher rates of 3GCr

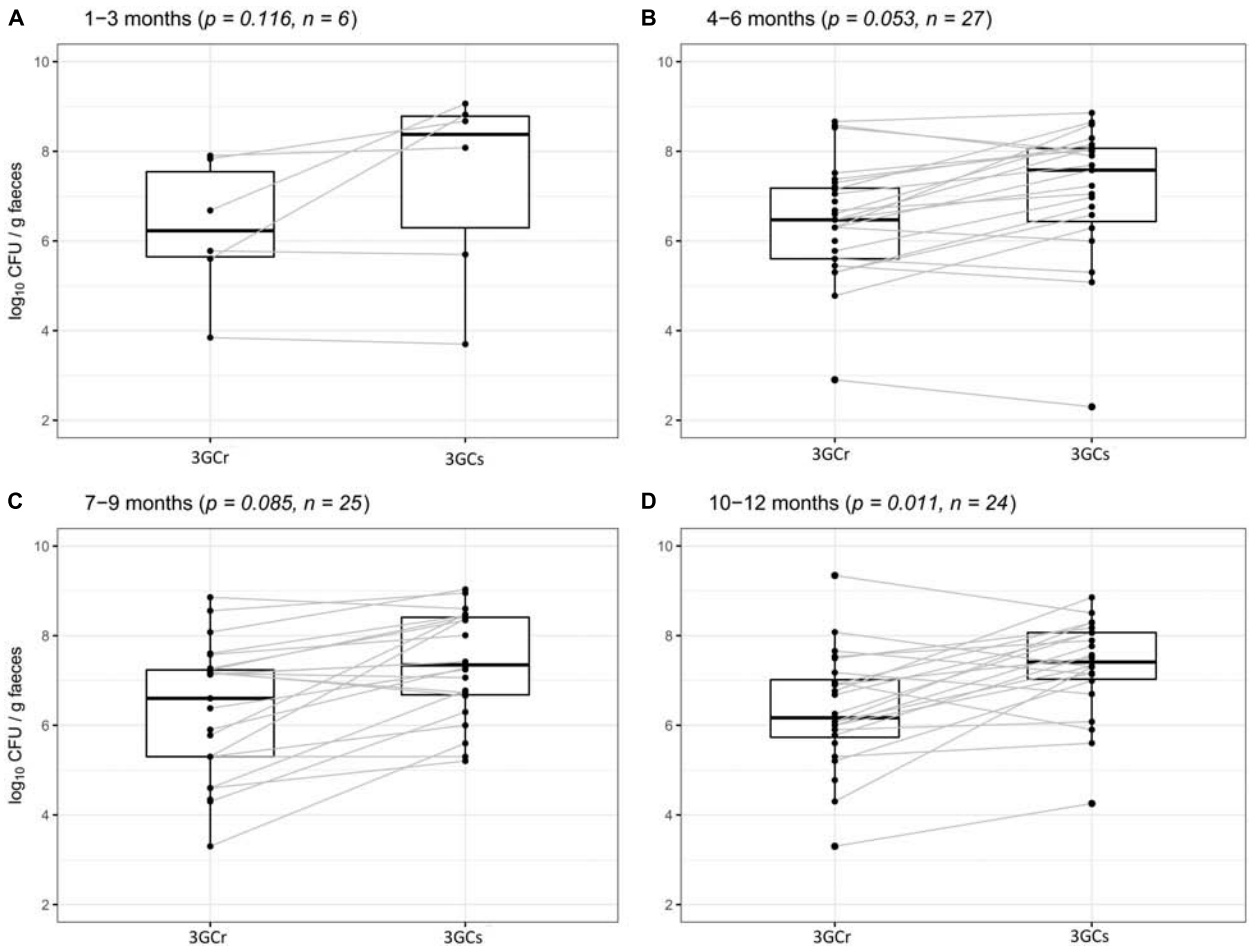

FIGURE 4 | Comparative analysis of carriage rates (in CFU/g wet weight of stool) of 3GCr and 3GCs *E. coli* within similar age group of infants. **(A)** 1–3 months of age, **(B)** 4–6 months of age, **(C)** 7–9 months of age, and **(D)** 10–12 months of age. 3GCr, third generation cephalosporins resistant; 3GCs, third generation cephalosporins sensitive; CFU, colony forming unit.

E. coli as a proportion of pSBI infections. Further research linking fecal carriage of resistant bacteria to risks of resistant infections is warranted.

Apart from increasing the risk of resistant infections, high carriage of 3GCr Enterobacteriaceae in the gut results in shedding through infant stool and thus contributes to an elevated risk of exposure to nearby people and animals. There is a common belief among illiterate or less literate mothers in the community that infant stool is not a health hazard or harmful, especially in comparison to adult stool (Yeager et al., 1999; Gil et al., 2004). According to a national survey during the period from 2012 to 2013 in Bangladesh, feces of about 61% children of age 0–2 years were disposed unsafely where the percentage was much higher in rural areas (67%) compared to urban areas (40%) (Bangladesh Bureau of Statistics [BBS] and United Nations Children's Fund [UNICEF], 2014). Thus household members including mothers or caregivers are exposed to fecal MDR bacteria through unsafe disposal of infant feces. Similarly, improper disposal of infant's stool in front yards or nearby ditches may contribute to transmission of these resistant bacteria to domestic and wild birds and/or other animals (Hasan et al., 2012).

The 3GCr *E. coli* isolates in this study were predominantly resistant to azithromycin and ciprofloxacin, among other antibiotics (**Figure 1**). Azithromycin is the first line of choice for treatment of shigellosis in children (Centers for Disease Control and Prevention [CDC], 2006) and a second line of choice for treatment of shigellosis in adults (World Health Organization [WHO], 2005). Although no infants were reported to be suffering from shigellosis during the study period, shigellosis has been identified as a major contributor to moderate-to-severe diarrheal disease in neighboring Mirzapur (Kotloff et al., 2013). Management of this infection might be complicated due to the high prevalence of azithromycin resistance in the study community. Among other antibiotics, ciprofloxacin resistance was found among 37% of 3GCr isolates, which is even higher than a previous report that showed resistance in 19% of *E. coli* obtained from healthy children (Gurnee et al., 2015). Interestingly, only 29% of *E. coli* isolates in this study were resistant to tetracycline, a first generation antibiotic which is less commonly used for the

treatment of *E. coli* infections in the community (Calva et al., 1996; Domínguez et al., 2002). Tetracycline is not prescribed in children due to its effect on the growth of bones and teeth (Sánchez et al., 2004). It suggests that by reducing the use of antibiotics in humans and animals, it is possible to reduce the burden of resistant microorganisms and this can eventually restore the efficacy of the existing antibiotics even in a setting like Bangladesh where overuse of antibiotics and burden of AMR, both are way too high.

Our study demonstrated that infant's guts serve as a reservoir of *E. coli* resistant to multiple antibiotics including 3GCr and fluoroquinolones, which are critical for the treatment of many infectious diseases in humans (World Health Organization [WHO], 2017). High rates of ESBL-producers among 3GCr isolates in the present study is alarming because patients with community acquired infections as well as their household members carrying ESBL-producing Enterobacteriaceae may spread resistance to other people in their community (Valverde et al., 2008). This can be explained by the overall high prevalence of ESBL *E. coli* infections in the community. A recent study in Bangladesh has reported that 34% of all clinical isolates of *E. coli* from patients with extra-intestinal infections were ESBL-producing (Khan et al., 2018).

The probable cause of colonization with ESBL-producing Enterobacteriaceae among pre-school children in Laos was reported due to misuse of antibiotics (Stoesser et al., 2015). In our study, we did not observe this: there was no significant association between colonization and reported previous use of antibiotic treatment among infants. Previous studies have suggested that acquisition of antimicrobial resistant bacteria or antimicrobial resistant genes in the infant gut might occur during and/or after the delivery (Zhang et al., 2011). Specifically, mothers' flora during normal vaginal delivery or environmental flora during caesarian (C-section) delivery colonize the infant gut (Zhang et al., 2011). Therefore, AMR bacteria from the mother or hospital environment may contribute to infant carriage. For example, a study carried out in Tunisia showed that 20% of patients acquired 3GCr *E. coli* in their gut due to nosocomial infection (Maamar et al., 2016). In our study, we did not find any significant differences in the level of colonization with 3GCr *E. coli* between infants with normal vaginal delivery and infants delivered through C-section (**Tables 1**, **2**). In Bangladesh, a recent study showed that delivery by C-section increased from 3.5 to 23% between 2004 and 2014 (Khan et al., 2017) and it is a common practice that prophylactic antibiotics are used before and after the surgery. During post-operative care mothers start to breastfeed the newborn while still on antibiotic treatment. This leads to transfer of antibiotics in its active form to infants and thus their gut microbiota may shift to survive in an antibiotic selective environment (Mathew, 2004). Further in Dhaka, Bangladesh, a significant proportion of newborns (98%) receive antibiotics (sulfonamides, fluoroquinolones, metronidazole, penicillins, etc) before 6 months of age (Rogawski et al., 2017), which renders the selective environment for antibiotic-resistant bacteria. Even in a very low concentration of antibiotics, fitness cost for microorganisms to become resistant is lower than becoming antibiotic susceptible (Sandegren, 2014). Surprisingly, in our study neither history of antibiotic use or previous record of hospitalization was associated with 3GCr colonization nor were gender, religion and feeding practices. The lack of association may be due to the high rate of colonization combined with relatively small sample size (n = 100). The low proportion of infants without 3GCr limits statistical analyses. Therefore, further studies, particularly focused on larger sample sizes, are needed to identify the causes of the high rate of antibiotic resistance carriage among infants under 1 year old in this setting.

CONCLUSION

The high rate of intestinal carriage with MDR microorganisms among infants in rural Bangladesh is a serious concern that can jeopardize the management of infectious diseases. In addition, shedding of high number of MDR microorganisms through infant feces increases the risk of widespread transmission of these microorganisms in the community and environment. This study raises important questions about how the acquisition of resistant microorganisms takes place in infants' guts within the first 3 months of life, what are the major drivers of acquisition, and what are the implications on infant health and well-being. Future studies should explore the source of acquisition of resistance in infants, to understand whether such resistance is primarily acquired from the environment, vertically from the child's mother, or through selective pressure from pediatric antibiotic use.

AUTHOR CONTRIBUTIONS

MAI and TJ conceived the development. MAI, TJ, MK, KL, TN-D, and MM designed the study and developed the protocol. MA, SR, and MBA contributed to the experiments, collection, and assembly of the data. MRI, MBA, and TJ contributed to the data entry and statistical analysis. MAI and MBA performed the first draft of the manuscript. TJ, MK, KL, TN-D, MM, MA, and MAI revised the manuscript and prepared the final draft of the manuscript.

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The Role of Plasmids in the Multiple Antibiotic Resistance Transfer in ESBLs-Producing *Escherichia coli* Isolated From Wastewater Treatment Plants

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Li Q, Chang W, Zhang H, Hu D and Wang X (2019) The Role of Plasmids in the Multiple Antibiotic Resistance Transfer in ESBLs-Producing Escherichia coli Isolated From Wastewater Treatment Plants. Front. Microbiol. 10:633. doi: 10.3389/fmicb.2019.00633

We compared the diversity of extended-spectrum β-lactamases (ESBLs) producing *Escherichia coli* (*E. coli*) in wastewater of a municipal wastewater treatment plant. This was done by analyzing multiple antibiotic resistant phenotypes and genotypes. Also, we investigated the antibiotic resistance transfer mechanism of the plasmid by comparing the antibiotic resistance gene linked transfer using a conjugative test, and by analyzing the full-length DNA sequence of one plasmid. The results showed that 50 ESBLs-producing *E. coli* isolates were isolated from 80 wastewater samples at the rate of 62.5% (50/80), out of which 35 transconjugants were obtained with the multiple antibiotic resistant transfer rate as high as 70.0% (35/50). Multiple antibiotic resistance was shown in all transconjugants and donor bacteria, which were capable of resistance to 11 out of 15 kinds of antibiotics. Both transconjugants and donors were capable of resistance to the Ampicillin and Cefalotin at a rate of 100.00% (35/35), while the total antibiotic resistant spectrum of transconjugants narrowed at the rate of 94.29% (33/35) and broadened at the rate of 5.71% (2/35) after conjugate to the donor bacteria. PCR showed that the resistant genotypes decreased or remained unchanged when compared to donor bacteria with transconjugants while the bla_{TEM} and bla_{CTX-M} genes were transferred and linked at a rate of 100.00% (35/35) and the bla_{SHV} gene was at the rate as high as 94.29% (33/35). However, the *qnrS* gene was transferred at a low rate of 4.17% (1/24). In addition, the major resistance gene subtypes were bla_{TEM-1}, bla_{SHV-11}, and $bla_{CTX-M-15}$ according to sequencing and Blast comparison. Plasmid wwA8 is a closed-loop DNA molecule with 83157 bp, and contains 45 predicted genes, including three antibiotic resistant resistance genes, $bla_{CTX-M-15}$, bla_{TEM-1} and *qnrS1*, which can be transferred with *E. coli in vitro*. This study shows that *E. coli* isolated

from wastewater was capable of transferring resistance genes and producing antibiotic resistant phenotypes. The plasmids containing different resistance genes in *E. coli* play an important role in the multiple antibiotic resistant transfer. Most importantly, antibiotic resistant resistance genes have different transfer efficiencies, the bla_{TEM} and $bla_{CTX\text{-}M}$ genes transferred at a rate of 100.00% and linked transfer in all 35 transconjugants.

Keywords: *Escherichia coli*, ESBLs, multiple antibiotic resistant, transconjugants, plasmid

INTRODUCTION

Enterobacteriaceae, particularly *Escherichia coli* (*E. coli*), are among the most important zoonotic pathogens. They are widely distributed in aquatic environments and can cause infectious disease in most animals and humans, such as urinary tract infections, diarrhoea, enteritis, and septicaemia (Lewis et al., 2007; Ang et al., 2016). Abuse and overuse of antibiotics in the clinic has resulted in the emergence of multiple antibiotic resistant bacteria strains (Goldman, 2004). In addition, an increase in the prevalence of multiple antibiotic resistant *E. coli* isolates has been reported worldwide. In recent decades, beta-lactams, as well as fluoroquinolones have been used as important therapeutic choices against bacterial infection. Therefore, the selective pressure resulting from their use and sometimes misuse contributes to antibiotic resistance (Ben Said et al., 2016; Correia et al., 2016). One of the most important mechanisms is the plasmid-mediated production of extended-spectrum β-lactamases (ESBLs), which can hydrolyze β-lactams (Ramos et al., 2013). ESBLs is a group of enzymes that can hydrolyze penicillin and also can hydrolyze the first, second, and third generations of antibiotics, such as Cephalosporins and Aztreonam. ESBLs can be inhibited by enzyme inhibitors, which are sensitive to antibiotics, such as Cephamycin and Carbapenem. Bacteria that carry this enzyme can hydrolyze the corresponding antibiotics, leading to the failure of some treatments. Over the past several years, the dissemination of *E. coli* isolates produces ESBLs and pAmpC, which has been reported in different settings, including in food, food-producing animals, and different types of aquatic environments, especially wastewater (Diwan et al., 2012; Divesh et al., 2014; Warjri et al., 2015). In addition, wastewater can also provide favorable conditions for the growth of a diverse bacterial community, which constitutes a basis for the further selection and spread of antibiotic resistance (Ben Said et al., 2016).

Wastewater treatment plants (WTPs) are important reservoirs of human and animal micro-organisms that can enter into the environment again through the plant outlet, such as with water and food, and are likely to infect humans and animals. "The main transport pathways of antibiotics into the ambient environment are via WTPs, where they may be only partially eliminated" (Xu et al., 2007). So in this ecosystem, antibiotics in wastewater may exert a selective pressure that promotes the spread of the resistant microorganisms to other environments (Schlüter et al., 2007; Amos et al., 2014). In addition, WTPs' wastewater contains a large number of bacteria, which is conducive to the bonding between bacteria, and this promotes transfer of multiple antibiotic resistance genes carried by movable elements. The discovery of R plasmid confirms that not only do the bacteria contain natural resistance genes, but also that they can acquire resistance to defend against survival pressures. This resistance is not only vertically transmitted, but it is also transmitted between species (i.e., horizontal transmission). The major factor in the spread of resistance is thought to be the ability of bacteria to acquire and transmit foreign genes through movable elements, such as plasmids and transposons (Mokracka et al., 2012).

The purpose of this study was to analyze the distribution of ESBLs-producing *E. coli* in municipal WTPs, to isolate ESBLs-producing *E. coli* strains, and then to elucidate the multiple antibiotic resistance linked transfer using a conjugative test. The resistant phenotypes and multiple antibiotic resistant genotypes were compared in transconjugants, donor and recipient strains. At last, we investigated the role of plasmids in the multiple antibiotic resistance transfer mechanism in *E. coli* by analyzing its full-length sequence.

MATERIALS AND METHODS

WTPs and Sample Collection

The wastewater samples were taken from a municipal WTPs, located in Tai'an county, China, in September 2016. The WTPs employed an activated sludge process. The wastewater was taken from a hospital and a multi-species slaughterhouse. The samples used for research were taken from (i) raw wastewater in the primary sedimentation tank (intake), (ii) treated water (aeration tank), and (iii) final treated wastewater (outlet). In each sampling event, the samples were taken simultaneously from the three sites. The samples were collected in sterile containers at the depth of 0.3 m and the distance of 1 m from the side of the respective sampling sites as previously described (Mokracka et al., 2012). Each sample was refrigerated and then transported to the lab and analyzed within 12 h.

Isolation and Identification of ESBLs-Producing *E. coli*

The isolation and the identification of *E. coli* were done following previously described methods (Mokracka et al., 2012). Briefly, the samples were diluted serially in 0.9% NaCl, inoculated onto Brilliance ™ *E. coli*/Coliform Selective Agar (Oxoid) and incubated at 37°C for 24 h. Then the single colony was passaged three times for the further experiments. Identification of bacteria was done with API 20E kit (bioMerieux), dedicated to identifying *E. coli* and other Gram-negative bacteria using biochemical tests.

The suspected ESBLs-producing *E. coli* isolates were confirmed by phenotypic confirmatory tests using cefotaxime (30 ug), cefotaxime/clavulanic acid (30 ug/10 ug), ceftazidime (30 ug), and ceftazidime/clavulanic acid (30 ug/10 ug) (Kim et al., 2017; Zhang et al., 2017).

Conjugation and Identification of Transconjugants

In order to prove the antibiotic resistance gene in *E. coli* has the ability to transfer *in vitro*, 50 ESBLs-producing *E. coli* strains were isolated from the WTPs, which were resistant to cefotaxime and sensitive to sodium azide. *E. coli* J53 was resistant to sodium azide and sensitive to most antibiotics, which was donated by Professor Yu-Song Yu from Zhejiang University School of Medicine. Conjugative testing was performed using the filter mating method (Wei et al., 2014; Knudsen et al., 2018). The suspected colonies were identified and the positive strains were passaged three times from the culture plates to a new antibiotics selective medium plate by scribing. They were then preserved in glycerol for subsequent experiments (Zhang, 2006; Knudsen et al., 2018).

Detection of Antibiotic Susceptibility and Antibiotic Resistant Genotypes

Susceptibility analysis to 16 antibiotics Florfenicol (FFC), Sulfamethoxazole (SXT), Ampicillin (AMP), Aztreonam (AZT), Kanamycin (KAN), Cefalotin (KF), Cefepime (FEP), Norfloxacin (NOR), Streptomycin (STR), Ciprofloxacin (CIP), Imine imipenem (IPM), Chloramphenicol (C), Erythromycin (E) and Gentamycin (CN), Tetracycline (TE) was carried out by disk-diffusion method (Clinical and Laboratory Standards Institute [CLSI], 2013). *E. coli* ATCC 25922 was used as a reference strain (Silva et al., 2010). All screen-positive ESBLs-producing strains and transconjugants were from plasmids and genomic DNA extraction. They were then examined for the presence of *CTX-M*, *OXA*, *SHV*, *TEM*, *qnrA*, *qnrB,* and *qnrS* genes by multiplex PCR with the same method and primers as our earlier research, and the primers described in **Table 1** (Li et al., 2017). DNA sequencing using purified PCR products was provided by ABI PRISM 3730XL Analyzer (Applied Biosystems, Foster City, CA, United States) in Shanghai Sangon Biotech, Co., Ltd., China. The database similarity searches for nucleotide sequences performed using the BLAST tool at the National Center for Biotechnology Information (NCBI) website[1].

TABLE 1 | Sequences of primers used for PCR.

Gene	Primer sequence (5′–3′)	Product length/bp
bla_{SHV}	F: GGGTTATTCTTATTTGTCGCT	913
	R: GGGTTAGCGTTGCCAGTG	
bla_{TEM}	F: GAGACAATAACCCTGGTAAATG	886
	R: AATGATTAATCAGTGAGGC	
bla_{CTX-M}	F: AAGAAAAGTGAAAGCGAA	548
	R: GTGAAGTAAGTGACCAGAATC	
qnrA	F: TCAGCAAGAGGATTTCTCA	627
	R: GGCAGCACTATTACTCCCA	
qnrB	F: ATGACGCCATTACTGTATAA	562
	R: GATCGCAATGTGTGAAGTTT	
qnrS	F: ACCTTCACCGCTTGCACATT	576
	R: CCAGTGCTTCGAGAATCAGT	
OXA	F: CTGTTGTTTGGGTTTCGCAAG	591
	R: CTTGGCTTTTATGCTTGATC	

TABLE 2 | Antibiotic resistance phenotypes of donor strains and transconjugants.

Transconjugant	Donor strains
AMP-KF-STR	AMP-KF-STR-C-FFC-CN-TE-KAN
AMP-AZT-KF-FEP-STR-E-TE	SXT-AMP-AZT-KF-FEP-STR-C-AZT-CIP-CN-TE-NOR
SXT-AMP-KF-FEP-STR-C-TE	AMP-KF-STR-C-CN-TE-KAN-FFC-IPM
SXT-AMP-AZT-KF-STR	AMP-AZT-KF-NOR-FEP-E-CIP-IPM
SXT-AMP-AZT-KAN-KF-STR-CIP-C	SXT-AMP-AZT-KAN-KF-CN-TE-FEP-E-IPM
SXT-AMP-AZT-KAN-KF-STR-E	SXT-AMP-AZT-KF-E-TE
SXT-AMP-AZT-KF-STR-C-TE	AMP-AZT-KF-TE-CN-NOR
SXT-AMP-AZT-KF-STR	SXT-AMP-AZT-KF-CN-TE-NOR-KAN-C-FFC
SXT-AMP-AZT-KF-STR	SXT-AMP-AZT-KF-STR-CN-TE-KAN-C-E-CIP-FFC
AMP-AZT-KF-STR	SXT-AMP-AZT-KAN-KF-STR-CN-TE-NOR-C-E-CIP-FFC
SXT-AMP-AZT-KF-STR	SXT-AMP-AZT-KF-CN-TE-FEP-C-E-IPM
SXT-AMP-AZT-KAN-KF-STR	SXT-AMP-AZT-KF-STR-TE-FEP
AMP-AZT-KF-STR-E	SXT-AMP-AZT-KF-STR-E-TE
SXT-AMP-AZT-KF-STR	SXT-AMP-AZT-KF-CN-TE-IPM
AMP-AZT-KAN-KF-STR	SXT-AMP-AZT-KAN-KF-STR-CN-TE
AMP-AZT-KF-FEP-STR	AMP-AZT-KF-STR-NOR-IPM-KAN
AMP-AZT-KF-STR	SXT-AMP-AZT-KF-STR-CN-NOR-CIP-IPM
SXT-AMP-AZT-KF-STR	SXT-AMP-AZT-KF-STR-CN-FEP
AMP-AZT–KF-STR	SXT-AMP-AZT-KF-STR-TE
AMP-AZT-KF-STR	SXT-AMP-AZT-KF-STR-CN-TE-NOR-C-CIP-FFC
AMP-KF-FEP–STR	SXT-AMP-AZT-KF-FEP-TE
AMP-AZT-KF-STR	SXT-AMP-AZT-KF-CN-TE
AMP-AZT-KF-FEP–STR	SXT-AMP-AZT-KF-FEP-STR-TE-E
AMP-AZT–KF-FEP-STR	SXT-AMP-AZT-KF-FEP-STR-IPM-TE
AMP-KF-FEP-STR	AMP-KF-STR-AZT-IPM
AMP-AZT-KF-STR	SXT-AMP-AZT-KF-FEP-E-IPM
AMP-AZT-KF-STR	SXT-AMP-AZT-KF-STR-TE-E-IPM
AMP-AZT-KF-FEP-STR	SXT-AMP-AZT-KF-FEP-STR-CN-TE-E-IPM
AMP–KF-STR	SXT-AMP-KF-AZT-STR-CN-TE-NOR-KAN-C-CIP-FFC-IPM
SXT-AMP-AZT-KF-FEP-STR	SXT-AMP-AZT-KF-STR-TE-IPM
SXT-AMP-AZT-KF-FEP	SXT-AMP-AZT-KF-FEP-IPM-TE
AMP-AZT-KF	SXT-AMP-AZT-KF-CIP-IPM-TE
AMP-AZT-KF	SXT-AMP-AZT-KF-E-IPM-TE-STR
AMP-AZT-KF	SXT-AMP-AZT-KF-FEP-TE-IPM
AMP-AZT-KF	SXT-AMP-AZT-KF-TE-STR-IPM

NAL, nalidixic acid; E, erythromycin; CIP, ciprofloxacin; FFC, florfenicol; IPM, imipenem; AML, amoxicillin; SXT, cotrimoxazole; AMP, ampicillin; CN, gentamicin; TE, tetracycline; STR, streptomycin; NOR, norfloxacin; KAN, kanamycin; FEP, cefepime; KF, cefalotin; AZT, aztreonam; C, chloramphenicol; CTX, cefotaxime; CAZ, ceftazidime.

[1] http://www.ncbi.nlm.nih.gov/BLAST

Analysis of the Ligated Plasmids

After plasmid electrophoresis analysis, all plasmids were successfully extracted from all *CTX-M* and *TEM* gene-positive binders. Strains showed great variation in banding numbers and distance, containing 1 to 6 plasmids (∼2 to >120 kb). *E. coli* A8 showed only one about 83 kb plasmid carrying *CTX-M-15*, *TEM-1* and *qnrS* and therefore was used as an analysis target. Plasmid wwA8 was extracted with TIAGEN company plasmid extraction kit by following the instructions and was sent to Sangon company for analysis of the whole DNA sequence. After sequencing was completed, the open reading frame of the plasmid sequence was predicted using the Bacterial Annotation System and the result was confirmed with DNAMAN 5.2.10 software (BASys[2]; Van et al., 2005). Each predicted protein was compared to all protein databases using BlastP[3]. The gene sequence was further aligned with the GenBank database by BLAST, and the sequence homology plasmid resembled the reference plasmid[3]. *E. coli* strain PGR46 plasmid pPGRT46 (GenBank Accession No. KM023153.1) was used as a reference plasmid for WWA8 annotation. Plasmid maps were drawn using SnapGene Viewer 3.2.1.

RESULTS

Distribution of ESBLs-Producing *E. coli*

Seventy *E. coli* strains were isolated from 80 wastewater samples with a separation rate of 87.5%. Among them, 25 out of 25 (100%) strains were isolated from intake, 30 out of 30 (100%) strains from aeration tank, and 15 out of 25 (60%) strains from outlet. ESBLs-producing strains could be identified according to the CLSI2009 standard, the ESBLs-producing strains were confirmed by phenotypic confirmation. A total of 50 ESBLs-producing isolates were obtained from 70 isolates of *E. coli*, with the isolation rate as high as 71.4%, of which 22 out of 25 (88%) were from water intakes, 20 out of 30 (66.7%) from aeration tanks and 8 out of 15 (53.3%) from water outlets.

Identification of Conjugation

After the conjugative test using the filter mating method, the ERIC-PCR, and the selective plate assay, it was judged according to the conjugative screening test (Zhang, 2006). Fifty strains of ESBLs-producing resistance to Cefotaxime were used as donor bacteria, and 35 transconjugants were obtained successfully with the transfer rate as high as 70%.

Resistant Phenotype of Donor Bacteria and Transconjugants

The resistant phenotypes of 35 transconjugants for 15 kinds of antibiotics compared to the donor strains were shown in **Table 2**. The results showed that all transconjugants and donor strains were capable of multiple antibiotic resistance for three or more antibiotics compared to recipient strain *E. coli* J53, which is sensitive to the above-mentioned 15 antibiotics. Both transconjugants and donors were capable of resistance to the AMP and KF at a rate of 100.00% (35/35). Among them, transconjugants had transferred STR, SXT, E, and KAN resistance compared to donors at a rate of 90.91% (20/22), 34.48% (10/29), 16.67% (2/12), and 22.22% (2/9). However, the capability of resistance to STR, SXT, E, and KAN in transconjugants broadened at a rate of 76.92% (10/13), 50.00% (3/6), 4.35% (1/23), and 7.69% (2/26). So transconjugants which had a narrowed antibiotic resistance spectrum, lost one or several antibiotic resistances which were present in the donor bacteria, or had a broadened antibiotic resistance spectrum and gained one or several antibiotic resistances which were not present in the donor bacteria. In a word, the antibiotic resistant spectrum of

TABLE 3 | The multiple antibiotic resistant genotypes of 35 strains of donors and transconjugants.

Transconjugant	Donor strain
$bla_{\text{TEM-135}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-135}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-135}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-135}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$\text{bla}_{\text{CTX-M-15}}$–*qnrS*	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-40}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-40}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-181}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-181}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$
$bla_{\text{TEM-181}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-55}}$	$bla_{\text{TEM-181}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-55}}$
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-56}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-6}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrB*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$
$bla_{\text{TEM-181}}$–$bla_{\text{SHV-40}}$–$bla_{\text{CTX-M-55}}$	$bla_{\text{TEM-181}}$–$bla_{\text{SHV-40}}$–$bla_{\text{CTX-M-55}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-56}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-56}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-55}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-55}}$
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-55}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-55}}$
$bla_{\text{TEM-116}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-116}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-79}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-79}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-55}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-55}}$–*qnrB*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$–*qnrS*
$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$–$bla_{\text{SHV-11}}$–$bla_{\text{CTX-M-15}}$

[2]http://wishart.biology.ualberta.ca/basys/cgi/submit.pl

[3]http://blast.ncbi.nlm.nih.gov/Blast.cgi

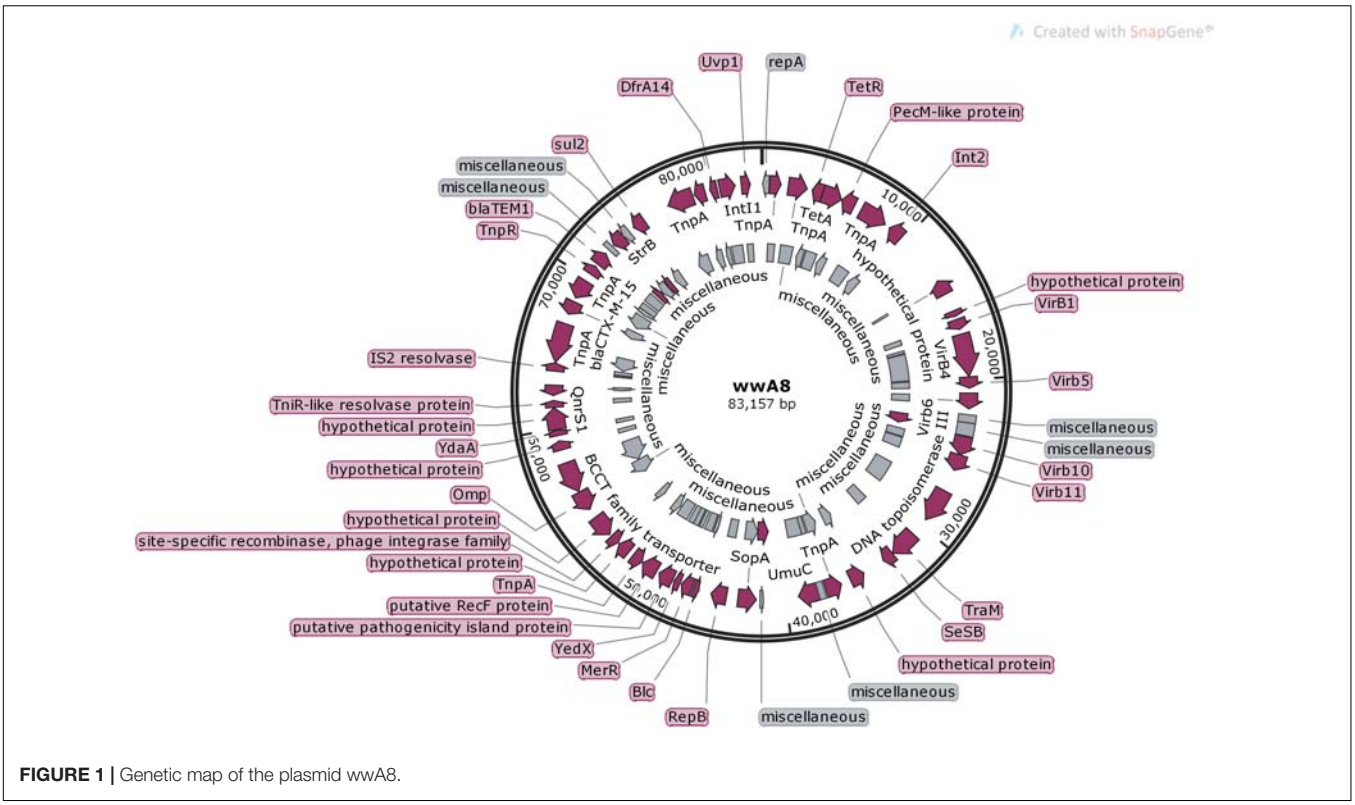

FIGURE 1 | Genetic map of the plasmid wwA8.

transconjugants narrowed after exposure to the donor bacteria at the rate of 94.29% (33/35) and broadened at the rate of 5.71% (2/35).

Antibiotic Resistant Genotypes of Donor Bacteria and Transconjugants

The resistant gene phenotypes of 35 transconjugants compared to its donor strains by PCR were shown in **Table 3**. The results showed that the bla_{TEM} and $bla_{\text{CTX-M}}$ genes were all transferred successfully at the rate 100.00% (35/35). The bla_{SHV} gene was transferred successfully at the rate 94.29% (33/35). However, only one strain of the *qnrS* gene was transferred at the rate of 4.17% (1/24). Blast comparison results showed that the gene subtype of the major resistance was $bla_{\text{TEM-1}}$, $bla_{\text{SHV-11}}$ and $bla_{\text{CTX-M-15}}$, and at the rate of 82.86% (29/35), 85.71% (30/35), and 85.71 (30/35), respectively.

Analysis of the Transferred Plasmid

A plasmid harbored in *E. coli* A8 was named wwA8 (GenBank MG773378), and its pattern map drawing with the whole DNA sequence was displayed in **Figure 1**. Plasmid wwA8 is a closed-loop DNA molecule with 83157 bp and GC content at the rate of 52.74%. The plasmid wwA8 contains 45 predicted genes (**Table 4**), carries three known antibiotic resistance genes, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, *qnrS1*, which can be transferred in *E. coli in vitro*. The sequence analyzing results of the plasmid showed that *E. coli* isolated from wastewater had the proficiency of resistance genes transferring. The basic structure of plasmid wwA8 is very homologous to plasmid IpPGRT46 (GenBank KM023153.1).

DISCUSSION

Escherichia coli are important opportunistic pathogens that cause urinary tract infections and sepsis in animals and humans (Lewis et al., 2007). The prevalence of multiple antibiotic resistant Enterobacteriaceae in the world has been increasing in recent decades. β-lactams and fluoroquinolones have been selected as important therapeutic agents. The selective pressure created by the abuse of these agents has led to the development of multiple antibiotic resistant bacteria. One of the mechanisms by which multiple antibiotic resistant bacteria are produced is the production of plasmid-mediated ESBLs which hydrolyze β-lactam (Cantón et al., 2008). ESBLs can hydrolyze β-lactam and propagate through bacteria in a plasmid-mediated manner, which is one of the main reasons for Gram-negative bacilli resistance. The gene coding for ESBLs is located on the plasmid, which has many genotypes such as $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and *OXA* types. Bacterial genes encoding ESBLs are often located on the same plasmid with other antibiotic resistance genes, leading to multiple bacterial resistances, causing great difficulties in clinical treatment of infectious diseases (Ben-Shahar et al., 2012).

The genes encoding ESBLs are located on the plasmids. There is diversity in genotypes of ESBLs including $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, *OXA*, etc. Due to the different geographical and antibiotic habits, the prevalence of genotypes in different countries, regions,

TABLE 4 | Open reading frames identified in wwA8.

Gene name	Nucleotide position	Function encoded
repA	72–487	IncN replicase gene distrupted by insertion of IS26
TnpA	539–1255	Transposase IS26
TnpA	1720–2940	Transposase for transposon Tn1721
TetR	3272–3949	Tetracycline repressor protein
TetA	3953–5227	Tetracycline efflux protein
Int2	8775–9752	Integrase/recombinase
VirB1	16090–16752	Type IV secretory pathway VirB1 component
VirB4	17072–19822	Type IV secretion system protein virB4
Virb5	19841–20557	P-type DNA transfer protein VirB5
Virb6	20871–21878	VirB6 plasmid conjugal transfer protein
Virb8	22088–22774	Type IV secretion system protein virB8
Virb9	22767–23660	P-type conjugative transfer protein VirB9
Virb10	23657–24853	Type IV secretion system protein virB10
Virb11	24857–25906	P-type DNA transfer ATPase VirB11
DNA topoisomerase III	27345–29546	DNA topoisomerase III family protein
TraM	30755–32551	Mobilization protein
SeSB	32566–33312	Mobilization protein
Hypothetical protein	34775–35740	Antirestriction protein
TnpA	36357–37415	Transposase of ISL3
UmuC	37941–39212	UV protection
SopB	40935–41906	Plasmid-partitioning protein
SopA	41906–43081	Plasmid partition protein SopA
RepB	43813–44823	Initiator replicase protein FIB-like replicon
Blc	45614–45973	Outer membrane lipoprotein precursor
MerR	46076–46801	Transcriptional regulator MerR
YedX	46901–47311	Hydroxyisourate hydrolase
TnpA	49670–50386	Transposase of IS26
Omp	54416–55708	Putative membrane protein
YdaA	59566–59925	Resolvase-like protein, YdaA
QnrS1	62133–62789	Quinolone resistance gene
TnpA	64242–66791	Transposase for transposon Tn3
blaCTX-M-15	67430–68305	Beta-lactamase enzyme family
TnpA	68561–69823	ISEcp1 transposase
tnpA	70005–70382	Fragment
blaTEM1	71127–71987	Beta lactamase TEM-1
TnpA	72197–72736	Transposase of ISVsa3
StrB	72708–73544	Streptomycin resistance protein B
StrA	73544–74035	Aminoglycoside phosphotransferase
sul2	74408–75223	Dihydropteroate synthase
TnpA	76994–78700	Tn3 family transposase
TnpA	78812–79528	Transposase of IS26
DfrA14	79842–80324	Dihydrofolate reductase DfrA14
IntI1	80471–81484	Class 1 integron integrase
TnpM	81423–81737	Transposon Tn21 modulator protein
Uvp1	81877–82446	Resolvase of the R46 plasmid

and environments varies (Fabre et al., 2009). Animal-derived ESBLs-producing *E. coli* has been reported (Alexy et al., 2006), but less ESBLs-producing *E. coli* is reported in wastewater. In this paper, ESBLs-producing *E. coli* were isolated from WTPs, and then *E. coli* J53 was as recipient bacteria performed plasmid conjugation, the multiple antibiotic resistance phenotype and the multiple antibiotic resistant genotypes test were carried out. One of the plasmids in transconjugants was sequenced to detect the transfer of the plasmids in the bacteria. In this experiment, 50 isolates of ESBLs-producing *E. coli* were isolated from 80 wastewater samples and the isolation rate was very high. Therefore, ESBLs-producing *E. coli* has been widespread in the environment. Among them, the outlet ESBLs-producing *E. coli* separation rate is 32%, and at the intake the separation rate is 88%. Although WTPs can significantly reduce the microbial load in water, it cannot completely eliminate antibiotic resistance bacteria. On the contrary, these selective pressures increase the resistance of certain bacteria. The ESBLs-producing *E. coli* in the outlet water cannot be completely eliminated. It will enter the local environment, resulting in the spread of resistant bacteria. On the other hand, untreated wastewater overflow into the surface during rainstorms may be one of the sources of ESBLs-producing *E. coli* (Diallo et al., 2013).

In this experiment, 50 ESBLs-producing *E. coli* strains were isolated from municipal WTPs in Tai'an City, 35 strains were successfully transferred. The detection of antibiotic resistant ESBLs-producing genes showed that three genotypes of *blaCTX-M*, bla_{SHV} and bla_{TEM} were detected, which was consistent with the previous study (Cohen Stuart et al., 2010; Sima et al., 2016). No *OXA* genotype was detected in this study and a small amount of the fluoroquinolone resistance gene was detected. The bla_{TEM} and $bla_{\text{CTX-M}}$ genes were transferred successfully in all strains, except for the bla_{SHV} only in which only one strain transferred successfully. With the increasing use of β-lactam antibiotics, especially the third-generation cephalosporins, it is important to monitor the production of $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} strains. In particular, it is important to monitor the surveillance of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} genotype transmission in order to provide a reliable basis for clinical use of antibiotics.

The mechanism of bacterial resistance is quite complex. However, great progress has been made in the research of this topic. In particular, research of the R plasmid confirms that the genetic material contains the natural resistance gene in bacteria. Acquired antibacterial resistance is gained via selective stress. Conjugation is the most common way genetic information is transferred and plays a very important role in the spread of multiple antibiotic resistance genes. 35 conjugations of *E. coli* J53 were finally obtained, and the success rate of conjugation was as high as 70%. The results show that under certain selective pressures, the plasmid is very easily transferred between *E. coli*, leading to the spread of antibiotic resistance, which is very harmful to clinical treatment (Cavaco et al., 2007).

The antibiotic resistant spectrum of transconjugants narrowed compared to the donor bacteria at the rate of 94.29% (33/35). This could mean that the antibiotic resistance gene may be located in the movable elements such as plasmids rather than the genomes (Park et al., 2017), or that different strains carry different plasmids, some of which are not compatible. However, the antibiotic resistance spectrum of transconjugants broadened

compared to donor bacteria at the rate of 5.71% (2/35). In addition, transconjugants which lost one or more antibiotic resistances also added one or more antibiotic resistances at the rate of 48.6%. These are why antibiotics should be used with caution so as not to cause an increase in antibiotic resistance. At the same time, there was a significant increase in the resistance to STR, which may be caused by the enhanced expression of *aadA1* and *aadA2* gene cassettes located on the transferred plasmid, showing resistances that are not in donor bacteria (Zhao et al., 2011). The transfer rate of AMP and KF in ESBLs-producing *E. coli* was 100%. This proved that the plasmids in *E. coli* play an important role in the multiple antibiotic resistant transfer.

CONCLUSION

This study shows that *E. coli* isolated from wastewater was capable of resistance gene transfer and of producing antibiotic resistance phenotypes. The resistance genes are located on plasmids which have the ability to transfer *in vitro*, and the plasmids in *E. coli* play an important role in the multiple antibiotic resistance linked transfer.

AUTHOR CONTRIBUTIONS

QL performed the experiments and analyzed the data. WC drafted the manuscript. HZ and DH collected wastewater samples and some data. XW designed and supervised the study and performed manuscript editing.

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Piperacillin-Tazobactam (TZP) Resistance in *Escherichia coli* Due to Hyperproduction of TEM-1 β-Lactamase Mediated by the Promoter *Pa/Pb*

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TEM-1, mediated by plasmid and transposon, is the most commonly encountered β-lactamase in Gram-negative bacteria. Four different promoters upstream of bla_{TEM}-related genes have been identified: the weak *P3* promoter, and the strong promoters *Pa/Pb*, *P4*, and *P5.* In this study, we investigated the genetic basis of a clinical strain of *Escherichia coli* (RJ904), which was found to be resistant to BLBLIs (β-lactam/β-lactamase inhibitors), including amoxicillin-clavulanate, ticarcillin-clavulanate (TCC), and piperacillin-tazobactam (TZP) but sensitive to third-generation cephalosporins. The conjugation test and S1-nuclease pulsed-field gel electrophoresis (S1-PFGE) demonstrated that transfer of this resistance was mediated by a ca. 100 kb plasmid. The transformant with TZP resistance was screened out with the shortgun cloning. Sequence analysis revealed that the recombinant plasmid contained a bla_{TEM-1b} gene with the strong promoter *Pa/Pb*. Different plasmids were cloned based on the clone vector pACYC184 with the insertion of the bla_{TEM-1b} gene with promoters *Pa/Pb* or *P3*. Susceptibility to TZP was determined by the *E*-test, agar dilution, and broth microdilution. The level of bla_{TEM-1b}-specific transcription was determined by quantitative real-time PCR. Substitution of *Pa/Pb* for *P3* resulted in a 128-fold decline of the MIC value of TZP, from >1024 mg/L to 8 mg/L, and a significantly lower bla_{TEM-1b} expression level. Hyperproduction of TEM-1 β-lactamase mediated by the promoter *Pa/Pb* was responsible for high resistance to TZP in *E. coli*. Our data show possible risks of resistance development in association with the clinical use of TZP. The bla_{TEM} promoter modifications should be considered for whole genome whole-genome sequencing-inferred bacterial antimicrobial susceptibility testing.

Keywords: TZP resistance, *Escherichia coli*, Pa/Pb, β-lactamase, antimicobial

INTRODUCTION

The production of β-lactamases is the predominant cause of resistance to β-lactam antibiotics in Gram-negative bacteria (Bonnet, 2004), including the hyperproduction of plasmid-mediated TEM-1 β-lactamases, production of extended-spectrum beta-lactamases (ESBLs), plasmid-mediated AmpC enzymes (Caroff et al., 1999) and carbapenem-hydrolyzing β-lactamases (carbapenemases)

(Wu et al., 1994; Jacoby and Munoz-Price, 2005). Combining β-lactam and a β-lactamase inhibitor (BLBLIs) was a common strategy to overcome resistance (Chaibi et al., 1999; Perez-Llarena and Bou, 2009). However, resistance to BLBLIs has also been regularly observed (Pérez-Moreno et al., 2010; Waltner-Toews et al., 2011).

TEM-1 was described in the early 1960s as the first plasmid-mediated β-lactamase in Gram-negative bacteria (Datta and Kontomichalou, 1965). Being plasmid and transposon-mediated has facilitated its spread to other species of bacteria and it is now the most commonly encountered β-lactamase in Gram-negative bacteria (Bradford, 2001). The subgroups were defined and designated a, b, and c for a given bla_{TEM} gene derivative, because of their relation to a certain number of nucleotide differences in their structural gene sequence (Leflon-Guibout et al., 2000). The corresponding $bla_{\mathrm{TEM-1b}}$ gene derives from $bla_{\mathrm{TEM-1a}}$ by three base pair changes: C226T, C436T, and G604T, silent base pair change. $bla_{\mathrm{TEM-1c}}$ gene differs from $bla_{\mathrm{TEM-1a}}$ by the nucleotide substitution C436T, which is also silent. $bla_{\mathrm{TEM-2}}$ differs from $bla_{\mathrm{TEM-1a}}$ at position 317, where a A-to-C substitution leads to Gln39Lys (Goussard and Goussard, 1991). Previous studies identified four bla_{TEM} promoters: the weak *P3* promoter, and the strong promoters *Pa/Pb*, *P4*, and *P5* (Lartigue et al., 2002). *P3* corresponds to the promoter of the bla_{TEM} gene located in a Tn*2* or Tn*3* transposon (Sutcliffe, 1978; Lartigue et al., 2002; Partridge and Hall, 2005). A single-base pair mutation (C32T) results in the stronger overlapping promoters *Pa/Pb*, first found upstream of the gene $bla_{\mathrm{TEM-2}}$, and produces larger amounts of the enzyme compared with the promoter *P3* (Chen and Clowes, 1987a,b). Thus, an updated bla_{TEM} gene nomenclature was proposed on the basis of the sequences of structural bla_{TEM} genes and their promoters (Goussard and Courvalin, 1999).

Lartigue et al. (2002) assessed and compared the respective impact of the four promoters on β-lactam resistance. Among the recombinant plasmids, one with a $bla_{\mathrm{TEM-1b}}$ gene driven by a *Pa/Pb* promoter resulted in resistance to AMC and ticarcillin-clavulanate (TCC), but susceptibility to piperacillin-tazobactam (TZP) with a MIC value of 2 mg/L. In this study, the mechanism of TZP resistance was investigated in *Escherichia coli* RJ904, a clinical isolate containing the $bla_{\mathrm{TEM-1b}}$ gene with a *Pa/Pb* promoter. Experimental and genomic data support a role for *Pa/Pb* promoter regulation, leading to $bla_{\mathrm{TEM-1b}}$ hyperproduction, as the primary basis for TZP resistance in this isolate.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the ethics committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China and the Review Board exempted the requirement for written informed consent because this retrospective study only focused on bacteria and did not affect the patients.

Bacterial Strains and Growth Condition

The clinical strain *E. coli* RJ904 was obtained from the blood specimen of a hospitalized patient in Shanghai, China (Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University) in 2005. Ceftazidime was used for the medication. The patient's condition improved after the treatment and the patient was discharged. The isolate was identified using VITEK2 automated systems (BioMérieux, France). All of the plasmids used in this study are listed in **Supplementary Table S1**. All cloning procedures were carried out in *E. coli* (DH5α), and antibiotics were used with suitable concentrations for plasmid selection when necessary. All the *E. coli* strains were routinely grown in Luria-Bertani (LB) broth (Oxoid) and incubated overnight at 35°C.

Antimicrobial Susceptibility Testing

Susceptibility testing of all the antibiotics for the clinical strain RJ904, transconjugant RJ904C, and recombinant vectors RJ904-PA/PB was determined using the *E*-test (bioMérieux, France). The antibiotic susceptibility of the strains to piperacillin with a fixed concentration of tazobactam (TZP, 4 mg/L) was determined using three methods: *E*-test, agar dilution, and broth microdilution method. The results were interpreted based on the guidelines of the CLSI (2014).

Conjugal Transfer Experiments and S1-Nuclease Pulsed-Field Gel Electrophoresis (S1-PFGE)

Conjugal transfer experiments were performed in broth culture using the strain RJ904 as the donor and the sodium azide-resistant strain *E. coli* J53Azi^{r} as the recipient. Selection was performed with piperacillin (100 mg/L), tazobactam (4 mg/L), and sodium azide (100 mg/L). The plasmid DNA of RJ904 and its transconjugant RJ904C was examined using S1-PFGE as previously described (Barton et al., 1995).

Plasmid Construction

The principle features of all plasmids are listed in **Supplementary Table S1**.

First, the fragment of $bla_{\mathrm{TEM-1b}}$ gene was screened by the shortgun cloning. In brief, plasmid DNAs of pRJ904 were extracted with the Plasmid DNA Mini Kit (Omega). pRJ904 and the clone vector pACYC184 were digested with restriction enzymes *BamH*I and *Hind*III (Thermo Fisher Scientific) and ligated to construct a DNA library, which was used to transform the competent cells. Selection was then performed with piperacillin (100 mg/L), tazobactam (4 mg/L), and chloramphenicol (50 mg/L). The new cloned plasmid was named pRJ904-PA/PB.

The recombinant vector was cloned as described by Lartigue et al. (2002) using the same primers (BamHI-P-F and BamHI-P-R), clone vector, and restriction enzyme digestion site. pRJ904-PA/PB and p749 (MH491004) served as templates, respectively. p749 was a plasmid from *E. coli* retained by our laboratory that contained the $bla_{\mathrm{TEM-1b}}$ gene and promoter region with 99% base pair identity to pRJ904, except a point

mutation (T32C) in the promoter region of bla_{TEM-1b}, resulting in substitution of the promoter *Pa/Pb* for *P3*. The PCR products were purified and digested with *BamH*I (Thermo Fisher Scientific) and cloned into pACYC184 to construct plasmids pRJ904-PA/PB-P and pRJ904-P3-P. Both plasmids were cloned based on pACYC184, and the bla_{TEM-1b} gene was inserted; however, pRJ904-PA/PB-P contained the *Pa/Pb* promoter while pRJ904-P3-P contained the *P3* promoter.

After cloning, all of the plasmids were transformed into *E. coli* DH5α cells by using standard techniques (Denman, 1983). Selection was performed on an LB agar plate containing ampicillin (100 mg/L) and chloramphenicol (50 mg/L). Proper integration of all the constructs were verified by PCR amplification with the primers 184-F and 184-R binding on pACYC184, followed by sequencing of the PCR product. The direction of the bla_{TEM-1b} fragments in all the constructs were opposite to the *tetR* gene of pACYC184 in order to rule out the possible expression of the *tetR* gene.

Transcriptional Analysis of bla_{TEM-1b}

For real-time PCR, the indicated *E. coli* strains were grown in LB broth and harvested at an OD600 of 1. The RNA was extracted using RNeasy Mini Kit (Qiagen), and then used to generate cDNA with PrimeScript™ RT Master Mix (TaKaRa). RT-PCR was performed using SYBR green PCR master mix (Applied Biosystems) with the primer pair TEM-F and TEM-R (**Supplementary Table S2**) on a cobas z480® system (Roche) (Her and Schutzbank, 2018). Amplification of the 16S rRNA gene (as an endogenous control) was performed to standardize the amount of sample RNA or DNA added to a reaction. Relative quantification was determined by the $2^{-\Delta\Delta CT}$ method. Each assay was performed in triplicate with three independent cultures. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Holm-Sidak tests to compare selected data pairs. Values of $P < 0.05$ were considered statistically significant.

Nucleotide Sequence Accession Number

The nucleotide sequence containing a bla_{TEM-1b} gene with the promoter *Pa/Pb* from the clinical strain RJ904 has been deposited in the GenBank sequence database under accession number MH357372.

RESULTS

Plasmid-Mediated Transfer of the Resistance to β-Lactam and β-Lactamase Inhibitor Combinations

The clinical isolate RJ904 was determined by *E*-test and found to be highly resistant to BLBLIs, including AMC, TCC, and TZP (MICs > 256 mg/L), but was susceptible to third-generation (**Table 1**). Resistance to TZP was transferable using the broth mate conjugation assay. Although the transconjugant RJ904C showed a decreased MIC to third-generation cephalosporins, the MIC values of BLs and BLBLIs were all significantly higher than that of the recipient strain *E. coli* J53Azi^r. The results of S1-PFGE confirmed the presence of a ca. 100 kb plasmid in both the donor strain RJ904 and the transconjugant RJ904C (**Supplementary Figure S1**).

TABLE 1 | Antibiotic susceptibilities of *E. coli* strains RJ904, RJ904C, RJ904-PA/PB, RJ904-P3.

Antibiotics	MIC (mg/L)				
	J53	DH5α	RJ904	RJ904C	RJ904-PA/PB
Amoxicillin	4	4	> 256	> 256	> 256
Piperacillin	2	2	> 256	> 256	> 256
Amoxicillin-clavulanate	4	2	> 256	> 256	> 256
Ticarcillin-clavulanate	2	1	> 256	> 256	> 256
Piperacillin-tazobactam	1	0.5	> 256	> 256	> 256
Cefazolin	4	4	> 256	> 256	> 256
Cefuroxime	4	4	32	4	8
Cefoperazone	0.125	0.064	> 256	32	256
Cefotaxime	0.032	0.032	0.5	0.064	0.25
Ceftazidime	0.125	0.125	2	0.5	2
Cefoxitin	4	4	64	4	4

Hyperproduction of TEM-1b β-Lactamase Mediated by the Promoter *Pa/Pb*

The shortgun cloning and sequence analysis revealed that the recombinant vector pRJ904-PA/PB contained a DNA insertion of approximately 3.9 kb containing the bla_{TEM-1b} gene, located on the resolvase gene (*tnpR*) of Tn2, and the promoter upstream the bla_{TEM-1b} gene was *Pa/Pb* (**Figure 1**). The MIC value of BLs and BLBLIs of *E. coli* RJ904-PA/PB was similar to that of the transconjugant RJ904C (**Table 1**).

The level of bla_{TEM-1b}-specific transcription was determined by quantitative RT-PCR. As shown in **Figure 2**, RJ904-PA/PB demonstrated a significantly higher relative bla_{TEM-1b} expression level than RJ904-P3-P ($P < 0.01$).

Expression of TEM-1b for pRJ904-PA/PB-P and pRJ904-P3-P

To further confirm that the resistance to TZP is caused by the promoter *Pa/Pb* and for comparison with the results of Lartigue et al. (2002), the plasmids pRJ904-PA/PB-P and pRJ904-P3-P were constructed.

The MIC value of TZP for all strains was determined by three different methods (**Table 2**). The MIC values of RJ904-PA/PB and RJ904-PA/PB-P were >256 mg/L in the *E*-test and were ≥024 mg/L in agar dilution and broth microdilution tests, indicating no difference from the susceptibility profile of the original strain RJ904 and the transconjugant RJ904C. However, RJ904-P3-P demonstrated significantly declined MIC values of 8 mg/L (agar dilution and *E*-test) or 16 mg/L (broth microdilution test), and 4 mg/L (agar dilution and *E*-test) or 8 mg/L (broth microdilution test), respectively. Consistently, RJ904-PA/PB-P demonstrated a significantly higher bla_{TEM-1b} expression level than RJ904-P3-P.

FIGURE 1 | Schematic representation of the 3.9-kb *BamH*I and *Hind*III-digested embedded TEM-1b fragment (black arrow) and the promoter *Pa/Pb* (white rectangle). Truncated *tnpA* of Tn2 was placed on both sides of the resistance gene (gray). The sites for primers BamHI-P-F and BamHI-P-R for PCR clone are also indicated.

DISCUSSION

The conjugation experiment demonstrated that resistance to TZP can be transferred from RJ904 to J53Azi^r. The short gun method was used to screen out a strain that was highly resistant to TZP, and sequence analysis revealed that the plasmid harbored a 3.9-kb insertion embedded in the $bla_{\mathrm{TEM-1b}}$ gene with the strong promoter *Pa/Pb*. The mutant strain RJ904-P3-P with the weak promoter *P3* demonstrated substantially declining MIC values to TZP. Moreover, RJ904-PA/PB and RJ904-PA/PB-P demonstrated a higher $bla_{\mathrm{TEM-1b}}$ expression level than RJ904-P3-P. Altogether, these data provide strong functional evidence that the acquisition of TZP resistance was due to the hyperproduction of TEM-1b β-lactamases mediated by the strong promoter *Pa/Pb*.

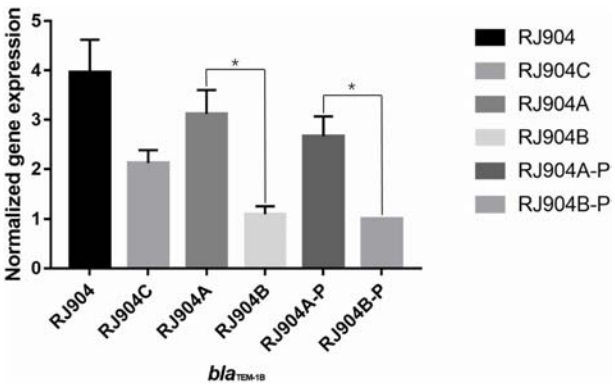

FIGURE 2 | Relative $bla_{\mathrm{TEM-1b}}$ expression levels of all the strains. *$p < 0.01$. RJ904-P3-P served as a reference. RJ904-PA/PB and RJ904-PA/PB-P also demonstrated a significantly higher $bla_{\mathrm{TEM-1b}}$ expression level than RJ904-P3-P.

TABLE 2 | Susceptibility testing results of *E. coli* strains to piperacillin with 4 mg/L of tazobactam (TZP).

Strain	*E*-test (mg/L)[a]	Agar dilution (mg/L)	Broth microdilution (mg/L)
ATCC25922	2	2	1
J53	1	1	1
DH5α	0.5	1	2
RJ904	> 256	≥ 1024	≥ 1024
RJ904C	> 256	≥ 1024	≥ 1024
RJ904-PA/PB	> 256	≥ 1024	≥ 1024
RJ904-PA/PB-P	> 256	≥ 1024	≥ 1024
RJ904-P3-P	4	4	8

[a]*MIC breakpoint (mg/L): S ≤ 16/4; I: 32/4–64/4; R ≥ 128/4 (CLSI).*

Lartigue et al. (2002) suggested that the $bla_{\mathrm{TEM-1b}}$ gene with a *Pa/Pb* promoter could contribute to the resistance to AMC and TCC but not to TZP with a MIC value of 2 mg/L, suggesting the potential importance of this promoter for β-lactam resistance. However, we found that strain RJ904-PA/PB, which also contained the $bla_{\mathrm{TEM-1b}}$ gene with a *Pa/Pb* promoter, was highly resistant to TZP with a MIC value >256 mg/L. To identify possible causes of the difference, we replicated the experiment of Lartigue et al. (2002) using the exact same primers, clone vector, and restriction enzyme digestion site to clone the plasmid with the $bla_{\mathrm{TEM-1b}}$ gene and *Pa/Pb* promoter (pRJ904-PA/PB-P), which was compared to a plasmid with the *P3* promoter (pRJ904-P3-P). We next determined the MIC value of TZP of all strains. Since several authors have claimed that the MIC determination of TZP can be method-dependent and strains exhibited discordant behavior and heterogeneous resistance in different methods (Creely et al., 2013; Shubert et al., 2014), we used three methods for susceptibility testing to avoid the methodological impact: broth microdilution, agar dilution, and *E*-test. Several studies have compared the results of TZP susceptibility testing with broth microdilution and agar dilution methods for isolates of various species,(Thomson et al., 2008; Creely et al., 2013; Steensels et al., 2013; Shubert et al., 2014) and broth microdilution showed a tendency toward higher MIC values than agar dilution (Steensels et al., 2013). In the present study, there was no difference in the MIC values of RJ904-PA/PB-P to those of strains RJ904, RJ904C, and RJ904-PA/PB regardless of the method used. All these strains with a promoter *Pa/Pb* demonstrated high resistance to TZP unlike Lartigue's transformants, while strains with a promoter *P3* (RJ904-P3 and RJ904-P3-P) demonstrated a significantly declined MIC value ultimately becoming susceptible to TZP, which is consistent with the findings of Lartigue's transformants with a *P3* promoter. *E. coli* DH5α was used as the recipient rather than *E. coli* NM554. However, RJ904, the transconjugant RJ904C (*E. coli* J53), and RJ904-PA/PB-P (*E. coli* DH5α) all demonstrated high resistance to TZP. These results indicate that the recipient will not have a great impact on the expression of drug-resistant genes.

Nevertheless, when we repeated the experiment, we reached a different conclusion. The strains with promoter *Pa/Pb* in our study demonstrated high resistance to TZP while Lartigue's transformants was susceptible to TZP. Although the reason for this discrepancy is not yet clear, our results from several independent assessments all indicate that the resistance to TZP

was due to hyperproduction of TEM-1b β-lactamases mediated by the strong promoter *Pa/Pb*. However, overexpression of *bla*TEM-1 can lead to resistance, including clavulanate and sulbactam (Stapleton et al., 1995; Waltner-Toews et al., 2011). bla_{TEM-1} hyperproduction resulting from an increase in bla_{TEM-1} gene dosage has also been documented (Wu et al., 1995; Waltner-Toews et al., 2011). Schechter et al. (2018) claimed that tandem bla_{TEM-1} gene amplification, leading to bla_{TEM-1} hyperproduction, as the primary basis for TZP resistance in *E. coli*. These results indicated that bla_{TEM-1} hyperproduction can lead to BLBLIs resistance, including TZP.

Whole-genome sequencing (WGS) can help to infer antimicrobial susceptibility accurately using a single assay (Ellington et al., 2017). However, most existing databases focus only on the commonly known resistance loci while neglecting the role of promoters. Our finding should be considered for the acquisition of more accurate WGS-inferred bacterial antimicrobial susceptibility testing. Importantly, these data add to the growing body of evidence that the same resistance gene with different promoters will result in completely different susceptibility testing results. Thus, when performing WGS-inferred AST, we should not only assess the resistance genes but should also analyze their promoter sequences simultaneously. Our finding also shed light on the possibility of a fast identification using a simple PCR and sequencing to identify strong promoters and weak promoters and to infer antimicrobial susceptibility.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00833/full#supplementary-material

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Antimicrobial Resistance Genes, Cassettes, and Plasmids Present in *Salmonella enterica* Associated With United States Food Animals

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The ability of antimicrobial resistance (AR) to transfer, on mobile genetic elements (MGEs) between bacteria, can cause the rapid establishment of multidrug resistance (MDR) in bacteria from animals, thus creating a foodborne risk to human health. To investigate MDR and its association with plasmids in *Salmonella enterica*, whole genome sequence (WGS) analysis was performed on 193 *S. enterica* isolated from sources associated with United States food animals between 1998 and 2011; 119 were resistant to at least one antibiotic tested. Isolates represented 86 serotypes and variants, as well as diverse phenotypic resistance profiles. A total of 923 AR genes and 212 plasmids were identified among the 193 strains. Every isolate contained at least one AR gene. At least one plasmid was detected in 157 isolates. Genes were identified for resistance to aminoglycosides (*n* = 472), β-lactams (*n* = 84), tetracyclines (*n* = 171), sulfonamides (*n* = 91), phenicols (*n* = 42), trimethoprim (*n* = 8), macrolides (*n* = 5), fosfomycin (*n* = 48), and rifampicin (*n* = 2). Plasmid replicon types detected in the isolates were A/C (*n* = 32), ColE (*n* = 76), F (*n* = 43), HI1 (*n* = 4), HI2 (*n* = 20), I1 (*n* = 62), N (*n* = 4), Q (*n* = 7), and X (*n* = 35). Phenotypic resistance correlated with the AR genes identified in 95.4% of cases. Most AR genes were located on plasmids, with many plasmids harboring multiple AR genes. Six antibiotic resistance cassette structures (ARCs) and one pseudo-cassette were identified. ARCs contained between one and five resistance genes (ARC1: *sul*2, *strAB*, *tetAR*; ARC2: *aac3-iid*; ARC3: *aph*, *sph*; ARC4: *cmy-2*; ARC5: *floR*; ARC6: *tetB*; pseudo-ARC: *aadA*, *aac3-Vla*, *sul*1). These ARCs were present in multiple isolates and on plasmids of multiple replicon types. To determine the current distribution and frequency of these ARCs, the public NCBI database was analyzed, including WGS data on isolates collected by the USDA Food Safety and Inspection Service (FSIS) from 2014 to 2018. ARC1, ARC4, and ARC5 were significantly associated with cattle isolates,

while ARC6 was significantly associated with chicken isolates. This study revealed that a diverse group of plasmids, carrying AR genes, are responsible for the phenotypic resistance seen in *Salmonella* isolated from United States food animals. It was also determined that many plasmids carry similar ARCs.

Keywords: *Salmonella*, plasmids, antimicrobial resistance, agriculture, integrons

INTRODUCTION

Non-typhoidal *Salmonella enterica* is one of the most common causes of foodborne illnesses globally, with an estimated 1.2 million cases each year in the United States alone (CDC, 2013). Symptoms range from self-limiting gastrointestinal illness to sepsis. These infections can lead to death unless treated with antibiotics (Crump et al., 2015). Unfortunately, antimicrobial resistance (AR) has been increasing since the 1980s (Crump et al., 2015). The Center for Disease Control and Prevention (CDC) considers drug-resistant non-typhoidal *Salmonella* to be a serious level threat to human health, and currently reports that 8% of *Salmonella* infections are either multidrug resistant (resistant to three or more classes of antimicrobials), or resistant to an antibiotic used for treatment, such as ceftriaxone and ciprofloxacin (CDC, 2013).

Up to 94% of United States *Salmonella* infections are estimated to be foodborne, demonstrating the importance of investigating *Salmonella* isolated from food animals (Scallan et al., 2011). The National Antimicrobial Resistance Monitoring System (NARMS) tracks antimicrobial susceptibility of bacteria associated with animals, retail meat, and foodborne illness in humans. In 2015, 21.3% of animals tested by NARMS were positive for *Salmonella* with individual sources as low as 8% in beef cattle and as high as 50% in sows, based on cecal sampling. Retail meat isolates in 2015 were positive for *Salmonella* at a lower percentage in all sources (4.3%). Individual sources ranged from 0.4% (ground beef) to 6.2% (ground chicken). Of the *Salmonella* isolated by NARMS, 35.3% of the animal samples, and 57.7% of the retail meat samples, were resistant to at least one antibiotic (FDA, 2015).

For many *Salmonella*, AR genes are carried on a mobile genetic element (MGE) (Carattoli, 2003). MGEs, like plasmids, have been shown to be extremely important in the expansion of AR genes in *Salmonella* and other Enterobacteriaceae, such as *Klebsiella pneumoniae* and *Escherichia coli* (Carattoli, 2013; Gillings, 2014). Plasmids specifically have been identified carrying AR genes in hospital-acquired infections, community-acquired outbreaks, and have also been associated with AR genes in isolates from animals raised for consumption (Conlan et al., 2016; Folster et al., 2017; Tate et al., 2017).

Salmonella are capable of harboring multiple, large, conjugative plasmids that can carry AR genes encoding resistance to several classes of antibiotics, including β-lactams, tetracyclines, aminoglycosides, and quinolones (Johnson et al., 2010; Glenn et al., 2011; Jain et al., 2018). However, while one cell can harbor multiple plasmids, they must be of different incompatibility groups. Plasmids of the same incompatibility group are unlikely to persist in the same isolate, while plasmids of different groups can usually coexist without issue (Novick, 1987). Incompatibility can be predicted by typing plasmids based on the replicon-associated genes they contain (Carattoli et al., 2005). Plasmids of several different incompatibility groups have been associated with multiple AR genes in *Salmonella* and other bacteria (Carattoli, 2009). For example, IncA/C plasmids isolated from *Salmonella* have been associated with genes conferring resistance to aminoglycosides, β-lactams, chloramphenicol, sulfisoxazole, tetracyclines, and trimethoprim (Hoffmann et al., 2017). Recently analyzed human infection isolates from the 1960s implicate F, I1, X1, and N type plasmids as early carriers of β-lactam resistance genes in *Salmonella* (Tran-Dien et al., 2018).

Integrons have also been shown to be important to the spread of AR in both clinical and agricultural isolates of *Salmonella* (Kaushik et al., 2018). Integrons have a well-defined structure consisting of: an integrase gene, which catalyzes the integration of new genes, the *att*I recombination site where the new genes integrate, and a promoter to express incorporated genes. The incorporated genes are called gene cassettes and are often AR genes (Gillings, 2014). The arrangement of these genes is used to assign them numbers based on the Integrall database of known integron sequences (Moura et al., 2009). While not independently mobile, integrons can be mobilized by other elements, like plasmids or transposons (Partridge et al., 2018).

Despite the established link between plasmids and AR genes, there is less known about the prevalence and characteristics of plasmids containing AR genes in isolates from food animals (Carattoli, 2003). Considering the high incidence of foodborne infection in the United States, and increasing AR, understanding the complete picture of AR in *Salmonella* is crucial. To investigate this relationship, 193 animal-associated *S. enterica* isolates of diverse serotypes and phenotypic resistance profiles, collected by NARMS from 1998 to 2011, were selected for this study. Whole genome sequence analysis (WGS) identified plasmids, AR genes, integrons, and AR cassettes (ARCs) present in these isolates. To determine the current relevance of these ARCs, publicly available genomic data of *S. enterica* from food animals collected by the USDA Food Safety and Inspection Service (FSIS) from 2014 to 2018 (n = 6681), were analyzed for the presence of the ARCs. Their association with plasmid replicons was determined. This is the first WGS analysis of isolates from the NARMS animal collection, which represent the first 15 years of this United States program. Combined with analysis of WGS data from the most recent 5 years of HAACP FSIS isolates, this is the most comprehensive nationwide study of AR in *Salmonella* associated with food animals. The associations of ARCs and MGEs identified in this study improve our understanding of AR in United States food

animals, and may help us predict and prevent further spread of AR in *Salmonella*.

MATERIALS AND METHODS

Isolates

One hundred and eighty nine *S. enterica* isolates, with collection dates ranging from 1998 to 2011, were selected from the NARMS animal isolate collection for the retrospective part of this study (Gupta et al., 2016a,b,c,d,e,f,g,h; Karp et al., 2017). In addition, four serotype Heidelberg isolates from a 2011 outbreak in humans were selected from the California Department of Health (Hoffmann et al., 2012). To maximize the AR gene diversity of the *Salmonella* in the retrospective study, isolates were selected based on differences in phenotypic AR profile, serotype, and the uncommon nature of their Pulsed-Field Gel Electrophoresis (PFGE) patterns within the PulseNet database. Eighty-six different serotypes and serotype variants were represented in this isolate set. These bacteria were isolated from various animal and animal associated sources, such as carcass rinses and swabs, ground product, the processing environment, sick animals, and infected humans. Animal associated sources included poultry, swine, cattle, horses, wild reptiles, wild mammals, companion animals, and associated processing environments (**Supplementary Table S1**). All patient information was blinded for the human isolates to insure confidentiality.

Additionally, WGS data of *S. enterica* isolates recently collected for Hazard Analysis and Critical Control Points (HACCP) verification testing by USDA-FSIS from chicken, turkey, pork, or beef products, were evaluated. Isolation procedures are described in the USDA-FSIS Microbiology Laboratory Guidebook (MLG) Chapter 4 (Dey and Lattuada, 1998). Only WGS data was used from these isolates as phenotypic data was not available. Isolates were selected based on publicly available data in NCBI's Pathogen Detection Isolate Browser[1].

Phenotypic Antimicrobial Susceptibility Testing

For the 193 retrospective isolates collected from 1998 to 2011, phenotypic susceptibility to 14 different antibiotics (**Supplementary Table S1**) was determined by broth-microdilution. The Sensititre semi-automated antimicrobial susceptibility system (TREK Diagnostic Systems Inc., Cleveland, OH, United States) was used to inoculate the Sensititre custom NARMS plate CMV3AGNF per manufacturer's instruction. The minimum inhibitory concentration (MIC) and classification as resistant, susceptible, or intermediate for each of the 14 antibiotics were assigned using breakpoints set by the Clinical and Laboratory Standards Institute (CLSI, 2016). For antibiotics without CLSI established breakpoints, NARMS breakpoints were used[2].

Genome Sequencing, Assembly, AR Gene, and Integron Identification

Total DNA was extracted using a Sigma GenElute kit (Sigma Life Sciences, St. Louis, MO, United States). Libraries were prepared according to the Illumina protocol using the Nextera XT DNA sample preparation kit. Isolates were sequenced using an Illumina HiSeq2500 (Illumina, San Diego, CA, United States) at The Genome Institute at Washington University in St. Louis, MO, United States. Reads were assembled into draft sequences using A5 with default settings, including quality trimming (Tritt et al., 2012). Draft genomes were annotated with Prokka using default settings (Seemann, 2014). All sequences had greater than 40× coverage, an average N50 of greater than 350,000, and an average of 116 contigs (median of 97 contigs) (**Supplementary Table S2**). AR genes were identified using ARG-ANNOT V3 (Gupta et al., 2014). Integrons were identified using Integrall (Moura et al., 2009).

Regulatory isolates collected and sequenced by the USDA-FSIS from 10/31/2014 to 4/16/2018 were also included for analysis. WGS data was generated from MiSeq libraries prepared using the Nextera XT library prep kit (Illumina, San Diego, CA, United States) and sequenced on the Illumina MiSeq platform using either 300 Cycle or 500 Cycle Version 2 chemistries. The raw files were assembled using either CLC Genomics Workbench v8 or v11 (Qiagen) or SPAdes version 3.7.0[3] (St. Petersburg, Russia).

Plasmid Identification

Plasmid replicon-associated genes were detected using BLASTN to identify the target sequence in the genomes of each isolate (Camacho et al., 2009). Target sequences were selected based on plasmid replicon typing as well as relaxase typing schemes (Carattoli et al., 2005; Villa et al., 2010; Compain et al., 2014). Additional contigs belonging to plasmids not identified in the replicon and relaxase BLAST were identified using BLASTN against a custom plasmid BLAST database. The custom database was created by extracting all plasmids from NCBI that were associated with Enterobacteriaceae as of March 2015 (Coordinators, 2017). The additional plasmid contigs were confirmed using the following criteria: First, contigs that were identified in the replicon/relaxase BLAST were used to identify the primary reference plasmid, meaning, the plasmid in the custom database that aligned to the initial BLAST identified contig with the greatest coverage and percent identity. Second, large contigs (>10,000 bp) not identified in the initial BLAST that aligned with high identity (>70%) and coverage (>40%) to the primary reference plasmid for a specific replicon and did not have substantial homology with another replicon were binned as part of the same plasmid. For these large contigs, a 70% cut-off for identity was chosen based on the range of percent identities of the primary reference plasmids to the contigs containing the replicon or relaxase genes. A 40% cut-off for coverage was chosen to allow for contigs that were continuous where the reference sequence was not, i.e., for cases where the reference plasmid and the contig

[1] https://www.ncbi.nlm.nih.gov/pathogens/isolates#/search/

[2] https://www.cdc.gov/narms/antibiotics-tested.html

[3] http://cab.spbu.ru/software/spades/

TABLE 1 | Resistance genes identified and associated with plasmids in the retrospective isolates (n = 193).

Antibiotic resistance gene	Antibiotic Class to which resistance is conferred	Number of genes identified in n = 193 isolates	Predicted resistance conferred*	Number of genes associated with plasmids
aac(6′)-I	Aminoglycosides	189	Gen	0
*str*B	Aminoglycosides	67	Str	57
*str*A	Aminoglycosides	65	Str	56
*aad*A (*ant*(3′′)Ia)	Aminoglycosides	54	Str	38
*aac*3-Via	Aminoglycosides	22	Gen	20
aph(3′)Ib	Aminoglycosides	22	Kan	16
*aph*A (*aph*(3′)IIa)	Aminoglycosides	13	Kan	13
sph (*aph*(6)Ic)	Aminoglycosides	13	Str	13
*aac*3-Iid	Aminoglycosides	11	Gen	11
*aad*B (*ant*(2′′)Ia)	Aminoglycosides	7	Gen	5
aac-IVa	Aminoglycosides	4	Gen	2
aph(4)Ia	Aminoglycosides	4	(Hygromycin)	1
ant(3′′)Ia	Aminoglycosides	1	Str	0
bla_{CMY-2}	β-lactams	44	Amp, Fox, Axo Amo, Tio	44
bla_{TEM-1}	β-lactams	37	Amp	27
bla_{CARB-3}	β-lactams	3	Amp	0
*tet*A	Tetracyclines	61	Tet	49
*tet*R**	Tetracyclines	64	Tet	50
*tet*B	Tetracyclines	35	Tet	30
*tet*C	Tetracyclines	7	Tet	7
*tet*G	Tetracyclines	2	Tet	7
*tet*M	Tetracyclines	2	Tet	0
*sul*1	Sulfonamides	48	Sul	41
*sul*2	Sulfonamides	41	Sul	36
*sul*3	Sulfonamides	2	Sul	0
*flo*R	Phenicols	27	Chl	24
*cml*A	Phenicols	11	Chl	8
*cat*A	Phenicols	4	Chl	0
*fos*A2	Fosfomycin	48	(Fosfomycin)	0
*mph*A	Macrolides	2	Azi	2
*ere*A	Macrolides	2	(Erythromycin)	0
*mef*B	Macrolides	1	Azi	0
*dfr*A	Trimethoprim	8	Trimethoprim	5
*arr*2	Rifampicin	2	(Rifampicin)	0

**For drugs which phenotypic testing was available, only tested drugs are listed. Resistance to other drugs are possible. Drug names in parentheses were not tested. Antibiotic abbreviations are as follows: Gen, gentamicin; Kan, kanamycin; Str, streptomycin; Amp, ampicillin; Fox, cefoxitin; Axo, ceftriaxone; Amo, amoxicillin; Tio, ceftiofur; Tet, tetracycline; Sul, sulfonamide; Chl, chloramphenicol; Azi, azithromycin. **Indicates regulatory gene usually associated with resistance.*

being queried began in different places and there was a large gap between homologous sequences resulting in two different BLAST hits for the identified contig. Third, smaller contigs (3,000–10,000 bp), that aligned to reference plasmids, different than the primary, but of the same replicon type, and those with lower identity were also binned if they matched the reference plasmid or a plasmid of the same replicon type. Contigs binned together were extracted and used to create a plasmid draft. Contigs were included in drafts only if they could not be associated with another plasmid of a different replicon type. Single contigs that aligned with an entire plasmid in the BLAST analysis, but were not identified in the initial BLAST, were considered separate plasmids. ColE replicons were not processed into draft sequences due to the short length of contigs and difficulty in assembly. However, contigs that contained both a ColE replicon and an AR gene were analyzed. Drafts were annotated with RAST (Overbeek et al., 2014). Replicon types with an established Plasmid Multi Locus Sequence Type (pMLST) scheme were typed by querying the pMLST database[4] (Garcia-Fernandez et al., 2008, 2011; Garcia-Fernandez and Carattoli, 2010; Jolley and Maiden, 2010; Hancock et al., 2017). Contig coverage was also analyzed for each sequence using Bowtie2 and Qualimap (Garcia-Alcalde et al., 2012; Langmead and Salzberg, 2012).

Antibiotic Resistance Cassette Identification

For AR genes that were identified in multiple retrospective isolates, the contig containing the gene was aligned with

[4]https://pubmlst.org/plasmid/

the contigs containing the gene from other isolates using SnapGene[5]. Homologous sequence among these isolates immediately adjacent to the resistance gene was considered an Antibiotic Resistance Cassette (ARC). ARC sequences were defined as the sequence including an identical AR gene with identical flanking sequence, allowing for up to five base pair mismatches, in multiple unrelated isolates. ARC sequences were compared to retrospective isolates containing the AR gene, but not the entire ARC, using BLASTN to identify additional isolates containing the ARC sequence split onto multiple contigs (Camacho et al., 2009). ARC sequences were compared to the NCBI non-redundant (nr) database using BLASTN to identify matching sequences, and to identify the species and prevalence of sequenced isolates containing these ARC sequences.

Antibiotic resistance cassette sequences were also compared to the USDA-FSIS *Salmonella* isolates, using BLASTN. Isolates were only included in the comparison if they were predicted to contain the ARC. Predictions were based on the presence of the ARC AR genes in each isolate as presented by the Pathogen Detection Isolate Browser. Isolates were considered to contain the ARC if the whole ARC sequence was present or if the sequence was overlapping on multiple contigs.

Statistics

Ratio of FSIS isolates containing ARCs (animal source and serotype) were compared using 95% confidence intervals (95% CI) calculated in R. Conditional probabilities were calculated in Excel for isolates containing multiple ARCs using the following formulas:

$$P(A|B) = \frac{P(A \text{ and } B)}{P(A)}$$

$$P(A|B|C) = \frac{P(A \text{ and } B \text{ and } C)}{P(A \text{ and } B) * P(A)}$$

RESULTS

Phenotypic and Genotypic Antimicrobial Resistance

The retrospective study utilized WGS to analyze 193 isolates collected from 1998 to 2011. Phenotypic AR was known prior to sequencing and was used to help select the isolates for this study. Selected isolates (n = 119) exhibited phenotypic resistance to at least one antimicrobial tested and 67 of those were multi-drug resistant (resistant to three or more classes of antimicrobial). Resistance was observed for 13 of 14 antimicrobials tested in at least one isolate, with no resistance seen to ciprofloxacin. The most common ARs in the data set were to tetracycline, streptomycin, ampicillin, and sulfamethoxazole or sulfisoxazole (**Supplementary Table S1**).

A total of 923 AR genes were identified from the sequences (**Table 1**). All 193 retrospective isolates contained at least one AR gene (**Supplementary Table S1**). The most frequently identified AR gene was *aac*(6′)-I, an aminoglycoside acetyltransferase gene, variants *aac*(6′)-I-y (n = 159) and *aac*(6′)-I-aa (n = 30) that was present in almost every isolate. Setting aside *aac*(6′)-I, other genes for resistance to aminoglycosides were still the most numerous followed by genes for resistance to tetracyclines and β-lactams (**Table 1**). AR gene presence corresponded with phenotypic AR for 95.4% (618/648) of genes for which phenotypic testing was completed (**Supplementary Table 1**). One hundred and twenty six isolates were considered MDR as they contained multiple AR genes for multiple classes of antimicrobials (**Supplementary Table S1**).

Integrons

Sixty-one isolates contained a complete integron (In). Fourteen different complete previously named integrons were identified, and six novel integrons were identified. Novel integrons were defined as an arrangement not previously sequenced and assigned a new number. In2, containing *aad*A1, was the most numerous (n = 21). Two isolates also contained In0, which contains no gene cassettes, but an otherwise complete integron structure. Forty-eight integrons were determined to be associated with plasmid sequences (**Table 2**).

TABLE 2 | Number of integrons identified and integron gene cassette content in retrospective isolates (n = 193).

Integron	Number of isolates containing integron	Number located on plasmids	Integron gene cassettes arranged 5′–3′			
In2	21	19	*aad*A1a			
In740	9	9	*aad*A1bs			
In571	5	5	*aad*B	*gcu*E2	*gcu*8	*cml*A1g
In363	4	4	*dfr*A1	*gcu*C		
In27	2	1	*dfr*A12	*gcu*F	*aad*A2	
In0	2	0				
In142	2	2	*aad*A7			
In167	2	0	bla_{CARB-2}			
In287	2	2	*aad*A6D2			
In839	2	1	*aad*A1bx			
In45	1	1	*aad*A12			
In127	1	0	*aad*A2			
In191	1	0	*dfr*A14b			
In862	1	1	*est*X-3	*aad*A1a		
In1581*	1	0	*aad*A7g			
In1582*	1	0	*dfr*A16c	bla_{CARB-3}	*aad*A2	*ere*A1c
In1583*	1	1	*aad*A31			
In1584*	1	1	*est*X-6	*gcu*116		
In1585*	1	0	*aad*A2	*cml*A1g	*aad*A1a	*qac*H2
In1586*	1	1	*aad*A1D13			

Integron gene cassettes are listed in order of arrangement within the DNA. gcu indicates gene cassette of unknown function (hypothetical protein). (*) indicates a new integron number.

[5]http://www.snapgene.com/

TABLE 3 | Genotypic profiles and metadata of A/C plasmids.

Isolate	pMLST type	Serotype	Source	Plasmid genotypic
80	U	Copenhagen*	C	*str*AB, bla_{CMY-2}, *sul*2, *flo*R
169	ST1	IIIa		N/A
106	ST1	IIIa18:z4,z32:-	T	N/A
15	ST2	Copenhagen*	CH	*tet*AR, *sul*1
19	ST3	Agona	S	*str*AB, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R, *dfr*A1
158	ST3	Agona	C	*aph*3-Ia, *str*AB, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R, *dfr*A1
99	ST3	Bardo	C	*str*AB, bla_{CMY-2}, *tet*AR, *sul*2, *flo*R
75	ST3	Bredeney	T	*str*AB, bla_{CMY-2}, *tet*AR, *sul*2
27	ST3	Dublin	C	*aph*3-Ia, *str*AB, *aad*B, bla_{CMY-2}, *tet*AR, *sul*2, *flo*R, *cml*A
140	ST3	Dublin	C	*str*AB, bla_{CMY-2}, *tet*AR, *sul*2, *flo*R
150	ST3	Dublin	C	*aph*3-Ia, *str*AB, bla_{CMY-2}, bla_{TEM-1}, *tet*AR, *flo*R
72	ST3	Give	C	*aac*3-VIa, *str*AB, *aad*A, bla_{CMY-2}, *tet*R, *sul*1, *sul*2, *flo*R
3	ST3	Heidelberg	C	*aph*3-Ia, *str*AB, *aad*B, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R, *cml*A, *dfr*A1
43	ST3	Heidelberg		*str*AB, *aad*B, bla_{CMY-2}, bla_{TEM-1}, *tet*AR, *flo*R, *cml*A
86	ST3	Heidelberg	CH	*str*AB, *aad*B, bla_{CMY-2}, bla_{TEM-1}, *tet*AR, *sul*2, *flo*R, *cml*A
103	ST3	Heidelberg	T	*str*AB, bla_{CMY-2}, *tet*AR, *sul*2, *flo*R
126, 175, 185, 187	ST3	Heidelberg	T	*str*AB, bla_{CMY-2}, *tet*AR, *sul*2
62	ST3	IIIa 18:z4,z23:-	T	*aph*3-Ia, *str*AB, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R, *dfr*A1
111	ST3	Kinshasa**	C	*str*AB, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R
14	ST3	Newport		*str*AB, bla_{CMY-2}, *tet*AR, *sul*2, *flo*R
83	ST3	Newport	H	*aac*3-VIa, *str*AB, *aad*A, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R
139	ST3	Newport	C	*str*AB, bla_{CMY-2}, *tet*AR, *sul*2, *flo*R
161	ST3	Ohio		*str*AB, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R, *dfr*A1
40	ST3	Reading	C	*aac*3-VIa, *str*AB, *aad*A, bla_{CMY-2}, bla_{TEM-1}, *tet*A, *sul*1, *sul*2, *flo*R
17	ST3	Typhimurium	T	*aad*B, bla_{CMY-2}, *tet*AR, *flo*R, *cml*A
125	ST3	Typhimurium	C	*str*AB, *tet*AR, *sul*2, *flo*R
132	ST3	Typhimurium	C	*str*AB, *aad*A, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R
39	ST3	Uganda	C	*str*AB, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R
50	ST3	Uganda		*str*AB, bla_{CMY-2}, *tet*AR, *sul*1, *sul*2, *flo*R

*Isolate numbers correspond to isolate numbers with CRJJGF prefixes. Blank indicates source is unknown. U indicates unknown pMLST type due to inability to calculate. Animal source abbreviations are as follows, C, cattle; Ch, chicken; T, turkey; S, swine; H, horse. N/A indicates the plasmid contained no resistance genes. *Typhimurium variant. **Uganda variant.*

Plasmid Replicons Detected and Linkage to AR Genes

At least one plasmid replicon-associated gene was detected in 157 of the 193 isolates; multiple replicons were detected in 91 isolates (**Supplementary Table S1**). The most common types of replicon-associated genes detected were ColE followed by I1, F, X, and A/C. Additionally, HI1, HI2, Q1, and N were also detected at lower levels. A total of 212 draft plasmid sequences were created; 124 of them contained at least one AR gene and 102 contained multiple AR genes with 57 containing five or more AR genes (**Tables 3–10**). In total, 81.5% of AR genes were associated with a plasmid replicon (**Table 1**).

With the exception of ColE plasmids, detection of a replicon associated gene correlated with the presence of additional plasmid sequence in 100% of cases. ColE plasmids were not further characterized because the plasmids were too small to be reliably assembled. However, AR genes were detected in a few cases on the same contig with the ColE replicon, including four ColE plasmids homologous to pSC101 that contained the *tet*C gene (**Supplementary Table S3**).

A/C Replicons

A/C replicon-associated genes were detected in 32 isolates, 30 of which were associated with AR genes. Eighteen different combinations of AR genes were present among these plasmids and five of the AR gene profiles were located on multiple A/C plasmids. According to the A/C pMLST scheme 27 plasmids were type ST3; the remaining four included two ST1, one ST2, and one untypable plasmid. Plasmids were present in 16 different serotypes and isolated from five different host sources. However, 14/32 plasmids were isolated from cattle sources and 9/32 were isolated from turkey sources (**Table 3**). These sources represented 21% and 15% of the total isolates, respectively, (**Supplementary Table S1**).

F Replicons

Forty-three isolates contained at least one F type replicon-associated gene (**Table 4**). Because F-type plasmids can contain multiple replicon-associated genes of different types, all contigs identified as belonging to an F-replicon plasmid were considered to belong to the same plasmid. F, FII, FIIs, FIA, FIB, FIBs, FIC, and FV replicons were identified. Fourteen of

TABLE 4 | Genotypic profiles and metadata of F plasmids.

Isolate	Replicons present	Serotype	Source	Plasmid genotype
46	F, FII	Braenderup	CH	*str*AB, *tet*AR
94	F, FII	Orion	CH	*aph3″*-Ia
17	F, FII, FIA, FIB	Typhimurium	T	*aph3″*-Ia, *str*AB, *tet*B, *sul*2
1, 53,	F, FII, FIB	Kentucky	PE	*str*AB, *tet*B
77, 109, 116	F, FII, FIB	Kentucky	CH	*str*AB, *tet*B
79	F, FII, FIB	Minnesota	T	*aph3″*-Ia, *str*AB, *tet*B, *sul*2
80	FIB, FIIs	Copenhagen*	C	*aph3″*-Ia, bla_{TEM-1}, *tet*AR
52	FIBs, FIIs	Choleraesuis	S	N/A
16	FIBs, FIIs	Copenhagen*	C	*aph3″*-Ia, *aad*A, bla_{TEM-1}, *tet*AR, *sul*1
5	FIBs, FIIs	Enteritidis	PE	N/A
6, 7, 8, 9, 11, 12, 13, 129, 130	FIBs, FIIs	Enteritidis	CH	N/A
10	FIBs, FIIs	Enteritidis	RTE	N/A
56	FIBs, FIIs	Enteritidis	R	N/A
131, 171	FIBs, FIIs	Enteritidis	WA	N/A
137	FIBs, FIIs	Enteritidis		N/A
29	FIBs, FIIs	I 4,[5],12:i:-	T	N/A
148	FIBs, FIIs	Kunzendorf***	S	N/A
18, 96	FIBs, FIIs	Typhimurium	CH	N/A
51	FIBs, FIIs	Typhimurium	S	N/A
35	FIC	Mbandaka		*aad*A, *tet*AR, *sul*1, *dfr*A
169	FIIs	IIIa		N/A
155	FIIs	Pullorum****	AV	N/A
125	FIIs	Typhimurium	C	*aad*A, bla_{TEM-1}, *sul*1
28	FIIs, X1	Dublin	CH	N/A
140, 150	FIIs, X1	Dublin	C	N/A
149	FIIs, X1	Dublin	C	bla_{TEM-1}
164	FV	Binza*****	T	N/A
115	FV	II 48:d:z6**	E	N/A

*Isolate numbers correspond to isolate numbers with CRJJGF prefixes. Animal source abbreviations are as follows, C, cattle; Ch, chicken; T, turkey; S, swine; PE, poultry environment; E, environmental food contact surface; RTE, ready to eat product; AV, avian; WA, wild animal; R, wild reptile. Blank source indicates unknown. N/A indicates no resistance genes were present. *Typhimurium variant. **Hagenbeck. ***Cholersuis variant. ****Gallinarum variant. *****Orion.*

these 43 draft plasmids contained AR genes. Eight different combinations of AR genes were present among these 14 isolates; five of these plasmids that contained *str*AB and *tet*B, were found in *Salmonella* Kentucky isolates from poultry. A total of eight different combinations of replicons were identified (**Table 4**).

HI Replicons

Four isolates contained a HI1 plasmid and 20 isolates contained a HI2 plasmid, all of which contained AR genes. All four HI1 plasmids were from different sources, but all carried the *tet*B resistance gene. Six HI2 plasmids belonged to one resistance gene profile containing *aph*, *sph*, *str*A, *str*B, and *tet*B, while six other HI2 plasmids had unique AR gene profiles. Based on the HI1 pMLST typing scheme, two HI1 plasmids were ST2, one was ST7, and one was untypable (due to a missing allele). By the HI2 pMLST scheme, three plasmids were ST1, four ST2, and the rest untypable due to a mutation in one of the alleles used for typing (**Table 5**).

TABLE 5 | Genotypic profiles and metadata of HI plasmids.

Isolate	HI Type	Serotype	Source	Plasmid genotype
28	HI1	Dublin	CH	*str*AB, bla_{TEM-1}, *tet*B
149	HI1	Dublin	C	*tet*B
154	HI1	Krefeld	S	*tet*B
152	HI1	Rubislaw	E	*aph*3Ia, *str*AB, *tet*B, *sul*1, *sul*2, *cml*A, *mph*A
164	HI2	Binza*	T	*tet*B
70	HI2	Bovismorbificans	S	*aac*-Iva, *aph*4-Ia
63	HI2	Brandenburg	C	*aac*3-Via, *aph*3Ia, *aad*A, *tet*B, *sul*1
75	HI2	Bredeney	T	*aad*A, *aad*B, *tet*C, *sul*1
126	HI2	Heidelberg	T	*aph*A, *sph*, *aph3″*Ia, *tet*B
128	HI2	Heidelberg	H	*aph*A, *sph*, *str*AB, *tet*B
145	HI2	Heidelberg	S	*aph*A, *sph*, *str*AB, *tet*B
174, 184	HI2	Heidelberg	CH	*aph*A, *sph*, *str*AB, *tet*B
175	HI2	Heidelberg	T	*aph*A, *sph*, *aph3″*Ia, *tet*B
180, 181	HI2	Heidelberg	T	*aph*, *sph*, *str*B, *tet*B
185, 187	HI2	Heidelberg	T	*aph*A, *sph*, *tet*B
186, 194	HI2	Heidelberg	T	*aph*A, *sph*, *str*AB, *tet*B
81	HI2	Livingstone	E	*str*AB
156	HI2	Ouakam	CH	*tet*B
110	HI2	Putten	S	*aac*3-Iid, *aac*-Iva,*str*AB, *aad*A, *tet*B, *sul*1
159	HI2	Putten		*aad*A, *tet*B, *sul*1

*Isolate numbers correspond to isolate numbers with CRJJGF prefixes. Animal source abbreviations are as follows, C, cattle; Ch, chicken; T, turkey; S, swine; H, horse; E, environmental food contact surface. *Orion variant.*

I1 Replicons

Sixty-two isolates contained an I1 replicon-associated gene, yielding 62 draft plasmid sequences. Fifty of those plasmids contained AR genes. Sixteen plasmids contained only bla_{CMY-2} and 15 plasmids contained only three AR genes, *aad*A, *aac*3, and *sul*1 (**Table 6**). On 14 of those 15 plasmids; the resistance genes were associated with the integron In2; on the remaining plasmid, the genes were associated with a novel integron, In1586. Nine different I1 pMLST types were present, with ST12 (n = 13) and ST26 (n = 20) being the most represented (**Tables 6**, **7**). Fourteen plasmids could not be typed by pMLST, due to missing alleles. Twenty-one plasmids were isolated from turkey sources and thirteen from chicken (**Table 6**).

N Replicons

Four isolates contained N replicon-associated genes leading to four draft plasmids. Three plasmids contained AR genes. IncN

TABLE 6 | Genotypic profiles and metadata of I1 plasmids containing resistance genes.

Isolate	pMLST Type	Serotype	Source	Plasmid genotype
15	ST12	Copenhagen*	CH	bla_{CMY-2}
74	ST12	Havana	S	bla_{CMY-2}
102	ST12	Heidelberg	cat	bla_{CMY-2}
178	ST12	Heidelberg	T	bla_{CMY-2}
182	ST12	Heidelberg	CH	bla_{CMY-2}
30	ST12	Infantis	CH	bla_{CMY-2}
44	ST12	Johannesburg	S	bla_{CMY-2}
154	ST12	Krefeld	S	bla_{CMY-2}
78	ST12	Minnesota	S	bla_{CMY-2}
37	ST12	Saintpaul	T	bla_{CMY-2}
120	ST12	Thompson	CH	bla_{CMY-2}
116, 109	ST12, U	Kentucky	CH	bla_{CMY-2}
141	ST155	Worthington	S	*tet*B
152	ST20	Rubislaw	E	bla_{CMY-2}
53	ST201	Kentucky	PE	*tet*AR
142	ST222	Albany	T	*aac*3VIa, *aad*A, *sul*1
187	ST222	Heidelberg	T	*aac*3VIa, *aad*A, *sul*1
33	ST222	Schwarzengrund		*aac*3VIa, *aad*A, *sul*1
41	ST23	Cerro	C	bla_{CMY-2}
143	ST25	Manhattan	S	*aad*A, *sul*1
23	ST26	Hadar	E	*aad*A, *sul*1
134	ST26	Hadar	T	*aac*3VIa, *aad*A, *sul*1
174, 175, 179, 180, 183, 185	ST26	Heidelberg	CH	*aac*3VIa, *aad*A, *sul*1
192	ST26	Heidelberg	T	*aac*3IId, *str*AB, *aad*A, bla_{TEM-1}, *tet*AR
194	ST26	Heidelberg	T	*aac*3IId, *aad*A, *tet*AR
188, 189, 190, 193, 195	ST26	Heidelberg	HU	*aac*3IId, *aad*A, bla_{TEM-1}, *tet*AR
191	ST26	Heidelberg	T	*aac*3IId, *str*A, *aad*A, bla_{TEM-1}, *tet*AR
29	ST26	I 4,[5],12:i:-	T	*aac*3VIa, *aad*A, *sul*1
59	ST26	IIIa 18:z4,z23:-		*aac*3VIa, *aad*A, *sul*1
87	ST26	Litchfield	CH	*aac*3VIa, *aad*A, *sul*1
126, 103	ST26, U	Heidelberg	T	*aac*3VIa, *aad*A, *sul*1
40	ST4	Reading	C	*tet*CR
85	ST4	Hartford	H	*tet*C
113	U	Anatum	T	*aac*3VIa, *aad*A, bla_{TEM-1}, *tet*B, *sul*1
45	U	Berta	T	tem
22	U	Derby	T	*tet*AR
105	U	Minneapolis**	T	*aac*3IId, *aad*A, bla_{TEM-1}, *tet*AR
94	U	Orion	CH	bla_{CMY-2}
124	U	Senftenberg	C	*aph*A, *sph*, *sul*1

*Isolate numbers correspond to isolate numbers with CRJJGF prefixes. U indicates unknown ST type due to inability to calculate. Animal source abbreviations are as follows, C, cattle; Ch, chicken; T, turkey; S, swine; PE, poultry environmental; E, environmental food contact surface; H, horse; HU, human. *Typhimurium variant. **Anatum variant.*

pMLST results identified two plasmids that were ST1, one was ST3, and one was untypable. Isolates were four different serotypes and sources (**Table 8**).

TABLE 7 | Genotypic profiles and metadata of I1 plasmids containing no resistance genes.

Isolate	pMLST	Serotype	Source
20	ST12	Agona	RTE
21	U	Montevideo	CH
31	U	Infantis	S
52	U	Choleraesuis	S
57, 77, 127	U	Kentucky	CH
75	ST80	Bredeney	T
117	U	Fresno	R
118	U	Sandiego	R
128	U	Heidelberg	H
139	U	Newport	C

Isolate numbers correspond to isolate numbers with CRJJGF prefixes. U indicates unknown ST type due to inability to calculate. Animal source abbreviations are as follows, C, cattle; Ch, chicken; T, turkey; S, swine; RTE, ready to eat product; H, horse; R, wild reptile.

TABLE 8 | Genotypic profiles and metadata of IncN plasmids.

Isolate	pMLST	Serotype	Source	Plasmid genotype
89	N/A	Tennessee	S	N/A
133	ST1	Montevideo	C	*tet*AR
110	ST1	Putten	S	bla_{TEM-1}
82	ST3	Javiana		*str*AB, bla_{TEM-1}, *sul*1, *sul*2, *cml*A, *mph*A

Isolate numbers correspond to isolate numbers with CRJJGF prefixes. Animal source abbreviations are as follows, C, cattle; S, swine; blank, unknown.

TABLE 9 | Genotypic profiles and metadata of IncQ1 plasmids.

Isolate	Serotype	Source	Plasmid genotype
91	Alachua	S	*str*AB, *tet*AR, *sul*2
177	Derby	S	*str*AB, *tet*AR, *sul*2
148	Kunzendorf*	S	*str*AB, *sul*2
65	London	S	*aph*3-Id, *str*AB, *tet*AR, *sul*2
143	Manhattan	S	*str*AB, *sul*2
48	Meleagridis	C	*str*AB, *tet*AR, *sul*2
42	Muenchen	T	*str*AB, *tet*AR, *sul*2

*Isolate numbers correspond to isolate numbers with CRJJGF prefixes. *Cholersuis variant. Animal source abbreviations are as follows, C, cattle; T, turkey; S, swine.*

Q1 Replicons

Q1 replicon associated genes were identified in seven isolates yielding seven draft plasmids containing AR genes. All Q1 plasmids contained AR genes for aminoglycosides and sulfonamides and three also contained *tet*AR genes for resistance to tetracycline. In addition to these five genes, one Q1 plasmid contained an additional aminoglycoside resistance gene, *aph*3-Id. Plasmids were found in isolates of seven different serotypes, and five plasmids were from swine sources (**Table 9**).

TABLE 10 | Genotypic profiles and metadata of IncX plasmids.

Isolate	Serotype	Source	X type	Genotype
3	Heidelberg	C	X4	bla_{TEM-1}
24	Hadar	C	X1	bla_{TEM-1}
27	Dublin	C	X1	bla_{TEM-1}
129	Enteritidis	CH	X1	bla_{TEM-1}
134	Hadar	T	X1	bla_{TEM-1}
186	Heidelberg	T	X1	bla_{TEM-1}
139	Newport	C	X2	*aph3″*Ia
8, 13	Enteritidis	CH	X1	
25, 43	Heidelberg		X1	
37	Saintpaul	T	X1	
40	Reading	C	X1	
45	Berta	T	X1	
65	London	S	X1	
86, 95, 176	Heidelberg	CH	X1	
98	IIIb 38:(k):z35	R	X1	
102	Heidelberg	cat	X1	
103, 191, 192, 194	Heidelberg	T	X1	
104	Minneapolis*	S	X1	
165, 166	IIIb 61:–:1,5,7	C	X1	
188, 190, 193, 195	Heidelberg	HU	X1	

*Isolate numbers correspond to isolate numbers with CRJJGF prefixes. Animal source abbreviations are as follows, C, cattle; Ch, chicken; T, turkey; S, swine; HU, human; R, wild reptile. Blank indicates unknown source. *Anatum variant.*

TABLE 11 | The co-occurrence of replicons with additional replicons within the same isolate from the retrospective isolates set (n = 193).

	AC	F	HI1	HI2	I1	N	Q1	X	pSC101	ColE
AC	32	6	0	5	9	0	0	9	0	14
F	6	43	2	2	7	0	1	7	0	8
HI1	0	2	4	0	2	0	0	2	0	0
HI2	5	2	0	21	9	1	0	2	0	16
I1	9	7	2	9	62	0	1	14	1	34
N	0	0	0	1	0	4	0	0	0	1
Q1	0	1	0	0	1	0	7	1	0	3
X	9	7	2	2	14	0	1	35	0	17
pSC101	0	0	0	0	1	0	0	0	4	2
ColE	14	8	0	16	34	1	3	17	2	76

Gray boxes indicate the total number of replicons identified.

X Replicons

Thirty-three isolates contained an X1 replicon-associated gene, one contained an X2 replicon-associated gene, and one contained an X4 replicon-associated gene, yielding 29 draft X plasmid sequences (**Table 10**). The other four isolates with X1 replicons were serotype Dublin, which can contain a virulence plasmid with two replicons, FIIs and X1; therefore, those plasmids were counted as F type (**Table 4**) (Mohammed et al., 2017). Five of the X1 plasmids and the one X4 plasmid contained bla_{TEM-1}. The X2 plasmid contained *aph*3″-Ia.

Co-occurrence

Multiple replicon-associated genes of different types were detected in 92 of 155 isolates containing plasmids (**Supplementary Table S1**). Incidence of co-occurrence varied by replicon type, but more than half of all plasmids were present with additional replicons in the same isolate. Replicons with the highest frequencies of co-occurrence were X1 (94.2%), HI1 (100%) and HI2 (85%), I1 (75.8%), and Q1 (85.7%) (**Table 11**). There were three cases of two different replicons present not only in the same isolate but on the same contig, all of which were FIIs replicons with an X1 replicon in *S.* Dublin isolates.

Antibiotic Resistance Cassettes (ARCs)

Six ARCs and one pseudo-ARC, as defined in materials and methods, were identified (**Figures 1**, **2** and **Table 12**). ARC1 (5627 bp), consisting of *tet*A, *tet*R, *str*A, *str*B, *sul*2, was found in 27 isolates on A/C plasmids and five isolates on Q1 plasmids. ARC2 (5868 bp), consisting of *aac*3-IId and *tmr*B, was present in 11 isolates and located on ColE (1), HI2 (1), and I1 (9) plasmids. ARC3 (1902 bp), consisting of *aph* and *sph*, and was found on eleven HI2 plasmids and two I1 plasmids while ARC4 (3911 bp), containing bla_{CMY}, *hyp*, and *sug*E, was found on 16 I1 and 28 A/C plasmids. ARC5 (4173 bp), consisting of *flo*R and genes of unknown function, was present on 24 A/C plasmids. ARC6 (4462 bp), containing *tet*B, was located on six F plasmids, 17 HI2, and two HI1 plasmids. ARC6 was also found in two additional isolates but could not be confirmed as associated with a plasmid. The final ARC, designated pseudo-ARC, was an integron (In2 In237, In839, In1581, and In1583), containing *aac*3-Via, *aad*A, and *sul*1 (**Figure 2**). This ARC was designated pseudo because there was no consensus sequence due to variation in sequence. However, the ARC was still included in the characterization because the genes were identified together on the same contig, all within an integron structure, and in the same order in 22 isolates.

Antibiotic resistance cassettes sequences were identified in *Salmonella*, isolated from 2014 to 2018, sequenced by USDA-FSIS (n = 6681) (**Figure 3**, **Table 13**, and **Supplementary Table S4**). ARC1 was found in 242 isolates, 79.8% of which were from cattle. Thirteen different serotypes were represented among the 242 isolates, and the ARC was identified on a contig also containing a plasmid replicon in 43 isolates. ARC2 was found in 11 isolates that were from five serotypes and three different sources. Only one was on a contig with an F plasmid replicon, a serotype Kentucky isolate from chicken. ARC3 was found in 20 isolates. All isolates were serotype Heidelberg except one isolate from swine that was serotype Mbandaka. Two were associated with a plasmid sequence, both HI2 from serotype Heidelberg. ARC4 was found in 259 isolates of 19 different serotypes. Sixty-three were associated with plasmids, types: A/C, F, K, and I1. ARC5 was identified in 142 isolates, of 15 different serotypes, and was associated with a plasmid in 17 isolates. ARC6 was identified in 355 isolates of 23 different serotypes, 78% of which were serotype Kentucky. ARC6 was present on a plasmid in 274 isolates (**Table 13**). Two hundred and five USDA-FSIS isolates contained multiple ARCs (**Figure 3**).

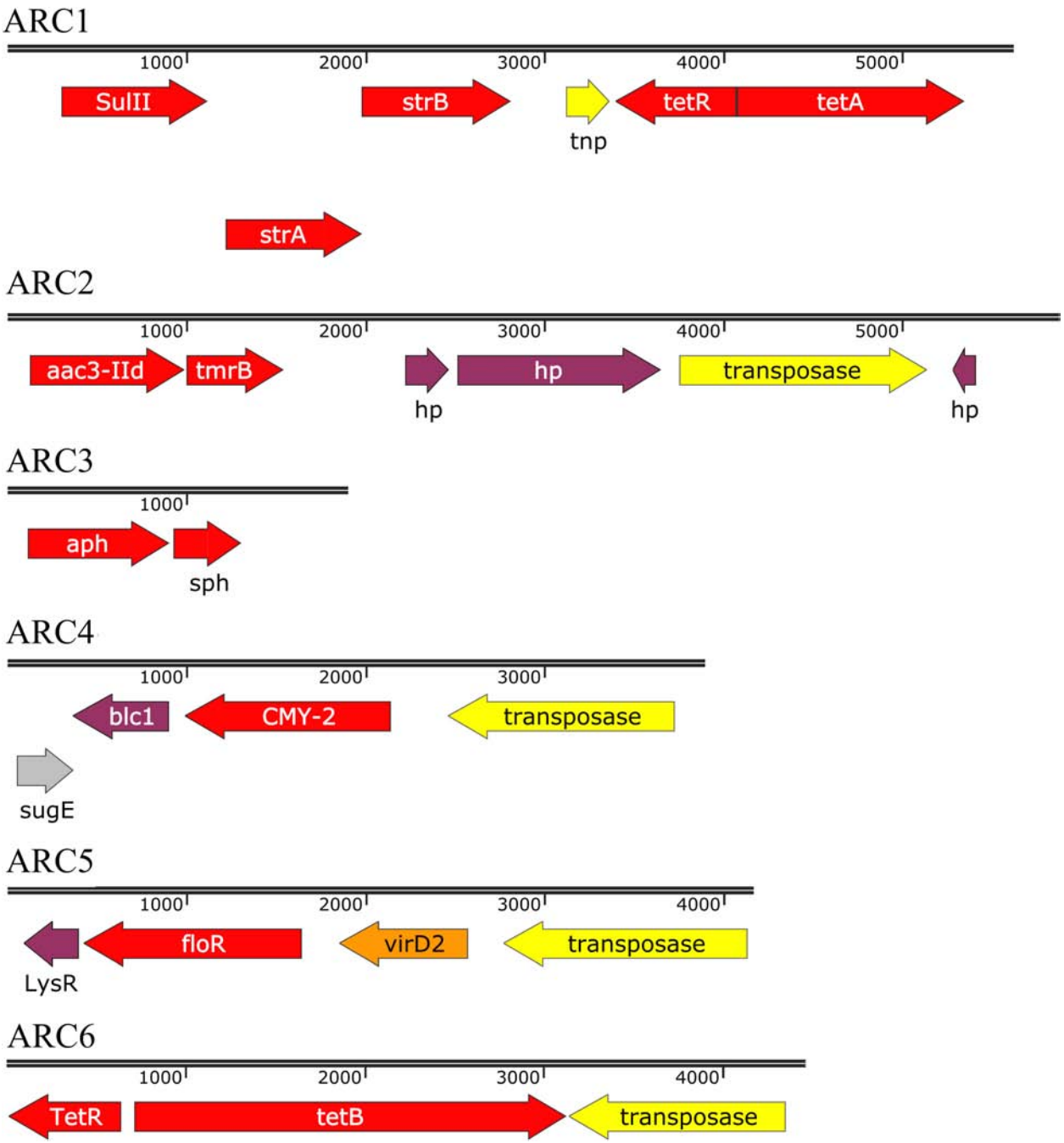

FIGURE 1 | Sequences of antibiotic resistance cassettes (ARCs) identified. Length of sequences are proportional. Arrow color indicates gene classification. Red, AR gene; Yellow, mobile element gene; Gray, metal resistance gene; Orange, relaxase gene; Purple, other gene. Gene abbreviations as follows: tnp, transposase; hp, hypothetical protein.

Among these FSIS isolates, animal sources and serotypes were significantly more likely to contain certain ARCs than others. Isolates from cattle sources were significantly more likely to contain ARC1 than any other source (95% CI: 0.18–0.23). Isolates from turkey sources were more likely to contain ARC1 than isolates from chicken and swine (95% CI: 0.06–0.11, **Supplementary Data**). Isolates from cattle were also significantly more likely to contain ARC4 and ARC5 than any other source (95% CI: 0.1–0.14, 0.12–0.15), while isolates from chicken were significantly more likely to contain ARC6 than other sources (95% CI: 0.06–0.08, **Figure 4**). Serotype Dublin isolates, which were only identified from cattle sources, and serotype Newport isolates were significantly more likely to contain ARC1 (95% CI: 0.78–0.91, 0.53–0.69) and ARC5 (95% CI: 0.41–0.59, 0.3–0.46)

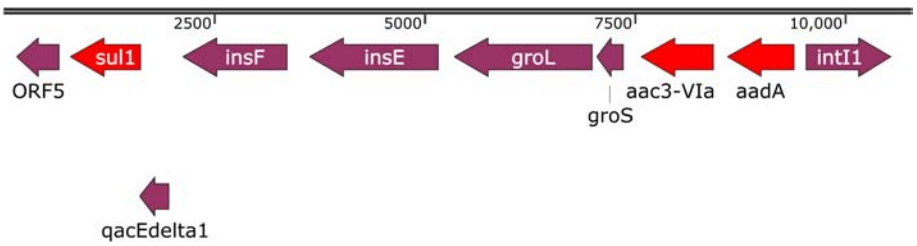

FIGURE 2 | Example sequence of one of the pseudo-ARC variant sequences. Red arrows are AR genes, purple arrows are other genes.

than isolates of other serotypes identified (**Supplementary Data**). Isolates of serotype Reading were also significantly more likely to contain ARC1 than other serotypes identified, except for Newport and Dublin (95% CI: 0.23–0.44, **Supplementary Data**). Serotype Newport isolates were also significantly more likely to contain ARC4 than all other serotypes (95% CI: 0.49–0.65, **Figure 5**).

Antibiotic resistance cassettes were also associated with each other in certain animal sources. Isolates from cattle containing ARC4 had a 90% probability of also containing ARC1, while isolates from chicken only had a 1.8% probability. Isolates from cattle containing ARC5 had a 94% probability of also containing ARC1; however, isolates from cattle that were positive for ARC1 only had a 52 and 58% probability of containing ARC4 and ARC5, respectively. Probabilities of ARC co-occurrence are shown in **Supplementary Data**.

Antibiotic resistance cassettes sequences were also compared with the NCBI non-redundant database to identify other isolates containing the ARC sequences. ARC1 was found in 88 isolates of 12 different species, 17 types of sources, 14 different countries, and present on A/C, I1, F, HI2, and Q1 plasmids, as well as on the chromosome and on integrative conjugative elements (ICE). ARC2 was identified in 16 different species from 15 countries and in 12 different source types. ARC2 was associated with the highest number of different replicon types including A/C, F, I1, HI1, HI2, L/M, and N. ARC3 was identified in 3 different species, 4 different countries, and from 2 sources, but associated with four different replicon types, F, I1, HI2, and N. ARC4 was identified in 12 different species, 20 different countries, and from 11 sources, but in only three identifiable plasmid types, A/C, I1, and K. ARC5 was identified in 13 different species, 17 different countries, and from 17 sources. Unlike in the retrospective dataset, ARC5 was found in four different replicon types, A/C, F, I1, and HI2, as well as ICEs (n = 14). ARC6 was identified in 26 different species, 21 different countries, from 10 sources, associated with four different replicon types F, HI1, HI2, and K (**Supplementary Tables S5–S10**).

TABLE 12 | AR genes contained in each antibiotic resistance cassette (ARC) and their associated replicons from the retrospective isolate set (n = 193).

ARC	Associated replicons
ARC1: *tet*AR, *str*AB, *sul*2	A/C(27), Q1(5)
ARC2: *aac*3-IId	ColE(1), I1(9), HI2(1)
ARC3: *aph*, *sph*	HI2(11), I1(2)
ARC4: bla_{CMY-2},	A/C(28), I1(16)
ARC5: *flo*R	A/C(24)
ARC6: *tet*B	F(7), HI1(2), HI2(15)
Pseudo-ARC: *aac*3-Via, *aad*A, *sul*1	A/C(4), HI2(1), I1(16)

Genes listed are not the only genes contained within the AR ARCs.

DISCUSSION

With a goal of investigating the relationship between AR genes and plasmids in *S. enterica* isolates associated with food animals, 193 isolates were sequenced to identify their AR genes and plasmids. The isolates for this retrospective study were selected to represent a great level of diversity, therefore, prevalence of plasmids, ARCs, AR genes, etc. in these retrospective isolates cannot be used to imply their overall prevalence in *Salmonella* associated with animals. Nevertheless, many conclusions can be made with this fact in mind.

More than 80% of AR genes identified were located within a plasmid sequence. The number and diversity of plasmids identified in the set of retrospective isolates indicated that many different plasmids were involved in AR in *Salmonella* among food animals. At least one plasmid of every replicon type identified contained an AR gene. Although certain replicon types were more prevalent than others, no single type was responsible for encoding the majority of the AR genes.

Although *aac6-I* was the most frequently identified gene, these genes are commonly chromosomal genes in *Salmonella* rendered silent by a deletion in the promoter. However, expression can be increased by a fusion of genes upstream (Magnet et al., 1999). No isolates from the retrospective study contained this fusion, despite three isolates showing resistance to gentamicin that lacked any other genes for gentamicin resistance. It is possible that these isolates contain an unknown gene or mutation that confers gentamicin resistance.

A/C plasmids, as a whole, contained more AR genes per plasmid than any other replicon type. Approximately 25% of the total AR genes identified were located on an A/C plasmid despite A/C plasmids only representing 15% of the total number of plasmids identified. Conversely, I1 was the most prevalent replicon type (aside from ColE), accounting for 29% of the total plasmids identified, but only contained 13% of the total AR genes. These findings are consistent with previous studies that isolated A/C and I1 plasmids (Cao et al., 2018). A/C plasmids containing

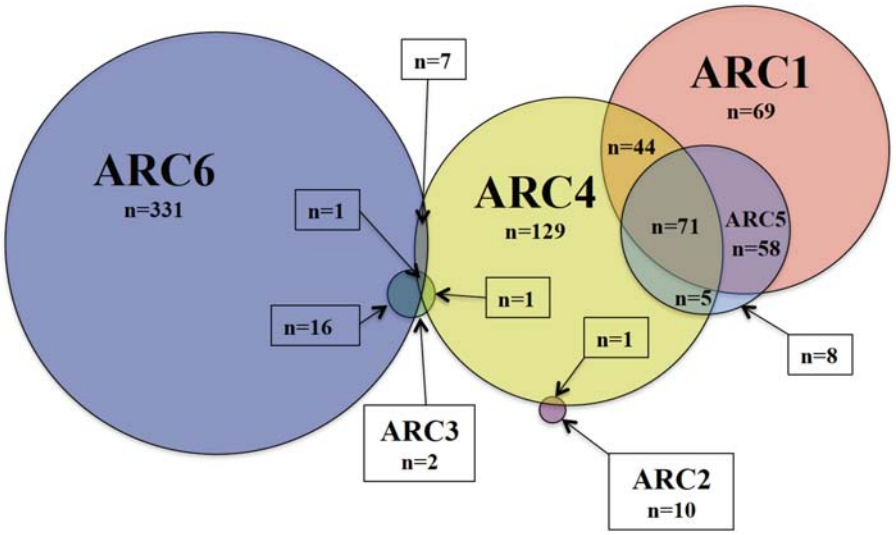

FIGURE 3 | Number of FSIS isolates containing the six ARCs or combination thereof. Total isolates containing each ARC are as follows: ARC1 = 242, ARC2 = 11, ARC3 = 20, ARC4 = 258, ARC5 = 142, ARC6 = 355.

TABLE 13 | Isolates from FSIS containing the six antibiotic resistance cassette (ARCs) described.

ARC	Total isolates	Animal source	Serotypes	Plasmid types
1	242	C: 193	Dublin, Heidelberg, Newport, Ohio, Reading, Typhimurium	A/C: 17 I1: 1 Q1: 25
		Ch: 2	Heidelberg, Infantis	
		T: 31	Agona, Heidelberg, I,4,[5],12:i:-, Infantis, Reading, Senftenberg Agona, Derby, Heidelberg, I,4,[5],12:i:-,	
		S: 15	Infantis, London, Muenchen, Ohio, Reading, Typhimurium	
		P: 1	Dublin	
2	11	Ch: 5	Kentucky, Schwarzengrund	F: 1
		T: 5	I,4,[5],12:i:-, London, Schwarzengrund	
		S: 1	Senftenberg	
3	20	C: 1	Heidelberg	HI2: 2
		Ch: 16	Heidelberg	
		T: 1	Heidelberg	
		S: 2	Heidelberg, Mbandaka	
4	259	C: 112	Dublin, Heidelberg, I,4,[5],12:i:-, Newport, Ohio, Reading, Typhimurium	A/C: 16 F: 1 I1: 44 K: 2
		Ch: 113	Cerro, Heidelberg, I,4,[5],12:i:-, Infantis	
		T: 14	Kentucky, Litchfield, Typhimurium Agona, Heidelberg, I,4,[5],12:i:-, Infantis, Litchfield, Liverpool, Montevideo	
		S: 19	Agona, Anatum, Derby, Heidelberg, I,4,[5],12:i:-, Infantis, London	
		E:1	Typhimurium, Uganda, Worthington Typhimurium	
5	142	C: 120	Anatum, Dublin, Meleagridis, Muenster, Newport, Ohio, Reading, Typhimurium	A/C: 16 I1: 1
		Ch: 7	Heidelberg, Infantis, Rough O:r:1,5	
		T: 6	Agona, Heidelberg, Infantis, Senftenberg	
		S: 9	Agona, Derby, I,4,[5],12:i:-, Infantis, Typhimurium	
6	355	C: 11	Anatum, Cerro, Heidelberg, Kentucky, Montevideo	F: 245 I1: 25 HI2: 4
		Ch: 295	8,20:-:z6, Heidelberg, Kentucky, Mbandaka, Oranienburg, Schwarzengrund	
		T: 13	4,[5],12:d:-, 4,[5],12:r:-, Agona, Albany, Berta, 1,4,[5],12:i:-	
		S: 35	Agona, Bovismorbificans, Braenderup, Brandenburg, Derby, Heidelberg, I,4,[5],12:i:-, Infantis, Johannesburg, Kentucky, London, Mbandaka, Uganda	
		RTE: 1	Derby	

Animal source abbreviations are as follows, C, cattle; Ch, chicken; T, turkey; S, swine; P, unidentified poultry; E, environmental food contact surface; RTE, ready to eat product. The number of isolates containing an ARC associated with a plasmid are total for the isolate set, not per commodity.

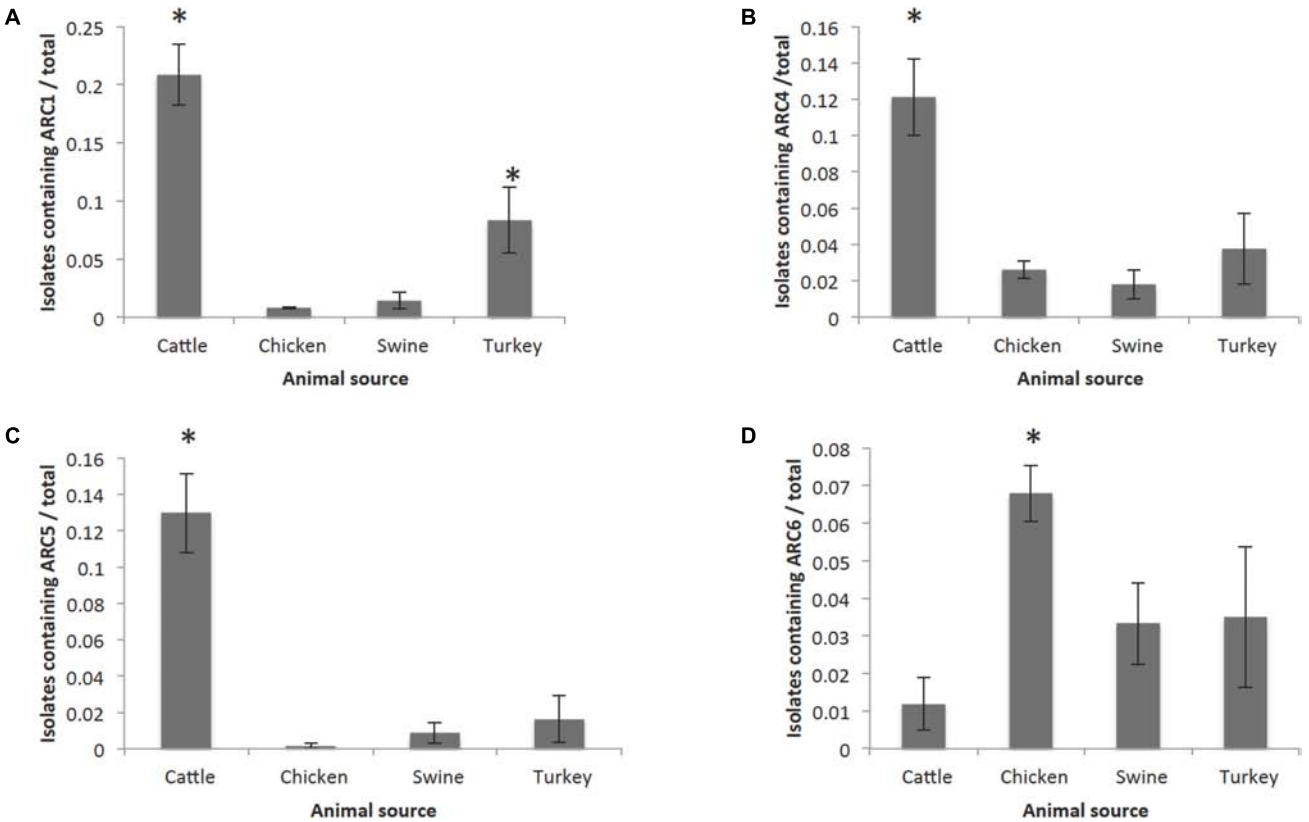

FIGURE 4 | Frequency of animal sources containing each cassette compared to other animal sources. Error bars reflect 95% Confidence intervals (95% CI). Only graphs for ARCs with significant (*) associations are shown. **(A)** Frequency of isolates containing ARC1. **(B)** Frequency of isolates containing ARC4. **(C)** Frequency of isolates containing ARC5. **(D)** Frequency of isolates containing ARC6.

up to 13 AR genes have been identified in isolates from animals in other studies (Hoffmann et al., 2017). I1 plasmids have been seen with similar gene profiles to the profiles detected in this study as well, especially the profile containing the single bla_{CMY-2} gene (Folster et al., 2012; Kaldhone et al., 2018). The single pMLST ST2 A/C plasmid found in this study was similar to a previously described ST2 A/C plasmid in that it contained approximately 22,500 base pairs of the *Yersinia pestis* chromosome (Hoffman et al., 2013). These genes from *Y. pestis* encoded a siderophore, methyltransferase, adenylase, as well as other virulence associated functions. The isolate identified in this study was serotype Typhimurium var 5 – from a chicken-associated source, isolated in 2004. It has been recently suggested that IncA/C plasmids are actually two separate incompatibility groups: IncA and IncC (Ambrose et al., 2018). By that classification, all A/C plasmids from the retrospective study would be considered IncC.

Interestingly, many A/C containing isolates also harbored an additional replicon, which could increase the transferability of AR genes from these isolates to others (Han et al., 2018). A/C plasmids occurred with additional replicons 23/32 times and did not occur with HI2 plasmids unless an I1 and a ColE replicon was also present. Those five isolates were the only isolates to have more than two large plasmids in the same isolate. All five of those isolates were from a turkey source and four were of serotype Heidelberg with the fifth being serotype Bredeney. Fourteen of the 23 isolates contained both an A/C and an additional plasmid of a different replicon. The additional plasmid contained AR genes different and in addition to those on the A/C plasmid. As suggested in Han et al. (2018), carriage of multiple plasmids may positively affect transfer of AR genes. It may also affect the transferability of A/C plasmids, including those without the genes required for transfer. While the study by Han et al. (2018) was only conducted in A/C positive isolates, it is possible this effect is present among isolates containing other combinations of replicons.

Although F type plasmids had one of the lower percentages of plasmids containing AR genes, these are of particular interest because several virulence plasmids belong to the F incompatibility group. Certain *Salmonella* serotypes, like Typhimurium and Enteritidis, usually contain an F replicon characterized by the *spv* genes for enhanced virulence as seen in the pSLT plasmid of *S. enterica* serovar Typhimurium strain LT2 (Boyd and Hartl, 1998; Silva et al., 2017). Of the 14 F plasmids identified with AR genes, four of those are variants of *Salmonella* virulence plasmids. In five isolates containing F-type plasmids, the plasmid was a variant of an avian pathogenic *E. coli* (APEC) plasmid that has been seen previously in *Salmonella* serotype Kentucky (Fricke et al., 2009; Johnson et al., 2010). Predictably,

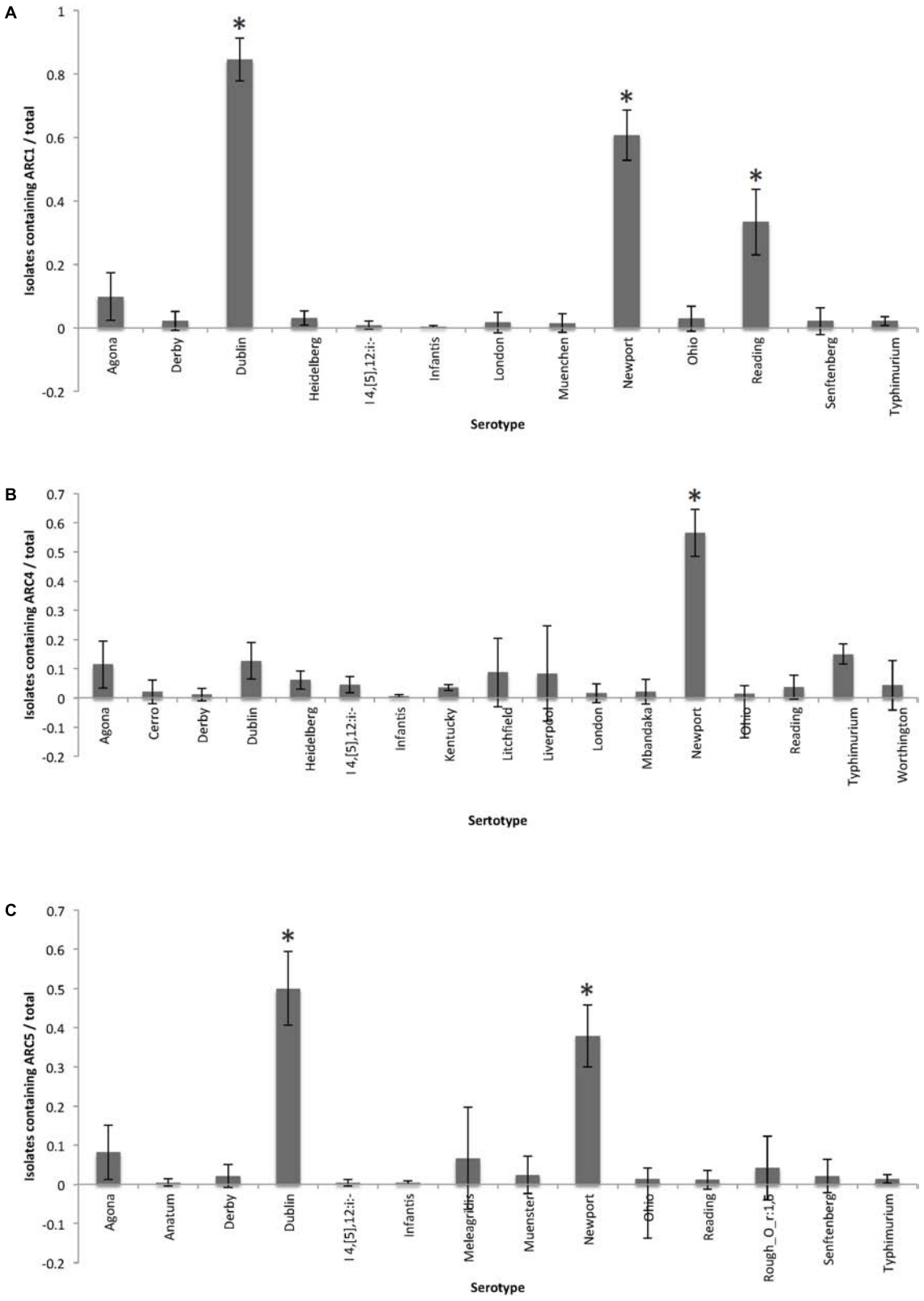

FIGURE 5 | Frequency of serotypes containing each cassette compared to other serotypes for each cassette. Error bars reflect 95% Confidence intervals (95% CI). Only graphs for ARCs with significant (*) associations are shown. **(A)** Frequency of isolates containing ARC1. **(B)** Frequency of isolates containing ARC4. **(C)** Frequency of isolates containing ARC5.

these five isolates were also serotype Kentucky and came from poultry sources. Additionally, one plasmid appears to be similar to a virulence plasmid of the fish pathogen, *Edwardsiella tarda* (Yu et al., 2012).

All HI type plasmids identified contained AR genes. HI1 and HI2 plasmids both contained *tet*B associated with ARC6, which is a portion of Tn10. This is also consistent with previous findings indicating an association between Tn10 and HI type plasmids (Cain and Hall, 2012a,b). However, HI2 plasmids identified in this study were largely untypable by pMLST despite containing every gene used in the scheme, due to a mutation in one of the alleles. This predicts that these plasmids belong to a new sequence type and may indicate a new lineage of HI2 plasmids, different from the sequenced plasmids used to develop the pMLST scheme (Garcia-Fernandez and Carattoli, 2010).

The seven Q1 plasmids identified were consistent with previously reported plasmids with the exception of additional AR genes found on the Q1 plasmids in this study. Q1 plasmids generally have a well-conserved structure with the differences being confined primarily to the AR genes (Loftie-Eaton and Rawlings, 2012). Five of the plasmids contained *tet*AR genes for tetracycline resistance, which are rare in Q1 plasmids, but have been seen in Europe and the United States (Oliva et al., 2017). The plasmids isolated were mostly from swine sources, but were also found in ground beef as well as one unknown source. Five of the Q1 plasmids isolated contained ARC1, which was also present on A/C plasmids. Interestingly, only three Q1 plasmids co-occurred in isolates with potentially conjugative plasmids. Since Q1 plasmids cannot transfer unless another conjugative plasmid is present, this likely indicates that four of the seven Q1 plasmids would be unable to transfer to other bacteria without the acquisition of a conjugative plasmid (Frey et al., 1992).

With the exception of ARC5 which was found only on IncA/C plasmids, all ARCs were present on multiple replicon types, indicating that the prevalence of these ARCs is not due to the expansion of a single clonal plasmid. In the NCBI databases, ARC5 was associated with multiple replicon types and therefore cannot be considered exclusive to the A/C replicon. In the retrospective isolate set, every plasmid-associated *flo*R gene was a part of ARC5. Two additional isolates contained the *flo*R gene but as part of *Salmonella* Genomic Island One (SGI-1) which did not share the ARC structure. ARC1 was the only ARC not associated with a transposase gene, possibly indicating that the MGE structure originally associated with ARC1 has been lost or that the MGE was lost in assembly.

In contrast to the retrospective isolates, the isolates collected by USDA-FSIS can be used to predict the frequency of the ARCs in the *Salmonella* population found currently among food animals over the past 4 years. More than 75% of the isolates containing ARC1 and more than 80% of isolates containing ARC5 were isolated from cattle associated sources. However, only around 40% of the isolates containing ARC4 were associated with cattle despite many of the isolates containing both ARC1 and ARC4 or all three ARCs. A higher percentage of chicken-associated isolates containing ARC4 was responsible for that reduction in percentage, with 37% of ARC4 isolates coming from chicken-associated sources as compared to 2% and almost 4% for ARC 1 and ARC5.

Cattle isolates from USDA-FSIS had a significantly higher chance of containing ARC1, ARC4, and ARC5 than all other sources. This is to be expected, as these three ARCs were associated with A/C plasmids when identified together in the retrospective isolate set. In the USDA-FSIS samples, 12 isolates had ARC1, ARC4, and ARC5 associated with an A/C plasmid. A/C plasmids carrying multiple AR genes have been frequently shown to be associated with isolates from cattle (Carattoli, 2009; Lindsey et al., 2009).

Chicken sources, however, had a significantly higher chance of containing ARC6 than other sources. This could be due to an association with serotype Kentucky, which was the most commonly isolated serotype from the FSIS isolate set. While not significantly more likely to contain the ARC than all the other serotypes, serotype Kentucky did have the third highest frequency of isolates containing the ARC, but was also the most frequently isolated serotype in the isolate set. *Salmonella* Kentucky isolates containing an APEC colV plasmid have been identified that contain ARC6 on that plasmid (Fricke et al., 2009; Johnson et al., 2010).

ARC2 and ARC3 were both detected infrequently in the FSIS isolate set. ARC2 was not found in any isolates from cattle but the 11 isolates were from five different serotypes. In contrast, the 20 isolates containing ARC3 were only comprised of two serotypes, Heidelberg and Mbandaka. Similarly, the majority of isolates in the retrospective isolate set that contained ARC3 were serotype Heidelberg.

The plasmids associated with each ARC in the FSIS sequences were also consistent with those identified in the retrospective isolate set; however, additional plasmid replicon types were associated with the ARCs. ARC1 was associated with A/C and Q1 as in the retrospective isolate set, but was also associated with one I1 plasmid. ARC4 was found on A/C, I1, K, and F plasmids, whereas ARC4 was seen only on A/C and I1 in the retrospective isolates. While only a fraction of the identified ARCs could be associated with a plasmid sequence, this does not mean that the ARCs identified in other isolates were not associated with plasmids. Further characterization of those isolates including assembly of plasmid sequences would be necessary to determine the location of all ARCs. However, the ARCs that were associated with plasmids indicated similarity between the retrospective isolates and the isolates recently collected by FSIS. Whether serotype or source is the correlating factor for plasmids identified cannot be determined without further investigation.

Every ARC identified in this study was also found in other bacteria when compared to the NCBI NR database. While the species represented are limited by what has been sequenced by others, the presence of the ARCs in these organisms indicates that these ARCs are not limited to *Salmonella* and have the ability to persist and confer AR to a diverse group of bacteria belonging to at least two orders, enterobacteriales and vibrionales. ARC1, ARC4, and ARC5 in

particular were identified in A/C plasmids from *E. coli* isolates in a 2011 study by Fernandez-Alarcon et al. (2011). This study also suggested that in A/C plasmids, ARC1 and ARC5 may be adjacent.

In contrast to the retrospective isolate set, some of the ARCs in isolates from NCBI were not plasmid associated, but instead associated with ICEs or incorporated into the chromosome. ARCs were also present in other isolates with varying frequencies. ARC4, ARC5, and ARC6 were found in over 100 isolates, while ARC2 was found in less than 10. While this is similar to what was identified in both the retrospective and FSIS isolates, this may reflect sequencing bias rather than infrequent presence of ARC2 and ARC3.

Overall, the plasmids identified in this study showed diversity, but also showed similarities among replicon types. While the plasmids shared homologous sequence with previously sequenced plasmids, there were also novel sequences. Additional investigation is needed into individual plasmids to further characterize each replicon type. It still remains to be determined why some AR genes were found on some replicon types, but not others, as well as if the plasmids that did not contain AR genes harbored other genes beneficial to the host bacterium. Answering these questions will further advance the knowledge of how AR genes are spreading in *Salmonella* as well as in agricultural environments.

AUTHOR CONTRIBUTIONS

EM, LW, CJ, JW, MS, GT, MM, and JF contributed to the conception and design of the experiments. EM, SG, TJ, LH, TW, JB, JW, and MS contributed to the generation and analysis of data. EM wrote the manuscript. All authors contributed to the revision of the manuscript and approved the submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00832/full#supplementary-material

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Polymorphisms of Gene Cassette Promoters of the Class 1 Integron in Clinical *Proteus* Isolates

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Objective: To describe the polymorphisms of gene cassette promoters of the class 1 integron in clinical *Proteus* isolates and their relationship with antibiotic resistance.

Methods: Polymorphisms of the gene cassette promoter in 153 strains of *Proteus* were analyzed by PCR and nucleotide sequencing. Variable regions of atypical class 1 integrons were detected by inverse PCR and nucleotide sequencing. Enterobacterial repetitive intergenic consensus (ERIC)-PCR was used to analyze the phylogenetic relationships of class 1 integron-positive clinical *Proteus* isolates. Representative beta-lactamase genes (*bla*), including bla_{TEM},bla_{SHV},$bla_{CTX\text{-}M\text{-}1}$,$bla_{CTX\text{-}M\text{-}2}$,$bla_{CTX\text{-}M\text{-}8}$,$bla_{CTX\text{-}M\text{-}9}$,$bla_{CTX\text{-}M\text{-}25}$ and $bla_{OXA\text{-}1}$, and plasmid-mediated quinolone resistance (PMQR) genes including *qnrA, qnrB, qnrC, qnrD, qnrS, oqxA, oqxB, qepA,* and *aac(6′)-Ib* were also screened using PCR and sequence analysis.

Results: Fifteen different gene cassette arrays and 20 different gene cassettes were detected in integron-positive strains. Of them, *aadB-aadA2* (37/96) was the most common gene cassette array. Two of these gene cassette arrays (*estX-psp-aadA2-cmlA1, estX-psp-aadA2-cmlA1-aadA1a-qacI-tnpA-sul3*) have not previously been reported. Three different Pc-P2 variants (PcS, $PcW_{TGN\text{-}10}$, PcH1) were detected among the 96 *Proteus* strains, with PcH1 being the most common (49/96). Strains carrying the promoters PcS or $PcW_{TGN\text{-}10}$ were more resistant to sulfamethoxazole, gentamicin and tobramycin than those carrying PcH1. Strains with weak promoter (PcH1) harbored significantly more intra- and extra-integron antibiotic resistance genes than isolates with strong promoter ($PcW_{TGN\text{-}10}$). Further, among 153 isolates, representative beta-lactamase genes were detected in 70 isolates ($bla_{TEM\text{-}1}$, 54; $bla_{OXA\text{-}1}$, 40; $bla_{CTX\text{-}M\text{-}3}$, 12; $bla_{CTX\text{-}M\text{-}14}$, 12; $bla_{CTX\text{-}M\text{-}65}$, 5; $bla_{CTX\text{-}M\text{-}15}$, 2) and representative PMQR genes were detected in 87 isolates (*qnrA*, 6; *qnrB*, 3; *qnrC*, 5; *qnrD*, 46; *qnrS,* 5; *oqxA*, 7; *aac(6′)-Ib,* 13; *aac(6′)-Ib-cr,* 32).

Conclusion: To the best of our knowledge, this study provides the first evidence for polymorphisms of the class 1 integron variable promoter in clinical *Proteus* isolates,

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which generally contain relatively strong promoters. Resistance genotypes showed a higher coincidence rate with the drug-resistant phenotype in strong-promoter-containing strains, resulting in an ability to confer strong resistance to antibiotics among host bacteria and a relatively limited ability to capture gene cassettes. Moreover, strains with relatively weak integron promoters can "afford" a heavier "extra-integron antibiotic resistance gene load". Furthermore, the gene cassettes *estX*, *psp* and the gene cassette arrays *estX-psp-aadA2-cmlA1, estX-psp-aadA2-cmlA1-aadA1a-qacI-tnpA-sul3* have been confirmed for the first time in clinical *Proteus* isolates. Beta-lactamase genes and PMQR were investigated, and $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ were the most common, with *qnrD* and *aac (6′)-Ib-cr* also being dominant.

Keywords: integron, gene cassettes, promoter, beta-lactamase genes, PMQR

INTRODUCTION

P. mirabilis is an important causative pathogen of various community and healthcare-associated infections, such as wound infections, primary bacteremia, pneumonia and urinary tract infections, particularly among patients with anatomical or functional urinary tract abnormalities or indwelling urinary catheters (Ahn et al., 2017). The incidence of antimicrobial resistance to *P. mirabilis* has increased, and the prevalence of *P. mirabilis* strains producing extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases, carbapenemases or integrons has increased worldwide (Rzeczkowska et al., 2012). However, the impact of these elements in *P. mirabilis* infections on antimicrobial resistance is unclear. The extensive use of antibiotics leads to increased selection pressures, resulting in the emergence of antibiotic-resistant bacterial strains. Integration of exogenous antibiotic resistance genes (Guerin et al., 2009; Grieb et al., 2017) via site-specific recombination is an important pathway in the development of clinical antibiotic-resistant strains. Class 1 integrons are highly mobile and repetitive bacterial elements that integrate foreign gene cassettes and promote the expression of genes in the gene cassettes (Frumerie et al., 2010; Loot et al., 2012; Nivina et al., 2016). In addition, class 1 integrons can be integrated into chromosomes, plasmids, or transposons, carrying resistance genes with them, therefore play an important role in the formation and dissemination of drug-resistant bacterial strains (Collis et al., 2002; Ghazi et al., 2015; Makena et al., 2015; Moyo et al., 2015). The classical structure of class 1 integrons includes an integrase gene *intI1*, a recombination site *attI1*, an integrase gene transcription promoter, a *lex*A-binding site that regulates integrase gene expression, and a variable region gene cassette promoter (Collis et al., 1998, 2002; Collis and Hall, 2004; Demarre et al., 2007).

Gene cassettes in the class 1 integron usually do not include their own promoter, and their transcription depends on the common promoters Pc and P2 (Subedi et al., 2018). Several kinds of Pc variants have been defined in class 1 integrons based on their −35 and −10 hexamer sequences, and the relative strengths of these Pc variant promoters have been verified experimentally. In addition to the Pc promoter, some class 1 integrons also contain a second co-promoter P2, located about 90 bp downstream of Pc, which inserts three G residues between the −35 and −10 hexamer sequences, thus increasing the number of spaced bases to 17 bp, representing an active P2 promoter (Lévesque et al., 1994; Brizio et al., 2006; Papagiannitsis et al., 2009; Vinue et al., 2011; Moura et al., 2012). A recent study reported a new P2 promoter variant, P2m3, with a similar strength to the $\text{PcW}_{\text{TGN-10}}$ variant (Lin et al., 2017). Jove et al. (2010) described variants of various types of Pc promoters, and noted that promoter polymorphisms could result in changes in the amino acid species in the IntI1 sequence, with the magnitude of the change in the excision activity of the mutant integrase being greater than the magnitude of the change in its integration activity. In addition, given identical Pc promoters, the integration efficiency is significantly reduced if the P2 promoter is located before the *attI1* site (Guerin et al., 2011). Guerin et al. (2011) carried out a detailed study of the transcriptional interference relationship between the *intI1* promoter PintI1 and the Pc or Pc-P2 combination and showed that higher gene cassette transcription levels inhibited expression of the integrase in class 1 integrons. The Pc and P2 co-promoter of class 1 integrons therefore not only play an important role in driving the transcription of downstream gene cassettes or gene cassette arrays, but also have a close relationship with the resection and integration phenomena that occur during the capture of exogenous gene cassettes. However, no promoter-related studies of class 1 integrons in clinical isolates of *Proteus* have yet been reported. In this study, we investigated the polymorphisms of the co-promoter of class 1 integrons and their association with the antibiotic resistance phenotype in clinical isolates of *Proteus*.

MATERIALS AND METHODS

Bacterial Strains and Susceptibility Testing

We previously obtained 153 strains of *Proteus* from patient samples from Zhejiang Province (Wei et al., 2014). These clinical isolates included 140 *P. mirabilis* isolates, 12 *Proteus vulgaris* isolates and 1 *Proteus penneri* isolate. Among these, 96 class 1 integron positive strains were studied further. *Escherichia coli* ATCC25922 and *E. coli* DH5α were also maintained in our laboratory. Antibiotic susceptibility was determined by disk diffusion and broth dilution. *E. coli*

ATCC25922 was used as a control strain. The tested antibiotics included: amikacin, gentamicin, tobramycin, sulfamethoxazole, chloramphenicol, Meropenem, Imipenem, Ciprofloxacin, Levofloxacin, Aztreonam, Cefepime, Ceftriaxone, Ceftazidime, Cefotetan, and Cefazolin. The results were interpreted in accordance with the guidelines of the Clinical and Laboratory Standards Institute.

Structural Analysis of Atypical Class 1 Integrons

Bacterial DNA preparation and class 1 integron analysis were conducted and reported as previously (Wei et al., 2014). Variable regions of atypical class 1 integrons that could not be amplified conventionally were detected by inverse PCR analysis of genomic DNA using the primer pairs INTRR and INTRF, followed by verification by electrophoresis and sequencing (**Table 1** and **Figure 1**). For *aac(6′)-Ib* gene positive isolates, the variable regions were also amplified through overlap PCR using the primer pairs intF and aacR, aacF and 3CS. PCR products were analyzed by sequencing. All sequencing results were aligned using the BLAST program[1].

Characterization of Pc and P2 Promoters of Class 1 Integrons

For typical class 1 integrons, the type of promoter upstream of the variable region was identified by direct sequencing. For atypical class 1 integrons, Pc and P2 promoters were identified by sequencing the PCR products amplified using the primer intF combined with specific primers for the downstream gene cassettes. For strains that cannot be successfully amplified using intF and specific primers for the downstream gene cassette, the class 1 integron-mixed common promoter was amplified only by intF and P2R2 primer pairs (some strains may contain multiple integrons). All of them were sequenced using the primer intF after electrophoresis validation, and the variable region promoter type was interpreted based on the sequence.

Polymerase Chain Reaction Detection and Sequencing of Beta-Lactamase Genes

To determine the genotype of beta-lactamase, we performed PCR amplification with bla_{TEM},bla_{SHV},$bla_{\text{CTX-M-1}}$,$bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$,$bla_{\text{CTX-M-9}}$,$bla_{\text{CTX-M-25}}$, and $bla_{\text{OXA-1}}$. Specific primers that were designed to detect beta-lactamase gene markers (**Table 1**) were used to screen for beta-lactamase antibiotic resistance gene in bacterial isolate template DNA. The total volume of the PCR mixture was 20 μl, containing 1 μl of genomic DNA template, 0.4 μl of each primer (10 pmol), 10 μl of Premix-rTaq PCR solution (TaKaRa, Japan), and 7 μl of distilled water. PCR was carried out using an ABI Veriti Thermal Cycler (Applied Biosystems, Singapore). The template was initially denatured at 94°C for 4 min, followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 40 s, with a final extension at 72°C for 5 min. PCR products were verified by electrophoresis

[1]http://www.ncbi.nlm.nih.gov/BLAST

TABLE 1 | Primers used for PCR amplification.

Primer	Primer sequence(5′–3′)	References
intF	CCAAGCTCTCGGGTAACATC	Wei et al., 2011
P2R2	CCCGAGGCATAGACTGTA	Sunde, 2005
ERIC2	AAGTAAGTGACTGGGGTGAGCG	Tsai et al., 2018
3CS	AAGCAGACTTGACCTGA	Sunde, 2005
INTRF	TCGGCCATTCCGACGTCTCTAC	Sunde, 2005
INTRR	TGCAAGTAGCGTATGCGCTC	Sunde, 2005
CMLF	AAACGCGCTTGGTACGACAGC	This study
CMLR	ATTACTTTCCTCGCGACCTGC	This study
AADA2F	CGATGAGCGAAATGTAGTG	This study
AADA2R	AAGACGGGCTGATACTGG	This study
ESTXF	AGGTCAGGCTCCATATTCC	This study
ESTXR	TGAATGTTGTCAGGATATTC	This study
QACF	TTGGTGAGGTCGTCGCAAC	This study
QACR	TGCGCTGACCTTGGATAGC	This study
SUL3F	GAGCAAGATTTTTGGAATCG	This study
PSPF	TCGATGGCACAATTACCAC	This study
QD14R1	CCTGAGCGGGTAACGAC	This study
IS26R	TTGCGTAGTGCACGCATCACC	This study
CMLF2	TAGGTTTGGGCATGATC	This study
TEMF	TCGGGGAAATGTGCG	Velasova et al., 2019
TEMR	TGCTTAATCAGTGAGGCACC	Velasova et al., 2019
SHVF	GCCTTTATCGGCCTTCACTCAAG	Velasova et al., 2019
SHVR	TTAGCGTTGCCAGTGCTCGATCA	Velasova et al., 2019
CTX-M-1F	CAGAGATTTTGCCGTCTAAG	Velasova et al., 2019
CTX-M-1R	GGCCCATGGTTAAAAAATCACTGC	Velasova et al., 2019
CTX-M-2F	CTCAGAGCATTCGCCGCTCA	Velasova et al., 2019
CTX-M-2R	CCGCCGCAGCCAGAATATCC	Velasova et al., 2019
CTX-M-8F	ACTTCAGCCACACGGATTCA	Velasova et al., 2019
CTX-M-8R	CGAGTACGTCACGACGACTT	Velasova et al., 2019
CTX-M-9F	GTTACAGCCCTTCGGCGATGATTC	Velasova et al., 2019
CTX-M-9R	GCGCATGGTGACAAAGAGAGTGCAA	Velasova et al., 2019
CTX-M-25F	GCACGATGACATTCGGG	Velasova et al., 2019
CTX-M-25R	AACCCACGATGTGGGTAGC	Velasova et al., 2019
OXA-1-F	GGCACCAGATTCAACTTTCAAG	Che et al., 2014
OXA-1-R	GACCCCAAGTTTCCTGTAAGTG	Che et al., 2014
qnrAF	AGAGGATTTCTCACGCCAGG	Kim et al., 2016
qnrAR	GCAGCACTATKACTCCCAAGG	Kim et al., 2016
qnrBF	GGMATHGAAATTCGCCACTG	Kim et al., 2016
qnrBR	TTTGCYGYYCGCCAGTCGAA	Kim et al., 2016
qnrCF	GGGTTGTACATTTATTGAATC	Kim et al., 2016
qnrCR	TCCACTTTACGAGGTTCT	Kim et al., 2016
qnrDF	CGAGATCAATTTACGGGGAATA	Kim et al., 2016
qnrDR	AACAAGCTGAAGCGCCTG	Kim et al., 2016
qnrSF	GCAAGTTCATTGAACAGGCT	Kim et al., 2016
qnrSR	TCTAAACCGTCGAGTTCGGCG	Kim et al., 2016
oqxAF	GACAGCGTCGCACAGAATG	Wong et al., 2014
oqxAR	GGAGACGAGGTTGGTATGGA	Wong et al., 2014
oqxBF	CGAAGAAAGACCTCCCTACCC	Kim et al., 2016
oqxBR	CGCCGCCAATGAGATACA	Kim et al., 2016
qepAF	CTGCAGGTACTGCGTCATG	Wong et al., 2014
qepAR	CGTGTTGCTGGAGTTCTTC	Wong et al., 2014
aacF	ATCTCATATCGTCGAGTGG	This study
aacR	TGCGTGTTCGCTCGAATGC	This study

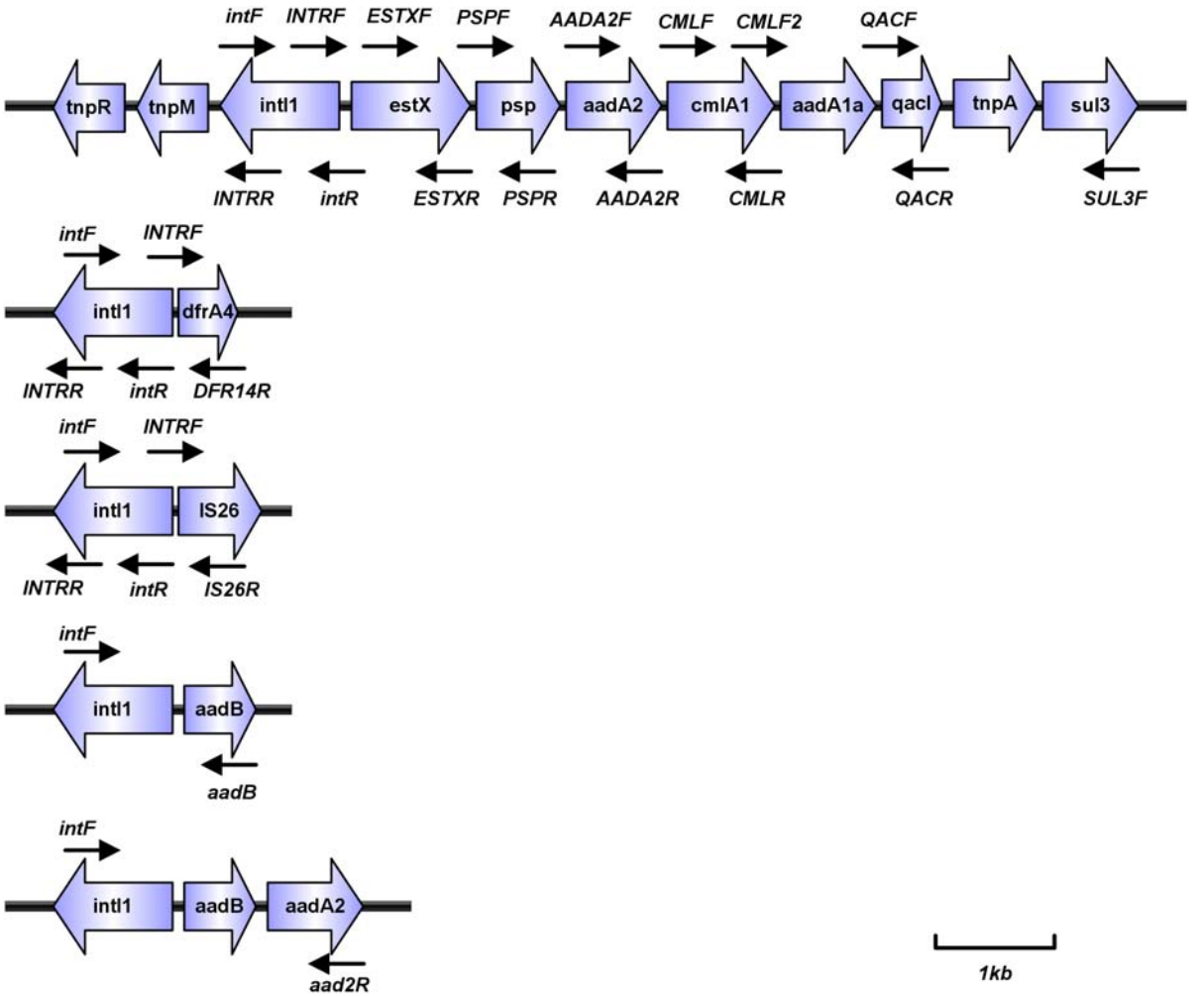

FIGURE 1 | PCR scheme (thin black arrows indicate the position of primer; thick blue arrows represent different genes).

and sequencing (**Table 1**). All sequencing results were aligned using the BLAST program.

Multiplex PCR Detection of Plasmid-Mediated Quinolone Resistance Genes

To determine the genotype of plasmid-mediated fluoroquinolone resistance genes, we performed PCR amplification with the *qnrA* (length = 619 bp), *qnrB* (length = 264 bp), *qnrC* (length = 447 bp), *qnrD* (length = 582 bp), *qnrS* (length = 428 bp), *oqxA* (length = 339bp), *oqxB* (length = 240 bp), *qepA* (length = 403 bp), and *aac(6′)-Ib*, *qnrA*/*qnrB*/*qnrC* as the first multiplex PCR amplification system, and *qnrD*/*qnrS*/*oqxA*/*oqxB* as the second multiplex PCR amplification system. *qepA* and *aac(6′)-Ib* were separately amplified. Specific primers which were designed for fluoroquinolone resistance maker (**Table 1**) were used to screen for antibiotic resistance genes in bacterial isolate template DNA. PCR amplification components and cycling conditions were identical to those used for the detection of BLA antibiotic resistance genes described above, followed by verification by electrophoresis. All *aac(6′)-Ib* positive products were then sequenced (**Table 1**). All sequencing results were aligned using the Vector NTI Advance 11 (Invitrogen, United States).

Determination of Phylogenetic Groups of *Proteus*

We analyzed the phylogenetic population of the 96 integron-positive *Proteus* strains based on the Enterobacterial repetitive intergenic consensus (ERIC)-PCR method (Wilson and Sharp, 2006). Phylogenetic groups of *Proteus* strains were determined according to the electrophoresis patterns of the PCR product by NTSYSpc 2.1e software (clustering program).

Statistical Analysis

All statistical analyses were performed using SPSS software, version 22.0. To compare the two groups, the Student's *t*-test or Mann-Whitney *u*-test, depending on the validity of the normality assumption, was used for continuous variables. The chi-squared

test or Fisher's exact test was used to assess categorical variables. Values of $p < 0.01$ were considered to indicate significance.

RESULTS

Antimicrobial Susceptibility

In this study, 153 strains of *Proteus* were isolated mainly from patients in the Internal Medicine surgery ward [53.6% (82/153)], the ICU [37.3% (57/153)] and the Outpatient clinic [9.1% (14/153)]. The cohort of 153 patients had a mean age of 67.2 years, which a range of 5–91. 104 (68.0%) patients were over 60 years old. The main sources of *Proteus* were from genital secretions [17.6% (27/153)], urine [41.2% (63/153)], sputa [32.7% (50/153)], hydrothorax and ascite [5.9% (9/153)], and blood [2.6% (4/153)].

The *in vitro* antimicrobial susceptibilities of the *Proteus* isolates showed that most isolates were susceptible to Imipenem (60%), Meropenem (55.6%), Ciprofloxacin (40.5%), Levofloxacin (52.3%), Cefepime (63.4%), Ceftriaxone (58.8%), Ceftazidime (58.2%), Cefazolin (41.8%), Aztreonam (79.1%), Amikacin (81.7%), Gentamicin (47.1%), Tobramycin (45.6%), Sulfamethoxazole (43.1%), and Chloramphenicol (61.4%). Moreover, all of the isolates were susceptible to Piperacillin/Tazobactam and Cefotetan.

Characterization of Gene Cassettes and Arrays

Of 96 class 1 integrin-positive strains, 70 variable regions of typical integrons were previously detected in *Proteus* strains (Wei et al., 2013). Variable regions in 26 atypical class 1 integrons were analyzed using inverse PCR. For *aac(6′)-Ib* gene positive isolates, the variable regions were amplified through overlap PCR. A total of 15 different types of variable region gene cassette arrays and 20 different gene cassettes were detected. These gene cassette arrays were divided into types A–K, of which type K included K1 and K2 (**Figure 2**). The most common antibiotic resistance gene cassettes were *aadA2* (72/96), *aadB* (38/96), and *aadA1a* (22/96), all of which conferred resistance to aminoglycoside antibiotics. Five trimethoprim-resistance gene cassettes [*dfrA17* (17/96), *dfrA12* (6/96), *dfrA32* (4/96), *dfrA1* (2/96), *dfrA14* (1/96)] conferred resistance to trimethoprim antibiotics; in addition, we also found *aac(6′)-Ib* gene cassettes (16/96) in the integron variable region, and a chloramphenicol-resistance gene cassette *cmlA1* (2/96). The gene cassette arrays were partly detected in strain NO.47685 (*IS26*) and strain NO.50772 (*dfrA14*), but variable regions were not detected in strain NO.45016 (**Table 2**). The most common gene cassette arrays were *aadB-aadA2*, *estX-psp-aadA2-cmlA1-aadA1a-qacI-tnpA-sul3*, and *dfrA17-aadA5*, which were detected in 37, 22, and 17 isolates, respectively.

Class 1 Integron Promoter Variants

We analyzed the promoters of class 1 integrons. All bacterial strains are shown in **Table 2**. Three common types of promoters were detected among the 96 clinical isolates of integron-positive *Proteus* strains. The most common promoter was PcH1, which was a relatively weak promoter occurring in 51% (49/96) of class 1 integron-positive strains (Wei et al., 2011), while PcS was the second most prevalent promoter, present in 41.6% (40/96), and the PcW_{TGN-10} was detected in only 7.3% (7/96) of class 1 integron-positive strains. An inactive P2 promoter unable to drive the expression of downstream gene cassettes was detected in all class 1 integron-positive strains.

Regarding the relationship between gene cassettes or gene cassette arrays and specific common promoters, PcH1 could drive the expression of *estX-psp-aadA2-cmlA1-aadA1a-qacI-tnpA-sul3*, *dfrA17-aadA5*, *dfrA32-ereA-aadA2*, and *estX-psp-aadA2-cmlA1* gene cassette arrays, PcS could drive *aadB-aadA2*, *aadB*, and *aadA2* gene cassette arrays, and PcW_{TGN-10} could drive the expression of *dfrA1-orfC* and *aacA4-cmlA1* gene cassette arrays. In addition, all three types of promoters (PcS, PcH1, and PcW_{TGN-10}) could drive the expression of the gene cassette array *dfrA12-orfF-aadA2*.

Associations Between Common Promoter Variants and Phylogenetic Groups of *Proteus*

We analyzed the phylogenetic relationships between clinical isolates of *Proteus*. We divided the 96 clinical isolates of class 1 integron-positive *Proteus* into seven groups (a1, a2, b, c1, c2, d1, and d2) according to the ERIC-PCR results. Among these, two strains belonged to group a1 [PcW_{TGN-10} (2/2)], 39 to group a2 [PcS (39/39)], 24 to group b [PcH1 (20/24), PcW_{TGN-10} (3/24), PcS (1/24)], six to group c1 [PcH1 (4/6), PcW_{TGN-10} (2/6)], one to group c2 (PcH1), 23 to group d1 [PcH1 (23/23)], and one to group d2 (PcH1) (**Figure 3**). The a1, a2, and d1 groups each included a single promoter type. The c2 (strain NO.45016) and d2 groups (strain NO.47685) each included only one strain, among which the integron variable region of 45016 could not be detected and the integron variable region of 47685 was an insertion sequence (*IS26*), which was different from that of other strains.

Relationships Between *Proteus* Pc and Pc-P2 Promoters and Resistance Phenotype

We tested the 96 class 1 integron-positive *Proteus* strains for antibiotic susceptibility, to clarify the relationship between the integron variable region promoter and the antibiotic-resistance phenotype in clinical isolates. Integron-positive strains containing relatively strong promoters had higher resistance rates to amikacin, gentamicin, and tobramycin, but low resistance to chloramphenicol (**Table 3**). There was no significant difference in sulfamethoxazole and chloramphenicol resistance rates between strains with relatively strong and weak promoters. However, strains with strong promoters still had higher MIC_{50} values for chloramphenicol than strains with weak promoters. We performed a more detailed analysis of the promoters and antibiotic-resistance phenotypes in the seven strains of bacteria with strong promoters (PcW_{TGN-10}) and showed that resistance phenotype was associated with the presence of a strong promoter (PcW_{TGN-10}), while

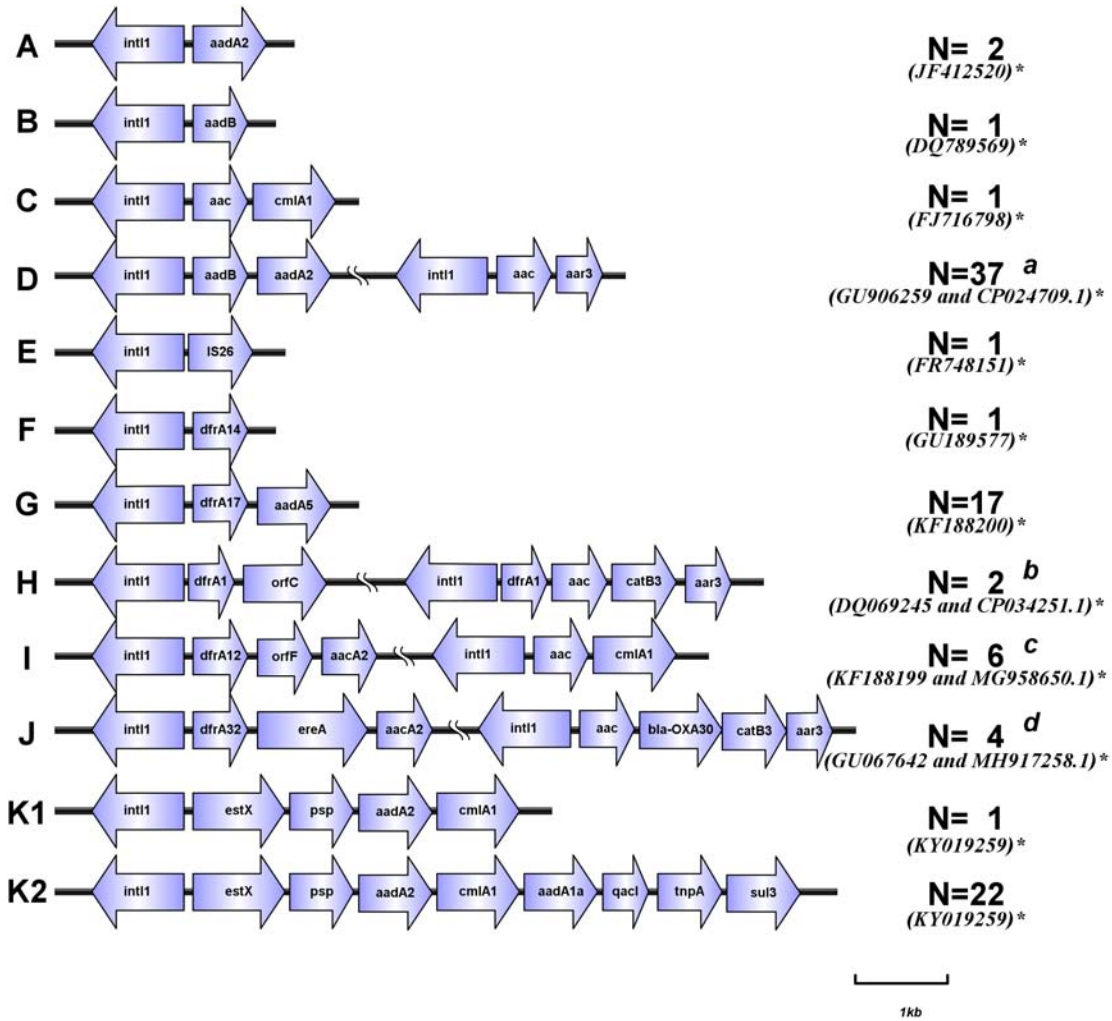

FIGURE 2 | Schematic representation of different types of gene cassette arrays (*aac* equal to *aac(6′)-Ib* or *aac(6′)-Ib-cr*). *Accession numbers in Genbank. [a]Among the 37 isolates, 25 of the integron variable regions were *aadB-aadA2*, and the other 12 were *aadB-aadA2* and *aac(6′)-Ib-aar3*. [b]Among the 2 isolates, 1 of the integron variable regions were *dfrA1-orfC*, and the other 1 was *dfrA1-orfC* and *dfrA1- aac(6′)-Ib -catB3-aar3*. [c]Among the 6 isolates, 5 of the integron variable regions were *dfrA12-orfF-aacA2*, and the other 1 was *dfrA12-orfF-aacA2* and *aac(6′)-Ib -cmlA1*, the second array (*aac(6′)-Ib -cmlA1*) is the same as C(*aac(6′)-Ib -cmlA1*). [d]Among the 4 isolates, 3 of the integron variable regions were *dfrA32-ereA-aacA2*, and the other 1 was *dfrA32-ereA-aacA2* and *aac(6′)-Ib -bla*$_{OXA-1}$*-catB3-aar3*. (Sequences of PCR products were analyzed with BLAST to identify target homologous sequences and their GenBank accession numbers. https://blast.ncbi.nlm.nih.gov/Blast.cgi).

this phenomenon was not observed in other promoter types (**Figure 4**).

Genotypes of Beta-Lactamase Genes

Among the beta-lactamase producing strains, we found 55 isolates that were positive for *bla*$_{TEM}$, 15 isolates positive for the *bla*$_{CTX-M-1}$ group, 17 isolates positive for the *bla*$_{CTX-M-9}$ group and 40 isolates positive for the *bla*$_{OXA-1}$ group. Using nucleotide sequence analysis, we found that 55 *bla*$_{TEM}$ positive isolates carried *bla*$_{TEM-1}$. Of 15 *bla*$_{CTX-M-1}$ group positive isolates, 12 had *bla*$_{CTX-M-3}$ and 3 carried *bla*$_{CTX-M-15}$. Meanwhile, of 17 *bla*$_{CTX-M-9}$ group positive isolates, 12 had *bla*$_{CTX-M-14}$ and 5 had *bla*$_{CTX-M-65}$. All 40 *bla*$_{OXA-1}$ group positive isolates were found to carry *bla*$_{OXA-1}$. Meanwhile, all 153 isolates were negative for *bla*$_{SHV}$, *bla*$_{CTX-M-2}$ group, *bla*$_{CTX-M-8}$ group and *bla*$_{CTX-M-25}$ group. Statistical analysis of the drug-sensitive phenotypes of the beta-lactamase positive and negative-positive groups revealed that the beta-lactamase positive group was significantly less sensitive to Ceftriaxone (35.7% vs. 77.1%, $p < 0.01$), Ceftazidime (31.4% vs. 80.7%, $p < 0.01$), Cefazolin (38.6% vs. 84.3%, $p < 0.01$), Imipenem (37.1% vs. 79.51%, $p < 0.01$), and Meropenem (35.71% vs. 72.3%, $p < 0.01$) than the beta-lactamase negative group (**Table 4**).

Plasmid-Mediated Quinolone Resistance Gene

Among 153 *Proteus* samples, we found 6 isolates positive for *qnrA*, 3 isolates positive for *qnrB*, 5 isolates positive for

TABLE 2 | Gene cassette arrays and their common promoters in 96(class 1 integrons)*Proteus* strains.

Gene cassette array	Type promoter			Total
	PcH1	PcW_{TGN-10}	PcS	
*aadA2**			2	2
*aadB**		1		1
*aac(6′)-Ib -cmlA1**		1		1
*aadB-aadA2**			37	37
IS26[a]	1			1
dfrA14[a]	1			1
*dfrA17-aadA5**	17			17
*dfrA1-orfC**		2		2
*dfrA12-orfF-aadA2**	2	3	1	6
*dfrA32-ereA-aadA2**	4			4
estX-psp-aadA2-cmlA1	1			1
estX-psp-aadA2-cmlA1-aadA1a-qacI-tnpA-sul3	22			22
Unknown[b]	1			1
aac(6′)-Ib -aar3			12	12
aac(6′)-Ib -bla_{OXA-1}-catB3-aar3	1			1
dfrA1- aac(6′)-Ib -catB3-aar3		1		1
Total	50	8	52	110[c]

[a]*Class 1 integrons for which only partial sequences of gene cassette arrays were amplified (IS26 detected in NO.47685 strain, dfrA14 detected in NO.45016 strain).* [b]*Class 1 integrons for which PCR failed to amplify the gene cassette array.* [c]*A total of 110 integrants were detected from 96 integron positive strains.* **The gene cassette combination that our research group has previously reported.*

qnrC, 46 isolates positive for *qnrD*, 5 isolates positive for *qnrS*, 7 isolates positive for *oqxA* and 45 isolates positive for *aac (6′)-Ib*, while all 153 isolates were negative for *oqxB* and *qepA*. All *aac (6′)-Ib* positive products were detected using nucleotide sequence analysis, and we found two types of the *aac (6′)-Ib* gene, which were *aac (6′)-Ib* (13/45) *and aac (6′)-Ib-cr* (32/45). Statistical analysis of the drug-sensitive phenotypes of the PMQR positive and negative groups showed that the PMQR positive group was significantly less sensitive to Ciprofloxacin (21.8% vs. 65.2%, $p < 0.01$) and Levofloxacin (33.3% vs. 77.3%, $p < 0.01$) than the PMQR negative group (**Table 4**).

Relationships Between Various Promoters and Antibiotic Resistance Gene Load

We compared the antibiotic resistance gene load of different promoters of 96 integron positive strains. We found that the relatively weak promoter (PcH1) strains carried 6.88 resistance genes on average, of which 5.35 resistance genes were located in the integrons, and there were 1.53 resistance genes not located on the integrons (including: 1.12 beta-lactamase genes, 0.41 PMQR). The relatively strong promoter (PcW_{TGN-10} and PcS) strains carried 3.57 and 3.88 resistance genes on average, respectively. Simultaneously, on average, 2.57 and 2.55 antibiotic resistant genes were located on integrons, while 1 (including: 0.85 beta-lactamase genes, 0.15 PMQR) and 1.3 (including: 0.9 beta-lactamase genes, 0.4 PMQR) antibiotic-resistant genes were not located on integrons, respectively (**Table 5**).

DISCUSSION

Integrons are genetic elements with a specific functional configuration that have evolved in bacteria and which can capture and express exogenous gene cassettes via site-specific recombination. In this study, 96 strains containing class 1 integrons were detected among 153 clinical isolates of *Proteus*, indicating that this evolutionary platform is common among clinical strains. Additionally, we detected 20 different gene cassettes, most of which conferred resistance to antibiotics. Antibiotics such as trimethoprim, chloramphenicol, and erythromycin were discovered in the early and mid-20th century and are now used extensively in clinical applications. However, during the process of bacterial evolution, antibiotic resistance gene cassettes have spread throughout clinical strains due to integration subsystems and high selection pressure imposed by the combined action of a large number of antibiotics, allowing the survival of bacteria carrying the appropriate antibiotic-resistance genes.

In contrast to previous research on Pc promoter polymorphisms in *E. coli* (Wei et al., 2013), the three promoters identified in the current study were relatively strong promoters (PcS, PcW_{TGN-10}, and PcH1), with the stronger promoters (PcS, PcW_{TGN-10}) accounting for 49% of all integron-positive strains. The variety of integron variable region gene cassettes was also shown to be more complicated, with *estX* and *psp* being detected for the first time in clinical isolates of *Proteus*. Integrons usually spread between strains with the help of plasmids or transposons. Additionally, we detected the same array of gene cassettes in different phylogenetic groups of clinical isolates of *Proteus*, and the upstream promoters also remained stable. This may be due to the class 1 integrons being embedded in larger transposons or plasmids, or may be recombined in a conserved region of the class 1 integron 5CS, such that the gene cassette array is combined with the same promoter.

This article reveals that strains with strong promoters have higher rates of antibiotic resistance than strains with weaker promoters, especially in amikacin, gentamicin, and tobramycin. This may be explained by the presence of a strong promoter in the variable region of the class 1 integron causing high expression of the relevant antibiotic-resistant genes. Interestingly, the antibiotic-resistant genotypes and phenotypes were highly matched among the seven strains with the strong promoter PcW_{TGN-10}, while strains containing other types of promoters do not show this phenomenon. In the phylogenetic analysis (**Figure 3**), we found that these 7 strains clearly belong to different colony

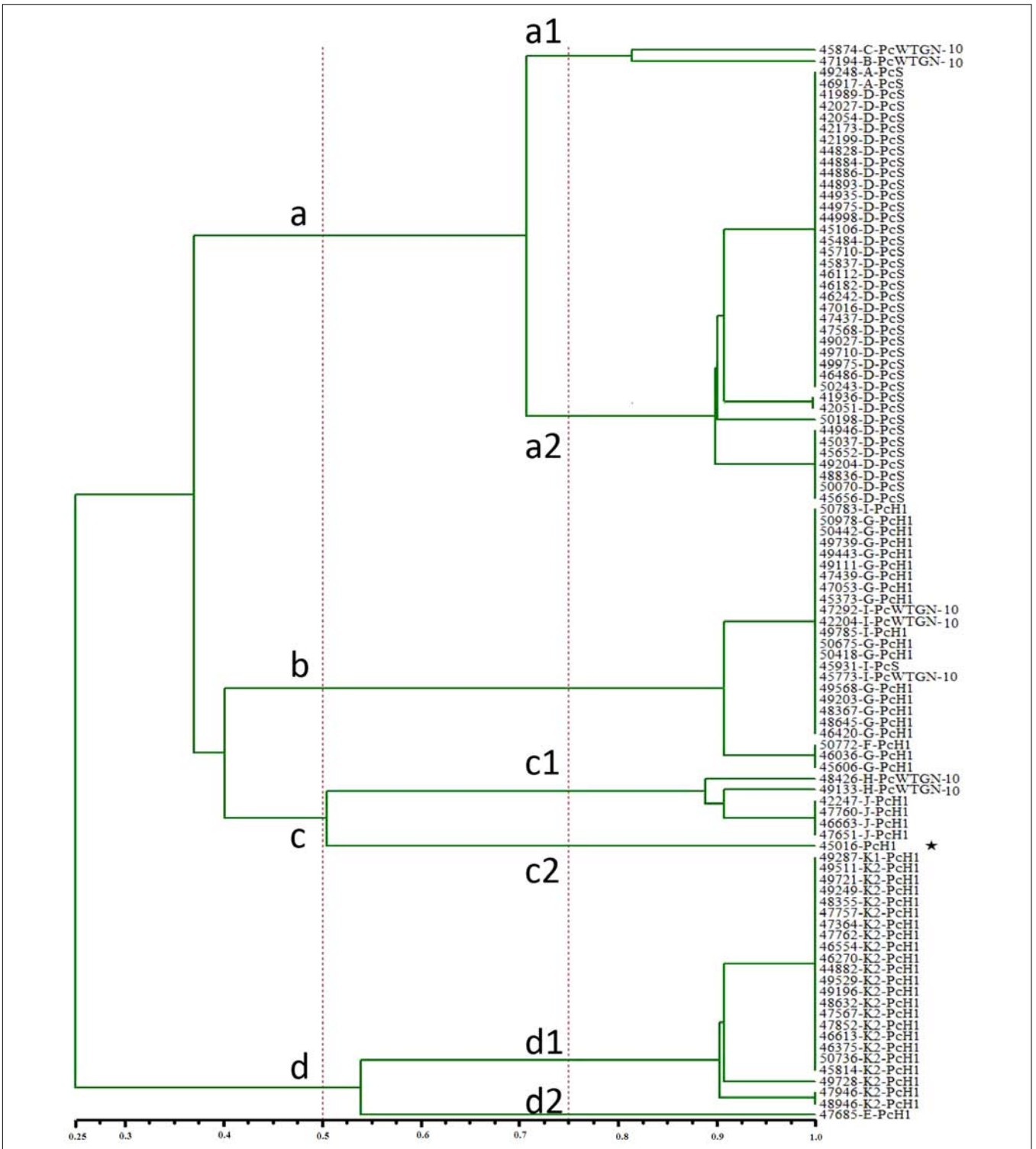

FIGURE 3 | Cluster analysis of 96 strains of *Proteus* based on ERIC-PCR. (50% homology gathers as a main class, and 75% homology gathers into a subclass, ★Class 1 integrons for which PCR failed to amplify the gene cassette array).

groups. In summary, antibiotic genes are located close to the promoter, making it relatively easy for the promoter to regulate their expression. However, the current results were only relevant to the individual strains studied, and clinical strains with different genetic backgrounds may present more complex phenomena.

TABLE 3 | Associations of promoter variants with antibiotic-resistance phenotypes.

Promoter	Total no. of isolates	No. (%) of isolates with resistance to[a]				
		AMK	GEN	TOB	SXT	CHL
Total	96	12 (12.5)	51 (53.1)	39 (40.6)	69 (71.9)	39 (40.6)
Strong promoter	47	12 (25.5)	38 (82.8)	37 (78.7)	37 (78.7)	17 (36.2)
PcW_{TGN-10}	7	1 (14.3)	5 (71.4)	5 (71.4)	6 (85.7)	1 (14.3)
PcS	40	11 (27.5)	33 (85.2)	32 (80)	31 (77.5)	16 (40)
MIC_{50} (μg/ml)		≤ 2	≥ 16	≥16	≥ 16	≥64
Weak promoter	49	0 (0)	13 (26.5)	2 (4.1)	32 (65.3)	22 (44.9)
PcH1	49	0 (0)	13 (26.5)	2 (4.1)	32 (65.3)	22 (44.9)
MIC_{50} (μg/ml)		–	≤ 4	≤4	≥ 16	16
p-value[b]		0.000	0.000	0.000	0.144	0.384

[a]*AMK amikacin, GEN gentamicin, TOB tobramycin, SXT Trimethoprim/Sulfamethoxazole, CHL chloramphenicol* [b]*The chi-squared test was used to assess strong promoter and weak promoters.*

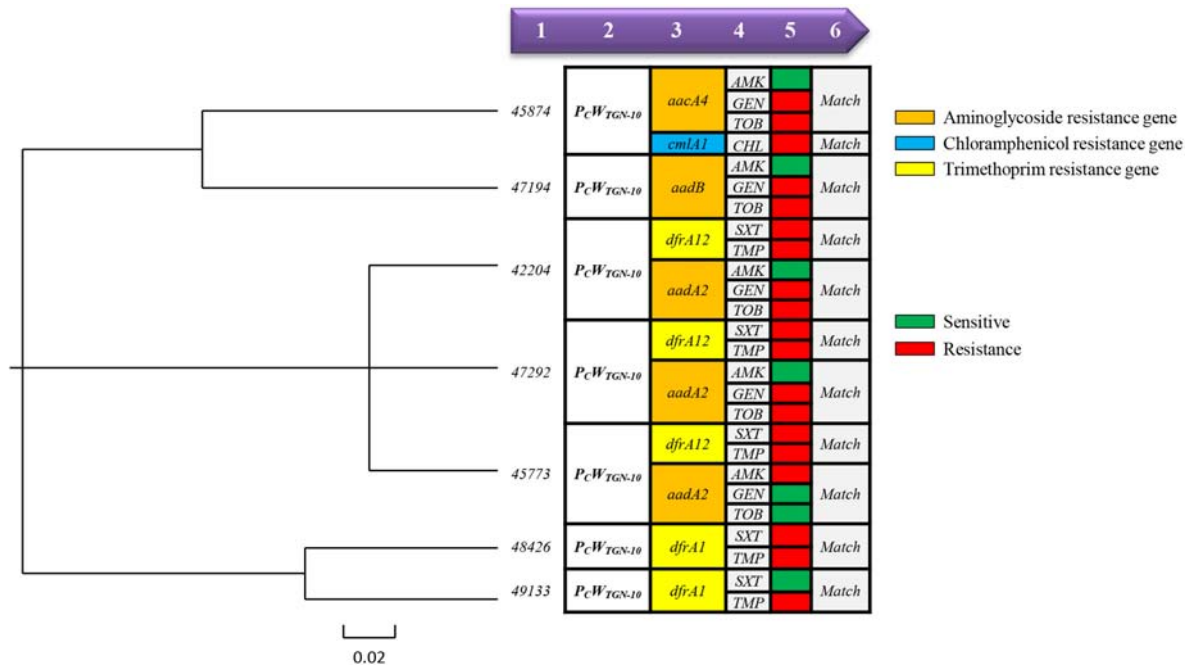

FIGURE 4 | Relationships between resistance gene and resistance phenotype in *Proteus* strains with strong promoters. (On the left side of this image is a phylogenetic tree based on the DNA sequence of each strain promoter using Vector NTI Advance 11 software, while the right side of this image shows the relationship between the variable region genotype and the resistant phenotype of each strain.) [1]Strain number. [2]Promoter type. [3]Integron variable region genotype (aminoglycoside resistance gene shown in orange, chloramphenicol resistance gene shown in blue and trimethoprim resistance gene shown in yellow). [4and5]Phenotype (sensitive shown in green, resistance shown in red). [6]Indicates whether the genotype matches the resistant phenotype (at least one genotype corresponds to a resistant phenotype and is considered to be a "*Match*").

In this article, we elucidated the relationship between beta-lactamase genes and integrons that were carried in strains. Therefore, we screened the beta-lactamase resistance gene of 153 *Proteus* isolates, and found that the positive rate reached 45.8%, which was significantly higher than previous reports (Ahn et al., 2017). A crucial argument shown by the statistical results is that there is a significant difference ($p < 0.01$) in the difference in drug resistance gene carrying between beta-lactamase genes and integrons in *Proteus* strains (**Table 6**). As a result, we studied their impact on antibiotic resistance and attempted to explain the association between the antibiotic resistance genes carried by these strains and the integron promoter. Moreover, we found that beta-lactamase genes were significantly more detectable in ICUs and surgical wards than in other wards, as most ICU patients had severe disease, reduced immunity, and long-term use of antibiotics, all of which helped improve detection rate. For patients undergoing urologic surgery, the higher detection rate is related to its own physiological

TABLE 4 | *In vitro* antimicrobial susceptibility of *bla* and PMQR.

Antimicrobial agent	No. (%) of susceptible			No. (%) of susceptible		
	***bla* positive**	***bla* negative**	***p*-value**	**PMQR positive**	**PMQR negative**	***p*-value**
	***N* = 70**	***N* = 83**		***N* = 87**	***N* = 66**	
Imipenem	26 (37.1)	66 (79.5)	0.000	52 (59.8)	40 (60.6)	0.917
Meropenem	25 (35.7)	60 (72.3)	0.000	55 (63.2)	40 (60.6)	0.742
Ciprofloxacin	22 (31.4)	40 (48.2)	0.035	19 (21.8)	43 (65.2)	0.000
Levofloxacin	33 (47.1)	47 (56.6)	0.242	29 (33.3)	51 (77.3)	0.000
Cefepime	27 (38.6)	70 (84.3)	0.000	57 (65.5)	40 (60.6)	0.532
Ceftriaxone	25 (35.7)	64 (77.1)	0.000	57 (65.5)	32 (48.5)	0.034
Ceftazidime	22 (31.4)	67 (80.7)	0.000	60 (68.9)	29 (43.9)	0.002
Cefazolin	20 (28.5)	44 (53.0)	0.002	36 (41.4)	28 (42.4)	0.897
Aztreonam	51 (72.8)	70 (84.3)	0.082	73 (83.9)	48 (72.7)	0.092
Amikacin	54 (77.1)	71 (85.5)	0.181	78 (89.7)	47 (71.2)	0.003
Gentamicin	28 (40.0)	44 (53.0)	0.108	39 (44.8)	33 (50.0)	0.526
Tobramycin	28 (40.0)	42 (50.6)	0.190	39 (44.8)	31 (47.0)	0.792
Sulfamethoxazole	18 (25.7)	48 (57.8)	0.000	30 (34.5)	36 (54.5)	0.013
Chloramphenicol	41 (58.6)	53 (63.9)	0.504	47 (54.0)	47 (71.2)	0.031
Piperacillin/Tazobactam	70 (100)	83 (100)	–	87 (100)	66 (100)	–
Cefotetan	70 (100)	83 (100)	–	87 (100)	66 (100)	–

TABLE 5 | Associations of promoter variants with gene load.

Promoter		Total no. of isolates	No. of antibiotic resistance genes				
			No. of genes	**Located on integrons**	**Not located on integrons**	***bla***	**PMQR**
"Strong" promoter	PcW_{TGN-10}	7	3.57[a]	2.57[a]	1[a]	0.85[a]	0.15[a]
	PcS	40	3.85[a]	2.55[a]	1.3[a]	0.9[a]	0.4[a]
"Weak" promoter	PcH1	49	6.88[a]	5.35[a]	1.53[a]	1.12[a]	0.41[a]

[a]*Average value.*

TABLE 6 | The relationship between carriage of integron and *bla*, PMQR.

Genotypes	No.(%) of carried		*p*-value
	Integron positive	**Integron negative**	
	***N* = 96**	***N* = 57**	
bla	57	13	0.000
PMQR	48	39	0.026

structural characteristics, one of which is mainly urinary tract obstruction, which is conducive to bacterial reproduction, in addition to urinary catheterization, further increasing the chance of infection. Furthermore, we found that most beta-lactamase producing strains occur in the elderly or women. Among the strains studied, we did not find other significant differences in gene carriers. This may be due to the low immunity of the elderly and the vulnerability of the female urethra to infection, so that some strains or resistance genes can be transmitted horizontally.

TEM is the main type of β-lactamases, and the TEM-1 group is the most common. The CTX-M enzyme is a new group of plasmid-mediated beta-lactamase genes that have dominated in Europe, and have increased dramatically in many countries over the past decade (Mohd et al., 2019). Antibiotic consumption and different risk factors may also contribute to the current epidemiology of CTX-M enzymes in different geographic regions. In recent years, China has also presented an increasing trend, and there are few reports of beta-lactamase genes in *Proteus* isolated from Chinese hospitals. Interestingly, our research found that bla_{TEM-1}, bla_{OXA-1}, and $bla_{CTX-M-14}$ were carried in the same strains, and they are resistant to third-generation cephalosporin, which may be synergy between them, increasing the ability of bacteria to hydrolyze cephalosporin. Drug susceptibility test data showed that *Proteus* producing beta-lactamase genes was significantly less sensitive to most third-generation cephalosporins (**Table 4**). If the patient is infected by a beta-lactamase producer, Cefotetan, Cefmetazole or Imipenem may be preferred prior to the results of the antibiotic susceptibility test, but if the patient is in a critical state, we should choose carbapenem antibiotics. These findings lead us to conclude that we should pay attention to the use of antibiotics in outpatient, inpatient and community hospitals, and reduce the chance of dissemination of β-lactamase gene levels due to antibiotic selection pressure.

The PMQR genes discovered in recent years, such as *qnrA, qnrB, qnrC, qnrD, qnrS, aac (6′)-Ib-cr,* and *qepA* resistance genes, are an important mechanism for bacteria to resist quinolone. In this study, we explored the relationship between quinolone resistance genes and integrons in *Proteus*, and we also screened quinolone resistance genes in 153 *Proteus* isolates, with a positive rate of up to 56.9%, mainly carrying *qnrD* and *aac(6′)-Ib*. Notably, *aac(6′)-Ib* is not resistant to quinolones, only variant *aac(6′)-Ib-cr* is resistant to quinolone. Among them, we studied *aac(6′)-Ib* in depth. The nucleic acid sequence of *aac(6′)-Ib* was found to contain Asp181Tyr (G541T) and Trp104Arg (T310C or T310A) in 32 strains of *aac(6′)-Ib* (Hidalgo-Grass and Strahilevitz, 2010). The variant *aac(6′)-Ib-cr* can confer bacterial resistance to Ciprofloxacin or Levofloxacin. In general, *aac(6′)-Ib* is mainly located in integrons and spreads horizontally with the spread of integrons. In this study, only 16 strains of *aac(6′)-Ib* were located in integrons (*aac(6′)-Ib-aar3*,12; *aac(6′)-Ib-bla*$_{OXA-1}$*-catB3-aar3*,1; *aac(6′)-Ib-cmlA1*,1; *dfrA1-aac(6′)-Ib-catB3-aar3*,1; *aac(6′)-Ib -cmlA1*,1), and all *aac(6′)-Ib-cr* variants were located on the integrons. However, *aac(6′)-Ib*, which cannot confer PMQR, was carried by another 29 strains and may be located on other mobile elements, such as transposons or insertion sequences, although its specific mechanism of action needs further study. In the end, the experimental results were contrary to our hypothesis. There was no statistically significant difference in the quinolone resistance gene and integron carrying in the *Proteus* strains ($p > 0.01$) (**Table 6**).

In this study, multiple resistance genes were detected in isolates, and we also compared the antibiotic resistance "gene load" of strains with different promoters. As such, it further explains the fitness of the clinical bacteria. These results demonstrate that strains with relatively weak integron promoters can "afford" a heavier intra- and extra-integron antibiotic resistance gene load. Although many antibiotic resistance genes are not in the integrons, such as *bla* and PMQR, and are not directly related to the integron promoter, only a few representative *bla* and PMQR genes were investigated in this study, which have certain limitations. However, the drug resistance genes detected in this experiment also illustrates the principle of "gene load." Some studies have shown that the "super-integration antibiotic resistance gene load" may affect the fitness of pathogens, which is consistent with our research conclusions (Guo et al., 2012; Darmency et al., 2015).

CONCLUSION

In conclusion, to the best of our knowledge, this study provides the first evidence for polymorphisms within the variable region promoter of class 1 integrons in clinical Proteus isolates. The results indicated that the gene cassette in the integron in Proteus strains confers antibiotic resistance to aminoglycosides, trimethoprim, and chloramphenicol. Class 1 integron-positive Proteus strains generally have strong promoters, and strains with strong promoters are more resistant to amikacin, gentamicin, and tobramycin than strains with weaker promoters, strains with relatively weak integron promoters can "afford" a heavier intra- and extra-integron antibiotic resistance gene load. Importantly, this study also provides the first evidence for the gene cassettes estX and psp in clinical isolates of Proteus. In addition, beta-lactamase genes and PMQR are widely prevalent in clinical isolates of Proteus, mainly blaTEM-1, blaOXA-1 and qnrD and aac (6′)-Ib-cr. Interestingly, it was also found that in Proteus aac(6′)-Ib-cr may be located on transposons, insertion sequences or other mobile genetic elements rather than on integrons, suggesting multiple pathways in its dissemination.

AUTHOR CONTRIBUTIONS

LX and QW conceived the study. WL coordinated the study. XW, NK, MC, and LZ performed the experiments. LX and QW analyzed the data and wrote the manuscript. QW and WL revised the manuscript.

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Microbial Diversity and Antimicrobial Resistance Profile in Microbiota From Soils of Conventional and Organic Farming Systems

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Soil is one of the biggest reservoirs of microbial diversity, yet the processes that define the community dynamics are not fully understood. Apart from soil management being vital for agricultural purposes, it is also considered a favorable environment for the evolution and development of antimicrobial resistance, which is due to its high complexity and ongoing competition between the microorganisms. Different approaches to agricultural production might have specific outcomes for soil microbial community composition and antibiotic resistance phenotype. Therefore in this study we aimed to compare the soil microbiota and its resistome in conventional and organic farming systems that are continually influenced by the different treatment (inorganic fertilizers and pesticides vs. organic manure and no chemical pest management). The comparison of the soil microbial communities revealed no major differences among the main phyla of bacteria between the two farming styles with similar soil structure and pH. Only small differences between the lower taxa could be observed indicating that the soil community is stable, with minor shifts in composition being able to handle the different styles of treatment and fertilization. It is still unclear what level of intensity can change microbial composition but current conventional farming in Central Europe demonstrates acceptable level of intensity for soil bacterial communities. When the resistome of the soils was assessed by screening the total soil DNA for clinically relevant and soil-derived antibiotic resistance genes, a low variety of resistance determinants was detected (resistance to β-lactams, aminoglycosides, tetracycline, erythromycin, and rifampicin) with no clear preference for the soil farming type. The same soil samples were also used to isolate antibiotic resistant cultivable bacteria, which were predominated by highly resistant isolates of *Pseudomonas, Stenotrophomonas, Sphingobacterium* and *Chryseobacterium* genera. The resistance of these isolates was largely dependent on the efflux mechanisms, the soil *Pseudomonas* spp. relying mostly on RND, while *Stenotrophomonas* spp. and *Chryseobacterium* spp. on RND and ABC transporters.

Keywords: organic and conventional farming, soil microbiota, antibiotic susceptibility, resistance genes, efflux pumps

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INTRODUCTION

Microbiota of the soil is greatly important for life on our planet, including its role in the cycling of carbon, nitrogen and other nutrients (Jansson and Hofmockel, 2018). Bacteria and other soil microorganisms are the agents of biotransformation of soil organic matter and nutrients and of most key soil processes. Their activities are influenced by both soil physico-chemical processes and ecological interactions (Powlson et al., 2001). As a habitat for microorganisms, soil is a very diverse and complex substrate on the planet. Conventional approaches based on isolation of the cultivable microbes and techniques based on the analysis of the total DNA in the soil show an enormous diversity in the microorganism composition (Torsvik et al., 1990). Culture-based methods suggest that a gram of soil contains for about one hundred species of microorganisms (Dunbar et al., 1999), but such data are underestimated because multiple lines of evidence indicate that fewer than 1% of the species in soil are presently cultivable (Amann et al., 1995). DNA based methods revealed that soils typically contain 10^9 to 10^{10} microorganisms per gram, which may represent thousands of bacterial species (Gans et al., 2005). Therefore, metagenomic and other next-generation sequencing based studies might be very useful for the studying the soil microbiome for understanding soil microbial functioning (Baveye, 2009; Raynaud and Nunan, 2014; Mandal et al., 2015).

Soil serves a range of different functions and it is the basis for forestry and agriculture and the importance of this role to be expected to increase (Fischer and Heilig, 1997). Although it is important to keep the soil microbiome stable, agricultural intensification carries dangers including the possibility of damaging soil functions. Latest studies have shown that anthropogenic activities, such as intensification of agriculture and land use change, reduce bacterial numbers and the overall diversity of soil microorganisms. During the past years studies had largely focused on the effects of specific microbial groups, such as fungi, soil bacteria and soil fauna. However, interactions of soil organisms are very complex and therefore changes in diversity within one trophic group or functional guild may alter the diversity, prevalence and functioning of another (Wagg et al., 2014).

Antimicrobial resistance is one of the biggest problems in human and animal medicine at present. Since a high percentage of antibiotics are discharged from the human or animal body without degradation, this means that different habitats, from the human body to river water or soils, are polluted with antibiotics (Martínez, 2017). Antibiotics from treatment of farm animals can accumulate in the farm sludge, which is afterward spread as a fertilizer on the farmland (Larsson, 2014), however, there is limited knowledge of antimicrobial concentrations that might exert selection for resistant bacteria in the environment (Bengtsson-Palme and Larsson, 2016). The concentrations of antibiotics in soils usually are low in most ecosystems, but even low concentrations may trigger specific bacterial responses, and analysis of such responses is a topic of interest (Martínez, 2017). Even though the usage of antibiotics is considered one of the most important risk for the development of antimicrobial resistance, the emergence of the resistance in clinical environment can also be based on the theory about a pre-existing pool of antibiotic resistance genes in natural environmental reservoirs and a transferability of these genes (Nesme and Simonet, 2015).

The aims of this study were twofold: (1) to investigate and compare microbiomes in soils of organic and conventional farming systems and (2) to analyze antimicrobial resistance profiles in soil microbiota.

MATERIALS AND METHODS

Soil Selection and Sampling

The soil samples were collected from six farming fields in Lithuania (located at the borderline of the zones Dfb and Cfb according to the Köppen climatic zones (Peel et al., 2007) during the year 2016. The six collection points of the soil represented two different types of farming, organic and conventional (intensive), and three agrocultures grown in the field during the year of collection (winter wheat, rapeseed, maize). The organic farming sites were known not to use inorganic fertilizers or pesticides for the time period of over 20 years and were fertilized only with organic fertilizers (farmyard manure and slurry). The conventional farming fields were fertilized with inorganic NPK fertilizers (3–4 times a year) and the cultures were regularly sprayed with herbicides, insecticides and fungicides. The pairs (organic and conventional) of farming soil samples were collected from two winter wheat fields, located 1.8 km apart (coordinates: 54.925416, 24.464575 and 54.933504, 24.488816) in October 2016; two rapeseed fields, located 17 km apart (54.921779, 24.463984 and 54.807963, 24.640339) and two maize fields, located 2.3 km apart (55.423267, 24.166897 and 55.41869, 24.202844) in December 2016. The type of the soil in the winter wheat and rapeseed fields was sandy loam whereas in the maize fields – sandy clay loam. In each field, samples were collected from 10 places all over the plot area from the depth of 20 cm using tubular soil sampler. Samples then were placed into sterile plastic bags and delivered to the laboratory during the time of 2 h, where the material was pooled and mixed. The samples were kept at +2°C until the next day for the cultivation of bacteria or aliquoted and frozen at −80°C for the DNA extraction.

DNA Extraction

For microbial community analysis total DNA was extracted using Quick-DNA Fecal/Soil Microbe kit (Zymo Research, United States) according to the manufacturer's instructions. For resistance gene detection by PCR total soil DNA was extracted by FastDNA^{TM} SPIN Kit for Soil (MP Biomedicals, United States), which was then additionally purified as described elsewhere (Young et al., 1993). DNA material for identification of species of cultivable soil bacteria and determination of antimicrobial resistance genes was obtained after bacterial lysis according to the extraction protocol prepared by the EU Community Reference Laboratory for Antimicrobial Resistance with modifications as described previously (Ruzauskas et al., 2014).

Soil Microbial Community and Data Analysis

Metagenomic sequencing of 16S rRNA and microbial profiling analysis was performed as described previously (Merkeviciene et al., 2017). Alpha diversity indexes were calculated with EstimateS (v. 8.2). The prevalence of separate taxonomic units of bacteria in soils of organic and conventional farming was given as the percentage from the total number of DNA reads. The differences among the prevalence of bacteria of the most abundant taxonomic units in organic and conventional soils were compared using Fisher's Exact Test for Count Data. Comparison of the taxonomic distribution of resistant isolates from organic and conventional farming was assessed using Fisher's Exact Test for Count Data. Statistical analysis was performed using IBM SPSS Statistics 20 package. Results were considered statistically significant if $p < 0.05$.

Selection of Resistant Isolates

For the isolation of antibiotic resistant bacteria the soil samples were suspended in water (1:2) and inoculated onto solid media Tryptone Soy Agar (Thermo Scientific, United Kingdom) supplemented with the following antimicrobial agents: ciprofloxacin, gentamicin, imipenem, trimethoprim, ceftazidime, and chloramphenicol. Only a single antibiotic was used per plate. As there are no clinical breakpoints set for most of the soil bacteria, the concentrations of antimicrobials in media were used as clinical breakpoints set by EUCAST for *Pseudomonas*, *Acinetobacter*, and *Enterobacteriaceae* for isolation and selection of Gram-negative bacteria as well as for *Enterococcus* in case of Gram-positive microbiota. The concentrations of antibiotics in media for resistance screening were as follows: ciprofloxacin – 2 μg/mL for gram-negatives and 8 μg/mL for gram-positives; gentamicin – 8 μg/mL; imipenem – 16 μg/mL; trimethoprim – 8 μg/mL; ceftazidime – 16 μg/mL and chloramphenicol, which breakpoint was taken from CLSI standard – 32 μg/mL. Plates were incubated for 72 h at + 22°C. After incubation, separate predominant colonies were selected for further purification to obtain pure cultures of different bacterial species from each soil sample.

Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was performed on selected isolates by broth micro-dilution method suing Sensititre®plates and the ARIS 2X automated system (Thermo Scientific, United States). Interpretation of results was carried-out using manufacturers software (SWIN®). The minimum inhibitory concentrations (MIC) of tested antibiotics are presented in **Supplementary Table S1**.

Identification of the Isolated Soil Bacteria

Identification of bacteria isolates was based on 16S rRNA fragment sequencing. For this purpose PCR using universal primers 27F and 515R (**Supplementary Table S2**) was performed as described previously (Kim et al., 2012) using DNA extracted from bacteria isolates. PCR products then were purified using DNA Clean and Concentrator-5 Kit (D4010, Zymo Research, United States) and identification of the isolates was performed after sequencing and analysis using Molecular Evolutionary Genetic Analysis software (MEGA, version 6). Basic local alignment search tool (BLAST) was used for comparison of obtained sequences with sequences in the database of National Center for Biotechnology Information (NCBI, United States). Species were identified by matching obtained sequences with a sequence showing the highest maximum identity score from the GenBank database. If the identity of the best match was < 99% and query cover < 96% only genus was assigned.

Antibiotic Resistance Gene Detection

The presence of genes encoding antibiotic resistance determinants was assessed by PCR at the same conditions as described earlier (Seputiene et al., 2012). Two sets of genes were screened in this study: the first set included clinically relevant ARGs, that have been previously shown to be important in the antibiotic resistance of pathogenic bacteria (the genes tested and specific primers used are described in **Supplementary Table S2**).

The other set comprised ARGs, naturally occurring in soil bacteria and chosen for analysis (**Supplementary Table S2**) based on their reported occurrence in metagenomes of soil samples obtained from different geographical locations (Allen et al., 2009; Torres-Cortés et al., 2011; McGarvey et al., 2012; Wichmann et al., 2014) and on the abundance in different species (presence in minimum three different species, non-identical hits) according to the BLAST (NCBI BLASTN, Bacteria domain, Nucleotide collection (nr/nt)) search. Of 149 ARGs analyzed bioinformatically, 10 mostly widespread genes were selected for further analysis (**Figure 2**). Primers for amplification of their DNA were designed by the alignment of homologous sequences of different species using Clustal Omega and identification of the conservative regions. To expand the sensitivity of detection, degenerative primers were designed (**Supplementary Table S2**).

A PCR amplifying 16S rDNA fragment (primers Frrs/Rrrs) was used in parallel as amplification control.

The Efflux Pump Activity Detection

To elucidate the contribution of multidrug resistance efflux pumps to bacteria antibiotic resistance, synergistic assays with antibiotics and specific efflux pump inhibitors were performed. First, the MICs of antibiotics and inhibitors was accessed by Broth microdilution method (Wiegand et al., 2008) for each isolate tested. Then MIC of the antibiotic was evaluated with 1/2 of inhibitor MIC present in the mix. Microtiter plates were incubated at 28°C for 19 h.

RESULTS

Composition of Bacterial Community in Organic and Conventional Farming Soil

The organic and conventional winter wheat fields, located 1.8 km apart, were chosen for the analysis. Both soils had neutral pH (7.08 and 6.58 for organic and conventional farming), humus

content of 2.8 and 1.5%, and amounts of phosphorus (P_2O_5) of 320 mg/kg and 130 mg/kg in organic conventional farming soils, respectively. Total DNA was extracted from both soils and used for 16S rRNA gene sequencing in order to analyze the microbial community composition. In total 93,212 and 192,939 sequences were obtained, with Good's coverage indexes of 0.995 and 0.998, indicating that sufficient number of reads was obtained to evaluate the bacterial diversity for the both respective soils. Alpha diversity of the samples was: Shannon index 5.87 and 6.07, and Chao1 2364.04 and 2735.3 for organic and conventional wheat field soil, respectively.

The 97 and 98 % of sequences were identified as DNA belonging to kingdom *Bacteria* in both samples, respectively. The relative abundance of the main bacterial phyla (comprising > 1% of reads) is presented in **Figure 1** (all the species detected are presented in **Supplementary Data Sheets S1, S2**). The predominant phylum in the soil samples from of organic and conventional wheat field was *Proteobacteria* (30–33%), followed by *Actinobacteria* (22–17%), *Acidobacteria* (11–9%), *Firmicutes* (8–10%) and *Bacteroidetes* (7–10%), respectively. No obvious differences could be detected among the main phyla.

Distributions of the most prevalent genera (with prevalence above 0.5 % from the total bacteria) in the soils of organic and conventional farming sites are presented in **Supplementary Table S3**. Although *Acidobacterium* and *Bacillus* statistically significantly were the most predominant genera ($p < 0.001$), their prevalence in general was under 5 % from a total population of microbiota in both soils. As could be seen from the **Supplementary Table S3**, the same genera were most prevalent in both soils and had only limited amount of difference in organic and conventional soils. The highest statistically reliable differences were among *Bacillus*, *Gemmatimonas* which prevalence was higher in the conventional soil as well as between *Holophaga*, *Acidobacteriaceae*, *Hyphomicrobium*, *Flavobacterium* and *Nocardioides* which were more abundant in the organic soil ($p < 0.05$).

As an increase in the relative abundance of phylum *Actinobacteria* could be observed in the organic wheat soil, we therefore checked which of the lower taxa were contributing most to the change. The more abundant (over 1% relative abundance) orders of *Actinobacteria*, *Rubrobacterales* (with the most abundant family *Gaiellaceae*), *Acidimicrobiales* (family *Acidimicrobiaceae*) and *Solirubrobacterales* (family *Conexibacteraceae*) constituted 5.83% in organic farming soil, which was two-fold higher than in conventional soil. The more abundant genera (**Supplementary Table S3**) in the organic farming soil that were overrepresented comparing to conventional farming soil were also mostly of phylum *Actinobacteria* (genera *Gaiella*, *Ilumatobacter*, *Iamia*), but also *Holophaga* of phylum *Acidobacteria* was also abundant. In conventional farming soil an increase in the abundance of order *Sphingobacteriales* (with the most abundant family *Sphingobacteriaceae*) was observed. Several genera were also more abundant, *Rhodanobacter* was only detected in conventional soil, while genera *Rhizobium*, *Agrobacterium*, *Devosia* (phylum *Alphaproteobacteria*) and genus *Paenibacillus* (phylum *Firmicutes*) were more abundant in the conventional farming soil.

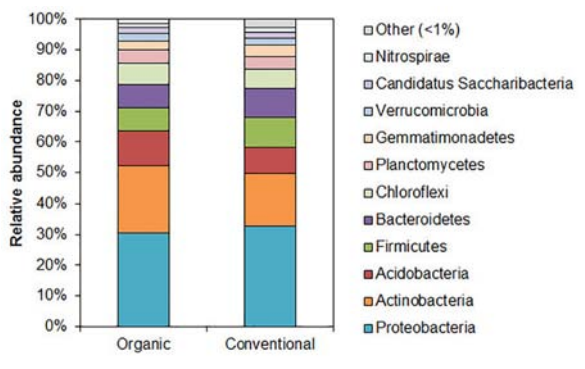

FIGURE 1 | Relative abundance of bacterial phyla in organic and conventional wheat farming soils. Bacterial community composition determined using 16S rRNA sequencing-based analysis. Only the phyla that were present in relative abundance of > 1% are indicated.

Detection of Antibiotic Resistance Genes (ARGs) in the Soil DNA

The differences in the microbial community composition of the two farming type soils were observed only between the smaller taxa. The overall composition was comparable between the tested soils, as well as similar to the composition of various soils around the world (Fierer et al., 2009). However, we were interested if the prevalence of ARGs in the soils of different farming systems differed. Genes, commonly found in the clinically important bacteria and conferring resistance to the different classes of antibiotics used in the human and veterinary medicine, were included in the study. In addition, ARGs, naturally found in the soil bacteria and conferring resistance to β-lactams, aminoglycosides, tetracycline and rifampicin were screened.

The total DNA was purified from the six soils of organic and conventional farming type, as described in "Materials and Methods." Winter wheat soils, described previously, were used and in addition organic and conventional pairs of rapeseed and maize soils were selected. The measured pH of the soils was 7.16 and 7.95 for rapeseed, and 8.15 and 7.81 for respective farming types of maize. The purified DNA was used for PCR with the gene-specific primers listed in the **Supplementary Table S2**. Primers targeting soil bacteria-specific resistance genes were designed as described in "Materials and Methods." The gene screen identified the extended spectrum β-lactamase (ESBL) coding gene *shv* in the organic farming rapeseed field soil (**Figure 2**). No other clinically relevant β-lactamase coding genes were observed. From the genes of known clinical relevance, only those coding for aminoglycoside modifying enzymes were found. The *ant*(6)*I*, *ant*(3″)*Ia* and *ant*(3″)*Ib*, genes, coding for streptomycin modifying nucleotidyltransferases and conferring streptomycin resistance (Vakulenko et al., 2003) were detected in the organic farming wheat field soil. The *ant*(3″)*Ib* gene was also found in a soil DNA from conventional farming field, together with the *ermC* gene coding for rRNA methylase

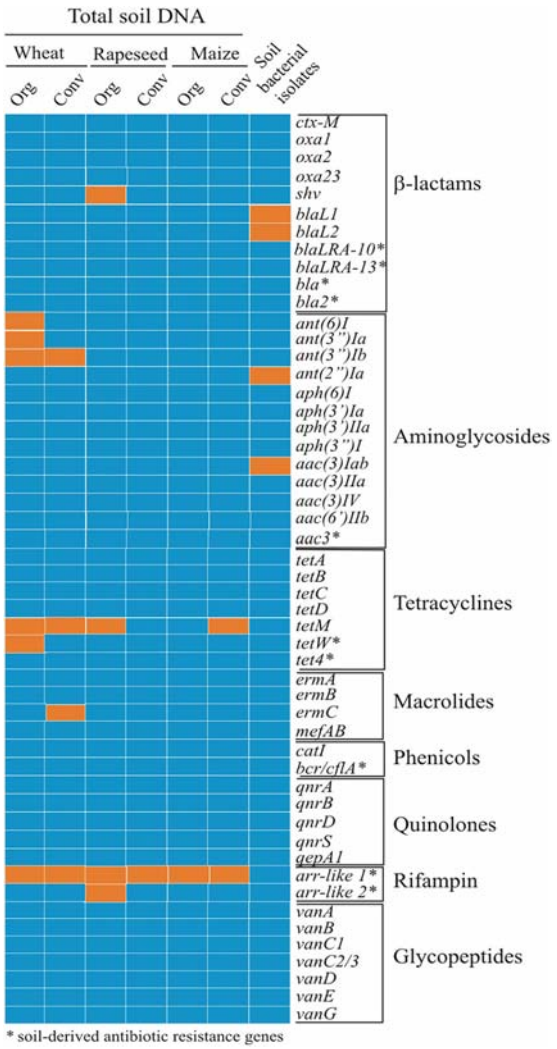

FIGURE 2 | The resistance genes in the total DNA and in bacterial isolates obtained from organic and conventional farming soils. The names of genes tested are listed on the right in groups regarding their mediated resistance to specific antibiotic class. Total DNA from soils of organic (Org) and conventional (Conv) farming sites and cultivable bacterial isolates (shown in a single column) were screened. Orange panel denotes gene present; blue panel – gene not detected. Resistance genes identified in soil bacteria were found as follows: blaL1–in a single S. *maltophilia* isolate from the soil of conventional rapeseed farming; blaL2–in four S. *maltophilia* isolates from the same field; ant(2′′)Ia – in four *Pseudomonas* spp. (one organic wheat and organic rapeseed soil and two from conventional maize soil) and one Sphingobacter sp. isolate (conventional rapeseed soil); aac(3)Iab – in five *Pseudomonas* spp. from organic maize field.

conferring erythromycin resistance. Tetracycline resistance gene *tetM* encoding ribosome protection protein (Burdett et al., 1982) was more common and found in the soil of four fields out of six tested (**Figure 2**).

In the next series of the soil resistance gene screen, we targeted the genes, which were previously detected by screening the metagenomic libraries constructed using DNA from a broad range of geographic locations and several types of environmental sources (soil and manure). The 10 selected genes (**Figure 2**) coded for the proteins of five families, including aminoglycoside acetyltransferases, β-lactamases, rifampin ADP-ribosyltransferases, transporters of tetracyclines and chloramphenicol. Aminoglycoside 3-N-acetyltransferase coding gene *aac3* (resistance to gentamicin), β-lactamase gene *bla* (resistance to ampicillin) and *bcr/cfl* gene coding efflux pump (resistance to chloramphenicol), were obtained from metagenomics libraries from agricultural soils from Spain (Torres-Cortés et al., 2011). Two *arr*-like genes (named here *arr*-like 1 and *arr*-like 2) coding for rifampin ADP-ribosyltransferase variants(rifampin resistance) showing highest similarity to the homologs from *Oscillatoria* sp. isolate and *tet4*gene (tetracycline resistance), coding for ABC transporter with the highest similarity to a homolog from *Paenibacillus curdlanolyticus* were identified in metagenomic libraries of soil from urban environment in Seattle, United States (McGarvey et al., 2012). Screening of the metagenomic libraries from a dairy cow manure (United States) (Wichmann et al., 2014) revealed *bla2* gene (resistance to carbenicillin) showing high sequence identity to a β-lactamase previously found only in *Firmicutes*. Ribosome modifying *tetW* gene demonstrated resistance to tetracycline and had homologs in both *Firmicutes* and *Actinobacteria*. And finally, functional metagenominc library from DNA extracted from the remote Alaskan soil (Allen et al., 2009) discovered $bla_{\mathrm{LRA-10}}$ and $bla_{\mathrm{LRA-13}}$ genes, which demonstrated highest homology to a class C β-lactamases from *Mycobacterium smegmatis* and *Shewanella baltica*, respectively.

Our PCR screening of this gene set in DNA from all soils identified *arr*-like gene variant 1, coding for rifampin-modifying ADP-ribosyltransferase and conferring resistance to rifampicin. Other above listed genes were not detected with the exception of another *arr*-like gene variant 2 and *tetW* gene in single soil (**Figure 2**).

The Abundance of Antibiotic Resistant Species in the Soils

To further access the prevalence of the antibiotic resistance in bacteria from soils of organic and conventional farming, we have isolated cultivable resistant bacteria as described in section "Materials and Methods." In total 151 isolates were recovered from the six soils. The majority of the isolates in all the soils belonged to the genus *Pseudomonas* (n = 79). Other more abundant genera included *Stenotrophomonas* (n = 13), *Bacillus* (n = 13), *Sphingobacterium* (n = 9) and *Cryseobacterium* (n = 8) (**Figure 3A**).

The MIC values were calculated as described in section "Materials and Methods." The isolate was designated as resistant if MIC value matched EUCAST clinical breakpoints (v. 7.0, 2017) for the bacteria belonging to *Pseudomonas*, *Acinetobacter* genera and *Enterobacteriaceae*. If the breakpoints were not available, the PK/PD (non-species related) breakpoints were assigned. The majority of the strains showed resistance to more than one antibiotic tested or even to several antibiotic classes. We calculated the average number of antibiotics, to which isolates recovered from the each soil, were resistant (**Figure 3B**). The

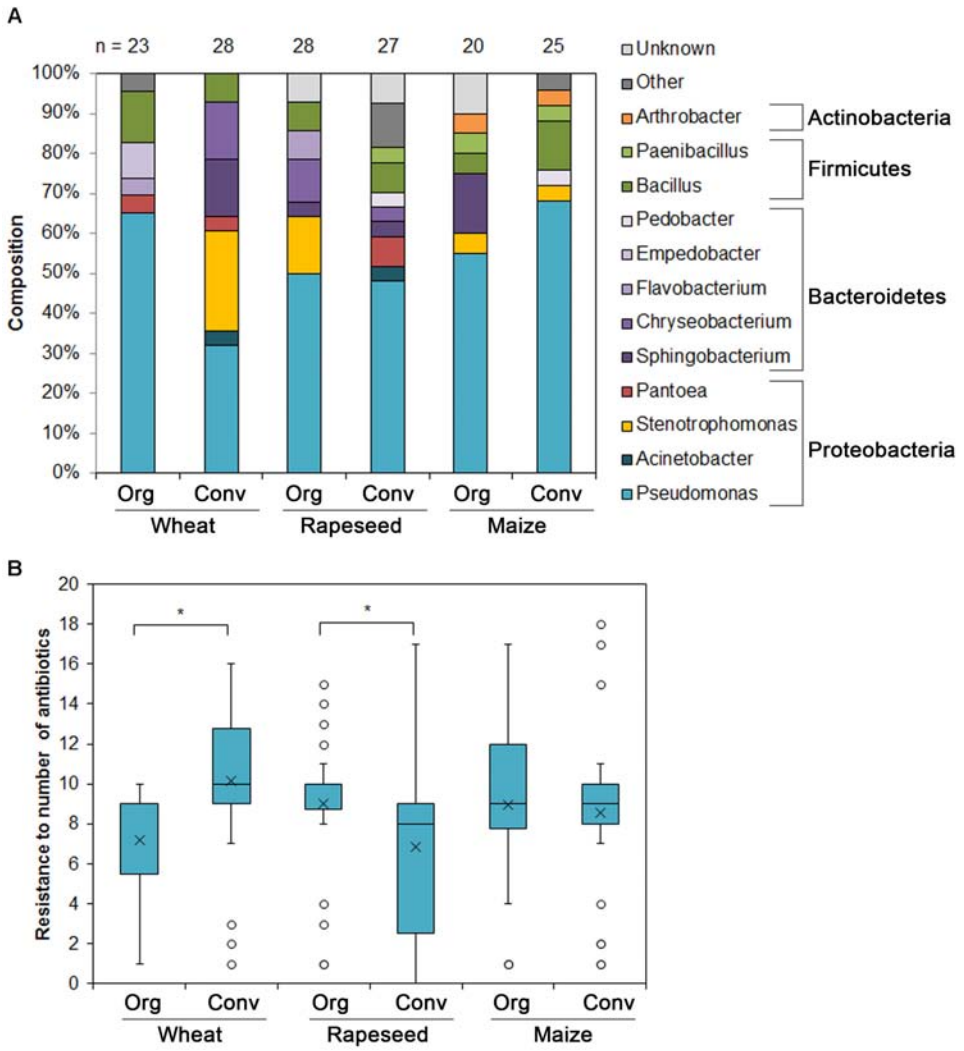

FIGURE 3 | Cultivable antibiotic resistant soil bacteria isolated from six soils under different farming styles. **(A)** The abundance of resistant bacterial genera isolated from soils under different farming styles. **(B)** The abundance of antibiotic resistant bacteria isolated from various soils. Bacteria were designated resistant if the MIC values met with EUCAST clinical breakpoints. Boxes indicate upper and lower quartiles, whiskers indicate minimum and maximum values excluding outliers, circles depict outliers and crosses indicate mean values. *Indicates statistical significance calculated as non-parametric Mann-Whitney test for two independent samples ($p < 0.05$; one-tailed).

bacteria from the conventional farming wheat field soil were more antibiotic resistant compared with those recovered from the organic farming site and the difference was significant. On the contrary, the bacteria isolated from the rapeseed field soil of organic farming were more antibiotic resistant compared with those recovered from the soil of conventional farming site. The differences between the soils where maize was cultivated were not significant.

Detection of Clinically Relevant ARGs in Cultivable Bacteria

The resistant isolates were screened by PCR for the presence of clinically relevant ARGs. The results in **Figure 2** show that only genes responsible for aminoglycoside resistance were found. Interestingly, *aac(3)Iab* gene, coding for the member of N-acethyltransferase superfamily, was found in five *Pseudomonas* sp. isolates, all derived from ecological maize field soil. Different MIC profiles indicated they are not the same strain. The other aminoglycoside resistance gene *ant(2")Ia*, coding for aminoglycoside O-nucleotidyltransferase, commonly encoded in transposons and plasmids (Vakulenko and Mobashery, 2003), was found in four *Pseudomonas* sp. and one *Sphingobacterium* isolate from soils of various origins (**Figure 2**). The aminoglycoside resistance genes observed in isolated bacteria differed from the ones found in total soil DNA. We also checked for species specific *Stenotrophomonas maltophilia* gene bla_{L1} coding for metallo-β-lactamase and the gene bla_{L2}, coding for serine-β-lactamase (Flores-Treviño et al., 2014) in isolates identified as the latter species (n = **6**) (**Supplementary Table S1**). The bla_{L2} gene was present in

four *S. maltophilia* isolates, all recovered from intensive wheat farming soil; one of the four also had bla_{L1} gene. None of cultivable bacterial isolates contained naturally occurring antibiotic resistance-related genes (**Figure 2**).

Resistance Due to Efflux Pumps

Our observation, that most abundant groups of soil bacterial isolates, exhibiting a high antibiotic resistance, carried rather a limited number of genes coding for modifying enzyme-based resistance mechanisms, prompted as to test the impact of efflux pumps (EPs) on the resistance displayed by these isolate groups. Most research has been focused upon *P. aeruginosa* and resistance nodulation-cell division (RND) superfamily exporters, which play the major role in the drug expulsion (Li X.Z. et al., 2015). As the majority of cultivable antibiotic resistant isolates from the soil in this study were of the genus *Pseudomonas*, we firstly investigated the impact of RND EPs.

Twenty four *Pseudomonas* spp. isolates from wheat farming soils were examined for the resistance to chloramphenicol, which is known as a substrate of RND EP (Li X.Z. et al., 2015). To access the influence of EPs we have used specific inhibitors and examined their impact on the antibiotic MIC value as described in "Materials and Methods." The phenylalanine-arginine-β-naphthylamide (PAβN) it is most active and best studied inhibitor of RND EPs (Rampioni et al., 2017).

The initial chloramphenicol MIC varied between 0.5 and 32 μg/ml, and the difference of MIC values between the *Pseudomonas* spp. isolates of different soil origin was not statistically significant (data not shown). However, all the isolates tested showed drastic reduction of resistance to chloramphenicol after addition of PAβN, the average MIC reduction being 89 % (the least reduction of MIC was 50%, while the highest −99%), indicating the major role of RND EPs (**Figure 4A**). We then checked how the initial resistance is related to the RND activity and observed that isolates with high initial chloramphenicol MIC were more RND-EPs-dependent compared with those with low initial resistance level and this difference was significant (**Figure 4B**).

Investigation of the impact of EPs on *Pseudomonas* spp. resistance to ampicillin, again, showed a considerable reduction of antibiotic MIC levels in the presence of PAβN in all bacterial isolates, clearly demonstrating an important role of RND pumps. However, as the resistance of the isolates to ampicillin was often very high (unmeasurable under the protocol used), therefore it was impossible to calculate MIC reduction accurately (data not shown).

Next, we accessed the role of other prominent efflux system, ABC transporters, in the bacterial susceptibility to chloramphenicol by using an inhibitor of ABC EPs verapamil (Li et al., 2016). The decrease of chloramphenicol MIC after addition of verapamil was low to absent (data not shown), indicating that ABC efflux transporters are not the main cause of antibiotic resistance in *Pseudomonas* spp. recovered from soil. However, a substantial synergistic effect of combined action of PAβN and verapamil on antibiotic MIC was observed, suggesting that operation of low-efficient ABC pumps may be masked in the background of active RND pumps (data not shown).

Other clinically relevant bacteria of the soil origin (*Stenotrophomonas* spp. and *Chryseobacterium* spp.) which showed resistance to a high number of antimicrobials (**Supplementary Table S1**) were checked for the activity of RND and ABC types of EPs by using pump-specific inhibitors. *Stenotrophomonas* spp. were affected by inhibition of RND pumps (average reduction being 62%), especially when initial chloramphenicol MIC values for isolates were high (**Figure 5A**). However, some isolates exhibited MIC reduction comparable to the *Pseudomonas* spp. (up to 94 %), while one did not show any chloramphenicol MIC changes after EP inhibitor addition. Similar tendency of greater importance of RND efflux pumps could be observed for the more initially resistant isolates. Inhibition of ABC EP also substantially affected the resistance to chloramphenicol (average MIC reduction being 59 %, and maximum reduction of 87%) (**Figure 5A**). Two strains did not show a change in chloramphenicol MIC after addition of verapamil, one of them was the same strain that exhibited the trait with PAβN. Similar effect was also observed for *Chryseobacterium* spp. (**Figure 5B**). Therefore, we show that antimicrobial resistance in the most prevalent cultivable soil bacteria is largely mediated by the efflux pumps.

DISCUSSION

Soil is a very complex structure which includes organic particles as well as thousands of living organisms from different taxa including worms, arthropods, fungi, bacteria and some other eukaryotic and prokaryotic organisms. Bacteria are one of the most important living parts of the soil ecosystem (Fierer, 2017; Sun et al., 2017). Many of them are decomposers, the other helps to assimilated nitrogen for plants as well as they serve as a food for protists. Recent study demonstrates that high abundances of beneficial bacteria are related with soil quality, which is indicated by better plant growth, lower outbreaks of diseases, higher soil pH and better nutrient activities (Wang et al., 2017). The findings also suggest that soil pH is the primary determinant and it is more important factor than addition of nutrients for bacterial community (Wu et al., 2017; Zhang et al., 2017). We have investigated near-neutral soils (pH 6.58–7.08) and found a wide variety but similar microbial composition in soils of different farming types. The relative abundance of most bacterial phyla is higher in near-neutral than in acidic or alkaline soils (Zhang et al., 2017). *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Firmicutes* and *Bacteroidetes* were the most abundant phyla in our study. The recent data demonstrates that those bacteria are more prevalent in near-neutral pH except *Acidobacteria* which are diverse and specific acidobacterial subgroups are adapted to distinct pH conditions (Lauber et al., 2009; Bartram et al., 2014; Zhang et al., 2017). The chemical soil composition, particularly the amount of phosphorus is also important factor for microbial load (Liu et al., 2013) but it is unclear the relation between amount of phosphorus and microbial variety. In our experiments we did not detect any significant changes in microbial composition at the genera level when different amount of phosphorus (130 mg/kg vs. 320 mg/kg) was presented in

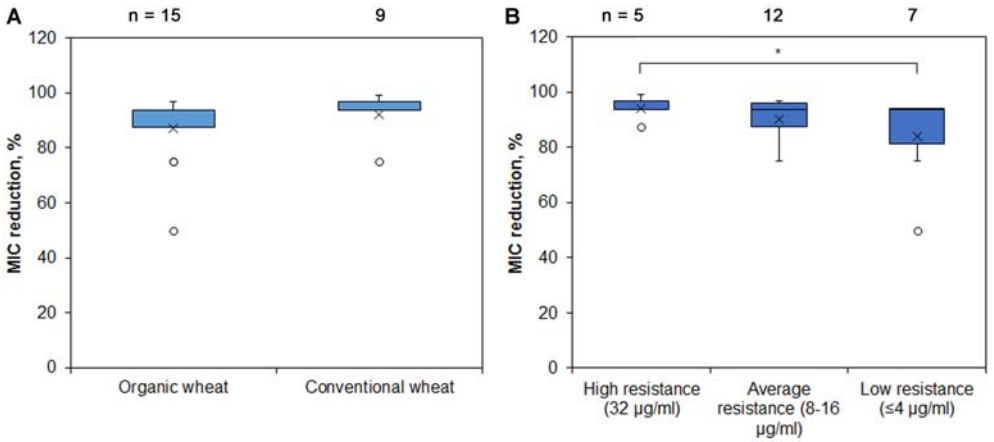

FIGURE 4 | MIC reduction of *Pseudomonas* spp. of different soil origin after addition of RND EP inhibitor PAβN. *Pseudomonas* spp. isolates were grown with or without RND EP inhibitor PAβN and their MIC of chloramphenicol was assessed. Blue boxes indicate upper and lower quartiles, whiskers indicate minimum and maximum values excluding outliers, circles depict outliers and crosses indicate mean values. * Indicates statistical significance calculated as non-parametric Mann-Whitney test for two independent samples ($p < 0.05$; one-tailed). **(A)** *Pseudomonas* spp. isolates from two farming sites of different style did not show significant differences in MIC reduction after addition of RND EP inhibitor. **(B)** *Pseudomonas* spp. with the higher initial resistance to chloramphenicol were more dependent on EP than the isolates with low initial resistance.

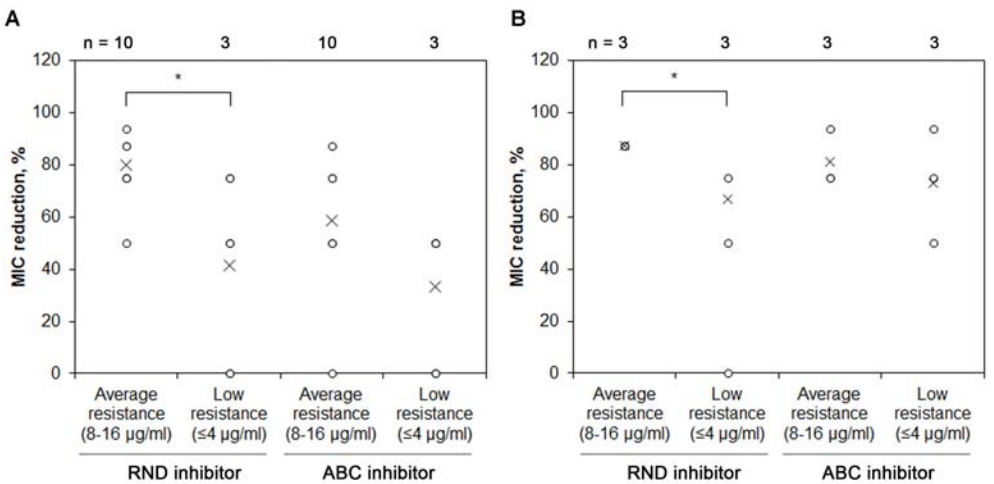

FIGURE 5 | MIC reduction of *Stenotrophomonas* spp. and *Chryseobacterium* spp. after addition of RND or ABC efflux pump inhibitors. **(A)** *Stenotrophomonas spp*. or **(B)** *Chryseobacterium* spp. isolates were grown with or without RND EP inhibitor PAβNor ABC inhibitor verapamil and their MIC of chloramphenicol was assessed. Each value is indicated as a circle, crosses indicate mean values.* Indicates statistical significance calculated as non-parametric Mann-Whitney test for two independent samples ($p < 0.05$; two-tailed).

a soil of different farming. Within the most prevalent genera the highest difference was among the prevalence of the genus *Holophaga* which number was almost two times higher in the soil of organic crops. *Holophaga* are homoacetogenic bacteria that degrades methoxylated aromatic compounds which are natural products of plants, animals and microorganisms (Liesack et al., 1994), however, more investigations are necessary to determine the reason of such difference. The stability of soil microbiome composition is very important for N and S cycles but certain pesticides and other chemicals may affect the composition of bacteria therefore, making serious ecological disturbances in living ecosystems (García-Delgado et al., 2018; Karas et al., 2018). At the same time there are some data that application of different herbicides including glyphosate, glufosinate, paraquat, paraquat-diquat and triasulfuron had no effect on the diversity and structure of soil bacteria and archaea (Pose-Juan et al., 2017; Dennis et al., 2018).

In this study we aimed to analyze the soils from two farming systems: conventional and organic (which were certified as organic farming for at least 20 years). Both conventional and to a lesser extent organic farming depend on pesticides, though the systems are subjected to different regulations. Organic farming exclusively allows the use of pesticides which are of natural origin, whereas synthetically produced products may be applied

in conventional farming systems (Lori et al., 2017). Analysis of the bacterial diversity in soils from different farming systems showed only slight differences among the main taxonomical units of microorganisms. The main prevalent phyla included *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Firmicutes* and *Bacteroidetes* in the soils from both farming systems. Both soils had a similar composition to the soil detected all around the globe (Fierer et al., 2009).

Only rarely detected lower taxons were different between the soils. From the genera that were present in significantly different quantities (higher in conventional farming), *Sphingomonas* and *Gemmatimonas* were observed previously to be increased in farming with mineral fertilizers (Ma et al., 2018). We also found *Rhodanobacter* genus, which was previously connected with denitrification of soil (Green et al., 2012), present only in conventional farming soil (0.37% relative abundance) and absent from organic farming soil.

Acidobacteria are related to nutrient-wise poor soil (Chaudhry et al., 2012), and therefore their abundance would be an indicator of poor quality of soil. The relative abundance of *Acidobacteria* was not high in both soils we have investigated, indicating both farming systems are able to retain soil quality. A relative abundance of *Firmicutes* has been previously connected with manure application to the soil (Hartmann et al., 2015; Wepking et al., 2017). Yet in our analysis we have also found higher relative abundance of *Firmicutes* in the conventional farming soil.

We also observed that the continuous pesticide use on the field did not affect the soil community composition, confirming a similar observation made previously (Hartmann et al., 2015). Increased diversity and richness of the microbial community has been previously observed in the organic farming, which is mostly due to the fertilization using manure, while continuous fertilization using mineral fertilizers decreases the diversity (Li et al., 2012; Hartmann et al., 2015; Lupatini et al., 2016). In our case, we did not observe significant differences between the two types of farming soils.

High variety and similarities of microorganisms in the soils from different farming systems indicates the stability of microbial populations that might be associated with the evolutionary ability of soil microorganisms to adapt the different environment and to survive among other organisms and different chemical substances which usually are originated from microorganisms like fungi, themselves.

This study also indicates the high diversity of microorganisms in soil as the highest number of the most predominant genus distribution was less than 5%. The presence of multiple genera and high diversity of the species within the soil could be one of the reasons for high soil sustainability as an external or internal influence, for instance, suppression of one or few bacterial genera probably will not affect the whole microbiome itself.

Soil is one of the most favorable settings for acquisition and selection of antimicrobial resistance, due to the abundance of antibiotics-producing microorganisms. Chemicals that are used in conventional farming have potential to induce resistance development (Kleiner et al., 2007). On the other hand, during organic farming manure as a fertilizer is used, therefore antimicrobial resistant bacteria originated from gut of the animals may spread into soil ecosystems and increase resistance (Li B. et al., 2015; McKinney et al., 2018). Different animal pathogens as well as commensal microbiota have potential for horizontal transmission of the resistance genes (von Wintersdorff et al., 2016) therefore, resistance transfer of antimicrobial resistant bacteria may occur in both directions – from animals to soil and vice versa – from soil to animals because soils also contain an autochtonous bacterial microbiota which harbors resistance genes (Rizzo et al., 2013; Marti et al., 2014). Once bacteria have acquired ARGs, they may exist in the environment for a long time, even after the selection pressure (Tamminen et al., 2012).

In this study we have detected only single genes encoding antimicrobial resistance from the DNA of soil microbiomes in all tested samples regardless of the farming system. They conferred resistance mechanisms to β-lactams, aminoglycosides, tetracycline and erythromycin. All these antimicrobials are used in human and veterinary medicine and our previous studies demonstrated that animal microbiota contain a wide variety of clinically important genes encoding antimicrobial resistance (Seputiene et al., 2012; Klimiene et al., 2016). There was no recorded history about the origin of the manure in the organic farming fields, therefore we could expect the variety of resistance genes to differ between the various animal farms depending on the treatment of animals, which could be reflected in the amount of resistance genes reaching the fields with manure.

The recent data from functional metagenomics reveals novel genetic determinants that could be potentially foreseen as indicators of soil resistome and its dynamics (Torres-Cortés et al., 2011; McGarvey et al., 2012; Wichmann et al., 2014). We have shown in our study that *arr*-like 1 gene conferring rifampin resistance was present in all soils, whereas other determinants were sporadic or absent. Moreover, all soil samples except two contained *tetM* gene, which has been reported to be abundantly present in the microbiomes of various origin and the gene was proposed to be an indicator for the co-occurrence of other antibiotic resistance genes (Li B. et al., 2015).

Recent soil metagenome studies show the relative dominance of determinants encoding bacterial efflux systems among ARGs compared to other resistance mechanisms such as enzyme-mediated drug modification or drug target binding (Li B. et al., 2015; Van Goethem et al., 2018). We therefore analyzed the EP activity of cultivable isolates of three genera (*Pseudomonas*, *Stenotrophomonas* and *Chryseobacterium*). The genera were chosen as they are increasingly associated with infections and raise a threat due to their high intrinsic resistance (Ho et al., 2010; Brooke, 2012; Mukerji et al., 2016). *Pseudomonas aeruginosa* has been continuously shown to use RND EPs to counteract antibiotics, the presence of the same mechanisms are also shown for environmental *Pseudomonas* strains (Poole, 2001). Our research confirms that resistant isolates of soil origin also efficiently use RND EP. *Stenotrophomonas* spp. environmental strains have been demonstrated to possess similar ARGs as clinical strains (Youenou et al., 2015; Wang et al., 2018). In our EP inibition test we have observed similar action of EP in *S. maltophilia* and *Stenotrophomonas* of other species, indicating the EP that are present (the RND and ABC in our study) are

able to cause resistance. Interestingly, we have found that efflux is also used by *Chryseobacterium* spp. of soil origin, thought these bacteria were mostly know to be resistant by drug modification mechanisms (Lin et al., 2012).

Hence, our resistance mechanisms studies of the most prevalent groups of soil cultivable bacteria from soils of different farming systems support the significant role of RND and ABC EPs in mediating resistance. The efficient efflux-mediated mechanisms in soil bacteria, therefore, might present a source for multidrug resistance spread including horizontal transfer (Dolejska et al., 2013; Walsh and Duffy, 2013).

According to this study it may be outlined that soil microbiota is a stable component as it were detected similar composition of microorganisms in soil both in organic as well as in conventional farming systems with similar soil structure and pH. The different amount of phosphorus in soils had no influence on bacterial variety at a genera level although more investigations would be useful to investigate changes among separate species. During evolution microorganisms adapted to survive in ecosystems independently of certain changes and probably serve as a buffer for ecological niches. It is unclear, however, what level of intensity can change microbial composition but current conventional farming in Central Europe demonstrates acceptable level of intensity for one of the most important ecological component of soils. Analysis of antimicrobial resistance in soils demonstrates that microorganisms did not acquire a plethora of genetic determinants encoding resistance mechanisms to the antimicrobials used in human and animal medicine as only a small number and low variety of clinically important genes encoding resistance to those antimicrobials were detected. However, the antibiotic resistance of the cultivable agricultural soil bacteria, including clinically relevant species, is largely mediated by the drug efflux mechanisms.

AUTHOR CONTRIBUTIONS

JA, JS, MR, EB, ES, IK, and VK designed the experiments. JA, JS, RK, EB, and RŠ performed the experiments. JA, JS, RK, EB, and SK analyzed the data. JA, ES, EB, and MR wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00892/full#supplementary-material

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Multiomics Assessment of Gene Expression in a Clinical Strain of CTX-M-15-Producing ST131 *Escherichia coli*

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Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* strain C999 was isolated of a Spanish patient with urinary tract infection. Previous genotyping indicated that this strain presented a multidrug-resistance phenotype and carried beta-lactamase genes encoding CTX-M-15, TEM-1, and OXA-1 enzymes. The whole-cell proteome, and the membrane, cytoplasmic, periplasmic and extracellular sub-proteomes of C999 were obtained in this work by two-dimensional gel electrophoresis (2DE) followed by fingerprint sequencing through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). A total of 602 proteins were identified in the different cell fractions, several of which are related to stress response systems, cellular responses, and antibiotic and drug responses, consistent with the multidrug-resistance phenotype. In parallel, whole genome sequencing (WGS) and RNA sequencing (RNA-Seq) was done to identify and quantify the genes present and expressing. The *in silico* prediction following WGS confirmed our strain as being serotype O25:H4 and sequence type ST131. The presence of proteins related to antibiotic resistance and virulence in an O25:H4-ST131 *E. coli* clone are serious indicators of the continued threat of antibiotic resistance spread amongst healthcare institutions. On a positive note, a multiomics approach can facilitate surveillance and more detailed characterization of virulent bacterial clones from hospital environments.

Keywords: bacteria, antimicrobial resistance, public health, genomics, transcriptomics, proteomics

Abbreviations: 2DE, two-dimensional gel electrophoresis; ACN, acetonitrile; DTT, dithiothreitol; ESBL, extended-spectrum β-lactamase producing; FPKM, fragments per kilo base per million mapped reads; IPG, ImmobilineTM pH Gradient; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel; RNA-Seq, RNA sequencing; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; WGS, whole genome sequencing.

INTRODUCTION

Rates of Gram-negative healthcare-associated infections have been increasing since 1998, mostly caused by antimicrobial resistant *Enterobacteriaceae*. A strikethrough recent worldwide survey revealed the prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* in 14% of healthy individuals, a rate predicted to increase by 5.38% year on year overall (Karanika et al., 2016; Bassetti et al., 2017). Patients hospitalized in intensive care units and in long-term care facilities, or those who are immunocompromised have a higher risk of acquiring multidrug-resistant Gram-negative infections (Kunz and Brook, 2010). ESBLs are enzymes encoded by plasmid-borne genes, typically from the TEM, SHV, CTX-M families, that mediate resistance to oxyimino-beta-lactam antibiotics, third-generation cephalosporins and aztreonam (Rice, 2009; Drawz and Bonomo, 2010). For years, *Escherichia coli* producing the CTX-M-15 variant have been frequently implicated in human infection (Ewers et al., 2010). It has also been noted that the $bla_{\mathrm{CTX-M-15}}$ gene is located 49 bp downstream of insertion sequence IS*Ecp1*, a well-known highly efficient mobile element playing a major role in the expression and spread of CTX-M beta-lactamases, the most common in Europe (Peirano and Pitout, 2010; Guiral et al., 2011). Throughout the years, the ciprofloxacin-resistant CTX-M-15-producing O25:H4-ST131 *E. coli* clonal group is known to have caused major outbreaks worldwide (Nicolas-Chanoine et al., 2008; Ewers et al., 2010; Johnson et al., 2017). Classed as a member of the virulent phylogenetic group B2 and having the multidrug-resistant profile of the sequence type (ST) 131 clonal group, the O25:H4-ST131 clone represents a major public health problem as it makes it more complicated to select an appropriate therapy to administer, with a higher risk of increased costs and use of "last resort" antibiotics (Vimont et al., 2012). ST131 is therefore seen as being at the leading edge of a deeply concerning set of increasingly challenging infection agents (Vimont et al., 2012; Johnson et al., 2017). In the present work, we studied an ESBL-producing *E. coli* strain of human clinical origin, designated C999, that was previously studied and characterized by Ruiz et al. (2012). C999 was resistant to fluoroquinolones and third generation cephalosporins because of CTX-M-15 ESBL and belonged to phylogenetic group B2 and ST131. According to the genomic profile of *E. coli* C999 we assumed that this strain is related to the hazardous intercontinental O25:H4-ST131 clone. In our research, we took a multiomics approach to more deeply characterize this significant clinical strain. Whole-genome sequencing (WGS) and RNA sequencing (RNA-Seq) analysis were conducted to confirm if the *E. coli* C999 strain belongs to the O25:H4 serotype and identify/quantify the genes expressed. In parallel, proteomic maps of C999 were produced by two-dimensional gel electrophoresis (2DE) of whole-cell and fractionated extracts followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) of peptides (Vlaanderen et al., 2010). This allowed us to monitor how resistance mechanisms affect the proteomes of the membrane and cytoplasmic compartments.

MATERIALS AND METHODS

Whole-Genome Sequencing

Total DNA was extracted from *E. coli* C999 using a silica-based automatic DNA extractor EasyMag (BioMérieux Inc., Durham, United States). A sequencing library was generated using the Nextera XT DNA library preparation kit (Illumina Inc., San Diego, CA, United States) and sequenced on a MiSeq (Illumina Inc., San Diego, CA, United States) using paired-end reads (2×150 bp), according to the manufacturer's instructions. FastQC[1] and Trimmomatic[2] software tools were used for read quality analysis and improvement, respectively (D'Antonio et al., 2015; Williams et al., 2016). Genome assembly and annotation were done using SPAdes[3] and RAST annotation[4], respectively. Finally, putative antimicrobial resistance genes were predicted using Comprehensive Antibiotic Resistance Database (CARD[5]) (Jia et al., 2017). WGS raw reads were submitted to the European Nucleotide Archive under the accession numbers ERR3013427.

RNA Library Preparation and Sequencing

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) with RNase-free DNase treatment on the column (Qiagen), followed by bacterial rRNA depletion using a Ribo-Zero rRNA Removal Kit (Illumina Inc., San Diego, CA, United States). The 2100 Bioanalyzer (Agilent, Santa Clara, CA, United States) was used to evaluate the concentration and quality of RNA samples pre- and post-depletion. For RNA-Seq analysis, a library was prepared with rRNA-depleted samples using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina). RNA was sequenced on a MiSeq using paired-end (2×75 bp) reads (Illumina), according to the manufacturer's instructions.

Transcriptomic Data Analysis

The quality of raw RNA-Seq data was evaluated using FastQC analysis. The sequence reads were then mapped against the obtained whole-genome sequence of strain C999 using the Bowtie2 algorithm[6] (Version 2.1.0). The expression level of each transcript was calculated using the Cufflinks software[7] (Version 2.1.1) by normalizing data as fragments per kilobase of coding sequence per million mapped reads (FPKM).

Whole-Cell Protein Extraction

Cells were grown in brain heart infusion agar for 24 h and afterward cultivated in brain heart infusion broth (15 ml) for 4 h (Goncalves et al., 2014). Exponentially growing cells were then harvested by centrifugation (3 min, 10,000 g, 4°C) and resuspended in 4 ml of phosphate-buffered saline at room temperature, centrifuged again, then resuspended in 0.2 ml of 10% (w/v) sodium dodecyl sulfate (SDS), 12% (w/v) Tris (Celis

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[2]http://www.usadellab.org/cms/?page=trimmomatic
[3]http://bioinf.spbau.ru/spades
[4]http://rast.nmpdr.org/
[5]https://card.mcmaster.ca/
[6]http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
[7]http://cufflinks.cbcb.umd.edu/

and Gromov, 1999). Cells were disrupted by sonication with an ultrasonic homogenizer (Vibra-Cell™ VCX130, Sonics & Materials Inc., Newtown, United States) in three 10 s bursts at 40% of full power, then cell debris was removed by centrifugation (14,000 g, 30 min, 4°C). The clear supernatant was collected then mixed with an equal volume of cold 20% (w/v) trichloroacetic acid (TCA; Merck, Darmstadt, Germany) in acetone (Sigma-Aldrich, St. Louis, MO, United States) and was kept at −20°C for 1 h. The precipitate was collected by centrifugation at 13,000 g for 30 min at 4°C. The precipitated protein was washed thrice with acetone to remove traces of TCA. Residual acetone was removed by air-drying. Protein pellets were solubilized in thiourea/urea lysis buffer. The resulting solutions were stored at −80°C for further analysis.

Extracellular Protein Extraction

Extracellular proteins were prepared as previously described with some modifications (Nandakumar et al., 2006; Xia et al., 2008; Goncalves et al., 2014). Cells were removed from brain heart infusion broth culture by centrifugation at 5500 g for 10 min at 4°C. The clear supernatant was collected, passed through a 0.22 μm filter, mixed with an equal volume of cold 20% (w/v) TCA in acetone, and kept at −20°C for 1 h. The precipitate formed after centrifugation at 13,000 g for 30 min at 4°C was washed thrice with acetone to remove traces of TCA, and residual acetone was removed by air-drying. Dried protein pellets were solubilized in thiourea/urea lysis buffer [2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT), 2% (v/v) carrier ampholytes (pH 3–10) and 10 mM Pefabloc® proteinase inhibitor], then stored at −80°C for further analysis.

Periplasmic and Cytoplasmic Protein Extraction

To extract periplasmic and cytoplasmic protein from bacterial cultures, the Epicentre Peripreps™ Periplasting kit (Epicentre, WI, United States) was used with a few modifications to the kit protocol. The bacterial cell culture was centrifuged at 5500 g for 10 min and the supernatant discarded. The pellet was resuspended by pipetting in 2 ml of PeriPreps™ Periplasting buffer (200 mM Tris-HCl pH 7.5, 20% sucrose, 1 mM EDTA, and 30 U/μl Ready-Lyse lysozyme) for each gram of cell pellet. The sample was incubated for 5 min at room temperature. Osmotic shock was induced by rapidly adding 3 ml of ice-cold water for each gram of original cell pellet, mixing the sample by inverting the centrifuge tubes. The sample was kept on ice for 10 min then centrifuged at 12,000 g for 2 min to separate the pellet (spheroplasts and intact cells) from the supernatant, the periplasmic fraction. Spheroplasts in the pellet were lysed by adding a detergent lysis buffer (10 mM KCl, 1 mM EDTA, and 0.1% deoxycholate) and the pellet was resuspended in 5 ml of PeriPreps lysis buffer for each gram of original cell pellet and incubated for 5 min at room temperature. The sample was sonicated with 2 s bursts at 40% of full power for a total of 1 min. Cell debris was removed by centrifugation at 12,000 g for 15 min at 4°C. The supernatant was removed and centrifuged as before. The supernatant recovered was the cytoplasmic fraction. Equal volumes of cold 20% (w/v) TCA in acetone were mixed with both periplasmic and cytoplasmic fractions that were then kept at −20°C Tris-HCl pH 7.5, 50 for 1 h. The precipitates collected after centrifugation at 13,000 g for 30 min at 4°C were washed thrice with acetone to remove traces of TCA. Residual acetone was removed by air-drying. Protein pellets were solubilized in thiourea/urea lysis buffer and stored at −80°C for further analysis.

Membrane Protein Extraction

Membrane proteins were isolated by a previously described method with some modifications (Taddei et al., 2011). Bacterial cells were recovered from liquid culture by centrifugation at 10,000 g for 3 min at 4°C and the pellet was resuspended in phosphate-buffered saline pH 7.4 (Gorg et al., 2004). After a second similar centrifugation step, the pellet was resuspended in 25 ml of 10 mM Tris buffer pH 8.8 with 1 mM phenylmethylsulfonylfluoride (Sigma-Aldrich). Cells were disrupted with 3 cycles of 20 s bursts of sonication at 40% of full power and the cell debris was removed by centrifugation at 12,000 g for 2 min at room temperature. The supernatant was centrifuged at 49,500 g for 60 min at 4°C (in a 3–30KS centrifuge with rotor no.12158, Sigma GmbH, Osterode am Harz, Germany) and the pellet was treated with 1.67% N-lauroylsarcosine sodium salt (Sigma-Aldrich) for 20 min at room temperature. The membrane proteins were recovered by centrifugation at 23,000 g for 90 min at 4°C and the pellet was solubilized in thiourea/urea lysis buffer. Samples were stored at −80°C for further analysis.

Protein Quantification

Protein concentration was determined using the 2-D Quant kit (GE Healthcare, Buckinghamshire, United Kingdom) following the manufacturer's instructions. In this procedure proteins are quantitatively precipitated leaving other substances in solution. The precipitated proteins are then resuspended in a copper-containing solution with the unbound copper being measured with a colorimetric agent. Color density (absorbance at 480 nm) is thus inversely related to the protein concentration and accurately reflects the protein concentration of the sample.

One-Dimensional and Two-Dimensional Electrophoresis

One-dimensional electrophoresis was done with SDS-polyacrylamide (SDS-PAGE) gels (T = 12.52%, C = 0.97%) in a HoeferTM SE 600 Ruby® unit (GE Healthcare, Chicago, United States) as described by Laemmli (1970) with some modifications (Igrejas, 2000). Whole-cell protein extract (15 μg) was resuspended in an equal volume of buffer containing 0.5 M Tris HCl pH 8.0, glycerol, SDS and bromophenol blue. After protein separation at 30 mA, gels were stained for 24 h in Coomassie Brilliant Blue R-250 and washed in water overnight. Gels were then fixed in 6% TCA for 4 h and in 5% glycerol for 2 h (Gorg et al., 2009). Two-dimensional electrophoresis (2DE) was performed according to the principles of O'Farrell but with Immobiline™ pH Gradient (IPG) technology (O'Farrell, 1975; Gorg et al., 2009). For the first dimension of isoelectric focusing,

precast 13 cm IPG strips with linear gradients of pH 3–10 (GE Healthcare) were passively rehydrated for 12–16 h in a reswelling tray with 250 μl of rehydration buffer (8M urea, 1% CHAPS, 0.4% DTT, and 0.5% carrier ampholyte IPG buffer pH 3–10) at room temperature. IPG strips were covered with Drystrip Cover Fluid (Plus One, GE Healthcare). Lysis buffer [9.5 M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3–10), and 10 mM Pefabloc® proteinase inhibitor] was added to *E. coli* protein extracts to achieve a concentration of 1 μg/μl of protein. Samples containing a total of 100 μg of protein were cup-loaded onto the rehydrated 13-cm IPG strips (Gorg et al., 2009). To optimize running conditions, isoelectric focusing replicate runs were performed according to Gorg et al. (2009) and the GE Healthcare protocol for 13 cm IPG strips pH 3–10 on an Ettan™IPGPhor II™ (GE Healthcare). The optimized 13 h run was as follows: sample proteins were focused at 500 V for 1 h, followed by a gradient up to 1000 V for 8 h, then a gradient up to 8000 V for 3 h, finally remaining at 8000 V for 1 h. Focused IPG strips were then stored at −80°C in plastic bags. Before running the second dimension of electrophoresis, strips were equilibrated twice for 15 min in equilibration buffer [6M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05M Tris-HCl buffer (pH 8.8) with bromophenol blue] with 1% DTT included in the first equilibration and 4% iodoacetamide in the second one. The equilibrated IPG strips were briefly rinsed with SDS electrophoresis buffer, blotted to remove any excessive buffer, and then loaded onto 12.52% polyacrylamide gels in a Hoefer™ SE 600 Ruby® unit (GE Healthcare). The SDS-PAGE technique previously reported by Laemmli (1970) was modified to increase the resolution with the proper insertion of the IPG strips in the stacking gel (Laemmli, 1970; Igrejas, 2000). SDS-PAGE was run at 440 V for 3 h. Gels were fixed in 40% methanol, 10% acetic acid for 1 h, then stained overnight in Coomassie Brilliant Blue G-250 (Gorg et al., 2009). Coomassie-stained gels were scanned on a flatbed scanner (UmaxPowerLook 1100, Freemont, CA, United States) and the digitized images were analyzed using Lab Scanner Image Master 5.0 software (GE Healthcare). Protein molecular weights were estimated by comparison with an internal calibration marker.

Protein Identification by MALDI-TOF/MS

For each extraction method, gels were analyzed and compared with each other. Spots that were expressed in all gels were manually excised from the gels and analyzed using MALDI-TOF/MS. Gel pieces were rehydrated twice in 200 μl Milli-Q water and washed twice with 25 mM ammonium bicarbonate, 50% acetonitrile (ACN), once with 50 μl ACN, then dried in a SpeedVac (Thermo Fisher Scientific, Waltham, MA, United States). To digest the proteins, 15 μl of trypsin solution [0.02 μg/μl trypsin (Promega, Madison, WI, United States), 12.5 mM ammonium bicarbonate, 2% (v/v) can] was added to the dried gel pieces, which were then kept on ice for 1 h before adding 30 μl of 12.5 mM ammonium bicarbonate and incubating them overnight at 37°C. Tryptic peptides were extracted by adding 20 μl of 5% formic acid, 50% ACN and then 25 μl of 50% ACN, 0.1% trifluoroacetic acid followed by threefold lyophilization in a SpeedVac (Thermo Fisher Scientific). Tryptic peptides were resuspended in 10 μl of 0.3% formic acid. Samples were mixed (1:2, v/v) with 1 μl of a saturated matrix solution of 5 mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% (v/v) trifluoroacetic acid, 50% (v/v) ACN, 8 mM ammonium phosphate). Aliquots of samples (0.5 μl) were spotted onto the MALDI sample target plate (384-spot ground-steel plate). Peptide mass spectra were obtained from a MALDI-TOF/MS Ultraflex mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in positive ion reflectron-mode. Spectra were acquired in the m/z range of 600–3500 Da at a laser frequency of 50 Hz. For each spot analyzed, a data-dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide, for subsequent MS/MS data acquisition. Mass spectra were internally calibrated with self-digested trypsin peaks (MH+: 842.5, 2211.42 Da) allowing a mass accuracy of better than 25 ppm. External calibration was performed with the [M + H]+ monoisotopic peaks of bradykinin 1–7 (m/z 757.3992), angiotensin II (m/z 1046.5418), angiotensin I (m/z 1296.6848), substance P (m/z 1758.9326), ACTH clip 1–17 (m/z 2093.0862), ACTH18–39 (m/z 2465.1983), and somatostatin 28 (m/z 3147.4710).

Bioinformatics Analysis for Proteomics

Spectra were processed and analyzed using the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v 2.1.04, Matrix Science, London, United Kingdom) to search for peptide mass fingerprints within MS/MS data. The Swiss-Prot non-redundant protein sequence database (Release 10 of October 2014, 546790 entries) and NCBI Reference Sequence Database (RefSeq release 68 of November 2014, 46968574 protein entries) were used to search *E. coli* protein sequences. The database search parameters were as follows: carbamidomethylation and propionamide of cysteine (+71 Da) and oxidation of methionine (+16 Da) as variable modifications, allowance for up to two missed tryptic cleavages, peptide mass tolerance of 50 ppm, and fragment ion mass tolerance of 0.3 Da. Positive identifications were accepted above 95% of confidence level. Protein identifications were considered as reliable when the MASCOT score was >70% calculated as $-10 \times \log P$, where P is the probability that the observed match is a random event. This is the lowest score indicated by the program as being significant ($P < 0.05$) below which proteins are likely to be incorrectly identified.

RESULTS AND DISCUSSION

E. coli C999 Strain Profile

Genomics and Transcriptomics

ESBL-producing *E. coli* strain C999, implicated in a urinary infection of a Spanish patient was collected in 2007 and used in this study, thus characterized in relation to the phenotype and genotype of antimicrobial resistance and to molecular typing (Ruiz et al., 2012). This strain was resistant to ampicillin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, naladixic acid, ciprofloxacin, tobramycin, kanamycin, streptomycin, tetracycline, sulfamides and trimethoprim-sulfametoxazole, and

carried the $bla_{CTX-M-15}$, bla_{OXA-1}, and bla_{TEM-1b} β-lactamase genes. Other resistance genes observed in strain C999 were *aac(6')-Ib-cr* (ciprofloxacin resistance), *tet(A)* (tetracycline resistance) and *sul1* (sulfametoxazole resistance). The gene cassette array *dfrA17* + *aadA5* was observed in strain C999 and mutations were also found in genes encoding GyrA (Ser83Leu + Asp87Asn) and ParC proteins (Ser80Ile + Glu84Val) (Ruiz et al., 2002). C999 was classified in the phylogenetic group B2, mostly implicated in extraintestinal infections (Clermont et al., 2000), and it belongs to sequence type ST131, as previously detected (Ruiz et al., 2012). To better understand the nature of the C999 strain we produced a comprehensive survey of its genome, transcriptome and proteome. WGS allowed comprehensive characterization of the genetic makeup of the bacterial strain, including the identification of antibiotic resistance genes, consistent with the known pathological nature of this strain previously determined by Ruiz and colleagues (Ruiz et al., 2012). In fact, *in silico* prediction was applied to the WGS assay using SerotypeFinder 2.0, thus confirming the O25:H4 serotype which can lead us to acknowledge our strain as a member of the O25:H4-ST131 *E. coli* clonal group (Joensen et al., 2015). **Supplementary Table S1** display all the identified genes related to antibiotic resistance such as *aac(6')-Ib-cr*, *tet(A)*, *sul1*, *aadA5* gene cassette and genes related to toxin-antitoxin addiction systems of plasmids *pemK*, *ccdA/ccdB*, *vagC/vagD,* and *sok*, as well as β-lactamase genes bla_{TEM-1}, bla_{OXA-1}, and $bla_{CTX-M-15}$. It is important to also highlight the presence of several stress response and oxidoreductase genes. This perspective of the C999 transcriptome gives an overview of all its cellular mechanisms (**Supplementary Table S2**).

Proteomics

The 2DE gels of the whole-cell proteome and four sub-proteomes of *E. coli* strain C999 were compared (**Figures 1**, **2**). From all the gels, a total of 564 protein spots were collected for analysis using MALDI-TOF/MS and identified by correlating the output with bioinformatics databases[8]. A total of 602 different proteins were identified from 471 different spots, which corresponds to 83.51% of the total spots collected (**Supplementary Tables S5–S9**). The proteins identified were related to different functions within bacterial cell metabolism, the most frequent being enzyme activity, transport and molecule/protein biosynthesis, followed by the stress response, the SOS response and antibiotic resistance (**Figures 3–5**). Proteins related to glycolysis and molecule biosynthesis were indeed well represented in all proteomes (**Figure 6**). In fact, 282 different proteins were identified as involved in biological processes of regular cell functioning and 42 proteins were found to be related to stress response mechanisms, as has been previously described (Micevski and Dougan, 2013; Delmar et al., 2014).

Comparison of RNA and Proteins Expressed in *E. coli* C999

With the use of RNA-Seq the abundance of all transcripts was quantified, thus allowing to compare the gene expression levels to the proteomic data (**Supplementary Table S3**; Han et al., 2015; Salipante et al., 2015). **Supplementary Table S4** summarizes the relevant genes identified with their lengths and abundance in FPKMs, juxtaposed with the proteomic data obtained and corresponding protein score. Taking an overview of all the data obtained, it is interesting to see that among the top-100 most highly expressed genes only 25 corresponded to detected proteins, whereas only 80 detected proteins were among the top-500 expressed genes. In fact, gene $bla_{CTX-M-15}$ was identified with an expression level of 355 FPKM being placed in the top-1000 although not being detected at the proteome level. The lack of correlation between mRNA and protein expression was already referred in previous studies, where different possibilities were advanced to explain this matter (Haider and Pal, 2013; Koussounadis et al., 2015; Liu et al., 2016). Considering the most highly expressed genes which did correlate well with the proteomic data in our survey, we can highlight the antibiotic resistance-related gene bla_{TEM} and also elongation factor *tufA*, as well as stress response genes *dps*, *clpB*, *dnaK,* and *groEL* (**Supplementary Table S3**). According to the genomic and transcript sequences, various expressed genes are related to multidrug resistance mechanisms. One example is the efflux pump AcrA-AcrB-TolC located in the intermembrane structure of Gram-negative bacteria, which ejects antibiotics and other compounds from the cell, thus playing an important part in the survival of pathogenic microorganisms (**Supplementary Table S2**; Tikhonova and Zgurskaya, 2004; Wang et al., 2009; Du et al., 2014). Adaptor protein (AcrA) and outer membrane channel (TolC) transcripts were both detected in RNA-Seq, and the AcrA homolog AcrE, the transcriptional repressor AcrR, and the potential AcrA-repressor AcrS were all expressed but at different levels (**Supplementary Table S3**). AcrE is very similar to AcrA and can substitute for AcrA function in multidrug transport, while AcrR can repress acrAB operon expression (Hirakawa et al., 2008; Hayashi et al., 2016). The *acrS* gene is upstream of *acrE*, and the protein binds to the same sequence on the AcrA promoter that is recognized by AcrR, thus potentially acting as an AcrA repressor negatively regulating kanamycin resistance (Hirakawa et al., 2008). As expected, the TolC protein was identified in the membrane sub-proteome, expressed at low levels (**Supplementary Table S4**). Outer membrane channel TolC is involved in various efflux and drug transportation systems like the tripartite systems EmrAB–TolC and MdtABC-TolC/MdtEF-TolC, and other resistance efflux systems that confer the capability to resist and expel a wide range of antibiotics, detergents and chemical solvents (Tanabe et al., 2009; Lennen et al., 2013; Anes et al., 2015). Genes *emrA*, *emrB*, *emrD*, *emrE*, *emrK,* and *emrR* were identified in our RNA-Seq survey at low expression levels (**Supplementary Table 3**, below 124 FPKMs), while genes *mdtA*, *mdtB*, *mdtC*, *mdtD*, *mdtE*, *mdtG*, *mdtI*, *mdtJ*, *mdtK*, *mdtL*, *mdtM*, *mdtN*, *mdtO,* and *mdtP* showed slightly higher expression levels (above 274 FPKMs). Except for TolC, the above efflux system components were not detected in the proteome. The lipid A-Ara4N pathway is involved in polymyxin resistance because Ara4N (4-amino-4-deoxy-L-arabinose) is added to phosphate groups of

[8]http://www.ncbi.nlm.nih.gov/

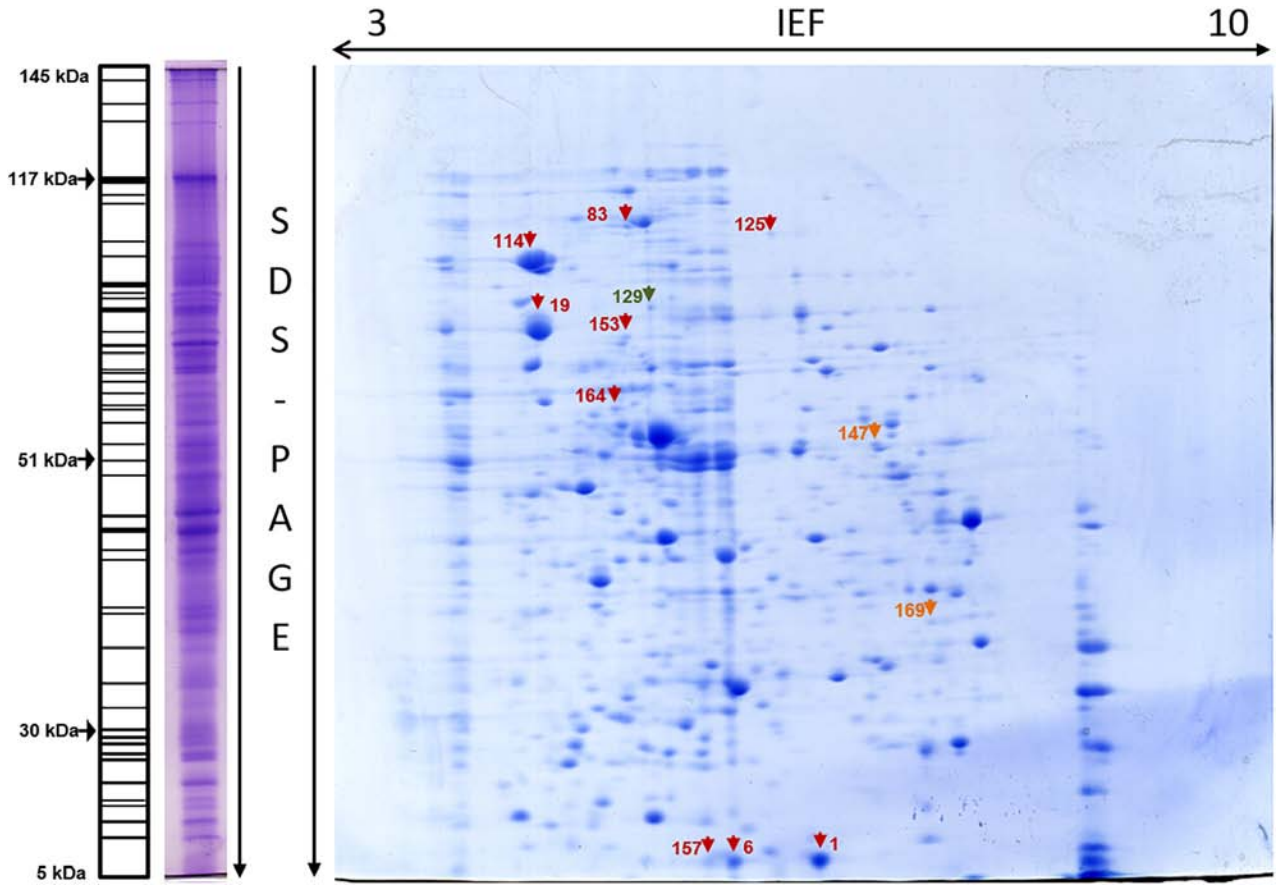


FIGURE 1 | Whole-cell proteome of *E. coli* C999. One-dimensional SDS-PAGE gel profile stained with Coomassie R-250 (left) with schematic profile showing molecular weights. Two-dimensional gel stained with Coomassie G-250 with spots marked (right). Protein spot color key: dark red, stress response; orange, SOS response; dark green, antibiotic resistance.

lipid A. Genes encoding lipid A-Ara4N pathway components are well represented among the RNA-Seq data with *arnC*, *arnE*, *arnF*, and *arnT* expressed between 64 and 45 FPKMs (**Supplementary Tables S3**, **S4**; Gatzeva-Topalova et al., 2005b). Even though the expression levels of the latter transcripts were similar, only bifunctional polymyxin resistance protein ArnA was expressing at low level in the membrane sub-proteome. Polymyxin resistance is also triggered by the up-regulation of operon *arnBCADTEF*, which is directly involved in the activation of the two-component system PmrA/PmrB that is represented in the RNA-Seq readout (Olaitan et al., 2014). Genes *pmrA*, *pmrB*, *pmrD*, *pmrG*, *pmrJ*, *pmrL*, and *pmrM* were expressed at under 178 FPKMs whilst the respective peptides they encode were not detected. The presence of these resistance mechanisms in clinical isolates with increased virulence raises concern for the spontaneous polymyxin resistance phenomena thus indicating why such bacteria reveal high pathogenic potential. Some other abundant transcripts did not have corresponding proteins in the proteomic data, which may be due to a number of factors like post-translational mechanisms of regulation and differential protein stability that can be influenced by a protein's location and/or interaction with other proteins, or even due to limitations within the proteomic techniques (Yoon et al., 2003; Hack, 2004; Kumar et al., 2016). In fact, the correlation between transcript and protein levels may vary according to specific patterns (Yoon et al., 2003). Immobilized pH gradient 2DE is widely used for protein separation and identification but have shown some limitations in resolving highly charged, long chain and insoluble proteins (Hack, 2004). Such proteins may therefore remain undetected with 2DE and MS techniques or displaying levels of expression below the define threshold, even when the corresponding genes and transcripts are identified through WGS and RNA-Seq, respectively. This reinforces the need to compare proteomic and transcriptomic results in order to fully characterize a given bacterial strain.

Proteins Related to Bacterial Resistance Mechanisms

Antibiotic Resistance

Following several reports of the identification and expression of antibiotic resistance genes around the world (Lavigne et al., 2007; Mitsou et al., 2010; Barguigua et al., 2011; Kim et al.,

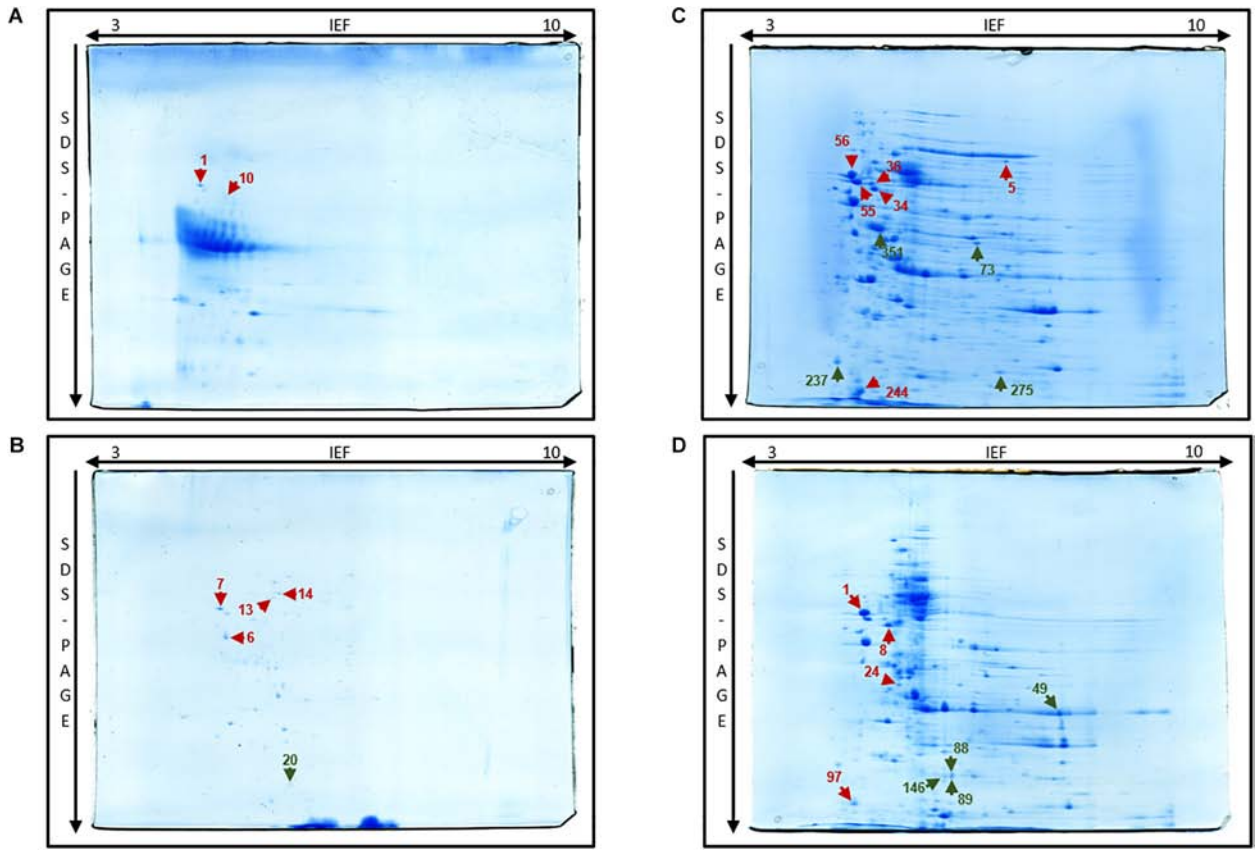


FIGURE 2 | Two-dimensional gel of *E. coli* C999 of extracellular **(A)**, periplasmic **(B)**, membrane **(C),** and cytoplasmic **(D)** proteome fractions stained with Coomassie G-250 with spots marked (right). Protein spot color key: dark red, stress response; orange, SOS response; dark green, antibiotic resistance.

2011), the dynamics of the proteome and the mechanism(s) of bacterial antibiotic resistance need to be considered in the context of the spread of bacterial pathogens (Cash, 2011). Elongation factor Tu, encoded by *tufA*, was identified in spot 46 [molecular weight (MW) 41636, isoelectric point (pI) 5.00] of the cytoplasm fraction (**Figure 2**). Present in most enterobacterial genomes, TufA is responsible for binding and transporting an appropriate codon-specified aminoacyl-tRNA to the ribosome aminoacyl site, and it also influences the assembly and stability of cytoskeletal polymers and is implicated in protein folding and protection from stress (Caldas et al., 1998; Isabel et al., 2008). Levels of TufA protein and transcripts were found to be elevated in C999 which is relevant and consistent with previous reports of *tufA* upregulated expression in the presence of antibacterial peptide polymyxin B, regulated by the *pmrA/pmrB* two-component system (**Supplementary Table S2**; Isabel et al., 2008; Ribeiro et al., 2013). Another one of the most expressed genes is β-lactamase TEM-1, which was present in the periplasmic sub-proteome [MW 31666, pI 5.60] (see **Figure 6** and **Supplementary Tables S3**, **S9**). Plasmid-encoded β-lactamases are among the most critical acquired resistance determinants emerging in members of *Enterobacteriaceae* such as *E. coli* (Hooff et al., 2012). The detection of this protein is noteworthy, even though the level of expression was low and poorly correlated with the mRNA levels determined by RNA-Seq. It is also relevant to note that not any other β-lactamase protein was found expressing, unlike the corresponding gene $bla_{\mathrm{CTX-M-15}}$ frequently found carried in ST131 *E. coli* clones accompanied by quinolone resistance gene *aac(6')-Ib-cr* (Chong et al., 2018). Similar uncorrelated levels of RNA and protein expression were found for outer membrane protein TolC [spot 351], a component of the efflux pump system which rids the cell of antibiotics like tetracycline (to which *E. coli* C999 is resistant) and chloramphenicol (Weatherspoon-Griffin et al., 2014). The antibiotic resistance related FabI protein, an enoyl-[acyl-carrier-protein] reductase [NADH], was detected in spot 129 [MW 28074, pI 5.58] of the whole-cell proteome and in spots 88, 89, and 146 of the cytoplasmic sub-proteome. The detection of FabI in various spots, although it occurs at low levels of protein expression, suggests the existence of post-translational modification affecting protein stability (Maier et al., 2009). FabI is a homo-tetrameric enzyme responsible for the catalysis of the last reductive step of fatty acid biosynthesis, and it is a critical target for antibacterials commonly used mediating resistance to *E. coli* enterohemorrhagic serotypes (see **Supplementary Table S1**). In *Staphylococcus aureus*, FabI is known to be inhibited by triclosan, a broad-spectrum antibacterial additive and hexachlorophene, which results in

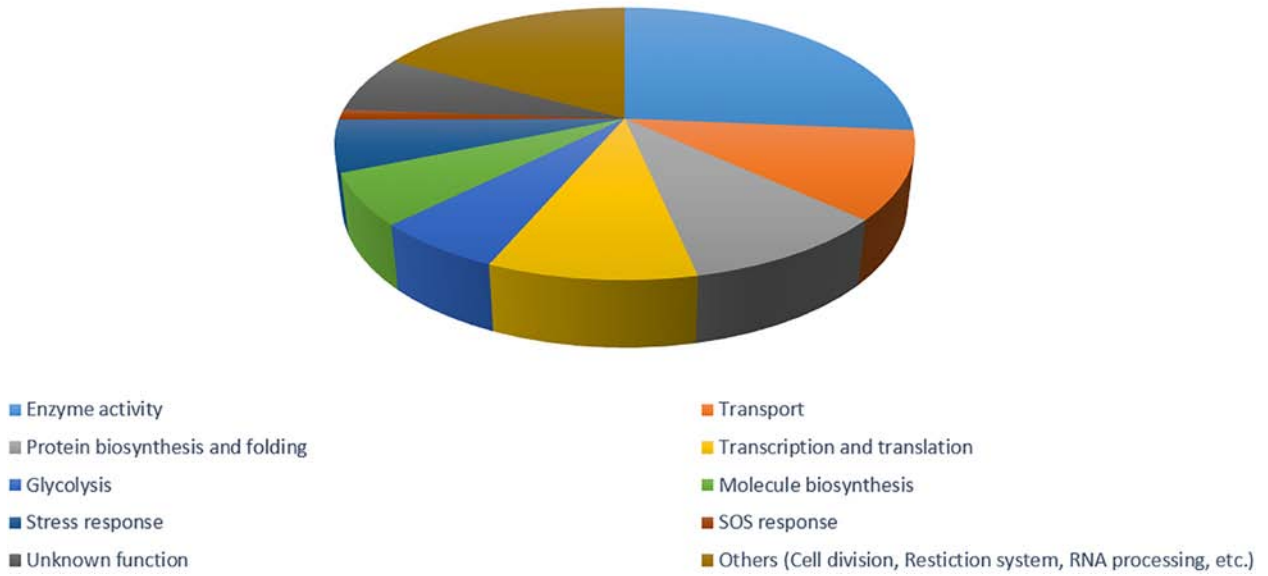


FIGURE 3 | Distribution of *E. coli* strain C999 proteins according to their predicted function in biological processes.

FabI being less effective toward Gram-negative bacteria (Heath et al., 2000; Schiebel et al., 2014). The previously cited bifunctional polymyxin resistance protein ArnA [spot 275] is a pathway-specific enzyme possessing a C-terminal domain which catalyzes the NAD+-dependent oxidative decarboxylation of UDP-GlcA to UDP-β-(L-*threo*pentapyranosyl-4″-ulose) (UDP-4-keto-pentose) (Gatzeva-Topalova et al., 2005b). This pathway is implicated in the pathophysiological effects associated with Gram-negative bacterial infections (Gatzeva-Topalova et al., 2005a). Aminoglycoside 3′-phosphotransferase AphA [spot 73] is reported to be involved in resistance to kanamycin and structurally-related aminoglycosides like tobramycin (Shi et al., 2013). Knowing that kanamycin and tobramycin were tested when phenotyping C999, the detection of the AphA protein confirms that the corresponding resistance is expressed at the proteome level. It is interesting that while ArnA transcript levels were consistently low, the AphA transcripts were not detected, which suggests that some regulatory mechanisms remain to be discovered. In the periplasmic fraction was detected the presence of two hits of Ferrous iron transport protein A, a known virulent factor, but under a very low protein score so that its identification is not validated (**Supplementary Table S9**).

Stress Response, Oxidoreductase, and SOS Response

The environmental stress response is a defense mechanism found in all bacteria in which many different factors regulate gene and protein expression according to the specific stress encountered (Calabrese et al., 2012). The analysis of both the transcriptome and proteomes of C999 revealed the presence of several genes related to stress response mechanisms that increase the survival rate of bacteria, a relevant factor when considering non-commensal bacteria that will therefore endure. Stress response associated Dps (DNA protection during starvation) protein [spots 1, 6, and 157; MW 18684 and pI 5.70], another factor contributing to the bacteria's survival, was highly expressed in both the whole-cell proteome and transcriptome (**Figures 1**, **2** and **Supplementary Table S2**). Very similar to ferritins, Dps has a compact and stable shell-like structure assembled from twelve identical subunits, with the lysine-rich N-termini of each monomer conferring flexibility. When present in stationary phase cells, Dps can bind DNA to form a highly stable DNA-Dps complex, which protects bacteria from oxidative stress or nutritional deprivation caused by harmful environmental stimuli (Stephani et al., 2003; Calhoun and Kwon, 2011). The highly stable protein conformation is also known to influence *E. coli* attachment to abiotic surfaces (Goulter-Thorsen et al., 2011). Expression of chaperone proteins ClpB [MW 95697, pI 5.37], DnaK (HSP70) [MW 69130, pI 4.83], and 60 kDa chaperonin GroEL1 [MW 57464, pI 4.85] is associated with the stress response. DnaK (HSP70) is an ATP-dependent molecular chaperone operating in thermal resistance in bacteria (Miot et al., 2011). In conjunction with ClpB, the DnaK/HSP70 chaperone system, is able to dissolve protein aggregates to protect bacterial cells from the effects of protein inactivation and aggregation caused by great heat stress (Doyle et al., 2007). ClpB is an ATP-dependent molecular chaperone from the AAA+ ATPase superfamily essential for bacterial thermotolerance that was found in the C999 proteomes (see **Supplementary Tables S5**, **S6**, **S9**; del Castillo et al., 2010; Miot et al., 2011). Another

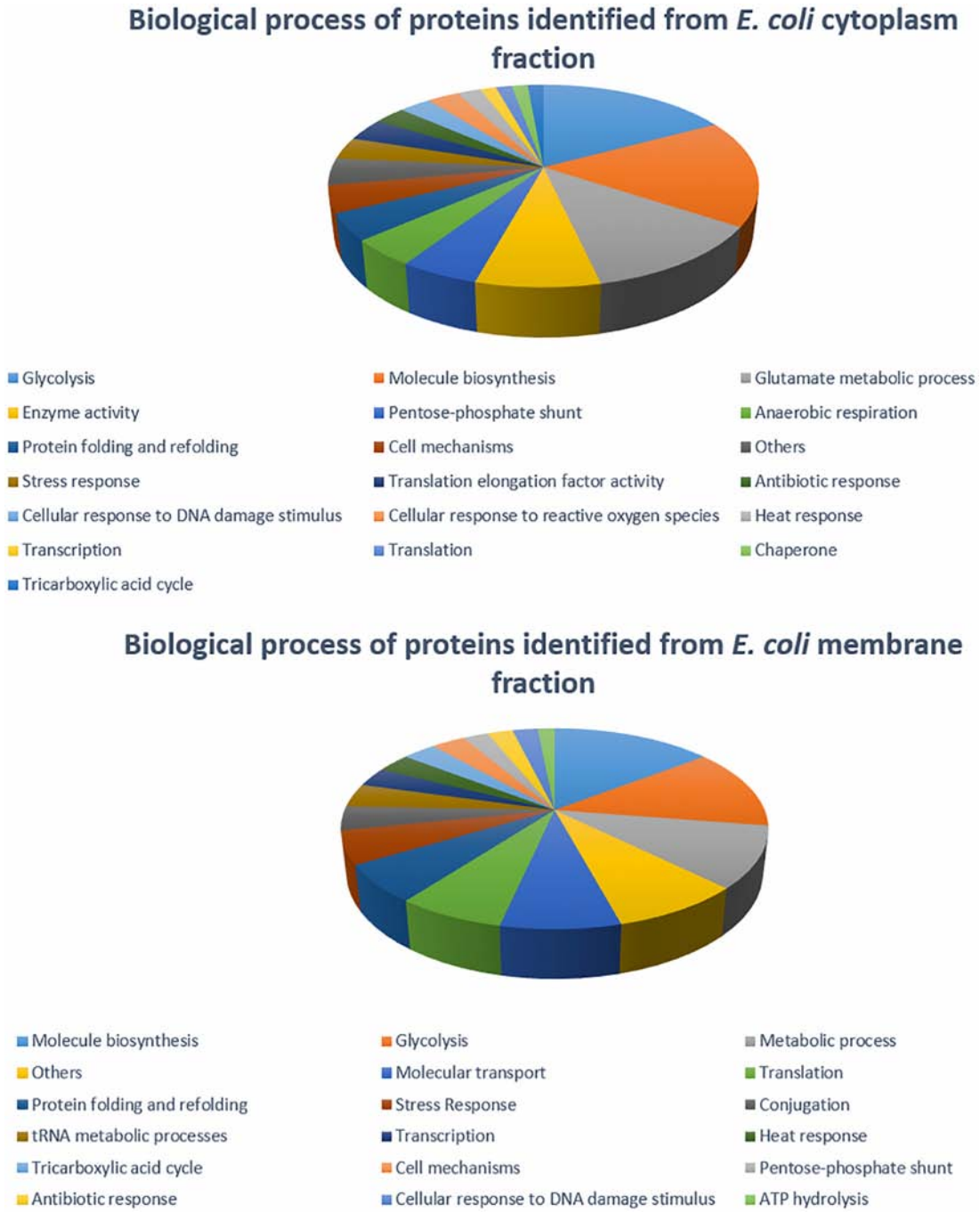


FIGURE 4 | Distribution of *E. coli* strain C999 proteins from cytoplasm and membrane fractions according to their predicted function in biological processes.

major *E. coli* chaperone, GroEL1, was found in whole-cell [spot 19] and also in the cytoplasm [spot 3], periplasm [spot 6] and membrane fraction [spots 18, 19, and 20] (Richter et al., 2010). GroEL belongs to the HSP60 class and plays an important role in protein folding and heat stress resistance. In fact, all three types of chaperones have similar biochemical structures and are involved in protecting cells by resisting heat stress at different stages of the bacterial chemical response (Kyratsous and Panagiotidis, 2012). In terms of oxidative stress defense, oxidoreductase function related proteins SodA [MW 23083, pI 6.44], AhpC [MW 20862, pI 5.03], and thiol peroxidase protein (Tpx) [MW 17995, pI 4.75] were identified in the C999 whole-cell proteome and AhpC was also identified in the C999 membrane fraction (see **Figure 1** and **Supplementary Tables S5**, **S8**). Superoxide dismutase (SodA) removes superoxide leading to the generation of hydrogen peroxide (H_2O_2) which is then removed

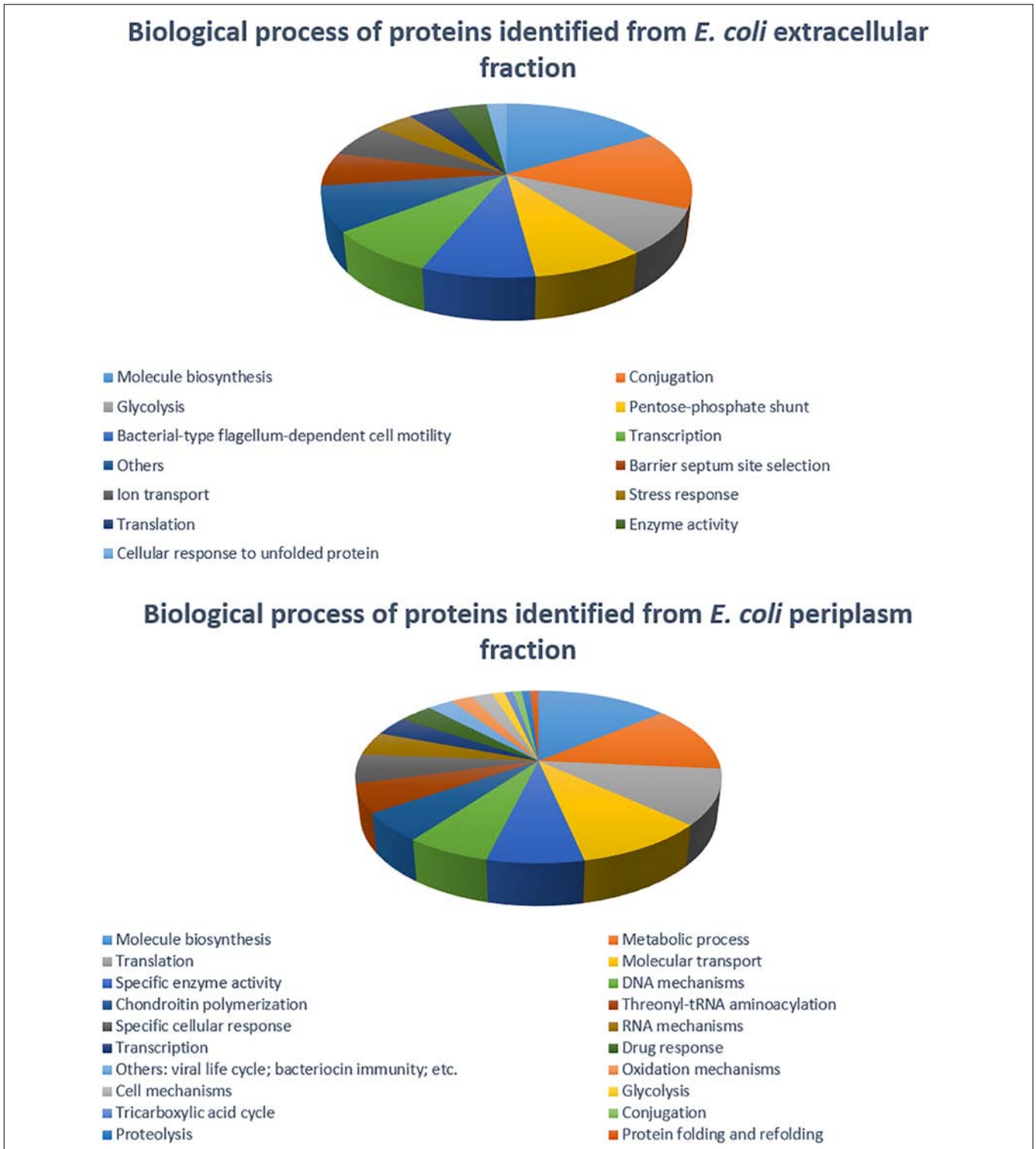
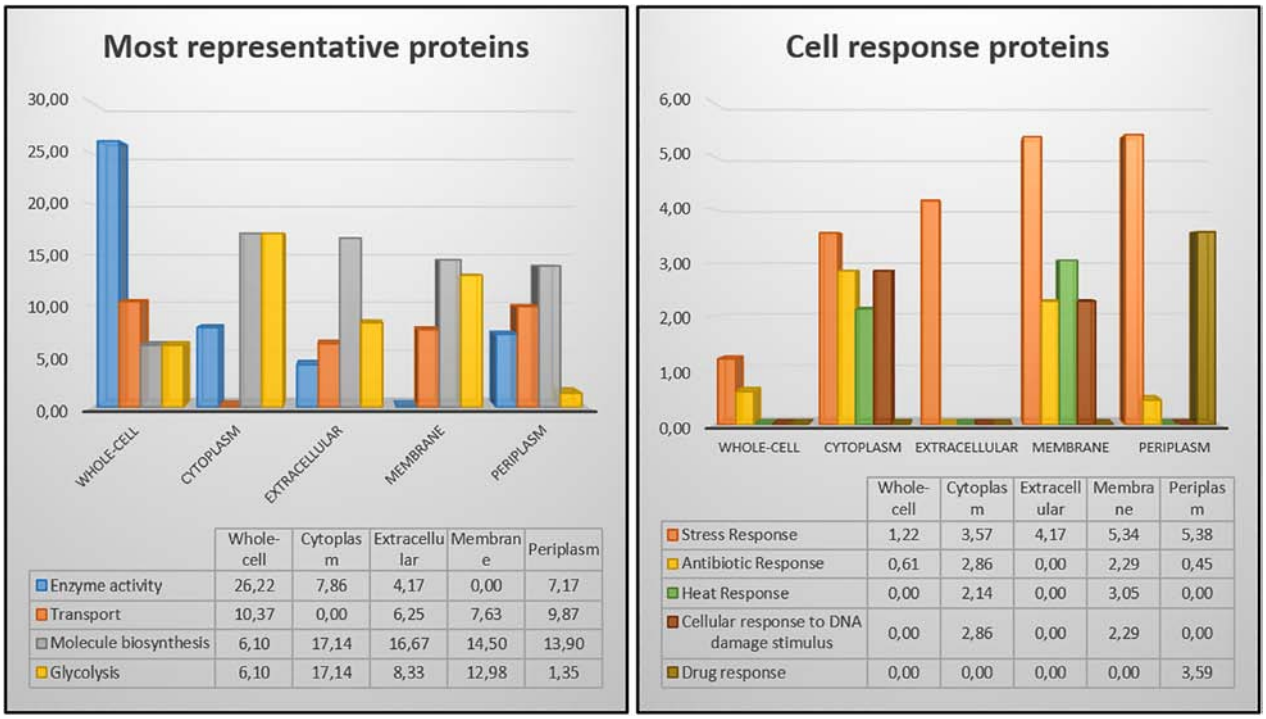


FIGURE 5 | Distribution of *E. coli* strain C999 proteins from extracellular and periplasm fractions according to their predicted function in biological processes.

by catalases like KatE and peroxidases like AhpC, the latter being a very extensively studied bacterial peroxiredoxin system (Jung and Kim, 2003; Seib et al., 2006; Dubbs and Mongkolsuk, 2007). Tpx is involved in the formation of biofilms alongside superoxide dismutase (SodC) in Shiga toxin *E. coli* O157:H7 where these periplasmic oxidative defense proteins are more highly expressed under biofilm-inducing conditions (Kim et al., 2006). Peroxidases Tpx and AhpC were also found to be expressed in *Salmonella*



	Whole-cell	Cytoplasm	Extracellular	Membrane	Periplasm
Enzyme activity	26,22	7,86	4,17	0,00	7,17
Transport	10,37	0,00	6,25	7,63	9,87
Molecule biosynthesis	6,10	17,14	16,67	14,50	13,90
Glycolysis	6,10	17,14	8,33	12,98	1,35

	Whole-cell	Cytoplasm	Extracellular	Membrane	Periplasm
Stress Response	1,22	3,57	4,17	5,34	5,38
Antibiotic Response	0,61	2,86	0,00	2,29	0,45
Heat Response	0,00	2,14	0,00	3,05	0,00
Cellular response to DNA damage stimulus	0,00	2,86	0,00	2,29	0,00
Drug response	0,00	0,00	0,00	0,00	3,59

FIGURE 6 | Comparison of protein distribution among the different cellular fractions and whole-cell extract.

enterica where a *tpx* mutant is more susceptible to exogenous H_2O_2 and is less able to degrade it than the wild type. Tpx therefore contributes to the defense system of this pathogen enabling it to survive oxidative stress (Horst et al., 2010). Another bacterial stress response mechanism involves RNA polymerase sigma factor RpoH, previously described as the main regulator of the heat stress response. RpoH is induced by protein unfolding and cytoplasmic stress in response to heat, DNA damage or antibiotic exposure (see **Supplementary Table S8**; Narberhaus and Balsiger, 2003; Foster, 2007). In our survey, RpoH mRNA was expressed at a high level, but protein expression was low, which might be expected as most heat induced mechanisms are post-trancriptional (Narberhaus and Balsiger, 2003). Also related to the general stress response is the two-component system connector protein sensor-associating-factor A (SafA), a 65-amino-acid membrane protein in whole cells [spots 125 and 153] and in the periplasm [spot 13] that is involved in the acid response network of two-component signal transduction systems. In *E. coli* there are 14 gene products and at least 15 regulators implicated in acid response (AR) biochemistry, where GadE is the main activator protein of resistance genes like gadA and gadE. Regulation of GadE in turn involves several regulators like EvgA and PhoP (Masuda and Church, 2003). EvgS/EvgA is the major system for acid resistance in exponentially-proliferating cells, inducing SafA and thus interacting and activating another connected regulating system, the PhoQ/PhoP system (Eguchi et al., 2011). Also relevant are the chaperone-related curved DNA-binding protein and the Mdh oxidoreductase identified in whole-cell [spot 76], cytoplasm [spot 73], and membrane [spot 305] fractions, and ATP-dependent protease ATP-ase subunit HslU, characteristic of *E. coli* O139:H28 (enterohemorrhagic strain E24377A), found in the whole-cell [spot 164] and cytoplasm [spot 24] (Marzan and Shimizu, 2011). SOS response components figured among our results. The LexA repressor [spot 147; MW 22344, pI 9.64], one of the main proteins regulating the SOS response, was expressed at a low level even though its mRNA levels were high (**Figure 3** and **Supplementary Tables S2**, **S5**). LexA represses the transcription of several genes involved in DNA damage repair to a basal level when a bacterial cell is exposed to UV or to widely used antibiotics, like β-lactams, fluoroquinolones and trimethoprim (Guerin et al., 2011; Yaguchi et al., 2011). Genes *lexA*-regulated have been shown to exhibit phenotypic heterogeneity with different levels of expression detected in different cell subpopulations. The heterogeneous expression is related to differential binding affinity of LexA to SOS boxes when DNA is damaged by external factors invoking the SOS response (Kamensek et al., 2010). On the subject of DNA UV damage, DNA replication and repair protein RecF [spot 169; MW 40717, pI 6.78] was also found. The functional *recF* gene is implicated in several forms of replication such as stable DNA replication and linear plasmid multimer replication, as well as in the recovery of replication in UV-irradiated *E. coli* cells. The RecF protein binds preferentially to single-stranded or linear DNA that arises during DNA metabolism such as replication and normal SOS induction, and repairs DNA breaks and gaps resulting from UV or other stresses. Cells lacking RecF pathway are thus hypersensitive to UV-induced damage (Handa et al., 2009; Ona et al., 2009).

CONCLUSION

In order to find a solution to the concern multidrug-resistance in humans it is vital that researchers possess precise knowledge of the gene and protein expression of the clinical bacterial strains and whether they are related to pandemic strains such as O25:H4-ST131, allowing to understand the dynamic framework surrounding the expansion and endurance of such organisms. In our study, we followed a previous genomic profile of clinical strain *E. coli* C999 revealing characteristics of the extraintestinal pathogenic CTX-M-15 producing *E. coli* clonal group O25:H4-ST131 and exhibiting fluoroquinolone resistance as other plasmid-mediated resistances. Through transcriptomics tools we were able to confirm our strain to be O25:H4-ST131 and also identify several genes related to antibiotic resistance and survival-related processes like stress and SOS response. Proteomics allowed the identification and quantification of several proteins regarding also antibiotic resistance and stress response, within some degree of correlation to the RNA expression. While the proteomics data is very valuable, transcriptomics using RNA-Seq provide precise transcript quantification so mRNA and protein levels can be compared. However, the lack of correlation between mRNA and protein expression (or the difficulty in detecting it) indicates there is much to discover about cellular mechanisms of gene regulation that could advance our understanding of antibiotic resistance. It will be necessary to investigate such relationships, particularly in terms of specific stimuli, by increasing sampling frequency in a metaomics approach, for example. In summary, omics-based studies of the metabolic pathways of antibiotic resistance should continue to be done if answers and sustainable solutions are to be found.

AUTHOR CONTRIBUTIONS

LP carried out laboratory work, data analysis, and drafted the manuscript. CT, PP, CG, JN-M, and GI implemented data analyses and helped to draft the manuscript. GI, PP, and CG conceived the study and revised the manuscript. VB, JG, CS, and GI helped interpret compiled data. HS, JP, LV, and GI provided facilities and helped implementing the laboratory work. CS and LV performed WGS and RNAseq wet-lab procedures. VB and JG performed nucleic acids extraction/depletion for WGS and RNAseq, and associated bioinformatics analyses. All the authors reviewed and contributed to the manuscript, approving its submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00831/full#supplementary-material

TABLE S1 | Antibiotic resistance genes of ESBL-producing *E. coli* isolate C999 identified by whole-genome sequencing and prediction using CARD software.

TABLE S2 | Summary of the metabolic pathways activated according to the total RNA sequencing of *E. coli* isolate C999.

TABLE S3 | Full integration of proteomic and transcriptomic data.

TABLE S4 | Integration of proteomic data of interest with corresponding transcriptomic data.

TABLE S5 | Identification of protein spots from 2DE gels of whole-cell extracts of ESBL-producing *E. coli* isolate C999 based on MALDI-TOF/MS sequencing results.

TABLE S6 | Identification of protein spots from 2DE gels of cytoplasmic extracts of ESBL-producing *E. coli* isolate C999 based on MALDI-TOF/MS sequencing results.

TABLE S7 | Identification of protein spots from 2DE gels of extracellular extracts of ESBL-producing *E. coli* isolate C999 based on MALDI-TOF/MS sequencing results.

TABLE S8 | Identification of protein spots from 2DE gels of membrane extracts of ESBL-producing *E. coli* isolate C999 based on MALDI-TOF/MS sequencing results.

TABLE S9 | Identification of protein spots from 2DE gels of periplasm extracts of ESBL-producing *E. coli* isolate C999 based on MALDI-TOF/MS sequencing results.

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REVIEW
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Antimicrobial Effects on Swine Gastrointestinal Microbiota and Their Accompanying Antibiotic Resistome

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Antimicrobials are the most commonly prescribed drugs in the swine industry. While antimicrobials are an effective treatment for serious bacterial infections, their use has been associated with major adverse effects on health. It has been shown that antimicrobials have substantial direct and indirect impacts on the swine gastrointestinal (GI) microbiota and their accompanying antimicrobial resistome. Antimicrobials have also been associated with a significant public health concern through selection of resistant opportunistic pathogens and increased emergence of antimicrobial resistance genes (ARGs). Since the mutualistic microbiota play a crucial role in host immune regulation and in providing colonization resistance against potential pathogens, the detrimental impacts of antimicrobial treatment on the microbiota structure and its metabolic activity may lead to further health complications later in life. In this review, we present an overview of antimicrobial use in the swine industry and their role in the emergence of antimicrobial resistance. Additionally, we review our current understanding of GI microbiota and their role in swine health. Finally, we investigate the effects of antimicrobial administration on the swine GI microbiota and their accompanying antibiotic resistome. The presented data is crucial for the development of robust non-antibiotic alternative strategies to restore the GI microbiota functionality and guarantee effective continued use of antimicrobials in the livestock production system.

Keywords: antimicrobial, gastrointestinal, microbiota, swine, resistome

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INTRODUCTION

Recently, the swine industry has focused on sustainable pork production which maximizes value over production costs and represents a shift away from antimicrobial usage. There is an urgent need not only for higher production efficiency to meet consumer expectations, but also for the development of new phenotypes related to host vitality and robustness (Merks et al., 2012). Phenotypic development in swine is a complex multistage process, starting from conception stage and continuing throughout the entire production cycle (Pluske, 2016). There are four major criteria that drive the phenotypic development and ultimately impact swine health, including host factors, management inputs, stable microbial ecosystem, and surrounding physical environment **(Figure 1)**. Some human data and animal experiments have revealed that the crosstalk and interaction between microbial environment and other phenotypic drivers are the key distinguishers of host health (Blaut and Clavel, 2007; Metzler and Mosenthin, 2008). The swine microbial ecosystem is composed of rich and diverse populations that harbor thousands of different microbial

species (aerobic, facultative anaerobic, and strictly anaerobic), dwelling in different anatomical biogeographic locations (Metzler and Mosenthin, 2008; Holman et al., 2017). These mutualist populations have a wide range of functions, including providing colonization resistance against potential pathogens, absorbing different kind of nutrients, modulation of the host's immune system, metabolizing indigestible polysaccharides, and regulating the host's metabolism (Bischoff, 2011; Venable et al., 2016). Therefore, alteration of the swine microbial environment may detrimentally influence the host's health status and inhibit the pathogens colonization (Marchesi et al., 2016). Understanding the mechanistic pathways and abundance of these alternations are required to discover new and different management practices to promote growth rate, increase efficiency of feed utilization, and improve overall swine health.

With recent advances in our understanding of swine microbial ecosystem structures and functions, we are becoming increasingly aware of the impacts of antimicrobial on mucosal microbiota and how its use negatively impacts the host's health (Zeineldin et al., 2018b). Equally important is the potential enrichment of antimicrobial resistome between the commensal microbiota as a result of antimicrobial use, which is one of the most vital public health issues that we currently face (Wright, 2007). The detrimental impacts of antimicrobial on the GI microbiota and host health are summarized in **Figure 2**. Traditionally, the impacts of antimicrobial administration on GI microbiota structures and development of antimicrobial resistance were largely characterized by culture-based techniques and/or a PCR-based approach, both of which underestimate the presence of novel ARGs (Zhu et al., 2013). Consequently, culture independent platforms (real-time PCR quantification, next generation sequencing, and functional metagenomics) have been used to efficiently quantify and assess the resistant opportunistic pathogens and emergence of antimicrobial resistome (Gerzova et al., 2015). While antimicrobial intervention disrupts GI microbiota structures and function, we are just beginning to estimate the relative contribution of its use on emergence of the antimicrobial resistome.

In this review, we present an overview of antimicrobial use in the swine industry and its association with the emergence of antibiotic resistance genes (ARGs). Additionally, we review our current understanding of GI microbiota and its role in swine health. Finally, we explore the effects of antimicrobial use on the swine GI microbiota and their accompanying antimicrobial resistome. The presented data is vital for the development of robust non-antibiotic alternative strategies to restore the GI microbiota functionality and guarantee effective continued use of antimicrobial in the livestock production system.

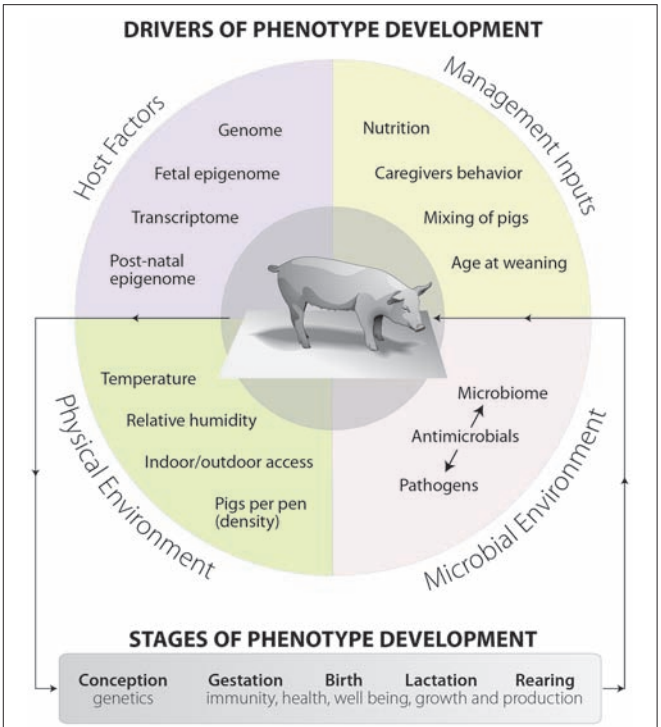

FIGURE 1 | The drivers and main components of phenotype development in swine production systems. Phenotype development is made up of four separate but often blended sets of factors and overlapping components. These components are host factors, management inputs, microbial environment, and physical environment. The dashed arrows represent interactions between drivers and can occur between any drivers. This diagram also shows the stages of phenotype development, starting from conception and moving toward rearing.

ANTIMICROBIAL USE IN SWINE MANAGEMENT SYSTEM

In the swine industry, antimicrobial has four potential uses: (1) disease treatment, (2) disease control, (3) disease prevention, and (4) increased the growth performance (O'Neill, 2014). It is therefore unsurprising that antimicrobial is the most commonly prescribed drug in the swine industry (Dumas et al., 2016). It is estimated that all food-producing animals consume more than 70% of antimicrobial produced worldwide. The pigs are usually raised in groups, in close proximity to one another. Many production systems use all-in, all-out management to control and prevent infectious disease outbreaks (Dewey et al., 1999). However, high contact rates provide optimal conditions for the spread of infectious diseases, many of which require the use of antimicrobials to minimize economic losses and welfare concerns. Estimates range from 62% of nursery production units and 44% of grower/finisher units (McEwen and Fedorka-Cray, 2002) to 33% of nursery units and 30% of grower/finisher units use of antimicrobial for growth promotion (Holman and Chénier, 2015). Data collected in 2001 by the USDA for US herds found that 70% used antimicrobials in starter feeds, 59% used them in grower/finisher feeds, and 46% used them in sow feeds (Cromwell, 2002), which were higher than the estimates of McEwen and Fedorka-Cray in similar populations (McEwen and Fedorka-Cray, 2002). According to the Food and Drug Administration (FDA), the available antimicrobial classes and chemotherapeutic agents (chemically synthesized agents with antimicrobial activity) for use in swine are listed in **Table 1** (FDA, 2017). Certain classes of these antimicrobial are approved

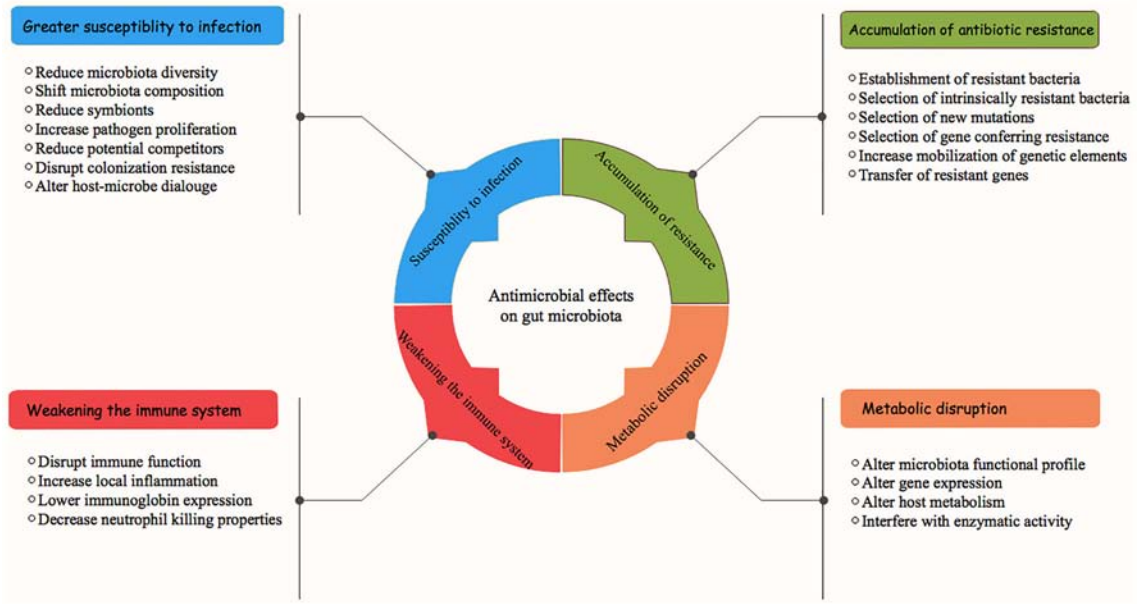

FIGURE 2 | Antimicrobial effects on swine gastrointestinal microbiota and associated health consequences.

and validated for their ability to be successfully combined with other antimicrobials (e.g., chlortetracycline, penicillin, and sulfamethazine), whereas others cannot be combined with other antimicrobials.

The antimicrobial spectrum, administration dosage, pharmacokinetics and pharmacodynamics vary greatly according to different antimicrobial classes and their chemical structures (Cromwell, 2002). Some antimicrobials are easily absorbed after both therapeutic and subtherapeutic administration (e.g., oxytetracyclines and sulfonamides), whereas other antimicrobials are poorly absorbed after administration (e.g., bacitracin). In swine industry, the duration of antimicrobial administration typically ranges from 20 to 40 days for disease prevention and control (Stone et al., 2009). Alternatively, for growth promotion, antimicrobials are generally used for a long period of time at relatively low concentrations. While the mode of action of antimicrobial growth promotion remains poorly characterized, several potential mechanisms have been proposed. These mechanisms include decreased production of harmful metabolites (metabolic effect), increased absorption of available dietary nutrients (nutritional effect), and reduction of endemic subclinical diseases (disease control effect; Dibner and Richards, 2005). It is remarkable that antimicrobial use as a growth promoter in younger pigs is consistently efficacious while little to no response is seen in older animals (Cromwell, 2002; Skinner et al., 2014). In growing piglets, the average duration of antimicrobial use for growth promotion ranges from 22.7 to 76.8 days (Dewey et al., 1997). This prolonged exposure to sub-therapeutic antimicrobial concentrations provides ample opportunity for antimicrobial resistance to develop, particularly when compared to therapeutic use (Aarestrup et al., 2008). Consequently, there is increasing consumer desire to make sub-therapeutic antimicrobial use less frequent in livestock production (Sommer et al., 2017).

Several published studies have addressed the safety of antimicrobials, all of which could not identify a direct link between antimicrobial use in livestock and human health (Phillips et al., 2004; Chang et al., 2015). In contrast, a systematic review about restricting antibiotic use in animals and its association with antibiotic resistance in human beings concluded that antimicrobial use in food-producing animals is recognized as one of the major contributors to development of resistant organisms that result in life-threatening human infections (Landers et al., 2013). But, in general, it seems inevitable that antimicrobial administration in animals and its relationship to human health remain unquantified.

ASSOCIATION BETWEEN ANTIMICROBIAL USE AND ANTIMICROBIAL RESISTANCE

Since the discovery of antimicrobials, the main goal of its use in the swine industry has been to eliminate pathogenic microbes, thereby facilitating growth and restoration of beneficial microbial communities (Holman and Chénier, 2015). However, these goals are routinely complicated by presence and dissemination of ARGs among microbes (McEwen and Fedorka-Cray, 2002). Resistance to antimicrobials is a natural occurrence, developed by the microbes to help in their survival against other antibiotic-producing microorganisms in the surrounding environment (Phillips et al., 2004). In many cases, detection of clinical signs for a disease in an individual animal provokes prophylactic treatment for the whole herd (Founou et al., 2016). This approach can increase abundance of resistant bacterial strains and elevate the expression of ARGs (Langdon et al., 2016).

TABLE 1 | Available antimicrobial classes and chemotherapeutics agents for use in swine.

Antimicrobial class	Antimicrobial drug	Spectrum and mode of action	Importance to human
Aminocoumarins	- Novobiocin	Narrow-spectrum antimicrobial that may be bacteriostatic or bactericidal at higher concentrations, that act by inhibiting bacterial DNA gyrase and work by targeting the GyrB subunit of the enzyme involved in energy transduction	Not medically important
Aminoglycosides	- Dihydrostreptomycin - Gentamicin - Neomycin - Spectinomycin	Broad-spectrum and potent bactericidal antimicrobials that act by inhibiting bacterial protein synthesis	Medically important
Amphenicols	- Florfenicol	Broad spectrum, bacteriostatic antimicrobial that acts by binding to the 50S ribosomal subunit of susceptible bacteria, preventing bacterial protein synthesis. It may be bactericidal against some very susceptible organisms	Medically important
Cephalosporins	- Ceftiofur hydrochloride - Cephapirin - Ceftiofur crystalline free acid	Broad-spectrum, bactericidal antimicrobials that act by disrupting the synthesis of the peptidoglycan layer forming the bacterial cell wall	Medically important
Diaminopyrimidines	- Ormetoprim	Broad-spectrum, bacteriostatic antimicrobial that acts by mimicking the substrate of respective enzymes and inhibiting the enzyme by blocking the active site of the enzyme	Medically important
Fluoroquinolones	- Danofloxacin - Enrofloxacin	Broad-spectrum, bactericidal antimicrobials that act by inhibiting DNA synthesis	Medically important
Ionophores	- Lasalocid - Monensin - Narasin - Salinomycin	Broad-spectrum biologically active molecules produced by microorganisms (mainly spore-forming bacteria) that act by specifically increasing the ion permeability of the cell membrane	Not medically important
Lincosamide	- Lincomycin - Pirlimycin	Broad-spectrum and bacteriostatic antimicrobials that act by interfering with the synthesis of proteins	Medically important
Macrolides	- Erythromycin - Gamithromycin - Tilmicosin - Tulathromycin - Tylosin	Broad-spectrum antimicrobials, dependent on concentration and bacterial species, that are either bactericidal or bacteriostatic; which act by inhibiting protein synthesis	Medically important
Penicillins	- Amoxicillin - Ampicillin - Cloxacillin - Penicillin	Narrow-spectrum bactericidal antimicrobials that act by specifically inhibiting the transpeptidase enzyme that catalyzes the final step in cell wall biosynthesis, the cross-linking of peptidoglycan.	Medically important
Polymyxins	- Polymyxin B (colistin)	Narrow-spectrum bactericidal antimicrobial that acts by disruption of both the outer and inner membranes of bacteria.	Medically important
Polypeptides	- Bacitracin	Narrow-spectrum, bacteriostatic antimicrobial; may be bactericidal, depending on the antimicrobial concentration and the susceptibility of the specific organism. Bacitracin acts by inhibition of the incorporation of amino acids and nucleotides into the cell wall	Not medically important
Quinoxalines	- Carbadox	Bactericidal synthetic antimicrobial that is primarily effective against gram-positive bacteria, with little efficacy against some gram-negative bacteria. The mechanism of carbadox action is not known	Not medically important
Streptogramins	- Virginiamycin	Bacteriostatic antimicrobial that acts by inhibition of cell growth of gram-positive bacteria and by inhibition of protein synthesis in gram-negative bacteria	Medically important
Sulfonamides	- Sulfadimethoxine - Sulfamethazine	Broad-spectrum, bacteriostatic antimicrobials that act by interfering with folic acid synthesis by preventing addition of para-aminobenzoic acid into the folic acid molecule through competing for the enzyme dihydropteroate synthase	Medically important
Tetracyclines	- Oxytetracycline - Chlortetracycline	Broad-spectrum, bacteriostatic antimicrobials that act by inhibition of bacterial protein synthesis	Medically important

Traditionally, the impacts of antimicrobial treatment on emergence of antimicrobial-resistant bacteria have focused only on pathogenic bacteria (e.g., *Salmonella, E. coli, Shigella,* and *Enterobacter*; Founou et al., 2016). Many researches have investigated the association between antimicrobial use in livestock and development of antimicrobial resistance across the resident microbiota (Everaert et al., 2017; Johnson et al., 2017). When an antimicrobial is administered, it eliminates the susceptible microbial populations, leaving behind resistant strains that continue to evolve and multiply in its number (Founou et al., 2016). Selective pressure from antimicrobial exposure is exploited by antimicrobial-resistant microbes, providing them with an evolutionary advantage (Brandl et al., 2008). The resistant microbes, in presence of antimicrobials, also have a competitive advantage which facilitates its spread among other microbial populations in the surrounding ecosystem (Holmes et al., 2016). The dissemination of ARGs requires acquisition or transfer of genetic elements

encoding antimicrobial resistance between the bacterial strains. The resistant bacterial populations transmit their genetic resistance pools to their progeny through vertical evolution or to other adherent bacterial species through horizontal transmission (D'Costa et al., 2007). Vertical gene transfer occurs during cell division, where resistant genes either on chromosomes or plasmids transfer to the progeny cells, leading to bacterial resistance (Lawrence, 2004). Alternately, horizontal gene transfer involves genetic pool exchange within and between the microbial populations, where genetic density and complexity of the commensal microbial community stimulate the spread of ARGs among microbes (Founou et al., 2016). The resistant genetic material is usually acquired by microbes either through conjugation, transformation, and/or transduction (Holmes et al., 2016). It is then possible for new mobile genetic element-associated transmission of antimicrobial resistance determinants to be incorporated into the bacterial chromosome or replicate independently (Sommer and Dantas, 2011). The presence of mobile genetic elements (plasmids, integrative conjugated elements, transposons, and integrons) are therefore important in transmission of antimicrobial resistance among microbes (D'Costa et al., 2007). The reservoirs of antimicrobial-resistant bacteria are ubiquitous and can merge with the GI resident microbiota through two different mechanisms (Holman and Chénier, 2015). First, the resistant bacteria can be acquired directly by the host and colonize the GI mucosal epithelium; secondly, a previously susceptible bacterial species can become resistant through induction of antibiotic-resistant mutants or through resistant gene transfer events (Crofts et al., 2017). While there is a clear association between the use of antimicrobial and emergence of antimicrobial resistance, this relationship is complex and influenced by multiple confounding factors (e.g., pathogen-host interactions, pathogen–drug interactions, rate of mutation, rate of transmission, cross-resistance, and co-selection of resistance to unrelated drugs; Holmes et al., 2016).

EFFECT OF ANTIMICROBIAL INTERVENTION ON SWINE GASTROINTESTINAL MICROBIOTA

The term microbiome is widely used to describe the resident populations of different organisms (bacteria, viruses, fungi, archaea, and protists) that live and/or colonize the body of multicellular host and their genetic material (Turnbaugh et al., 2007). Swine GI microbiota is not uniform and differs drastically between individuals, even individuals raised in the same management system. Additionally, the relative abundance of specific bacteria differ according to different GI biogeographic locations (Leser et al., 2002; Maradiaga et al., 2018; Yeoman et al., 2018), with richer and more diverse communities in the colon compared to the ileum and stomach (Holman and Chénier, 2015). Understanding how GI microbiome composition affects swine health is an emerging area of research (Isaacson and Kim, 2012; Zeineldin et al., 2017a). However, the exact mechanisms of how GI microbiota contributes to swine health are still unclear. There are new studies endeavoring to increase our understanding about this mechanism (Pluske et al., 2018). GI mutualistic microbiota play an important function in bile salt recycling, volatile fatty acid production, cellulose digestion, metabolism of undigested carbohydrates, and nutrient recovery (Bischoff, 2011). Additionally, GI microbiota contribute to resistance against colonization of pathogenic microbes through competition for binding sites, nutrient utilization at mucosal epithelium, and modification of local environment (Mach et al., 2015). Therefore, understanding different factors that shape swine GI microbiota and their composition, particularly in early life, are required to discover new targets and/or develop novel management practices to promote optimal GI microbiota development.

With the advancement of methodologies to assess microbiota composition (Zeineldin et al., 2017b), several considerations have been raised regarding the impact of antimicrobial administration on the resident microbial populations in swine (Bokulich et al., 2016). There are several reports and longitudinal studies that attempt to understand the impacts of antimicrobial intervention on swine GI microbiota (Gerzova et al., 2015; Holman and Chénier, 2015; Oultram et al., 2015; Bokulich et al., 2016; Founou et al., 2016; Holman et al., 2018; Zeineldin et al., 2018a). **Table 2** lists a summary of the existing metagenomic studies on the impacts of antimicrobial administration on swine GI microbial communities. Commonly, antimicrobial is given to wipe out pathogenic microbes during acute infection (Dewey et al., 1999). However, several antimicrobial classes are not specific, and consequently wipe out a wide range of resident GI microbiota that are beneficial and pivotal for health (Neuman et al., 2018). Recently, a comprehensive review by Langdon et al. revealed that short and long term antimicrobial intervention in humans drastically changes both adult and neonatal microbiota structure (Leibovitz et al., 2003; Langdon et al., 2016). This shift has been associated with an increased chance of subsequent GI disease (Pettigrew et al., 2012). Although the shifts in microbiota composition occurred after antimicrobial administration, some populations have returned to a pretreatment state within 4 weeks following a single-dose treatment. Other taxa, meanwhile, failed to return to pretreatment levels even after 6 months following treatment (Jernberg et al., 2010). Similarly, shifts in the GI microbiota in other animals after antimicrobial administration (a combination of metronidazole, amoxicillin and bismuth) dissipated after cessation of treatment (Schmidt et al., 2009). The precise components responsible for GI microbiota recovery after antimicrobial administration are still undefined. Recognition of different factors that promote microbiota recovery after antimicrobial administration open up new opportunities for development of novel therapies that promote the GI health.

It is important, when quantifying the impacts of antimicrobial intervention on swine GI microbiota structure, to consider the ages of the studied populations, route of administration and the class of the administered antimicrobial (Neuman et al., 2018). While there are some similarities between the effects of antimicrobial administration on GI microbiota structure in growing and neonatal piglets, there are also significant dissimilarities due to distinct characteristics of the neonatal microbial composition. A recent study of 16 42-day-old ileal-cannulated pigs demonstrated that oral administration of ampicillin, gentamicin, and metronidazole treatment modified

GI microbial population structure and function (Gao et al., 2018b). More precisely, use of ampicillin, gentamicin, and metronidazole decreased the *Lactobacillus* and *Bifidobacterium* abundance and increased the abundance of *Shigella* species by 256-fold compared to the control pigs (Gao et al., 2018b). Similarly, early life amoxicillin administration in neonatal piglets during the first 14 days of life exerted transient impacts on developing gut microbiota and decreased the genes involved in short-chain fatty acid signaling and pancreatic development (Li J. et al., 2017). In neonatal piglets, early life antimicrobial administration also resulted in differential dysbiosis of GI microbiota, with major alteration between different geographical locations. For instance, a mixture of olaquindox, kitasamycin, and oxytetracycline calcium administration decreased the relative abundance of beneficial *Lactobacillus* species and increased the relative abundance of potentially pathogenic *Streptococcus suis* in both the small intestine and stomach lumen (Mu et al., 2017). In growing piglets, antimicrobial administration also induced microbiota compositional changes in both abundant and less abundant GI microbiota. For example, tylosin-treated piglets showed higher relative abundance of *Lactobacillus, Eggerthella, Acetanaerobacterium,* and *Sporacetigenium* genera compared to control piglets (Kim et al., 2012). A mixture of amoxicillin and colistin sulfate treatment in post-weaning piglets also resulted in different digestive microbiota profiles along the entire gastrointestinal tract (Soler et al., 2018). Similarly, in-feed administration of colistin sulfate and bacitracin zinc in weaned piglets caused a significant shift in GI microbiota composition along different biogeographic gut locations (Li K. et al., 2017).

Published data also suggested that different classes of antimicrobial disrupt GI microbiota in different ways. This should be included in the decision-making process for antimicrobial prescription in livestock management systems. When assessing the impacts of in-feed sub-therapeutic concentrations of two common antimicrobials (tylosin and chlortetracycline) on swine GI microbiota composition, tylosin administration resulted in a major shift in the relative abundance of several taxa, while chlortetracycline administration only resulted in minor alterations (Holman and Chénier, 2014). Similarly, oral vancomycin and metronidazole have different effects on *Clostridium difficile,* where only vancomycin had an obvious impact on microbial community structure (Lewis et al., 2015). The simplest mechanistic explanation for variation in the swine GI microbiota response to antimicrobial intervention is due to differences in antimicrobial spectrum, route of administration, and degree of antimicrobial resistance (Kim et al., 2012; Looft et al., 2014a,b; Schokker et al., 2015; Mu et al., 2017; Soler et al., 2018).

GASTROINTESTINAL MICROBIOTA AS A RESERVOIR OF ANTIMICROBIAL RESISTOME

The concept of the antimicrobial resistome was proposed by Gerard Wright in 2007 as a means of describing the collection of all known ARGs in the microbial ecosystem and their precursors at multiple levels (e.g., environment, pathogenic, and non-pathogenic microbes; Wright, 2007). Historically, determination of ARGs have primarily relied on conventional culture-based methods, with a focus on major pathogens that are readily cultured (Isaacson and Kim, 2012). While beneficial, these protocols do not provide information on the total amount of ARGs in the bacterial community as most species in that community cannot be cultivated, likely underestimating the complexity of the antimicrobial resistome (Henriksson et al., 1995). Although the antimicrobial resistome is theoretically accessible to all bacteria, the GI microbiota harbor a distinct antimicrobial resistome (Sundin and Wang, 2018). The known ARGs are likely to represent just a small portion of actual antimicrobial resistome populations. It is reasonable to assume that with the explosion of bacterial genome sequencing and functional metagenomics, many novel ARGs that were previously of unknown function and unrecognizable by sequence alone will be identified (D'Costa et al., 2007). The generation of more information about ARGs will be helpful in understanding the relationship between the resident microbial communities and their accompanying resistome (Boolchandani et al., 2019).

In parallel with the consecutive development of GI microbiota, the antimicrobial resistome is established during first few days of life or perhaps during prenatal phase even without prior exposure to antimicrobial treatment (Wright, 2007; Zeineldin et al., 2019). This concept endorses the theory that resistant bacteria and their antimicrobial resistome are established shortly after birth and are acquired either directly from the mother or through direct contact with resistant bacteria in surrounding environment (Gonzales-Marin et al., 2012). The GI microbiota has a large and diverse genetic pool that facilitates transmission of resistance between and within the resident commensal species (Sengupta et al., 2013). The effects of different antimicrobial intervention on emergence of the antimicrobial resistome has been extensively demonstrated (Wright, 2007; Enwemeka, 2013). In people, when the infants received antimicrobial treatment in the first 3 years of life, the GI microbiota expressed high levels of antimicrobial resistance compared to the control (Yassour et al., 2016). Similarly, the abundance of 149 ARGs conferring resistance to different classes of antimicrobials were detected in the swine feces from production units that used different antimicrobials either orally or via intramuscular injection (Zhu et al., 2013). Emergence of antimicrobial resistance determinants in pigs without prior antimicrobial administration has been also demonstrated previously (Pakpour et al., 2012; Agga et al., 2015), with the largest resistance category being against tetracycline antibiotic (Chambers et al., 2015). For instance, several tetracycline resistance genes (e.g., tetO, tetW, tetM, tetX, and tetQ), and macrolide resistance genes (e.g., ermG, ermF, and ermB) were frequently identified in the swine facilities in the absence of antimicrobial exposure (Looft et al., 2012). Similarly, our recent study showed that the neonatal piglets displayed a high frequency of ARGs without prior exposure of antibiotics

TABLE 2 | Summary of the existing metagenomic studies on the impacts of antimicrobial intervention on the swine gastrointestinal microbiota.

Study population	Population age	Samples type	Sequencing platform and 16S rRNA gene hypervariable region	Antimicrobial class	Route of administration & duration	Data availability accession number	Finding	References
20 piglets	8 weeks	Fecal swabs	Illumina MiSeq (V1–V3)	Ceftiofur crystalline free acid, ceftiofur hydrochloride, oxytetracycline, procaine penicillin G and tulathromycin	Parenteral-Single dose	PRJNA323521	Analysis revealed a pronounced, antimicrobial-dependent shift in the composition of fecal microbiota over time from day 0. By day 14, the fecal microbial compositions of the treatment groups had returned to a distribution that closely resembled that observed on day 0, but differences were still evident.	Zeineldin et al., 2018a
16 ileal-cannulated piglets	42 days	Fecal and ileal samples	Illumina MiSeq sequencing (V3–V4)	Ampicillin, gentamicin, and metronidazole	In feed for 13 days	SRP115601	Antibiotics administration increased abundance *of Escherichia/ Shigella* and decreased abundance of *Lactobacillus* and *Bifidobacterium.*	Gao et al., 2018b
30 piglets	Post-weaning	Feces	Illumina MiSeq sequencing (V1–V2)	Amoxicillin and colistin sulfate	In feed for 30 days	PRJNA445806	Antibiotics administration had a positive effect in the endogenous microbiota of post-weaning pigs with clear increase in abundance of *Bacillus* and *Lactobacillus* Spp.	Soler et al., 2018
16 litters of suckling piglets	7 days	Ileal and cecal digesta	Illumina MiSeq sequencing (V3–V4)	Olaquindox, oxytetracycline and kitasamycin	In feed from days 7 until days 23 of old	SRP 132384	Antibiotics administration significantly decreased bacterial diversity and richness in ileum. Antibiotics significantly reduced the abundance of *Lactobacillus* in both ileum and cecum, increased the abundance of *Streptococcus, Enterococcaceae, Fusobacteriales*, and *Corynebacterium* in ileum, and increased the abundance of *Ruminococcaceae* and *Erysipelotrichaceae* in cecum.	Yu et al., 2018
12 weaned piglets	45 days	Fresh digesta from the proximal ileum and feces	Illumina MiSeq sequencing (V3–V4)	Ampicillin, gentamicin, and metronidazole	Infused the antibiotic mixture with 10 mL saline through T-cannula in distal ileum for 25 days	SRP124814	The antibiotic infusion did not change the proximal ileal microbial composition, but it markedly altered the fecal microbial composition and increased aromatic amino acid metabolism.	Gao et al., 2018a
69 crossbred barrows	21 days	Intestinal contents from the ileum and colon	Illumina MiSeq sequencing (V3–V4)	Chlortetracycline and colistin sulfate	In feed for 28 days	SRP095386	In-feed antibiotic supplementation significantly increased *Spirochaetes, Tenericutes, Euryarchaeota, Verrucomicrobia, TM7* and reduced *Chlamydiae* in ileal digesta.	Yu et al., 2017

(Continued)

TABLE 2 | Continued

Study population	Population age	Samples type	Sequencing platform and 16S rRNA gene hypervariable region	Antimicrobial class	Route of administration & duration	Data availability accession number	Finding	References
4 litters of crossbred piglets	After birth	The entire intestine was subsequently extracted.	Illumina MiSeq sequencing (V1–V3)	Amoxicillin	Oral feeding- twice daily at birth until day 14	None	Antibiotic administration exerted a transient impact on postnatal gut microbiota colonization and microbial metabolite production.	Li J. et al., 2017
60 weaning piglets	28-d-old	Luminal contents of the jejunum, ileum, cecum, and colon	Illumina MiSeq sequencing (V3–V4)	Colistin sulfate	In-feed-28 days	None	Antibiotic treatment resulted in modulation of gut microbiota with decreased abundance of *Acholeplasma, Arcobacter, Caldicoprobacter, Desulfotomaculum, Ignatzschineria, KSA1, Leptolyngbya, Natronincola_Anaerovirgula, Pseudomonas, Pseudoramibacter_Eubacterium, Tepidimicrobium*, and *Tissierella_Soehngenia.*	Li K. et al., 2017
187 piglets	7 days	Luminal content and scraping from stomach, duodenum, jejunum, ileum, cecum, and colon	Illumina MiSeq sequencing (V3–V4)	Olaquindox, oxytetracycline, kitasamycin	In feed from day 7 to 42	SRP102481	The early-life antibiotic intervention decreased the abundance of *Lactobacillus* in the stomach, increased the abundance of potentially pathogenic *Streptococcus suis* in the small intestine, increased the abundance of *Treponema* in the colonic lumen and increased abundance of *Faecalibacterium* in the ileal mucosa.	Mu et al., 2017
150 recently weaned pigs	25 days	Fecal samples	Illumina MiSeq sequencing (V4)	Bacitracin zinc, chlortetracycline and colistin	In feed for 28 days	None	Antibiotics caused shifts in microbiota composition and affected composition of gut microbiota in the different gut locations of the weaning piglets.	Li P. et al., 2017
9 weaned piglets	30 days	The contents of three intestinal segments (jejunum, colon, and cecum)	MiSeq sequencing (V3–V4)	Chlortetracycline	In feed for 10 days	SRP071319	Chlortetracycline treatment resulted in elimination of several taxa and increased abundance of *Firmicutes* and the genus *Prevotella.*	Zhang et al., 2016
All piglets of 16 sows	4 day	Jejunal content	Pig Intestinal Tract Chip (PITChip) version	Tulathromycin	Parenteral- single dose	Available upon request	Antibiotic treatment resulted in long-lasting effects on gut microbiota composition and host intestinal gene expression.	Schokker et al., 2015
6 piglets	3 weeks	Fresh fecal samples	Illumina MiSeq sequencing (V4)	Chlortetracycline, sulfathiazole, and penicillin (2:2:1)	In feed for 9 weeks	SRP045387	Antibiotics administration showed no growth-promoting effect but resulted in inhibition of the growth of potential pathogens.	Unno et al., 2015

(Continued)

TABLE 2 | Continued

Study population	Population age	Samples type	Sequencing platform and 16S rRNA gene hypervariable region	Antimicrobial class	Route of administration & duration	Data availability accession number	Finding	References
6 piglets (3 male and 3 female)	3-19 weeks	Fecal swabs	Illumina MiSeq V4	Tylosin and Chlortetracycline	In feed for 70 days	SRP041290	Tylosin treatment resulted in a shift in the relative abundance of several taxa and in 26 operational taxonomic units, while chlortetracycline treatment resulted in minor alterations.	Holman and Chénier, 2014
12 piglets	3 months	Scraping and luminal content of ileum, cecum and mid-colon	Illumina MiSeq sequencing (V1–V3)	Chlortetracycline, sulfamethazine and penicillin (ASP250)	In feed for 3 months	PRJNA72355	ASP250 resulted in increased *Escherichia coli* populations in the ileum, *Lachnobacterium* spp. in all gut locations, and resistance genes to antibiotics not administered.	Looft et al., 2014a
6 piglets	3 weeks	Fecal samples	Illumina MiSeq sequencing (V1–V3)	Carbadox	In feed for 3 weeks	PRJNA237795	Carbadox treatment caused striking effects within 4 days of administration, with significant alterations in both community structure and bacterial membership, with increased *Prevotella* populations.	Looft et al., 2014b
24 pregnant sows	-	Feces	Roche 454 GS-FLX sequencer (V3–V5)	Mixture of: lincomycin, chlortetracycline, and amoxicillin.	In feed before delivery	None	Antimicrobial administration resulted in shifts in microbial community structure, increased proportion of resistant bacteria and genes.	Sun et al., 2014
4 pigs	28 days	Fecal material	Roche 454 GS-FLX sequencer (V1–V3)	Chlortetracycline	In feed for 4 weeks	None	No differences in alpha or beta diversity, nor at the taxa-level. Higher concentrations of chlortetracycline may be required to observe a shift in the gastrointestinal flora in swine feces compared with the low-level dose in this study.	Poole et al., 2013
10 pigs	10 weeks	Fecal samples	Roche 454 GS-FLX sequencer (V3)	Tylosin	In feed for 3 weeks	None	Tylosin administration caused shifts in both abundant and less abundant microbial species.	Kim et al., 2012
6 pigs	Recently weaned piglets	Freshly voided feces	Roche 454 GS-FLX sequencer (V1–V3)	ASP250	In feed for 18–21 days	None	ASP250 treatment resulted in a shift in bacterial phylotypes after 14 days of treatment, with the medicated pigs showing an increase in *Escherichia coli*, microbial functional genes related to energy production, and antibiotic-resistant genes.	Looft et al., 2012
12 piglets	3-7 weeks	Fecal samples	Roche 454 GS-FLX sequencer (V1–V3)	Carbadox and ASP250	In-feed- 1 weeks	PRJNA72355	ASP250, but not carbadox, caused significant population shifts in bacterial communities with non-significant changes in the abundance of antibiotic-resistant genes.	Allen et al., 2011

TABLE 3 | Currently available alternatives to antimicrobials in swine industry.

Antimicrobial alternative	Advantages	Possible disadvantages	Mechanism of action	References
Phage therapy	- Phages are self-replicating - Lack of cross-resistance - Potential for modification - Low inherent toxicity - Biofilm clearance - Single and low dose potential - Relatively low cost	- Can be discovered by the host's immune system as a potential invader and may therefore rapidly be eliminated from the systemic circulation - Pharmacokinetic characteristics of phages are barely known - Phage therapy is time-sensitive - Bacteria can develop resistance to phages by mutation	Targets bacteria	Pires et al., 2017
Lysins	- Can quickly kill susceptible strains with a wider antibacterial spectrum - Selective toward specific strains of bacteria - Not prone to resistance development	- High cost - Easily degraded and lose activities during use and storage - Poor efficacy against gram-negative bacteria	Targets bacteria	Love et al., 2018
Antibacterial vaccine	- Inexpensive in production - Stable in storage	- Lack of relevant protective antigens - Lack of safety due to potentially harmful components - Killed vaccines require the use of adjuvants, which limits the delivery options for the vaccines	Primes host's immune response	Hoelzer et al., 2018
Antimicrobial peptides	- Not prone to resistance development - Broad-spectrum and bactericidal activity	- High production cost - Potentially toxic to cells - Unstable during transportation - Easily hydrolyzed by proteases in the gut	Targets bacteria	Wang et al., 2016
Phytobiotics	- Nutritional effect - Easy availability	- High variability - Pharmacokinetic characteristics of most of plants are not well-known - High risk of toxicity	Targets bacteria and improves gut health	Mohammadi Gheisar and Kim, 2018
Inhibitors for bacterial quorum sensing	- Not prone to resistance development	- The majority of QSIs cannot be widely applied because of their toxicity to eukaryotic cells - Only narrow-spectrum activity - High chance of degradation	Targets bacteria	Cheng et al. et al., 2014
Probiotics	- Easy availability - Relatively cheap - Not prone to resistance development	- Lack of standards - Causes several potential problems (animal poisoning, allergies, and diarrhea) - Cannot withstand low pH and bile acids in gastrointestinal tract - Difficult to reach high sufficient number of viable cells to colonize in the intestine	Improves gut health	Collins and Gibson, 1999
Prebiotics	- Promote immune functions - Show anti-viral activity - Have no residue - Not prone to resistance development	- Cannot inhibit and kill pathogens - Feeding large quantity of prebiotics may cause bloating, diarrhea, and other adverse reactions	Improves gut health	Collins and Gibson, 1999

(Zeineldin et al., 2019). Emergence of these ARGs without direct exposure to a known antibiotic also reveals that the swine GI antimicrobial resistome may not be affected by a reduction in antimicrobial administration in the swine industry (Holman and Chénier, 2015).

ANTIMICROBIAL ALTERNATIVES IN SWINE INDUSTRY

The current efforts to define the complex composition of GI microbiota and how that community responds to antimicrobial intervention would improve our ability to develop novel non-antibiotic strategies to prevent GI infection in food-producing animals, subsequently increasing animal productivity (Marchesi et al., 2016). Considering this information, different management strategies are required to reduce the deleterious consequences of antimicrobials, particularly when its administration is needed to control bacterial infections. Broad discussions of possible antimicrobial alternatives have been summarized in **Table 3** and were mentioned elsewhere (Potter et al., 2008; Allen et al., 2013, 2014; Papatsiros, 2013; Czaplewski et al., 2016). In this section, we will only focus on bacteriophage therapy as an important and promising example of available antimicrobial alternatives in the swine industry.

Bacteriophage (phage) therapy involves the use of bacterial viruses (phages) to attack specific bacterial species, or a narrow group of microbes, without harming the resident autochthonous microbial communities (Kutateladze and Adamia, 2010). Because of their ubiquity in all natural environments and commercial

swine facilities, as well as their specific action against pathogens, phages have been suggested as a promising antimicrobial alternative for use in swine (Zhang et al., 2015). Recent studies based on high throughput next-generation sequencing approaches highlighted the importance of phages in microbial evolution and bacterial community control (Pratama and van Elsas, 2018). In addition to GI microbiota inhabitants, the GI tract harbors diverse phage communities that have a synergistic effect along with the resident microbial communities to maintain GI health (Allen et al., 2013). Subsequent research studies demonstrated that bacteriophages attacks bacteria by attaching to the cell wall and injecting their genetic material into bacterial cytoplasm with subsequent integration into the bacterial genome. Phage populations are extensively diverse and generally grouped according to their morphological properties and life cycle into temperate (lysogenic) or virulent (lytic) phages. Virulent bacteriophages are natural predators of their bacterial hosts, they replicate using the host machinery, and complete their lifecycle by lysis of the host cell (Calero-Cáceres et al., 2019). In contrast, temperate bacteriophages integrate into the host's chromosome and produce a stable genetic relationship with the host during the process of lysogeny without creating new phage particles (Zhang et al., 2015). Despite the growing evidence that supports the medical importance of virulent bacteriophages, their functional potential in swine is not yet well-defined.

In the swine industry, bacteriophage intervention strategies have been extensively used to control various *Salmonella* serovars, *E. coli O157:H7*, *enterotoxigenic E. coli*-induced diarrhea and *Campylobacter* species (Lee and Harris, 2001; Nisbet et al., 2010; Harvey et al., 2011; Hooton et al., 2011; Cha et al., 2012). These studies have shown that phages can be effectively utilized against these pathogens. Most recently, a phage cocktail was used to reduce *Salmonella typhimurium* in artificially-infected market-weight swine (Wall et al., 2010; Hooton et al., 2011). Similarly, phage treatment in weaned piglets challenged with *S. typhimurium* via oral gavage reduced fecal and cecal *Salmonella* populations in phage-treated piglets compared to control piglets (Nisbet et al., 2010). Several other experiments have evaluated the antimicrobial ability of phages against *E. coli* infections. Oral administration of a phage cocktail was capable of reducing morbidity and mortality in enterotoxigenic *E. coli*-challenged pigs, even when used at the onset of clinical signs (Atterbury, 2009). Smith and Huggins also investigated the efficacy of a mixture of two phages against an enteropathogenic strain of *E. coli* in neonatal pigs. The results of this work indicated that phages which targeted adherence pili were more effective in controlling porcine *E. coli* than phages that target other pili (Smith and Huggins, 2009). Phage therapy was also associated with increased prevalence of beneficial microbes (e.g., *Bifidobacterium* and *Lactobacillus*) and decreased relative abundance of coliforms and *Clostridium* species in post-weaning piglets (Hosseindoust et al., 2017).

Since their discovery in 1915, phages have been proven to be harmless to humans, animals and plants. Compared to antimicrobial, phages are highly effective in killing their target bacteria without harming the rest of the microbiota in the ecosystem. Additionally, phages are relatively cheap, self-replicating, easy to isolate, and have low inherent toxicity (Sillankorva et al., 2012). Despite these advantages, there are many technical limitations in the implementation of phage therapy for treatment of infectious diseases in human and animals (Allen et al., 2014). Commercially available phages have a limited microbial range, are unstable, sensitive to temperature, have a narrow range of hosts, require rapid administration after infection, and could be neutralized by the host's immune system (Papatsiros, 2013; Zhang et al., 2015). Similarly to antimicrobial resistance, recent studies suggest that bacteriophages play a crucial role in the acquisition and emergence of the antimicrobial resistome (Calero-Cáceres et al., 2019). Phage genomes can harbor several antimicrobial resistomes belonging to different antimicrobial classes. Phage-resistant strains are believed to be generally less virulent than the phage susceptible wild types, but the use of a number of different phages in combination (phage cocktails) against many serotypes will likely alleviate this problem (Kutateladze and Adamia, 2010; Harvey et al., 2011). Therefore, high-throughput next-generation sequencing and genetic engineering will be necessary to create a more reasonable phage to optimize impact and create the best alternative to antimicrobial treatment.

CONCLUSION

The application of both high-throughput next-generation sequencing and functional metagenomics have clarified the effects of antimicrobial administration on commensal populations as well as on emergence of ARGs. There is, therefore, a great interest in understanding the origins, evolution and totality of antimicrobial resistance, not just in pathogenic microbes but also in whole resident microbial environment. The evidence that the commensal population harbors a previously underappreciated antimicrobial resistome should shift the paradigm of what judicious use of antimicrobials in livestock means. In addition, it raises exciting questions about the acquisition and transfer of antimicrobial resistance cross GI microbiota. A better understanding of the impacts of specific antimicrobial intervention strategies on GI microbiota and their accompanying antimicrobial resistome could open the door to the development of a novel therapeutic approach in swine production systems.

AUTHOR CONTRIBUTIONS

MZ wrote the manuscript. BA and JL revised it. All authors have approved the manuscript submission.

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Antibiotic Resistance of *E. coli* Isolated From a Constructed Wetland Dominated by a Crow Roost, With Emphasis on ESBL and AmpC Containing *E. coli*

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Information on the dissemination of antibiotic resistance mechanisms in the environment as well as wild life is needed in North America. A constructed wetland (where ∼15,000 American crows roost) was sampled on the University of Washington Bothell Campus for the presence of antibiotic resistant *E. coli* (ARE). Crow droppings from individual birds and grab samples of water were collected in 2014–2015. *E. coli* were isolated by selective agar plating. The most frequent antibiotic resistance (AR) of the fecal isolates was to ampicillin (AMP) (53%), followed by amoxicillin-clavulanic acid (AMC) (45%), streptomycin (S) (40%), and nalidixic acid (NA) (33%). Water isolates had similar AR pattern and ∼40% were multidrug resistant. Isolates from water samples collected during storm events showed higher resistance than isolates from no rain days to tetracycline, AMP, AMC, NA, and gentamycin. Extended spectrum beta lactamase (ESBL) containing *E. coli* with the $bla_{\text{ctx-M}}$ was found in three water and nine fecal isolates while $bla_{\text{cmy-2}}$ in 19 water and 16 fecal isolates. Multilocus Sequence Typing analysis (MLST) yielded 13 and 12 different sequence types (STs) amongst fecal and water isolates, many of which could be correlated to livestock, bird, and humans. MLST identified ESBL *E. coli* belonging to the clinically relevant ST131 clone in six fecal and one water isolate. Three STs found in feces could be found in water on the same dates of collection but not subsequently. Thus, the strains do not appear to survive for long in the wetland. Phylogenetic analysis revealed similar distribution of the water and fecal isolates among the different phylo-groups, with the majority belonging to the commensal B1 phylo-group, followed by the pathogenic B2 phylo-group. This study demonstrates that corvids can be reservoirs and vectors of ARE and pathogenic *E. coli*, posing a significant environmental threat.

Keywords: wetland, crows, ST131, ESBL, multi-drug resistant *E. coli*, antibiotic resistant genes, $bla_{\text{ctx-M}}$, $bla_{\text{cmy-2}}$

INTRODUCTION

The spread of antimicrobial resistance has reached proportions of global magnitude and poses a threat to the effective treatment of several infectious diseases (Centers for Disease Control and Prevention [CDC], 2013; WHO, 2014). The environment is increasingly being recognized as a reservoir of antibiotic resistant (AR) bacteria as well as antibiotic resistant genes (ARG). Such resistance may arise by the release of fecal bacteria from humans and animals including birds, which then allows antibiotic resistance genes to be transferred to non-resistant indigenous microorganisms in the environment (Aminov, 2011; Guenther et al., 2011). Antibiotics or other chemicals and contaminants present in environmental matrices, contribute to this further by offering selective pressure, thus allowing for their survival and expansion (Martinez, 2009). Fecal contamination of surface water, river water, wetlands, and even drinking water have been implicated in the spread of such resistance (Baquero et al., 2008; Coleman et al., 2013; Li et al., 2014; Rodriguez-Mozaz et al., 2015; Vivant et al., 2016). On the other hand, constructed wetlands have also been shown to remove such bacteria (Ibekwe et al., 2016; Vivant et al., 2016).

Free living birds can be a significant contributor to the pollution of water bodies. Although they may not be directly exposed to antibiotics like humans or farm animals, they can acquire antibiotic resistance by being in close contact to humans, their farm animals and pets, and subsequently be vectors for their spread (Verbeek and Caffrey, 2002; Guenther et al., 2011; Jamborova et al., 2015). In addition, crows can acquire AR bacteria by foraging on a variety of wastes such as garbage dumps, hospital and animal wastes, and animal feed lots (Verbeek and Caffrey, 2002; Guenther et al., 2011). Several recent studies have reported crows and rooks shedding bacteria that were resistant to one or more antibiotics (Literak et al., 2007; Hasan et al., 2015; Jamborova et al., 2015, 2018). *E. coli*, which lives as a harmless commensal in the gut of all animal and birds, has proved to be not only an indicator of fecal coliform but also of antibiotic resistance present in the environment (van Den Bogaard et al., 2000; Dolejská et al., 2009; Guenther et al., 2011; Jamborova et al., 2015, 2018). From the United States, only one study investigating antibiotic resistance in *E. coli* in crows has been reported (Jamborova et al., 2017). In this study, which was a survey from four different states, 13% (n = 590) of *E. coli* from American crows (*Corvus brachyrhyncos*) possessed AmpC and ESBL phenotypes, while 15% (n = 590) were resistant to Ciprofloxacin (Jamborova et al., 2017). Two other studies reported on vancomycin resistant enterococci shed by crows in United States (Oravcova et al., 2014; Roberts et al., 2016). These studies specifically selected for cefotaxime or ciprofloxacin or vancomycin resistant bacteria. The overall antibiotic resistance pattern of the crow isolates was not reported.

In this study, samples collected within the University of Washington Bothell/Cascadia College (UWB/CC) campus (where more than 15,000 crows roost in the autumn and winter months) were tested for the resistance of *E. coli* isolates to thirteen antibiotics represented three classes of antibiotics. Extended Spectrum beta lactamase (ESBL) and AmpC beta lactamase containing *E. coli* were additionally targeted because the presence of these genes continue to hinder the efficacy of beta lactams (Pitout et al., 2007). The spread of ESBL resistance by crows has been documented in other parts of the United States, but not in Washington State (Jamborova et al., 2017). Multi Locus Sequence Typing (MLST) and phylogenetic characterization of the isolates was performed in order to have an idea of the source and pathogenicity of the isolates.

MATERIALS AND METHODS

Sample Collections

All samples were collected within the 58-acre wetland restoration area of the UWB/CC campus. Begun in 1997 with the construction of campus, this restoration project converted pastureland and a straightened and deepened reach of North Creek into a more natural, meandering stream channel and a fully functioning forested floodplain ecosystem. It serves as a natural filter for campus stormwater runoff that is discharged in various locations to the wetland prior to flowing into North Creek (see **Figure 1**). The campus runoff contributes to the wetness of the wetland, as do a high water table, plentiful rain between October and June, and occasional flood events when North Creek spills over its banks (∼2–4 times a year).

Crow fecal samples were collected between August 2014 and April 2015 from the crow roost areas within the wetland. Samples were collected by spreading plastic sheets on the ground underneath the trees where the crows roosted in the evening. Fresh fecal samples from free flying crows were collected the following morning with sterile swabs and placed in sterile vials kept on ice as described previously (Sen et al., 2018). Sixty one samples were collected in five rounds of sampling. On the days that fecal samples were collected, surface water samples were also collected within the wetland at four different sites. Two sites, NC5 and SW2 were within the roost area while RP3 and NC6 were located in areas bordering the roost (**Figure 1**). Twenty water samples were collected altogether during this period. Water samples were collected again from June, 2016–April, 2017 from the sites designated as SW8, SW2, NC6, RS1, and RS2 to compare *E. coli* collected during "no rain" versus "rainy" days. The NC prefix of sampling sites indicates North Creek water. SW indicates a surface water tributary to North Creek. RS indicates discharge of campus runoff into a runoff bioswale. To qualify as a rainy day, more than 0.05 inches of cumulative rain for that day had to be recorded at the 21 Acres weather station approximately 1.5 miles away[1]. No rain days not only had no rain that day, but were also preceded by 72 h without rain.

Isolation and Enumeration of *E. coli*

Approximately 100 mg of fecal sample was diluted in 500 ml Phosphate Buffered Saline until a fluid suspension was obtained. Ten to twenty microliters were directly plated onto Eosin Methylene Blue (EMB) Agar and incubated at 37°C for 24 h. Colonies with metallic green sheen were isolated as putative

[1]http://weather.wsu.edu/index.php?page=station_details&UNIT_ID=330026

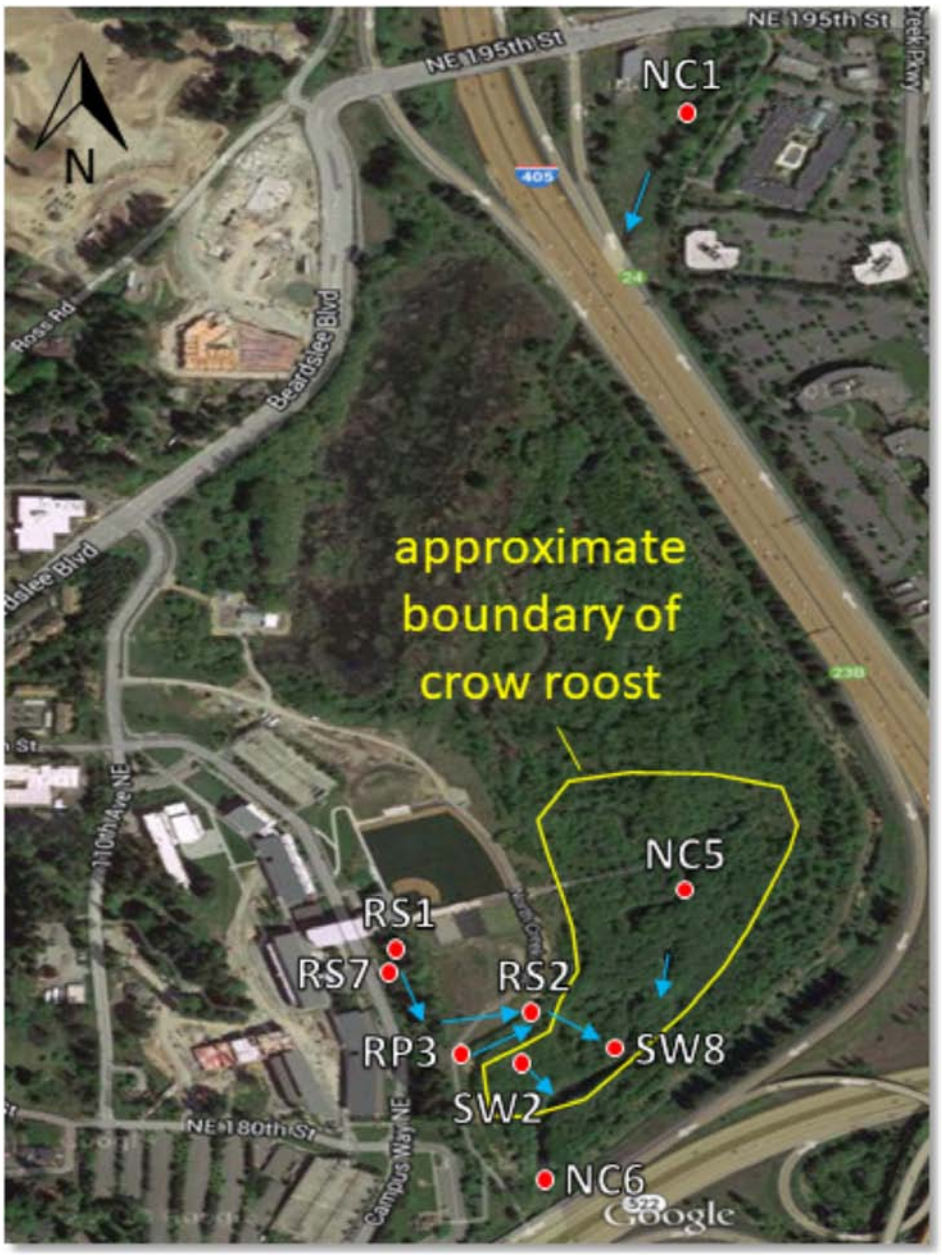


FIGURE 1 | Sampling site map showing North Creek, the UW Bothell/Cascadia College campus, and the 58 acre restored floodplain wetland. Red dots indicate locations of surface water sampling sites. Blue arrows indicate direction of water flow. Water sampled at RS1, RS7, and RP3 flows to these locations in a series of catch basins and pipes from the upland (western) portion of the campus. The crow roost boundary fluctuates year to year, though the southern part by the sampling sites is relatively stable. Aerial photograph from Google.

E. coli. They were further verified by the presence of the malate dehydrogenase (*mdh)* gene as described below. From the 61 samples, 49 samples were positive for *E. coli.* Four isolates from each sample were stored at −70°C in Tryptic Soy Broth containing 16–20% glycerol until ready for use.

Water samples were collected in 120 ml IDEXX polyethylene terephthalate vessels and subsequently filtered through 0.45 micron Millipore S-Pak filters. *E. coli* and other coliform bacteria colonies were allowed to grow on the filters by placing them on m-ColiBlue24 broth following US EPA method 10029 (Hach Company 2018)[2]. Triplicate samples were collected at each site. Most of the water samples required dilution in order to generate countable filters.

Blue Colonies were counted for determination of total number of *E.coli* in colony forming units (CFU)/100 ml of sample. The *E. coli* isolated by this method were verified on EMB agar and

[2]https://www.hach.com/asset-get.download-en.jsa?id=7639984023

further by the presence of the *mdh* gene. Four *E. coli* isolates were stored at −70°C from each sample until ready for use. For ESBL isolation one set of filters from each site was extracted with PBS as described below.

Antibiotic Susceptibility Testing

Colonies grown on Mueller Hinton (MH) agar were used in antibiotic susceptibility testing by the Disk Diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI) (ClSI, 2012). The CLSI clinical breakpoints for an antibiotic toward enterobactericiae were used to assign isolates sensitive or resistant status. Altogether 98 isolates from the fecal samples and 184 isolates from the water samples were analyzed. Thirteen antibiotics were tested: ampicillin (AMP or A) 10 μg, amoxicillin-clavulanic acid (AMC) 20 μg, ceftazidime (CAZ) 30 μg, ceftiofur (XNL) 30 μg, tetracycline (T or TE) 30 μg, ciprofloxacin (CIP) 5 μg, enrofloxacin (ENO) 5 μg, chloramphenicol (C) 30 μg, streptomycin (S) 10 μg, spectinomycin (SPT), sulfamethaoxazole/trimethoprim (SXT) 25 μg, nalidixic acid (NA) 30 μg, and neomycin (N) 5 μg. For some of the isolates (pre and post rain) gentamycin (G) 10 μg and kanamycin (K) 30 μg were also evaluated.

ESBL Selection

Filters obtained from water samples were washed with 300 μl of PBS and the washings were plated onto three MacConkey agar (MCA) plates supplemented with 4 μg/ml Cefotaxime and incubated overnight at 37°C (Durso et al., 2016). Pink colonies obtained were further verified on EMB agar for confirmation as *E. coli*, as described above. Initially CTX was added at a concentration of 1 μg/ ml on the plates, but most of the isolates turned out to be false positives since they failed to regrow on these plates. In addition, all *E. coli* isolates from mColiBlue filters that tested resistant to AMP and CAZ but were susceptible to AMC in disk diffusion assays were further evaluated for ESBL presence by the double disc method (DDST) originally described by Jarlier et al. (1988), with slight modifications. Briefly, a disk containing amoxicillin/Clavulanic acid (AMC) was placed in the center of a MH agar plate spread with the test isolate. At 20 mm apart (center to center) from the AMC disk ceftriaxone (CRO), cefotaxime or ceftazidime were placed on three sides. For several of the isolates Cefoxitin (FOX) was included on a 4th side. The test was considered positive if, after 24-h incubation at 37°C, the zone of inhibition between one or more of the disks was enhanced.

Fecal samples were plated directly on MCA + Cefotaxime plates and pink colonies were saved as putative ESBL containing *E. coli*. They were further tested and confirmed as above. In addition isolates obtained on EMB agar that tested resistant to AMP and CAZ but were susceptible to AMC, were further tested for ESBL phenotype. We also tested for the presence of $bla_{\text{ctx-M}}$ gene in all water and fecal isolates that were resistant to AMP, CAZ/CTX as well as AMC, as described below.

All procedures were conducted under strict biosafety guidelines laid out by University of Washington Environmental Health and Safety office[3].

[3]https://www.ehs.washington.edu/

DNA Isolation and PCR

A 1–2 mm size colony from an overnight culture plate was suspended in 10 μL of Prepman Ultra Sample Preparation Reagent (Life Technologies, Foster City, CA, United States). Alternatively, 1 mL of an overnight culture broth of an isolate was centrifuged at 10,000 *g* for 5 min. The supernatant was removed, and the pellet was re-suspended in 200 μL of Prepman Ultra Sample buffer. In either case, the suspensions were heated at 95°C for 10 min, cooled, and centrifuged at 10,000 *g* for 2 min. Two microliters of the supernatant was directly used in a 20 μL PCR reaction. The supernatants were stored at 4°C if they were to be used within the week otherwise at −20°C. Extracts stored at −20°C performed as well as a fresh preparation in a qPCR or PCR reaction, 20 months later (data not shown).

Antibiotic Resistance Gene Detection

All isolates that showed antibiotic resistance by phenotypic methods were tested for the respective genetic determinant. Strains that showed ESBL phenotype by the double disc method were tested for $bla_{\text{ctx-M}}$ bla_{shv} and bla_{tem} by a qPCR method (Birkett et al., 2007; Angeletti et al., 2013) cefotaxime and/or ceftazidime resistant isolates that were also resistant to AMC were tested for the $bla_{\text{cmy-2}}$ gene (Alali et al., 2009) as well as $bla_{\text{ctx-M}}$. The later was tested to eliminate the possibility of an ESBL carrying isolate being missed, by the phenotypic method. For sequencing we used 453–510 bp PCR products obtained by primers Cottell CTX M- F 5′-CCG CTG CCG GTY TTA TC-3′ and Cottell CTX-M R-5′-ATG TGC AGY ACC AGT AA-3′ described earlier (Cottell et al., 2013). We also used another PCR product of 554 bp obtained with forward primer 5′ATG TGC AGY ACC AGT AAR GTK ATG GC-3′ and reverse primer 5′TGG GTR AAR TAR GTS ACC AGA AYS AGC GG-3′ (Hedman et al., 2019). The last set of primers allowed us to distinguish between $bla_{\text{ctx-M27}}$ and $bla_{\text{ctx-M14}}$. Tetracycline resistance genes were measured by the method of Ng et al for *tet* (A), *tet* (B), *tet* (C), *tet* (D), *tet* (E),*tet* (G), *tet* (j), *tet* (k), *tet* (L), *tet* (M), *tet* (O), *tet* (Q), *tet*(S), *tet* (X) (Ng et al., 2001). Additionally qPCR assays were also used for rapid detection of *tet* (M) *and tet* (W) as described earlier (Walsh et al., 2011) Streptomycin resistance was measured by testing for *strA*, *strB*, and *aadA* (Walsh et al., 2011). All qPCR reactions were performed in a Mini-opticon icycler (BioRad). For SYBR green PCR, iTaq$^{\text{TM}}$ Universal SYBR green mastermix and for TaqMan$^{\text{TM}}$ PCR, iTaq$^{\text{TM}}$ Universal Probes Supermix (Bio-rad, Hercules, CA, United States) was used. The cycling parameters for Taqman qPCR was as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C, with a final cycle of 5 min at 72°C. For tetracycline resistance genes controls were obtained from Dr. Lisa Durso, USDA, NE, United States (Durso et al., 2016) and Dr. Marilyn Roberts (University of Washington). A D-block synthesized by IDT (IDT Inc.) that contained the sequences of the $bla_{\text{ctx-M1}}$, $bla_{\text{ctx-M2}}$, and $bla_{\text{ctx-M9}}$ PCR products as described in Birkett et al. (2007) was used as control for $bla_{\text{ctx-M}}$ in the initial TaqMan$^{\text{TM}}$ PCR. $bla_{\text{ctx-M}}$ isolates identified thus were then used as positive controls for the other regular PCR reactions.

bla$_{shv}$, *bla*$_{tem}$, *strA*, *strB*, and *aadA* controls were developed in house from strains that tested positive by PCR and subsequent sequencing. The sequences obtained for *bla*$_{ctx-M}$ gene from the different isolates have been deposited in the GenBank and their accession numbers are: MK78174 to MK781784.

Grouping Isolates Based on *mdh* Gene Sequence and MLST Studies

A 825 bp region of the *mdh* gene was amplified and sequenced for several feces and water isolates using the published primers: mdhF: 5′TGAAAGTCGCAGTCCTCGG-3′ and mdhR 5′-TCC ACGCCGTTTTTACCC-3′ as described before (Ivanetich et al., 2006). A 282 bp region from this was trimmed, aligned and a phylogenetic tree obtained using the Maximum Likelihood method. Epidemiological relatedness of the isolates was tested using seven *E. coli* housekeeping genes, utilizing MLST. MLST was performed according to the methods specified at the MLST website http://enterobase.warwick.ac.uk/species/index/ecol. The PCR products from the seven housekeeping genes were sequenced using the same primers used to generate the fragments. Sanger sequencing was performed by Eurofins Genomics (Louisville, KY, United States). *E. coli* STs were assigned using the above databases as well as that developed by Keith Jolley [33], at University of Oxford Site: https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef&page=profiles.

Phylogenetic Studies

The quadruplex PCR method of Clermont et al. (2013) was used to assign the *E. coli* isolates to one of the eight phylo groups. After initial placement into groups, based on the results of the quadruplex, strains belonging to phylo-groups A and C or D and E were further identified by using C and E specific primer sets, as per Clermont et al. (2013).

Statistical Analysis

One sided proportional *Z* test was used to identify significant differences between count data which is represented as percentages, such as percent antibiotic resistant and percent presence of a phylo-group. The *P* values corresponding to the differences are reported in the tables below the graphs.

RESULTS

E. coli Loading in the Wetland Roost Area

Total number of *E. coli* in CFU/100 ml was determined at RS2 site where runoff water from the campus enters the wetland roost area and at the SW8 site where the water exits the roost area, flowing into North Creek (**Figures 1**, **2**). Thus, the number of isolates collected at the RS2 site indicate collection from an area not directly influenced by the crow roost, while SW8 is an area under the direct influence of crows. (**Figure 1**) The apparent impact of the short journey through the roost zone on the runoff as it flowed from the RS2 site to the SW8 site was an order of magnitude increase in the average *E. coli* count (**Figure 2**).

Antibiotic Susceptibility of Crow and Water Isolates

The fecal *E. coli* isolated in 2014–2015, were compared with *E. coli* water isolates from the same period for their susceptibilities against 13 antibiotics. 65 and 70% of the isolates from water and crow fecal samples, respectively, were resistant to one or more antibiotics. Ampicillin resistance was the most prevalent, followed by Amoxicillin Clavulanic acid (**Figure 3**). Multiple drug resistance (three or more of different classes) was found in 40% of the water isolates as well as the crow fecal isolates. Resistance to four antibiotics was most common in water isolates (20%), while among fecal isolates resistance to 4–5 antibiotics was more common (12%). Six fecal isolates showed resistance to seven antibiotics (**Table 1**). Overall the wetland water isolates showed a similar pattern of susceptibility as that of the fecal isolates for 12 of the 13 antibiotics tested at p value 5% or less (**Figure 2**). Neomycin was the only antibiotic against which the resistance was significantly different between the water and fecal isolates ($p \leq 0.0019$), with that in fecal being higher. Among the *tet* and *str* genes tested, *tet* (A), *tet* (B), or *tet* (M) were the genes responsible for >95% of isolates to show the resistance phenotype, while *strA* and/or *strB* was responsible for streptomycin resistance phenotype. *aadA* was detected in a couple of isolates together with *strB*. *tet* (C) along with tet (D) was present in one fecal isolate. *tet* (M) was usually present with *tet* (A) (15 isolates). Two isolates had *tet* (A), *tet*(B) and *tet* (M) while *tet* (A) and *tet*(B) co-occurred in six isolates. For sulfamethaoxazole/trimethoprim (SXT) resistance the *sul1* gene was tested and it was present in 100% of the isolates that showed the phenotype.

Antibiotic Susceptibility of *E. coli* Isolates Before (No Rain) and After Rainfall (Rain)

Altogether 65 isolates from no rain and 67 from rain days were tested for their susceptibility to 11 antibiotics (**Figure 4**). There was a significant difference in resistance to TE, AMP, AMC, NA, and gentamycin with rain days demonstrating a higher level of resistance to these antibiotics. No resistance was observed to Ciprofloxacin, and only one isolate each were resistant to gentamycin and kanamycin post rain. For the remaining three antibiotics the difference was not as significant at $p < 0.05$.

ESBL and Beta Lactamase (*ampC*) Containing Isolates

Only two ESBL containing *E. coli* were isolated from the water samples collected between 9/17/14 and 4/05/2015, and one more from collections made between 2016–2017 (**Table 2**). These isolates were obtained initially on m-ColiBlue24 broth and based on their antibiotic profile were plated on MCA+ Cefotaxime. Among the fecal isolates, 7 of the 98 (7.1%) isolates carried ESBL. Except for one, all the fecal isolates were obtained non-selectively on EMB agar for *E. coli*. Since they were ampicillin resistant but AMC susceptible, they were further tested and purified on MCA + CTX and subjected to ESBL verification. Two additional isolates had *bla*$_{cmy-2}$ and *bla*$_{ctx-m}$ and thus 9 of 98 (8.9%) can be considered as ESBL *E. coli*. All ESBL isolates were multi drug resistant with resistance to at least Amp, Caz/Ctx, S, SXT, TE.

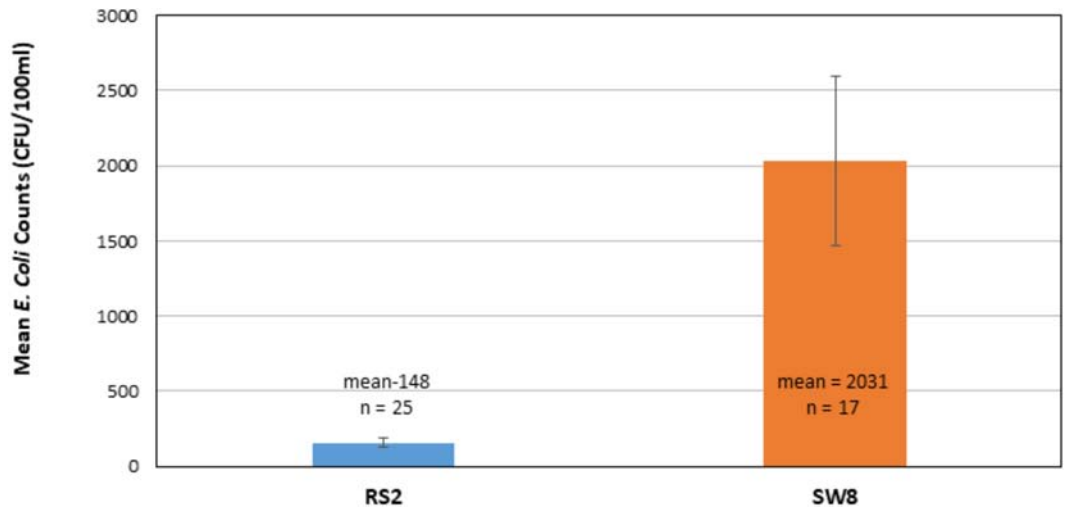
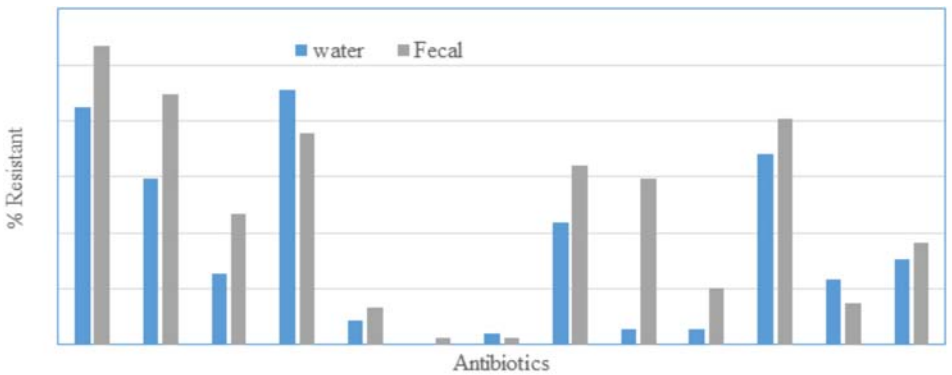


FIGURE 2 | Comparison of mean *E. coli* counts in runoff as it enters (RS2) and leaves the wetland roost zone (SW8). The mean of counts in CFUs, determined 25 times between 2014–2017 at RS2 and 17 times at SW8, is shown. Triplicate samples were collected at each site each time. The error bars represent one standard deviation around the mean for the respective data sets.



	AMP	AMC	CAZ	XNL	C	CIP	ENO5	NA30	N30	SPT	S10	SXT	TE
Z statistics	0.871	1.347	1.314	-0.688	0.575	0.727	-0.413	1.057	2.834	1.389	0.58	-0.7	0.392
P value	0.19	0,09	0.1	-0.8	0.28	0.22	-0.66	0.15	0.0019	0.09	0.28	-0.76	0.35

FIGURE 3 | Percentage of *E. coli* in water (n = 49) and fecal (n = 98) isolates showing non-susceptibility to 13 selected antimicrobials. AMC, amoxicillin/clavulanic acid; AMP, ampicillin; XNL, ceftiofur; C, chloramphenicol; CAZ, Ceftazidime; CIP, ciprofloxacin; ENO, Enrofloxacin; NA, Nalidixic acid; N, Neomycin; STR, streptomycin; SPT, spectinomycin; TE, tetracycline; SXT, trimethoprim/sulfamethoxazole. Table indicates significant difference in antibiotic resistance between water and fecal isolates for 10 antibiotics according to Z-test.

The $bla_{\text{cmy}-2}$ gene was present in 16 of 98 (16.3%) fecal isolates and 9 of 49 (18.36%) water isolates in the collections from 2014 and 2015. All of these isolates were first non-selectively isolated for *E. coli* on EMB agar. AMP, AMC, and ceftifuor resistance indicated testing for $bla_{\text{cmy}-2}$. Seven of the 16 $bla_{\text{cmy}-2}$ containing isolates were MDR in the fecal isolates. bla_{shv} co-occurred with $bla_{\text{cmy}-2}$ in one instance and with bla_{tem} in two instances. For bla_{tem} a 189 bp sequence was obtained that had 100% homology with classA ESBL – TEM1, while for bla_{shv}, a 193 bp sequence was obtained that had 100% homology to ESBLs – SHV12, SHV-61, SHV-5.

E. coli Sequence Types in Water and Fecal Samples

A total of 39 isolates, 23 fecal and 16 water, were selected for MLST. This was based on presence of $bla_{\text{ctx}-\text{M}}$, $bla_{\text{cmy}-2}$, bla_{tem}, or bla_{shv} gene. Care was taken to see that there were

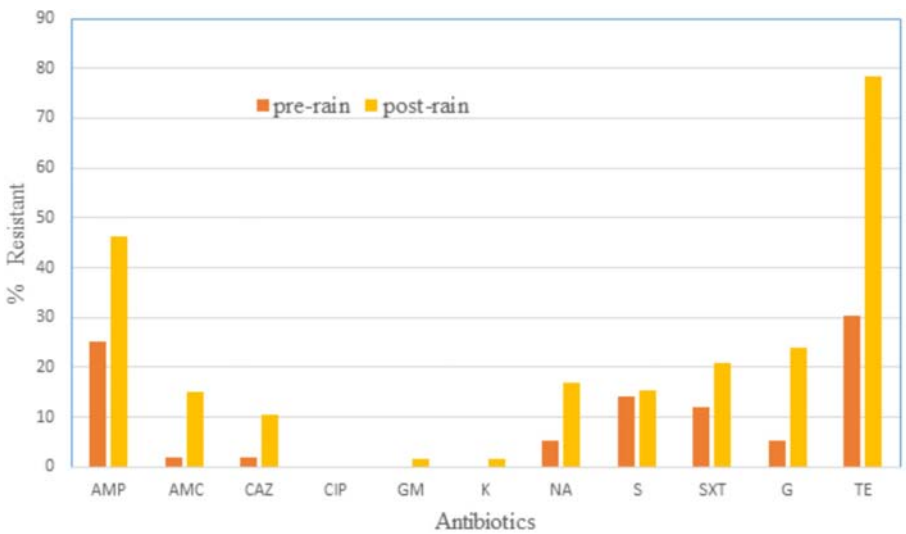
TABLE 1 | Percentage of water (n = 49) and fecal (n = 98) isolates resistant to one or more antibiotics.

No. of Antibiotics	Water (%)	Fecal (%)
0	17 (34)	29 (29.6)
1	8 (16.3)	17 (17.3)
2	3 (6.12)	8 (8.16)
3	2 (4.08)	5 (5.1)
4	10 (20.4)	12 (12.2)
5	5 (10.2)	12 (12.2)
6	4 (8.16)	9 (9.18)
7	0 (0)	6 (6.12)
Multidrug resistant (≥3 classes)	19 (39.58)	39 (39.8)

representative isolates from different collection dates, both from water and fecal. A phylogenetic tree based on presence of 282 bp of the *mdh* gene alone was obtained for 30 crow fecal and 29 water isolates from 2014–2015, **Supplementary Figure S1** as described earlier (Ivanetich et al., 2006). The isolates were randomly chosen, however, isolates from each collection were included for determination of *mdh* presence. Eight clusters (a cluster was formed if three isolates had identical 282 bp region) were obtained. Where water and fecal isolates clustered together, a bigger region of the *mdh* gene that encompassed the 452 bp region, that is used for MLST analysis, was aligned and if the same allele was obtained then sequencing of the remaining six housekeeping genes was undertaken. For example, F35.1 had *mdh* gene corresponding to allele 16, while the ESBL isolates in this cluster had an *mdh* gene with allele 36, and thus F35.1 was not subjected to MLST. In this manner F14.1 and NC6.2 (R2) were selected and identified as ST58 and F32.1 and NC6.7 (R2) as ST10. Two isolates from the fecal samples F11 and F13 were analyzed because their antibiotic resistance phenotype was a little different although they both had the $bla_{\text{ctx-M-27}}$ gene.

Multilocus Sequence Typing analysis showed high diversity in the sequence types obtained from the different collection dates. 13 different STs were obtained for the fecal isolates and 10 for the water isolates. STs of 4 of the 39 isolates could not be determined (**Table 2**). Within one collection date, although there was genetic diversity, several identical STs were obtained within the fecal isolates. Thus 6 of 16 isolates from the 9/15/14 collection belonged to ST131, while 2 of 12 from 11/10/14 collection belonged to ST68. All ST131 isolates had the $bla_{\text{ctx-M}}$ gene and sequencing of the gene showed them to be $bla_{\text{ctx-M-27}}$.

When STs from water and fecal isolates were compared, in three instances a common ST was found in the water and fecal. Thus one ST131 isolate, NC 5.1 ctx, found in a water sample from NC5 site (**Figure 1**) on 9/14/15, was found in several (six) fecal isolates from the same date (**Table 2**). Fecal isolate F32.1 from 1/12/15 had ST10, a ST which was also found in a water sample NC 6.7 from site NC6 on the same date. Similarly, ST58



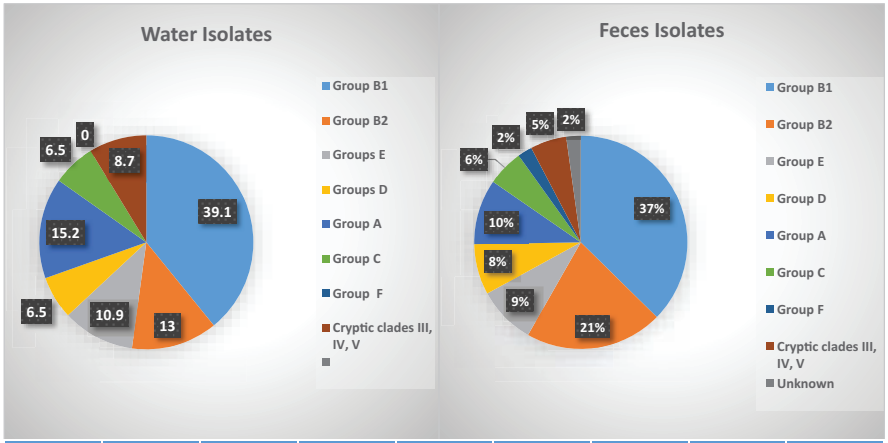
	AMP	AMC	CAZ	Gm	K	NA30	S10	SXT	G	TE
Zstatistics	2.031	2.62	2.02	1.032	1.057	1.954	0.173	0.912	2.716	3.68
P value	0.01	0.01	0.06	0.15	.0.14	0.03	0.43	0.19	0.01	0.001

FIGURE 4 | Percentage of *E. coli* isolates in water on no rain days (n = 62) and post-rain days (n = 63) showing non-susceptibility to 11 selected antimicrobials. AMC, amoxicillin/clavulanic acid; AMP, ampicillin; C, chloramphenicol; CAZ, Ceftazidime; CIP, ciprofloxacin; GM, Gentamycin; K, Kanamycin; NA, Nalidixic acid; STR, streptomycin; TE, tetracycline; SXT, trimethoprim/sulfamethoxazole. Table indicates significant difference in antibiotic resistance between no rain and rain days by Z test of proportionality.

TABLE 2 | Sequence type, antibiotic genetic determinant, and phylo-group of fecal and water isolates that had bla_{ctx-M} or bla_{CMY-2}, bla_{tem} or bla_{shv} genes.

Strain	Round	ST	ESBL phenotype	Resistance Phenotype	AR Genes	Phylo-grou
Fecal						
F4.1	RI	ST 2614	ND	AMP-AMC-CTX	bla_{tem}	B2
F7.1	RI	ST5914	Negative	AMP-AMC-CF-N	bla_{tem}	BI
F2.7	RI	ST8371	Negative	AMP-AMC	bla_{tem}	D
F11.I	R2	ST131	CAZ,CTX,CRO, FOX, CF	AMP-CTX-CF-XNL-S-SXT-T	bla_{ctx}, *strA*, *sul1*, *tet* (A), *tet*(M)	B2
F11.2	R2	ST131	CAZ,CTX,CRO, FOX, CF	AMP-CAZ-CF-XNL-S-SXT-T	bla_{ctx}, *strA*, *str* B, *sul1*, *tet*(A), *tet* (B), *tet* (M)	B2
F13.1	R2	ST131	CAZ,CTX,CRO, FOX, CF	AMP-CAZ-CF-XNL-NA-S-SPT-SXT-T	bla_{ctx}, *str* A, *str* B, *sul1*, *tet*(A)	B2
F13.2	R2	ST131	CAZ,CTX,CRO, FOX, CF	AMP-CAZ-CF-XNL-S-SPT-SXT-T	bla_{ctx}, *str* A, *str* B, *sul1*, *tet*(A), *tet* (M)	B2
F14.1*	R2	ST 58	ND	No resistance	No resistance	BI
F15.2	R2	ST131	CAZ,CTX,CRO, FOX, CF	AMP-CAZ-XNL-N–S-SXT-SPT-T	bla_{ctx-M}, *str* A, *str* B, *sul1*, *tet* (A), *tet* (M), *tetB*	B2
F16.2	R2	ST131	CAZ,CTX,CRO, FOX, CF	AMP-CAZ-CF-XNL-NA-SXT-T	bla_{ctx-M}, bla_{CMY-2}, *str* A, *str* B, *sul1*, *tet*(A),	B2
F20.3**	R2	ST68	CAZ,CTX,CRO, FOX,CF	AMP-AMC-CAZ-XNL-N-S-T	bla_{ctx}, bla_{CMY-2}, bla_{shv}, *str* A, *str* B, tet(A)	F
F27.2**	R2	ST68	CAZ,CTX,CRO, FOX, CF	AMP-AMC-CAZ-XNL-T	bla_{ctx}, bla_{CMY-2}, bla_{tem}, tet (A) *tet* (M)	F
F31.1	R2	unknown	Negative	AMP-AMC-CTX-XNL-	bla_{CMY-2}_ *strB*, *tet(B)*,	B2
F32.1	R2	ST10	Negative	AMP-CAZ-XNL-C-NA-S-T	bla_{CMY-2}, *str* A, *tet* (A)	A
F34.2	R3	ST7348	Negative	AMP-AMC-XNL-C	Bla_{CMY-2} *tet (M)*	BI
F35.2	R3	ST2541	CAZ,CTX,CRO	AMP-CAZ	bla_{ctx}	A
F 42.2	R4	Unknown	Negative	AMP-AMC-CAZ-XNL-NA-N-S	bla_{CMY-2} *strB*	BI
F43.1	R4	ST7207	Negative	AMP-AMC-XNL-C-NA-N-S-T	bla_{CMY-2} *strB*, *tet(B)*, *tet(M)*	A
F44.2	R4	Unknown	Negative	AMP-CAZ-XNL-NA-N	bla_{CMY}	E
F46.2	R4	ST357	Negative	AMP-CAZ-NA-N-S-T	bla_{CMY-2} *strB*, *tet(B)*	B2
F47.2	R4	ST58	Negative	AMP-AMC-XNL-ENO-NA-N-S	bla_{CMY-2}, bla_{tem}, *str* A	BI
F48.1	R4	ST3727	Negative	AMP-AMC-XNL-NA-N	bla_{CMY-2}	C
F52.2	R5	ST195	Negative	AMP-AMC-CAZ-C	bla_{CMY-2}	C
Water						
NC5.1ctx	R2	ST131	CAZ,CTX,CRO, FOX	AMP-CAZ-XNL-S-SXT–T	bla_{ctx}, *str* B, *sul1*, *tet*(A), *tet* (M)	B2
SW2.3 ctx	R2	ST1625	Negative	AMP-AMC-CTX-C-AZ-XNL	bla_{CMY-2}	BI
NC5.3	R3	ST2721	Negative	A-AMC-XNL-CF-S-T	bla_{CMY-2} *str* B, *tet* (A)	Cryptic Clade III,IV,V
NC6.2*	R3	ST58	ND	No resistance	No resistance	BI
NC 6.7	R3	ST10	Negative	AMP-CAZ-XNL-C-NA-S-T	$bla_{CMY-2}bla_{shv}$, *strB*, *tet(B)*	A
SW2.4	R3	ST83	Negative	AMP-AMC-CAZ-XNL-NA-SPT-S-T	bla_{cmy-2}*strB*	B2
NC5.2	R3	ST1204	Negative	AMP-AMC-XNL-CF-S-T	bla_{CMY-2} bla_{shv}, *str* A,*strB*, *tet* (M)	D
RP3.1ctx	R3	Unknown	CAZ-CTX-CRO, FOX	AMP-CTX-XNL-NA-SPT-S-SXT-T	bla_{ctx}, *aadA*,*sull*, *tet(M)*	E
RP3.1 (Box)	R3	ST5463	Negative	AMP-CTX-XNL-CF-NA-SPT-S-SXT-T	bla_{shv}, *str* A, *sul1*, *tet* (B)	E
RP3.2*	R4	ST58	Negative	AMP-AMC-CTX-XNL-ENO-NA-N	bla_{CMY-2}	BI
RP3.5 (CAT)	R4	ST469	Negative	AMP-XNL-CF-NA-S	bla_{tem}, *strA*	BI
SW2.2	R4	ST1065	Negative	AMC-CTX-CF-S	*bla*, *strA*,*strB*	B2
SW2.4	R5	ST1850	Negative	AMP-AMC-CAZ-CF-XNL-S-SXT-C	$bla_{CMY.2}$ *strB*, *sull*	A
RP3.6	R5	Unknown	Negative	CF-XNL	bla_{CMY}	C
RSI.3	6/16/2017	Unknown	CAZ-CTX-CRO-CF	AMP-CAZ-CF-XNL-S-SXT-C	bla_{ctx}, *tet*(B), *str* A *sul1*	E
RS2.1 Mcoli	4/6/2018	ST297	CAZ-CTX-CRO-FOX-CF	AMP-AMC-CAZ-CF-CRO	bla_{ctx}	B1

**Selected because they had same mdh (282 bp) sequence (Ivanetich et al., 2006). AR, antibiotic resistance; ST, Sequence Type. R1, R2, R3, R4, R5 are collection dates from 8/20/14, 9/17/14, 1/21/15, 2/27/15, 4/5/15, respectively. RS2.1 Mcoli was an isolate with the bla ctx gene that was found in the summer of 2018 (June–August) from two rounds of sampling that screened 15 isolates. **Indicate isolates that had both bla ctx-M and bla cmy-2 genes.*



Phylo-groups	B 1	B2	E	D	A	C	F	Cryptic clades
Z statistics	0.152	-1.022	0.377	-0.246	0.857	0.229	-1.000	0.229
P value	0.44	0.11	0.36	0.41	0.19	0.41	0.16	0.41

FIGURE 5 | Phylo-grouping of the Isolates. Percentage of *E. coli* isolates from water (n = 46) and feces (n = 92) belonging to each phylo-group. Different groups are represented by different colors. Among the water isolates there were no unknowns. Table indicates significant difference in presence of each phylo-group, between water and fecal samples.

was found in a fecal isolate F47.2 as well as water isolate RP3.2, both isolated on 2/27/15.

The fecal and water isolates were phylo-typed by the method of Clermont et al. (2013). The largest percentage of *E. coli* isolates from both crow fecal (n = 91) and surface water (n = 46) samples belonged to the non-pathogenic, commensal phylo-group B1, followed by the pathogenic B2 group (**Figure 5**). Statistical analysis revealed no significant difference in the presence of any of the phylo-groups across the water and fecal isolates ($p > 0.05$). Although the B2 and D phylo-groups, the two groups where most of the ExPEC strains are expected to belong, have a slightly more representation among the fecal isolates, the numbers are not statistically significant.

DISCUSSION

Several studies have reported that the environment imposes its own selection on the population of *E.coli* following fecal deposition from its primary habitat within the intestine of animals (Gordon et al., 2002; Bergholz et al., 2011; Jang et al., 2017). As a result a new genomic diversity may develop with species that are stress tolerant and are able to adapt locally to that particular matrix being amplified and over represented. To what extent this will happen is a subject of much debate and study, nonetheless, it is generally agreed that fecal deposition is the major predictor of the population structure of the matrix (Bergholz et al., 2011; Jang et al., 2017). Thus, while there were differences in the genetic diversity of the *E. coli* isolated from the crow fecal isolates in our wetland, from the limited sequence typing we performed, the finding of similar antibiotic resistance pattern between the water and crow isolates is not unexpected.

The fecal population showed no significant difference in the overall resistance to twelve of the 13 antibiotics tested, when compared to that of the water population. Some of the drug resistance genetic determinants may be on mobile genetic elements, e.g., plasmids were isolated from F20.3, F46.1, and RP3.5 ctx, F15.2 (results not shown) and these have the ability to be transmitted to the indigenous bacteria in the wetland (Aminov, 2011; Wellington et al., 2013). The number of isolates resistant to at least one antibiotic in the crows (70%) and water (65%) was high in our study. In 97% of our isolates we were able to find the corresponding genetic determinant of the phenotypic antibiotic resistance displayed by an isolate. The distribution of isolates based on their phylo-group, proved to be similar between the fecal and water samples, providing additional support that crow fecal deposition drives the distribution of the strains in water. The high proportion of B1 phylo-group (37% in fecal and 39% in water) in our isolates agrees well with one other recent study which found high percentages of the commensal *E. coli* phylo-group B1 in the fecal (38%) and soil (40%) samples collected in a recreational meadow (Bergholz et al., 2011). They correlated phylo-group B1 *E. coli* with the presence of feces from wild and domestic animals. In our study, however, presence of the B2 phylo-group cannot be ignored because of their potential to cause disease. 21 and 13% of the fecal and water isolates, respectively, belonged to the B2 phylo-group, which is expected to contain the majority of the extra intestinal pathogenic *E. coli*

(ExPEC) strains and may come from a human source (Picard et al., 1999; Tenaillon et al., 2010). The D group which contains some ExPEC strains was also represented in the fecal and water samples. Further characterization of the virulence genes from these isolates are in progress.

We found a predominance of bla_{cmy-2} gene in the AmpC phenotype in the crow (16.8%) and water (18.36%) isolates. bla_{cmy-2} has been shown to be the most common plasmid borne beta lactamase in human, animal, and environmental bacterial isolates, and that includes large corvids in United States and Canada (Pitout et al., 2007; Mataseje et al., 2010; Folster et al., 2011; Martin et al., 2012; Jamborova et al., 2017, 2018). In a recent report 18.7% of Corvids from Canada were shown to carry the bla_{cmy-2}, which was substantially more than that reported from Corvids from European countries (4.4%). The authors suggested a difference in population dynamics of antimicrobial resistance in *E. coli* between the two continents. While our sample size and survey is small, this may be true for the United States as well, especially since another report on *E. coli* isolated from different cities of the United States from the same species of corvid as ours, viz., *C. brachyrhynchos*, described 15–19% presence of bla_{cmy-2} (Jamborova et al., 2017). bla_{cmy-2} is not very commonly isolated among clinical isolates in the United States (Castanheira et al., 2013). The bla_{cmy-2} isolates in this study could have come from any number of sources. MLST analysis revealed a genetic diversity within and between the fecal and water *E. coli* isolates possessing the bla_{cmy-2}. Sequence types frequently isolated from companion animals as well as livestock and farm animals, besides humans, were found in these isolates. Thus, ST7207, ST5914, ST2721, ST2541, ST1204 found in our study were shown to have been isolated from livestock and water sources[4]. Agricultural and rural lands are abundant in the nearby Snohomish County, WA and it is possible that the crows acquired some of these strains from the farm animals that live there. Other bla_{cmy-2} possessing sequence types found in this study, viz., ST58, ST 83, ST357, which have been shown to belong to Avian Pathogenic *E. coli* (APEC) group, have been reported to be found in birds including crows, poultry, companion animals, as well as humans (Dissanayake et al., 2014; Jamborova et al., 2017). In humans they been described as ExPEC strains capable of causing urinary tract infections among other infections, but can be present as non-pathogens as well. Two ST58 strains in our study had no virulence genes or antibiotic resistance genes. Two other fecal isolates, ST8371 and ST2614, have previously been reported to be isolated only from humans[4].

The most unexpected finding was the presence of an isolate (NC5.3 ctx) belonging to ST131 from the North Creek site within the roosting area of the wetland. ST131, a pandemic clone, has been shown to be responsible for severe extra intestinal infections in animals and humans, besides being MDR (Johnson et al., 2010). In the United States it was first reported in 2007 (Johnson et al., 2010). The wetland is situated within the UWB/CC campus which has a maximum population of 6000 students and thus is not crowded. The campus septic wastewater is entirely piped offsite for treatment and there are no septic systems or porta-potties on campus. However, North Creek originates in the highly urbanized City of Everett flowing 12.6 miles southward through suburban areas of the cities of Mill Creek and Bothell before reaching the UWB/CC campus, passing the roost area, and draining into the Sammamish River. There are many houses with septic systems in the North Creek drainage basin (City of Bothell 2019) and the creek has received raw sewage discharges multiple times between 2012 and 2018 during peak rainfall events (King County, 2014). Overbank flooding from North Creek did not occur during sampling, so North Creek water did not impact any of the wetland water samples. Nor were water samples collected during or shortly after the sewage overflow events (eight between 11/24/16 and 3/18/17) from upstream manhole 54 of the North Creek Interceptor sewer line. Isolate NC5.3 ctx had an antibiotic resistance phenotype that matched with the fecal isolate F11.1 which also was ST131. It is tempting to speculate that the water ST131 came from one of the crows. The omnivorous feeding habit of the crows, together with their synanthropic behavior may very well allow them to be colonized by MDR bacteria. This has been shown in other studies as well (Jamborova et al., 2017). In addition, these North American crows can fly as far as 40 miles per day away from their roosting site in non-breeding seasons (Link, 2005) to acquire food, and these may include agricultural and rural areas as well (Roberts et al., 2016). All of the ST 131 isolates belonged to the phylo-group B2, indicating the isolates may be virulent strains.

By grouping the isolates based on the *mdh* gene (**Supplementary Figure S1**) and performing MLST on selected isolates within a cluster, we were able to find two more sequence types from the water that matched with those of crows and both were collected on the same respective dates as the fecal isolates. The phylo-group and antibiotic resistance phenotype matched in both cases. Both STs have been reported to be found from crows as well as humans. Analysis by techniques such as Pulsed-field gel electrophoresis or repetitive sequence-based PCR or Whole Genome sequencing can further firmly establish the clonal relationship of these isolates. Interestingly, the ST131 strain found in both fecal and water samples in September, 2014 was not found again in subsequent isolations from 2014, 2015, 2016, or 2017. This was also true for the other two isolates with matching STs. Only one ST58 (F14.1) found in September, 2014, was seen in water collection of February, 2015 (RP3.2). Their AR phenotype matched, but the exact clonal relationship needs to be confirmed. Thus, it appears that most of these strains may not be able to survive for long in the environment. *E. coli* abundance is known to decline over months in water and soil matrices, although persistent strains may remain (Avery et al., 2004; Vivant et al., 2016). It can be speculated that the isolates are not able to survive in the crow gut either for any length of time, since the crows are known to roost in the same area repeatedly (Link, 2005) and the STs were not recovered in the following months. Further studies are needed to understand how long they persist in the gastrointestinal tracts of the birds. We continued to monitor for ESBL *E. coli* in water through 2016, 2017 and spring 2018 at the roosting sites. We were able to find only two more ESBL containing isolates, one of which belonged to ST297, and for the other we were not able to find a ST, even though we

[4]http://enterobase.warwick.ac.uk/species/ecoli/search_strains

found a matching allele for each of the seven genes in both of the MLST data bases that we used.

Increase in antibiotic resistant *E. coli* in storm water runoff has been reported by Salmore et al. (2006) and increase in ARGs due to storm water loading was recently reported by Garner et al. (2017). Our study also detected additional ARE following rainfall, with tetracycline resistance increasing the most. While the crows deposit the bulk of their feces in the roost area, they gather for short periods each dusk and dawn all over the campus leading to widespread deposition of feces. During dry periods, the crow feces and the bacteria contained within them accumulate on campus. During rain events, these bacteria are mobilized, flowing in the storm water system. It is also possible septic systems within the North Creek watershed overflow during a storm event, contributing additional bacteria. An increase in overall *E. coli* count was also observed at the sampling sites, both within and outside the roost area in response to rain events (**Supplementary Figure S2**).

CONCLUSION

In conclusion, although most of the crow deposited strains may not be able to survive for long in the wetland, there appears to be a constant addition of AR bacteria, and most of them appear to be coming from the crows because the overall pathogenicity and AR pattern of the wetland water isolates were very similar to that of the birds' fecal isolates over the course of 9 months that they were tested. Regardless, the crows do drink this water and ingest the *E. coli* during their daily visitation to the wetland. They are thus potential vectors for transmission of the multiple drug resistant strains (as well as non-virulent and non-AR ones) to various places during their daytime scavenging activities. They are also partially migratory, with populations moving to more southern latitudes of North America during the winter and thus these strains may be carried even further during the winter months (Verbeek and Caffrey, 2002), posing an overall public health risk. This first report from one of the largest crow roost areas within the state of Washington, highlights the risks that the crows may pose for the spread of antibiotic resistance and the need for remedial measures.

AUTHOR CONTRIBUTIONS

KS designed and supervised all experiments and did data analysis, performed some experiments and prepared the manuscript. RT identified sites in the wetland to collect samples, determined coliform and *E. coli* counts and helped with manuscript preparation. TB collected samples, determined AR by phenotypic and genotypic methods, performed MLST and data analysis. MS collected samples, did AR studies, *mdh* sequence based phylogenetic studies, and data analysis. BT did MLST and phylo-grouping studies. YM determined all *tet* genes and *sul1* gene presence. MF determined *str* gene presence, *tem* and *shv* sequences and plasmid isolation. LK did phylo-grouping studies. JL helped with manuscript preparation and some analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01034/full#supplementary-material

FIGURE S1 | Molecular Phylogenetic analysis of the crow and water isolates based on *mdh* 282 bp region. A 825 bp region of the mdh gene was amplified and sequenced for 32 fecal and 29 water isolates. For Fecal samples 11 and 13, named F11 and F13, respectively, two isolates were sampled. A 282 bp region from this was trimmed, aligned, and a phylogenetic tree was obtained using the Maximum Likelihood method based on the Tamur-Nei model. Eight clusters (at least three isolates with the same sequence) were obtained as marked. The different rounds of collection are denoted as: 8-20-14 (R1) 9-5-15 (R2), 1-21-15 (R3), 2-27-15, (R4), 4-5-15(R5). Accession numbers of the *mdh* sequences deposited in GenBank are: MK564267 to MK564325.

FIGURE S2 | Impact of Rain events on total counts of *E. coli*. Total number of *E. coli* in CFUs was determined at three of the sites, RS1, RS2, and SW8 before and after a rainfall event. The number of times (N) this was determined at each site is indicated in the figure.

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Sub-Inhibitory Effects of Antimicrobial Peptides

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Antimicrobials, and particularly antimicrobial peptides (AMPs), have been thoroughly studied due to their therapeutic potential. The research on their exact mode of action on bacterial cells, especially at under sublethal concentrations, has resulted in a better understanding of the unpredictable nature of bacterial behavior under stress conditions. In this review, we were aiming to gather the wide yet still under-investigated knowledge about various AMPs and their subinhibition effects on cellular and molecular levels. We describe how AMP action is non-linear and unpredictable, also showing that exposure to AMP can lead to antimicrobial resistance via triggering various regulatory systems. Being one of the most known types of antimicrobials, bacteriocins have dual action and can also be utilized by microorganisms as signaling molecules at naturally achievable sub-inhibitory concentrations. The unpredictable nature of AMP action and the pathogenic response triggered by them remains an area of knowledge that requires further investigation.

Keywords: antimicrobial peptides, AMP, sub-inhibitory effects, virulence, factor of pathogenicity

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INTRODUCTION

Antimicrobial peptides (AMPs) are protective molecules of innate immunity in living organisms (Zasloff, 2002).

In general definition, antimicrobial peptides are a diverse group of naturally derived or synthetically obtained molecules, which have antimicrobial properties because of their specific physical properties (antivirus and/or antitumor properties, in several cases). Attempts to classify antimicrobial peptides interfere with the structural diversity of existing substances. In a general, there are two ways in which peptides are synthesized; this fact underlies their structural and functional diversity. Natural-derived AMPs can be formed by ribosomal synthesis and can be produced from non-ribosomal peptide synthesis. Ribosomally synthesized peptides are produced by almost all organisms, their classification is based on the secondary structure formed in aqueous solutions. Thus, distinguish α-helical, β-sheet, peptides with extended/random-coil structure (Hancock and Chapple, 1999; Bahar and Ren, 2013; Mahlapuu et al., 2016).

In turn, the greatest diversity is inherent in microbial antimicrobial peptides, since microorganisms are capable not only of non-ribosomally synthesis (Hancock and Chapple, 1999), but also of post-translational/co-translational modifications (Arnison et al., 2013). Extensive post-translational modifications give peptides additional properties, for example, better recognition of targets and increased stability, which expands their functionality as compared to ribosomally synthesized peptides of animals (Arnison et al., 2013). These peptides have been classified within the bacteriocins, the most recent classification of which is given in review (Acedo et al., 2018).

As of now, the nature of antimicrobial peptides has been thoroughly investigated. All data accumulated to date can be summarized in simple statistics. For instance, upon a query, an antimicrobial peptide database (October 2018) returns extracted data on three thousand peptides with annotated structures (Wang et al., 2016).

In addition, the number of articles dedicated to the study of antimicrobial peptides exceeds 350,000[1]. Such a heightened interest in this topic does not seem unreasonable, since antimicrobial peptides remain an attractive alternative to conventional antibiotics. AMPs have a unique ability to overcoming pathogenic virulence and defense, primarily by targeting highly conserved structures of the microbial cell (Brogden, 2005; Omardien et al., 2016). Due to the unique properties of AMPs, they can and should be used for the benefit of humanity in the face of the antibiotic resistance catastrophe (Ventola, 2015). Existing efforts of scientific research are directed toward searching for more effective bactericides and studying of their mode of action (Cytryńska and Zdybicka-Barabas, 2015). Even though such investigations are necessary, there are some aspects of this problem that are poorly addressed by research. This includes the under-investigated effects of sub-inhibitory concentrations (sub-MIC) of AMPs on the physiology of the bacterial cells. Often, produced peptides dilute in the environment medium. Thus, it appears that the peptide concentration necessary for bactericidal of fungicidal effect is not always achievable in natural conditions.

Regarding conventional antibiotics, their effects at sub-inhibitory concentrations have been studied for a substantially long period of time (Lorian, 1975; Andersson and Hughes, 2014). It has been shown that sub-inhibitory concentrations of antibiotics can trigger unexpected reactions from the bacterial population. For example, fluoroquinolones can stimulate bacterial adaptation to different stresses, including effects of antibiotics (López and Blázquez, 2009).

By the way the AMP's action on eukaryotic cells also have concentration dependent features (Baindara et al., 2017).

Generally, the antimicrobial action of peptides is exhibited via compromising the integrity of the microbial cell's barrier structures. However, other intracellular targets for peptides are known (Hale and Hancock, 2007), which leads to the conclusion about peptide's multifunctional nature (Le et al., 2017). In this review, we are summarizing the currently available data on the sub-inhibitory concentrations effects (sub-MIC effects) of antimicrobial peptides on bacteria. Our main interest is directed toward peptides' ability to trigger various effects on subcellular (expression of virulence genes) and cellular (phenotypic manifestation of the response) levels. It is important to note that the response of a bacterial population to AMP's treatment can be both positive and negative for humans. Positive effects include changes in the morphofunctional properties of bacteria that, lead to a decrease in their pathogenicity. Negative effects are comprised of increased bacterial aggression after being exposed to antimicrobial peptides.

[1]www.pubmed.gov

The remaining questions are as follows:

1. What are the triggering mechanisms behind sub-MIC effects?
2. Is it possible to predict the nature of the bacterial response to sub-MIC action of an AMPs?
3. How exactly does AMP structure determine its sub-MIC action?

Given the therapeutic potential of antimicrobial peptides in addition to the known data on the sub-MIC effects of conventional antibiotics, this review aims to encourage the investigation on the non-killing effects of antimicrobial peptides.

SUB-INHIBITION CONCENTRATION EFFECTS OF AMPs AT SUBCELLULAR LEVEL

The Molecular Mechanisms of Peptide Reception and Response to Sub-Inhibitory Action

Antimicrobial peptides have physical and chemical properties necessary to be able to interact with bacterial membranes (Datta et al., 2015). Interaction of cationic peptides is promoted through electrostatic interaction, while interaction of anionic peptides is driven by hydrophobicity (Phoenix et al., 2013; Travkova et al., 2017). Membrane damage is the main cause of cell death, since it disrupts the work of many subsystems, associated with the membrane's integrity. If membrane damage is not fatal, the cell is able to respond to external stress.

Bacterial genomic machinery responds with the expression of various genes within several minutes after the moment of exposure to stress factors. One of the first works on sub-MIC effects of AMPs was dedicated to cecropin A and *E. coli* cells (**Table 1**). It was found that cecropin A caused a significant change in the transcript levels for 26 bacterial genes (Hong et al., 2003); the sub-MIC of colistin altered expression of 30 genes of *P. aeruginosa* (Cummins et al., 2009); LL-37 affected expression of several 100 genes of *P. aeruginosa* (Overhage et al., 2008).

Thus, antimicrobial peptides in the non-killing concentration has a strong restructuring effect on of a genome's functionality.

Can the Direct Peptide-DNA Interaction Affect Bacterial Transcriptome?

What is the mechanism of signal reception and transmission? It may be a direct interaction of the peptide molecule with bacterial DNA. It is known that many AMPs have a dual mode of action (**Table 2**). At high peptide concentrations they cause damage to cell membranes, eventually breaking it down, but at lower concentrations, peptides translocate to the cytoplasm and electrostatically interact with DNA or ribosome (Gottschalk et al., 2015; Polikanov et al., 2018). For example, a number of synthetic peptides can interact

TABLE 1 | The physico-chemical properties of antimicrobial peptides described in the review.

Peptide	Mol. weight, Da[1]	Type of structure	Charge[2]	μHrel[2]	GRAVY[1]	The sources of the structural data
LL-37	4490.6	Alpha-helix conformation	+6	0.499	−0.72	www.anaspec.com
Cecropin A	4004.82	Alpha-helix conformation	+6	0.202	−0.07	www.anaspec.com
Indolicidin	1700	Random coil	+4	0.190	−1.07	www.anaspec.com
Fallaxin analog FL9	2717	Alpha-helix	+2	0.275	0.51	Nielsen et al., 2007
C18G	2043	Alpha-helix	+7	0.604	−0.19	Kohn et al., 2018
α-defensin HNP-1	3448	β-strand, β-turn	+3	0.028	0.30	www.anaspec.com
β-defensin hBD-1	3934	Alpha-helix and triple-stranded antiparallel β-sheet	+4	0.348	−0.27	www.anaspec.com
β-defensin hBD-2	3885.9	Alpha-helix and triple-stranded antiparallel β-sheet	+6	0.246	−0.10	http://bpsbioscience.com/bd-2-90107-b
Bovicin HC5	3525		+2	0.163	0.28	http://bactibase.hammamilab.org
Subtilosin A	3425	Cysteine sulfur to α-carbon bridges	−2.2	0.08	0.69	Acedo et al., 2018, http://bactibase.hammamilab.org
Plantaricin A	2683	Alpha-helix conformation	+5	0.321	−0.24	http://bactibase.hammamilab.org
Subtilin	3465	Fivefold-stranded antiparallel β-sheet and alpha-helices	+2	0.151	0.19	http://bactibase.hammamilab.org
Nisin Z	3475	Alpha-helices and β-turn	+3	0.084	0.41	http://bactibase.hammamilab.org
Polymyxin B	1203.50	Cyclic	+5	ND	ND	Fernández et al., 2012
Colistin	1156.0	Cyclic	+5	ND	ND	Fernández et al., 2012
Hemoglobin-derived Hbg-1	2495	Random coil	+1	0.053	−0.56	Merriman et al., 2014
Hemoglobin-derived Hbg-2	2495	Random coil	+1	0.220	−0.56	Merriman et al., 2014
Dipeptides cyclo(L-Phe-L-Pro)	245.35	ND	ND	ND	ND	Li et al., 2011

[1]The properties were calculated using the web-tool, which is available at https://www.thermofisher.com/ru/ru/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html. The grand average of hydropathicity (GRAVY) of a peptide is the sum of the hydropathy values of all the amino acids divided by the number of residues in the peptide or protein sequence. [2]The properties were calculated using the web-tool, which is available at http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py. The relative hydrophobic moment (μHrel) is the hydrophobic moment of a peptide relative to that of a perfectly amphipathic peptide.

with DNA and induce a SOS-response. During this process, peptide's action increases the expression of the α-haemolysin (Gottschalk et al., 2015). A similar effect was shown for indolicidin, which disturbed a membrane at MIC and induced the SOS-response at sub-MIC (Vasilchenko et al., 2017). The direct mutagenic effect of the cationic peptide is known (Limoli et al., 2014). However, it should be noted that mutagenesis and SOS-response are observed only at concentrations close to MIC, whereas a change in the transcriptome is usually observed at doses that are many times smaller (Farris et al., 2010).

Thus, changes in gene expression caused by the DNA-peptide interaction should be considered exceptional and not as a general rule.

Recently a novel approach for precisely prevention of pathogenicity of Gram-negative bacteria was described, which is based on blocking a specific gene transcription by cationic peptide. The authors designed and synthesized cationic hydrocarbon stapled alpha-helical peptides based on a DNA-interacting a helix of σ54. The treatment of bacteria with synthesized peptides blocked the interaction between endogenous σ 54 and its target DNA sequence (Payne et al., 2018).

Thus, deciphering the molecular mechanisms of interaction of peptides with intracellular targets is a bridge between the fundamental knowledge and the practical use of the knowledge gained.

Peptide Sensing?

In addition to nucleic acids, there are other intracellular targets for antimicrobial peptides. In particular, the bacterial cell envelope contains a variety of sensory regulatory systems, which sense environmental signals and regulate a genes expression accordingly.

Two-component systems (TCS) are widely distributed among bacteria and are diverse in structure and function. The presence of about one hundred thousand identified and classified TCS allows bacterial cells to recognize many different stressors and respond to them (Tiwari et al., 2017). In general, a TCS is comprised of a sensor protein (histidine kinase) and its corresponding response regulator. The sensor kinase attaches to the bacterial cytoplasmic membrane that has a sensing domain on its extracellular side.

Antimicrobial peptides can have an effect on bacterial genomes both indirectly and directly. Indirect action

TABLE 2 | The mode of action and sub-inhibitory effects of peptides described in the review.

Peptide	The cell's targets	Negative sub-MIC effects*	Positive sub-MIC effects*	References
LL-37	Membranes permeabilization; direct DNA binding	Promote mucoidy phenotype in Gr-bacteria; overproduction of virulence factors; promote resistance to antimicrobials	Inhibites biofilm formation *P. aeruginosa* and *S. aureus*	Fernández et al., 2010; Dean et al., 2011; de la Fuente-Núñez et al., 2012; Strempel et al., 2013; Limoli et al., 2014
Cecropin A	Membranes permeabilization	Unknown	Unknown	Silvestro et al., 2000; Hong et al., 2003; Rangarajan et al., 2013
Indolicidin	Membranes permeabilization; direct DNA binding	Promote resistance to antimicrobials	Prevent biofilm development of MRSA *S. aureus*	Fernández et al., 2010, 2012; Mataraci and Dosler, 2012
Fallaxin analog FL9	Membranes permeabilization; direct DNA binding	Increase production of α-haemolysin	Unknown	Gottschalk et al., 2015
C18G	Membranes permeabilization	Increased expression of the virulence factor of *S. typhimurium*	Unknown	Yu and Guo, 2011
α-defensin HNP-1	Membranes permeabilization; lipid II binding; target the ExPortal of *S. pyogenes*	Unknown	inhibition of secretion of SpeB cysteine protease and the streptolysin O	Vega and Caparon, 2012
β-defensin hBD-2	Membranes permeabilization	Unknown	Regulatory of gut homeostasis	Marzani et al., 2012; Dicks et al., 2018
Bovicin HC5	Membranes permeabilization	Unknown	Prevents biofilm formation of *S. aureus*	Mantovani et al., 2002; Pimentel-Filho Nde et al., 2014
Subtilosin	Membranes permeabilization	Unknown	Prevents biofilm formation of Gram-negative bacteria	Algburi et al., 2017
Plantaricin A	Membranes permeabilization at high (*in vitro*) concentration and pheromone at low (in natural) concentration	Unknown	Involved in the formation of a sustainable animal microbiome	Anderssen et al., 1998; Hauge et al., 1998; Kristiansen et al., 2005; Sturme et al., 2007; Calasso et al., 2013
Nisin	Membranes permeabilization; inhibites peptidoglycan sintesis; pheromone	Unknown	Inhibites bacterial biofilm formation	Mahdavi et al., 2007; Shin et al., 2015
Polymyxin B	Membranes permeabilization	Promote resistance to antimicrobials	Inhibites of secretion of SpeB cysteine protease and the streptolysin O	Fernández et al., 2012; Vega and Caparon, 2012
Colistin	Membranes permeabilization	Resistance; promote biofilm formation; pyocyanin production		Cummins et al., 2009; Fernández et al., 2012
Hemoglobin-derived peptides (Hbg-1, 2 and other)	Membranes permeabilization	Promote *S. aureus* surface colonization	Inhibites production of TSS toxin-1, enterotoxin C, α, δ hemolysin of *S. aureus*	Schlievert et al., 2007; Pynnonen et al., 2011
Dipeptides cyclo(L-Phe-L-Pro)	Unknown	Unknown	Inhibites production of TSS toxin-1	Li et al., 2011

**Is meant the reactions of the bacterial population, which has a final positive or negative effect on macroorganism (animal, plant, etc.).*

occurs in response to a violation of the structural integrity of cell barriers (**Table 2**). For example, Rcs regulon controls the expression of many specific virulence factors in bacteria belonging to the Enterobacteriaceae family. According to a model proposed by Farris et al. (2010), the sensory molecule RcsF is anchored to the outer membrane, sequestered from its signaling partners in the "off state." During the cellular envelope disorganization, conformational or spatial change promote direct non-covalent interaction of RcsF with periplasmic domains of signaling constituents, leading to Rcs activation. A more detailed molecular mechanism is described in the review (Guo and Sun, 2017; **Figure 1**).

Interaction of antimicrobial peptides with bacterial membranes in some cases led to an indirect activation of several genes regulated through "Quorum Sensing" (QS). It is known that

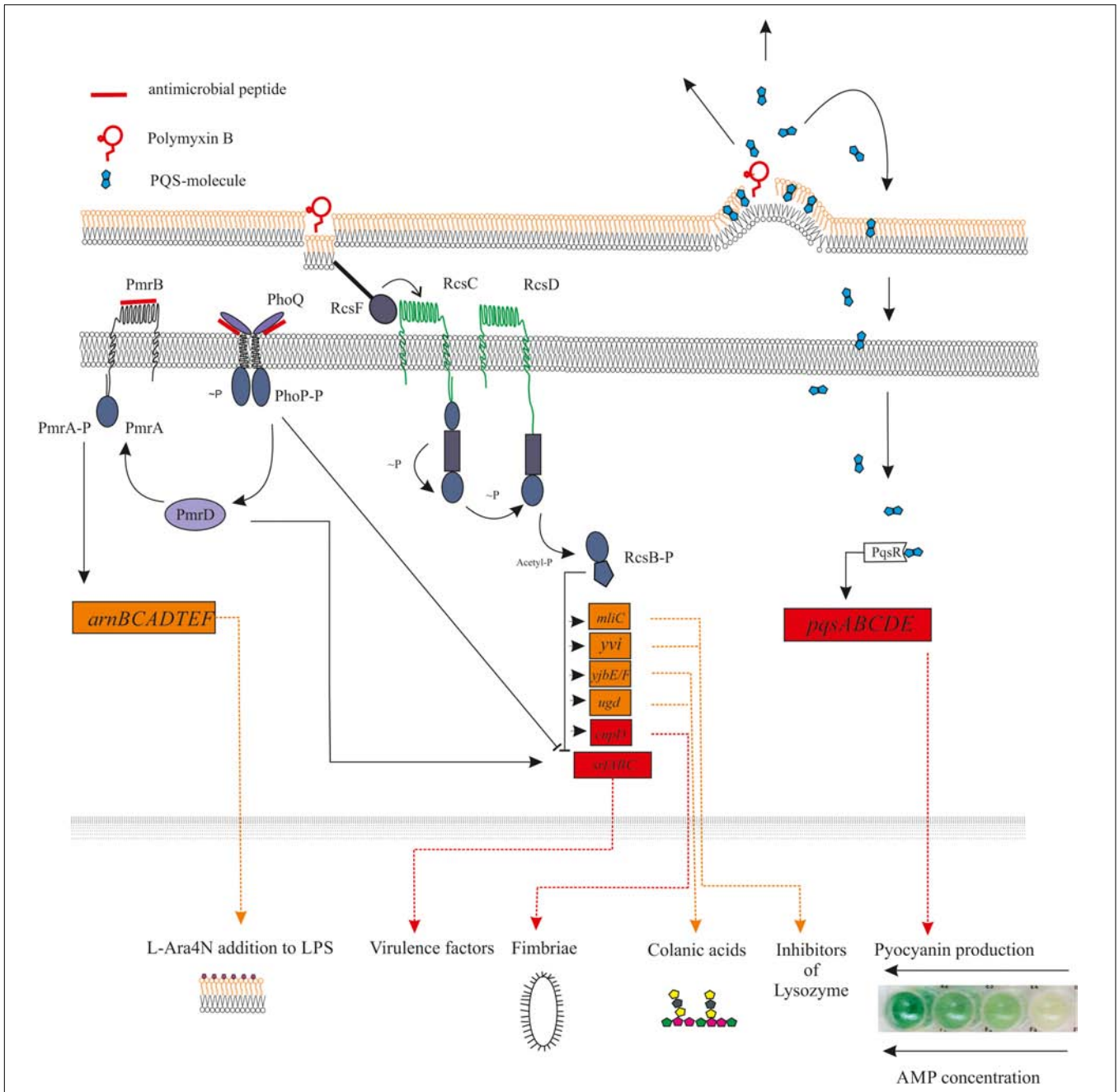

FIGURE 1 | Diagram showing the main receptors of antimicrobial peptides and the relationship between them. The mechanisms of signal transmission from the activated receptor to the corresponding genes are shown. Antimicrobial peptides is capable to direct (PhoQ, PmrAB, and other) or indirect (RcsF) activation of the histidine kinase sensors, which led to regulation of activity of appropriated genes. At the same time, the expression of some genes can simultaneously be under the positive and/or negative regulation of different TCS. For example, the effect of polymyxin B on the bacterial outer membrane can activates the RcsF sensors, what leads to inhibition of expression of virulence genes in *srfABC* operon. Interestingly, the same operon is activated by another TSC PmrAB, for which "Peptide Sensing" was revealed (for example, for LL-37).

some hydrophobic QS-autoinducers such as PQS are trafficked between cells via membrane vesicles (Mashburn-Warren et al., 2008). In this case, the peptide's membrane-permeabilizing action releases accumulated PQS molecules, which can triggers the expression of the virulence genes associated with quorum sensing (Cummins et al., 2009; **Figure 1**).

Another example of TCS being indirectly activated by AMPs is the PhoQP two-component system, which controls the development of resistance to AMPs. The periplasmic domain of the PhoQ sensor is in conjunction with Mg^{2+} cations. Reducing the available amount of magnesium leads to electrostatic repulsion between PhoQ and the inner membrane domain (Cho

et al., 2006). The resistance of *Salmonella* to polymyxin B is formed through this mechanism, since this AMP is able to displace Mg^{2+} cations from their binding site in the PhoQ sensor (Santos et al., 2017; **Figure 1**).

The majority of antimicrobial peptides have cationic properties that allow them to interact directly with the extracellular loop of sensors activating them (Li et al., 2007b; Gryllos et al., 2008). The possibility of such direct interaction was convincingly demonstrated in the study examining the ability of the LL-37 to activate the expression of streptococcal virulence factors, which are under control of the CsRS (CovRS) two-component system (Gryllos et al., 2008). Streptococci have cell surface-associated histidine kinases CsrS that can directly sense peptide molecules (Tran-Winkler et al., 2011). It turned out that a 10-amino acid residue fragment of the LL-37 did not exhibit any antimicrobial activity, but it determined the direct interaction of the LL-37 molecule with the sensory part of CsrS, according to the principle of ligand-receptor interaction (Velarde et al., 2014). Presumably, such interactions are determined by electrostatic forces, since the sensor domain of a two-component system has periplasmic loops which are usually negatively charged (Fernández et al., 2010).

Thus, there is strong evidence for the fact that bacteria have some kind of "Peptide Sensing." It is only left to find out how sensitive is the "Peptide Sensing." Does the "Peptide Sensing" recognize the specific structure of a peptide or does it responds to peptides as stress agents in the whole? These questions are not easy to answer, and more research is still needed. However, it is already clear that bacteria have sensory systems and mechanisms, which respond specifically to positively charged amphiphilic molecules with a certain amino acid composition.

Qualitative and Quantitative Response of Sensory Regulatory Systems on Antimicrobial Peptides

Sensory systems can be categorized depending on their ability to recognize peptide structural features. The sensory systems are triggered by molecules with cationic and amphiphilic properties and constitute the first level of defense, since the primary result of their activation is the development of resistance to AMPs. For example, Rcs phosphorelay systems are activated through outer membrane disturbance only by hydrophobic substances like most antimicrobial peptides (Farris et al., 2010). In turn, the sensory part of the *aps* three-component system of staphylococci can recognize a variety of cationic, but not anionic AMPs (Li et al., 2007a).

The second level consists of sensory systems, which are possibly activated with a wide range of different peptides. Their quantitative properties are crucial. For example, the PhoQP TCS is activated by peptides with various structures, but the more charged and hydrophobic the peptide is, the greater activation is achieved by the exposure to it (Shprung et al., 2012). Thus, it was shown that LL-37, but not polymyxin B, activates the expression of virulent genes, which are under the control of PhoQP/PmrAB (Shprung et al., 2012). The used peptide's sub-MIC concentrations are also important for the final result. For example, sub-MIC effect of LL-37 on *Pseudomonas aeruginosa* PAO1 at 4 μg/mL was down-regulation of QS-gene (*pqsE*) and other (production of rhamnosyltransferase, phenazine, etc.) (Overhage et al., 2008), but increase its expression at 20 μg/mL (Strempel et al., 2013).

It would be an interesting attempt to circumvent the undesirable sub-inhibitory effects by tuning of physic-chemical properties of designed synthetic peptides. Unfortunately, today there is no complete understanding to predict which of TCS will be activated. Various TCS have a different susceptibility to AMPs. Thus, using a bioluminescent reporter strain, it was shown that ParRS TSC was activated after being treated with colistin/polymyxin B and indolicidin, while other cationic peptides (including LL-37) did not activate it (Fernández et al., 2012). Additional experiments with 19 peptides, different in charge and hydrophobicity, did not reveal a clear correlation between peptides' properties and their activation ability (Fernández et al., 2012). New targeted researches aimed to study the sub-inhibitory effects of AMPs in the structure-function aspect, with appropriate mathematical processing, would allow answering many questions.

Thus, these facts allow us to conclude that different sensory systems have different levels of sensitivity and the ability to recognize specific stressors. Ultimately, this determines the various responses of bacterial cells to different AMPs. However, it can be assumed that the main reaction of bacterial genome and its metabolic apparatus is developing resistance, while all other effects may be secondary. Probably, in stress conditions, this is the most adequate response of bacteria to the antimicrobial action of peptides, which, however, can be followed by others.

Bacterial Defense Network Is Activated by AMPs

Numerous different genes that are directed toward following a forming network and regulate a comprehensive strategy of protection and response to external influences are under the control of one master regulator. The GraSR TCS of *S. aureus*, which are involved in AMPs resistance, and are indirectly associated with pathogenesis, control pathways through connections with Agr signal transduction network (Kraus et al., 2008; Falord et al., 2011). Bacterial Rcs phosphorelay is a well-known signaling system that regulates virulence and persistence of Enterobacteriaceae (Erickson and Detweiler, 2006). The Rcs, simultaneously with PhoQP and PmrAB TCS, is involved in regulation of several genes, whose expression maintained integrated resistance of bacteria to polymyxin B (Llobet et al., 2011; **Figure 1**).

There is a large number of similar examples, which shows a close interweaving of different ways of signal transmission and responding. Often, stress activates a variety of regulatory systems that overlap closely. Thus, while being surrounded by antimicrobial peptides, bacterial cells experience stress, the first response to which will be self-protection.

Concerning the peptides themselves, there is no doubt that their exclusive physicochemical properties are important.

However, a more detailed investigation of structure-function relationships still needs to be conducted.

EFFECTS OF SUB-INHIBITORY CONCENTRATIONS OF ANTIMICROBIAL PEPTIDES AT CELLULAR LEVEL

When used in their non-lethal concentration, antimicrobial peptides have a powerful effect on the functioning of a bacterial genome, which ultimately leads to a change in the entire behavior of the bacterial population, provoking negative or positive effects for interrelated living organisms.

The bacterial envelope is the first protective structure on the pathway of antimicrobial peptides. AMP's interaction with bacterial shells changes their surface architecture provoking undesirable effects. Thus, *Shigella flexneri* can use cationic proteins produced by neutrophils to increase self-adhesion and promote invasion inside epithelial cells (Eilers et al., 2010; Ni et al., 2015). LL-37 at sub-inhibitory concentration was proven to change *Streptococcus pyogenes* surface architecture, provoking the formation of extracellular vesicles, which contain numerous factors of streptococcal virulence (Uhlmann et al., 2016).

In Gram-positive bacteria, some virulence factors are assembled and attached to the cell wall by sortase enzymes, which are localized on one or two sides in the cell membrane. Several antimicrobial peptides can interact with focal sites and disrupt the localization of some proteins necessary for secretion and virulence factor assembly (Kandaswamy et al., 2013). For example, polymyxin B and HNP-1 at sub-MIC concentrations can bind to the anionic lipids of so-called ExPortal. It leads to structural disorder and effects cysteine protease and cytolysin secretion (Vega and Caparon, 2012).

The process of a microorganism's conquest of a new habitat is accompanied by an appropriate reorganization of its metabolic processes. The presence of antimicrobial peptides at this point can either trigger the secretion of virulence factors that enhance the aggressiveness of the pathogenic microorganism, or decrease the metabolic activity and the appearance of persisters aimed surviving under the stress.

AMP-dependent sequential activation of PhoQP > PmrAB > ArnC leads to modification of lipid A (development of AMP-resistance) and at the same time, increased expression of the virulence factor PagC, necessary for bacterial persistence within macrophages (Yu and Guo, 2011; Tsai et al., 2016). The presence of LL-37 at sub-MIC led to the diversification of the *P. aeruginosa* population to the mucoid type, which increased their persistence and subsequently promoted chronic infection (Limoli et al., 2014). A similar result was revealed for *P. aeruginosa* population, growing in sputum of cystic fibrosis under sub-inhibitory concentrations of colistin (Wright et al., 2013). Another example of bacterial persistence is the induction of protective substances the function of which is inactivation of host defense antimicrobial proteins. For example, the human serum has numerous antimicrobial peptides and proteins, including lysozyme. The inhibition of lysozyme activity is one of the main causes of bacterial persistence (Bukharin et al., 1987). It was proven that the ability for induction of the main lysozyme inhibitor proteins Ivy and MliC is widespread in bacterial world and is under control of Rcs-regulon (Callewaert et al., 2009; **Figure 1**).

In addition, a good illustration of non-linearity and unpredictability of AMPs' effects is the inhibition of toxin production in bacteria. *S. aureus* is one of the main pathogens of nosocomial infections, and methicillin-resistant strains are a serious problem in antimicrobial therapy. *S. aureus* is able to secrete a set of different virulence factors that allow it to colonize a different habitat. However, it has been observed that staphylococci growing on a blood-containing medium did not produce any toxins (Schlievert et al., 2007). It was hypothesized that human blood contains a factor that suppresses toxin-production. Today, it is known that animals' blood is a source of various peptides including hemocidins, which are the cationic peptide fragments derived from hemoglobin (Mak et al., 2000; Arroume et al., 2008; Vasilchenko et al., 2016). Further studies of the antitoxic effects of hemoglobin showed the ability of globin chains to inhibit all known types of Agr-quorum sensing systems of *S. aureus*. Surprisingly, downregulation of *agr*-genes allows *S. aureus* to colonize nasal passages (Liu et al., 2013). It turned out that *S. aureus* cells reduce production of some Agr-regulated proteases to avoid generation of hemoglobin-derived antimicrobial peptides.

Finally, it is worth noting cases when the change in gene expression does not lead to the expected phenotypic changes. For example colicin M induces an envelope stress response of *E. coli* which upregulated numerous biofilm-associated genes. Nevertheless, the induction of neither biofilm formation nor of colonic acid production was observed (Kamenšek and Žgur-Bertok, 2013). Inducing the expression of virulence genes, did not cause any expected phenotypic changes indicating that several cellular targets were affected. So, colicin M induced the up-regulation of numerous biofilm-associated genes of *E. coli*. At the same time, it promoted the hydrolysis of lipid II, which limited its availability for exopolysaccharide biosynthesis, including colanic acid (Liu et al., 2013).

ANTIMICROBIAL PEPTIDES AS SIGNALING MOLECULES

Dual Function of Small Oligopeptides: Antimicrobial QS-Autoinductors

A shift in AMP's function from antibiotic to signaling is one of the side-effects of diluting to sub-inhibitory concentrations. It is known that β-lactam antibiotics in sub-MIC have quorum-inducing activities, which triggers the synthesis of quorum sensing-dependent pathogenicity factors (Liu et al., 2013; Deryabin and Inchagova, 2017). However, the reverse scenario is also possible, when the autoinducer exhibits bactericidal properties (Qazi et al., 2006).

The quorum sensing-dependent process of regulation of gene expression usually takes place in four stages, one of which receives the signal molecule, which provide a possibility to interference

between cognate and non-cognate autoinducers (Ji et al., 1997). It makes sense, since autoinducers work not only within a single population, but are also involved in interspecies signal transduction (Lowery et al., 2008).

Among the various existing autoinducers, within the framework of this review, the most interesting group are small autoinducing peptides molecules (AIP). The chemical structure of AIPs is diversified into several types, such as small oligopeptides and cyclic lactone/thiolactone peptides (Singh et al., 2016). Thus, cyclic oligopeptides often combine an antimicrobial and a signal activity (Prasad, 1995). Some Lactobacilli produce a variety of antimicrobial small dipeptides, which inhibit the viability of bacteria, fungi and viruses, while also suppressing the production of bacterial exotoxins (Kwak et al., 2017). In particular, the culture filtrate of *Lactobacillus* contained numerous dipeptides including cyclo (L-Phe-L-Pro) having antifungal activity (Kwak et al., 2014). The ability of such molecules to suppress exotoxin production is related to their interference with cognate QS-autoinducers. It was shown that cyclo (L-Phe-L-Pro) dipeptide suppress the production of staphylococcal exotoxins (TSST-1) by interfering with the agr QS-system (Li et al., 2011).

This class of substances is relatively poorly studied, and aggregated information concerning they biological activity can be found in remarkable reviews devoted to precisely these substances (Prasad, 1995).

Dual Function of High-Molecular-Weight Peptides: Antimicrobial Pheromones

As for ribosomally synthesized antimicrobial peptides, considering their role in signal transduction, it is first of all worth considering bacteriocins. Many bacteriocins are synthesized in a quorum-dependent manner (Kleerebezem and Quadri, 2001; Quadri, 2002). It is also known that co-incubation of several different strains significantly enhances production of bacteriocins (Maldonado et al., 2004). Apparently, the induction of bacteriocin synthesis in a mixed culture is widespread in nature, however, the role of inducers is usually taken by proteins or peptides that do not themselves have antimicrobial properties (Chanos and Mygind, 2016).

Can bacteriocins affect production of defense peptides in other species? To date, several bacteriocins that combine both antimicrobial and signaling properties are known, since their own biosynthesis is a quorum-dependent bacteriocin (Kuipers et al., 1995; Kleerebezem et al., 2004). The most studied one in this respect is plantaricin A (Hauge et al., 1998). The mechanisms of plantaricin A's function as a pheromone and antimicrobial are different. The pheromone action of plantaricin A is initiated by electrostatic interaction with membrane lipids. Subsequent events include the spatial arrangement of the plantaricin A molecule in the lipid/aqueous phase interface, which allows the N-terminal residues to engage in a chiral interaction with its histidine kinase receptor (Kristiansen et al., 2005). Bactericidal activity of plantaricin A is realized when plantaricin's concentration is increasing, which leads to a rearrangement into a alpha-helical conformation and penetration of a bacterial cell wall (Di Cagno et al., 2010). Nevertheless, the main function of plantaricin A is signaling, because concentrations, which are exhibited required for antimicrobial action are not achieved in nature (Dicks et al., 2018).

As expected, the spectrum of processes which are activated by bacteriocin' autoinducers includes only synthesis pathways. However, proteomic studies of bacteria co-incubated with bacteriocin (plantaricin A, nisin) revealed a change in the production of proteins and peptides, which are involved in increasing the adaptive capacity of the strain in a multi-species community (Calasso et al., 2013; Mukherjee and Ramesh, 2015) and overcome a bacteriocin-containing environment (Miyamoto et al., 2015).

In addition, bacteriocin production stimulates the synthesis of human-defensin-2 (HBD-2) by the cells of the host intestine (Marzani et al., 2012), which also increases the colonization potential of certain species and provides ability for intra- and interspecies competition (Anderssen et al., 1998; Dicks et al., 2018; **Figure 2**). Thus, bacteriocins of one species can initiate the production of their own bacteriocins in another similar species. However, it seems that this induction of synthesis is caused by indirect action, since even insignificant structural differences between bacteriocins are critical for ligand/receptor interaction. Thus, subtilin does not interact with the histidine kinase NisK, which normally senses nisin, due to the differences between these bacteriocins in the structure of their N-terminal part (Spieß et al., 2015).

Describing the role of bacteriocins in microbial communities, it is necessary to mention the ability of bacteria to form biofilms. Biofilm is one of the characteristic forms of the existence of the multimicrobial community in nature (Sutherland, 2001). In nature, microbial cells exist in the attached state more often than in a free-floating planktonic state. Biofilms are structured by masses of microorganisms embedded in the matrix of polysaccharides, proteins, extracellular DNA and other molecules (Gillor, 2007). The development of bacterial biofilm is a quorum dependent phenomenon that ensures the viability of a bacterial population under adverse conditions.

It is known that bacteriocins have an important role in biofilm development. Bacteriocins inhibit the fixing of bacterial cells and the development of biofilms of competitive species when high local concentration is achieved (Gillor, 2007). At sub-inhibitory bacteriocin concentration a similar goal is also achieved, but in a slightly different way. For example, biofilm formation of *S. aureus* was abolished at sub-inhibitory concentrations of bovicin HC5 and nisin, because normal expression of genes associated with quorum sensing was affected (Pimentel-Filho Nde et al., 2014). Taken at sub-inhibitory concentration, subtilosin reduced biofilm formation of a conditionally pathogenic species *C. violaceum*. It was shown that subtilosin acts as a proton pump inhibitor in Gram-negative bacteria, which prevents efflux of a synthetized QS-autoinducer (Algburi et al., 2017). For more information about anti-biofilm properties of bacteriocins, the readers can be addressed to the recent review (Mathur et al., 2018).

There is an interesting point related to the fact that the action of bacteriocins, unlike most eukaryotic AMPs, is mediated through interaction with the corresponding receptors

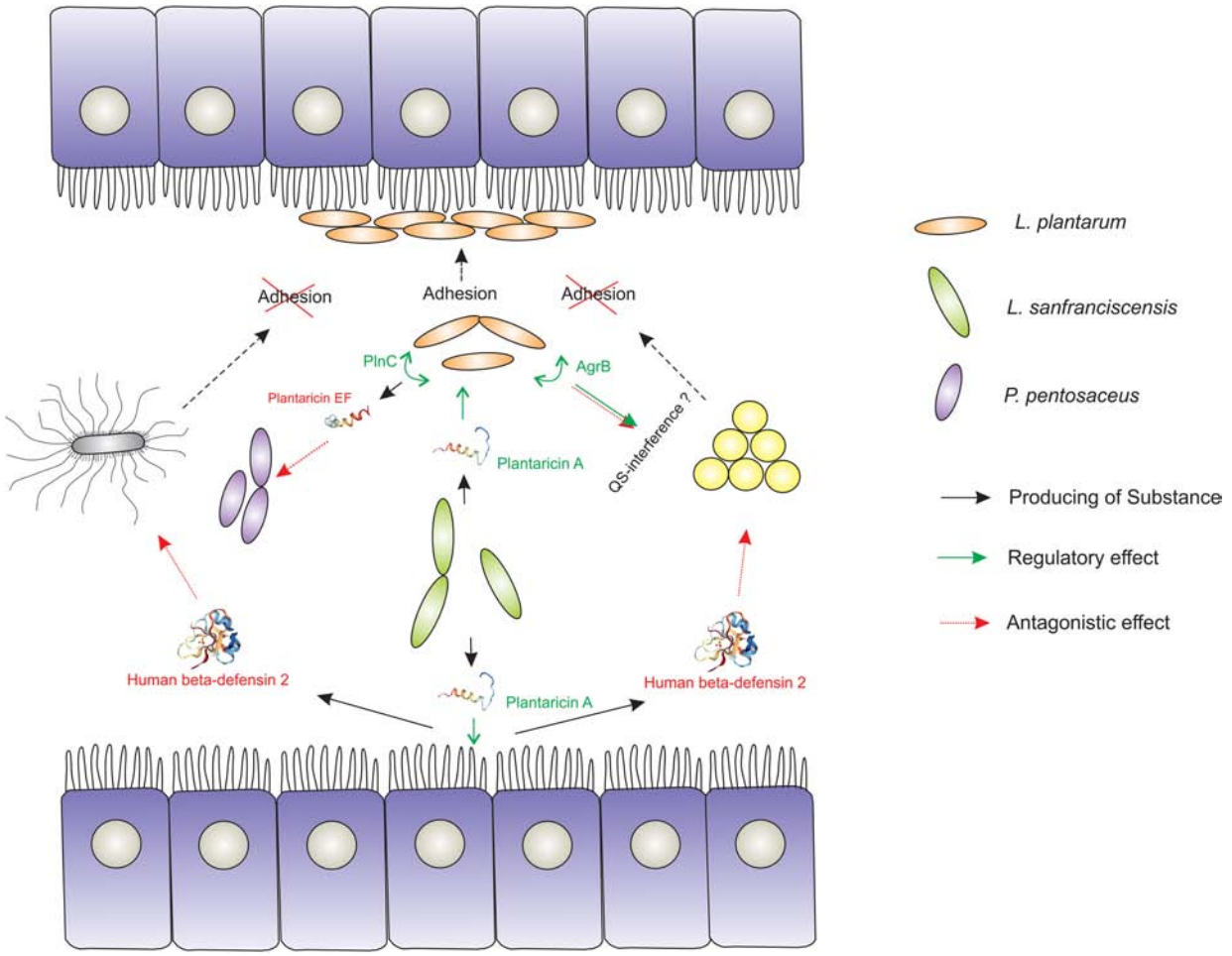

FIGURE 2 | Demonstration of the signaling role of bacteriocins in the formation of a sustainable mammalian gut microbiome. The *Lactobacillus* strain producing plantaricin A triggers the expression of genes and the production of metabolites that enhance the colonizing ability (adhesion and biofilm formation) of another *Lactobacillus* strain. The occupied econish is no longer available for pathogenic and conditionally pathogenic microorganisms (*S. aureus* and *S. typhimurium*). In addition, certain (PlnC) activators of bacteriocin production (Plantaricin EF, for example) and some component of the agr QS-system (AgrB) are launched in susceptible to PlnA Lactobacilli cells, which has a certain antagonistic effect on the competitor species. Also plantaricin A triggers the production of human β-defensin 2 of the intestinal epithelium, which potentially has an antagonistic effect on a number of pathogenic and conditionally pathogenic microorganisms.

(Cotter, 2014). Numerous receptors, such as lipid II, are universal for a wide range of bacteriocins. In turn, certain molecules are receptors only for certain bacteriocins. Thus, lasso bacteriocin streptomonomicin interacts with WalR, a response regulator involved in cell wall metabolism and cell division (Acedo et al., 2018). Some thiopeptides interfere with protein synthesis either by binding to the 50S ribosomal subunit or elongation factors (Acedo et al., 2018). It is not yet clear what reactions can be triggered at the genome or secretome level when exposed to sub-inhibitory concentrations of such bacteriocins. Although it is known some antibiotics that inhibit protein biosynthesis in sub-inhibitory concentrations induce biofilm formation (Hoffman et al., 2005). There is also evidence that sub-inhibitory concentrations of glycopeptide vancomycin [cellular target is lipid II (De Moura et al., 2015)] change the expression of a several genes associated with virulence *E. faecalis* (Breukink and de Kruijff, 2006).

Thus, the main conclusions are:

1. Only cognate bacteriocins-pheromones can interact with appropriated receptors of regulatory systems.
2. The main function of such pheromones is the initial production of its own bacteriocins, and their antimicrobial properties is an additional feature.
3. However, it is possible that the range of biological effects initiated by bacteriocin-pheromones can be significantly wider than the production of its own bacteriocins (Xu et al., 2014). This presents a productive possibility for future research.

CONCLUSION

In view of the above, the basic mechanisms for regulation of bacterial virulence factors have become more understandable.

However, it is not yet possible to say exactly what happens with bacterial cells when sub-inhibitory doses of AMPs are exposed. Bacterial reaction on sub-MIC of AMPs can be non-linear. Yes, peptides are able to inhibit the production of any toxins, but it turns out that, subsequently, this ability is either restored, or one toxin is replaced by the production of another. Hemocidins reduce intracellular amounts of TSST-1, hemolysins, and lipase for *S. aureus* cells. However, the production of the virulence factor protein A is increased (Schlievert et al., 2007).

The presence of a multitude of sensory systems that are intertwined with each other allows bacteria to adapt to any stress. Thus, the reaction of bacterial pathogens to protective peptides consists of two parts: on one hand, the initial presence of a certain amount of AMP reduces the production of aggression factors and various exotoxins. On the other hand, a decrease in the microbe's enzymatic activity provokes their persistence.

Throughout their evolutionary pathway bacteria have demonstrated a highly adaptive potential compared to other living organisms. In part, this has been the cause behind the current problem of antibiotic resistance, against which the efforts of many scientific groups are directed. Previously, it was believed that bacteria are significantly less resistant to the action of antimicrobial peptides than to conventional antibiotics, but today it is known to be not entirely true. Bacterial populations often respond to stressful effects unpredictably, and peptide action can both weaken the virulent potential of microbes as well as substantially increase it. The specific scenario will depend on the peptide's properties and its local concentration. These factors are very poorly studied. For the realization of antimicrobial peptides' potential as therapeutic agents, it is necessary to study their non-lethal effects on the physiology and behavior of microorganisms in the same way as the mechanisms of lethal action.

AUTHOR CONTRIBUTIONS

AV designed the review and wrote the first draft of the manuscript. ER reviewed and edited the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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flaA-SVR Based Genetic Diversity of Multiresistant *Campylobacter jejuni* Isolated From Chickens and Humans

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Campylobacter jejuni is one of the most common causes of human foodborne bacterial infections worldwide. The objective of this study was to assess the molecular diversity, using *flaA* sequencing, of 602 *C. jejuni* isolated from chicken food chain, i.e., chicken feces (*n* = 151), chicken carcasses (*n* = 150), chicken meat (*n* = 150), and from humans (*n* = 151) and to determine antimicrobial multiresistant profiles of the isolates as well as to analyze the relationship of the isolate genotypes with their antimicrobial resistance profiles and source of isolation. Multidrug resistant patterns were identified in 110 (18.3%) *C. jejuni* isolates recovered from all sources and most isolates were resistant to ciprofloxacin (CIP), nalidixic acid (NAL), streptomycin (STR), and tetracycline (TET) (92; 15.3%) or ciprofloxacin, streptomycin, and tetracycline (13; 2.2%). Only a few isolates were multiresistant to ciprofloxacin, nalidixic acid, tetracycline, and erythromycin (3; 0.5%) or ciprofloxacin, nalidixic acid, streptomycin, tetracycline, and erythromycin (2; 0.3%). A total of 79 *flaA*-SVR subtypes were identified, including 40 (50.6%) unique to the isolates' origins, with the most common sequence types 16, 54, 36, 34, and 287 which covered 56 (9.3%), 50 (8.3%), 48 (8.0%), 35 (5.8%), and 32 (5.3%) of *C. jejuni* isolates, respectively. It was found that 13 isolates had the novel *flaA*-SVR subtypes which were not present in the pubMLST database. These isolates were recovered from chicken feces (6 isolates), carcasses (2 isolates), meat (one isolate) and from humans (4 isolates). Multiresistant *C. jejuni* were classified into 26 different sequence subtypes. Among the most numerous multidrug resistant profile CIP+NAL+STR+TET 21 different *flaA*-SVR subtypes, with total of 92 isolates, were identified. Most of them were classified to 287 (18; 19.6% isolates), 100 (13; 14.1%), 34 (9; 9.8%), 208 (8; 8.7%), and 781 (8; 8.7%) molecular variants. Isolates resistant to CIP, STR and TET (13 isolates) were mainly from chicken feces (12 isolates) and classified into 5 *flaA*-SVR sequence types, with the most common 36 (8 isolates). The obtained results show a broad molecular diversity of multiresistant *C. jejuni* isolates and suggest chickens as a possible source of human *Campylobacter* infections in Poland.

Keywords: *Campylobacter jejuni*, chicken food chain, humans, antimicrobial resistance, *flaA*-SVR sequencing

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INTRODUCTION

Campylobacter, especially *Campylobacter jejuni*, is one of the most common causes of foodborne bacterial infections worldwide (Allos, 2001; Bolton, 2015; Kaakoush et al., 2015; Tresse et al., 2017). Campylobacteriosis is also the most commonly reported zoonosis in the European Union with 246,158 confirmed cases and a notification rate of 64.8 per 100,000 population in 2017 (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2018). However, it has been estimated that the real number of *Campylobacter* infections occurring yearly may be several millions and the annual cost of the disease is almost one billion of United States dollars (Havelaar et al., 2009; Silva et al., 2011; Kaakoush et al., 2015). Several studies showed that one of the most important transmission routes of *C. jejuni* to humans is handling, preparation and consumption of contaminated food of poultry origin (Allos, 2001; Park, 2002; Humphrey et al., 2007). *C. jejuni* colonizes chicken intestines at the number of 10^8 cells per gram of cecal contents or greater without causing disease (Beery et al., 1988; Sahin et al., 2002). After colonization of the first birds in a flock, the bacteria rapidly spread throughout the flock and remain present until slaughtering (Wagenaar et al., 2006). *C. jejuni* colonizes the mucus mainly of the cecal epithelial cells and the small intestine but may also be found in other parts of the gut (Newell and Fearnley, 2003). Transmission from chickens to humans most commonly occurs through consumption and handling of chicken meat and meat products contaminated with these bacteria during slaughter and carcass processing (Kaakoush et al., 2015). It has been estimated that the chicken reservoir as a whole is estimated to be responsible for up to 80% of human campylobacteriosis cases (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2018).

Most campylobacteriosis cases are usually self-limiting and do not require antimicrobial treatment. However, severe infections occasionally require antimicrobial therapy often with macrolides (erythromycin or azithromycin) and, to a lesser extent, with fluoroquinolones, tetracyclines, or gentamicin when infection becomes systemic (Iovine, 2013). A major concern with regard to treating campylobacteriosis in humans is antimicrobial resistance, particularly resistance of *C. jejuni* to fluoroquinolones and macrolides, which has increased significantly over the past two decades (Melero et al., 2012; Piccirillo et al., 2013; Wieczorek et al., 2013; Han et al., 2016; Mäesaar et al., 2016; Olkkola et al., 2016; Post et al., 2017; Woźniak-Biel et al., 2018). It has been suggested that food of animal origin, especially poultry meat, may represent a vehicle of transmission of resistant *Campylobacter* to humans (Aarestrup et al., 2008). Ciprofloxacin and erythromycin are the antimicrobials of choice for treatment of human campylobacteriosis (Ge et al., 2013; Iovine, 2013). The intensive use of antimicrobials in animals and in humans has led to an increase in the antibiotic-resistant *Campylobacter* population (Humphrey et al., 2007; Ge et al., 2013). Thus, monitoring of resistance of *C. jejuni* derived from infected patients and food of animal (poultry) origin is highly relevant to public health.

Molecular typing is an important tool for evaluation of diversity and transmission routes of *Campylobacter* isolates contaminating the food chain and isolated from patients with diarrhea.

Several studies of *C. jejuni* demonstrated that this microorganism is genetically diverse, predominantly as a result of frequent intra- and interspecies genetic recombination, within a weakly clonal population structure (Dingle et al., 2001; Suerbaum et al., 2001; Manning et al., 2003). In order to investigate the epidemiology of *C. jejuni*, molecular subtyping methods with enhanced discriminatory power are used (Wassenaar and Newell, 2000; Wieczorek et al., 2017). One of them is direct sequencing of PCR-amplified short variable regions (SVRs) products of the flagellin-encoding A (*flaA*) gene (Wassenaar et al., 1995; Harrington et al., 1997). It was shown that the SVR region is located between 450 and 600 base positions in the *C. jejuni flaA* encoding gene (Meinersmann et al., 1997). Several studies have demonstrated that direct sequencing of PCR-amplified short variable regions (SVRs) of the *A* gene is a useful tool for *Campylobacter* genotyping, offering similar or higher discriminatory power than multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) (Meinersmann et al., 1997; Wassenaar et al., 2009; Wirz et al., 2010; Magnússon et al., 2011; Gomes et al., 2016). Additionally, sequences of *flaA*-SVR nucleotide alleles are stored in the pubMLST database[1], and allow open access to the *flaA*-SVR types of *Campylobacter* strains isolated around the world. Furthermore, it has been shown that *flaA*-typing provides sufficient discrimination for its use as a subtyping method for *C. jejuni* (Suerbaum et al., 2001; Manning et al., 2003). Despite the observation that recombination rates in *C. jejuni* may potentially have an adverse impact on the reliable interpretation of *flaA*-typing (Wassenaar et al., 1995; Harrington et al., 1997), several studies showed that in the majority of *C. jejuni* isolates some regions of the flagellin-encoding A gene are genetically stable over long periods and may be used for molecular typing and differentiation of the isolates (Burnens et al., 1995; Owen et al., 1995).

The objectives of the present study were: (i) to determine antimicrobial multiresistant profiles of a collection of *C. jejuni* isolates recovered from chicken food chain and from humans with diarrhea, (ii) to assess the genetic relatedness of these isolates using *flaA* sequencing, and (iii) to examine the relationship of the isolate genotypes with their antimicrobial resistance profiles and source of isolation.

MATERIALS AND METHODS

Isolation of *C. jejuni*

Sampling and isolation of *C. jejuni* from the poultry food chain and from humans with diarrhea were performed as described previously (Wieczorek et al., 2018). The detailed information on all 602 isolates are included in the **Supplementary Material** (**Supplementary Table S1**). In case of chicken *C. jejuni* (a total of 451 isolates), the samples were collected during years

[1]http://pubmlst.org/campylobacter/

2010–2016 according to the monitoring plan prepared in the Polish National Reference Laboratory for *Campylobacter*. Intact ceca from 10 birds were taken after evisceration, the content was pooled and one loop-full (10 μl) of the material was streaked directly onto Karmali agar (*Campylobacter* Agar Base + *Campylobacter* Supplement; Oxoid, United Kingdom) and *Campylobacter* blood-free agar (Oxoid) with CCDA selective supplement (Oxoid) and incubated at 41.5°C ± 1°C for at least 48 h ± 2 h in a microaerobic atmosphere generated using a CampyGen kit (Oxoid). From each fecal sample one presumptive *Campylobacter* isolate was then confirmed by PCR as described previously (Wieczorek et al., 2013). Briefly, one bacterial colony was suspended in 1 ml of redistilled water and centrifuged at 13,000 × *g* for 1 min. The pellet was resuspended in 100 μl of 10 mM Tris buffer (A&A Biotechnology, Gdańsk, Poland) and DNA was isolated with the Genomic – Mini kit according to the manufacturer's instruction (A&A Biotechnology). The PCR was performed using the following program: 94°C for 5 min (initial denaturation) followed by 30 cycles: 94°C for 1 min, 58°C 2 min, 72°C for 1 min. The final extension step was performed at 72°C for 5 min. The used PCR primers amplified fragments of the *mapA* (specific for *C. jejuni*) and the *ceuE* (characteristic for *C. coli*) *Campylobacter* genes. The amplification of the *16S rRNA* gene fragment, presents in both *C. jejuni* and *C. coli*, which allowed to evaluate the PCR reaction and the bacterial DNA, was also performed. A total of 151 *C. jejuni* isolates from chicken feces were used in the present study. The detailed information on the chicken samples are included in the **Supplementary Material** (**Supplementary Table S1**).

The swab samples from the neck skin and the skin surface under the wings of chicken carcasses were collected directly after immersion chilling (0–4°C) but before further processing and immediately transported to the laboratory in Amies transport medium with charcoal (Medlab, Poland). *Campylobacter* bacteria were isolated as described (Wieczorek et al., 2013). Briefly, the swabs were placed in 5 ml of Bolton enrichment broth (Oxoid) supplemented with vancomycin, cefoperazone, trimethoprim, and amphotericin B and incubated as described for fecal samples. The cultures were then plated onto Karmali agar (Oxoid) and *Campylobacter* blood-free agar with CCDA selective supplement (Oxoid) and incubated under the same conditions. From each sample one presumptive *Campylobacter* isolate was confirmed using PCR as described previously (Wieczorek et al., 2013). A total of 150 *C. jejuni* from chicken carcasses were collected for the current investigation.

The *Campylobacter* isolates from chicken meat (n = 150) were recovered using the ISO 10272-1 standard and *C. jejuni* were confirmed with the PCR method as described for the chicken carcasses.

A total of 151 *C. jejuni* isolates were obtained from patients with diarrhea using standard culturing techniques. Rectal swabs were directly streaked onto mCCDA agar (Oxoid) and incubated at 41.5°C ± 1°C for 48 h ± 2 h under microaerobic conditions. Then, typical *Campylobacter* colonies were selected for further investigation using standard biochemical tests. *C. jejuni* was identified with PCR as described previously (Vandamme et al., 1997). All *C. jejuni* identified in patients with diarrhea were isolated by regional diagnostic laboratories located in five voivodeships (administrative regions of Poland) during years 2011–2016 and confirmed by PCR at the National Veterinary Research Institute in Pulawy. The detailed information on the human samples are included in the **Supplementary Material** (**Supplementary Table S1**). The authors declare that the study did not need any recommendation or approval of an ethics committee nor written consent from the people from whom *C. jejuni* were isolated.

Altogether, 602 *C. jejuni* were isolated and stored at −80°C until further analysis.

Antimicrobial Resistance

A microbroth dilution method was used to establish the minimum inhibitory concentrations (MICs) of six antimicrobials (gentamicin [GEN], streptomycin [STR], erythromycin [ERY], ciprofloxacin [CIP], nalidixic acid [NAL], and tetracycline [TET]) to *C. jejuni* isolates using EUCAMP2 Sensititre® custom susceptibility plates (Trek Diagnostics, United Kingdom). The dilution ranges and cut-off values are presented in **Supplementary Table S2** (Wieczorek et al., 2018). The isolates were sub-cultured twice on Columbia agar (Oxoid) at 41.5°C for 48 h under microaerobic conditions. The minimum inhibitory concentration of the antimicrobial agents was determined using Mueller-Hinton broth (Oxoid) supplemented with 2–2.5% horse blood (Trek). The plates were incubated at 37°C for 48 h under microaerophilic conditions and read using the Vision® system (Trek). The antimicrobials and cut off values used for the interpretation of the MIC results were in accordance with EUCAST (Sifré et al., 2015) and the European Union Reference Laboratory for Antimicrobial Resistance. Multidrug resistance of the isolated *C. jejuni* was defined as resistance to at least three classes of antimicrobials used in the study (Magiorakos et al., 2012).

DNA Extraction and *flaA*-SVR Sequencing

One bacterial colony was suspended in 1 ml of sterile, DNase- and RNase-free water and centrifuged at 13,000 *g* for 1 min, and DNA was extracted using the Genomic-Mini kit (A&A Biotechnology, Poland) according to the manufacturer's instruction. DNA was then utilized as a template in PCR with the forward 5′-ATGGGATTTCGTATTAACAC-3′ and reverse 5′-CTGTAGTAATCTTAAAACATTTTG-3′ primers (Wassenaar and Newell, 2000), using the following amplification conditions: initial DNA denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. The final extension step was performed at 72°C for 1 min. Purification and sequencing of the amplified products was performed by an external company (Genomed, Warsaw, Poland) using the BigDye Terminator v. 3.1 kit (Applied Biosystems, United States). The sequencing products were separated in a 3730 × l DNA Analyzer capillary sequencer (Applied Biosystems) and the DNA sequences were then imported and checked for quality using the BioNumerics v. 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium). The sequences were then assigned

allelic numbers based on the data present in the *Campylobacter flaA*-nucleotide database using sequence query *Campylobacter* locus/sequence definitions (see text footnote 1). If exact match was identified the number was assigned to the isolates. When any mismatches of DNA sequences to those present in the database were found, which suggested possible new *flaA*-SVR alleles, the isolates were sequenced once again and then submitted to the database administrator for confirmation. The sequences of all *C. jejuni* isolates examined in the present study are now present in the mentioned above database and could be identified by the number of *flaA*-SVR sequence type.

Statistical Analysis

The chi-squared test with Yates' correction was used to examine differences in the prevalence of *flaA*-SVR subtypes among *C. jejuni* isolated from different sources and $P < 0.05$ was considered significant. The genetic diversity of *C. jejuni* within the populations of isolates recovered from different sources was assessed by Simpson's diversity index (ID) as described previously (Hunter and Gaston, 1988) using the online tool "Comparing Partitions" from the website http://www.comparingpartitions.info (Carriço et al., 2006). The data was directly transfer from the excel file to the online tool (**Supplementary Table S3**). The column corresponding to a different partition assignment and each value to a cluster identifier. The first row contains the columns titles. The proportional similarity index (PSI) was applied to compare sequence types distribution among *C. jejuni* isolates from various sources (Hunter and Gaston, 1988; Garrett et al., 2007). The frequency distributions of the different sources were estimated by calculating their similarity using the following equation: $PSI = 1–0.5\Sigma_i |p_i - q_i| = \Sigma_i \min (p_i, q_i)$, where p_i and q_i are the proportion of isolates from group p and q, respectively, belonging to type i. PSI ranges from zero to one, where one indicates that two groups are identical and zero means they share no types. Around 95% confidence intervals (CI) were computed using bias-corrected and accelerated non-parametric bootstrap. Calculations were performed using R, ver. 3.1.3 and @RISK for Excel, ver. 6.0.1 (Palisade Co., Ithaca, NY, United States). An index greater than 0.90 is considered desirable if the typing results are to be interpreted with confidence (Hunter and Gaston, 1988).

RESULTS

flaA-SVR Sequence Types

A total of 79 *flaA*-SVR subtypes were identified, including 40 (50.6%) sequences unique to the isolates' origin, with 15 sequences found only in *C. jejuni* from chicken feces, 12 subtypes in isolates from chicken carcasses, 7 sequences in chicken meat, and 6 subtypes detected only in isolates recovered from humans. Additionally, 24 different *flaA*-SVR subtypes were found in *C. jejuni* from all sources which cover 76.2% (459 out of 602) isolates (**Supplementary Table S1**). The most common sequence types identified among all 602 isolates tested were 16, 54, 36, 34, and 287 which included 56 (9.3%), 50 (8.3%), 48 (8.0%), 35 (5.8%), and 32 (5.3%) of *C. jejuni* isolates, respectively (**Table 1**). Among isolates from the chicken food chain (n = 451), 50 sequence types were identified in *C. jejuni* from feces, 47 variants from carcasses, and 39 types from meat, respectively. Most of them were classified to 16, 54, and 36 variants (total 103 out of 451 isolates; 22.8%). In the human bacterial population (n = 151) 37 different *flaA*-SVR sequence alleles were detected, mainly belonging to subtypes 16, 54, and 14 (total 55; 36.4% isolates) (**Table 1**).

Distribution of the most prevalent *flaA*-SVR genotypes in relation to the sources of the isolates is shown in **Table 2**. Among *C. jejuni* from the chicken food chain, the most numerous subtypes were classified into sequence variants 36 (41; 9.1% isolates), 16 (33; 7.3% isolates), and 54 (29; 6.4% isolates), whereas human isolates mainly belonged to genotypes 16 (23; 15.2% isolates) and 54 (21 (13.9% isolates).

It was also found that 13 isolates had an *flaA*-SVR subtype which was not present in the pubMLST database. These isolates were recovered from chicken feces (6 isolates with the new sequences 1662, 1663, 1666, 1667, 1669, and 1673), chicken carcasses (2 isolates with the sequences 1670 and 1672), chicken meat (1 isolate with the sequence 1674), and humans origin (4 strains with the sequences 1664, 1665, 1668, and 1671). All these novel alleles were submitted to pubMLST database.

Overall, the *flaA*-SVR typing method was highly discriminative for all *C. jejuni* used in the study since the Simpson's diversity index (D) achieved value 0.968, indicating considerable diversity in the bacterial population tested, although isolates collected from the chicken food chain displayed a higher genetic diversity than isolates from humans (**Table 3**). Taking into account the number of the *flaA*-SVR sequences, no significant difference of diversity was observed between isolates recovered from chicken feces, carcasses, and meat. The lowest genetic diversity was identified among *C. jejuni* isolates with multidrug resistance profiles, although the number of such isolates was lower than the total number of campylobacters identified in each tested group.

The PSIs were calculated to assess the similarity of *flaA*-SVR sequences distributions between different *C. jejuni* sources, i.e., humans and three stages of chicken food chain, i.e., feces, carcasses, and meat (**Table 3**). The *flaA*-SVR subtypes identified in the chicken samples were highly similar (PSIs above 0.8) and the similarity of the chicken and human isolates was also calculated at the comparable levels.

Antimicrobial Resistance

The results of antimicrobial resistance of the *C. jejuni* showed that most of the isolates were resistant to ciprofloxacin (total 556; 92.4% isolates), nalidixic acid (538; 89.4%) and, to a lesser extent, tetracycline (412; 68.4%). Isolates from the chicken food chain were more often resistant to CIP than those from human patients. A similar relationship was observed for TET where the isolates from chicken feces were more often resistant than *C. jejuni* of carcasses and meat origin. A low number of isolates, irrespective of the origin, were resistant to STR (111; 18.4%). It was also found that only 5 of 624 isolates (0.8%) displayed resistance to ERY and all of them were recovered from the chicken food chain.

Multiresistance patterns were identified among 110 out of 602 (18.3%) *C. jejuni* isolated from all sources (**Table 4**). The

TABLE 1 | Prevalence of the most numerous *flaA*-SVR sequence types in *C. jejuni* tested.

Source of isolates		***flaA*-SVR sequence allele and number of isolates in each sequence type**																	
		16*	**54**	**36**	**34**	**287**	**14**	**49**	**100**	**239**	**278**	**21**	**78**	**208**	**222**	**5**	**10**	**57**	**Other (No. of different alleles)**
Chicken	Feces	9	14	13	12	8	3	10	7	1	5	2	1	3	4	0	4	2	53 (34)
	Carcasses	12	10	11	10	5	6	4	6	3	6	2	5	7	3	3	0	1	56 (31)
	Meat	12	5	17	4	12	5	5	6	14	2	6	0	1	2	6	7	7	39 (23)
Human		23	21	7	9	7	11	4	2	2	4	6	8	3	4	3	1	2	34 (20)
Total		56	50	48	35	32	25	23	21	20	17	16	14	14	13	12	12	12	182 (62)

*Other includes 62 different sequence types, counting from 9 to one isolate; among them are 13 isolates with novel flaA-SVR sequences. *Statistically significant differences between the presence of the following flaA-SVR sequences of C. jejuni isolates from different sources were identified: 54 from carcasses and humans ($P < 0.005$); 239 from feces and meat ($P < 0.005$), from carcasses and humans ($P < 0.05$), from meat and humans ($P < 0.005$); 78 from feces and humans ($P < 0.05$), from meat and humans ($P < 0.05$); 5 from feces and meat ($P < 0.05$); 10 from carcasses and meat ($P < 0.05$). In case of the remaining flaA-SVR sequences no statistically significant differences between sources of the isolates were detected.*

TABLE 2 | Prevalence of *flaA*-SVR sequence types in *C. jejuni* isolated from different sources.

Chicken feces																	
Sequence types	54	36	34	49	16	287	100	117	278	781	10	222	Other**				
No. (%) of isolates*	14 (9.3)	13 (8.6)	12 (7.9)	10 (6.6)	9 (6.0)	8 (5.3)	7 (4.6)	5 (3.3)	5 (3.3)	5 (3.3)	4 (2.6)	4 (2.6)	55 (36.4)				
Chicken carcasses																	
Sequence types	16	36	34	54	208	14	100	278	78	161	287	410	22	49	67	265	Other**
No. (%) of isolates*	12 (8.0)	11 (7.3)	10 (6.7)	10 (6.7)	7 (4.7)	6 (4.0)	6 (4.0)	6 (4.0)	5 (3.3)	5 (3.3)	5 (3.3)	5 (3.3)	4 (2.7)	4 (2.7)	4 (2.7)	4 (2.7)	46 (30.7)
Chicken meat																	
Sequence types	36	239	16	287	10	57	5	21	100	14	49	54	34	320	1424	Other**	
No. (%) of isolates*	17 (11.3)	14 (9.3)	12 (8.0)	12 (8.0)	7 (4.7)	7 (4.7)	6 (4.0)	6 (4.0)	6 (4.0)	5 (3.3)	5 (3.3)	5 (3.3)	4 (2.7)	4 (2.7)	4 (2.7)	36 (24.0)	
Humans																	
Sequence types	16	54	14	34	78	36	287	21	49	222	278	Other**					
No. (%) of isolates*	23 (15.2)	21 (13.9)	11 (7.3)	9 (6.0)	8 (5.3)	7 (4.6)	7 (4.6)	6 (4.0)	4 (2.6)	4 (2.6)	4 (2.6)	47 (31.1)					

More than 2% of isolates belonging to each sequence types are shown. **Other includes sequences with equal or less than 2% of isolates as shown in* ***Supplementary Table S1**.*

TABLE 3 | Simpson's diversity (DI) and proportional similarity (PSI) indexes of *flaA*-SVR sequencing within *C. jejuni* isolates from different sources.

Type of *C. jejuni* isolates (No. of isolates)		No. of *flaA*-SVR sequences	DI (95% CI*)	PSI (95% CI)			
				Chicken			Human
				Feces	Carcasses	Meat	
Chicken	Feces (n = 151)	50	0.962 (0.952–0.972)	1**	0.849 (0.815–0.885)	0.856 (0.822–0.889)	0.859 (0.824–0.891)
	Carcasses (n = 150)	47	0.968 (0.960–0.976)	0.849 (0.812–0.882)	1	0.856 (0.823–0.887)	0.860 (0.825–0.892)
	Meat (n = 150)	39	0.955 (0.943–0.966)	0.856 (0.822–0.888)	0.855 (0.820–0.886)	1	0.867 (0.835–0.901)
Human (n = 151)		37	0.940 (0.922–0.957)	0.860 (0.821–0.891)	0.862 (0.822–0.895)	0.868 (0.832–0.901)	1
Total (n = 602)		79	0.961 (0.956–0.966)		NA***		
Multiresistant (n = 110)		26	0.922 (0.902–0.942)		NA***		

**CI, confidence intervals with 95% confidence level. **1 = maximal similarity; 0 = maximal difference. ***Not applicable.*

TABLE 4 | Relationship between multiresistance and *flaA*-SVR sequence types in *C. jejuni* tested.

Antimicrobial resistance pattern (No. of isolates)	Source of isolates	No. of isolates with *flaA*-SVR sequence allele																								
		5	14	16	18	21	22	34	36	54	67	100	105	121	136	208	265	269	278	287	410	423	781	1523	1556	1662*	1663*
CIP+NAL+STR+TET (n = 92)	Chicken feces							5				6			1	1	2	2	2	5			5	2	1		
	Chicken carcasses		2	2	1	1		2	1	1	1	4	1			5	3		1	4			1				
	Chicken meat							1	6			2								5	2	1	1				
	Human			1				1			1	1				2				4			1	1			
CIP+STR+TET (n = 13)	Chicken feces						2		7					1												1	1
	Human								1																		
CIP+NAL+TET+ERY (n = 3)	Chicken carcasses			1																							
	Chicken meat	1															1										
CIP+NAL+STR+TET+ERY (n = 2)	Chicken feces																		1								
	Human																		1								

*CIP, ciprofloxacin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline; ERY, erythromycin. *New allele not present in the pubMLST database.*

vast majority of such isolates were resistant to CIP, NAL, STR, and TET (92 out of 110 isolates; 83.6%) and they were mainly recovered from the chicken food chain (80; 72.7% isolates). Detailed information on antimicrobial resistance of each *C. jejuni* isolate tested in the study, including the minimum inhibitory concentrations (MICs), is shown in **Supplementary Table S1**.

Multidrug Resistance and *flaA*-SVR Subtypes

All 110 multiresistant isolates were classified into 26 different *flaA*-SVR sequence subtypes, mainly 287 (18; 16.4% isolates), 100 (13; 11.8%), and 34 (9; 8.2%) (**Table 4**). Among 13 *C. jejuni* resistant to CIP, STR and TET two new allele types (1662 and 1663) found in the isolates from chicken feces were identified for the first time and submitted to the pubMLST database.

DISCUSSION

During the present study a significant *flaA*-SVR diversity among 602 *C. jejuni* isolated from the chicken food chain and from humans with diarrhea was identified. The isolates were collected during a broad range of time (2011–2017) and were obtained in 15 and 5 of 16 voivodeships (administrative regions) of Poland in case of chicken and human *C. jejuni*, respectively. Such representative material may reflect the prevalence and characteristics of the *C. jejuni* isolates all over the whole country. The large numbers of sequence profiles generated may be due to the high variability of the *Campylobacter* genome caused by its instability (Wittwer et al., 2005). It has been previously shown that the *flaA* flagellar gene undergoes spontaneous mutations during the host infection that may play an important role in molecular variation (Guerry, 2007). Among the total of 79 *flaA*-SVR variants, several identical sequences were identified among both human and chicken isolates suggesting a possible chicken source for human infection. Furthermore, on overlap of several genotypes found between chicken isolates recovered from different stages of the food chain may suggest that *C. jejuni* isolates with such allele types are circulating along the chicken meat production chain and may result in transmission of the bacteria to man.

The high genetic diversity of *C. jejuni* tested by the *flaA*-SVR method was previously demonstrated by several authors (Meinersmann et al., 1997; Corcoran et al., 2006; Djordjevic et al., 2007; Wassenaar et al., 2009; Magnússon et al., 2011; Giacomelli et al., 2012; Sing and Kwon, 2013; Gomes et al., 2016). Wassenaar et al. (2009) identified 92 different alleles among 293 *C. jejuni* isolated from three different geographical regions and found that sequence types 36, 32, 34, 15, and 239 were predominant (38.1% of 293 strains tested). Most of these allelic variants (i.e., 36, 34, 15, and 239 were also identified in the present study. Some of these *flaA*-SVR types (e.g., 34 and 36) were previously found in poultry and human *C. jejuni* isolates in Ireland, Italy and Iceland (Corcoran et al., 2006; Magnússon et al., 2011; Giacomelli et al., 2012). It seems that these molecular variants are predominant in Europe and are rarely or never detected in other geographical regions (Sing and Kwon, 2013; Gomes et al., 2016).

Several isolates of chicken and human origins tested in the present study were multiresistant, especially to quinolones, streptomycin and tetracycline. The high potential for resistance to fluoroquinolones in the *Campylobacter* isolates of chicken origin may be associated with the use of these antimicrobials in poultry treatments, although information about antimicrobial usage in the flocks we examined was not available. However, the exceptionally high percentage of *C. jejuni* resistant to quinolones in Poland identified in the present and in previous studies may be due to broad use of these antimicrobials in animal husbandry (Wieczorek et al., 2013, 2015; Woźniak-Biel et al., 2018). According to the recent European Medicines Agency report on fluoroquinolone supply for veterinary medical use, in Poland in 2016 the sales this antimicrobial group (in mg for population correction unit, PCU) were 9.7 mg/PCU, while the average for 30 European countries described in the report in that year was 2.7 mg/PCU (EMA, 2018). Such frequent administration of these drugs may have an influence on the spread of fluoroquinolone-resistant gene determinants in population of these bacteria identified in humans (Aarestrup et al., 2008).

A correlation between specific *flaA*-SVR genotypes and antimicrobial multiresistance among *C. jejuni* tested was not clear and distinct. Isolates with the same resistance pattern were classified into different molecular subtypes whereas the *C. jejuni* with an identical *flaA*-SVR profile were resistant to different antimicrobials. Similarly, other authors likewise found no correlation between genotype and antibiotic resistance (Wittwer et al., 2005; Corcoran et al., 2006). Such difference can be explained by a frequent intra- and interspecies genetic mutation among *C. jejuni* which results with many different molecular variants as determined by the *flaA*-SVR typing. On the other hand, circulation of genetic determinants encoding resistance to more than one antimicrobial may be slower than molecular mutations resulted that such multiresistant isolates are less frequently identified among different *C. jejuni* genotypes (Aarestrup et al., 2008; Iovine, 2013).

In the present study, only a few *C. jejuni* of poultry origin possessed the same multidrug resistance patterns and genotypes as the isolates recovered from humans. This limited correlation may be due to the small number of multiresistant isolates recovered from patients (only 11 isolates) as compared to 99 chicken isolates. Furthermore, it has been shown that such multidrug resistant *C. jejuni* were recovered from patients in only two voivodeships (malopolskie and slaskie) whereas chicken isolates were identified in all over Poland. Therefore, it is difficult to drawn a clear conclusion whether the chicken meat was the source of human multidrug resistant *C. jejuni* infection.

CONCLUSION

An important step in control of campylobacteriosis in humans is identification and extensive investigation of *C. jejuni* isolated from the chicken food chain as well as acquisition of full

knowledge of their molecular makeup and determination of their resistance to antimicrobials used in treatment of the infection. In the present study a total of 79 different genetic *flaA*-SVR subtypes among 602 isolates were identified which. The obtained results highlighted the lower genetic diversity of human isolates compared with chicken *C. jejuni*. A total of 13 isolates had novel alleles which were not present in the pubMLST database. Some *C. jejuni* tested displayed a multiresistant pattern, mainly to CIP, NAL, STR, and TET and the vast majority of such resistant isolates were of the chicken food chain origin. These *C. jejuni* belonged to 21 different *flaA*-SVR types which shows their broad molecular diversity. Such campylobacters were recovered from the chicken food chain and from patients which may suggest the possible source of human infection.

AUTHOR CONTRIBUTIONS

KW and JO contributed to the conception and design of the study, provided samples from the chicken food chain, planned the study, analyzed the data, and drafted the manuscript. TW delivered the human *C. jejuni* isolates. KW and TW performed the experiments. All authors critically read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01176/full#supplementary-material

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Characterization of Phenotypic and Genotypic Diversity of *Stenotrophomonas maltophilia* Strains Isolated From Selected Hospitals in Iran

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Stenotrophomonas maltophilia is an environmental Gram-negative bacterium that has rapidly emerged as an important nosocomial pathogen in hospitalized patients. Treatment of *S. maltophilia* infections is difficult due to increasing resistance to multiple antibacterial agents. The purpose of this study was to determine the phenotypic and genotypic characterization of *S. maltophilia* isolates recovered from patients referred to several hospitals. A total of 164 clinical isolates of *S. maltophilia* were collected from hospitals in various regions in Iran between 2016 and 2017. Antibiotic susceptibility testing was performed by disc diffusion method and E-test assay according to the Clinical and Laboratory Standards Institute (CLSI) guideline. The ability of biofilm formation was assessed with crystal violet staining and then, biofilm-associated genes were investigated by PCR-sequencing method. The presence of *L1* (a metallo-β-lactamase), *L2* (a clavulanic acid-sensitive cephalosporinase), *sul1* and *sul2* (resistance to Trimethoprim/Sulfamethoxazole), Sm*qnr (*intrinsic resistance to quinolones), and *dfrA* genes (dihydrofolate reductase enzyme that contributes to trimethoprim resistance) was also examined by PCR-sequencing. Relative gene expression of *smeDEF* efflux pump was assessed by real-time PCR. Genotyping was performed using the multi-locus sequencing typing (MLST) and repetitive extragenic palindromic-PCR (Rep-PCR). Isolates were resistant to imipenem (100%), meropenem (96%), doripenem (96%), and ceftazidime (36.58%). Notably, 5 (3.04%) isolates showed resistant to trimethoprim-sulfamethoxazole (TMP-SMX), an alarming trend of decreased susceptibility to TMP-SMX in Iran. Minocycline and levofloxacin exhibited the highest susceptibility of 91.46 and 99.39%, respectively. Using the crystal violet staining, 157 (95.73%) isolates had biofilm phenotype: 49 (29.87%), 63 (38.41%),

and 45 (27.43%) isolates were categorized as strong-, moderate- and weak-biofilm producer while 7 isolates (4.26%) were identified a non-biofilm producer. Biofilm genes had an overall prevalence of 145 (88.41%), 137 (83.53%), and 164 (100%) of *rmlA*, *rpfF*, and *spgM*, respectively. *L1*, *L2*, *Smqnr*, *sul1*, and *sul2* resistance genes were detected in 145 (88.41%), 156 (96.12%), 103 (62.80%), 89 (54.26%), and 92 (56.09%) isolates, respectively. None of the *S. maltophilia* isolates were positive for *dfrA12*, *dfrA17*, and *dfrA27* genes. Gene expression analysis showed that *smeD* efflux system was overexpressed in two out of the five clinical isolates (40%) that showed resistance to TMP-SMX. Most of the isolates were genetically unrelated. Two new sequence types (ST139 and ST259) were determined. Our results showed that TMP-SMX was still an effective antibiotic against *S. maltophilia*. The findings of the current study revealed an increasing prevalence of antibiotic resistance and biofilm genes in clinical *S. maltophilia* isolates in Iran.

Keywords: antibiotic resistance genes, biofilm, efflux pump, sequence type, *Stenotrophomonas maltophilia*, trimethoprim-sulfamethoxazole

INTRODUCTION

The genus *Stenotrophomonas*, together with *Xanthomonas*, belongs to the γ-β subclass of proteobacteria (Anzai et al., 2000). *S. maltophilia* isolated in 1943 from pleural effusion of patients was first named as *Bacterium bookeri*. Later, it was reclassified as a member of the genera *Pseudomonas* and *Xanthomonas* in 1961 and 1983, respectively, until it was classified as a new genus, *Stenotrophomonas*, in 1993 (Al-Anazi and Al-Jasser, 2014).

S. maltophilia is a Gram-negative, non-fermentative, aerobic, motile bacillus that is abundant in the ubiquitous environment with a broad geographical distribution. This organism has emerged as an important opportunistic pathogen in humans worldwide. Although it is considered to have limited pathogenicity (Di Bonaventura et al., 2010), *S. maltophilia* causes various types of hospital- and community-acquired *infections,* especially in debilitated or immunocompromised patients, with the mortality rate of 37.5% (Falagas et al., 2009). The bacterium has been increasingly recognized as responsible for a number of clinical syndromes, such as pneumonia, sepsis, bacteremia, endocarditis, septic arthritis, meningitis, endophthalmitis, and urinary infections (Looney et al., 2009; Sumida et al., 2015; Hu et al., 2016).

During the last decade, *S. maltophilia* has been considered as one of the leading multi-drug resistant (MDR) organisms in hospital settings due to exhibiting high levels of intrinsic and acquired resistance to a broad array of antibacterial agents, including fluoroquinolones, aminoglycosides, and the most common of β-lactam antibiotics (Brooke, 2014). Different types of antimicrobial resistance mechanisms, such as expression of antibiotic hydrolyzing or modifying enzymes, membrane permeability alteration (Hu et al., 2008), and multi-drug efflux systems (Huang et al., 2014) have been identified in *S. maltophilia*.

This bacterium produces two chromosomal-mediated inducible β-lactamases, known as *L1* and *L2*. The *L1* belongs to molecular class B Zn^{2+}-dependent metallo-β-lactamase (MBL), is resistant to clavulanic acid and hydrolyses carbapenems, cephalosporins, and penicillins (Brooke, 2012; Chang et al., 2015). *The L2 serine-β-lactamase*, an *Ambler class* A enzyme, is an inducible cephalosporinase that hydrolyses cephalosporins, penicillins, and aztreonam (Flores-Trevino et al., 2014; Mojica et al., 2016). Two mechanisms are associated with resistance to quinolones among *S. maltophilia* strains, including *smeDEF*, *smeIJK*, *smeABC*, and *smeVWX* efflux pumps and a novel chromosomal quinolone resistance gene, *Smqnr*, encoding the pentapeptide repeat protein that protects both topoisomerase IV and gyrase from the quinolones (Sanchez et al., 2009; Chang et al., 2015; Kanamori et al., 2015).

Trimethoprim-sulfamethoxazole (TMP-SMX) is recommended as the *first choice* for *S. maltophilia* infections (Abbott et al., 2011; Chong et al., 2017). However, the increasing reports of resistance to TMP-SMX are a matter of concern and have complicated the treatment strategies (Brooke, 2014; Hu et al., 2016; Madi et al., 2016). Resistance to this antibiotic has been recognized due to the presence of *sul1* and *sul2* genes that are found in class 1 integrons and *insertion sequence common region* (ISCR) elements, respectively. *dfrA* gene cassettes are observed in class 1 integrons and encode for the dihydrofolate reductase enzyme, and TolCsm, smeDEF, smeYZ efflux pumps (Hu et al., 2011, 2016; Huang et al., 2013; Lin et al., 2015; Sánchez and Martínez, 2015).

Biofilms are multicellular communities usually held together by extracellular matrix molecules. These extracellular polysaccharides (EPS) produced by the bacteria usually function as highly organized multicellular communities of microorganisms (Bjarnsholt et al., 2009; Irie et al., 2017), appear to be preferred survival strategy of microbes, and confer tolerance to high doses of antimicrobial agents than non-biofilm forming bacteria (Bjarnsholt et al., 2009). In addition, they are increasingly recognized as a contributing factor in the pathogenesis of disease in respiratory diseases often caused by chronic bacterial infections. *S. maltophilia* strains are well-known biofilm-producing organisms with ability to adhere to biotic and abiotic surfaces (Pompilio et al., 2008). Few genes associated

with biofilm formation in *S. maltophilia* have been experimentally studied (Liu et al., 2017). More recently, the correlation between mutations in *rpfF* and *rmlA* genes, encoding enoyl-CoA hydratase and glucose-1-phosphate thymidyltransferase, respectively, and the less extensive biofilm formation have been reported (Huang et al., 2006; Fouhy et al., 2007). In addition, the *spgM* gene, responsible for the production of phosphoglucomutase (PGM) and phosphomannomutase, could be involved in biofilm-forming ability (McKay et al., 2003; Zhuo et al., 2014).

High genetic diversity was identified among *S. maltophilia* strains through the use of a variety of molecular biology techniques. Several genotypic profile methods have been used to compare and link clinical isolates to environmental sources, including whole genome sequencing analyses, amplified fragment length polymorphism (AFLP) fingerprinting, PCR-restriction fragment length polymorphism (PCR-*RFLP*), analysis of the *gyrase B* gene, PCR-based fingerprinting methods, such as BOX and repetitive extragenic palindromic (rep)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, pulsed-field gel electrophoresis (PFGE) analysis of *XbaI* genomic digests, and multi-locus sequence typing (MLST) (Gherardi et al., 2015). Rep-PCR technique is based on the fact that microbial genomes contain a variety of repetitive sequences. Although their function has mostly not been elucidated so far, most rep-PCR-based DNA fingerprinting studies have used short polytrinucleotides, such as $(GTG)_5$ 35–40 bp repetitive sequences, and 154 bp BOX element as priming sites for PCR, resulting in amplification of DNA sequences between the repetitive parts (Ishii and Sadowsky, 2009). MLST technique was developed for tracking the source of infections and the distribution of pathogens isolated from hospitalized patients, providing reliable epidemiological data. In addition, because of its accessible related international databases, the results from different laboratories by MLST can be compared (Cho et al., 2012).

The main purpose of this study was to evaluate the antimicrobial resistance patterns and different resistance mechanisms of the clinical *S. maltophilia* isolated from different regions of Iran. In addition, the ability of biofilm production as well as clonal and genetic diversity of isolates were examined.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences "IR.SBMU. MSP. REC.1397.579." In order to maintain patients confidentiality participants were anonymous and no personal information was collected or included in the study.

Bacterial Isolation and Species Identification

S. maltophilia isolates were collected from different hospitalized patients in selected hospitals in Iran over a 12-months period from May 2016 to May 2017. Laboratory identification of isolates was carried out using the standard biochemical methods, such as oxidase and catalase tests, and reactions in media, including deoxyribonuclease test agar (Merck Cat. No.1.10449.0500), triple sugar iron agar (Merck Cat. No 1.03915.0500), and SIM (Merck Cat. No1.05470.0500). Consequently, isolates were confirmed as *S. maltophilia* by using the 16S rRNA sequencing with specific primers (**Table 1**; Kettleson et al., 2013). All isolates were stored in LB with 20% glycerol at −70°C. *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *S. maltophilia* ATCC 13637 were used as the quality control strains.

Antimicrobial Susceptibility Testing

Susceptibility of isolates to different antibiotics was evaluated according to the criteria of the Clinical and Laboratory Standard Institute (Clinical and Laboratory Standards Institute (CLSI) (2016)). Kirby-Bauer disc diffusion method was used for susceptibility testing to imipenem (10 μg), meropenem (10 μg), doripenem (10 μg), levofloxacin (5 μg), minocycline (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), ceftazidime (30 μg), and tetracycline (30 μg) (Mast, Company). Minimal inhibitory concentration (MIC) was determined by MIC-Test Strip (Liofilchem; Roseto degli Abruzzi, Italy) for four selected antibiotics, including trimethoprim-sulfamethoxazole, chloramphenicol, ceftazidime, and ticarcillin-clavulanate. Quality control was performed using *E. coli* ATCC 35218 and *E. coli* ATCC 25922.

DNA Extraction

S. maltophilia isolates were grown on LB for 24 h at 37°C, and genomic DNA was extracted using the high pure PCR Template Preparation Kit (Roche, Germany, and Lot. No.10362400) according to the manufacturer's guidelines. The total DNA concentration was determined using the Nanodrop instrument (WPA Biowave II Nanospectrophotometer, USA).

PCR-Sequencing Technique

The presence of β-lactamase genes *L1* and *L2* as well as *dfrA12*, *dfrA17*, *dfrA27*, *sul1*, *sul2*, and *Smqnr* genes were examined using the primers shown in **Table 1** (Levesque et al., 1995; Hu et al., 2011, 2016; Liu et al., 2012; Kanamori et al., 2015). As described previously (Hu et al., 2011), PCR was conducted in a final volume of 25 μl containing 1 μl (20 ng) of DNA template and 12.5 μl of 2× Master Mix (SinaClon-Iran, CAT. No., PR901638), including 1× PCR buffer, 0.4 mmol/L dNTPs, 3 mmol/L $MgCl_2$, and 0.08 IU *Taq* DNA polymerase, 1 μl of 10 pmol of each primer and 9.5 μl of sterile distilled water. Amplification reactions were performed on a thermal cycler (Eppendorf, Master Cycler Gradient, Germany). PCR was initiated by denaturation for 5 min 94°C, followed by 36 cycles of 45 s at 94°C, annealing at 50–59°C, according to the primers for each gene for 45 s, and extension at 72°C for 45 s. PCR products were electrophoresed by 1–1.5% agarose gel, visualized by DNA Safe staining and photographed under UV light. The PCR products were purified using a PCR purification Kit (Bioneer Co., Korea) and then, nucleotide sequencing of amplicons was performed by an ABI PRISM 3700 sequencer

TABLE 1 | Oligonucleotide primers used in this study.

Primers	Sequences(5′_3′)	Target	References
16srRNA-F	AGTTCGCATCGTTTAGGG	16 s RNA	(Di Bonaventura et al., 2010)
16srRNA-R	ACGGCAGCACAGAAGAGC		
L1-F	AGCCGTTAAAATTAAGCCC	L1	(Flores-Trevino et al., 2014)
L1-R	CTTGATTGAAGGGTTGGGCG		
L2-F	CGACAATGCCGCAGCTAACC	L2	(Flores-Trevino et al., 2014)
L2-R	CAGAAGCAATTAATAACGCCC		
Smqnr-F	ACACAGAACGGCTGGACTGC	Smqnr	(Kanamori et al., 2015)
Smqnr-R	TTCAACGACGTGGAGCTGT		
sul1-F	ATGGTGACGGTGTTCGGCATTCTGA	sul1	(Hu et al., 2008)
sul1-R	CTAGGCATGATCTAACCCTCGGTC		
sul2-F	GAAGCGCAGCCGCAATTCAT	sul2	(Hu et al., 2008)
sul2-R	CCTGTTTCGTCCGACACAGA		
spgM-F	ATACCGGGGTGCGTTGAC	spgM	(Madi et al., 2016)
spgM-R	CATCTGCATGTGGATCTCGT		
rpfF-F	CACGACAGTACAGGGGACC	rpfF	(Madi et al., 2016)
rpfF-R	GGCAGGAATGCGTTGG		
rmlA-F	CGGAAAAGCAGAACATCG	rmlA	(Madi et al., 2016)
rmlA-R	GCAACTTGGTTTCAATCACTT		
dfrA12-F	TTAGCCGTTTCGACGCGCAT	dfrA12	(Hu et al., 2008)
dfrA12-R	ATGAACTCGGAATCAGTACGC		
dfrA17-F	GTTAGCCTTTTTTCCAAATCTGGTATG	dfrA17	(Hu et al., 2008)
dfrA17-R	TTGAAAATATTATTGATTTCTGCAGTG		
DfrA27-F	AAGAGTCTGATCGCCCATGCCG	dfrA27	(Hu et al., 2008)
DfrA27-R	TAAAGCAATAACTTACAATC		
SmeD-F	CGGTCAGCATCCTGATGGA	smeDEF	(Cho et al., 2012)
SmeD-R	TCAACGCTGACTTCGGAGAACT		
rDNA-F	TGACACTGAGGCACGAAAGC	smeDEF	(Cho et al., 2012)
rDNA-R	CATCGTTTAGGGCGTGGACTA		

(Macrogen Co., Korea). The sequenced data obtained was viewed in Chromas version 1.45 software. In addition, sequence alignment was conducted using the Nucleotide BLAST program[1].

Phenotypic and Genotypic Detection of Biofilm Formation

Biofilm formation was examined by crystal violet staining as previously described by Stepanović et al. (2007). All experiments were performed in triplicate. An overnight culture of *S. maltophilia* was adjusted to match the turbidity of a 1.0 McFarland standard. The cultures were then diluted 1:100 in 200 ml tryptic soy broth (TSB) and were transferred into the wells of a flat-bottom polystyrene plate (SPL, Korea). After 24 h incubation at 37°C, plates were washed three times with sterile phosphate buffered saline (PBS with pH 7.3). Adherent biofilms were fixed for 60 min at 65°C, stained for 10 min at room temperature with 250 ml modified crystal violet and then, rinsed with water and allowed to dry. Biofilm samples were destained by treatment with 250 ml 33% glacial acetic acid for 20 min and the optical density (OD) was read at 492 nm (OD492). Grouping of isolates was carried out according to the following criteria: strong-biofilm producer (4 × ODc < OD), moderate-biofilm producer (2 × ODc < OD _ 4 × ODc), weak-biofilm producer (ODc < OD _2 × ODc), and non-biofilm producer (OD _ ODc). In addition, the presence of *rpfF*, *spgM*, and *rmlA* genes was investigated by PCR with specific primers described in **Table 1** (Pompilio et al., 2011). Amplicons representing each studied gene was confirmed by sequencing analysis (Macrogen Korea). Obtained sequences were aligned in the NCBI database using BLAST program[2].

RNA Preparation and qRT-PCR

TMP-SMX-resistant isolates were assessed for expression of SmeDEF efflux pump. Cell suspensions were prepared and inoculated on LB broth (Cho et al., 2012). After an overnight growth, total RNA was extracted from the cell suspensions by using the RNX-Plus Kit (Cat. No., RN7713C, Sinaclon, Iran) according to the manufacturer's instructions. The contaminating DNA was removed by RNase-free DNase I (Fermentas, USA). The total RNA concentration was determined using the Nanodrop (WPA Biowave II Nanospectrophotometer, USA). DNase-treated RNA was reverse-transcribed into cDNA using the Takara Kit (Japan). The primers used for real-time PCR are shown in **Table 2**.

[1]http://www.ncbi.nlm.nih.gov/nucleotide/

[2]http://www.ncbi.nlm.nih.gov/nucleotide/

TABLE 2 | Antibiotic susceptibility of the *S. maltophilia* clinical isolates (n = 164).

Antimicrobial agents	MIC (μg/ml)			Disc diffusion Number (%)		
	Range	MIC_{50}	MIC_{90}	Susceptible	Intermediate	Resistant
Imipenem	–	–	–	–	–	164 (100%)
Meropenem	–	–	–	6 (3/65%)	–	158 (96%)
Doripenem	–	–	–	6 (3/65%)	–	158 (96%)
Ceftazidime	0.5–64	16	32	34 (20/73%)	16 (9/75%)	114 (69/51%)
Tetracycline	–	–	–	131 (79%)	–	33 (20%)
Minocycline	–	–	–	150 (91/46%)	14 (8/53%)	0 (0%)
Levofloxacin	–	–	–	163 (99/39%)	1 (0/6%)	0 (0%)
TMP/SMX	0.64– ≥ 32	0.5	≤2.38	155 (94/51%)	4 (2/43%)	5 (3/04%)
Chloramphenicol	0.5–128	16	≥32	–	–	–
Ticarcillin-clavulanate	0.5–128	16	64	–	–	–

Real-time PCR assay was performed on synthesized cDNA using the Power SYBR Green PCR Master Mix (Bioneer, Korea) on a Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia). Each amplification protocol included a first denaturation step of 10 min at 94°C, followed by 40 cycles of 20 s at 94°C and 45 s at 59°C. Samples were run in triplicate. Controls run without reverse transcriptase confirmed the absence of contaminating cDNA in any of the samples. The expression level of *smeD* gene was normalized using the *rDNA* housekeeping gene, and was calculated based on $2^{-\Delta\Delta CT}$ method. Results were obtained as the relative expression of the mRNA compared to that of *S. maltophilia* ATCC 13637. The parameter Ct was defined as the threshold cycle number at which the first detectable fluorescence generated by the binding of SYBR Green I dye to the minor groove of double-stranded DNA began to increase exponentially. Final results, expressed as n-fold differences in expression of *smeD* genes, were determined as follows:

$$n-\text{fold differences in gene expression} = \frac{\text{Ct } smeD \text{ sample}}{\text{Ct } rDNA \text{ sample}} / \frac{\text{Ct } smeD \text{ calibrator}}{Ct\ rDNA \text{ calibrator}}$$

Values of $n < 1$ were considered to indicate overexpression of the Sme efflux system.

Molecular Typing by Multi-Locus Sequence Typing

Multi-Locus Sequence Typing (MLST) technique was performed as the same as described by Kaiser et al. (2009). Briefly, PCR for seven housekeeping genes, including *atpD*, *guaA*, *gapA*, *nuoD*, *ppsA*, *mutM*, and *recA* was carried out. Amplicons were sequenced according to the PubMLST website recommendations[3]. Unique sequence (allele) number for each gene was assigned on the basis of the information in the *S. maltophilia* MLST database[4] to determine specific sequence type (ST). A combination of the allelic sequences of the seven genes yielded the allelic profile for each isolate.

Molecular Typing by Repetitive Extragenic Palindromic-Pcr

Rep-PCR analyses were conducted with the single primer BoxA1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) according to Versalovic et al. (1994). The PCR reaction mix consisted of 25 μl total volume with 12.5 μl of 2× Master Mix (Genet Bio Cat.No:G-5000) containing 1 unit of Taq polymerase in 2× reaction buffer, 10% dimethyl sulfoxide (DMSO), enzyme stabilizer, sediment, loading dye, 4 mM $MgCl_2$, pH 9.0 and 0.5 mM of each dNTP, 5 μM of primer, and 1 μl of cell extract. Thermal cycling was conducted with an initial denaturation at 94°C for 10 min, followed by 25 cycles of 94°C for 45 s, 50°C for 1.5 min, 65°C for 8 min each, and concluded by a final extension of 65°C for 16 min. To determine phylogenetic relationships, rep-PCR profiles were analyzed by GelCompar II software (Applied Maths, Belgium) using the Pearson's correlation coefficient with unweighted paired group method using arithmetic averages (UPGMA) as well as at the 80% similarity level (Adamek et al., 2011).

Statistical Analysis

Chi-squared test was performed on the association of TMP-SMX resistance phenotype and resistance genes using SPSS software, 20.0 (SPSS Inc., Chicago, IL, USA). The Pearson's correlation coefficient was calculated to determine the association between two variables. A significant level of $p = 0.05$ was considered statistically significant.

RESULTS

Patients and Bacterial Isolates

During 1-year period of study, 164 *S. maltophilia* isolates were collected from several hospitals in different regions of Iran (**Figure 1**).

Among the 164 isolates obtained, 88 were from males and 76 were from females (male:female ratio = 1.15).

[3]http://pubmlst.org/smaltophilia/
[4]http://pubmlst.org/smaltophilia/

FIGURE 1 | *S. maltophilia* strains isolated from Iran. 4 isolates from Birjand. 87 isolates from Tehran: Capital of Iran. 32 isolates from Ahwaz. 20 isolates from Shiraz. 14 isolates from Bandar Abbas. 4 isolates from Zahedan. 1 isolate from Kerman. 1 isolate from Gorgan. 1 isolate from Qom.

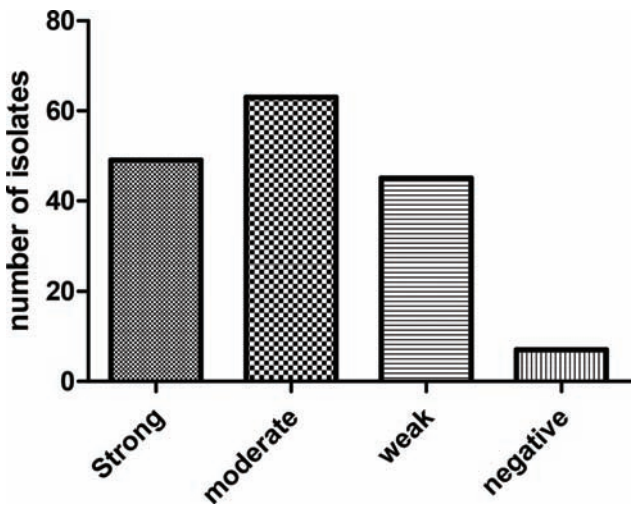


FIGURE 2 | Distribution of *S. maltophilia* isolates based on the biofilm formation in crystal violet staining assay.

The age range of patients was from 1 month to 85 years. The majority of the isolates were originated from blood (83.53%), followed by nose/throat secretions (5.48%), cough swabs (9.75%), sputum (0.6%), and CSF (0.6%).

Antibiotic Susceptibility Profile

Based on CLSI interpretive criteria (Clinical and Laboratory Standards Institute (CLSI) (2016)), isolates were resistant to imipenem (100%), meropenem (96%), doripenem (96%), and ceftazidime (36.58%). Interestingly, 5 (3.04%) isolates showed resistance to TMP-SMX. Minocycline and levofloxacin exhibited the highest susceptibility of 91.46 and 99.39%, respectively. The MIC ranges, MIC_{50}, MIC_{90}, and the percentages of isolates resistant, intermediate, or susceptible isolates to the six antimicrobial agents are shown in **Table 2**.

Biofilm Phenotypes and Genotypes

Biofilm phenotypes accounted for 157 out of 164 isolates (95.73%): 49 isolates (29.87%) produced strong biofilm, 63 isolates (38.41%) produced moderate biofilm, and 45 isolates (27.43%) produced weak biofilm; whereas, 7 isolates (4.26%) did not form biofilm (**Figure 2**). PCR-based typing of biofilm-related genes revealed an overall prevalence of 145 (88.41%), 137 (83.53%), and 164 (100%) of *rmlA*, *rpfF*, and *spgM*, respectively. In addition, the presence of *rmlA*, *rpfF*, and *spgM* had a close relationship with biofilm formation but did not significantly affect the mean amount of biofilm ($p \leq 0.05$). Some strong- and weak biofilm-producer phenotypes had mutations within the sequence of each *rpfF*, *spgM*, and *rmlA* genes.

Prevalence of Resistance Genes

Prevalence of resistance genes among 164 *S. maltophilia* isolates are shown in **Table 3**.

Of the 145 isolates that were positive for *L1*, all 145(100%) and 139(92.3%) showed resistance to imipenem and meropenem, respectively. Amongst 156 isolates carrying the *L2* gene, all (100%) were imipenem resistant and 150 (91.1%) were

TABLE 3 | Prevalence of resistance genes among 164 *S. maltophilia* strains isolated from Iran.

Resistance Genes, No. (%)							
L1	***L2***	***Smqnr***	***sul1***	***sul2***	***dfrA12***	***dfrA17***	***dfrA27***
145 (88.41%)	156 (96.12%)	103 (62.80%)	89 (54.26%)	92 (56.09%)	0 (0%)	0 (0%)	0 (0%)

TABLE 4 | Sequence type (ST) of TMP-SMX-resistant *S. maltophilia* clinical isolates recovered in the present study.

Number of isolates	***atpD***	***gapA***	***guaA***	***mutM***	***nuoD***	***ppsA***	***recA***	**ST**
3	allele 3	allele 4	allele 110	allele 46	allele 6	allele 38	allele 58	139
2	allele 26	allele 14	allele 140	allele 103	allele 3	allele 8	allele 11	259

meropenem-resistant ($p \leq 0.001$). In addition, 54.19% (89/155) and 58.70% (91/155) TMP-SMX-susceptible isolates and 100% (5/5) and 20% (1/5) TMP-SMX-resistant isolates were detected to contain the *sul1*, and *sul2* genes, respectively.

Gene Expression Analysis of *smeDEF*

Real-time PCR analysis was used to assess the expression of SmeDEF efflux system in TMP-SMX-resistant *S. maltophilia* isolates (MIC > 4/76 μg/ml). Results showed that *smeD* gene was overexpressed (5.47–7.87 fold) in two out of five isolates (40%) in comparison to the *S. maltophilia* ATCC 13637 standard strain.

MLST Analysis

As shown in **Table 4**, five TMP-SMX-resistant *S. maltophilia* isolates belonged to two different STs, ST139 and ST259. This is the first report on the detection of ST139 and ST259 from Iran. In addition, ST259 ($n = 2$) with allelic profile (26, 14, 140, 103, 3, 8, 11) was not previously reported. New allele sequences were deposited at the MLST Database hosted by the Shahid Beheshti University of Medical Science, Tehran, Iran[5].

Rep-PCR Fingerprinting

To evaluate the genetic diversity, all 164 *S. maltophilia* isolates were subjected to rep-PCR fingerprinting. As shown in **Figure 3,** isolates were divided into 16 common types (CT) containing 2–5 isolates and 114 single types (ST). Among these numerous clones, a dominant one was isolated from Ahwaz and from blood samples. The genotypic pattern of the dominant clone revealed that all isolates harbored *sul1* gene.

Nucleotide Sequence Accession Numbers

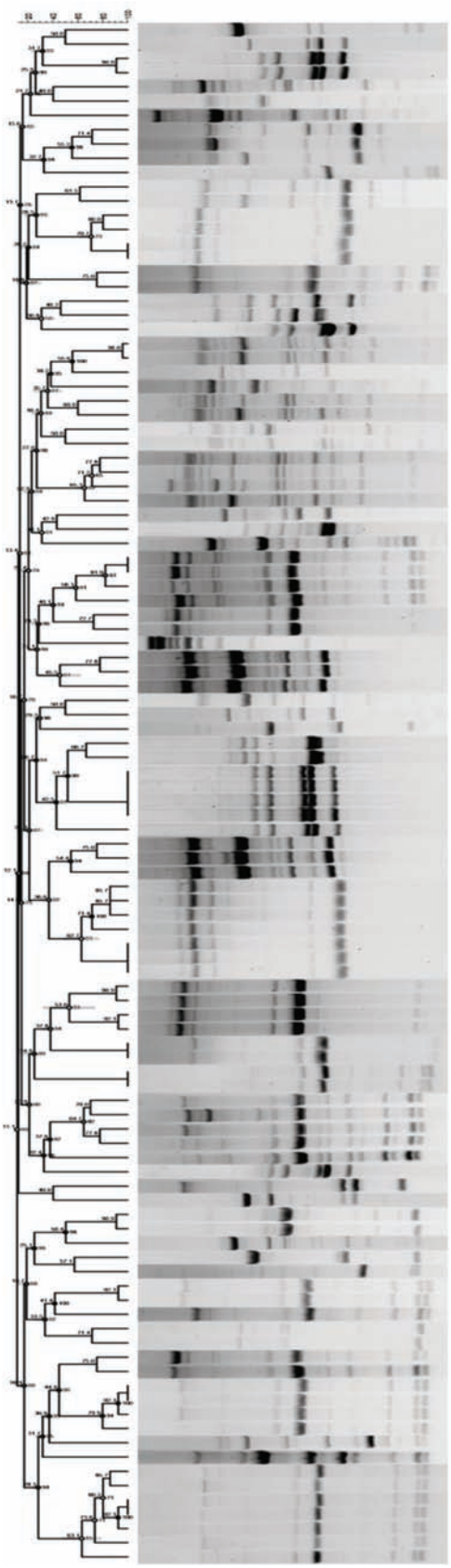
The nucleotide sequence data reported in this study were submitted to the GenBank sequence database and assigned under the accession numbers: MF458984, MF497329, MG601517, MG640120, MG648332, MG597493, MF805867, MG640120, MG560825, MG597494, MG640119, and MG601518 for the *L1*, *L2*, *sul1*, *sul2*, *smqnR*, *atpD*, *gapA*, *guaA*, *mutM*, *nuoD*, *ppsA*, and *recA* genes, respectively.

[5]http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_smaltophilia_isolates&page=query

DISCUSSION

The emergence of *S. maltophilia* as a nosocomial pathogen in hospitals with intrinsic resistance to multiple antibacterial agents, including carbapenems, aminoglycosides, β-lactams, and quinolones have caused great concern (Farrell et al., 2010). Additionally, some strains have acquired resistance, leading to limited antimicrobial options (Wang et al., 2013; Gholipourmalekabadi et al., 2016). In Iran, decades of misuse of antibiotics resulted in high prevalence of antibiotic resistance in bacteria (Habibzadeh, 2013; Saniee et al., 2018).

Global infectious disease surveillance stipulated that resistance rates for trimethoprim–sulfamethoxazole, ticarcillin-clavulanic acid, levofloxacin, and minocycline in *S. maltophilia* isolates are less than 4.7, 16.1, 6.5 and 5%, respectively (Sader and Jones, 2005). Among the 164 clinical isolates of *S. maltophilia* studied in the present study, a significant percentage was resistant to carbapenems ($p \leq 0.001$). Resistance to carbapenems in *S. maltophilia* occurs through several mechanisms, including intrinsic β-lactamase expression. In this study, 145 (88.41%) and 156 (96.12%) isolates harbored L1-and L2- β-lactamase genes, respectively. Also, the results indicate that the susceptibility rate of *S. maltophilia* isolates against ceftazidime was 20.73%, with the MIC_{50} and MIC_{90} of 8 and 32 μg/ml, a figure that was in agreement with previous findings (Nicodemo and Paez, 2007). A study by Jamali et al. showed that susceptibility of *S. maltophilia* against ceftazidime was 82% with the MIC_{50} and MIC_{90} values of 2 and 32 μg/ml, respectively (Jamali et al., 2011). Shahla et al. indicated that among 11 isolate of *S. maltophilia*, 91.4% were susceptible to ceftazidime (Shahla et al., 2012). In a study by Pfaller, the susceptibility in Canada, United States, and Latin America was respectively 27, 64.7, and 93.3% and Tatmanin Turkey showed the susceptibility of 67% for this drug (Pfaller et al., 1999; Tatman-Otkun et al., 2005). A study by Farrell et al. conducted in North America, Latin America, Europe, and Asian-Pacific reported a susceptibility rate of 27.0–46.1% to ticarcillin-clavulanate among *S. maltophilia* isolates (Farrell et al., 2010). The present study showed



Strain NO.	Specimen	Ward	City	Genotype
20	Blood	Urology	Bandar Abbas	sul1,qnr
41	Blood	Children	Ahvaz	qnr
62	Tracheal tube	Immunology	Tehran	qnr
64	Blood	Immunology	Tehran	qnr
163	Blood	ICU	Shiraz	sul1,sul2,qnr
7	Blood	CICU	Bandar Abbas	sul2
85	Blood	Children	Ahvaz	sul2,qnr
147	Blood	Men	Tehran	sul2
148	Blood	ICU	Tehran	sul1,sul2
26	Blood	CICU	Tehran	sul2
5	Blood	CICU	Bandar Abbas	sul2
155	Blood	ICU	Shiraz	sul1,sul2,qnr
156	Blood	ICU	Shiraz	sul2
28	Blood	CICU	Tehran	sul2
81	Blood	CICU	Birjand	qnr
75	Blood	Children	Ahvaz	sul1
76	Blood	Children	Ahvaz	sul1
110	Blood	CICU	Bandar Abbas	sul1,sul2,qnr
133	Blood	ICU	Kerman	sul1,sul2,qnr
58	Blood	Nephrology	Shiraz	sul1,sul2,qnr
59	Tracheal tube	ICU	Shiraz	sul1,qnr
71	Blood	Children	Ahvaz	qnr
140	Blood	Children	Ahvaz	sul2,qnr
141	Blood	CICU	Tehran	sul1,sul2,qnr
150	Throat secretions	Children	Shiraz	
105	Blood	ICU	Ahvaz	
142	Blood	CICU	Tehran	sul1,sul2,qnr
143	Blood	Children	Tehran	sul1,qnr
2	Blood	Children	Tehran	
3	Blood	ICU	Tehran	
116	Blood	Emergency	Tehran	sul1,qnr
134	Blood	CRP	Tehran	sul1,sul2
136	Blood	Children	Tehran	sul1,sul2,qnr
99	Blood	Children	Tehran	sul1
160	Throat secretions	Nephrology	Shiraz	sul1,sul2
66	Blood	Children	Tehran	sul1,qnr
49	Tracheal tube	Children	Ahvaz	sul1,qnr
86	Blood	ICU	Ahvaz	sul1
92	Blood	CICU	Tehran	sul1,sul2,qnr
104	Blood	ICU	Ahvaz	sul1,sul2,qnr
103	Blood	Urology	Bandar Abbas	sul1,sul2,qnr
117	Blood	Emergency	Tehran	sul1,qnr
122	Blood	Children	Tehran	sul1,sul2,qnr
REF				
79	Blood	Children	Ahvaz	sul2,qnr
82	Blood	Children	Ahvaz	sul2
80	Blood	CICU	Ahvaz	
1	Blood	ICU	Tehran	sul2
162	Blood	Nephrology	Shiraz	sul1,sul2,qnr
159	Tracheal tube	Nephrology	Shiraz	sul1,qnr
55	Blood	ICU	Bandar Abbas	sul1,sul2,qnr
56	Blood	NICU	Bandar Abbas	sul1,qnr
120	Blood	Nephrology	Tehran	sul1,sul2,qnr
125	Blood	Oncology	Tehran	sul1,sul2,qnr
53	Blood	ICU	Bandar Abbas	qnr
77	Blood	Children	Ahvaz	sul1,sul2
57	Blood	Immunology	Bandar Abbas	
108	Tracheal tube	ICU	Ahvaz	sul1,qnr
84	Tracheal tube	CICU	Ahvaz	qnr
83	Tracheal tube	ICU	Ahvaz	
10	Blood	ICU	Tehran	sul2
11	Blood	Digestive	Tehran	qnr
8	Blood	CICU	Bandar Abbas	
12	Blood	emergency	Kerman	sul2,qnr
13	Blood	Digestive	Tehran	qnr
14	Blood	CICU	Tehran	qnr
27	Blood	Men	Tehran	sul2
33	Blood	CICU	Tehran	sul2,qnr
34	Blood	CICU	Tehran	qnr
38	Blood	ICU	Tehran	sul1,sul2
95	Blood	Nephrology	Tehran	sul1
100	Blood	CICU	Tehran	sul1,sul2,qnr
101	Blood	CICU	Tehran	sul1,sul2,qnr
17	Blood	Nephrology	Ahvaz	
19	Blood	Children	Ahvaz	
112	Blood	NICU	Bandar Abbas	sul1,sul2,qnr
35	Blood	Nephrology	Ahvaz	sul1,sul2
119	Blood	Nephrology	Tehran	sul1,sul2,qnr
132	Blood	ICU	Bandar Abbas	sul1,sul2,qnr
37	Blood	Children	Ahvaz	sul1,sul2
69	Tracheal tube	Nephrology	Ahvaz	sul1
164	Blood	CICU	Shiraz	sul1,sul2,qnr
44	Throat secretions	Children	Ahvaz	qnr
72	Blood	Children	Ahvaz	sul1
73	Blood	Children	Ahvaz	sul2
21	Tracheal tube	ICU	Ahvaz	
68	Blood	CICU	Shiraz	
96	Blood	Nephrology	Tehran	
153	Blood	CICU	Shiraz	sul1,sul2,qnr
157	Blood	Children	Ahvaz	
139	Blood	Oncology	Bandar Abbas	
4	Blood	Emergency	Tehran	sul2
6	Blood	Immunology	Bandar Abbas	
107	Tracheal tube	Nephrology	Ahvaz	sul1,qnr
109	Blood	Emergency	Bandar Abbas	sul1,sul2,qnr
42	Blood	Emergency	Tehran	
70	Blood	Children	Bandar Abbas	
158	Blood	ICU	Tehran	sul1,sul2,qnr
98	Blood	CICU	Shiraz	sul1
161	Tracheal tube	ICU	Shiraz	sul2
111	Blood	CICU	Bandar Abbas	sul1,sul2,qnr
22	Blood	ICU	Ahvaz	sul1
51	Blood	CICU	Bandar Abbas	sul1,qnr
23	Blood	Immunology	Ahvaz	sul1
24	Blood	CICU	Ahvaz	sul1
25	Blood	CICU	Ahvaz	sul1
50	Blood	ICU	Ahvaz	sul1
45	Blood	ICU	Ahvaz	

FIGURE 3 | Continued

FIGURE 3 | Dendrogram based on Dice's coefficient of similarity using UPGMA method applied by the GelComparII program showing relationships between *S. maltophilia* strains according to BOX-PCR genotyping.

susceptibility rate of 57.92% to ticarcillin-clavulanate. MIC_{50} and MIC_{90} for ticarcillin-clavulanate was 12 and 128 μg/ml. A study in a Brazilian hospital showed the susceptibility pattern of *S. maltophilia* against chloramphenicol differs from 11.5 to 81.4% (Nicodemo and Paez, 2007). In our study, 7.31% of isolates were found to be susceptible to this antibiotic with MIC_{50} and MIC_{90} of 24 and 64 μg/ml. This variety in results designate that the susceptibility of *S. maltophilia* is variable in different countries and even in different hospitals. Other therapeutic alternatives, such as levofloxacin and minocycline, which have been reported as effective agents for treatment of invasive *S. maltophilia* infections (Wu et al., 2012, 2013; Cho et al., 2014), showed susceptibility rates of 99.39 and 96.41% in our study. Although the prevalence of minocycline and levofloxacin-resistant *S. maltophilia* is low worldwide, continued surveillance of resistance to such antimicrobials ensures their activity.

Historically, TMP-SMX is considered the first line of defense in *S. maltophilia* infections (Chung et al., 2015; Kaur et al., 2015). Results from the SENTRY Antimicrobial Surveillance Program in 2004 indicated that 3.8% of *S. maltophilia* isolates were resistant to TMP-SMX (Fedler et al., 2006). Moreover, the resistance rate reported for Latin America, Argentina, and Malaysia were approximately less than 4.5 and 1% (Barbolla et al., 2004; Farrell et al., 2010; Neela et al., 2012). Resistance rates vary geographically but are commonly less than 10% reported in several studies (Kaur et al., 2015). However, high and different rates of resistance have been reported in patients with cancer and cystic fibrosis (Valenza et al., 2008). In different studies by Shahla et al. (2012), Hu et al. (2016), Tatman-Otkun et al. (2005), Wang et al. (2004), Nicodemo et al. (2004), and Kaur et al. (2015), the susceptibility rates were reported 47.3, 61.3, 95.8, 60, 98.6, and 22.6%, respectively. Jamali et al. showed about 60% susceptibility rate for TMP-SMX and the MIC_{50} and MIC_{90} values were 0.5 and 2 μg/ml (Jamali et al., 2011). In our study, based on the CLSI recommended dose of TMP-SMX, the resistance rate of 3.04% and the $MIC_{50\%}$ and $MIC_{90\%}$ values of 2.38 and 4.76 were found, respectively. We believe that this resistance rate for TMP-SMX, as the treatment of choice for *S. maltophilia* infection, is sustainable, making necessary the future successive reevaluation of susceptibility to this antibiotic in Iranian hospitals.

The well-known mechanism responsible for TMP-SMX resistance is harboring the *sul1*, *sul2,* and/or*dfrA* resistance genes located either on a chromosome or plasmid (Hu et al., 2011). In this study, *sul1* and *sul2* genes were detected in both TMP-SMX-resistant and TMP-SMX-susceptible *S. maltophilia* clinical isolates. Additionally, antimicrobial efflux pump mechanisms have been increasingly recognized as sources of intrinsic and acquired resistance (Song et al., 2010; Hu et al., 2011; Gholami et al., 2015). As reported in other studies, the frequency of *sul2* gene in *S. maltophilia* strains is less than that of *sul1* gene (Song et al., 2010; Hu et al., 2011). These reports are contrary to the results of our study, where a higher percentage of *sul2* and *sul1* (56.9 and 54.26%, respectively) was observed. Furthermore, both *sul1* and *sul2* genes were found in TMP-SMX -susceptible and –resistant isolates. Similar to our study, Kaur et al. indicated that the percentage of *sul1* and *sul2* were 50 and 58.3%, respectively (Kaur et al., 2015). In addition, none of the isolates tested were positive for *dfrA12*, *dfrA17*, and *dfrA27*. In contrast, a study showed that 49.1% of TMP-SMX-resistant isolates and 10.3% of TMP-SMX-susceptible isolates were positive for *dfrA* genes, among them *dfrA12* and *dfrA17* genes were more prevalent (Hu et al., 2016). Previous reports indicated that overexpression of the SmeDEF efflux system in *S. maltophilia* plays a significant role in resistance to several antibacterials, including aminoglycosides, β-lactams, and quinolones (Chang et al., 2004; Cho et al., 2012). The results showed overexpression of *smeD* in 2 (40%) of the 5 TMP-SMX-resistant clinical isolates. Sanchez et al. showed that overexpression of the SmeDEF efflux pump decreases the susceptibility to TMP-SMX (Sánchez and Martínez, 2015).

An important feature of *S. maltophilia* is its ability to form biofilms on hospital surfaces as well as on human tissues; biofilms have been related to 65% of hospital-acquired infections (Zhuo et al., 2014). In this study, the majority of isolates were biofilm-producer as well as biofilm-related gene (*rpfF*, *rmlA* and *spgM*) carrier. In a study by Flores-Trevino et al., they showed that all *S. maltophilia* isolates were able to form biofilm and 47.9, 38.7, and 13.4% of the isolates were weak-, moderate-, and strong-biofilm producers, respectively (Flores-Trevino et al., 2014). Zhou et al. showed that the results of a biofilm formation assay on polystyrene was strong in 49 (29.87%) strains, moderate in 63 strains (38.41%), and weak in 45 (27.43%) strains, while nine strains (4.26%) were non-biofilm former. Furthermore, the presence of *rpfF* and *spgM* was significantly correlated to biofilm formation. Pompilio et al. reported that *spgM* gene played a significant role in formation of strong biofilm among *S. maltophilia isolates* (Zhuo et al., 2014). Similarly, the presence of *rmlA*, *rpfF*, and *spgM* genes in the present study improved significantly biofilm formation by *S. maltophilia* isolates tested ($p \leq 0.05$). Indeed, the isolates with $rpfF^{+}/spgM^{+}/rmlA^{+}$ genotype were associated with production of moderate or strong biofilm. In addition, amino acid substitution in genes encoding SpgM, RpfF and RmlA were found among some strains (Corlouer et al., 2017). However, it is still unclear which gene mutation results to change in biofilm formation.

High genetic diversity among *S. maltophilia* isolates has been described worldwide. Although occurrences of outbreaks within hospital settings have also been reported (Flores-Trevino et al., 2014). Recently, molecular epidemiologic studies, like MLST is developed for *S. maltophilia* strain-typing that focuses on conserved housekeeping genes (Corlouer et al., 2017). In the present study, MLST analysis was performed for determining genetic diversity

of five TMP-SMX-resistant isolates. The results revealed two STs (ST139 and ST259), of which ST259 was identified for the first time in this study. Similarly, studies in Spain in 2004, and Korea in 2010, a high rate of genetic diversity among *S. maltophilia* isolates despite their source in a single hospital (Valdezate et al., 2004; Cho et al., 2012; Corlouer et al., 2017). These findings indicate that *S. maltophilia* has a high potential for environmental distribution, although database analysis shows that there are noticeably fewer STs for *S. maltophilia* isolates than other bacterial isolates. Rep-PCR fingerprinting is a method with lower cost and the best time efficiency. According to the cluster analysis of *S. maltophilia* strains, this study detected high clonal diversity among the isolates. The only exception is the dominant common type including strains isolated from blood culture of patients hospitalized in Ahwaz. In addition, all these isolates harbored *sul1* gene. As a result, the presence and spread of these strains with resistance gene could be a significant threat.

CONCLUSIONS

This multi-institutional study revealed that *S. maltophilia* is an emerging MDR opportunistic pathogen in hospital settings, especially among immunocompromised patients. TMP-SMX remains the most effective antibacterial agent against *S. maltophilia*. So, a significant effort is required to maintain antibacterial properties of this antibiotic. Due to the low prevalence of resistance to two antibiotics levofloxacin and minocycline, clinical usage of these agents can be continued. The findings of this study showed an increasing presence of antibacterial resistance-and biofilm genes among the clinical isolates of *S. maltophilia* strains in Iran. Clinicians must consider that *S. maltophilia* as a co-pathogen or co-colonizer in polymicrobial infections can have negative effect on the success rate of antibacterial treatment and clinical outcome. In our opinion, this is significant medical problem, which should be of great concern.

AUTHOR CONTRIBUTIONS

NB, ZG, FF, and AH conceived and designed the experiments. NB, ZG, AH, AY, JM, AA, SS, ST, AA, FF, and AP performed the experiments and analyzed the data. NB, AA, and AH wrote the paper.

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Prevalence of Antimicrobial Resistance and Virulence Gene Elements of *Salmonella* Serovars From Ready-to-Eat (RTE) Shrimps

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Gastrointestinal illnesses continue to be a global public health risk. Exposure to foodborne *Salmonella* directly or indirectly through consumption of ready-to-eat seafood can be an important route of infection to humans. This study was designed to estimate the population cell density, prevalence, virulence gene signatures, and antibiotic resistance of *Salmonella* serovars from ready-to-eat shrimps. Ready-to-eat (RTE) shrimp samples were obtained from different open markets in Delta and Edo States, Nigeria from November 2016 to October 2017. We employed classical and polymerase chain reaction (PCR) approaches. The mean *Salmonella* species enumerated from the RTE shrimps ranged from −0.301 to 5.434 $\log_{10}$ cfu/g with 210/1440 (14.58%) of the RTE shrimp samples harbored *Salmonella* species. After biochemical and PCR approach, the identified isolates were *Salmonella* Enteritidis 11(24.4%), *Salmonella* Typhimurium 14 (31.1%) and other *Salmonella* spp. 20 (44.4%). All *Salmonella* species recovered were resistant to penicillin and erythromycin with 100% sensitivity to cefotaxime, cephalothin, colistin, and polymyxin B. Findings on the multidrug-resistant (MDR) profile showed that a total of 9/14 (64.3%) of *Salmonella* Enteritidis were resistant to 5 antibiotics which belongs to 3 different groups of antimicrobials with a multiple antibiotic-resistant (MAR) index of 0.21; while 3/11 (27.3%) of *Salmonella* Typhimurium were resistant to 11 antibiotics which belongs to 7 different groups of antimicrobials with a MAR index of 0.46. Virulence genes (*spi*A, *sip*B, *inv*A, *sif*A, *flj*B, and *sef*A) and resistance genes (class 1 and II integrase, *sul*2, *catB*3, *flor*, *tmp*, bla_{TEM}, *str*B, *dfr*1, and *tet*C) were also detected in some of the *Salmonella* species with variable percentage. This study indicates that ready-to-eat shrimps are probable reservoirs harboring *Salmonella* strains. The identified *Salmonella* isolates which exhibited virulence determinants and antibiotic-resistant coupled with high MAR index constitute a consumer health risk to the communities.

Keywords: multidrug resistant, salmonellosis, virulence determinants, seafood, health risk

INTRODUCTION

Shrimps constitute a large proportion of crustaceans which varies in sizes (Orji et al., 2016) and have been described as the most significant seafood traded on a global scale (Oosterveer, 2006). The world shrimp production for both farmed and captured shrimp is ∼6 million tons with 60% entering the world market. Shrimp has been reported to be the most essentially traded fishery product internationally as it translates to value. Yearly shrimp exports presently value above US$10 billion, or 16% of total fish product exports (Food Agriculture Organization of the United Nations, 2008). Shrimp makes up 20% value of exported fishery products for more than 20 years (CAC, 2002). Imports of shrimps into developed nations are responsible for about 40% trade of intra-developed countries, while approximately 60% comes from developing nations. From developing nation exports, 80% goes to developed nation with only 20% left behind (Josupeit, 2005). Shrimps are one of the important exported aquaculture products from the tropics. The interaction of microbial diversity that comes in connection with shrimps during harvesting and processing is a prospective public health threats as a consequence of disease and spoilage transmission.

The main disease causing serovars of *Salmonella enterica* subspecies *enterica* which are pathogenic to humans as a result of diverse seafood and non-seafood products include *Salmonella* Typhimurium and *Salmonella* Enteritidis (Ed-dra et al., 2017). Salmonellosis which is an infection of the intestinal epithelium is instigated by the *Salmonella* genus (Igbinosa and Beshiru, 2017; Beshiru et al., 2018). Within the United States more than 40,000 cases of salmonellosis are recounted yearly with seafood considered as one of the most significant source of *Salmonella* (Brands et al., 2005; Duran and Marshall, 2005). Contamination results when the salmonellae enter RTE food and replicate within the food, as a result of inadequate food preparation, poor storage temperatures, or cross-contamination (Skyberg et al., 2006). Hence, the occurrence of *Salmonella* in RTE seafood from open market is an important food safety risk.

Antimicrobial-resistant *Salmonella* serovars may result from the continuous usage of antimicrobials in food animal production, where these antimicrobial resistant *Salmonella* are therefore disseminated to humans, usually through contaminated food. The application of antimicrobials in aquaculture systems has led to the accumulation of antibiotic-resistance genes and antibiotic-resistant bacteria (Yano et al., 2011; Igbinosa, 2016). Antibiotics commonly used in agricultural/aquaculture systems in Nigeria are gentamycin, ivermectin, oxytetracycline, tylosin, septinomycin, and cephalosporin. Food and Drug Administration (FDA) has permitted the use of five different drugs (sulfamerazine, chorionic gonadotropin, florfenicol, oxytetracycline hydrochloride, oxytetracycline dihydrate, as well as combination of sulfadimethoxine and ormetoprim) in aquaculture so long as the seafood harbors less than the required maximum residue limit (Serrano, 2005). FDA has also approved two drugs: hydrogen peroxide and formalinwith no tolerance level set a (Serrano, 2005). Multidrug resistance (MDR) in *Salmonella* is of significant concern as treatment regimens may be challenging, thus making management of these disease difficult. *Salmonella* Typhimurium is one of the most widespread MDR *Salmonella* serovars recovered from humans and animals in the United States (Brunelle et al., 2013). The continuous rise and dissemination of antibiotic resistance phenotypes and determinants among *Salmonella* serovars has metamorphosed into a public health space. Notably, strains of *Salmonella* which have clinically phenotypic and genotypic resistance to antibiotic agents such as extended spectrum cephalosporins and fluoroquinolones, have been recovered from food animals (Li et al., 2013; Igbinosa, 2015). Within developing countries, overuse and misuse of antibiotics has led to the upsurge of MDR in *Salmonella* strains (Ed-dra et al., 2017).

Antibiotic-resistant *Salmonella* connected with cultivated *Litopenaeus vannamei* have been reported in Malaysia where *Salmonella enterica* serovar Corvallis recovered from shrimp revealed multiple and individual antibiotic resistance profiles (Banerjee et al., 2012). In Nigeria there are some reports that have revealed the occurrence of *Salmonella* species from numerous food types, with no study on the surveillance of *Salmonella* Typhimurium, *Salmonella* Enteritidis and other *Salmonella* spp. from RTE shrimps. Hence, the objective of this research was to determine the prevalence, multiple antibiotic resistance, virulence and antibiotic resistance genes of *Salmonella* serovars recovered from retail RTE-shrimps in Nigeria.

MATERIALS AND METHODS

Study Area

The RTE shrimp were obtained from major open markets in Delta and Edo States, Nigeria. There are 3 Senatorial Districts in each of Edo and Delta State. Six different markets were assessed from each state which makes it a total of 12 markets with 2 markets from each Senatorial District. In Delta State, markets include Ughelli main market, Sapele market (Delta Central Senatorial District), Ogbegonogo market, Ashafor market Aniocha Asaba market (Delta North Senatorial District), main market Oleh Isoko, and Igbudu market Warri (Delta South Senatorial District). For Edo State, markets include New Benin market, Oba market (Edo South Senatorial District), Igarra market, Jattu market (Edo North Senatorial District), Uromi main market and Ekpoma market (Edo Central Senatorial District). The respective markets were chosen based on the strategic locations in their respective communities and are highly dense due to the population of individuals that patronizes these markets. The RTE shrimp that were collected from these markets were mainly tiger shrimps (*Penaeus monodon*) and pink shrimp (*Penaeus notialis*) and included smoked shrimps, dried shrimps, fried shrimps, sauced shrimps, and boiled shrimps.

Sample Collection

One thousand four hundred and forty RTE shrimp samples were obtained between November 2016 and October, 2017. Ten samples each were obtained from each of the respective 12 selected open markets (6 each from Delta and Edo States) culminating in the 1440 RTE samples. Samples were obtained based on the type of RTE shrimps available with respect to the sampling location. The RTE shrimp samples were obtained from

the selected open markets with the aid of a sterile polythene bag. The polythene bags were immediately placed on ice pack and conveyed to the laboratory where microbiological analyses were carried out within 24 h after collection.

Enrichment, Enumeration and Isolation of *Salmonella* Species

This was carried out in line with the International Organization for Standardization (2017). Twenty-five grams of individual RTE shrimp samples was weighed and placed in a sterile homogenizer bag containing 225 mL of tryptone soy broth (TSB) (Merck, Darmstadt, Germany), as pre-enrichment and incubated at 37°C for 18–24 h. Before incubation, the stock suspension was serially diluted using sterile distilled water from 10^{-1} to 10^{-9}. Dilution with 100 μL of each diluent aseptically platted in triplicates into xylose lysine deoxycholate (XLD) agar (Lab M, Lancashire, United Kingdom) and hektoen enteric agar (HEA) (Lab M, Lancashire, United Kingdom). This was followed with incubated at 37°C for 24–48 h where presumptive *Salmonella* species which appear as distinct green colonies with or without black centers were enumerated and expressed in $\log_{10}$ colony forming units per gram ($\log_{10}$ cfu/g). After incubation with the pre-enrichment broth with TSB, 100 μL were inoculated into 9.0 mL of selenite cysteine F Broth (Lab M, Lancashire, United Kingdom) and incubated at 37°C for 18–24 h. After incubation, 100 μL of the turbid suspension was inoculated into XLD and HEA and incubated at 37°C for 18–24 h where a maximum of 2 presumptive *Salmonella* colonies were selected and sub-cultured on a fresh XLD and HEA and incubated at 37°C for 18–24 h. Distinct colonies were further purified on tryptone soy agar (TSA) (Merck, Darmstadt, Germany) incubated at 37°C for 18–24 h. Isolates were transferred into a 1 mL TSA in an Eppendorf tube and incubated at 37°C for 24 h and stored in the refrigerator at 4°C until ready for further use.

Presumptive Identification of *Salmonella* Species

All *Salmonella* species were screened via biochemical (oxidase, catalase, indole, and sugar fermentation test, citrate), morphological (Gram reaction with 3% KOH test), and cultural (colony) characterization. Analytical Profile Index 20E (API 20E) was also used for the *Salmonella* species respectively according to the manufacturer's instructions (BioMerieux, Marcy-l'Étoile, France) using API lab plus software (bioMerieux, Marcy l'Etoile, France).

Genomic Deoxyribonucleic Acid (gDNA) Extraction Procedure

Genomic DNA from *Salmonella* species were extracted via boiling method described by Igbinosa et al. (2017). The *Salmonella* isolates were inoculated in 5.0 mL TSB incubated at 37°C for 18–24 h (Beshiru et al., 2018). A 100 μL of the turbid suspension was combined with 100 μL of sterilized distilled water in a 2.0 mL Eppendorf tube and subjected to a dry bath (MK200-2, Shanghai, China) for 15 min at 100°C for cell lyses. The lysed cell mixture was then centrifuged with a mini centrifuge (Mini 14k, Zhuhai, Guangdong, China) at 14 500 r/min for 15 min. The cell fragments were carefully separated from the supernatant. The supernatant was stored at −20°C as the template gDNA.

Polymerase Chain Reaction Amplification Procedure

All reactions were carried out in 25.0 μL volume of reaction (10 × Buffer 2.5 μL; $MgCl_2$ 1.0 μL; dNTP-Mix 3.0 μL; Taq polymerase 0.2 μL; Reverse primer 1.25 μL; Forward primer 1.25 μL; sterile double distilled H_20 10.8 μL and gDNA 5.0 μL). Primers used for the detection of *Salmonella* species are shown in **Table S1**. The reaction was performed via a Peltier-based Thermal Cycler (BioSeparation System, Shanxi, China) with an initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 94°C for 60 s, primer annealing as indicated in **Table S1** and extension at 72°C for 90 s; final extension at 72°C for 10 min. *Salmonella enterica* serovar Typhymurium ATCC 14028, *Salmonella* Enteritidis ATCC 13076, were used as positive controls while deionized water was used as a negative control for each test procedure. Thermal cyclic conditions for the detection of antibiotic-resistance genes for *Salmonella* species were as follows; initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 1 min, annealing condition as in **Table S2** and extension at 72°C for 1 min with a final extension at 72°C for 10 min and cooling to 4°C. The PCR conditions for amplification of the virulence genes were as follows: 5 min of initial denaturation at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing as described in **Table S3**, and extension at 72°C for 60 s, ending with a final extension period of 72°C for 2 min. Electrophoresis of the amplified PCR products were loaded on 1.2% agarose gel (CLS-AG100, Warwickshire, United Kingdom) in 0.5× TAE buffer (pH 8.5, 20 mM Na acetate, 40 mM Tris-HCl, 1 mM EDTA) and allowed to run for 1 h at 100 V. The gels were viewed via a UV transilluminator (EBOX VX5, Vilber Lourmat, France).

Antimicrobial Susceptibility Profile of the *Salmonella* Isolates

Antimicrobial susceptibility profile of the *Salmonella* species was carried out using Kirby-Bauer disc diffusion method. Briefly, the purified isolates were inoculated in 5.0 mL Mueller-Hinton Broth (MHB) (Lab M, Lancashire, United Kingdom) and incubated overnight. The optical density (OD) of the turbidity of the broth was adjusted to McFarland standard 0.5 equivalent to 10^8 cfu/mL. Using a sterile swab sticks, respective broth cultures were aseptically swabbed on Mueller Hinton Agar (Lab M, Lancashire, United Kingdom). A total of 24 antibiotic discs (Mast Diagnostics, Merseyside, United Kingdom) which included kanamycin (30 μg), gentamycin (10 μg), streptomycin (25 μg), erythromycin (15 μg), tobramycin (10 μg), ampicillin (10 μg), amoxicillin (25 μg), imipenem (10 μg), ampicillin/sulbactam (30 μg), meropenem (10 μg), cefotaxime (30 μg), sulfamethoxazole (30 μg), cephalothin (30 μg), trimethoprim (25 μg), erythromycin (15 μg), amoxicillin/clavulanate (30 μg), colistin (20 μg), chloramphenicol (30 μg), penicillin G (10 μnits), polymyxin

B (300 units), oxytetracycline (30 μg), doxycycline (30 μg), tetracycline (30 μg), ofloxacin (5 μg), and ciprofloxacin (10 μg) were used for the susceptibility testing. The respective discs were also aseptically impregnated on the agar plates using a sterile forceps equidistant apart. Plates were allowed to stand at room temperature for 5 min and incubated at 37°C for 18–24 h. Resistance, intermediate or susceptibility profile of the isolates were elucidated by determining zone of inhibition and matched with the interpretative chart of Clinical Laboratory Standards Institute (2017) to determine the sensitivity, intermediate and resistance profiles of the isolates to the antibiotics used.

Statistical Analysis

All data were analyzed using the statistical package SPSS (Version 21.0) and Microsoft Excel 2013. Descriptive statistics were carried out to determine the mean population density and expressed in Log_{10} CFU/g. One Way Analysis of Variance was applied to the densities from open markets while Duncan Multiple Range test was used to show significant difference between mean variables. The $p < 0.05$ were considered statistically significant.

RESULTS

Population Cell Density of *Salmonella* Species From the RTE Shrimps

The mean *Salmonella* species counts from RTE shrimps obtained from open markets are presented in **Table S4**. The mean *Salmonella* species counts from the RTE shrimps are all expressed in log_{10} cfu/g. The values ranged from 0.079 to 3.516 (November), 0.613–3.817 (December), 0.255–4.492 (January), 0.602–4.841 (February), 0.959–4.822 (March), 1.562–5.118 (April), 1.573–5.434 (May), 2.003–5.274 (June), 2.001–5.356 (July), 1.782–4.555 (August), 0.944–4.754 (September), and −0.301 −3.748 (October) during the 12 month sampling regimen. Significant differences were observed across the respective markets as $p < 0.01$. For the respective markets, values ranged from 0.977 to 2.391 (Oba Market), 0.944–3.283 (New Benin Market), 0.613–3.231 (Jattu Market), 0.079–2.075 (Igarra Market), −0.301 −3.318 (Ekpoma Market), 1.272–3.484 (Uromi Market), 2.572–4.428 (Sapele Market), 3.053–4.481 (Ughele Market), 3.083–5.434 (Ogbegonogo Market), 3.185–5.205 (Ashafor Market), 3.161–4.435 (Igbudu Market), and 3.236–5.356 (Main Market, Oleh). Significant differences were observed across the respective months as $p < 0.01$.

Prevalence of Positive *Salmonella* Samples

The distribution of positive *Salmonella* samples from respective markets include, 14/120 (11.7%) for Oba market, 10/120 (8.33%) for New Benin market, 8/120 (8.33%) for Jattu market, 9/120 (7.5%) for Igarra market, 7/120 (5.83%) for Ekpoma market, 10/120 (8.33%) for Uromi market, 23/120 (19.17%) for Sapele market, 27/120 (22.5%) for Ughele market, 26/120 (21.67%) for Ogbegonogo market, 25/120 (20.83%) for Ashafor market, 27/120 (22.5%) for Igbudu market, 24/120 (20%) for main Market Oleh. Overall, 210/1440 (14.58%) were positive for *Salmonella* species.

Salmonella Detection From RTE Shrimps

This study revealed, 210/1440 (14.58 %) of the RTE shrimp samples were positive for *Salmonella* species. All the tentatively 210 *Salmonella* isolates were characterized via culture-based and biochemical procedures using Gram-reaction with 3% KOH test, oxidase, urease reactions, indole and motility tests. The *Salmonella* isolates that appear negative for urease, oxidase, indole and Gram-negative rods were selected as presumptive *Salmonella*. Only 67 *Salmonella* isolates were positive using this culture-based approach. Analytical profile index (API 20E) were further employed to confirmed the identity of 49 *Salmonella* isolates. From the 49 *Salmonella* isolates positive from the API test, *Salmonella* genus-specific primer was only positive for 45 isolates. This was further identified using the species-specific primer that target *Salmonella* Enteritidis 11 (24.4%), *Salmonella* Typhimurium 14 (31.1%) and other *Salmonella* spp. 20 (44.4%). In Oba Market, 1/4 (25%) were confirmed to be *Salmonella* Enteritidis, 1/4 (25%) were confirmed to be *Salmonella* Typhimurium, 2/4 (50%) were confirmed to be other *Salmonella* species (**Table S5**).

Antimicrobial Susceptibility Profiles of the *Salmonella* Species From RTE Shrimps

The distribution of antimicrobial susceptibility profile of *Salmonella* species is presented in **Table 1**. For *Salmonella* Enteritidis, 100% (14/14) were resistant to erythromycin and penicillin, 85.7% (12/14) were resistant to amoxicillin/clavulanate and ampicillin, 92.9% (13/14) were resistant to amoxicillin, 71.4% (10/14) were resistant to ampicillin/sulbactam. For *Salmonella* Typhimurium, 100% (11/11) were resistant to erythromycin and penicillin, 90.9% (10/11) were resistant to ampicillin and amoxicillin, 72.7% (8/11) were resistant to amoxicillin/clavulanate, 63.6% (7/11) were resistant to doxycycline. For other *Salmonella* species, 100% (20/20) were resistant to erythromycin and penicillin, 80% (16/20) were resistant to amoxicillin, 75% (15/20) were resistant to ampicillin, 70% (14/20) were resistant to amoxicillin/clavulanate, 65% (13/20) were resistant to streptomycin. Number of resistant + intermediate *Salmonella* species as shown in **Table 1** include 0/45 (cefotaxime, cephalothin, polymycin B and colistin), 45/45 (ampicillin, amoxicillin, erythromycin, penicillin, and amoxicillin/clavulanate), 38/45 (ampicillin/sulbactam), 37/45 (streptomycin), 36/45 (doxycline), 33/45 (tetracycline), 30/45 (oxytetracycline and ciprofloxacin), 26/45 (ofloxacin).

Distribution of Multiple Antibiotic-Resistance Characteristics of the *Salmonella* Species

The MDR and MAR index distribution of *Salmonella* species is presented in **Table 2**. A total of 9/14 (64.3%) of *Salmonella* Enteritidis were resistant to 5 antibiotics (AMP^R, AMX^R, AMC^R, ERY^R, PEN^R) which belonged to 3 different groups of antimicrobials with a MAR index of 0.21. Furthermore, 4/14 (28.6%) of *Salmonella* Enteritidis were resistant to 11 antibiotics (AMP^R, AMX^R, AMC^R, STR^R, SAM^R, CIP^R, OXY^R, TET^R, OFX^R, ERY^R, PEN^R) which belonged to 8 different groups of

TABLE 1 | Antimicrobial susceptibility profiles of the *Salmonella* species.

Antimicrobial class	Antibiotics	*Salmonella* species								
		Salmonella Enteritidis ($n = 14$)			*Salmonella* Typhimurium ($n = 11$)			Other *Salmonella* spp. ($n = 20$)		
		R	I	S	R	I	S	R	I	S
Aminoglycosides	GEN	7.1	42.9	50	0	27.27	72.72	10	15	75
	KAN	0	21.4	78.6	0	9.09	90.9	0	5	95
	STR	50	28.6	21.4	54.5	27.27	18.18	65	20	15
	TOB	7.1	42.9	50	0	54.54	45.45	5	25	70
Aminopenicillins	AMP	85.7	14.3	0	90.9	9.09	0	75	25	0
	AMX	92.9	7.1	0	90.9	9.09	0	80	20	0
B-lactam/Beta-lactamase Inhibitors	SAM	71.4	14.3	14.3	45.45	54.54	0	50	25	25
Carbapenems	IPM	7.1	14.3	78.6	18.18	9.09	72.72	5	5	90
	MEM	0	7.1	92.9	0	9.09	90.9	0	10	90
Cephalosporins	CTX	0	0	100	0	0	100	0	0	100
	CEF	0	0	100	0	0	100	0	0	100
Folate pathway inhibitors	SUL	0	21.4	78.6	0	54.54	45.45	0	35	65
	TMP	0	7.1	92.9	0	27.27	72.72	0	10	90
Macrolides	ERY	100	0	0	100	0	0	100	0	0
Penicillins	PEN	100	0	0	100	0	0	100	0	0
	AMC	85.7	14.3	0	72.72	27.27	0	70	30	0
Phenicols	CHL	7.1	28.6	64.3	9.09	9.09	81.81	0	50	50
Ploymyxins	CST	0	0	100	0	0	100	0	0	100
	PMB	0	0	100	0	0	100	0	0	100
Tetracyclines	DOX	35.7	50	14.3	63.64	9.09	27.27	45	35	20
	OXY	42.9	21.4	35.7	27.27	45.45	27.27	35	30	35
	TET	50	28.6	21.4	36.36	18.18	45.45	50	30	20
Quinolone	CIP	50	35.7	14.3	36.36	18.18	45.45	25	35	40
	OFX	42.9	21.4	35.7	45.45	9.09	45.45	35	20	45

GEN, Gentamycin (10 μg); KAN, Kanamycin (30 μg); STR, Streptomycin (25 μg); TOB, Tobramycin (10 μg); AMP, Ampicillin (10 μg); AMX, Amoxicillin (25 μg); SAM, Ampicillin/Sulbactam (30 μg); MEM, Meropenem (10μg); IPM, Imipenem (10 μg); CTX, Cefotaxime (30 μg); CEF, Cephalothin (30 μg); SUL, Sulfamethoxazole (30 μg); TMP, Trimethoprim (25 μg); ERY, Erythromycin (15 μg); PEN, Penicillin G (10 μunits); AMC, Amoxicillin/clavulanate (30 μg); CHL, Chloramphenicol (30 μg); CST, Colistin (20 μg); PMB, Polymyxin B (300 units); DOX, Doxycycline (30 μg); OXY, Oxytetracycline (30 μg); TET, Tetracycline (30 μg); CIP, Ciprofloxacin (10 μg); OFX, Ofloxacin (5 μg); R, Resistant; I, Intermediate; S, Sensitive.

antimicrobials with a MAR index of 0.46. A total of 9/11 (81.8%) of *Salmonella* Typhimurium were resistant to 4 antibiotics (AMP^R, AMX^R, ERY^R, PEN^R) which belonged to 3 different groups of antimicrobials with a MAR index of 0.17. Furthermore, 3/11 (27.3%) of *Salmonella* Typhimurium were resistant to 11 antibiotics (AMP^R, AMX^R, ERY^R, PEN^R, STR^R, AMC^R, DOX^R, SAM^R, TET^R, CIP^R, OFX^R) which belonged to 7 different groups of antimicrobials with a MAR index of 0.46. A total of 9/20 (45%) of other *Salmonella* spp. were resistant to 6 antibiotics (STR^R, AMP^R, AMX^R, ERY^R, PEN^R, AMC^R) which belonged to 4 different groups of antimicrobials with a MAR index of 0.25. Furthermore, 3/20 (15%) of other *Salmonella* spp. were resistant to 12 antibiotics (STR^R, AMP^R, AMX^R, ERY^R, PEN^R, AMC^R, SAM^R, DOX^R, TET^R, OXY^R, OFX^R, CIP^R) which belonged to 7 different groups of antimicrobials with a MAR index of 0.50.

Distribution and Proportion of Virulence Gene Elements Among the *Salmonella* Species

The distribution of virulence genes among *Salmonella* species is presented in **Table 3**. For *Salmonella* Enteritidis 10/14 (71.4%) harbored *spiA* (involved in both biofilm formation and virulence), 11/14 (78.6%) revealed *sipB* (allows easy entering of non-phagocytic cells and lysing of macrophages), 14/14 (100%) harbored *inv*A (*Salmonella* invasion gene), 12/14 (85.7%) revealed *sifA* (for the development of filamentous assemblies) and *fljB* (flagellin gene), 13/14 (92.9%) harbored *sefA* (fimbrial subunit of *Salmonella* antigen) (**Table 3**).

Distribution of Antibiotic-Resistant Elements Among the *Salmonella* Species

The distribution of antibiotic-resistant elements amongst *Salmonella* species is presented in **Table 4**. For *Salmonella* Enteritidis 9/14 (64.3%) harbored Class 1 integrase, 6/14 (42.9%) demonstrated Class 2 integrase, 8/14 (57.1%) revealed *sul*2 (sulphonamide resistance gene) and *catB*3 (group B chloramphenicol acetyltransferase gene), 10/14 (71.4%) revealed *flor* (florfenicol/chloramphenicol resistance gene), *tmp* (dihydrofolate reductase gene), bla_{TEM} (beta-lactamase resistant gene), 11/14 (78.6%) demonstrated *str*B (streptomycin inactivating enzyme), 12/14 (85.7%) harbored *dfr*1 (specific

TABLE 2 | Distribution of multiple antibiotic resistant characterizations of the *Salmonella* species.

Salmonella species	No of antimicrobial class	No of antibiotics	Resistance phenotypes	No of resistant species (%)	MAR index
Salmonella Enteritidis ($n = 14$)	3	5	AMP^R, AMX^R, AMC^R, ERY^R, PEN^R	9(64.3)	0.21
	6	8	AMP^R, AMX^R, AMC^R, STR^R, SAM^R, CIP^R, ERY^R, PEN^R	6(42.9)	0.33
	8	11	AMP^R, AMX^R, AMC^R, STR^R, SAM^R, CIP^R, OXY^R, TET^R, OFX^R, ERY^R, PEN^R	4(28.6)	0.46
Salmonella Typhimurium ($n = 11$)	3	4	AMP^R, AMX^R, ERY^R, PEN^R	9(81.8)	0.17
	5	7	AMP^R, AMX^R, ERY^R, PEN^R, STR^R, AMC^R, DOX^R	5(45.5)	0.29
	7	11	AMP^R, AMX^R, ERY^R, PEN^R, STR^R, AMC^R, DOX^R, SAM^R, TET^R, CIP^R, OFX^R	3(27.3)	0.46
Other *Salmonella* spp. ($n = 20$)	4	6	STR^R, AMP^R, AMX^R, ERY^R, PEN^R, AMC^R	9(45)	0.25
	6	9	STR^R, AMP^R, AMX^R, ERY^R, PEN^R, AMC^R, SAM^R, DOX^R, TET^R	8(40)	0.38
	7	11	STR^R, AMP^R, AMX^R, ERY^R, PEN^R, AMC^R, SAM^R, DOX^R, TET^R, OXY^R, OFX^R	5(25)	0.46
	7	12	STR^R, AMP^R, AMX^R, ERY^R, PEN^R, AMC^R, SAM^R, DOX^R, TET^R, OXY^R, OFX^R, CIP^R	3(15)	0.50

GEN, Gentamycin (10 μg); KAN, Kanamycin (30 μg); STR, Streptomycin (25 μg); TOB, Tobramycin (10 μg); AMP, Ampicillin (10 μg); AMX, Amoxicillin (25 μg); SAM, Ampicillin/Sulbactam (30 μg); IPM, Imipenem (10 μg); MEM, Meropenem (10 μg); CTX, Cefotaxime (30 μg); CEF, Cephalothin (30 μg); SUL, Sulfamethoxazole (30 μg); TMP, Trimethoprim (25 μg); ERY, Erythromycin (15 μg); PEN, Penicillin G (10 μunits); AMC, Amoxicillin/clavulanate (30 μg); CHL, Chloramphenicol (30 μg); CST, Colistin (20 μg); PMB, Polymyxin B (300 units); DOX, Doxycycline (30 μg); OXY, Oxytetracycline (30 μg); TET, Tetracycline (30 μg); CIP, Ciprofloxacin (10 μg); OFX, Ofloxacin (5 μg); R, Resistant; I, Intermediate; S, Sensitive; Values in parenthesis represent percentage; MAR, Multiple antibiotic resistance.

TABLE 3 | Distribution of virulence genes in the *Salmonella* species.

Salmonella species	Virulence determinants					
	spiA	***sipB***	***invA***	***sifA***	***fljB***	***sefA***
Salmonella Enteritidis ($n = 14$)	10(71.4)	11(78.6)	14(100)	12(85.7)	12(85.7)	13(92.9)
Salmonella Typhimurium ($n = 11$)	9(81.8)	10(90.9)	11(100)	10(90.9)	10(90.9)	10(90.9)
Other *Salmonella* spp. ($n = 20$)	16(80)	15(75)	20(100)	18(90)	19(95)	18(90)
Total ($n = 45$)	35(77.8)	36(80)	45(100)	40(88.9)	41(91.1)	41(91.1)

trimethoprim resistance), and *tet*C (tetracycline resistance protein) (**Table 4**).

DISCUSSION

Gastrointestinal illnesses continue to be a global and public health menace. Exposure to food borne *Salmonella* directly or indirectly via consumption of RTE seafood can be an important route of infection to humans. Findings from this study provide an estimation of the prevalence of *Salmonella* from RTE shrimps in open markets from south-south region in Nigeria. The prevalence of *Salmonella* positive samples was higher than a previous study from Turkey (Ikiz et al., 2016) (2%), Iran (Rahimi et al., 2013) (1.8%) and China (Yang et al., 2015) (13%). The prevalence of *Salmonella* spp. from the RTE shrimp samples assessed in this study was also lower compared to those detected from India (Kumar et al., 2008) (29.0%), Saudi Arabia (Elhadi, 2014) (39.9%), Vietnam (Nguyen et al., 2016) (49.1%), Thailand (Woodring et al., 2012) (21%), Brazil (Carvalho et al., 2013) (16.12%), China (Zhang et al., 2015) (29.7%) and India (Kumar et al., 2009) (26.7%); but higher than, findings by Koonse et al. (2005) from six different countries with participating countries not mentioned at their request (two countries are located in southeast Asia, one is in central Asia, one is in Central America, one is in North America, and one is an island in the Pacific Ocean) re-counted a prevalence rate of 1.6% in shrimp samples. It was also reported in Nigeria (Raufu et al., 2014) that a total of 23/200 (11.5%) samples were positive for *Salmonella*, with three serovars comprising *Salmonella* serovars Eko, 47: mt:-, and Hadar, recovered. In Brazil (Carvalho et al., 2013) reported that from a total of 186 confirmed *Salmonella* spp., five serovars were identified and they include: *Salmonella* Saintpaul, *Salmonella* Infantis, *Salmonella* Panama, *Salmonella* Madelia, and *Salmonella* Braenderup. Five different *Salmonella* serotypes including *Salmonella* Typhi, *Salmonella* Newport, *Salmonella* Paratyphi B, *Salmonella* Enteritidis, and *Salmonella* Typhimurium were recovered from seafood samples in Iran (Rahimi et al., 2013). The most prevailing *Salmonella*

TABLE 4 | Distribution of antibiotic-resistant genes in the *Salmonella* species.

***Salmonella* species**	**Antibiotic-resistant genes**									
	Class 1 integrase	**Class 2 integrase**	***sul*2**	***flor***	***Tmp***	***str*B**	***dfr*1**	***bla*$_{TEM}$**	***catB*3**	***tet*C**
Salmonella Enteritidis ($n = 14$)	9(64.3)	6(42.9)	8(57.1)	10(71.4)	10(71.4)	11(78.6)	12(85.7)	10(71.4)	8(57.1)	12(85.7)
Salmonella Typhimurium ($n = 11$)	7(63.6)	6(54.5)	7(63.6)	9(81.8)	10(90.9)	8(72.7)	10(90.9)	6(54.5)	4(36.4)	9(81.8)
Other *Salmonella* spp. ($n = 20$)	11(55)	5(25)	17(85)	16(80)	17(85)	16(80)	18(90)	12(60)	9(45)	17(85)
Total ($n = 45$)	27(60)	17(37.8)	32(71.1)	35(77.8)	37(82.2)	35(77.8)	40(88.9)	28(62.2)	21(46.7)	38(84.4)

serovars from China (Zhang et al., 2015) among the 730 seafood samples examined were *Salmonella* Typhimurium (4.1%), *Salmonella* Hvittingfoss (4.1%), *Salmonella* Schwarzengrund (4.6%), *Salmonella* Stanley (4.6%), *Salmonella* Singapore (5.5%), *Salmonella* Thompson (9.2%), *Salmonella* Wandsworth (12.0%), and *Salmonella* Aberdeen (18.4%).

The findings from Yang et al. (2015) reported a most probable number (MPN)/g of 0.3–10, with one sample exceeding 110 MPN/g which was somewhat similar to the *Salmonella* density in this study. The mean *Salmonella* density in this study varied across the sampling months as higher densities were observed in the wet season (March to October) compared to dry season (November to February) and from one open market to another particularly from open markets in Delta State. Siala et al. (2017) reported that the presence of *Salmonella* spp. in shrimps is an indicator of contamination in the shrimp industry which happens to be one of the most significant seafood commodities worldwide. The high rate of positive *Salmonella* species in RTE shrimps in Southern Nigeria is worrisome and a substantial risk to public health. Thus, it is imperative to manage *Salmonella* infection in the food production process by further strengthening the surveillance of aquatic food products to circumvent the contamination of RTE seafood products. The high prevalence of *Salmonella* in open markets in the present study indicates poor sanitary condition during processing as well as the environment and poor hygiene of the RTE shrimp handlers during preparation of the products. The difference in the densities and prevalence of *Salmonella* from RTE seafood could also be ascribed to geographical variation, contaminated raw materials and poor /inadequate detection methods.

Determination of *Salmonella* resistance to antibiotics is crucial for therapeutic regimen during outbreaks. *Salmonella* resistance to erythromycin, amoxicillin and penicillin in this study are of public health threat and thus be as a consequence of extensive usage of these antibiotics in the study area. Interestingly, no *Salmonella* serovars was resistant to cefotaxime, cephalothin, colistin, and polymyxin B. This is very important to public health as these antibiotics could be crucial in threating drug resistant *Salmonella* pathogens. Public education to enlighten individual not to misuse these antibiotics is essential to circumvent the occurrence and development of resistance to these antibiotics.

Akiyama et al. (2011) reported from the United States that none of the *Salmonella* isolates showed resistance to ampicillin, gentamicin, chloramphenicol, kanamycin, sulfisoxazole, tetracycline, and streptomycin. The highest antibiotic resistance *Salmonella* species form seafood observed by Elhadi (2014) from Saudi Arabia were amoxicillin-clavulanic acid (45%), ampicillin (70%) and tetracycline (90.71%). Percentage resistance to nalidixic acid (47.4%) was the predominant report from Iran by Rahimi et al. (2013), prior to others such as ciprofloxacin (5.3%), trimethoprim (15.8%), streptomycin (15.8%), and tetracycline (36.8%). From China, Yang et al. (2015) reported resistance for ampicillin (28.2%), tetracycline (35.9%), trimethoprim-sulfamethoxazole (25.2%), streptomycin (18.4%) and chloramphenicol (20.4%), with 34.0% being resistant to more than three antibiotics. These were somewhat in accordance to the findings in this study. Zhang et al. (2015) also reported resistance of *Salmonella* from China from retail aquaculture products to tetracycline (34.1%), sulfonamides (56.5%), streptomycin (28.6%) and ampicillin (23.5%) with lower levels of resistance for ciprofloxacin (2.3%), gentamicin (3.2%), ceftazidime (0.5%) cefepime (0.5%), and cefotaxime (0.9%) which was rather similar to the findings in this study. In addition, 43.3% of the *Salmonella* serovars from a finding of Zhang et al. (2015) were multidrug resistant which is reduced when compared to the results in this study. *Salmonella* serotypes such as Typhimurium and Enteritidis have historically been reported as the significant causes of non-typhoidal salmonellosis. Though, other serotypes have been revealed to be included to be prevalent with respect to difference in geographical regions (Brands et al., 2005).

The occurrence of resistance to ciprofloxacin in *Salmonella* serovars is of public health importance as it translates possible misuse in animals and over-prescription in humans. *Salmonella* isolates in this study that were resistant to ciprofloxacin were also observed to be multidrug resistant strains to other antibiotics which were in accordance to the finding of Vo et al. (2006) from the Netherlands. MDR *Salmonella* isolates in this study are prevalent in open markets, which necessitates that more attention be ascribed toward the control and supervision of antibiotic usage, particularly in human health care and agriculture divisions in Nigeria. Bacterial virulence is predisposed by both the occurrence of antibiotic resistance and virulence determinants. The advancement of *Salmonella* strains that are is based particularly on elements of biochemical and genetic mechanisms so as to heighten their survival via preservation of their antibiotic resistance genes. As regards the virulence determinants that were analyzed, *Salmonella* Enteritidis, *Salmonella* Typhimurium, and other *Salmonella* isolates represent a broader range of pathogenicity.

High MAR index was observed in this study which indicates high use/misuse of antibiotics in the study areas. MAR index of *Salmonella* isolates ranged from 0.14 to 0.45 for different

seafood in a study by Budiati et al. (2013) in Malaysia. From Brazil, Carvalho et al. (2013) reported that 23% of *Salmonella* serovars were resistant to ≤ 1 antibiotic, 20% were resistant to ≤ 2 antibiotics while 3 strains showed multi-resistance characteristics. These were lower compared to the findings of this study. The rapid development of bacterial resistance is ascribed to the selective pressure of antibiotics via evolutionary responses as a consequence of natural selection.

The dissemination of resistant elements in natural ecosystem can alter as well as change the physiology and population dynamics of resident microbial populaces (Igbinosa and Odjadjare, 2015). The emergence of antibiotic resistant determinants in pathogenic *Salmonella* species has made it more problematic due to the pervasiveness of horizontal gene transfer which is the procedure where bacteria obtain elements/determinants from the environment (Thomas and Nielsen, 2005). Most antibiotic resistance genes are found on intregons, plasmids or transposons, which can be transferred and mobilized to other bacteria of different or the same species. Integrons have been reported to be involved in the acquisition of antibiotic resistance elements. Class 1 integrons which contains numerous resistance elements could play vital roles in the maintenance and spread of antibiotic resistance in *Salmonella* species both in the absence and presence of selective pressure as reported in India (Deekshit et al., 2012). Meng et al. (2011) from China documented that class 1 integron showed empty regions from strains in serotypes Choleraesuis isolated from seafood. Findings by Meng et al. (2011) also suggest the possible dissemination of class 1 integrons from foodborne pathogens to human inhabited bacteria through horizontal gene transfer.

The occurrence and dissemination of resistant elements to pathogenic and commensal bacteria of human origin as well as gene transfer in human intestinal microbiome have been reported (Slayers et al., 2004). Antibiotic resistance genes such as *tet*A and *cat*A1 were present in 60 and 57.52%, of *Salmonella* isolates, respectively in a study by Deekshit et al. (2012) from India. Adesiji et al. (2014) reported that of the 20 tetracycline resistant isolates from India, 20(100%) *tet*A, 6(30%) *tet*B, 7(35%) *tet*C, and 10(50%) *tet*G encoded resistant elements, respectively. Of 18 cotrimoxazole-resistant strains, 4(22.2%), 14(77.7%), and 18(100%) had *sul*3, *sul*2, and *sul*1genes, respectively (Adesiji et al., 2014). Deekshit et al. (2013) reported the occurrence of three antibiotic resistance determinants *sul*1, *tet*G, and *flo*R from seafood some of which were also detected in this study.

Virulence determinants are involved in bacterial pathogenicity, and their occurrence in *Salmonella* can result in salmonellosis (). Findings from this study revealed that isolates of *Salmonella* Enteritidis and *Salmonella* Typhimurium demonstrated a diverse range of pathogenicity elements, which makes these serovars more virulent toward consumers of the RTE shrimp products especially immunocompromised individuals. Antibiotic resistance phenotypes and determinants have also been reported to be positively correlated with *Salmonella* virulence (Turki et al., 2014). Infections as a consequence of antibiotic-resistant *Salmonella* with virulence potential have been reported to take longer to recover from by been frequently fatal, when compared with ailments caused by antibiotic-susceptible strains of *Salmonella* with virulent capabilities.

The *spi*A gene of *Salmonella* is essential for its virulence and biofilm formation in host cells (Romling et al., 2003; Socher et al., 2005; Dong et al., 2011; Col et al., 2013; Beshiru et al., 2018). The *sip*B gene is required by *Salmonella* to form functional pores during *Salmonella* infection of erythrocytes for entry into the host cell through the host cell plasma membrane (Miki et al., 2004). The *sip*B gene is referred to as trans-locators as they translocate *Salmonella* effector proteins into host cells (consumers of RTE shrimps) which can cause typhoid fever and gastroenteritis (Galan and Wolf-Watz, 2006). The *sip*B gene in *Salmonella* serovars induces apoptotic macrophage either by activating or inducing autophagy and disruption of mitochondria, or by binding the proapoptotic enzyme caspase-1 which results in the discharge of interleukin-1 beta active form (Myeni et al., 2013).

A significant step in the cycle of facultative pathogenic intracellular *Salmonella* serovars on RTE shrimps and by extension the consumers is the incursion of the cells via the intestinal mucosa. Amplification of nucleotide sequences within the *inv*A gene of *Salmonella* has been evaluated as a means of detecting invasive *Salmonella* serovars (). The *inv*A gene of the *Salmonella* species allows the bacteria to invade the host and initiate infection, thereby increasing the degree of pathogenicity of the isolates. PCR analysis of 15 virulence genes by Yang et al. (2015) from retail seafood in China showed that all 103 *Salmonella* isolates had at least 4 virulence genes (*mgt*C, *ssa*Q, *sii*D, *bcf* C, and *sop*B), where the loci that remains were unevenly distributed. In addition, isolates of *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Salmonella* Weltevreden displayed a broader range of pathogenicity elements when compared with other *Salmonella* serovars by Yang et al. (2015) which was evident in this study.

A significant number of *Salmonella* serovars from RTE shrimps in this study harbored the *sif* A gene. The *sif* A gene plays a crucial role in *Salmonella* virulence. The degree of pathogenicity by *Salmonella* lies predominantly on the phenotypic manifestation of effector proteins released into the bacterial cell. *Salmonella* gains enterance into eukaryotic cells and exist in a vascular section with which some effector proteins (e.g., *sif* A) are located (Zhao et al., 2015).

Flagellin occurrence on RTE shrimps is a significant external antigen for numerous species which aids *Salmonella* virulence. Considerable heterogeneity of sequence exist within alleles which codes for different flagellar antigen from a previous study by McQuiston et al. (2004) while alleles which encodes similar antigenic flagella were homologous, signifying that flagellin determinants may be beneficial to targets for the genotypic resolve of flagellar antigenic type. Fimbriae are an important factor in *Salmonella* survival and persistence in the host (Kaur and Jain, 2012). The *sef* A gene encodes the *Salmonella* Enteritidis fimbrial protein (Mirmomeni et al., 2008). Studies have also revealed that the *sef* A gene plays a significant part in the adhesion of *Salmonella* Enteritidis to biotic surfaces (Lopes et al., 2006). Akiyama et al. (2011) reported that all *Salmonella* strains were positive for 14 virulence genes (*sif* A, *spi*A, *inv*A, *sop*E, *spa*N, *sip*B,

*msg*A, *iro*N, *pag*C, *prg*H, *org*A, *lpf*C, *tol*C, and *sit*C) and negative for three genes (*cdt*B, *spv*B, and *pef* A). Some of these genes detected by Akiyama et al. (2011) were also detected in this study. Antibiotic resistance is a major public health menace globally, and particularly persistent in developing countries, including Nigeria, where the problem of infectious disease is on the increase with decreased healthcare budget. Though the emergence and dissemination of antibiotic-resistant *Salmonella* is a significant concern to food processors, cinnamaldehyde and carvacrol which are effective plant-derived antimicrobials have been reported to inactivate antibiotic-resistant *Salmonella enterica* in oysters, buffer and celery (Ravishankar et al., 2010). Bacteriophages propose effective and highly specific bio-control of antibiotic-resistant *Salmonella* pathogens from RTE foods (Guenther et al., 2012). Although phage particles keep their infectious capabilities, they are immobilized freely by the RTE food, which result in loss of their capacity to infect and diffuse target cells. Short-chain fatty acids have found application in animal diets to manage pathogens with *Salmonella* serovars inclusive (Van-Immerseel et al., 2002). Another alternative to eliminating pathogens is the precise suppression of functions vital to cause infection in the host (Clatworthy et al., 2007). Gene regulation mechanism via quorum sensing, where bacteria regulate the manifestation of numerous genes in reaction to the occurrence of small signal molecules is also very crucial (Defoirdt et al., 2011).

Other management strategies for antibiotic resistance includes the following: limiting the non-therapeutic usage of antibiotics for agriculture; improved information to strengthen resolutions on standard therapeutic regimen, education, other actions, coupled with continuous monitoring and validating effectiveness of management strategies; strengthening infection control boards in hospitals; nutrient management and runoff control; and improved diagnostic procedures, which requires developmental variations and infrastructure upgrades, enhancements in microbiological laboratory equipment and personnel (Global Antibiotic Resistance Partnership - India Working Group, 2011; Pruden et al., 2013). These recommendations could assist in the reduced of antibiotic resistance, directly advance public health, advantageous to the populace and decrease pressure on healthcare system. Finally, enhancing the coverage and types of juvenile vaccines administered by government agencies would enormously decrease the disease burden and circumvent the misuse of antibiotics (Global Antibiotic Resistance Partnership - India Working Group, 2011).

CONCLUSION

Findings indicate that RTE shrimps act as reservoirs in harboring multiple *Salmonella* strains. The recovered *Salmonella* serovars which exhibits multiple virulence and antibiotic resistance genes coupled with high MAR index constitute a risk to consumers. Hence, it is crucial to monitor the usage of antibiotics and hygiene status in processing and post-processing handling to circumvent the acquisition and dissemination of virulent *Salmonella* serovars. Furthermore, maintenance, and implementation of control measures such as good manufacturing practices (GMP), and hazard analysis and critical control point (HACCP) coupled with education of the RTE shrimp processors is necessary, for reducing and/or spreading *Salmonella* contamination.

AUTHOR CONTRIBUTIONS

AB and II carried out the sampling, laboratory procedures, data interpretation, and writing of the manuscript. EI conceptualized, designed, and supervised the research, contributed in the laboratory methodologies and data interpretation, as well as the writing of the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Molecular Epidemiology of Multidrug-Resistant *Klebsiella pneumoniae* Isolates in a Brazilian Tertiary Hospital

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Multidrug-resistant (MDR) *Klebsiella pneumoniae* (Kp) is a major bacterial pathogen responsible for hospital outbreaks worldwide, mainly via the spread of high-risk clones and epidemic resistance plasmids. In this study, we evaluated the molecular epidemiology and β-lactam resistance mechanisms of MDR-Kp strains isolated in a Brazilian academic care hospital. We used whole-genome sequencing to study drug resistance mechanisms and their relationships with a *K. pneumoniae* carbapenemase-producing (KPC) Kp outbreak. Forty-three Kp strains were collected between 2003 and 2012. Antimicrobial susceptibility testing was performed for 15 antimicrobial agents, and polymerase chain reaction (PCR) was used to detect 32 resistance genes. Mutations in *ompk35*, *ompk36*, and *ompk37* were evaluated by PCR and DNA sequencing. Pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were carried out to differentiate the strains. Based on distinct epidemiological periods, six Kp strains were subjected to whole-genome sequencing. β-lactamase coding genes were widely distributed among isolates. Almost all isolates had mutations in porin genes, particularly *ompk35*. The presence of bla_{KPC} promoted a very high increase in carbapenem minimum inhibitory concentration only when *ompk35* and *ompk36* were interrupted by insertion sequences. A major cluster was identified by PFGE analysis and all isolates from this cluster belonged to clonal group (CG) 258. We have also identified a large repertoire of resistance genes in the sequenced isolates. A bla_{KPC-2}-bearing plasmid (pUFPRA2) was also identified, which was very similar to a plasmid previously described in the first Brazilian KPC-Kp (2005). We found high-risk clones (CG258) and an epidemic resistance plasmid throughout the duration of the study (2003 to 2012), emphasizing a persistent presence of MDR-Kp strains in the hospital setting. Finally, we found that horizontal transfer of resistance genes between clones may have played a key role in the evolution of the outbreak.

Keywords: Brazil, hospital outbreak, MLST, antimicrobial resistance, clonal group 258, whole-genome sequencing

INTRODUCTION

Multidrug-resistant *Klebsiella pneumoniae* (MDR-Kp) is recognized in healthcare settings as a cause of high morbidity and mortality among patients with severe infections. Some MDR-Kp isolates have evolved to become extensively drug-resistant (XDR) isolates that have few therapeutic options (Lee et al., 2016). In Brazil, the National Program for Monitoring Bacterial Resistance has reported increasing annual rates of carbapenem-resistant Kp isolated from bloodstream infections (Anvisa, 2017). Carbapenem resistance is attributed to a high expression of carbapenemases and extended spectrum β-lactamases (ESBLs) or AmpC β-lactamases coupled with modification of outer membrane permeability (Fernandez and Hancock, 2012). Kp produces an intrinsic β-lactamase, bla_{SHV}, and two major porins, OmpK35 and OmpK36, in addition to the major multidrug efflux pump AcrAB-TolC, which may also be related to this phenotype (Fernandez and Hancock, 2012; Lee et al., 2016).

Klebsiella pneumoniae carbapenemase-producing Kp (KPC-Kp) is a major bacterial pathogen responsible for hospital outbreaks worldwide (Lee et al., 2016), mainly via the spread of high-risk clones and epidemic resistance plasmids (Mathers et al., 2015). In general, these clones belong to clonal group 258 (CG258), which comprises 43 different sequence types (STs) (Chen et al., 2014) between single- and double-locus variants, based on multilocus sequence typing (MLST) (Chen et al., 2014; Bowers et al., 2015; Gaiarsa et al., 2015). Epidemiological data have reported that STs 11, 258, 340, 437, and 512 comprise most of the bla_{KPC} CG258 isolates (Chen et al., 2014; Bowers et al., 2015; Gaiarsa et al., 2015). Furthermore, epidemic resistance plasmids harboring bla_{CTX-M} and bla_{KPC}, often belong to incompatibility groups F and N and are common among members of the STs from CG258 (Mathers et al., 2015; Lee et al., 2016).

Here, we evaluated the molecular epidemiology and β-lactam resistance mechanisms of MDR-Kp strains isolated between 2003 and 2012 in a Brazilian academic care hospital. We also selected six MDR-Kp strains for whole-genome sequencing (WGS) to gather insights on their drug resistance mechanisms and association with a KPC-Kp outbreak.

MATERIALS AND METHODS

Study Setting

This study was performed at Complexo Hospital de Clínicas of the Universidade Federal do Paraná (CHC/UFPR), a 655-bed tertiary hospital located in Curitiba, Paraná, Southern Brazil. CHC/UFPR is a referral center which also supports other hospitals. The Institutional Ethics Review Board of the CHC/UFPR approved this study under reference number IRB#: 2656.263/2011-11.

Bacterial Strains and Phenotypic Tests

A total of 43 clinical isolates of Kp from different body sites of 32 patients were studied. These isolates were selected from a CHC-UFPR bacterial collection. Only isolates resistant to at least one carbapenem (ertapenem) by disk diffusion testing were included. These isolates were collected between August 2003 and February 2012, a time interval that we divided into three well-defined epidemiological periods, according to the prevalence of MDR-*Enterobacteriaceae*. The first period (2000–2009) was characterized by ESBL prevalence, resistance to fluoroquinolones and aminoglycosides, and low resistance to imipenem and meropenem (Nogueira Kda et al., 2014, Nogueira et al., 2015). The second period was defined by a KPC-Kp outbreak that occurred in June 2010 (Almeida et al., 2014), and the third period was characterized by a gradual increase in the prevalence of KPC-Kp and other *Enterobacteriaceae*.

Five isolates recovered from patients treated in four other hospitals were also included (C4, C5, C7, D1, and D5; **Figure 1** and **Table 1**). In all but six cases, a single bacterial specimen was isolated. However, from each of those six patients, between two and four bacterial samples were isolated (**Table 1**). Kp isolates recovered from clinical specimens were stored at −80°C in trypticase soy broth (TSB; HiMedia, Mumbai, India) containing 15% glycerol. Bacterial isolates were identified using a Vitek2 Compact instrument (BioMérieux S.A., Marcy l'Etoile, France) and mass spectrometer (Microflex LT; Bruker Daltonics, Bremen, Germany).

Antimicrobial susceptibility testing (AST) was performed for 15 antimicrobial agents (**Table 1**) by agar dilution, except for polymyxin which was tested by broth dilution, as recommended by the Clinical and Laboratory Standards Institute (CLSI). Minimal inhibitory concentrations (MICs) were interpreted according to CLSI standards (CLSI M100-S27 document, 2017[1]). Polymyxin, tigecycline, and fosfomycin breakpoints were interpreted using Brazilian Committee on AST and European Committee on AST standards (BrCAST-EUCAST[2]). Double-disk synergy (EUCAST, 2013[3]) and imipenem hydrolysis assay by spectrophotometry (Nicoletti et al., 2015) were performed to determine the carbapenem resistance phenotypes.

Antibiotic Resistance Characterization and Molecular Typing

The presence of bla_{MOX}, bla_{CMY}, bla_{LAT}, bla_{BIL}, bla_{DHA}, bla_{ACC}, bla_{MIR}, bla_{ACT}, bla_{FOX}, bla_{TEM}, $bla_{CTX-M-1}$, $-M-2$, $-M-8$, $-M-9$, $-M-25$, bla_{PER}, bla_{BES}, bla_{VEB}, bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{GIM}, bla_{SIM}, bla_{OXA-23}, bla_{OXA-48}, bla_{OXA-51}, bla_{OXA-58}, $bla_{OXA-143}$, and bla_{BKC} was investigated by polymerase chain reaction (PCR) using primers and amplification conditions indicated in **Supplementary Table 1**.

Mutations in *ompk35*, *ompk36*, and *ompk37* were evaluated by PCR and DNA sequencing (Kaczmarek et al., 2006; Nicoletti et al., 2015). PCR products were sequenced using a 3730XL DNA Analyzer (Applied Biosystems, Carlsbad, CA, United States). Nucleotide and protein sequences were compared to the reference proteins OmpK35 (GenBank accession no. AJ011501), OmpK36 (accession no. Z33506), and OmpK37 (accession no. AJ011502). Genes or promoter regions of porins truncated

[1]http://em100.edaptivedocs.info

[2]http://brcast.org.br/, accessed in January 2018.

[3]http://www.eucast.org/resistance_mechanisms

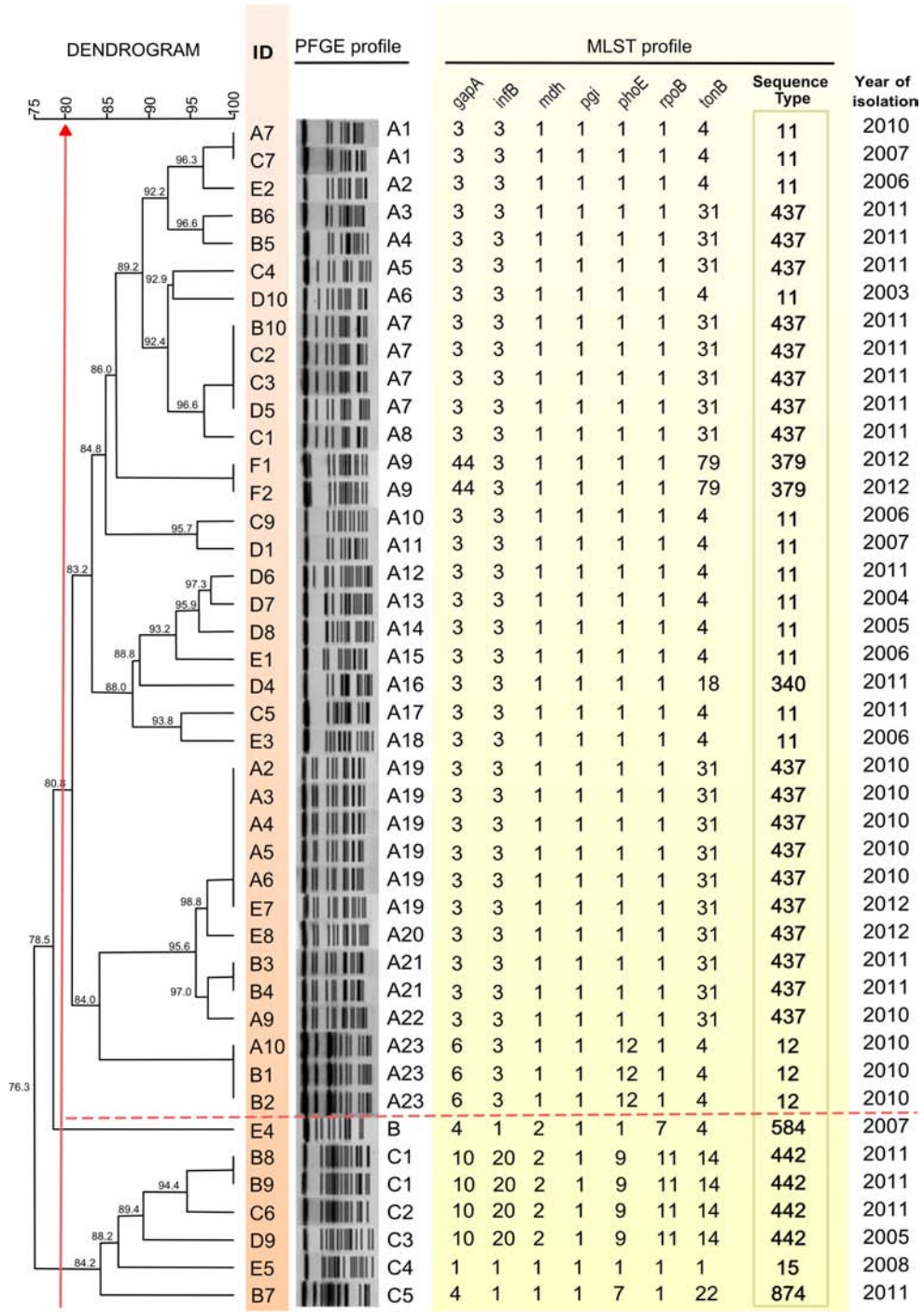

FIGURE 1 | Dendrogram constructed on the basis of PFGE patterns and MLST profile of 43 *K. pneumoniae* isolates. A dice coefficient similarity of at least 80% included two PFGE clusters designated as A and C, as indicated by the vertical red arrow crossing the dendrogram on the left. Isolate identifiers are shown aligned to the dendrogram tips in the column ID. A dashed line delimits the cluster A, which contains the largest numbers of PFGE profiles. Isolates with the same pulsotype designation (column PFGE profile) are genetically indistinguishable under this procedure. KpA2, KpA3, KpA4, KpA5, KpA6, and KpA9 are isolates of the Kp outbreak.

by insertion sequences (IS) were evaluated using ISFinder (Siguier et al., 2006).

Pulsed-field gel electrophoresis (PFGE) was performed according to Nogueira et al. (Nogueira Kda et al., 2014; Nogueira et al., 2015) to differentiate between isolates. Gels were analyzed with BioNumerics program version 6.6 (Applied Maths, Kortrijk, Belgium). The dice similarity coefficient was used to determine the similarity between each banding pattern.

TABLE 1 | Clinical data, antibiotic susceptibilities and molecular features of 43 *K. pneumoniae* isolates[a–d].

Patient	Isolate ID	Date of isolation	Clinical data			Minimal inhibitory concentration (mg/L)[g]														*bla* genes	Porin mutations[h]		
			Source[e]	Outcome	Clinic[f]	AMI	CAZ	CIP	CPM	CTX	DOX	ERT	FOS	GEN	IMI	LEV	MER	MIN	POL	TIG		OmpK35	OmpK36	OmpK37
P1	D10	2003.08.28	BAL	Death	ICU	16	64	>16	128	128	8	0.5	32	>64	0.25	2	0.06	4	>16	1	$bla_{CTX-M-2}$			
P2	D7	2004.12.04	TA	Death	ICU	64	32	>16	>128	>128	8	32	256	64	1	2	8	4	0.5	0.5	bla_{TEM}, $bla_{CTX-M-2}$	+(ii)		
P3	D8	2005.01.14	Blood	Recovery	ID	32	32	>16	64	128	8	0.25	32	16	0.25	8	0.06	4	0.5	0.5	bla_{TEM}, $bla_{CTX-M-2}$			
P4	D9	2005.01.27	Blood	Death	CT	32	32	>16	>128	>128	64	32	>512	4	1	8	4	>64	0.5	8	bla_{TEM}, $bla_{CTX-M-2}$	+(ii)	+(ii)	
P5	E1	2006.01.02	Blood	Death	ICU	2	32	>16	128	128	8	0.25	32	>64	0.12	2	0.12	2	0.5	0.5	$bla_{CTX-M-2}$			
P6	E2	2006.01.22	IC	Death	ICU	32	16	>16	16	64	8	0.12	16	16	0.25	2	0.06	4	0.5	1	$bla_{CTX-M-2}$	+(i)		
P7	E3	2006.02.24	Blood	Death	ICU	1	32	>16	64	128	32	0.25	128	0.25	0.25	1	0.06	32	0.5	2	$bla_{CTX-M-2}$	+(iii)	+(v)	
P8	C9	2006.11.18	Urine	Recovery	FMC	32	32	>16	>128	>128	64	32	>512	>64	4	4	8	16	>16	1	bla_{TEM}, $bla_{CTX-M-2}$	+(i)	+(ii)	
P9	C7	2007.01.31	PF	Death	ICU	128	32	>16	>128	>128	8	16	128	32	1	8	4	4	0.5	0.5	$bla_{CTX-M-2}$	+(i)	+(ii)	
P10	D1	2007.02.11	Blood	Death	ICU	128	32	>16	>128	>128	8	32	512	64	1	8	4	4	0.5	1	bla_{TEM}, $bla_{CTX-M-2}$	+(i)	+(ii)	
P11	E4	2007.10.22	Urine	Recovery	P-ICU	16	8	0.03	16	128	8	0.03	32	>64	0.5	0.004	0.06	2	0.5	0.5	bla_{TEM}, $bla_{CTX-M-2}$		+(i)	
P12	E5	2008.01.17	Blood	Death	ICU	>256	16	>16	64	128	32	1	64	>64	0.25	8	0.12	32	0.5	8	bla_{TEM}, $bla_{CTX-M-2}$			
P13	A2	2010.06.24	CSF	Death	ID	4	64	>16	32	128	>64	4	16	64	0.5	8	1	>64	0.5	2	bla_{TEM}, $bla_{CTX-M-1}$, bla_{KPC}	+(ii)		
P13	A3	2010.06.29	Rectal	Death	ID	4	>64	>16	>128	>128	>64	256	>512	>64	64	2	64	>64	0.5	2	bla_{TEM}, $bla_{CTX-M-1}$, bla_{KPC}	+(ii)	+(ii)	
P13	A4	2010.06.30	CSF	Death	ID	4	>64	>16	16	128	>64	4	16	64	2	8	2	>64	0.5	4	bla_{TEM}, $bla_{CTX-M-1}$, bla_{KPC}	+(ii)		
P13	A5	2010.07.01	CSF	Death	ID	4	>64	>16	32	64	>64	4	16	64	2	8	2	>64	0.5	4	bla_{TEM}, $bla_{CTX-M-1}$, bla_{KPC}	+(ii)		
P14	A6	2010.07.01	Rectal	Recovery	NS	4	>64	>16	32	128	64	4	16	>64	2	8	2	>64	0.5	4	bla_{TEM}, $bla_{CTX-M-1}$, bla_{KPC}	+(ii)		
P15	A7	2010.07.01	TA	Death	ICU	2	64	>16	>128	>128	64	64	512	64	4	2	8	4	0.5	1	$bla_{CTX-M-2}$		+(ii)	
P16	A9	2010.08.02	SW	Death	OT	4	>64	>16	32	64	>64	4	16	>64	1	8	2	>64	0.5	4	bla_{TEM}, $bla_{CTX-M-1}$, bla_{KPC}	+(ii)		
P17	A10	2010.09.01	Rectal	Death	N-ICU	8	1	0.06	1	0.5	8	0.25	>512	2	1	0.008	0.25	1	0.5	0.25	$bla_{CTX-M-25}$		+(ii)	
P17	B1	2010.09.09	Rectal	Death	N-ICU	8	4	0.25	8	4	32	4	>512	2	1	0.12	1	16	0.5	4	$bla_{CTX-M-25}$		+(ii)	
P17	B2	2010.09.11	Rectal	Death	N-ICU	8	32	0.5	32	4	32	16	>512	2	2	0.12	2	16	0.5	4	$bla_{CTX-M-25}$		+(ii)	
P18	B3	2011.03.11	Urine	Death	ICU	1	16	>16	4	2	>64	4	16	>64	2	8	2	>64	0.5	2	bla_{TEM}, $bla_{CTX-M-25}$, bla_{KPC}	+(ii)		
P19	B4	2011.03.16	Rectal	Death	ICU	4	64	>16	32	128	64	4	16	64	2	8	1	>64	0.5	4	bla_{TEM}, $bla_{CTX-M-1}$, bla_{KPC}	+(ii)		
P20	D6	2011.05.16	TA	Death	ICU	16	64	>16	>128	>128	8	64	>512	64	8	2	16	2	0.25	1	bla_{TEM}, $bla_{CTX-M-2}$			
P21	B5	2011.06.19	Blood	Death	FMC	1	16	>16	>128	>128	8	16	128	32	1	2	4	4	≤0.25	1	$bla_{CTX-M-2}$	+(i)		
P21	B6	2011.06.20	Urine	Death	FMC	1	32	>16	>128	>128	32	32	128	64	4	8	8	4	≤0.25	1	$bla_{CTX-M-2}$	+(i)	+(ii)	
P22	C5	2011.06.28	TA	Death	ICU	32	>64	>16	>128	>128	32	64	>512	32	2	>8	8	16	0.5	1	$bla_{CTX-M-2}$		+(ii)	
P23	D5	2011.07.10	Urine	Recovery	MMC	2	>64	>16	32	128	32	2	8	0.25	1	4	1	8	0.5	1	$bla_{CTX-M-1}$, bla_{KPC}	+(ii)		
P24	B7	2011.07.12	Urine	Recovery	LT	16	>64	>16	>128	>128	16	32	32	>64	2	2	8	4	>16	1	$bla_{CTX-M-1}$	+(i)	+(ii)	
P25	B8	2011.07.22	Blood	Death	ICU	2	>64	>16	>128	>128	8	8	64	64	0.5	4	2	2	0.5	0.25	bla_{TEM}, $bla_{CTX-M-2}$	+(i)	+(ii)	
P26	B9	2011.07.24	Blood	Death	ICU	4	>64	>16	>128	>128	8	4	64	64	0.5	2	1	2	0.5	0.25	bla_{TEM}, $bla_{CTX-M-2}$	+(ii)	+(iii, iv)	+(ii)
P27	B10	2011.07.27	Rectal	Recovery	P-ICU	2	>64	>16	>128	>128	32	256	256	0.5	256	2	128	16	0.5	2	$bla_{CTX-M-1}$, bla_{KPC}	+(ii)	+(ii)	
P28	D4	2011.07.28	IC	Death	ICU	16	>64	>16	64	>128	64	0.5	32	>64	0.5	8	0.12	>64	0.5	0.5	bla_{TEM}, $bla_{CTX-M-1}$	+(ii)		
P27	C1	2011.08.09	TA	Recovery	P-ICU	1	16	>16	32	16	32	256	256	0.25	256	2	128	4	0.5	1	$bla_{CTX-M-1}$, bla_{KPC}	+(ii)	+(ii)	
P29	C4	2011.08.10	SW	Recovery	OT	2	16	>16	4	4	8	8	32	0.25	1	2	2	4	0.5	1	$bla_{CTX-M-25}$, bla_{KPC}	+(ii)	+(v)	
P27	C2	2011.08.14	Blood	Recovery	P-ICU	2	>64	>16	>128	>128	32	256	64	0.25	256	2	128	16	0.5	2	$bla_{CTX-M-1}$, bla_{KPC}	+(ii)	+(ii)	

(Continued)

TABLE 1 | Continued

Patient	Isolate ID	Date of isolation	Clinical data			Minimal inhibitory concentration (mg/L)[g]													*bla* genes	Porin mutations[h]		
			Source[e]	Outcome	Clinic[f]	AMI	CAZ	CIP	CPM	CTX	DOX	ERT	FOS	GEN	IMI	LEV	MER	MIN	POL	TIG		OmpK35	OmpK36	OmpK37
P27	C3	2011.08.15	CB	Recovery	P-ICU	4	>64	>16	>128	>128	32	256	64	0.25	128	2	64	16	0.5	2	$bla_{CTX-M-1}$, bla_{KPC}	+(ii)	+(ii)	
P30	C6	2011.10.11	LB	Death	ICU	8	>64	>16	>128	>128	8	16	64	8	2	4	4	4	>16	0.5	bla_{TEM}, $bla_{CTX-M-2}$	+(ii)	+(ii)	+(ii)
P31	F1	2012.01.16	Urine	Recovery	ID	>256	16	>16	>128	>128	16	64	64	>64	8	8	16	8	0.5	0.5	$bla_{CTX-M-9}$, bla_{KPC}	*()	+(ii)	
P31	F2	2012.01.20	Urine	Recovery	ID	>256	32	>16	128	128	16	64	32	>64	8	8	16	8	0.5	0.5	$bla_{CTX-M-9}$, bla_{KPC}	+(ii)	+(ii)	
P32	E7	2012.02.15	Urine	Death	FMC	8	>64	>16	32	128	>64	4	32	64	2	4	2	>64	>16	4	bla_{TEM}, $bla_{CTX-M-25}$, bla_{KPC}	+(ii)		
P32	E8	2012.02.23	SW	Death	FMC	4	>64	>16	32	128	>64	4	32	>64	2	>8	1	>64	0.5	4	bla_{TEM}, $bla_{CTX-M-25}$, bla_{KPC}	+(ii)		

[a]Highlights in gray depict representative Kp isolates of the hospital outbreak. [b]Kp samples selected for genome sequencing (D8, C9, A2, A3, B10, and C2). [c]KpC4, KpC5, KpC7, KpD1, and KpD5 were recovered from patients of other four hospitals. [d]Identical designation in the "patient" column indicates that bacterial samples were isolated from the same patient. [e]Clinical specimen abbreviations: BAL, bronchoalveolar lavage; CB, catheter blood; CSF, cerebrospinal fluid; IC, intravenous catheter; LB, lung biopsy; PF, peritoneal fluid; SW, surgical wound; TA, tracheal aspirate. [f]Inpatient unit abbreviations: CT, chemotherapy; FMC, female medical clinic; ICU, intensive care unit; ID, infectious diseases; LT, liver transplantation; MMC, male medical clinic; N-ICU, neonatology intensive care unit; NS, neurosurgery; OT, orthopedics and traumatology; P-ICU, pediatric intensive care unit. [g]Antibiotic abbreviations: AMI, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CPM, cefepime; CTX, cefotaxime; DOX, doxycycline; ERT, ertapenem; FOS, fosfomycin; GEN, gentamicin; IMI, imipenem; LEV, levofloxacin; MER, meropenem; MIN, minocycline; POL, polymyxin B; TIG, tigecycline. [h]"+" indicates that porin loss or inactivation was a result of at least one of the following changes: (i) frameshift mutations caused by indels, (ii) fragmentation of the coding sequence or promoter regions, caused by insertion of transposons of the IS1, IS5, IS6, or IS1380 families, (iii) nonsense mutations resulting in premature stop codons, (iv) insertion of nucleotides in the loop 3 region, or (v) mutations of trinucleotides (not causing frameshifts). '"" indicates no PCR amplification.*

A dendrogram was constructed using the unweighted-pair group method with arithmetic averages. The values used for optimization and tolerance were 1.0 and 2.0%, respectively. Isolates with similarities greater than 80% were considered to belong to the same cluster, following previously proposed criteria (Tenover et al., 1995). Different PFGE profiles within clusters were numbered according to the order in the dendrogram (**Figure 1**). MLST was performed by PCR and sequencing of seven Kp housekeeping genes (i.e., *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) following the protocol available at the Pasteur MLST website (Diancourt et al., 2005).

Genome Sequencing, Assembly, and Annotation

Based on three previously defined epidemiological periods, antimicrobial resistance profiles, and body sites from which strains were isolated, six Kp isolates were selected for genome sequencing, including the index isolates KpA2 and KpA3 of the KPC outbreak. KpD8/KpC9 and KpB10/KpC2 were isolated before and after the outbreak, respectively (**Table 1**). KpA2 and KpA3 were isolated from different body sites of the same patient (P13), as were KpB10 and KpC2 (P27), while C9 and D8 were obtained from distinct patients (P8 and P3, respectively) (**Table 1**). Genomic DNA was extracted using a DNeasy 96 Blood & Tissue Kit (Qiagen, Silicon Valley, CA, United States) and sequenced at the Life Sciences Core Facility of the State University of Campinas (LaCTAD; São Paulo, Brazil).

Paired-end (PE) libraries with an average insert size of 550 bp fragments were generated using an Illumina TruSeq DNA PCR-free LT Kit (Illumina Inc., San Diego, CA, United States) and sequenced (PE, 2 × 150 bp) using a HiSeq 2500 instrument in RAPID run mode (Illumina Inc.).

Quality-based trimming and filtering were performed using Trimmomatic version 0.32 (Bolger et al., 2014). Paired-end reads were assembled using Velvet version 1.2.10 (Zerbino and Birney, 2008). Chromosomal and plasmid contigs were manually inspected and separated based on BLASTn results, considering the best hit for identity and coverage. Chromosomal contigs were scaffolded using SSPACE version 3.0 (Boetzer et al., 2011). To sort the chromosomal sequence, the scaffolds were ordered by synteny against a reference chromosome using Gepard version 5.0 (Krumsiek et al., 2007). For each isolate, the reference genome used for scaffold sorting was the publicly available genome with the most similar *k*-mer spectrum, which was determined by KmerFinder version 2.0[4], which was Kp HS11286 (GenBank accession no. CP003200.1) (Bi et al., 2015) for KpA2, KpA3, and KpD8 and Kp JM45 (accession no. CP006656.1) for KpB10, KpC2, and KpC9. Gaps within scaffolds were filled using GapFiller version 2.1.1 (Nadalin et al., 2012) and inspected by aligning PE reads against the scaffolds using Bowtie2 version 2.1.0 (Langmead and Salzberg, 2012). Draft chromosomes and plasmid contigs had their genes predicted with Prokka version 1.12

[4]https://cge.cbs.dtu.dk/services/KmerFinder

(Seemann, 2014). *In silico* sequence typing was defined by MLST version 1.8[5].

The presence of plasmids was also investigated using plasmidSPAdes version 3.10.0 (Antipov et al., 2016). The plasmid scaffolds obtained with plasmidSPAdes were compared against all plasmids available in GenBank (Updated 2016.11.03) and plasmid *rep* genes available in PlasmidFinder version 1.3[6]. We also used Bandage (Wick et al., 2015) to analyze graph structures (**Supplementary Table 2**). Furthermore, plasmids recognized by plasmidSPAdes were mapped against reads and contigs using GFinisher (Guizelini et al., 2016) to improve plasmid assemblies. The complete plasmid was annotated with Prokka and manually curated using similarity with sequences available in UniRef90[7]. Plasmid incompatibility groups were predicted using PlasmidFinder (**Supplementary Table 2**) and *oriT* region was annotated using oriTfinder (Li et al., 2018).

Profiling of Antibiotic Resistance-Related Genes

Chromosomal and plasmid antibiotic resistance genes were predicted by ResFinder database version 2.1[8] and Comprehensive Antimicrobial Resistance Database (CARD) version 1.1.8 (Jia et al., 2017). The Short Read Sequence Typing (SRST2) version 0.2.0 (Inouye et al., 2014) and Genefinder algorithms (Sadouki et al., 2017) were tested to detect resistance genes with both databases. Furthermore, for ResFinder, the following parameters were defined: all databases were set for the antimicrobial configuration, and the type of input was set to assembled genomes/contigs and minimum thresholds of 98% identity and 80% alignment coverage between query and hit sequences.

Nucleotide Sequence Accession Numbers

The genomes of the six MDR-*K. pneumoniae* subsp. *pneumoniae* isolates have been deposited at DDBJ/ENA/GenBank under the accession numbers: PYWQ00000000 (D8), PYWR00000000 (C9), PYWS00000000 (C2), PYWT00000000 (B10), PYWU00000000 (A3), and PYWV00000000 (A2). The complete nucleotide sequence of the pUFPRA2 plasmid was included under accession number PYWV00000000.

RESULTS

Clinical Patient Profiles

Patient outcomes and clinical data are summarized in **Table 1**. Blood (n = 10/43, 23%), urine (n = 9/43, 20%), rectal (n = 7/43, 16%), and tracheal aspirate (n = 5/43, 11%) specimens yielded the highest numbers of isolates. Most patients were in the intensive care unit (ICU), and a high mortality rate was observed (24 out of 32 patients died; **Table 1**).

Antimicrobial Susceptibility, β-Lactam Resistance Profile, and Molecular Typing

Table 1 summarizes the results of ASTs. All isolates (except A10 and E4) displayed increased MICs for at least three classes of antibiotics and were classified as MDR (Magiorakos et al., 2012). Nine isolates exhibited sensitivity to all carbapenems by agar dilution.

All Kp isolates had $bla_{\mathrm{CTX-M}}$ and co-occurrence of bla_{TEM} and $bla_{\mathrm{CTX-M}}$ was found in 48.8% (n = 21/43) of isolates. No class C β-lactamase or minor-ESBL (BES, GES, PER, and VEB) was detected. Among carbapenemases, 18 isolates possessed bla_{KPC}, although no class B or D carbapenemases were detected. Ciprofloxacin and gentamicin showed low activity against ESBL-producing isolates. For isolates co-producing ESBL and KPC, neither ciprofloxacin nor minocycline were effective. All isolates were resistant to doxycycline. Only amikacin, fosfomycin, polymyxin, and tigecycline showed good activity against KPC-ESBL-coproducing isolates.

Nearly 90% of isolates had mutations in porins (n = 38/43, **Table 1**); among them, 33 were carbapenem-resistant and five were carbapenem-sensitive (i.e., A10, D4, E2, E3, and E4). Out of the five remaining samples which did not show porin mutations, four were carbapenem-sensitive (D8, D10, E1, and E5) and one was carbapenem-resistant (D6). Mutations in either *ompk35* or *ompk36* were observed in 14 strains and 6 strains, respectively, while 18 isolates were identified as having mutations in both of these porin genes. Only two isolates had mutations in *ompk35*, *ompk36*, and *ompk37* (**Table 1**). Types of mutations identified in the porin genes included: frameshift mutations caused by indels (9 isolates), fragmentation of the coding sequence or promoter regions caused by insertion of the IS1-like, IS5-like, IS6-like, or IS1380-like transposons (34 isolates), nonsense mutations resulting in premature stop codons (2 isolates), insertion of nucleotides in the loop 3 region (1 isolate), and mutations of trinucleotides not causing frameshifts (2 isolates) (**Table 1** and **Supplementary Figure 1**).

The o*mpk37* truncation by an IS5-like IS did not result in increased carbapenem MICs (i.e., B9 and C6, **Table 1**). Moreover, higher carbapenem MICs were observed only when bla_{KPC} was associated to *ompk35* and *ompk36* interrupted by ISs. Different antimicrobial resistance profiles were observed in Kp isolated from different body sites of the same patient (**Table 1**; P13, P17, P21, P27, P31, and P32), justifying their inclusion in the study. In some of these patients, isolates from different body sites had the same *bla* genes, but a different set of porin mutations.

Two distinct clusters A and C (>80% similarity) were identified based on similarities observed in dendrogram analysis based on PFGE typing (**Figure 1**). Notably, the major part of cluster A isolates (n = 33) belong to CG258 (ST11, n = 12; ST340, n = 1; ST379, n = 2; and ST437, n = 18), except for three non-CG258 isolates (ST12, n = 3). The cluster C displayed STs that do not belong to CG258 (ST15, 442, 584, and 874) (**Figure 1**). KpB3, KpB4, KpE7, and KpE8 isolates showed more

[5]https://cge.cbs.dtu.dk/services/MLST
[6]https://cge.cbs.dtu.dk/services/PlasmidFinder
[7]https://uniprot.org
[8]https://cge.cbs.dtu.dk/services/ResFinder

than 95% similarity to outbreak isolates, although these strains were isolated in 2011 and 2012.

Genomic Diversity of Six Kp Isolates

Pulsed field gel electrophoresis results were not used to select samples for WGS, since most of them belong to a single cluster (cluster A). We performed WGS of six Kp strains from the previously defined epidemiological period and diversity of antimicrobial resistance: two strains isolated before the outbreak, with low (KpD8) and high (KpC9) carbapenem MIC; two strains from the outbreak, with low (KpA2) and high (KpC9) carbapenem MIC (KpA3), and two strains isolated after the outbreak, both with high carbapenem MIC (KpB10 and KpC2) (**Table 1**).

Each of the six sequenced isolates belonged to cluster A (**Figure 1**) and had the following distinct PFGE and MLST profiles: KpD8 (pulsotype A14, ST11), KpC9 (pulsotype A10, ST11), KpA2 and KpA3 (pulsotype A19, ST437), and KpB10 and KpC2 (pulsotype A7, ST437). A summary of the genomic features of the six MDR-Kp isolates is shown in **Supplementary Table 3**.

Resistance genes were widely distributed among isolates. In **Table 2**, we list resistance genes in the plasmids and chromosomes of the sequenced genomes, which were identified based on the results of manually inspected BLAST searches (see section "Materials and Methods" for details). In addition to the β-lactamases detected by PCR, narrow-spectrum oxacillinases were also found ($bla_{\mathrm{OXA-1}}$ and $bla_{\mathrm{OXA-2}}$). No discrepancies were found between PCR and genome sequencing data. Mutations in *ompk35* and *ompk36* were confirmed and mutations in *ompk26*, *lamB*, and *phoE* were not found. Various aminoglycoside-modifying enzymes (AMEs) were detected, even in isolates that showed sensitivity to amikacin and gentamicin (**Table 2**). However, this result was not supported by all used prediction tools, as we found some divergences in the identification of AMESs from ResFinder, CARD, SRST2, and Genefinder. Determinants of resistance to fluoroquinolones were: (i) mutations in *gyrA* and *parC*, (ii) presence of the acetyltransferase, AAC(6′)Ib-cr, and (iii) presence of *qnrB1* (**Table 2**). Resistance to levofloxacin emerged when there were more mutations in *gyrA* (Ser83Ile and Asp87Gly; in D8) or when QnrB was present (A2). KpC9 was unique regarding its resistance to polymyxin because the *mgrB* from this isolate was truncated by IS*Kpn13* (IS5 family), which was inserted in the opposite orientation, between nucleotides 75 and 76.

Due to intrinsic methodological limitations, it was not possible to obtain a complete view of the plasmid landscape of each isolate. However, PlasmidSPAdes provided important support for the presence of some plasmids (**Supplementary Table 2**), including the recovery of a complete conjugative plasmid, pUFPRA2 (**Figure 2**), which was identified in the index isolates KpA2 and KpA3 of the KPC outbreak, also in KpB10 and KpC2 isolated after the outbreak. This plasmid belongs to the IncN group and carries $bla_{\mathrm{KPC-2}}$ within a Tn*4401b* transposon. pUFPRA2 possesses 98% identity to pKPC_FCF/3SP (GenBank accession no. CP004367) and 95% identity to pKPC_FCF13/05 (GenBank accession no. CP004366), which are previously published plasmids. The region around ∼15 kbp contains *ardA*, an anti-restriction gene, lacking only in pKPC_FCF13/05. Furthermore, pUFPRA2 presented a Tn*4401b* sequence identical to pKPC_FCF/3SP, including the direct-repeat target site duplications (5′CTTCAG3′). We were able to independently recover the complete sequence of plasmid pUFPRA2 from the WGS of all KPC-producing isolates (A2, A3, and C2), although it was not possible to reconstruct the complete plasmid from KpB10 (**Supplementary Table 2**).

DISCUSSION

This study describes the gradual increase in antimicrobial resistance in Kp, including an outbreak of KPC and its spread in the hospital between August 2003 and February 2012. Our intention was to study the molecular epidemiology of Kp isolated from a period shift in resistance profile revealed by our hospital infection control service.

Antimicrobial resistance evolution in *Enterobacteriaceae* involved in outbreaks at CHC/UFPR was initially associated with expansion of ESBL-carrying strains co-expressing fluoroquinolone and aminoglycoside resistance genes (Toledo et al., 2012; Nogueira Kda et al., 2014, Nogueira et al., 2015). Since the 2000s, ESBL prevalence has led to an increase in carbapenem prescriptions, resulting in the emergence of ertapenem-resistant strains between 2004 and 2009 (Nogueira Kda et al., 2014, Nogueira et al., 2015).

Several functional studies have investigated the role of porins in antimicrobial resistance (Kaczmarek et al., 2006; Fernandez and Hancock, 2012; Sugawara et al., 2016). Here, we evaluated the distribution of *ompk35*, *ompk36*, and *ompk37* mutations and their correlation with other resistance markers. Our results are also consistent with a previous observation that loss of OmpK35 is more frequent than that of OmpK36, particularly among ESBL producers (Domenech-Sanchez et al., 2003). The higher frequency of OmpK35 loss could be explained by selection for a less permeable outer membrane, as suggested by the recent discovery that OmpK35 allows faster influx of β-lactams than OmpK36 (Sugawara et al., 2016). Considering the reported differences in the impact of each porin on permeability, loss-of-function mutations affecting *ompK35* are expected to be more rapidly fixed than those affecting *ompK36*. Among our samples, we found carbapenem-sensitive strains, although some of them were ESBL producers that lost one of the porins. Kp is extremely versatile, and compensation by other outer membrane proteins or changes in gene expression could explain these different resistance profiles (Garcia-Sureda et al., 2011a; García-Sureda et al., 2011b).

In this study, we observed different mutations in porins among isolates recovered from different body sites of the same patient (P13 and P21). Patient P13 had isolates from CSF that were resistant to a single carbapenem (ertapenem), whereas the isolate from the rectal specimen showed high MICs for ertapenem, imipenem, and meropenem. Similar trends were observed for patient P21 from different sources. Concomitant loss of both OmpK35 and OmpK36 was observed in the isolates that were most resistant to carbapenems. These isolates were also found at body sites that contained abundant and diverse

TABLE 2 | Resistance gene repertoire identified using ResFinder and CARD databases.

Sample ID	KpD8	KpC9	KpA2	KpA3	KpB10	KpC2
Plasmid-mediated						
Beta-lactams	*bla*$_{TEM-1}$	*bla*$_{TEM-1}$	*bla*$_{TEM-1}$	*bla*$_{TEM-1}$	*bla*$_{OXA-1}$	*bla*$_{OXA-1}$
	bla$_{CTX-M-2}$	*bla*$_{OXA-2}$	*bla*$_{OXA-1}$	*bla*$_{OXA-1}$	*bla*$_{CTX-M15}$	*bla*$_{CTX-M15}$
		bla$_{CTX-M-2}$	*bla*$_{CTX-M-15}$	*bla*$_{CTX-M-15}$	*bla*$_{KPC-2}$	*bla*$_{KPC-2}$
			bla$_{KPC-2}$	*bla*$_{KPC-2}$		
Aminoglycosides	*aac(3)-IIa*	*aac(3)-IIa*	*aac(3)-IId*	*aac(3)-IId*	*aadA2*	*aadA2*
	aadA1(2 copies)	*aadA2*	*aph(3′)-Ia*	*aph(3′)-Ia*	*aph(3′)-Ia*	*aph(3′)-Ia*
	aadA2					
	aph(3′)-Ia					
	aph(3′)-VIa					
Quinolones		*aac(6′)Ib-cr*	*aac(6′)Ib-cr*	*aac(6′)Ib-cr*	*aac(6′)Ib-cr*	*aac(6′)Ib-cr*
			qnrB1			
Fosfomycin	*fosA5/6*	*fosA5/6*	*fosA5/6*	*fosA5/6*	*fosA5/6*	*fosA5/6*
Sulphonamide	*sul1*	*sul1*	*sul1*	*sul1*	*sul1*	*sul1*
	sul3					
Trimethoprim	*dfrA12*	*dfrA12*	*dfrA5*	*dfrA5*	*dfrA5*	*dfrA5*
			dfrA30	*dfrA30*	*dfrA8*	*dfrA8*
					dfrA30	*dfrA30*
Chloramphenicol	*catA1*		*catB3*	*catB3*	*catA1*	*catA1*
	cmlA1				*catB3*	*catB3*
Chromosome-mediated						
Beta-lactams	*bla*$_{SHV-11}$	*bla*$_{SHV-11}$	*bla*$_{SHV-11}$	*bla*$_{SHV-11}$	*bla*$_{SHV-11}$	*bla*$_{SHV-11}$
				bla$_{CTX-M-15}$		
		ompk35, frameshift (Δ342C)	*ompk35*, disrupted by IS	*ompk35*, disrupted by IS	*ompk35*, disrupted by IS	*ompk35*, disrupted by IS
		ompk36, disrupted by IS		*ompk36*, disrupted by IS	*ompk36*, disrupted by IS	*ompk36*, disrupted by IS
Quinolones	GyrA (Ser83Ile, Asp87Gly)	GyrA (Ser83Ile)	GyrA (Ser83Ile)	GyrA (Ser83Ile)	GyrA (Ser83Ile)	GyrA (Ser83Ile)
	ParC (Ser80Ile)	ParC (Ser80Ile)	ParC (Ser80Ile)	ParC (Ser80Ile)	ParC (Ser80Ile)	ParC (Ser80Ile)
	oqxA	*oqxA*	*oqxA*	*oqxA*	*oqxA*	*oqxA*
	oqxB	*oqxB*	*oqxB*	*oqxB*	*oqxB*	*oqxB*
Polymyxin		*mgrB*, disrupted by IS				
Tetracycline		*tetA*	*tetA*	*tetA*	*tetA*	*tetA*
			tetD	*tetD*		

microbiota, which is interesting given the roles of the gut human microbiome in antibiotic resistance (Carlet, 2012). Changes in the gut microbiome, particularly those driven by antibiotics, could silently select for increasingly resistant bacteria. These microorganisms may remain for months in the gut of the carrier or translocate through the gut epithelium, promoting infections and cross-transmission to other patients, resulting in outbreaks that are hard to control.

Klebsiella pneumoniae carbapenemase-producing Kp were first described in Brazil in 2006 (Monteiro et al., 2009) and their incidence has significantly increased since that time. In 2010, a great dispersion of *bla*$_{KPC}$ was observed in Brazil (Seki et al., 2011; Pereira et al., 2013), including an outbreak in our hospital (Almeida et al., 2014). During 2011 and 2012, few KPC-producing *Enterobacteriaceae* were found in this same hospital (42 cases in 2 years). However, in 2013, the number of cases doubled, and the co-occurrence of *bla*$_{KPC}$ and *bla*$_{CTX-M}$ was widespread, mainly in the ICU. Interestingly, PFGE analysis showed a major cluster containing isolates recovered between 2003 and 2012, including both non-KPC and KPC-Kp. A previous study, also conducted in our hospital, investigated the distribution of ESBL-producing *Enterobacteriaceae* isolated between 2003 and 2008 (Nogueira et al., 2015). They reported that both Kp and *Enterobacter aerogenes* (recently renamed *Klebsiella aerogenes*) isolates were clustered, but clustering was not observed in *Escherichia coli*. Another study showed that 84% of 129 KPC-Kp isolates from different healthcare facilities in Curitiba belonged to two clusters, isolated between 2010 and 2012 (Arend et al., 2015), suggesting that a predominant lineage of Kp might have spread in the city.

Emerging technologies for rapid identification of resistance determinants, such as WGS, may lead to a shift from traditional

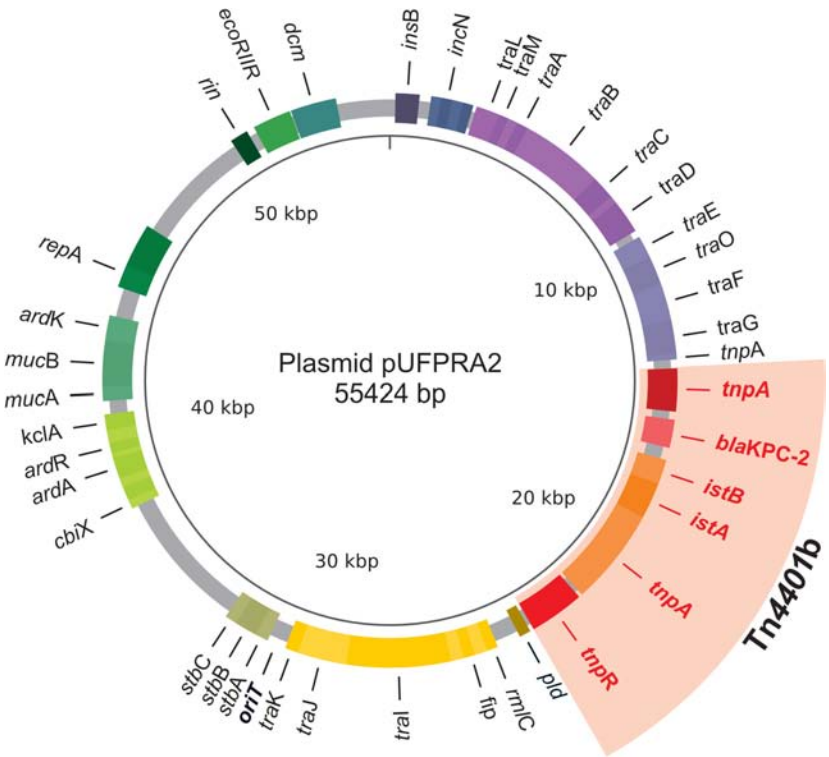

FIGURE 2 | Map of the 55-kb plasmid obtained from KpA2, the index isolate of the KPC outbreak at CHC/UFPR. The representative genes of the pUFPRA2 plasmid are shown in colored boxes. The area in red indicates the Tn*4401b* region. *Tra* genes confirm that it is a conjugative plasmid.

AST toward the analysis of genetic elements and discovery of emergent resistance mechanisms. By using this technology, we have found a large and diverse repertoire of resistance genes that accounts for most of the MDR phenotype obtained *in vitro*. The genetic MDR profile has been described by co-existence of beta-lactam (bla_{KPC}, bla_{CTX-M}, bla_{TEM}, bla_{OXA}), quinolone [*aac*(6′)-*Ib-cr*, *qnr*], aminoglycosides (AMEs-coding genes, methylases), tetracyclines (*tet*), sulfonamides (*sul*), and trimethoprim (*dfr*) determinants. These elements are frequently mobilized by a variety of mobile genetic elements (insertion sequences, transposons, and integrons) which are recombined in plasmids and/or chromosomes (Carattoli, 2013; Bi et al., 2015; Mathers et al., 2015; Shankar et al., 2017).

Most of the isolates studied here displayed a single genetic cluster under PFGE analysis and all are members of CG258, predominantly distributed among two different sequence types (ST11 and ST437). Kitchel et al. (2009) showed that all members of a single Kp cluster with more than 80% similarity by PFGE belonged to ST258, corroborating with our findings. Our results also revealed higher-than-expected genotypic diversity of isolates from different body sites of the same patient during a short period of antibiotic therapy, highlighting additional potential challenges for the treatment, diagnosis, and surveillance of MDR bacteria.

The bla_{KPC-2}-bearing plasmid identified in our Kp isolates (pUFPRA2) was similar to pKPP_FCF13/05 and pKPC_FCF/3SP, which were obtained from two distinct blood cultures of patients infected by Kp. The strain harboring FCF1305-Kp belonged to ST442 and was isolated for the first time in Brazil in 2005 from a patient living in the State of São Paulo; FCF3SP-Kp, also a member of ST442, was isolated in 2009 in the same state (Perez-Chaparro et al., 2014). The KPC-Kp outbreak at our hospital, located further South, in the State of Paraná, occurred in 2010. The presence of very similar plasmids in earlier isolates from the neighboring state of São Paulo indicates that these plasmids are successfully spreading among Kp strains in the Brazilian population.

In summary, our results indicate long-term stability of the same cluster and MLST clonal group of Kp that has been observed in hospitals since the rise of the ESBL endemicity period until the development of resistance to carbapenems, including the bla_{KPC} outbreak. A considerable amount of genetic variation, particularly in β-lactams resistance determinants, was observed among isolates. Porin mutations may play an important role in increasing carbapenem MIC. In several cases, they were shown to be even more effective than beta-lactamases at inducing carbapenem resistance. In addition, variation in resistance mechanisms between isolates from the same patient suggests selection and propagation of MDR bacteria in the patient's body and shows how challenging it is for healthcare teams to control and treat such infections. The remarkable transmissibility coupled with limited therapeutic options to fight MDR isolates drastically reduce the effective control of this pathogen in the nosocomial setting. The integration of

WGS technologies and computational analyses with diagnostic procedures can contribute to a better understanding of the co-occurrence of several distinct resistance mechanisms.

AUTHOR CONTRIBUTIONS

JP, AG, NV, TV, and LD-C conceived the idea and designed the study. JP carried out the sample collections and performed the wet lab experiments. JP, RdS, MS, H-PA, AG, and NV carried out the genome analysis. JP, RdS, TV, and LD-C interpreted the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01669/full#supplementary-material

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Occurrence and Characterization of *mcr-1*-Positive *Escherichia coli* Isolated From Food-Producing Animals in Poland, 2011–2016

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The emergence of plasmid-mediated colistin resistance (*mcr* genes) threatens the effectiveness of polymyxins, which are last-resort drugs to treat infections by multidrug- and carbapenem-resistant Gram-negative bacteria. Based on the occurrence of colistin resistance the aims of the study were to determine possible resistance mechanisms and then characterize the *mcr*-positive *Escherichia coli*. The research used material from the Polish national and EU harmonized antimicrobial resistance (AMR) monitoring programs. A total of 5,878 commensal *E. coli* from fecal samples of turkeys, chickens, pigs, and cattle collected in 2011–2016 were screened by minimum inhibitory concentration (MIC) determination for the presence of resistance to colistin (R) defined as $R > 2$ mg/L. Strains with MIC = 2 mg/L isolated in 2014–2016 were also included. A total of 128 isolates were obtained, and most (66.3%) had colistin MIC of 2 mg/L. PCR revealed *mcr-1* in 80 (62.5%) isolates recovered from 61 turkeys, 11 broilers, 2 laying hens, 1 pig, and 1 bovine. No other *mcr*-type genes (including *mcr-2* to *-5*) were detected. Whole-genome sequencing (WGS) of the *mcr-1*–positive isolates showed high diversity in the multi-locus sequence types (MLST) of *E. coli*, plasmid replicons, and AMR and virulence genes. Generally *mcr-1.1* was detected on the same contig as the IncX4 (76.3%) and IncHI2 (6.3%) replicons. One isolate harbored *mcr-1.1* on the chromosome. Various extended-spectrum beta-lactamase (bla_{SHV-12}, $bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{TEM-30}, bla_{TEM-52}, and $bla_{TEM-135}$) and quinolone resistance genes (*qnrS1*, *qnrB19*, and chromosomal *gyrA, parC,* and *parE* mutations) were present in the *mcr-1.1*–positive *E. coli*. A total of 49 sequence types (ST) were identified, ST354, ST359, ST48, and ST617 predominating. One isolate, identified as ST189, belonged to atypical enteropathogenic *E. coli.* Our findings show that *mcr-1.1* has spread widely among production animals in Poland, particularly in turkeys and appears to be transferable mainly by IncX4 and IncHI2 plasmids spread across diverse *E. coli*

lineages. Interestingly, most of these *mcr-1*–positive *E. coli* would remain undetected using phenotypic methods with the current epidemiological cut-off value (ECOFF). The appearance and spread of *mcr-1* among various animals, but notably in turkeys, might be considered a food chain, and public health hazard.

Keywords: WGS, *mcr-1*, colistin resistance, aEPEC, food animal, IncX4, IncHI2

INTRODUCTION

The worldwide increase in the occurrence of antimicrobial resistance (AMR) and prevalence of multidrug-resistant (MDR) Gram-negative *Enterobacteriaceae* challenge our ability to treat infections in humans and animals, thus resulting in a renewed interest in old drugs such as polymyxins. Colistin (polymyxin E), which has been used in veterinary practice for decades mainly for treating Gram-negative bacteria infections of the gastrointestinal tract in pigs, poultry and cattle, is nowadays considered a last-resort drug to treat human infections by multidrug-, and carbapenem-resistant Gram-negative bacteria. Together with third, fourth, and fifth generation cephalosporins, glycopeptides, quinolones, and macrolides, polymyxins are among the critically important antimicrobials (CIA) for human medicine (World Human Organization [WHO], 2017) and should be mainly used for treating the severest human infections to preserve their effectiveness. Antimicrobials are used in hospitals and care facilities as well as in veterinary clinics and on farms. Extensive use of antimicrobials is recognized as the most important factor selecting for AMR in bacteria (Centers for Disease Control and Prevention [CDC], 2013). According to the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) report, sales of veterinary antimicrobial agents in 2016 varied from 0.7 to 2,726.5 tons in the 30 participating countries (European Medicine Agency [EMA] and European Surveillance of Veterinary Antimicrobial Consumption [ESVAC], 2018). Notably, polymyxins were the fifth most sold group of antimicrobials in 2015–2016 (European Medicine Agency [EMA] and European Surveillance of Veterinary Antimicrobial Consumption [ESVAC], 2017, 2018). In Poland, colistin sales increased by 35% from 2011 to 2016, reaching their highest value of 5.94 mg per population correction unit (PCU) in 2015 and exceeding the recommended maximum sale target of 5 mg/PCU for this antimicrobial (European Medicine Agency [EMA] and European Surveillance of Veterinary Antimicrobial Consumption [ESVAC], 2016, 2017, 2018). Currently, there are 26 veterinary medicinal products containing colistin (*Colistini sulfas* or colistinum) registered in Poland as powders for oral solution, with six registered only in 2017[1].

In *Enterobacteriaceae*, resistance to polymyxines was theorized to be regulated by the two-component systems PhoP/PhoQ and PmrA/PmrB involved in LPS modifications (Olaitan et al., 2014). The emergence and spread of plasmid-mediated colistin resistance (the *mcr-1* gene), first described in China in 2015 (Liu et al., 2016), and poses a threat to the effectiveness of colistin. The *mcr-1* gene has been detected in several bacterial species (Li et al., 2017; Tian et al., 2017; Torpdahl et al., 2017) in association with different plasmid types such as IncI2, IncHI2, IncP, IncFIP, and IncX4 and also inserted into the bacterial chromosome (Liu et al., 2016; Zurfluh et al., 2016; Hadjadj et al., 2017; Sun et al., 2018). New *mcr* genes and their variants have also been identified: *mcr-2* (Xavier et al., 2016), *mcr-3* (Yin et al., 2017), *mcr-4* (Carattoli et al., 2017), *mcr*-5 (Borowiak et al., 2017), *mcr-6* (Abuoun et al., 2017), *mcr-7* (Yang et al., 2018), *mcr-8* (Wang et al., 2018), and *mcr-9* (Carroll et al., 2019).

Little is known about the prevalence of colistin resistance and the occurrence of *mcr* genes in livestock in Poland. In 2015, a single case of *mcr-1*–positive *Escherichia coli* was described from a human patient with a urinary tract infection (Izdebski et al., 2016). This might be the first evidence from Poland that *mcr*-mediated colistin resistance from animals has spread to humans, which would validate concerns over foodborne transfer of colistin-resistant bacteria to humans (Grami et al., 2016). Based on investigation of the occurrence of colistin resistance among *E. coli* isolated from food-producing animals in Poland over a 6-year period, the aim of the study was to determine the resistance mechanisms among the colistin-resistant isolates. Whole genome sequence analysis of the *mcr-1*–positive *E. coli* strains was made to elucidate the pathways of dissemination of *mcr-1* in food-producing animals in Poland and highlight possible animal and public health threats.

MATERIALS AND METHODS

Bacterial Isolates

A total of 5,878 commensal *E. coli* isolates were obtained from individual fecal samples collected from turkeys, chickens, pigs and cattle in 2011–2016, and tested for antimicrobial susceptibility by minimum inhibitory concentration (MIC) determination (Sensititre, TREK Diagnostic; EUMVS2 and EUVSEC plates). The isolates were screened to confirm the presence of microbiological resistance (*R*) to colistin ($R > 2$ mg/L). Additionally, available isolates with MIC = 2 mg/L (wild-type isolates) from 2014 to 2016 were included in the study because they represented colistin MIC values one dilution step from those considered as non-wild type (NWT). Isolates were collected as part of the multiannual national program (2011–2016) and the EU harmonized AMR monitoring program carried out in 2014–2016 (Decision 2013/652/EU).

[1]http://bip.urpl.gov.pl/pl/biuletyny-i-wykazy/produkty-lecznicze-weterynaryjne

Those programs are based on isolation of commensal *E. coli* from the cecal content of samples collected from random animals at slaughter. The sampling was carried out by veterinary officers on a by-slaughterhouse basis proportionally to the annual capacity of the slaughterhouse and at intervals distributed over the 6-year period. The antimicrobial susceptibility testing (AST) for ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, colistin, nalidixic acid, meropenem, sulfamethoxazole, tetracycline, tigecycline, and trimethoprim was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria describing epidemiological cut-off values (ECOFFs) for antimicrobials. The selected isolates were subjected to PCR targeting the *mcr-1* and *mcr*-2 genes (Cavaco et al., 2016). Subsequently, resistant isolates were whole-genome sequenced (WGS) as detailed below. PCR-negative strains were re-tested phenotypically to confirm the MIC to colistin and screened for the presence of *mcr-1*, *-2, -3, -4*, and *-5* using PCR (Rebelo et al., 2018).

Whole Genome Sequencing

DNA from bacterial cells of the 80 *mcr-1*–positive isolates was extracted from nutrient agar plate cultures using a Genomic Mini Kit (A&A Biotechnology) following the manufacturer's recommendations. Sequencing libraries were prepared with the Nextera XT DNA Sample Preparation Kit (Illumina) according to the manufacturer's protocol. Sequencing of the strains was performed using Illumina MiSeq 2 bp × 250 bp and 2 bp × 300 bp reads or Illumina HiSeq 2 bp × 150 bp reads, generating on average 398 Mb per sample (176–673 Mb), which corresponds to average coverage of 80× (35–135×) in a 5 Mb genome. The raw reads were processed using bbmerge v36.62 (Bushnell, 2018) to merge overlapping reads and Trimmomatic v0.36 (Bolger et al., 2014) to trim adapters and low quality reads. Merged reads and trimmed unmerged pairs were used to generate assembly contigs and scaffolds using SPAdes 3.9.0 (Bankevich et al., 2012). The mean N50 of assemblies was 178 kb (77–433 kb) and the average number of contigs longer than 1 kb was 102 (40–364). Six isolates where the *mcr* gene was not located on the same contig as a plasmid replicon were subjected to additional Pacific Biosciences long-read sequencing, three samples per SMRTcell. The raw PacBio reads were de-multiplexed to subreads using *lima* 1.0.0 (Pacific Biosciences) (Topfer, 2018) yielding on average 225 Mb per sample (72–390 Mb), which translates to average 45× coverage (14.4–78×) of a 5 Mbps genome. The mean subread length was 3,555 bp (3,183–3,929 bp) and mean basepair quality 13.1 (12.95–13.22). Subreads were used in a hybrid SPAdes assembly together with raw short Illumina reads. Assembly analysis with QUAST 4.5 (Gurevich et al., 2013) reported 8–13 contigs longer than 10 kb per sample and 2.1 Mb average N50 (0.91–3.9 Mb). The DNA sequences (reads) from the isolates were deposited in the European Nucleotide Archive (ENA) under project number PRJEB23993. Specific sequence numbers are included in **Supplementary Table S1**. *E. coli* strains which codes start from "U" were gathered within antimicrobial resistance monitoring according to 2013/652/EC and they are included in the annual EFSA/ECDC reports.

Bioinformatic Data Analysis

Sequences were analyzed for the presence of AMR genes, virulence genes and plasmid replicons by using the Center for Genomic Epidemiology (CGE)[2] ResFinder 3.1.0 (with database updated on September 10, 2018) (Zankari et al., 2012), VirulenceFinder 1.5 (February 18, 2016) (Joensen et al., 2014), PlasmidFinder 1.3 (December 15, 2017) (Carattoli et al., 2014), and pMLST v1.4 (December 15, 2017) (Carattoli et al., 2014) web-based tools for typing of IncHI2 plasmids. The criteria for these tools were: 90% threshold for identity with the reference and minimum 60% coverage of the gene length. Multi-locus sequence typing (MLST) of strains was performed using MLST 1.8 (Carattoli et al., 2014). The phylogenetic tree of 80 isolates was constructed by complete linkage clustering using a sequence similarity distance matrix. The distance matrix was generated by global pairwise MUMmer 3.23 (Kurtz et al., 2004) alignments between samples' scaffolds, automated by CONCOCT 0.4.0 (Alneberg et al., 2014). A phylogenetic tree of IncX4 plasmids was created in a similar way, using contigs carrying the IncX4 replicon and the *mcr-1* gene. The *mcr-1* carrying contigs were identified using BLAST (Altschul et al., 1990) and *mcr-1* sequence AKF16168.1. The iTol web-based tool (Letunic and Bork, 2016) was used to visualize the trees.

RESULTS

Occurrence of Colistin Resistance and *mcr-1*

Retrospective analysis of MIC data revealed a total of 128 (2.2%) out of 5,878 commensal *E. coli* fulfilling the selection criteria with colistin MICs ranging from 2 to 16 mg/L (**Figure 1**). They originated mostly from turkeys (63%) and chickens (23%). A slight temporal increase of microbiological resistance to colistin from very low to low (0.7–1.7%) was observed when considering all *E. coli* isolates detected in samples from 2011 to 2016 taken from Polish food-producing animals irrespective of their origin (**Figure 2**).

The *mcr-1* gene was detected in 80 (62.5%) out of the selected 128 isolates, deriving from 76 fecal samples recovered from turkeys (n = 61), broilers (n = 11), laying hens (n = 2), pigs (n = 1), and cattle (n = 1). Most of the *mcr-1*–positive *E. coli* originated from individual samples, but in four samples from turkeys (n = 3) and broilers (n = 1), two different isolates per sample were identified (**Supplementary Table S1**). An increase in occurrence of the *mcr-1*–positive *E. coli* was noted in turkey and chicken samples, respectively from 1.1 and 0.0% in 2011 to 11.6 and 1.7% in 2016 (**Figure 3**). The CGE ResFinder tool confirmed the presence of the *mcr-1.1* gene in all PCR-confirmed isolates. No *mcr-2, mcr-3, mcr-4,* or *mcr-5* was identified either from PCR or the genome analysis in *mcr-1*–positive isolates. Noteworthily, the *mcr-1.1* was mostly found (n = 53; 66.3%) in isolates with colistin MIC = 2 mg/L which is the EUCAST ECOFF and regarded as that of the wild-type

[2]https://cge.cbs.dtu.dk/services/

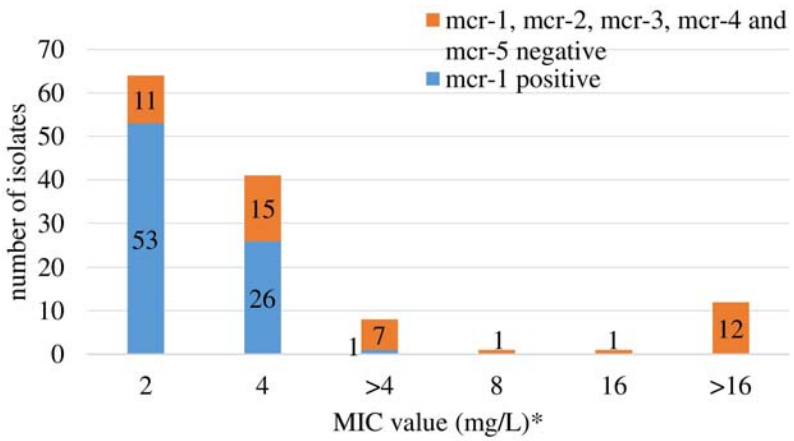

FIGURE 1 | Colistin MIC distribution and occurrence of the *mcr-1* and *mcr-2* gene among 128 *Escherichia coli* selected based on colistin MIC > 2 mg/L (2011–2013) and ≥2 mg/L (2014–2016). * only isolates with $MIC_{colistin}$ ≥ 2 mg/L were tested for *mcr-1* and *mcr-2* genes. Different MIC values (>4 and 8, 16, and >16) are result of changed MIC panel in plates. EUMVS2 plate (2–4 mg/L) was used in 2011–2013, EUVSEC plate (1–16 mg/L) in 2014–2016.

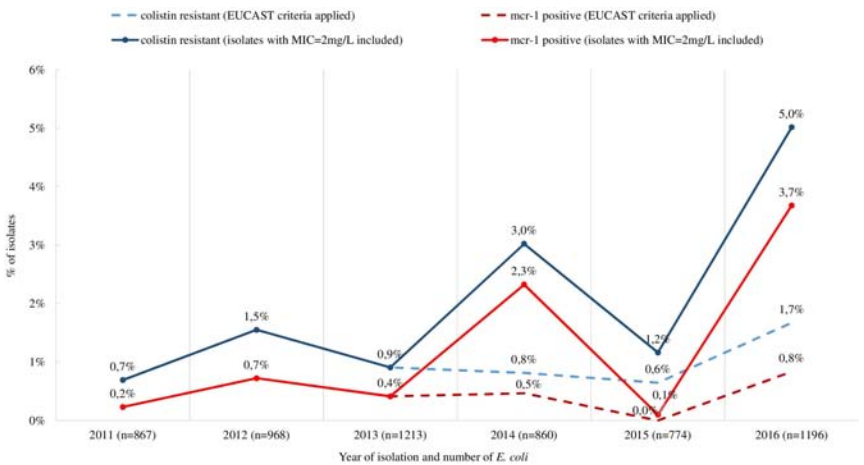

FIGURE 2 | Occurrence of isolates meeting the selection criteria (MIC > 2 mg/L for isolates in 2011–2013 and MIC ≥ 2 mg/L for isolates in 2014–2016) and *mcr-1* positive commensal and ESBL/ampC producing *E. coli* from all tested sources (turkeys, chickens, pigs, and cattle), 2011–2016. The occurrence of colistin resistant and *mcr-1* positive isolates when using exclusively EUCAST ECOFF was included (dashed line).

population. Most of these (n = 41; 77.4%) were sampled from turkeys. Additionally, a mutation in the chromosomal *pmrB* gene (Val161→Gly) was detected in one *mcr-1.1*–positive isolate with MIC = 2 mg/L.

As shown on the maps of the farm locations from which *mcr-1*–positive *E. coli* was isolated, the colonized farms were distributed over the country with no specific regional trend (**Figures 4–6**).

An MIC ≥ 2 mg/L for colistin could not be confirmed in any of the re-tested 48 isolates initially suspected but found negative for *mcr-1* and *mcr-2*, and none of the *mcr-1*, *-2*, *-3*, *-4*, or *-5* genes were identified by PCR. They were not investigated further as we considered them either false positives in the initial testing, or to have eventually lost the mechanisms over prolonged storage or handling.

Phylogeny and Epidemiology

The MLST revealed 49 ST among the sequenced isolates. In 64 *E. coli* from turkeys, 41 STs were identified, as were 10 in 14 chicken isolates. The most common types were ST354 and ST359, which were observed in five isolates each, ST48 and ST617 which were identified in four isolates each, and ST10, ST58, ST155, and ST1011 which were represented by three isolates each. Single isolates represented 32 ST (**Figure 7**).

The analysis showed high heterogeneity of *mcr-1*–positive *E. coli* independent of source and year of isolation. Isolates deriving from animals from 27 farms and slaughtered in 27 slaughterhouses (**Supplementary Table S2**) were clustered according to their ST. The majority of isolates belonging to the most numerous STs (i.e., ST48, ST88, ST359, and ST1011) derived from different animal species (**Figure 7**) slaughtered

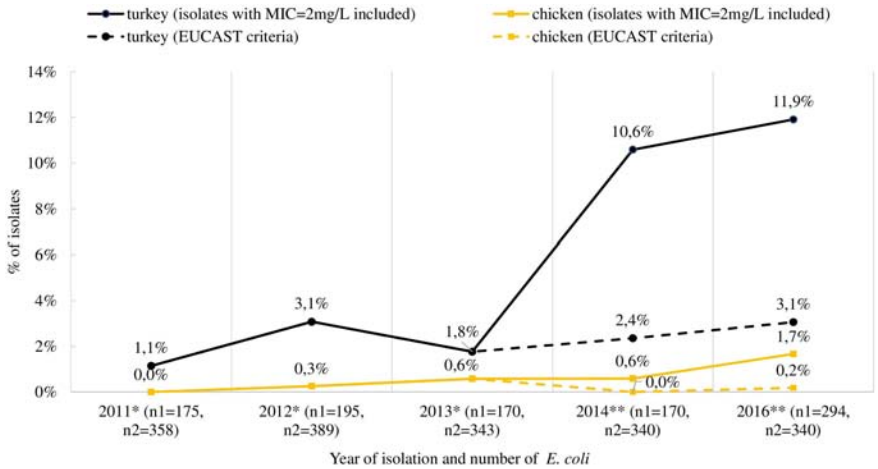

FIGURE 3 | Occurrence of *mcr-1*-positive *E. coli* in Poland isolated from turkey and chicken fecal samples. * isolates collected in multiannual governmental programs (according to of the Council of Ministers Decisions), ** isolates deriving from official monitoring according to Decision 2013/652/EC and thus not encompassing samples from turkeys and broilers in 2015; n1 and n2 indicate the total number of isolates tested for MIC determination from turkeys and chickens (both broiler and laying hens), respectively. The occurrence of *mcr-1* positive strains when using exclusively EUCAST ECOFF during selection of isolates was also included (dashed line).

in different slaughterhouses (**Supplementary Table S1**) and originating from different farms or flocks (data not shown). In cases where the same ST was found in animals from the same slaughterhouse and/or farm, the *mcr* localization and plasmid profile were often different, as for example with ST354 observed exclusively in turkeys where two isolates deriving from the same farm but slaughtered in different places had *mcr* localized on IncX4 (U16_0311), and chromosome (U16_0259) (**Supplementary Table S1**). In a few cases, the same ST (i.e., ST354, ST359, ST919, and ST1564) was present among strains isolated in different years.

Phenotypic and Genetic Traits of Microbiological Resistance to Additional Antimicrobials

The *mcr-1*–positive strains showed resistance to at least two and up to seven different classes of antimicrobials and had different resistance gene contents. Seventy-eight (97.5%) *mcr-1*-positive *E. coli* were classified as MDR isolates. Seventy-nine (98.8%) were resistant to ampicillin and 22 (27.5%) to cefotaxime and ceftazidime. Resistance to ciprofloxacin was confirmed in 70 (87.5%), to tetracycline in 61 (76.3%), to nalidixic acid in 50 (62.5%), to chloramphenicol in 27 (33.8%), to gentamicin in 16 (20.0%), and to tigecycline in 12 (15.0%). Four of the isolates had an azithromycin MIC $\geq$ 16 mg/L, which can be interpreted as resistance according to the tentative ECOFF for this antimicrobial. The strains were susceptible to meropenem and presented no resistance genes to carbapenems.

The whole-genome sequencing data revealed the occurrence of bla_{TEM-1} in the majority (n = 73; 92.4%) of the ampicillin-resistant isolates. The genes encoding extended-spectrum beta-lactamases (ESBLs) and AmpC-type cephalosporinases were identified in 18 (22.5%) *E. coli* belonging to 14 STs: bla_{SHV-12} was present in five isolates (ST58, ST69, ST359, and ST1011), $bla_{CTX-M-1}$, bla_{TEM-30}, and $bla_{TEM-135}$ in two each (respectively, ST617, ST1611, ST154, ST617, ST93, and ST5979), single isolates carried $bla_{CTX-M-15}$ (ST767), or $bla_{TEM-52C}$ (ST117) and bla_{CMY-2} was present in six strains (ST48, ST58, ST155, ST398, and ST1011) (**Figure 7**). Fifteen isolates carried extended-spectrum cephalosporin (ESC) resistance gene in combination with bla_{TEM-1}. Two isolates, U15_0035X (ST767) and U16_0016X (ST617), possessed simultaneously two ESC resistance genes, respectively, $bla_{CTX-M-15}$ with bla_{CMY-2} and $bla_{CTX-M-1}$ with bla_{TEM-30}. The swine isolate (U15_0035X) was the only one carrying the $bla_{CTX-M-15}$ gene.

Analysis of the genetic background of resistance to quinolones showed chromosomal mutations in the quinolone resistance-determining region (QRDR) of topoisomerase genes in 63.8% (n = 51) isolates, resulting in amino acid substitutions in the *gyrA* subunit [Ser83→Leu (n = 48); Asp87→Ans (n = 40), Asp87→Tyr (n = 2)], *parC* [Ser80→Ile (n = 37), Ser80→Arg (n = 4); Ser57→Thr (n = 1); Glu84→Gly (n = 3), Glu84→Lys (n = 2)], and *par*E [Leu416→Phe (n = 2), Leu460→Asp (n = 1)]. The *gyrB* gene remained unaltered. Several silent mutations irrelevant for quinolone resistance were also noted. Different patterns combining up to four simultaneous amino acid substitutions were noted among tested isolates with a combination of mutations in *gyrA* S83L, *gyrA* D87N, and *parC* S80I being the most frequent (n = 30) (**Figure 7**). Plasmid-mediated quinolone resistance (PMQR) genes were detected in 23 isolates, namely *qnrS1* (n = 12) and *qnrB19* (n = 11) (**Figure 7**). Four of the PMQR carriers also harbored QRDR chromosomal mutations. Eight isolates carried both ESBL/AmpC and PMQR determinants. The *aac(6′)Ib-cr* gene, conferring resistance to both quinolones and aminoglycosides, was identified in two strains, occurring along with *qnrS1* (U15_0035X), or the set

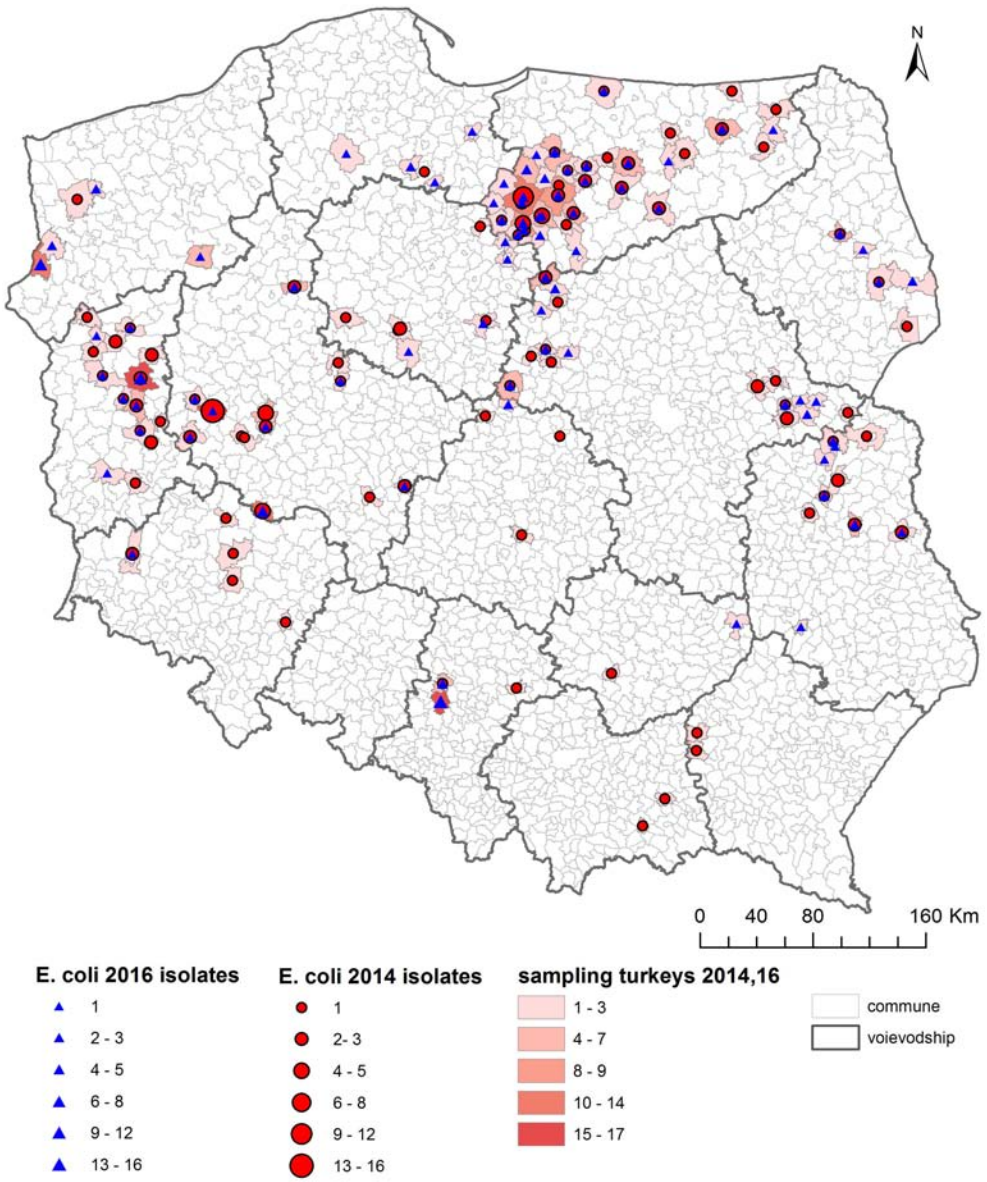

FIGURE 4 | Geographical distribution (commune level) of turkey sampling. Single turkey caeca was collected at slaughter and farm of origin was retrieved for verification of randomization of samples used for isolation of indicator *E. coli* in 2014 and 2016.

of mutations in *gyrA* (S83L and D87N) and *parC* (S80I and E84G) (U14_0810). A 21.3% portion of the isolates carrying quinolone resistance mechanisms were also confirmed as ESBL- or AmpC-producers.

A variety of gentamicin resistance genes was identified. The sequences revealed genes coding N-acetyltransferases catalyzing acetyl CoA-dependent acetylation of an amino group, like *aac(3′)-IIa* (n = 8) and *aac(3′)-IId* (n = 5), and O-phosphotransferases (APH) catalyzing ATP-dependent phosphorylation of a hydroxyl group, namely *aph(3′)-Ia* (n = 14) (**Supplementary Table S1**). Overall, there was 93.8% genotype–phenotype correlation for gentamicin resistance. The presence of genes coding adenyltransferases [*aadA1, aadA2, aadA5, aadA24*, and *ant(2")-Ia*] was identified in 50 isolates (**Supplementary Table S1**). WGS data showed the occurrence of three genes responsible for macrolide resistance: *mph*(B), *mph*(A), and *msr*(E)-*mph*(E) in single isolates with MICs equal to 8, 16, and 32 mg/L, respectively. Sixty isolates were resistant to sulfonamides due to *sul1* (n = 32), *sul2* (n = 39), or *sul3* (n = 18). In 5 isolates all three genes occurred simultaneously, while in 23 a set of two genes was found with *sul*1 and *sul2* being the most frequent (n = 19). Of the 49 trimethoprim-resistant *E. coli*, 45 harbored at least one of the following genes: *dfrA1* (n = 33), *dfrA12* (n = 2), *dfrA14* (n = 5), *dfrA15* (n = 1), *dfrA16* (n = 1), and *dfrA17* (n = 5).

At least one of the tetracycline resistance genes *tet*(A) or *tet*(B) was carried by 73 isolates, these genes being found, respectively

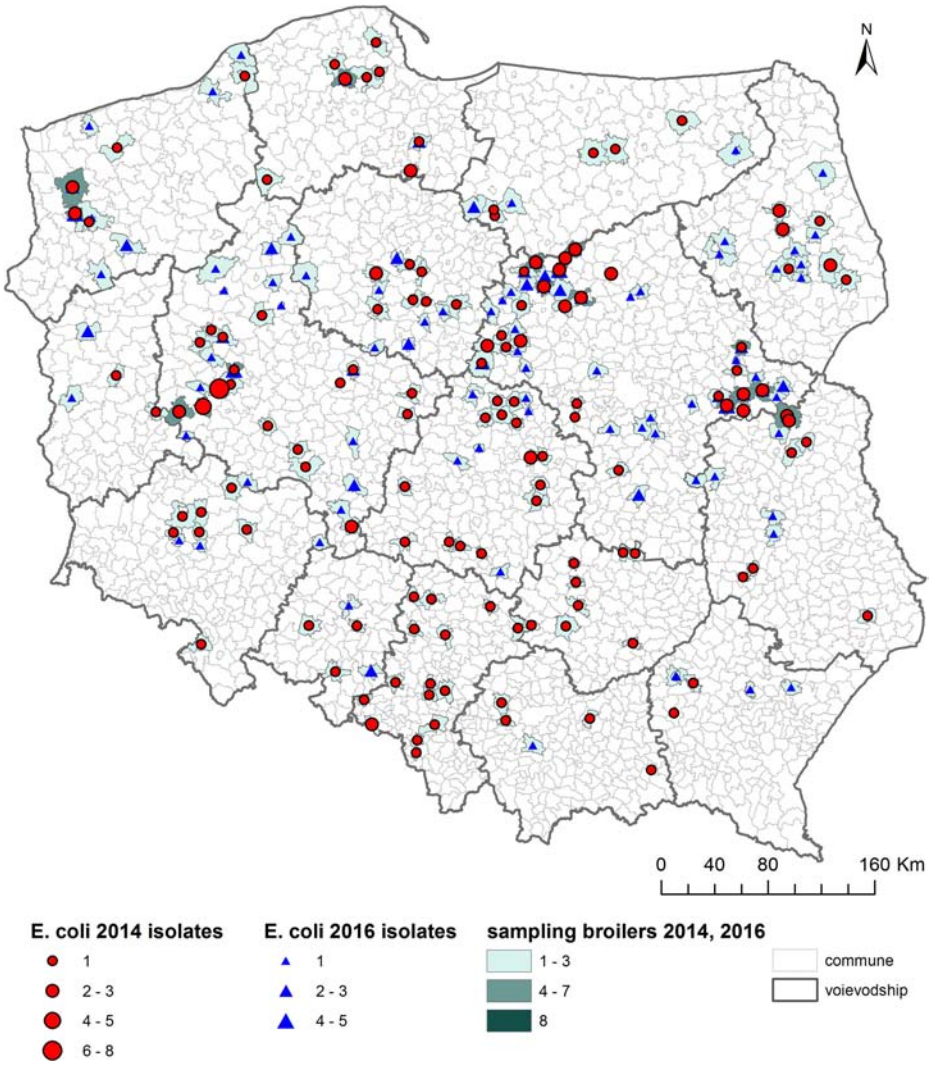

FIGURE 5 | Geographical distribution (commune level) of broiler sampling. Single broiler caeca was collected at slaughter and farm of origin was retrieved for verification of randomization of samples used for isolation of indicator *E. coli* in 2014 and 2016.

in 60 and 18 *E. coli*. In five isolates both genes were detected and *tet*(M) was additionally identified in one of them. Overall, there was 100% genotype–phenotype correlation for tetracycline resistance. In 31 isolates the presence of *catA1* (n = 13), *catB3* (n = 2), *cmlA1* (n = 18), and *floR* (n = 10) was confirmed. In four isolates the resistance genes were present despite a lack of phenotypic resistance to chloramphenicol. Two of them possessing the *cmlA1* gene had MIC = 16 mg/L, one isolate had two point mutations in *cmlA1* and in the last case a fragment of the *catB3* gene was missing (short contig length).

Plasmids and Location of the *mcr-1* Gene

Escherichia coli positive for *mcr-1* carried a wide variety of plasmid incompatibility group replicons in different proportions and ranging from 4 up to 11 replicons per strain (**Supplementary Table S1**). The most frequent were: IncFIB (AP001918) (n = 64), ColRNAI (n = 47), Col (MG828) (n = 43), IncFII (n = 40), IncI1 (n = 30), p0111 (n = 24), IncFIC (FII) (n = 21), Col156 (n = 17), IncX1 (n = 17), IncHI2A (n = 16), IncQ1 (n = 14), and IncN (n = 9). Plasmid replicons of all other identified plasmids are noted in **Supplementary Table S1**.

Sixty-one isolates out of the 80 *mcr-1*–positive *E. coli* (76.3%) harbored plasmids of the IncX4 group with the replicon located on the same contig of the *mcr-1* gene (hereafter IncX4–*mcr-1* contigs). In most cases, the *mcr-1* gene was the only resistance gene found on IncX4–*mcr-1* contigs, which ranged in size from 10772 to 39252 bp. The isolates U16_0149 and U16_0323 also contained the *qnrS1* and bla_{TEM-1} genes and IncX1 replicon located on the IncX4–*mcr-1* contig (contig sizes 76785 bp and 69841 bp, respectively). IncX4–*mcr-1* contigs were of high sequence similarity and clustered independently of the sample isolation source and sampling year (**Figure 8**).

In five of the *mcr-1*–positive *E. coli* isolates (6.3%), IncHI2 plasmids were found to be *mcr-1* carriers. In 4 out of 5 cases

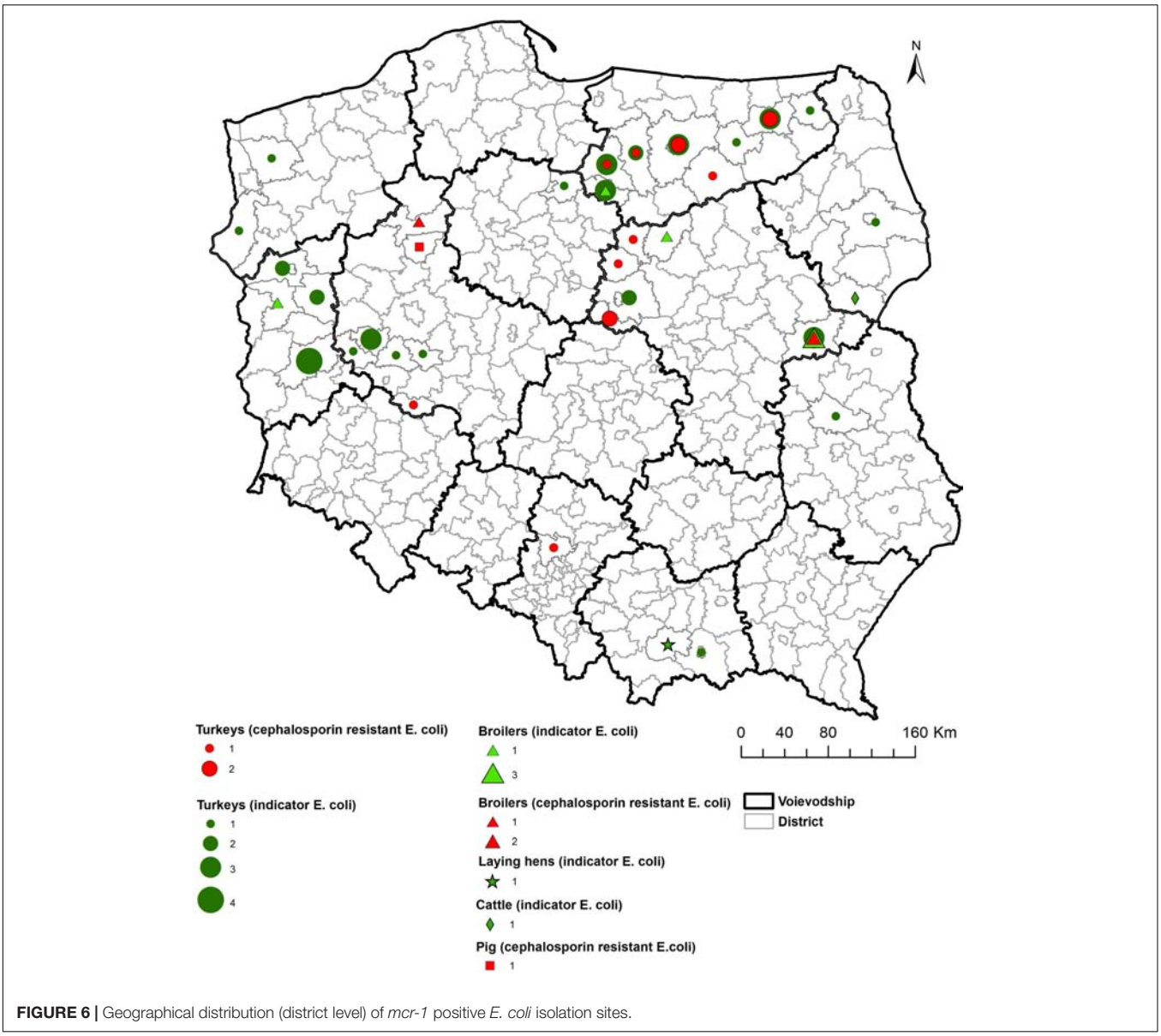

FIGURE 6 | Geographical distribution (district level) of *mcr-1* positive *E. coli* isolation sites.

the *mcr*-carrying plasmid was identified using PacBio data as it was not possible to link the plasmid replicon with the *mcr-1* gene using only short Illumina reads. All the IncHI2–*mcr-1* plasmids were subtyped as pST4. On the IncHI2–*mcr-1* contig (234213 bp) of the U16_0565X isolate additional resistance genes were identified as follows: *aadA1*, *aadA2*, bla_{TEM-1B}, *catA1*, *cmlA1*, *sul1*, and *tet*(A). This isolate possessed the $bla_{TEM-52C}$ gene located on the other contig (8917 bp). On the IncHI2–*mcr-1* contig (235356 bp) of U16_0288X, *aac(3)-IIa, aadA1, aadA2*, bla_{TEM-1C}, *sul1, cmlA1, dfrA1, tet*(A), *aph(3″)-Ib,* and *aph(6)-Id* were also found. The presence of the *aph(3′)-Ia* gene was confirmed on the relevant contig (201917 bp) of U16_0579. No other plasmid replicons except IncHI2 were annotated on those contigs.

Three isolates (3.7%) possessed both the IncHI2 and IncX4 replicons, but *mcr-1* was associated with IncX4. In one strain (U16_0259) a chromosomal location of the *mcr-1* gene was confirmed (data not shown). In 15.0% (n = 12) of isolates no plasmid replicons were found on contigs carrying the *mcr-1* gene (ranging in size from 2587 to 57048 bp) but the presence of the IncX4 or IncHI2 replicon in the assembly was confirmed. A curiosity is that in two *E. coli* (ID U16_0115 and U16_0115X) isolated from the same sample, the *mcr-1* genes were located on different incompatibility group plasmids (IncX4 or IncHI2).

Virulence Genes

The virulence genes were variable among isolates (**Supplementary Table S1**). Of the 80 *E. coli* sequences, six contained one virulence gene, whereas the remainder carried up to 10 virulence genes. The most common were: *gad* (n = 72), *iss* (n = 62), *iroN* (n = 56), *lpf*A (n = 41), *cma* (n = 28), *mchF* (n = 25), *astA* (n = 19), *air* (n = 15), *eilA* (n = 13), and *tsh* (n = 10),

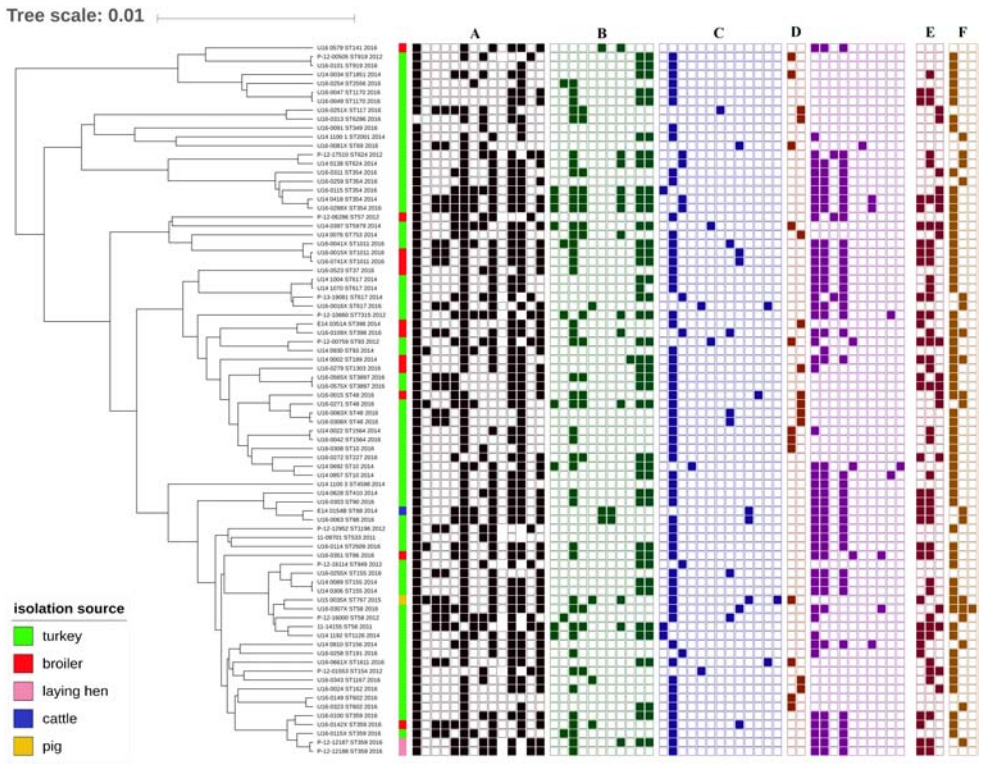

FIGURE 7 | Phylogeny of *mcr-1*-positive *E. coli* (sequence types, year and source of isolation, and map of phenotypic resistance and resistance genes). Full and empty square mean presence and absence of antimicrobial resistance (AMR) gene, respectively, whereas empty space means non-defined or not tested. **(A)** Resistance profiles (black): ampicillin, azithromycin, cefuroxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, colistin, nalidixic acid, meropenem, sulphonamides, tetracycline, tigecycline, and trimethoprim (Sensititre EUSVEC MIC panel). **(B)** Aminoglycoside resistance genes (green): *aac*(3)-IIa, *aac*(3)-IId, *aad*A1, *aad*A2, *aad*A5, *aad*A24, *aad*B, *aph*(3′)-Ia, *aph*(3′)-Ic, *aph*(3″)-Ib, and *aph(6)*-Id. **(C)** Beta-lactam resistance genes (blue): bla_{TEM-1A}, bla_{TEM-1B}, bla_{TEM-1C}, bla_{TEM-30}, $bla_{TEM-135}$, $bla_{TEM-52C}$, bla_{CMY-2}, bla_{SHV-12}, bla_{OXA-1},bla_{CARB-2}, and $bla_{CTX-M-1}$. **(D)** Quinolone resistance genes: plasmid-mediated quinolone resistance (PMQR, brown): *qnrS1*, *qnrB19*; mutations in quinolone resistance determining regions (QRDR, violet): *gyrA* S83L, *gyrA* D87N, *gyrA* D87Y, *parC* S80I, *parC* S80R, *parC* S57T, *parC* E84G, *parC* E84K, *par*E L416F, and *par*E L460D. **(E)** Sulphonamide resistance genes (dark brown): *sul*1, *sul*2, and *sul*3. **(F)** Tetracycline resistance genes (light brown): *tet*(A), *tet*(B), and *tet*(M).

whereas *cba, celb, ireA, vat, cap*U, *iha*, and *mcmA* were found in single isolates. We found no correlation of virulence genes with sample source or sampling year. Isolates were characterized with different sets of virulence genes. Notably, one isolate (U14_0002) presented a unique set of virulence genes (*cif, eae, espA, espB, espF, nleB, tccP*, and *tir*) that designated it as atypical enteropathogenic *E. coli* (aEPEC) group (**Supplementary Table S1**).

DISCUSSION

Based on screening of colistin MIC values in *E. coli* derived from various monitoring programs on AMR in 2011–2016, we collected extensive information about the occurrence of the *mcr-1* gene in *E. coli* isolated from food-producing animals in Poland. Detailed characterization of *mcr-1*–positive isolates from several hosts, different geographical locations, and a range of sampling years included analysis of the phenotypic AMR to a broad range of antimicrobials and its genetic background, the presence of virulence genes, plasmid replicons, and ST identification.

Many European countries reported the occurrence of colistin resistance in *E. coli* deriving from both humans and animals (Hasman et al., 2015; Irrgang et al., 2016; Malhotra-Kumar et al., 2016; Perrin-Guyomard et al., 2016; Carattoli et al., 2017; Duggett et al., 2017; Hartl et al., 2017; Kawanishi et al., 2017; Apostolakos and Piccirillo, 2018). In Poland, we observed a slight increase in colistin resistance in *E. coli* and also in the prevalence of *mcr*-positive isolates originating from healthy livestock from 0.7 to 1.7% and 0.2 to 3.7%, respectively in the analyzed time frame, irrespective of the animal of origin. The overall occurrence of colistin resistance in turkeys was higher than in chickens but it still remained low compared to data from some European countries (European Food Safety Authority [EFSA], 2018).

Escherichia coli totaling 53 *mcr*-positive *E. coli* were identified after including isolates with $MIC_{colistin}$ = 2 mg/L, which is the EUCAST epidemiological cut-off delimiting the wild-type population. Applying this criterion, the prevalence was 3.7% *mcr-1*–positive *E. coli* rather than 0.8% in Poland in 2016. Detection of the *mcr* gene in wild-type isolates was reported (Fernandes et al., 2016; Lentz et al., 2016; Hadjadj et al., 2017;

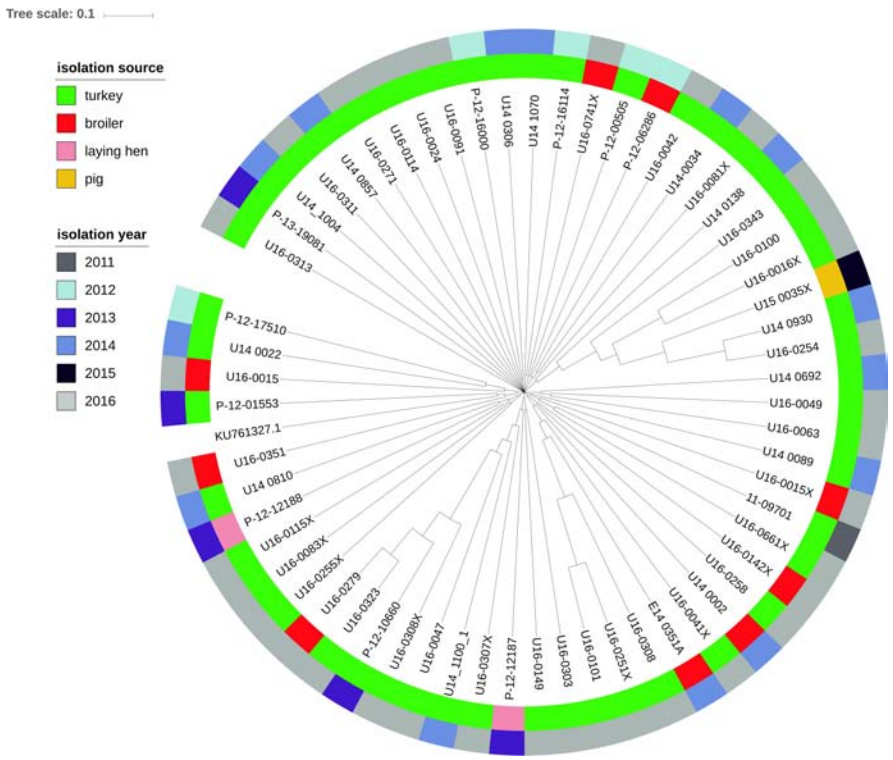

FIGURE 8 | Phylogenetic relationship of contigs with *mcr-1* and IncX4 plasmid replicon extracted from the genomes of 61 *E. coli* isolates by source and year of *E. coli* isolation. Complete sequence of IncX4 plasmid with accession number KU761327.1 from GenBank was used as the reference.

Wang et al., 2017; Zhou et al., 2017) and might result from a non-functional *mcr-1* gene (Terveer et al., 2017). Some reports indicate the possibility of deactivation of *mcr-1* by insertion of an IS1294b element and its reactivation by the loss of that element under colistin selection pressure (Zhou et al., 2018). In the current study, all of the *mcr-1* had the typical sequence of the *mcr-1.1* gene. In some cases, the wild-type concentration $MIC_{colistin}$ = 2 mg/L could result from a limitation in the MIC determination method where one dilution step difference is permissible. It should therefore be considered during selection of suspected isolates. Except for one isolate, the presence of *mcr-1* was not associated with a high level of resistance (MIC > 4 mg/L) to colistin and the presence of a chromosomal resistance mechanism in one of the isolates did not lead to elevated colistin MIC values either (MIC = 2 mg/L). There was a noted presence in Brazil of the *mcr-1* gene in wild type isolates derived from poultry confirmed as never exposed to polymyxin during their entire lives (Lentz et al., 2016).

In some cases the lack of genotype–phenotype correlation in isolates with resistance genes but without phenotypic resistance to chloramphenicol could result from the limitation of the MIC method. In others the reason could be substitutions found in the relevant gene. In an isolate carrying the *catB3* gene the lack of genotype–phenotype correlation could not be identified due to lack of a fragment gene at one end of the contig.

Despite several *mcr*-types and their variants being described in isolates from animals across Europe (Rebelo et al., 2018), our study suggests only *mcr-1.1* being present in Polish livestock, the first cases dating back to 2011. For yet unknown reasons, but in concordance with data from Germany and France, the highest occurrence of *mcr-1*–positive *E. coli* was detected in turkeys (Irrgang et al., 2016; Perrin-Guyomard et al., 2016). We speculate it could be related to the longer life span of these animals compared to chickens, and consequently to a longer length of exposure to selective pressure favoring antibiotic resistance. Colistin is used for treatment of gastrointestinal infections in animals, but in some countries low doses may be used as a growth promoter (Kempf et al., 2013; Fernandes et al., 2016). However, this practice is not allowed in Poland or the other EU countries (European Medicine Agency [EMA] and European Surveillance of Veterinary Antimicrobial Consumption [ESVAC], 2017). The proliferation of *mcr-1*–carrying *E. coli,* only occasionally found in 2011 but reaching a case count of several dozen by 2016, raises the question of the effects of excessive colistin use in animal husbandry. Worth noting is that in Poland, unlike other animal species, most of the turkey population is raised from imported one-day-old poults or hatching eggs and it might be an additional way for resistant isolates to be introduced to Polish farm environments. Some research indicates that the introduction of resistant bacteria may have been through imported breeding animals (Mo et al., 2014).

Horizontal transfer via plasmids plays an important role in the dissemination of antibiotic resistance genes. The IncX4 plasmid

is considered one of the most prevalent carriers of the *mcr-1* gene in *Enterobacteriaceae* (Johnson et al., 2012; Matamoros et al., 2017; Sun et al., 2017). Our study showed that *mcr-1* was associated with IncX4 plasmids in the vast majority (76.3%) of isolates, and with IncHI2 plasmids, another well-known *mcr-1* vector (Matamoros et al., 2017; Sun et al., 2018) in a few (6.3%) isolates. The fact that in one sample two different *E. coli* were found with *mcr-1* located on different plasmids (IncX4 and IncHI2) might evidence a parallel route of resistance spread but we cannot exclude transfer across the plasmids. Furthermore, occurrence of the *mcr-1* gene on the chromosome shows that plasmid-mediated colistin resistance genes might become fixed into specific *E. coli* populations and spread vertically.

Most of the tested isolates were genetically unrelated, which has also been observed in other reports on *mcr-1*–positive *E. coli* (Veldman et al., 2016). One of the reported *E. coli* ST 10, identified in 3 isolates, has been previously described in relation to *mcr-1* (Yang et al., 2017), and is considered a reservoir of this gene (Matamoros et al., 2017). The STs exhibited genetic diversity and were not related to animal source, geographic area, or isolation year. The identification of the same ST (i.e., ST919, ST354, and ST1564) in strains deriving from the same animal source but isolated in different years, or even in strains isolated from different species and in some cases harboring the *mcr-1* gene on different plasmids proves the wide dissemination of plasmid-mediated colistin resistance over the whole country. The study shows, in the light of the ESVAC data on colistin sales (European Medicine Agency [EMA] and European Surveillance of Veterinary Antimicrobial Consumption [ESVAC], 2017), that the phenomenon is probably a result of wide colistin selection pressure and plasmid dissemination, and not due to the spread of specific bacterial clones (El Garch et al., 2017; Wang et al., 2017). In Poland, sales of colistin still remain above the maximum sale target (European Medicine Agency [EMA] and European Surveillance of Veterinary Antimicrobial Consumption [ESVAC], 2018). External introduction, transmission of plasmids, and dissemination under selection pressure create the potential for the *mcr-1* gene to become established in Polish food-producing animals.

Of significance is that almost all *mcr-1.1*–positive isolates were MDR including the compounds considered CIA (World Human Organization [WHO], 2017). They carried a range of genes encoding resistance to cephalosporins and quinolones. Some reports have demonstrated the presence of the *mcr-1* gene together with ESBL genes (Robin et al., 2017; Yamaguchi et al., 2018). Therefore *mcr-1*–positive *E. coli* should be considered a reservoir not only of the colistin resistance gene, but also of those of PMQR, and ESBL or sets of other resistance genes carried along with *mcr-1* on some plasmids. This is supported by our finding of the genes encoding for resistance to beta-lactams, including cephalosporins, aminoglycosides, or sulphonamides located on the same contig as *mcr-1.1* and IncHI2 replicon. This is a serious concern for veterinary medicine and also for human health since direct transmission of resistant isolates from animals to humans has been confirmed (Marshall and Levy, 2011). The genes found in the current study did not differ from the ones identified previously in *E. coli* occurring in the healthy animal population (Wasyl, 2014; Lalak et al., 2016). The $bla_{CTX-M-15}$ gene, which occurs in isolates responsible for nosocomial infections in Poland (Empel et al., 2008) was found in this study in only a single pig isolate.

The aEPEC (atypical enteropathogenic *Escherichia coli*) isolates are a cause of diarrhea in both humans and animals (Afset et al., 2004; Almeida et al., 2012). Here, in the collection of non-clinical *E. coli* isolates from healthy animals, we identified *mcr-1.1* in a single chicken strain surprisingly carrying several virulence determinants of the aEPEC phenotype, namely EAST1, cell cycle inhibiting factor, intimin adherence protein Eae, secreted proteins EspA, EspB, and EspF type III secretion system effector NleB, Tir-cytoskeleton coupling protein, and translocated intimin receptor Tir. The strain carried also additional AMR genes combining to afford resistance to 4 classes. Since the *mcr-1.1*–positive, multidrug resistant aEPEC should be considered a vector of both resistance determinants and pathogens, this finding is worrisome for successive treatment of animals or humans.

CONCLUSION

The results highlight that poultry, especially turkeys, can be an important reservoir of *mcr-1.1*–carrying *E. coli* strains in Poland. Our findings indicate an increasing occurrence of *mcr-1.1* in *E. coli* from turkeys and, to a lesser extent, chickens in Poland from 2011 to 2016, whereas cases in pigs and cattle appear to be sporadic in the study period. The *mcr-1.1* gene occurred mainly on the IncX4 and IncHI2 plasmids in a wide diversity of *E. coli* harboring multiple resistance genes, virulence genes, and various plasmid replicons. Thus, dissemination of *mcr*-positive plasmids is a probable pathway for plasmid-mediated colistin resistance to spread in food-producing animals. The impressive genetic diversity of isolates as well as the association of colistin resistance with particularly relevant phenotypes (e.g., third-generation cephalosporin and fluoroquinolone resistance as well as aEPEC) call for urgent reduction in the use of colistin to avoid further selection of co-resistance in *E. coli* in animal production and possible animal and public health consequences. Definitely excluding isolates that are currently considered wild-type might contribute to silent dissemination of the *mcr*-positive ones. Great attention should be given to continuous phenotypic and genotypic surveillance of AMR and data collection in both human and veterinary settings, thus enabling intervention to counteract any rapid dissemination of *mcr-1.1*–positive *E. coli*.

AUTHOR CONTRIBUTIONS

MZ and DW designed the experiments. MZ, PS, DW, and AZ-B analyzed the resistance and genotypic data. MZ, PS, and AZ-B prepared the tables and figures. MZ, PS, and DW prepared the manuscript. All authors discussed the results, reviewed and edited the manuscript, read, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01753/full#supplementary-material

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Corrigendum: Occurrence and Characterization of *mcr-1*-Positive *Escherichia coli* Isolated From Food-Producing Animals in Poland, 2011–2016

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Combinatory Therapy Antimicrobial Peptide-Antibiotic to Minimize the Ongoing Rise of Resistance

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THE ANTIBIOTIC RESISTANCE CRISIS

Antibiotics are cytotoxic or cytostatic compounds, very effective, harmful and specific against pathogenic microorganisms that have saved millions of lives and increased human life expectancy and quality (Zaman et al., 2017). Nevertheless, we have almost reached a post-antibiotic era, where even simple infections have become untreatable due to the remarkable rise of resistance (Chaudhary, 2016).

Antibiotic resistance is the ability of microorganisms to withstand the effect of medicines. Although an inevitable natural phenomenon, the abusive use of antibiotics has provided constant selection pressure and accelerated the emergence of highly resistant strains (Richardson, 2017). It is perhaps not intuitive, but it is estimated that the vast majority of all antibiotics produced is used improperly in the food-animal sector to promote rapid growth and prevent infectious diseases, rather than in human medicine (Landers et al., 2012). This article focuses on the main aspects of the combinatory therapy antimicrobial peptide (AMP)-antibiotic to treat infectious diseases.

COMBINATORY THERAPY AMP-ANTIBIOTIC

Antibiotic resistance management is an attempt to decrease the resistance rate. It demands both the limitation of antibiotic use and the application of more efficient infection therapies. Since antibiotic exposure time correlates with the development of resistance, effective therapies should include drugs with rapid death kinetics and broad spectra of action. Such requirements are mainly found in combinatory therapies, which in contrast to monotherapies, simultaneously employs different drugs to treat a particular disease. Combining different drugs mainly leads to synergism or antagonism. In a synergistic response, the combination has a considerably stronger effect than single drugs would, more than just an additive effect. It meaningfully improves clinical outcomes and decreases the probability of resistance evolution since it is unlikely that a pathogen simultaneously develops resistance to multiple antibiotics (Xu et al., 2018). Correctly choosing the combination cocktail is a crucial step and AMPs have been increasingly recognized as a promising class of compounds to be used in combination with classic antibiotics for the treatment of various infections (Lewies et al., 2018).

AMPs are composed of amino acids, typically 5–50 residues, and produced by all classes of multicellular organisms as an essential part of the innate immune response. Usually, they target a broad range of essential metabolic processes of bacterial and fungal cells. The main characteristic of most AMPs is its positive net charge, which allows for the interaction with negatively charged components of the cell wall and plasma membrane. Following interaction, amphipathic AMPs insert into the membrane, a process driven by the presence of hydrophobic amino acids. Subsequent membrane disruption occurs by a variety of mechanisms, leading to loss of its integrity and,

ultimately, cell death (Carvalho et al., 2015). In addition to membrane-lytic activities, AMPs also exert intracellular inhibitory activity by interfering with diverse essential processes as protein biosynthesis, cell division, cell-wall biosynthesis and nucleic acid metabolism. Furthermore, in complement to antimicrobial activities, AMPs also modulate the immune response stimulating cytokine production, acting as chemokines, and promoting wound healing (Bechinger and Gorr, 2017).

Presumably, the crucial advantage of AMPs is that they are described as less prone to induce resistance as most have multiple targets and rarely interact with a specific receptor. Among those with a single target, most act on the membrane where resistance evolution is more unlikely to occur (Sierra et al., 2017). However, in exceptional cases involving specific protein interactions, the possibility of genetic mutation and resistance development is a significant event but unlikely in combinatory therapy AMP-antibiotic due to the magnitude of different targets involved. Moreover, this rare event can be overcome by slight structural modifications that is easy and rapid for AMPs due to the tremendous progress made in solid phase peptide synthesis. Such advance resulted from the availability of low-cost high-quality building blocks and coupling reagents, establishment of efficient approaches and protocols to speed up peptide assembly, and the development of fully automated synthesizers (Mijalis et al., 2017).

In the last few years, several studies have demonstrated the benefits and advantages of combinatory AMP-antibiotic therapy, which include the successful elimination of multidrug-resistant (MDR) and biofilm-forming organisms, a significant lower outcome of resistance development, reduction of single doses and a decrease in side effects (Lewies et al., 2018). Perhaps one of the leading causes of resistance development is the low microbial cell membrane permeability to antibiotics (especially the outer membrane of gram-negative bacteria that is primarily composed of polyanionic lipopolysaccharides) and since most AMPs act on membranes, perturbing their structures, the combinatory therapy AMP-antibiotic arises as an efficient tool to increase antibiotic bioavailability (Li et al., 2017). Indeed, recent studies have shown that in particular cationic AMPs, such as LL-37, piperacillin, buforin II, ceprocin P1, indolicidin, nisin, and magainin II, are remarkably effective in combination with antibiotics like polymyxin E, piperacillin, azithromycin, daptomycin, linezolid, and clarithromycin to enhance antibiotic bioavailability against highly multidrug-resistant gram-negative and methicillin-resistant *S. aureus* (MRSA) pathogens (Giacometti et al., 2000; Mataraci and Dosler, 2012; Lin et al., 2015). These studies are of enormous importance as increasing bioavailability reduces the required antibiotic concentration and, consequently, the probability of resistance development.

More than to enhance oral bioavailability, the strong membrane permeabilization capacity of AMPs can directly kill even dormant biofilm-forming cells in combination with classical antibiotics. An example demonstrating the efficacy of AMP-antibiotic therapy to remove biofilm is the treatment of *Pseudomonas aeruginosa* (*P. aeuruginosa*) with carbapenems. Such antibiotics belong to the class of broad-spectrum antimicrobials routinely used for the treatment of infections caused by multidrug-resistant *P. aeuruginosa* that leads to chronic diseases. Recently, a novel synthetic cyclolipopeptide analog of polymyxin (AMP38) was tested in combination with carbapenems, and the synergistic effect was observed to cause the killing of biofilm-forming and carbapenem-resistant *P. aeruginosa* (Rudilla et al., 2016). Since biofilm represents an enormous obstacle in antibiotic-therapy, this area has recently received increased attention from the scientific community, given the high number of reports demonstrating the benefits of combination AMP-antibiotics for the treatment of biofilm-forming organisms (Reffuveille et al., 2014; Ribeiro et al., 2015; Grassi et al., 2017).

Besides affecting membrane integrity, some AMPs also have intracellular targets. For instance, arenicin-1 in combination with a broad spectrum of antibiotics increases drug bioavailability and promotes oxidative stress by depletion of NADH (Choi and Lee, 2012). Similarly, buforin II was primarily shown to act on the membrane, but as it was demonstrated later, it also interacts with DNA, interrupting DNA and RNA metabolisms (Sim et al., 2017). It is important to note that since most AMPs have multiple cell targets, their mechanisms of action are strictly dependent on the concentration. For instance, studies conducted with pleurocidin has shown that, at its lowest inhibitory concentrations, this is less able to damage cell membranes but capable of inhibiting macromolecular synthesis (Patrzykat et al., 2002). Indeed, typically, AMPs cause membrane lysis at high concentrations and no-membrane lysis at low concentrations (Cudic and Otvos, 2002).

It is also essential to emphasize that the combinatory therapy AMP-antibiotic is effective to treat diseases caused by MDR organisms as for such proposes the essential requirement of the drug cocktail is to have components with different killing mechanisms. For example, the combination of the antimicrobial peptide DP7 with azithromycin or vancomycin was shown to eradicate some antibiotic-resistant bacteria like *Staphylococcus aureus* (*S. aureus*), *P. aeruginosa,* and *Escherichia coli* (*E. coli*) (Wu et al., 2017). Analogously, the AMP SET-M33 was extremely effective against a set of gram-negative MDR organisms as *Klebsiella pneumoniae* (*K. pneumoniae*), *P. aeruginosa* and *Acinetobacter baumannii* (*A. baumannii*), especially in combination with rifampin (Pollini et al., 2017). Even in cases where the AMP alone has just a moderate antimicrobial activity, its combination with antibiotics was effective against MDR organisms. Indeed, as recently shown, a combination of ASU014, a bivalent branched peptide with moderate activity against *S. aureus*, with oxacillin was also very efficient against MRSA. The synergism between both meaningfully improved the killing effect as compared to single drugs, so that lower peptide concentrations and sub-MIC doses of the antibiotic were required for the complete eradication of the pathogen (Lainson et al., 2017).

Closely related to resistance is persistence, a phenomenon in which microorganisms become insensible toward lethal antibiotic doses not due to genetic acquired modifications, but by entering in a dormant and drug-tolerant state. This state is transient and lasts as long as the stress condition endures. Consequently, persistence is directly related to chronic and

recurrent infectious diseases. The mediation of persistence occurs by the signaling molecule ppGpp in response to environmental stress as the presence of antibiotics (Pollini et al., 2017). A recent study of the synergistic effect between a broad set of AMPs and antibiotics like ciprofloxacin, meropenem, erythromycin, and vancomycin for treating infections caused by clinical hard-to-treat pathogens, including all ESKAPE (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter cloacae*) pathogens, revealed the ability of AMPs to elicit degradation of ppGpp, avoiding the entry in an energy-starved state. This is a significant finding as potentially all microorganisms react to antibiotic treatment mediating persistence, and the fact that some AMPs can prevent it opens new doors for the development of alternative therapies that effectively decrease the resistance rate (Pletzer et al., 2018).

In addition to all those benefits, some AMPs can confer protection by acting as potent immune regulators by means of chemokine, inhibiting pro-inflammatory cytokine production and modulating the response of the adaptive immune response via the regulation of T cells (Diamond et al., 2009). In this regard, LL-37 is probably the most tested AMP in combinatory therapy. A study performed to assess the antibacterial activity of amoxicillin with clavulanic acid and amikacin against different clinical isolates of *S. aureus* revealed that the killing effect of the antibiotic alone was strongly potentiated by the addition of synthetic LL-37 (Leszczyńska et al., 2010). Similarly, the anti-tuberculosis antibiotics isoniazid and rifampicin were shown to clear *Mycobacterium tuberculosis* (*M. tuberculosis*) from infected lungs, liver, and spleen, more efficiently in combination with the human neutrophil peptide (HNP)-1 in comparison to when they were employed alone (Kalita et al., 2004).

In summary, the benefits of AMPs associated with the potency of conventional antibiotics in combinatory therapy can very efficiently favor the resolution of infections caused by MDR and biofilm forming microorganisms, enhances the natural immune response and decreases the likelihood of resistance.

BARRIERS FOR THE THERAPEUTIC USE OF AMPs

In clinical therapy, the most desirable route of drug administration is orally due to the relatively low cost of production and patient compliance (Zhu et al., 2017). However, before any drug reaches the bloodstream, and consequently its target, it will typically face many obstacles that include the mouth environment and the harsh gastric tract containing digestive enzymes, highly viscose mucosal layers, epithelial cells preventing the direct contact with the capillary and tight junctions between the epithelial, blocking the paracellular passage. For peptides, all those barriers restrict their ease of administration due to their low cell membrane permeability and limited stability toward proteolysis (Lewis and Richard, 2015). In fact, according to THPdb (http://crdd.osdd.net/raghava/thpdb/), a database for therapeutic peptides and proteins, only 4 % of all approved therapeutic peptides and proteins are administered orally. The following sections focus on the stability issue and the low membrane permeability of peptides in general and present some successful strategies that overcome the practical limitations of peptides as orally administered drugs.

Except cyclic and D-amino acid composed AMPs, the majority is linear and formed by natural L-amino acids. Thus, they are similar to food peptide/proteins and substrates of several digestive enzymes. Nevertheless, most proteases exclusively recognize the 20 natural L-amino acids and, consequently, the stability of many AMPs can be enhanced by the addition of chemical modifications, replacement of L-amino acids by their D-isomers (Remuzgo et al., 2014) and chain cyclization. In contrast to conventional antibiotics, AMPs tolerate more modifications while maintaining their activity. As already discussed, most AMPs form disruptive pores in the membrane, an event that is primarily driven by physical properties like net charge rather than by amino acids conformation. Thus, changing L-amino acids to the corresponding D-isomers usually does not impair AMP activity. Even in cases involving specific receptor-AMP interaction, replacements and modifications might not necessarily impair AMP action. Unlike classic antibiotics, the interaction surface AMP-receptor is usually more extensive, and the replacement of natural amino acids by non-natural analogs is less pronounced and, in some cases, can even improve the affinity. A study comparing the impact of many modifications has revealed that the addition of alpha-methyl amino acids and D-analogs confers to the peptide the most pronounced protection from proteolysis without activity loss (Werner et al., 2016).

In addition to the stability problem, oral delivery of AMPs is also challenging due to their poor cell membrane permeability. Once orally administered, AMPs should cross the gastrointestinal epithelium to reach the bloodstream. However, this is not so simple for hydrophilic molecules exceeding 700 Da (Fosgerau and Hoffmann, 2015). Nonetheless, it has been shown that the successful transport of different molecules like proteins, peptides or DNA across the biological membrane could be achieved by the simultaneous addition or fusion of the molecule of interest with a class of transcellular enhancers known as cell penetrating peptides (CPPs). Such molecules are short peptides able to cross cellular membranes via an energy-dependent or independent mechanism. Their chemical nature is diverse, but most CPPs are positively charged; a primary or secondary amphipathic character can also be implicated but is not strictly required for internalization. In fact, it has been reported that even octa-arginine can mediate cellular uptake when co-administered or in conjugation with a cargo molecule (Dinca et al., 2016). Conjugation of CPPs with clinically relevant molecules was reported. Examples include the combination of lipo-polyarginine with insulin that was shown to enhance the transport through Caco-2/HT-29 cells almost two-fold (Garcia et al., 2018). Moreover, the conjugation or co-administration of TAT and polynonaarginin with the parathyroid hormone has sharply increased the transport through Caco-2 cells (Kristensen et al., 2015). However, in cases where the AMP should act in the gastrointestinal tract, low bioavailability is desired as the MIC value is more likely to be reached by lower doses. Thus, only proteolytic stability remains a possible issue. For instance, surotomycin is a cyclic lipopeptide antibiotic active against

Clostridium difficile (*C. difficile*), and as clinical evidence has shown, its low oral bioavailability allows the gastrointestinal tract concentrations to considerably exceed its MIC for the pathogen (Knight-Connoni et al., 2016).

PERSPECTIVES

The combinatory therapy AMP-Antibiotic has increasingly attracted attention within contemporary studies due to its diverse benefits. The number of combination studies involving AMP-antibiotic has therefore been exponentially growing over the last few years (Jorge et al., 2017). However, the peptide permeability/stability problem remains the main obstacle for the use of peptides in clinical therapy. Currently, no straightforward solution is available, but great efforts have been made to develop targeted AMPs and to turn peptides into more appropriate drugs for oral use. In addition, the standardization of methods used to determine the synergism between AMPs and antibiotics, their interactions and the creation of antimicrobial combination networks has been facilitating combinatory studies (García-Fuente et al., 2018; Pemovska et al., 2018). Given the promising results obtained so far, the trend shows that the appeal of using combinatory therapy AMP-antibiotic will become even greater. It could represent the beginning of a modern and efficient era in the battle against infectious diseases.

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LP-C and NO-S are doctoral students who contributed to the preparation of the article. All authors contributed equally to this work.

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Prevalence and Characterization of Fluoroquinolone Resistant *Salmonella* Isolated From an Integrated Broiler Chicken Supply Chain

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The objectives of this study were to investigate the prevalence and fluoroquinolone resistant *Salmonella* isolated from an integrated broiler chicken supply chain and their molecular characterization. In total, 73 *Salmonella* isolates were recovered from a broiler chicken supply chain in Shanghai. *Salmonella* isolates were tested for susceptibility to 11 antimicrobial agents using the broth dilution method and were characterized using pulsed-field gel electrophoresis (PFGE). Then, the *Salmonella* isolates were examined for mutations in quinolone resistance-determining region (QRDR) of *gyrA*, *gyrB*, *parC*, and *parE*, and were screened for plasmid-mediated quinolone resistance (PMQR) genes. Lastly, we sequenced the plasmids carrying *qnrS1* in six *Salmonella* isolates from three sources (two isolated per source). Among 73 *Salmonella* isolates, 45 isolates were identified as *S*. Indiana, 24 were *S*. Schwarzengrund, 2 were *S*. Enteritidis, and 2 were *S*. Stanleyville. In addition, high rates of resistance were detected for nalidixic acid (41.1%) and ciprofloxacin (37.0%), while resistance to other test agents was diverse (2.0–100%). *S*. Indiana and *S*. Schwarzengrund isolates from different sources exhibited the same PFGE pattern, suggesting that the *Salmonella* isolates possessed high potential to spread along the broiler chicken supply chain. *gyrA* and *parC* exhibited frequent missense mutations. Moreover, *qnrS1* was the most prevalent PMQR gene in the 73 *Salmonella* isolates, and it was found about a new hybrid plasmid. This study concludes a high prevalence of fluoroquinolone resistant *Salmonella* in chicken supply chain, threatening the treatment of *Salmonella* foodborne diseases. In particular, the emergence of a new hybrid plasmid carrying *qnrS1* indicates that the recombination of plasmid carrying resistance gene might be a potential risk factor for the prevention and control strategies of drug resistance.

Keywords: *Salmonella*, fluoroquinolone resistance, *qnrS1*, hybrid plasmid, broiler chicken supply chain

INTRODUCTION

Salmonellosis, caused by *Salmonella*, is one of the most frequently reported foodborne illnesses worldwide. *Salmonella* is divided into more than 2500 serovars by the White–Kauffman and Le Minor scheme. This classification scheme defines the serogroup according to expression of somatic lipopolysaccharide O antigens, and the serovar by the expression of flagellar H antigens. *Salmonella*, as an important human pathogen, is a potential public health risk.

It has been estimated that *Salmonella* causes about 1.2 million illnesses in the United States every year. Food is a major source of these infections, accounting for 1 million illnesses, 19,336 hospitalizations, and 378 deaths (Scallan et al., 2011). The majority of human infections caused by *Salmonella* is associated with the consumption of food products. Chicken, as one of the most widely consumed meats, is an important reservoir of *Salmonella* (Adu-Gyamfi et al., 2012; Truong Ha and Yamaguchi, 2012). Importantly, antibiotic-resistant bacteria of animal origin could be transmitted to humans (Piddock, 2002; Khemtong and Chuanchuen, 2008), which adversely affect the treatment of salmonellosis. Therefore, it is necessary to monitor the epidemiology and genetic characteristics of *Salmonella* in the food chain.

Fluoroquinolones are widely used to treat salmonellosis in human and animal (Folster et al., 2015). Currently, the main mechanism underlying quinolone resistance is the accumulation of mutations in quinolone resistance-determining region (QRDR) of *gyrA*, *gyrB*, *parC*, and *parE* and plasmid-mediated quinolone resistance (PMQR), which includes five major groups of *qnr* determinants (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*), *aac(6′)-Ib-cr* and quinolone extrusion such as *qepA* and *oqxAB* (Strahilevitz et al., 2009). Some studies focused on fluoroquinolone resistance-related determinants about PMQR and QRDR in *Salmonella* derived from humans and animals (Wasyl et al., 2014; Wong et al., 2014). However, comprehensive data regarding fluoroquinolone resistance determinants in *Salmonella* from chicken supply chain are lacking, despite the implication for human health.

Thus, the aims of this study were to investigate the prevalence of *Salmonella* and their molecular characteristics related to fluoroquinolone resistance determinants, including PMQR and QRDR, in the broiler chicken supply chain in Shanghai. These data provide insight into the quantitative risk of resistant *Salmonella* from chicken supply chain.

MATERIALS AND METHODS

Statement of Ethics

This study was carried out in accordance with the ethical guide lines of the College of Veterinary Medicine, China Agricultural University, Beijing. Moreover, before the initiation of this study, formal approval was obtained by the departmental committee of institute. Sampling was carried according to the standard protocols and with prior consent of the farmer/manager of the facilities.

Salmonella Strains and Antimicrobial Susceptibility Testing

Salmonella isolates were recovered from three sources including adult broilers, broiler carcasses and retail chicken, representing vertically integrated commercial broiler chicken supply chain in Shanghai City, China. One sample was collected from each animal or meat product as appropriate. Caecal samples from adult broilers were randomly collected at the abattoir. Whole carcasses or meat samples were aseptically obtained from chicken processing chain. Carcasses from the retail chicken source were sampled from the markets. All samples were immediately transported to the laboratory in an insulated ice boxes containing ice packs. Microbiological procedures were performed immediately upon arrival at the laboratory. All test strains were isolated in CHROMagar *Salmonella* agar (CHROMagar Company, Paris, France). Suspected *Salmonella* colonies were confirmed by a PCR assay targeting the *invA* gene (Rahn et al., 1992). *Salmonella* serotyping was conducted by performing the slide agglutination test, using *Salmonella* antisera (S & A Reagents Lab Ltd., Bangkok, Thailand) according to manufacturer's instructions.

Salmonella isolates were subjected to antimicrobial susceptibility tests using standard broth dilution method of minimum inhibitory concentrations according to the guideline of the Clinical and Laboratory Standards Institute [CLSI] (2015a). Antimicrobial agents included 11 antimicrobials (i.e., amoxicillin/clavulanic acid, nalidixic acid, ampicillin, cefazolin, doxycycline, gentamicin, trimethoprim/sulfamethoxazole, chloramphenicol, ciprofloxacin, meropenem, and ceftriaxone). *Escherichia coli* ATCC 25922 was used as a quality control strain. The interpretive category for each isolate (susceptible, intermediate, or resistant) was determined according to the CLSI recommendations (Clinical and Laboratory Standards Institute [CLSI], 2015b).

Identification of Fluoroquinolone Resistance-Related Determinant

The DNA templates of isolates were prepared using TIANamp Bacteria DNA Reagent Kit (Tiangen, Beijing, China). The extracted DNAs were amplified by PCR assay. The mutations in *gyrA*, *gyrB*, *parC*, and *parE* genes were analyzed as described previously (Eaves et al., 2004). *Salmonella* isolates were screened for *oqxA*, *oqxB*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr*, and *qepA* genes. The primers and amplification conditions were described previously (Chen et al., 2012). PCR products were sequenced and identified.

Pulsed-Field Gel Electrophoresis (PFGE), S1 Nuclease Pulsed-Field Gel Electrophoresis (S1-PFGE), Southern Hybridization, Conjugation and Sequencing by Illumina

All *Salmonella* isolates were analyzed by PFGE method according to a previous protocol for subtyping *Salmonella* (Cui et al., 2016). According to the PFGE profile, six *Salmonella* isolates

carrying *qnrS1* were randomly selected from three sources (two isolates per source) for analysis by S1-PFGE and Southern hybridization, as previously described (Zhang et al., 2015). For the conjugation assay, six selected *Salmonella* isolates were used as donor strains, and sodium azide-resistant *E. coli* J53 was used as recipient strains. Both the donor strain and recipient strain were mixed on Luria-Bertani agar at a ratio of 1:3, and 100 μL mixtures were incubated for 16 h at 37°C. Transconjugants were selected on LB supplemented with sodium azide (100 mg/L) and ciprofloxacin (0.5 mg/L). Plasmids DNA were extracted from six *Salmonella* isolates transconjugants using by Wizard® Plus SV Minipreps DNA Purification Systems (Promega, Madison, WI, United States), then been sequenced by Illumina HiSeq 2500 system.

RESULTS

Prevalence and Characteristics of *Salmonella* Isolates in the Chicken Supply Chain

A total of 73 (7.7%) *Salmonella* isolates were recovered from 715 samples. The highest prevalence of (17.5%, 48 isolates) detected in 200 broiler carcass samples, followed by 127 retail chicken samples (8.7%, 16 isolates), while lowest prevalence (2.3%, 9 isolates) of *Salmonella* isolates was observed in 388 adult broiler samples. Among these, 45 isolates were identified as *S.* Indiana, 24 were *S.* Schwarzengrund, 2 were *S.* Enteritidis, and 2 were *S.* Stanleyville. Furthermore, all 73 *Salmonella* isolates were evaluated for susceptibilities to eleven antibiotics. The rates of resistance were 41.1% for nalidixic acid and 37.0% for ciprofloxacin, while, resistance to other test agents varied substantially (amoxicillin/clavulanic acid: 43.8%, ampicillin: 42.5%, cefazolin: 47.9%, doxycycline: 95.9%, gentamicin: 6.8%, trimethoprim/sulfamethoxazole: 100%, chloramphenicol: 43.8%, meropenem: 2.0%, and ceftriaxone: 12.3%). PFGE profiles are shown in **Figure 1**. Notably, some *S.* Indiana isolates from different sources exhibited the same PFGE pattern. Likewise, some *S.* Schwarzengrund exhibited the same PFGE pattern, suggesting that *Salmonella* isolates have high potential to spread along the broiler chicken supply chain.

Fluoroquinolone Resistance Determinants in the Chicken Supply Chain

Based on the detail information in **Supplementary Table S1**, mutations within QRDR of *gyrA*, *gyrB*, *parC*, and *parE* are summarized in terms of serotype in **Table 1** The presented data indicated that missense mutations frequently occurred in *gyrA* and *parC*, whereas silent mutations were observed in *gyrA*, *gyrB*, *parC*, and *parE*. Among 73 *Salmonella* isolates from this broiler chicken supply chain, 47 *Salmonella* isolates carried the wild-type (no mutation) within *gyrA* gene, missense mutation (Thr57Ser) within *parC* gene, 15 of which did not exhibit implicated in fluoroquinolone resistance phenotypes. The remaining 26 isolates contained missense mutations within *gyrA* (Ser83Phe and Asp87Asn) and *parC* (Thr57Ser, Ser80Arg). In addition, **Table 2** shows the distribution of fluoroquinolone resistance genes (*oqxA*, *oqxB*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac (6′)-Ib-cr*, and *qepA*). Among them, *qnrS1* (30/73) was predominant gene, following by *qnrB1* (22/73), *oqxA* (1/73), *oqxB* (1/73), and *aac (6′)-Ib-cr* (1/73). The genes sequences of *qnrS1*, *qnrB1*, *oqxA*, *oqxB*, and *aac (6′)-Ib-cr* were deposited in GenBank. Accession numbers were MK990505, MK990506, MK990507, MK990508, and MK990509, respectively. However, the other fluoroquinolone resistance genes were not observed. In total, 53 of 73 *Salmonella* isolates carried fluoroquinolone resistance genes, and *qnrS1* gene was detected in most *S.* Indiana (23/45), *S.* Schwarzengrund (5/22), and *S.* Enteritidis (2/2) isolates, accounting for the majority *Salmonella* isolates in the broiler supply chain.

Novel Plasmid Associated With *qnrS1*

The *qnrS1* was predominant gene in the chicken supply chain, thus, six *Salmonella* isolates from three sources (two isolates per source) were randomly selected to analyze the transmission mechanism of *qnrS1* according to the PFGE profile. S1-PFGE and Southern blot analyses (**Figure 2**) indicated that *qnrS1* is located on a ∼40 kb plasmid, designated pSH-01. Conjugation experiments by filter mating revealed that *qnrS1* could be co-transferred from *Salmonella* isolates to *E. coli* J53. S1-PFGE and Southern hybridization confirmed that the DNA probes specific for *qnrS1* hybridized to the same plasmids with a size ∼40 kb in both *Salmonella* isolates and their transconjugants (**Figure 2**). Then, the plasmids were extracted from transconjugants and sequenced using the Illumina MiSeq system. The analysis of the sequences showed that the plasmids are extremely similar with more than 99% identity, indicating that the plasmids from the different strains are indeed the same plamids. The NCBI BLAST results showed that the hybrid plasmid carrying *qnrS1* is a new plasmid type (pSH-01, submission number KY486279.1). It was 43,257 bp in length and harbored 51 predicted open reading frames. Furthermore, a plasmid sequence analysis (Carattoli et al., 2014) of pSH-01 indicated that it is an IncR type hybrid plasmid.

According to the BLAST results of pSH-01 nucleotide sequence against the NCBI database, the two junctions (**Figures 3B,C**) were often occurred, indicating that the three fragments are usually associated and transferred as a whole. These fragments were almost derived from plasmids. Notably, the genetic features of pSH-01 showed that the fragment carrying *qnrS1* (2849–14429) is derived from plasmids of one *Shigella flexneri* and six *E. coli* isolates (blast query cover: above 99% and blast identity: 99%). Another fragment (nt14429–31031) was derived from plasmids of *Salmonella* and other bacterial isolates, and an additional fragment (30758–43257, 1–2851) was mostly derived from plasmids of *Klebsiella pneumoniae*. The origin of target plasmids covered different bacterial host. According to an alternative sequence analysis, for example, it was postulated that pSH-01 might be hybrids of three plasmids (**Figure 3A**); the region spanning nt 2849–14429 matched with plasmid pEBG1 (KF738053; nt 37915–26335) of the *E. coli* strain, nt 14429–31031 shared a nucleotide identity of 99% to the plasmid p33676 (CP012682; nt 25123–41725) from *S.* Typhimurium, and nt 30758–43257, 1–2851 shared a

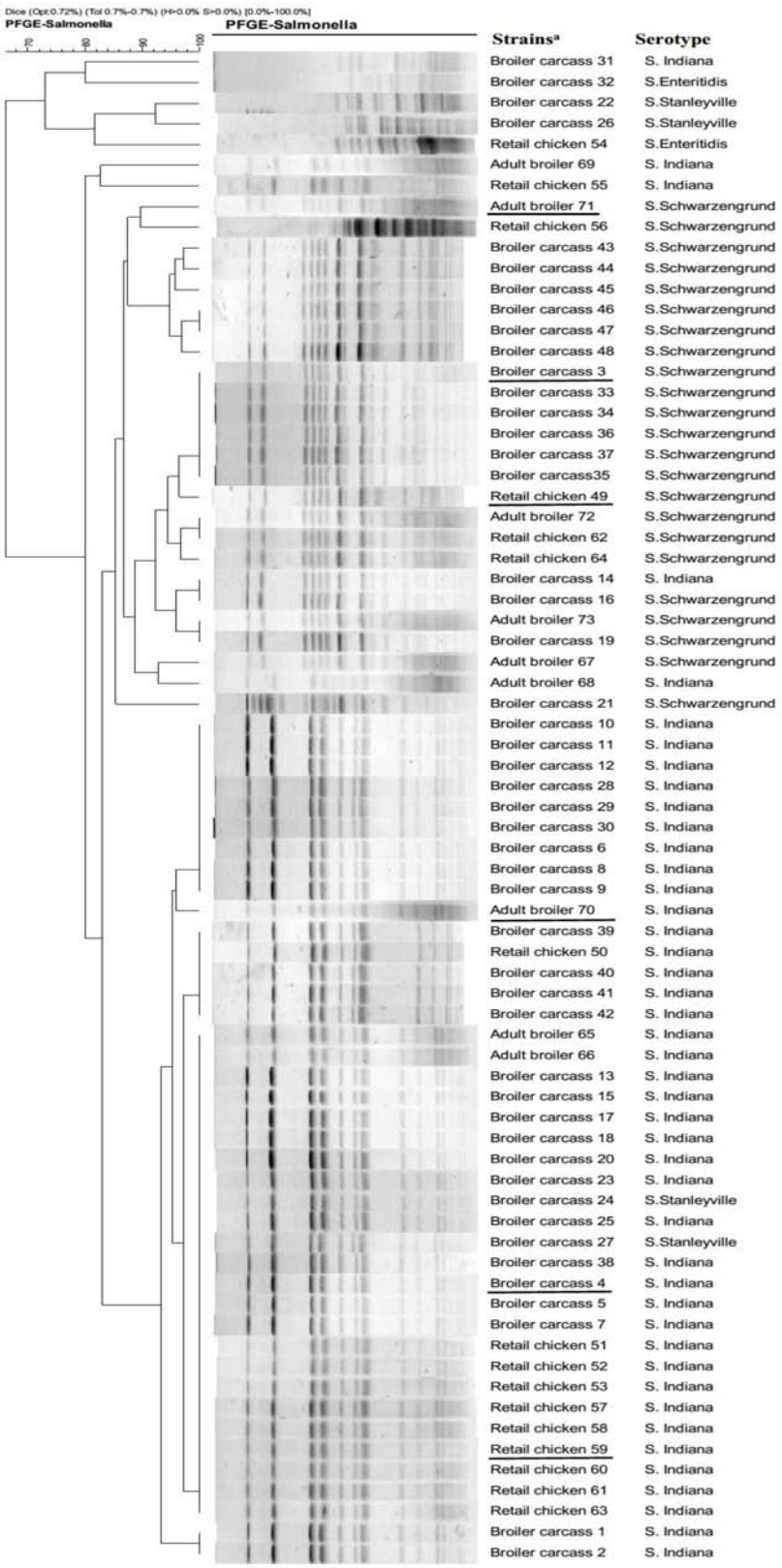

FIGURE 1 | PFGE profiles of *Salmonella* isolates from the broiler chicken supply chain. Strain codes indicate the source of broiler chicken supply chain and the isolate number. [a]Underlined strains were selected to been sequenced by the Illumina HiSeq 2500 system.

TABLE 1 | Mutations within QRDR of *gyrA*, *gyrB*, *parC*, and *parE* genes in *Salmonella* isolates from broiler chicken supply chain.

Gene	Mutation type	Serotype			
		***S.* Indiana (*n* = 45)**	***S.* Schwarzengrund (*n* = 24)**	***S.* Enteritidis (*n* = 2)**	***S.* Stanleyville (*n* = 2)**
gyrA	Wild type (*n* = 47)	32	13	2	0
	Ser83Phe, **Asp87Asn** (*n* = 26)	13	11	0	2
gyrB	Lys447, Leu451, Leu462, Ser464 (*n* = 47)	32	13	2	0
	Arg438, Lys439, Leu451, Leu462, Ser464 (*n* = 26)	13	11	0	2
parC	**Thr57Ser**, Val67, His75, His77, Asp101, Gly102, Gly104, Ala117, Ser123 (*n* = 47)	32	13	2	0
	Thr57Ser, Val67, His75, His77, **Ser80Arg**, Gly104, Ala117, Ser123 (*n* = 26)	13	11	0	2
parE	Glu460, His509 (*n* = 27)	14	11	0	2
	Glu460, Ie464, His509 (*n* = 46)	31	13	2	0

Missense mutations are marked in bold.

nucleotide identity of 99% to the plasmid tig00000005_pilon (CP021858; nt 17585–5015, 4958–1790) from *K. pneumoniae* AR_0125. Similar recombination junctions could also be found in another alternative sequence analysis. Therefore, based on sequence analyses of three recombination junctions (**Figure 3B**), it was postulated that Tn3, IS6 and homologous recombination played important roles in the formation of pSH-01.

DISCUSSION

In the broiler supply chain in China, there are geographical differences in the dominance of various *Salmonella* serotypes and in the prevalence of fluoroquinolones resistant *Salmonella*. In this study, *S.* Indiana was the most common serotype isolated from the broiler supply chain, which differs from previous results showing that *S.* Enteritidis is dominant in Qingdao and *S.* Weltevreden is dominant in Guangdong, China (Cui et al., 2016; Ren et al., 2016). In addition, several studies have indicated *Salmonella* could be transmitted along the food chain (Nogrady et al., 2008; Hauser et al., 2012). Our PFGE results showed that there is the potential for the transmission of *Salmonella* along the broiler chicken chain. With the emergence of antibiotic-resistant bacteria presenting a serious challenge in human and veterinary medicine globally, there is an abundance of evidence showing that the antimicrobial resistance of *Salmonella* in the chicken supply chain is more possibly attributed to the use of antibiotics in the animal husbandry (Cui et al., 2016). In particular, there are many reports of increasing prevalence of fluoroquinolone-resistant *Salmonella* (Piddock, 2002; Wasyl et al., 2014), which might be a potential risk for human health. In this study, resistance to ciprofloxacin was detected in 37.0% of the *Salmonella* isolates, and this resistance rate was relatively high compared to those of previous reports (Cui et al., 2016; Ren et al., 2016; Nhung et al., 2018).

TABLE 2 | The distribution of fluoroquinolone resistance genes about PMQR in *Salmonella* isolates from broiler chicken supply chain.

Genes	Serotype			
	***S.* Indiana (*n* = 45)**	***S.* Schwarzengrund (*n* = 24)**	***S.* Enteritidis (*n* = 2)**	***S.* Stanleyville (*n* = 2)**
qnrS1 (*n* = 30)	23	5	2	0
qnrB1 (*n* = 22)	12	9	0	1
oqxA (*n* = 1)	1	0	0	0
oqxB (*n* = 1)	1	0	0	0
aac-Ib-cr (*n* = 1)	0	1	0	0

qnrC, qnrD, qnrA, and qepA were not detected.

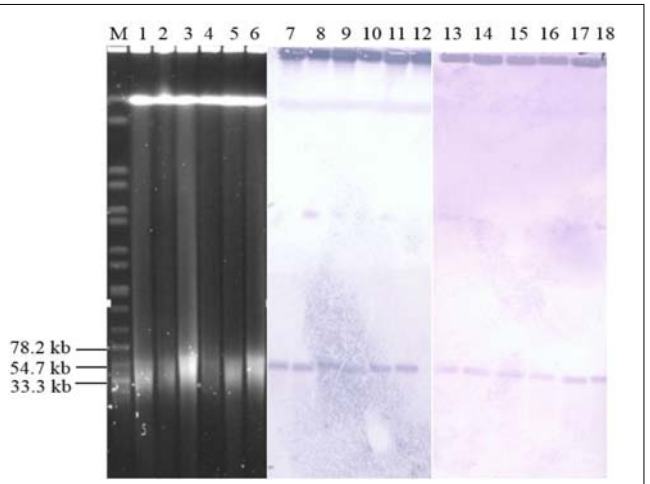

FIGURE 2 | Results of S1-PFGE and Southern blotting. Location of the *qnrS1*-carrying plasmid pSH-01 in *Salmonella* by S1-PFGE (lanes M, 1, 2, 3, 4, 5, and 6) and Southern blot hybridization (lanes M, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, and 18). Lane M, serotype Braenderup H9812; lane 1 and 7, Adult broiler 70; lane2 and 8, Adult broiler 71; lane 3 and 9, Broiler carcass 3; lane 4 and 10, Broiler carcass 4; lanes 5 and 11, Retail chicken 49; lanes 6 and 12, Retail chicken 59; lanes 13, Adult broiler 70 transconjugant; lane 14, Adult broiler 71 transconjugant; lane 15, Broiler carcass 3 transconjugant; lane 16, Broiler carcass 4 transconjugant; lanes 17, Retail chicken 49 transconjugant; and lanes 18, Retail chicken 5.

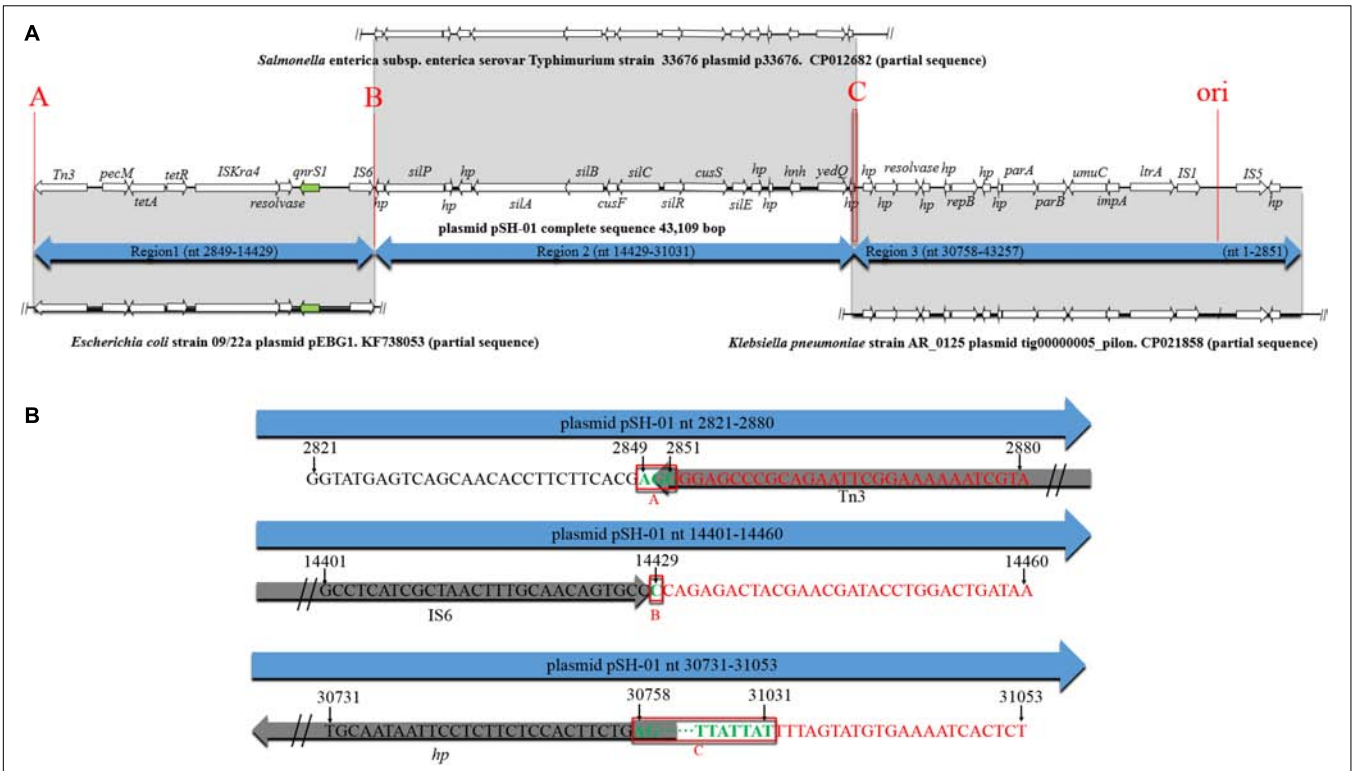

FIGURE 3 | Genetic environment of the *qnrS1* gene in plasmid pSH-01. (A–C) The potential recombination junctions in plasmid pSH-01. **(A)** A structural comparison was made with plasmid pEBG1 from *E. coli* 09/22a, plasmid p33676 from *S.* Typhimurium, and plasmid tig00000005_pilon from *K. pneumoniae* AR_0125. The arrows indicate the positions and directions of transcription of the genes. Regions of 99% nucleotide sequence identity are marked by gray shading. **(B)** Sequence analysis of three recombination junctions (A–C). The bases in black are upstream sequence of recombination junction in plasmid pSH-01. The bases in red are downstream sequence of recombination junction in plasmid pSH-01. The bases in green and in red boxes represent the common region shared by potential hybridized plasmids.

In this study, the same PFGE pattern was shared among the majority of *Salmonella* isolates (such as *S.* Indiana and *S.* Schwarzengrund isolates), which might suggest they are clones of *S.* Indiana and *S.* Schwarzengrund, respectively. Compared with the QRDR genotypes in non-clones *Salmonella* isolates (Eaves et al., 2004), the genetic diversity of *Salmonella* isolates in this study was lower. In addition, similar to previous investigation (Hopkins et al., 2005), our results indicate that missense mutations occurred frequently in QRDR of *gyrA* and *parC*, which are considered major quinolone resistance determinants in *Salmonella*. In this study, *gyrA* missense mutations (Ser83Phe and Asp87Asn) were detected in 26/73 *Salmonella* isolates and these are considered the major target-site mutations in *Salmonella* (Nüeschinderbinen et al., 2015). Thr57Ser *parC* substitution was frequently observed in the *Salmonella* isolates, and a second substitution (Ser80Arg) in *parC* was also detected in 26 *Salmonella* isolates. Importantly, Thr57Ser *parC* substitution was considered not or doubtfully associated to fluoroquinolone resistance phenotypes (Wasyl et al., 2014). The 15 *Salmonella* isolates with the Thr57Ser *parC* substitution in this study did not show fluoroquinolone resistance phenotypes, in agreement with previous report (Ceyssens et al., 2015). Although mutation types in *gyrA* and *parC* were similar to those in previous studies of *Salmonella*, it is worth noting the high frequency of silent site mutations in QRDR, which might be developed into potential missense mutations (Heisig, 1993; Hopkins et al., 2005). Furthermore, a recent study has shown that mutations in the target genes *gyrA* and *parC* are correlated with an increase of intrinsic fitness in *Salmonella* (Baker et al., 2013). This indicated that the potential risk that *Salmonella* isolates with mutations in *gyrA* and *parC* may naturally maintain during the broiler chicken supply chain, even if fluoroquinolone use was reduced.

In addition, the predominant PMQR gene varies among bacteria from different sources. The most common PMQR gene was *oqxAB* in *E. coli* from chicken (Chen et al., 2012) and in *Salmonella* from retail meat (Lin et al., 2015), *qnrB* in *Enterobacteriaceae* from crows (Halova et al., 2014), and *aac(6′)-Ib-cr* in bacteria isolated from sewage and surface water (Osinska et al., 2016). However, this study indicated that *qnrS* was commonly distributed in *Salmonella* isolates from the broiler chicken supply chain, consistent with the high reported rates in *Salmonella* isolated from animals, food, and feed (Wasyl et al., 2014).

The recombination of plasmid, to some extent, can provide a mechanism to improve the diversity of plasmids carrying resistance genes. Recombination of hybrid plasmids frequently occurs at insertion sequence (IS) location (Hudson et al., 2014). Recently, *NDM-5* and *mcr-1* were recombined in the plasmid

pCQ02-121 by recombination junctions: IS26 and the *nic* site of *oriT* (Sun et al., 2016). Similarly, in this study, the novel plasmid pSH-01 might arise via recombination junction IS6. The other two recombination junctions involve Tn3 and homologous recombination of sequences. Transposons in the Tn3 family can mediate gene reassortment and genomic plasticity owing to their modular organization, and they contribution substantially to antimicrobial drug resistance dissemination or to endowing environmental catabolic capacities (Nicolas et al., 2015).

Genetic features of pSH-01 showed that only region 2 (nt 14429–31031) could matched the plasmid sequences from clinical *Salmonella* isolates, and the sequences with matches in the other two regions (region 1 and 3) were derived from plasmids from non-*Salmonella* bacteria. The full-length of pSH-01 did not match an individual plasmid in NCBI. Therefore, this is a new plasmid in *Salmonella* isolates from the broiler chicken supply chain, suggesting that the diversity of plasmids carrying the resistance gene might be a potential risk factor for the dissemination of *qnrS1*.

It is worth noting that the new plasmid carrying *qnrS1* presented in the six *Salmonella* isolates (three *S.* Indiana and three *S.* Schwarzengrund) from different sources in the broiler chicken supply chain. This suggests that there was a potential epidemic spread of the plasmid in the *Salmonella* isolates of different serotype from different geographical origin, which is similar to the potential transmission of the plasmids among various serotype of *Salmonella* and diverse geographical location (Li et al., 2016; Wong et al., 2017). Therefore, we should carefully monitor the new plasmid carrying *qnrS1* along the chicken supply chain.

This study provided comprehensive data for the prevalence of *Salmonella* and their fluoroquinolone resistance determinants associated with QRDR and PMQR in the broiler chicken supply chain. Furthermore, we found that *qnrS1*, a transmissible PMQR gene, was prevalent in *Salmonella* isolates from the broiler chicken supply chain. Selective pressure from fluoroquinolones in animals may further promote the recombination and dissemination of the plasmid carrying PMQR genes.

AUTHOR CONTRIBUTIONS

CW and MC designed the experiments. MC, PZ, JL, and CS carried out the experiments. MC wrote the manuscript. MC, LS, CZ, and QZ reviewed and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01865/full#supplementary-material

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Altered Integrative and Conjugative Elements (ICEs) in Recent *Vibrio cholerae* O1 Isolated From Cholera Cases, Kolkata, India

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The self-transferring integrative and conjugative elements (ICEs) are large genomic segments carrying several bacterial adaptive functions including antimicrobial resistance (AMR). SXT/R391 family is one of the ICEs extensively studied in cholera-causing pathogen *Vibrio cholerae*. The genetic characteristics of ICE-SXT/R391 in *V. cholerae* are dynamic and region-specific. These ICEs in *V. cholerae* are strongly correlated with resistance to several antibiotics such as tetracycline, streptomycin and trimethoprim-sulfamethoxazole. We screened *V. cholerae* O1 strains isolated from cholera patients in Kolkata, India from 2008 to 2015 for antibiotic susceptibility and the presence of ICEs, and subsequently sequenced their conserved genes. Resistance to tetracycline, streptomycin and trimethoprim-sulfamethoxazole was detected in strains isolated during 2008–2010 and 2014–2015. The genes encoding resistance to tetracycline (*tetA*), trimethoprim-sulfamethoxazole (*dfrA1* and *sul2*), streptomycin (*strAB*), and chloramphenicol (*floR*) were detected in the ICEs of these strains. There was a decrease in overall drug resistance in *V. cholerae* associated with the ICEs in 2011. DNA sequence analysis also showed that AMR in these strains was conferred mainly by two types of ICEs, i.e., ICE^{TET} (comprising *tetA, strAB, sul2*, and *dfrA1*) and ICE^{GEN} (*floR, strAB, sul2*, and *dfrA1*). Based on the genetic structure, Kolkata strains of *V. cholerae* O1 had distinct genetic traits different from the ICEs reported in other cholera endemic regions. Transfer of AMR was confirmed by conjugation with sodium azide resistant *Escherichia coli* J53. In addition to the acquired resistance to streptomycin and trimethoprim-sulfamethoxazole, the conjugally transferred (CT) *E. coli* J53 with ICE showed higher resistance to chloramphenicol and tetracycline than the donor *V. cholerae.* Pulsed-field gel electrophoresis (PFGE) based clonal analysis revealed that the *V. cholerae* strains could be grouped based on their ICEs and AMR patterns. Our findings demonstrate the epidemiological importance of ICEs and their role in the emergence of multidrug resistance (MDR) in El Tor vibrios.

Keywords: cholera, *V. cholerae* O1, tetracycline, antimicrobial resistance, multidrug resistance, integrative conjugative element

INTRODUCTION

The Gram-negative pathogen *Vibiro cholerae* O1 has caused seven pandemics in the history of cholera and tends to cause several epidemics in developing countries (Lekshmi et al., 2018). This pathogen has more than 200 serogroups, but only the serogroups O1 and O139 are associated with epidemic cholera (Lekshmi et al., 2018). The ongoing seventh pandemic is linked with the El Tor biotype of serogroup O1 that has spread in the cholera endemic regions of the world (Lekshmi et al., 2018). The emergence and spread of antimicrobial resistant (AMR) *V. cholerae*, especially those resistant to nalidixic acid, tetracycline, and trimethoprim-sulfamethoxazole, has been reported since the 1980s (Ghosh and Ramamurthy, 2011). Resistance to these antimicrobials has been strongly associated with the presence of integrative and conjugative elements (ICEs) of the SXT/R391 family and its discovery has greatly changed the understanding of AMR in *V. cholerae*.

SXT/R391 ICEs have been characterized/classified based on the conserved core genes, and their integration into the 5′-end of the *prfC* gene that encodes peptide chain release factor 3 (Hochhut and Waldor, 1999). More than 1000 ICEs have been updated in the ICEberg database[1]. Mobility of SXT/R391 ICEs occurs between bacteria by conjugation, resulting in the transfer of several functions including AMR, resistance to heavy metals, regulation of motility and biofilm formation (Waldor et al., 1996; Bordeleau et al., 2010). Five insertion hotspots (H1 to H5) and four variable regions (VRI to VRIV) are also carried by the ICEs (Wozniak et al., 2009). The structure of ICEs changes periodically contributing to the differences in AMR profiles of *V. cholerae*. More than 50 ICEs have been grouped within the SXT/R391 family, of which 30 are reported in clinical and environmental *V. cholerae* strains (Pande et al., 2012). Between 1992 and 2001, 15 ICEs were identified in India and Bangladesh, of which six (SXT^{MO10}, ICE*Vch*Ind4, ICE*Vch*Ban5, ICE*Vch*Ban10, ICE*Vch*Ban9, and ICE*Vch*Ind5) were completely sequenced and annotated (Ceccarelli et al., 2011).

Tetracycline has been the drug of choice in treating cholera cases for a long time (World Health Organization [WHO], 2005). A sudden upsurge in the tetracycline resistance (Tet^R), from 1% in 2004 to 76% in 2007, was reported among *V. cholerae* in Kolkata and it decreased to about 50% in 2009 (Bhattacharya et al., 2011). Similar trends have been observed previously in large cholera epidemics in Tanzania and Madagascar due to extensive prophylactic use of tetracycline (Mhalu et al., 1979; Dromigny et al., 2002). Only a few studies have been carried out to understand the mechanisms of AMR due to ICEs in India (Roychowdhury et al., 2008; Bhattacharya et al., 2011; Kutar et al., 2013). In this study, we screened the AMR patterns of *V. cholerae* O1 Ogawa strains isolated from cholera patients in Kolkata, India from 2008 to 2015 and examined the type of ICEs present by analyzing their backbone genes. Our study revealed the differences between the sequence types of ICEs and recent changes in AMR patterns of *V. cholerae*.

[1]http://db-mml.sjtu.edu.cn/ICEberg

MATERIALS AND METHODS

Clinical Specimens and Bacterial Strains

Stool specimens were collected from the Infectious Diseases Hospital (IDH) and B. C. Roy Children Hospital (BCH), Kolkata, before the patients were treated with antibiotics. Clinical symptoms of diarrheal patients included loose/watery stools with or without dehydration, abdominal cramps, vomiting and fever. Dysentery patients had frequent passage of stool with blood/mucus and mild to severe abdominal pain. For the isolation of *V. cholerae*, all the stool specimens/rectal swabs were enriched in alkaline peptone water (pH 8.0) (Difco, Sparks, MD, United States) for 6 h, followed by inoculation and overnight incubation in thiosulphate citrate bile-salts sucrose agar (TCBS, Eiken, Tokyo, Japan) plates. Sucrose-positive strains were confirmed serologically using commercially available *V. cholerae* O1 poly and monovalent antisera (Denka-Seiken, Tokyo, Japan). To obtain the AMR pattern from 2008 to 2015, 546 out of 1591 strains were randomly selected covering each month of the study period. Sodium azide resistant (Az^R) *Escherichia coli* J53 (Martínez-Martínez et al., 1998) was used for the conjugation experiments. All the strains were preserved in Luria Bertani (LB) broth (Difco) containing 15% glycerol at −80°C. *E. coli* ATCC 25922 (Clinical and Laboratory Standards Institute [CLSI], 2014) was used as a control strain in antimicrobial susceptibility testing.

Antibiotic Susceptibility Testing

Susceptibilities of *V. cholerae* strains to ampicillin (AMP, 10 μg), ceftriaxone (CRO, 30 μg), chloramphenicol (CHL, 30 μg), nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg), ofloxacin (OFX, 5 μg), norfloxacin (NOR, 10 μg), imipenem (IPM, 10 μg), streptomycin (STR, 10 μg), azithromycin (AZM, 15 μg), tetracycline (TET, 30 μg), trimethoprim-sulfamethoxazole (SXT, 1.25 and 23.75 μg) and gentamicin (GEN, 10 μg), were determined by Kirby-Bauer disk diffusion technique using commercial disks (BD, Sparks, MD, United States) as per the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute [CLSI], 2014, 2015).

Detection of Antibiotic Resistance Encoding Genes

Total nucleic acid of *V. cholerae* strains was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The integrase gene (*int^{SXT}*) present in ICE was amplified by PCR using previously described primer pair int1-F and int1-B (Dalsgaard et al., 2001). Beside *int^{SXT}*, PCR was also performed to detect the presence of resistance encoding genes for chloramphenicol (*floR* and *cat*), streptomycin (*strA* and *strB*), and sulfonamide (*sul1* and *sul2*) (Sarkar et al., 2015a). Primer pairs VCtetA.F-(5′-ACGGTATCCTGCTGGCACTGTATG-3′) and VCtetA.R-(5′-CATCCATATCCAGCCATCCCAACT-3′) and VctetR.F-(5′-GAAGTGGGAATGGAAGGGCTGAC-3′) and VctetR.R-(5′-AGCCTCTGTGCCATCATCTTG-3′) were designed to detect the Tet^R encoding gene (*tetA*), and the repressor protein (*tetR*) for a regulatory portion of resistance cassettes, respectively.

Representative amplicons were purified using a PCR product purification kit (Qiagen) and sequenced using the ABI Big Dye terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems, Foster City, CA, United States) in an automated DNA sequencer (ABI 3730, Applied Biosystems). The sequences were assembled and analyzed using DNASTAR software (DNASTAR Inc., Madison, WI, United States).

Conjugation

To test the mobility of the ICEs, conjugation assay was carried out using a representative ICE-positive *V. cholerae* O1 strain as donor with *E. coli* J53 (Az^R, Martínez-Martínez et al., 1998). In brief, overnight cultures of the bacteria were mixed at 1:2 donor-to-recipient ratios in 1 ml of LB broth and allowed to grow overnight at 37°C. The donor and recipient suspensions were diluted serially in phosphate buffer saline (PBS) and plated on TCBS and MacConkey agar plates, respectively, to confirm the purity and count the number of colonies. To detect the conjugally transferred *E. coli* J53 (CT-*E. coli* J53), MacConkey agar supplemented with streptomycin (100 μg/ml) and sodium azide (AZD, 100 μg/ml) was used. Transconjugants were confirmed as ICE-positive by PCR analysis, followed by PCR amplicon sequencing. To confirm the resistance phenotype, antibiotic susceptibility patterns of the donor, recipient and transconjugants were determined after their growth on Mueller-Hinton (MH, Difco) agar by disk diffusion method. An increase in resistance of transconjugants was quantified by determining the MICs of CHL, STR, TET, and SXT using *E*-test strips (AB bioMérieux, Solna, Sweden).

Pulsed-Field Gel Electrophoresis (PFGE)

Clonal analysis of representative *V. cholerae* O1 strains isolated between 2008 and 2015 was made following the PulseNet protocol (Cooper et al., 2006). *V. cholerae* O1 strains were used after digesting the DNA with *Not*I [New England Biolabs (NEB), Ipswich, MA, United States]. *Xba*I (NEB) digested *Salmonella* Braendruff H-9812 was used as a DNA size marker. The PFGE run conditions were generated by the auto-algorithm mode of the CHEF Mapper system (Bio-Rad, Hercules, CA, United States). PFGE profiles were analyzed by the BioNumerics version 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient and unweighted pair group method using arithmetic averages (UPGMA).

Whole Genome Sequence Analysis

The whole genome sequences submitted from our previous study (Imamura et al., 2017) were used in the analysis. The open reading frames (ORFs) from the contigs were generated by contig integrator for sequence assembly (CISA) using Glimmer-MG program[2]. Nucleotide sequences and amino acid sequences were obtained from these ORFs and translated in the appropriate frame. The predicted ORFs were annotated using CANoPI (Contig Annotator Pipeline) that also includes BlastX search for each ORF sequence against the "nr" database of NCBI[3]. From the whole genome sequence data of representative strains (Tet^R IDH 1986 and Tet^S IDH 4268), we have used part of the ICE region in the analysis. The contigs were aligned, assembled and compared with SEQMAN, assembly module of DNASTAR's LASERGENE with published sequences like ICE*Vch*Ind5 (GQ463142), ICE*Vch*Ban5 (GQ463140), MO10 (AY055428), etc. For confirmation, PCR was performed targeting important short regions of the ICEs (*rumAB, traI, traC, setR, traA-traC*, and *traG*) with previously described primers (Bani et al., 2007). Published ICE sequences were used for homology search. ORF search and gene prediction were performed for the complete ICE region with EditSEQ, Lasergene software (DNASTAR), and pairwise alignment was analyzed by blastN and blastP homology search using the NCBI database.

Nucleotide Sequence Submission

The AMR encoding gene cassettes and their flanking sequences of representative ICE of Tet^R and Tet^S *V. cholerae* O1 have been submitted in GenBank (Accession numbers MK165649 and MK165650, respectively).

Ethics and Biosafety Statements

The Ethics and Biosafety Committees of National Institute of Cholera and Enteric Diseases, Kolkata approved this study (A:1/2015-IEC). Each participant/parent in the case of children gave written informed consent. All the experiments were performed following Biosafety Level-2 standards.

RESULTS

Prevalence of Cholera

During 8 years of surveillance from 2008 to 2015, the isolation rate of *V. cholerae* O1 Ogawa was about 11% (1591 of 14237 tested samples) (**Figure 1**). The incidence of this pathogen in BCH samples was very low (∼2%) but was found to be much higher (∼18%) in IDH samples. As shown in **Figure 1**, the mean incidence of cholera in IDH/BCH fluctuated between 4.9% (2014) and 27.2% (2009). Except for children ≤5 years, *V. cholerae* O1 remained one of the important bacterial pathogens. The incidence of *V. cholerae* O1 varies in certain extent from year to year (**Figure 1**).

Antimicrobial Resistance

All the *V. cholerae* O1 strains isolated were consistently resistant to NA. Tet^R gradually decreased from 58% in 2008 to 48% in 2009, followed by a further drop in 2010 (9%). Thereafter, all the strains isolated between 2011 and 2013 were found to be susceptible to TET (**Table 1**). Remarkably, Tet^R trait increased again in 2015 (56%). There was a marked change in AMP resistance each year with highest in 2010 (94%) and lowest in 2012 (21%) (**Table 1**). About three fourth of the strains were resistant to AMP in 2009 and 2011 (>76%). Thereafter, most of the *V. cholerae* isolated from 2013 to 2015 were found to be susceptible to AMP.

Throughout the study period, only three *V. cholerae* strains were found to be fully resistant to CHL and the rest of the

[2]http://www.cbcb.umd.edu/software/glimmer-mg

[3]www.scigenom.com/CANoPI

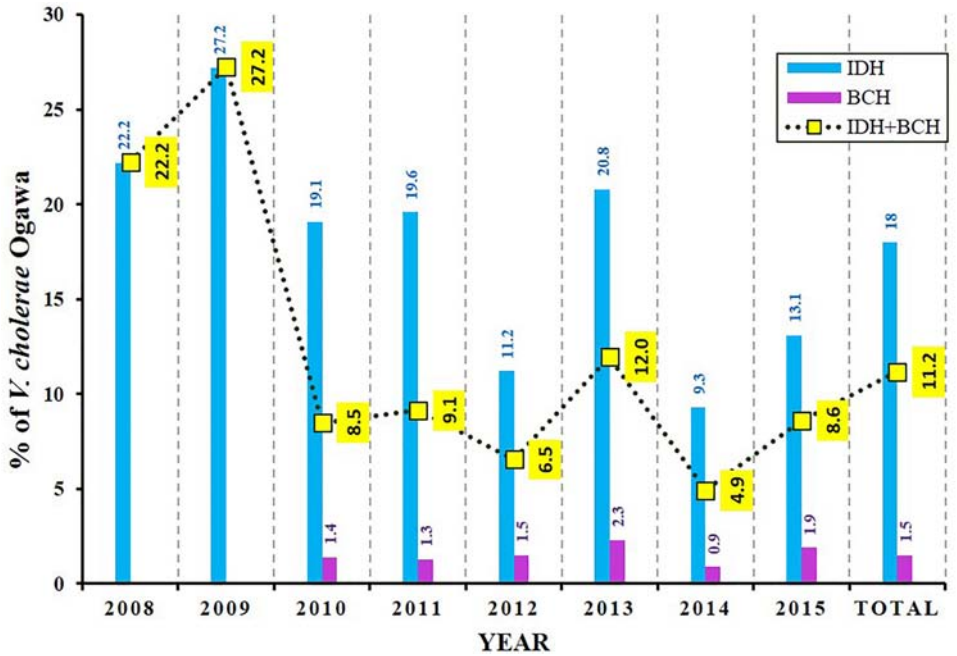

FIGURE 1 | Isolation rate of *V. cholerae* O1 Ogawa among diarrheal patients. BCH sample collection was started from 2010 onward. The dotted line with yellow boxes represent the mean incidence of cholera in IDH and BCH.

TABLE 1 | Resistance of *V. cholerae* O1 Ogawa against different antibiotics.

Year (n)	% of resistance					
	TET	CHL*	STR	SXT	AMP	NA
2008 (76)	58	33	92	92	53	100
2009 (120)	48	45	96	99	76	100
2010 (53)	9	91	98	98	94	100
2011 (52)	0	25	23	31	77	100
2012 (48)	0	58	67	69	21	100
2013 (87)	0	91	98	99	0	100
2014 (44)	2	86	91	93	0	98
2015 (66)	56	38	94	92	0	100

*Except three, rest of the strains were intermediate (i) to CHL.

floR containing strains showed intermediate resistance [CHL(i)] to this antibiotic. Interestingly, resistance to TET was found to be inversely proportional to CHL(i), i.e., strains showing Tet^R had intermediate resistance to CHL. The CHL(i) trait increased in 2010 (91%) when Tet^R was very low (9%) but dropped to 38% with the re-emergence of Tet^R in 2015 (56%). Resistance to STR and SXT were detected in most of the *V. cholerae* O1 strains. Resistance to these antimicrobials was >90% from 2008 to 2010 and 2013 to 2015. Interestingly, there was a sudden decrease in STR and SXT resistance (23 and 31%, respectively, in 2011) followed by an increase in 2012 (67 and 69%, respectively) (**Table 1**).

This study shows the changing profile of MDR in *V. cholerae* from Kolkata; MDR profiles NA-STR-SXT-TET-AMP and NA-STR-SXT-TET were predominant during 2008, 2009 and 2015 (**Table 2**), while from 2009 to 2010 and 2012 to 2014 the MDR profiles NA-STR-SXT-CHL(i), and NA-STR-SXT-CHL(i)-AMP were found in more than 50% of the *V. cholerae* O1 strains.

ICE Comprising Antimicrobial Resistance Genes

While analyzing the sequences of the resistance gene clusters, two types of ICEs could be detected, i.e., ICE^{TET} (Acc No. MK165649; Tet^R IDH 1986) and ICE^{GEN} (Acc No. MK165650; Tet^S IDH 4268). The superscript "GEN" stands for "general." Although the ICE^{GEN} was very similar to the ICE*Vch*Ind5 with 99% identity at 100% query coverage, the ICE^{TET} had only 99% identity at 70% query coverage. The structure of these two ICEs with ORFs is shown in **Figure 2**. The ICE^{GEN} was found to be larger (96.7 kb) than ICE^{TET} (91.5 kb). SXT and STR resistant *V. cholerae* O1 strains were positive for int^{SXT}. Detection of ICEs was >90% in 2008 and 2009, with highest in 2010 (98%), followed by an abrupt decrease in 2011 (23%). However, in 2012, 68% of the *V. cholerae* O1 strains harbored the ICEs. Interestingly, except for NA, the int^{SXT} negative strains were susceptible to most of the antimicrobials tested in this study. In the 1st type, ICE^{TET} carried a TET efflux pump encoding gene (*tetAR*; *tetA* is a gene encoding TET efflux pump and *tetR* is a repressor protein regulating the *tetA* expression) and in the 2nd type, ICE^{GEN} harbored CHL efflux pump encoding gene (*floR*). ICE^{GEN} has high similarity (99%) with the ICE*Vch*Ind5, the most common ICE detected among seventh-pandemic El Tor vibrios (Spagnoletti et al., 2014; Bioteau et al., 2018). This ICE also has very high similarity to the ICE*Vch*Hai1 from the Haitian *V. cholerae* lineage (Sjölund-Karlsson et al., 2011).

TABLE 2 | Percentage of resistance pattern in *V. cholerae* O1 strains during 8 years in Kolkata.

Resistance profile/Year	2008 (*n* = 76)		2009 (*n* = 120)		2010 (*n* = 53)		2011 (*n* = 52)		2012 (*n* = 48)		2013 (*n* = 87)		2014 (*n* = 44)		2015 (*n* = 66)	
NA-STR-SXT-TET-AMP	27.6	**58**	35.8	**48**	3.8	**9**							0.0	**2**	0.0	**53**
NA-STR-SXT-TET	30.3		12.5		5.7								2.3		52.9	
NA-STR-SXT-CHL(i)-AMP	18.4	**34**	40.0	**51**	88.7	**89**	19.6	**24**	6.4	**72**	0.0	**100**	0.0	**98**	0.0	**46**
NA-STR-SXT-CHL(i)	15.8		10.8				4.3		66.0		100		97.7		45.7	
NA-AMP	6.6	**8**	0.8	**1**	1.9	**2**	56.5	**76**	14.9	**28**					0.0	**1**
NA	1.3		0.0		0.0		19.6		12.8						1.0	

(i), intermediate resistance for CHL. Numbers in bold represents cumulative percentage of resistance patterns.

The ICE^{GEN} and ICE^{TET} had *sul2*, *strBA* in the AMR gene cluster conferring resistance to SXT and STR, respectively. Generally, in *V. cholerae*, the presence of *tet* alleles within the ICE gene clusters is uncommon. In the prototype SXT^{MO10}, resistance gene cluster comprised *dfr18, floR, strBA, sul2* encoding resistance to trimethoprim, CHL, STR, and sulfamethoxazole, respectively (**Table 3**). In ICE*Vch*Ind4, there was a major deletion of *dfr18* gene in the cluster. In IDH1986 and IDH14268 strains, a class 4 integron carrying the trimethoprim resistance encoding *dfrA1* was identified in H3 located within the *s073-traF* locus. Such arrangement exists in ICE*Vch*Ind5 backbone (**Figure 2**) and ICE*Vch*Ind1. But, *tetA* gene was absent in these ICEs.

Detection of ICE^{TET} in *V. cholerae* O1 decreased from 2008 (58% Tet^{R}) to 2010 (9% Tet^{R}). All the *V. cholerae* O1 strains isolated during 2011–2013 lacked ICE^{TET}. In 2015, however, the *tetAR* was again detected in a higher number of strains (56% Tet^{R}). In contrast, ICE^{GEN} was detected throughout the study period. AMR gene cassettes located within the *rumB* locus are also different. From 2011 to 2013, the *tetAR* locus in ICE^{TET} was replaced by *floR* gene of ICE^{GEN}. This feature marked the difference of ICE^{TET} from ICE*Vch*Lao1, where *floR* and *tetA* were concurrently present.

Based on the presence of the AMR encoding genes harbored by these elements, the genetic background of ICE^{TET} appears to be very different from the other ICEs carrying the *tet*. The ICE*Pda*Spa1 was found to have only the TET resistance determinant located within *rumBA* operon (**Table 3**). Whereas, in the ICE*Vch*Lao1, resistance genes of CHL *(floR)*, STR *(strBA)* and sulfamethoxazole *(sul2)* were present along with *tetA*. But, the ICE*Vch*Lao1 did not carry *dfrA1* or *dfr18* that confer resistance to trimethoprim in SXT^{ET} and SXT^{MO10}, respectively. Within the resistance gene cluster of 2008–2010 strains of *V. cholerae* in Kolkata, a deletion of *floR* gene, which was present upstream of the *tetA* gene in ICE*Vch*Lao1 and ICE*Vch*Ban9 was detected.

Genetic Structure of the ICEs

Generally, the genetic organization of ICE^{TET} and ICE^{GEN} was similar to that of the other members of this family. Many ORFs were commonly shared by these ICEs; most of them being in the conserved core genes (Beaber et al., 2002). Five conserved insertion hotspots are located between *s043 (traJ)* and *traL* (H1), *traA* and *s054* (H2), *s073* and *traF* (H3), *traN* and *s063* (H4), and *s025* and *traID* (H5) (Wozniak et al., 2009).

Five ORFs were found in the H1 of ICE^{TET} that include *tbp* (integrase catalytic subunit), a hypothetical protein (HP), transposase, ISPsy4 transposition helper protein and DNA helicase family protein. These ORFs present in H1 are unique compared to other reported ICEs. Instead of *mosA*, *mosT* that encode toxin-antitoxin reported in the H2 of other ICEs, the ICE^{GEN} and ICE^{TET} have 3 ORFs with *ynd* (transcriptional regulator with AbiEi antitoxin N-terminal domain), *ync* (nucleotidyl transferase AbiEii/AbiGii toxin family protein) and *dsbC* (disulfide isomerase DsbC). H3 of ICE^{GEN} and ICE^{TET} contains 7 ORFs with *bleR* (glyoxalase/bleomycin resistance), *araC* (AraC family transcriptional regulator; helix-turn-helix domain protein), a hypothetical protein, XRE family transcriptional regulators, a putative membrane protein, *dfrA1* (trimethoprim-resistance) and *intI4* (site-specific recombinase IntI4). Of these, AraC, XRE, and DFRA1 were reported in ICE*Vch*Moz10. H3 in ICE^{GEN} and ICE^{TET} is varied from ICE*Vch*Ind4, SXT^{MO10}, ICE^{R391} ICE*Vch*Mex1, ICE*Vfl*Ind1, ICE*Pmi*USA1, ICE*Spu*PO1 (Wozniak et al., 2009). H4 of ICE^{TET} was small with 2 ORFs, whereas the ICE^{GEN} had 5 ORFs with two SMC (structural maintenance of chromosome) domain proteins, *istB* (ATP binding domain), *istA* (integrase catalytic subunit) and deoxyribonuclease I. The ORF content of H4 in these ICEs is different from the others. In ICE^{GEN} and ICE^{TET}, the H5 has 10–11 gene combinations with the new ORFs of WYL domain protein, N-6 DNA methylase, restriction endonuclease subunit S, *Bst*XI (restriction endonuclease protein), ATPases associated with diverse cellular activities (AAA) family protein, McrC (putative protein) in ICE^{TET} and WYL domain-containing protein with three conserved amino acids, BrxC (BREX system P-loop protein), PglX (BREX-1 system adenine-specific DNA-methyltransferase) and abortive phage resistance protein in ICE^{GEN}. These changes in the hotspot regions may not have an obvious effect on the ICE, as they did not influence its transfer. VR-II has an insertion of single ORF, *mutL* similar to the ICE contigs circulating in India and Bangladesh. In the VRIII of ICE^{TET}, 12 ORFs [Tn3 (transposase), *tnpA* (transposase), *tnpB* (InsA transposase), truncated *virD2*, *tetA*, *tetR*, IS91 transposase, *strB*, *strA*, *sul2*, *tnpA* tn3 transposase, *s021*] were identified within

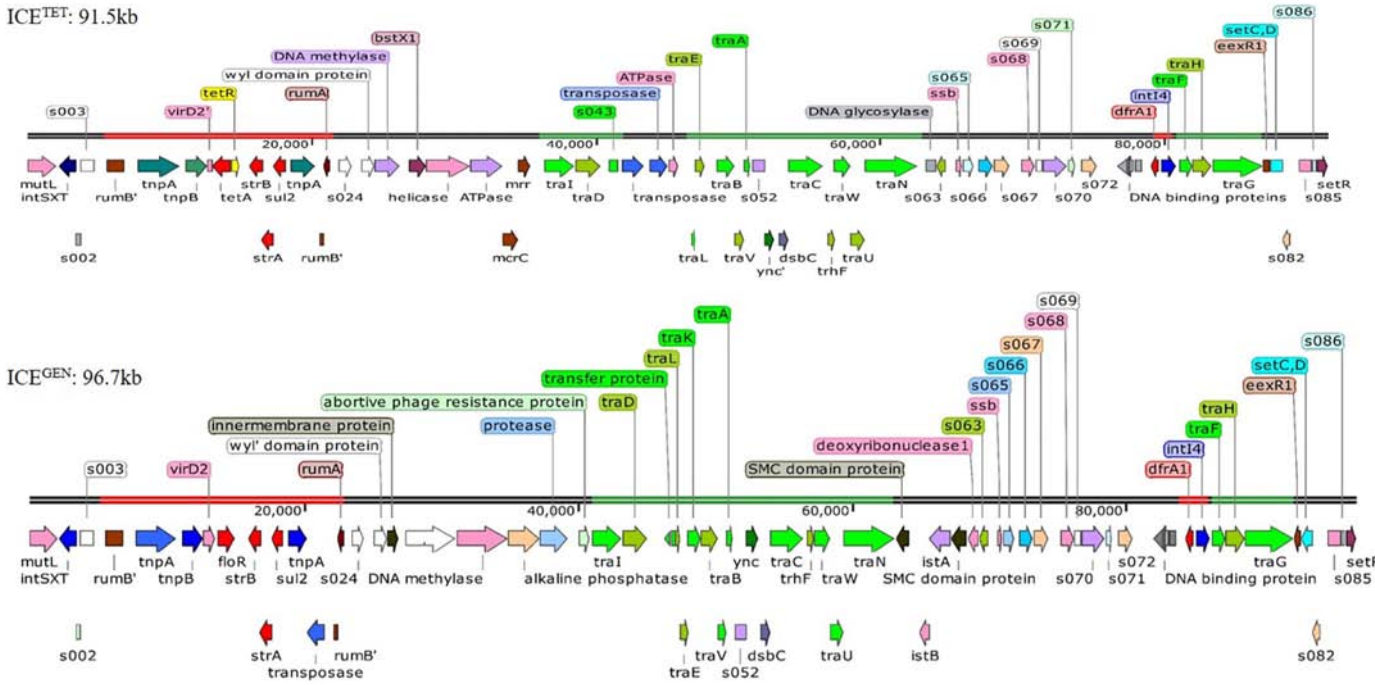

FIGURE 2 | Structure of the two ICEs found in MDR *V. cholerae* O1 Ogawa strains. The AMR genes are shown in red, the genes responsible for the transfer are presented in green, and transposases and integrases are shown in blue. The other shades represented miscellaneous features.

TABLE 3 | Comparison of the ICE gene cluster with the other SXT/R391 ICE family members.

ICE	Host strain	Country and year of isolation	Size (bp)	Resistance gene content	GenBank accession number	References
ICE*Vch*Mex1	*Vibrio cholerae* non O1-O139	Mexico 2001	82839	–	GQ463143	Burrus et al., 2006
ICE^{TET}	*Vibrio cholerae* O1 (IDH1986)	India 2009	91463	*tetAR, strBA, sul2, dfrA1*	MK165649	In this study
ICE^{GEN}	*Vibrio cholerae* O1 (IDH4268)	India 2012	96718	*floR, strBA, sul2, dfrA1*	MK165650	In this study
ICE*Vch*Ind4	*Vibrio cholerae* O139	India 1997	95491	*floR, strBA, sul2*	GQ463141	Wozniak et al., 2009
ICE*Vch*Ind5	*Vibrio cholerae* O1	India 1994	97847	*floR, strBA, sul2, dfrA1*	GQ463142	Ceccarelli et al., 2011
ICE*Vch*Ban5	*Vibrio cholerae* O1	Bangladesh 1998	102131	*floR, strBA, sul2, dfrA1*	GQ463140	Wozniak et al., 2009
ICE*Pal*Ban1	*Providencia alcalifaciens*	Bangladesh 1999	96586	*floR, strBA, sul2, dfrA1*	GQ463139	Wozniak et al., 2009
ICE*Vfl*Ind1	*Vibrio fluvialis*	India 2002	91369	*dfr18, floR, strBA, sul2*	GQ463144	Wozniak et al., 2009
ICE*Vch*Moz10/ICE*Vch*B33	*Vibrio cholerae* O1	Mozambique 2004	104495	*floR, strBA, sul2, tetA'*	ACHZ00000000	Taviani et al., 2009
ICE*Pmi*Usa1	*Proteus mirabilis*	United States 1986	79733	–	AM942759	Pearson et al., 2008
ICE*Vch*Ban9	*Vibrio cholerae* O1	Bangladesh 1994	106124	*floR, strBA, sul2, dfrA1, tetA'*	CP001485	Wozniak et al., 2009
ICE*Vch*Ban8	*Vibrio cholerae* non O1-O139	Bangladesh 2001	105790	–	NZ_AAUU00000000	Wozniak et al., 2009
SXT^{MO10}	*Vibrio cholerae* O139	India 2002	99452	*dfr18, floR, strBA, sul2*	AY055428	Beaber et al., 2002
R391	*Providencia rettgeri*	South Africa 1967	88532	*kanR, merRTPCA*	AY090559	Böltner et al., 2002
ICE*Pda*Spa1	*Photobacterium damselae*	Spain 2003	102985	*tetAR*	AJ870986	Juíz-Río et al., 2005
ICE*Spu*PO1	*Shewanella putrefaciens*	Pacific Ocean 2000	108623	–	CP000503	Wozniak et al., 2009

the two *rumB* portions. In the case of ICE^{GEN}, 14 ORFs [Tn3 (trnansposase), *tnpA* (transposase), *tnpB* (InsA transposase), *virD2* (relaxase), *floR*, LysR family protein, truncated transpoase, *strB*, *strA*, *sul2*, *tnpA* tn3 transposase, truncated *s021*, putative transpoase, truncated *mutL*] have been detected.

The restriction-modification system is composed of genes encoding the functions of DNA modification, recombination, and repair (Wozniak and Waldor, 2009). ICE^{GEN} and ICE^{TET} were found to have a type I restriction-modification system in the H5. In the ICE backbones, there were sequences in the ORFs located between *s024* and *traI* in Kolkata strains (**Figure 2**). In ICE^{GEN} carrying strains, after the *traN* locus, there was an insertion of *istBA* gene flanked by gene encoding SMC domain protein. This arrangement was not observed in *V. cholerae* strains with ICE^{TET}. Though these two types of ICEs had same *traFHG* locus, ORFs encoding transposases and ATPase were found incorporated between the *traD* and *traE* locus only in ICE^{TET}. In contrast, the ICE^{GEN} possessed an intact transfer region (**Figure 2**). In ICE*Vch*Ind4, there was a major deletion of *dfr18* gene in the cluster. In strains with ICE^{GEN} or ICE^{TET}, a class 4 integron carrying the trimethoprim resistance encoding *dfrA1* was identified in the H3 region located within the *s073-traF* locus. Similar gene configuration exists in the ICE*Vch*Ind1 and ICE*Vch*Ind5 backbones. In the 2008–2010 strains of *V. cholerae* in Kolkata, Tet^{R} in ICE was primarily due to *tetA*, whose presence was previously reported in ICE*Pda*Spa1 of *Photobacterium damselae*, ICE*Vch*Lao1 and ICE*Vch*Ban9 of *V. cholerae* O1 from Laos and Bangladesh, respectively (**Table 3**).

The *tra* loci appeared to be derived from a common ancestor and were mostly present in ICEs of *V. cholerae* strains. These loci are crucial for the transfer of ICEs and generating the conjugation machinery (Wozniak et al., 2009). Similar to the other ICEs backbone, the *tra* genes are arranged in four clusters in IDH1986 and IDH4268 strains, spanning more than 25 kb. Cluster 1 contains the genes and sequences necessary for transfer initiation, the nickase (encoded by *traI*), and the coupling protein (encoded in the *traD*). The mating pair formation function is controlled by three gene clusters: (i) *traLEKBVA*, (ii) *traC/trhF/traWUN*, and (iii) *traFHG* (**Figure 2**).

Comparison of Conserved Genes in the ICEs

ICE^{TET} and ICE^{GEN} shared the same exclusion group (EexR). This EexR system might have been transferred from R391 type ICEs (Marrero and Waldor, 2007). The site-specific integration

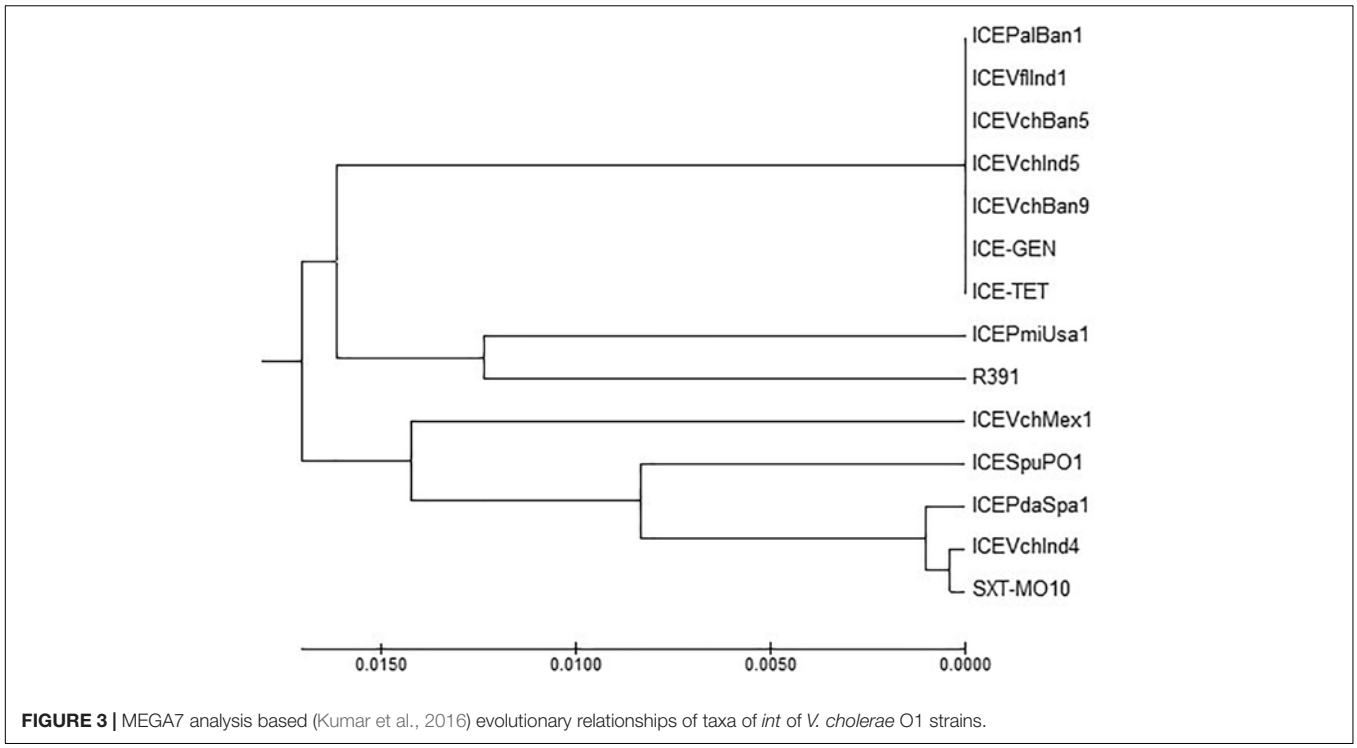

FIGURE 3 | MEGA7 analysis based (Kumar et al., 2016) evolutionary relationships of taxa of *int* of *V. cholerae* O1 strains.

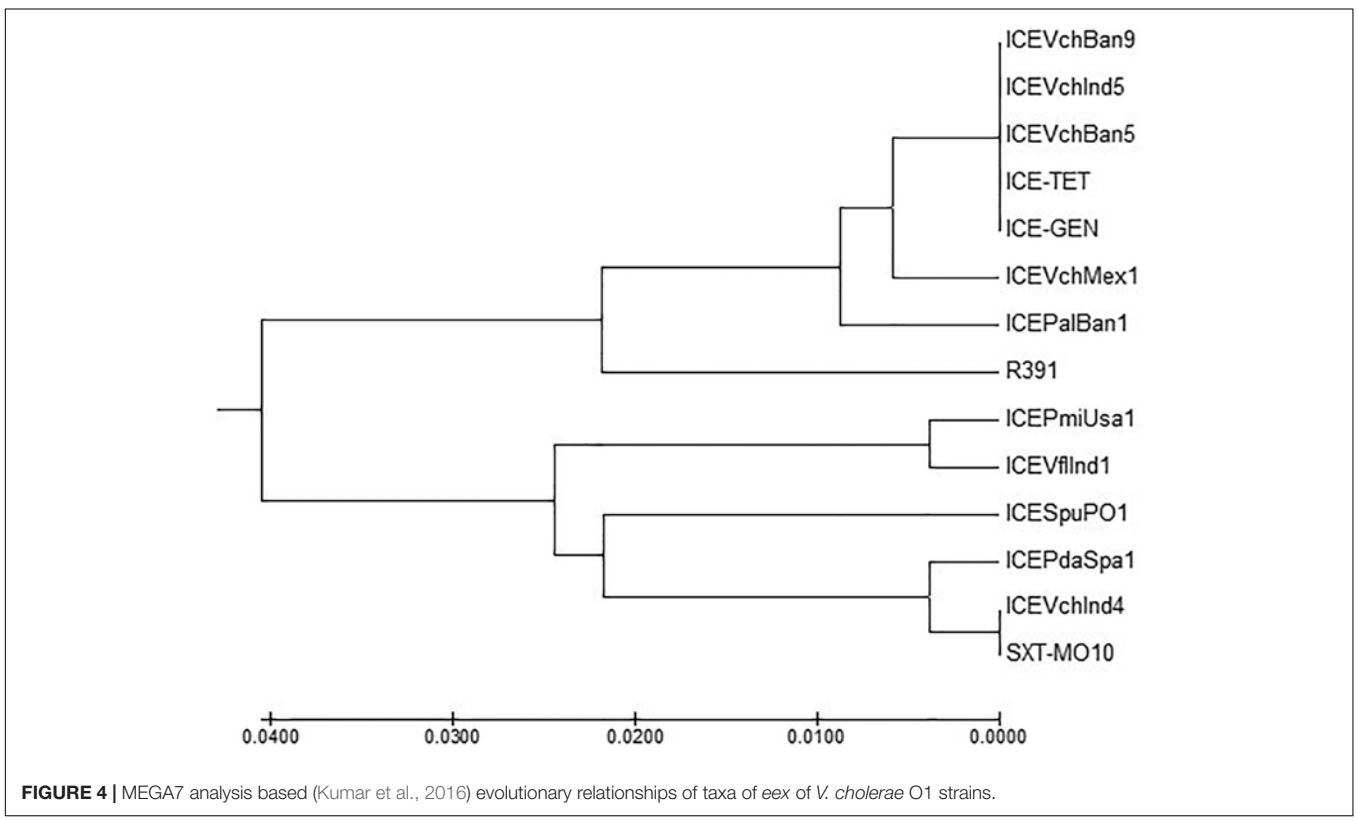

FIGURE 4 | MEGA7 analysis based (Kumar et al., 2016) evolutionary relationships of taxa of *eex* of *V. cholerae* O1 strains.

of the ICE is mediated through integrase enzyme encoded in the *int*. The *int* of ICE^{TET} and ICE^{GEN} harboring *V. cholerae* O1 is identical to those present in the strains that have ICE*Pal*Ban1 of *P. alcalifaciens*, ICE*Vf*Ind1 of *V. fluvialis* and ICE*Vch*Ban5, ICE*Vch*Ban9 and ICE*Vch*Ind5 of *V. cholerae* (**Figure 3**). These ICEs are distinct from those reported in *Proteus mirabilis*,

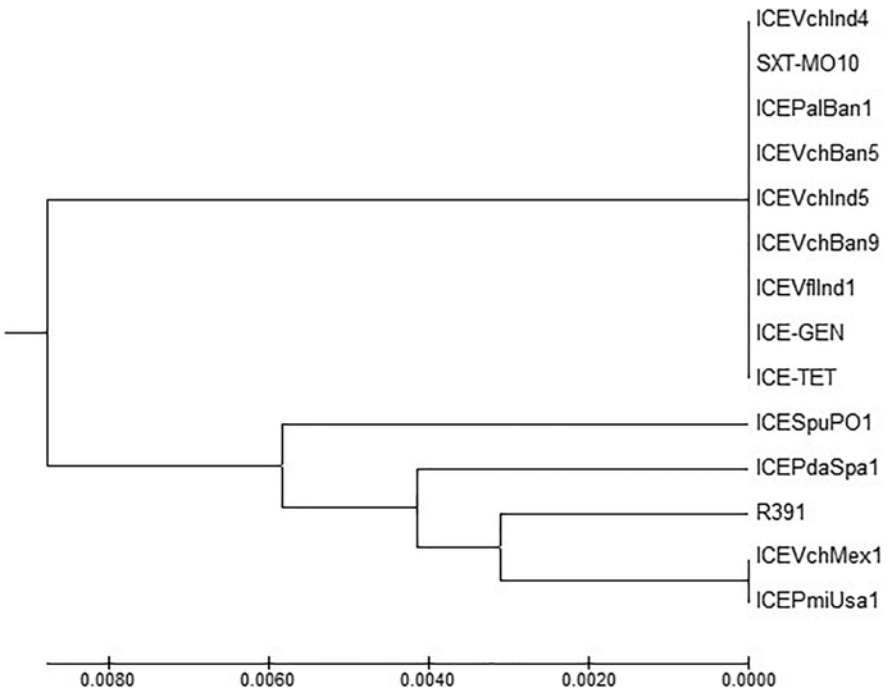

FIGURE 5 | MEGA7 analysis based (Kumar et al., 2016) evolutionary relationships of taxa of *setR* of *V. cholerae* O1 strains.

TABLE 4 | Increased resistance attributed by acquired ICE in transconjugants.

Strain	Resistance profile	MIC value (μg/ml)			
		SXT	STR	TET	CHL
IDH1986 (*V. cholerae* O1 Ogawa)	NA-TET-SXT-STR	>32	192	16	1
CT-*E. coli* J53/ICE^{TET} (Transconjugant)	TET-SXT-STR-AZD	>32 (>600 fold)	48 (24 fold)	24 (48 fold)*	3
E. coli J53 (Recipient)	AZD	0.047	2	0.5	3
CT-*E. coli* J53/ICE^{GEN} (Transconjugant)	CHL-SXT-STR-AZD	>32 (>600 fold)	64 (32 fold)	0.5	>256 (>85 fold)
IDH1439 (*V. cholerae* O1 Ogawa)	NA-SXT-STR-{CHL(i)}	>32	128	0.5	8

**Increase in fold compared to the recipient.*

Providencia rettgeri, Shewanella putrefaciens, *P. damselae* as well as in other *V. cholerae* with ICE*Vch*Mex1, ICE*Vch*Ind4, and SXT^{MO10}. SetR and SetC/D are the key regulators of ICEs, which are closely followed by the genes encoding for inner membrane proteins (Eex and TraG) of the donor and recipient cells. Eex and TraG facilitate entry-exclusion in the SXT/R391 family of ICEs. In the cluster tree, *eex* genes of the ICE^{TET} and ICE^{GEN} showed high homology with ICE identified in ICE*Vch*Ban5, ICE*Vch*Ban9, ICE*Vch*Ind5, but was distantly related to other ICEs of *V. cholerae* and other species (**Figure 4**). *setR* in the ICE^{TET} and ICE^{GEN} are identical with that in ICE*Vch*Ind4, ICE*Vch*Ind5, ICE*Vch*Ban5, ICE*Vch*Ban9, SXT^{MO10}, ICE*Vf*Ind1, ICE*Pal*Ban1 but different from ICE*Vch*Mex1 and ICEs of other species (**Figure 5**).

Transfer of ICEs

To test the transferability of the *V. cholerae* ICEs, we selected ICE^{TET} and ICE^{GEN} carrying strains (IDH1986 and IDH1439, respectively). Both the types of ICEs could be transferred to *E. coli* J53 by conjugation. The transconjugants acquired additional resistance against SXT and STR (**Table 4**). Remarkably, CT-*E. coli* J53 from ICE^{GEN} was highly resistant to CHL compared to the donor *V. cholerae* O1 strain, which showed reduced susceptibility to this antibiotic. Similarly, CT-*E. coli* J53 from ICE^{TET} expressed more resistance against TET than the donor *Vibrio* (**Table 4**). The frequency of transfer ranged from 3×10^{-5} to 5×10^{-6} transconjugants/recipient.

PFGE Analysis

Pulsed-field gel electrophoresis was performed to identify the clonal relationship between ICE^{TET} and ICE^{GEN} carrying *V. choleare* strains. It was found that the *V. cholerae* O1 strains displayed clonal clusters reflecting their MDR profile, which indirectly revealed the composition of AMR encoding genes in the ICEs (**Figure 6**). Cluster A represented *Vibrio* strains devoid of the ICEs. These strains were only resistant to NA. Strains with ICE^{GEN} were present in cluster B. These strains are resistant

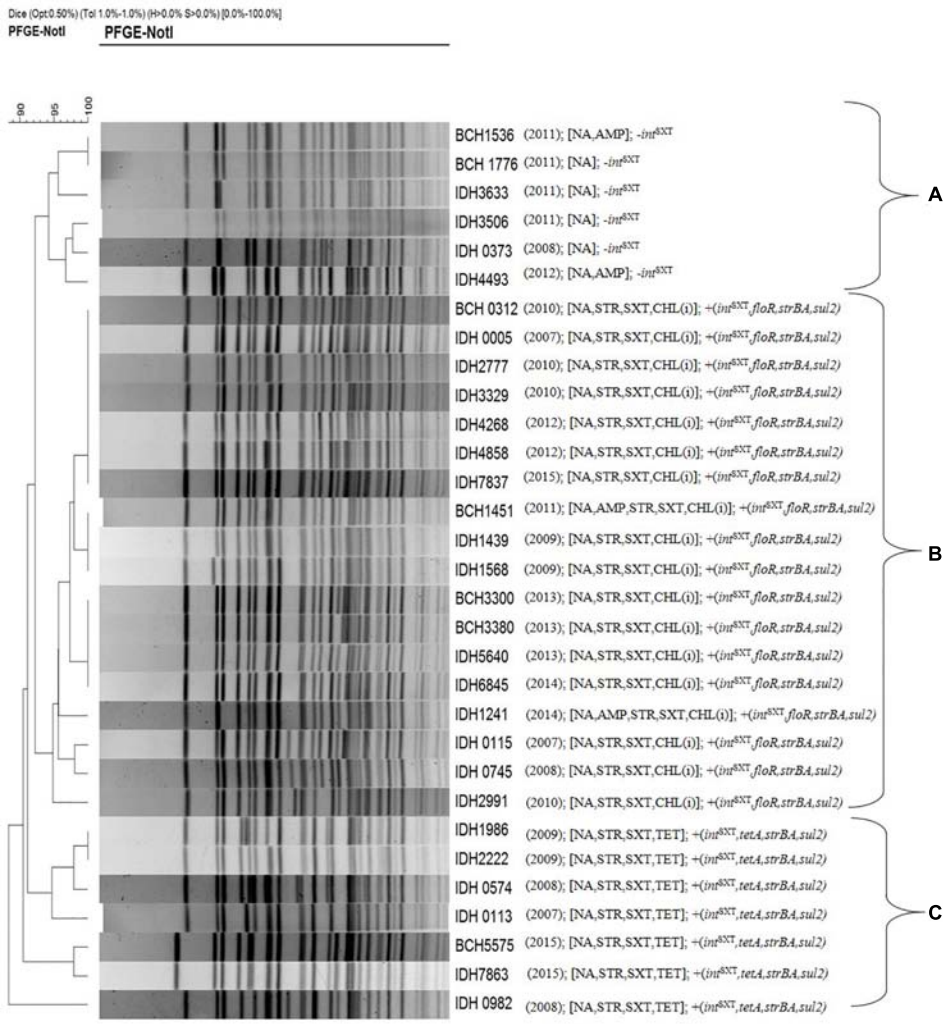

FIGURE 6 | PFGE profile of the representative *V. cholerae* O1 strains with their antimicrobial resistance. Cluster **A**, strains devoid of int^{SXT} or ICE; Cluster **B,** strains carrying ICE^{GEN}; Cluster **C,** strains having ICE^{TET}. Number in the parenthesis represents the year of isolation.

to NA, SXT and exhibited intermediate susceptibility to CHL. Cluster C contained the ICE^{TET} harboring strains that showed resistance to NA, SXT, and TET (**Figure 6**).

DISCUSSION

Cholera is endemic in the Indian subcontinent and it has spread to several other parts of the world (Mutreja et al., 2011). In Kolkata, MDR *V. cholerae* is associated with sporadic cholera for many years (Garg et al., 2000; Nair et al., 2010). *V. cholerae* O1 was susceptible to several antibiotics before 1980s, but developed resistance to SXT in the following years (Ghosh and Ramamurthy, 2011). *V. cholerae* O1 El Tor biotype that re-emerged in 1994 may have acquired SXT resistance phenotype from the O139 serogroup (Ramamurthy et al., 2003). Investigations conducted almost during the same period in several cholera endemic regions in India showed that the isolation rate of *V. cholerae* O1 was lesser than Kolkata, but the AMR pattern followed nearly the same trend, especially to tetracycline (Taneja et al., 2010; Das et al., 2011; Bhattacharya et al., 2012; Borkakoty et al., 2012; Mandal et al., 2012; Roy et al., 2012; Palewar et al., 2015; Bhuyan et al., 2016; Jain et al., 2016; Torane et al., 2016; Pal et al., 2018).

From 2010 to 2012, *V. cholerae* strains with AMR profiles of NA-STR-SXT-TET-AMP and NA-STR-SXT-TET were completely replaced with NA-STR-SXT-CHL(i)-AMP and NA-STR-SXT-CHL(i) along with NA-AMP and NA. Strains with the AMR profile of NA-STR-SXT-TET appeared again in 2015 (53%). Though the number of *V. cholerae* strains with the NA-SXT-STR-CHL(i) profile was highest from 2013 to 2014 (98–100%), it has reached to 46% with the re-emergence of Tet^R in 2015. The appearance of Tet^R in *V. cholerae* O1 Ogawa in 2008 has been reported from northern parts of India (Taneja et al., 2010). Tet^R has been previously reported mostly in Inaba

serotype (Jesudason, 2006; Roychowdhury et al., 2008). Presence of *tetA, floR, strBA, sul2, dfrA1* within the AMR gene cassettes has positive correlation with the phenotypic expression of drug resistance against TET, CHL, STR, and SXT (Dalsgaard et al., 2001; Hochhut et al., 2001; Wang et al., 2016). It is interesting to note that although *dfrA18* conferring resistance to trimethoprim was reported in MO10, later it was replaced by the *dfrA1* allele in a class IV integron located in the H3 (Wozniak et al., 2009).

In our study, *floR* and *tetA* genes were not found to coexist within the VRIII present in the *rumB* locus. Previous reports, however, had shown the presence of both *floR* and *tetA* in the *V. cholerae* ICE*Vch*Lao1 isolated from the Laos, ICE*Vch*B33 from Beira, Mozambique (Iwanaga et al., 2004; Taviani et al., 2009). Depending upon the presence of resistance cassettes in the ICEs, we found two types of ICEs in our study namely ICE^{GEN} and ICE^{TET}. Though the ICE backbone of ICE^{GEN} was similar to those of SXT^{MO10} and SXT^{ET}, it had 99% structural similarity to ICE*Vch*Ind5. Lineages of ICE*Vch*Ind5 of *V. cholerae* O1 strains causing epidemics in the Indian subcontinent might have spread to Africa (Valia et al., 2013).

ICE^{GEN} circulating in *V. cholerae* strains from Kolkata belonged to the group 1 ICE, which comprised ICE*Vch*Ind5 (India, 1994–2005), ICE*Vch*Ban5 (Bangladesh, 1998), ICE*Vch*Hai1 (Haiti, 2010), ICE*Vch*Nig1 (Nigeria, 2010), and ICE*Vch*Nep1 (Nepal, 1994) (Marin et al., 2014). Type I restriction-modification system systems of ICE^{GEN} and ICE^{TET} were also reported in the other ICEs families, such as ICE*Vch*Mex1 and ICE*Spu*PO1 (Burrus et al., 2006; Pembroke and Piterina, 2006). ICEs are constantly spreading in different geographical areas. ICE*Vch*B33, which is different from other ICEs of SXT/R391 was first identified in *V. cholerae* O1 strains from India in 1994 and then Mozambique in 2004 (Taviani et al., 2009). Similar to *V. cholerae* O1 from India with ICE*Vch*Ind1, the other ICEs identified in Vietnam, Laos, and Mozambique (ICE*Vch*Vie1, ICE*Vch*Lao1, and ICE*Vch*B33, respectively) lack the trimethoprim resistance encoding *dfr18*, but carried *virD2* and *floR*, conferring resistance to CHL (Taviani et al., 2009). Majority of the *V. cholerae* O1 isolated in Kolkata from 1989 to 1990 had STX^{MO10}/ICE*Vch*Ind4. This ICE was replaced by ICE*Vch*Ind5/ICE*Vch*Ban5 in the subsequent years (Weill et al., 2017, 2019).

In this study, the ICE^{TET} detected in *V. cholerae* O1 strains had significant structural dissimilarities with ICE*Vch*Ban9 (Bangladesh, 1994), ICE*Vch*Moz10 (Mozambique, 2004), ICE*Vch*B33 (Beira, 2004), and ICE*Vch*Lao1 (Iwanaga et al., 2004; Taviani et al., 2009; Marin et al., 2014). Nevertheless, structural variations, unstable core region, and the transfer region of both the ICEs found in our study were very much similar and shared a common ancestral backbone. In many ICEs, the core genes such as *int*, *bet, exo*, and *setR* are usually associated with phages, and genes such as *tra* are associated with plasmids (Wozniak et al., 2009; Armshaw and Pembroke, 2013). Having the same exclusion group (*eexR1*), ICE^{GEN} and ICE^{TET} were mutually exclusive and therefore did not co-exist in a strain. ICE sequences reconfirmed that there were two ICE types that kept emerging in different years. The key modifications between them indicated that they may have diverse origins or be derived from a common ancestor and could have later evolved independently.

We could transfer the ICE^{GEN} and ICE^{TET} from *V. cholerae* O1 to *E. coli* J53 by conjugation. The frequency of transfer observed was high (10^{-5} to 10^{-6}), indicating that the ICEs were promiscuous due to the presence of an active *tra* region (Kiiru et al., 2009; Pande et al., 2012). Our study showed that only the resistances conferred by genes present in ICE were transferable and that the level of expression was different, being more in the transconjugants with respect to the donor vibrios. This could be due to "gene dosage" effect or absence of repressor in the new genetic environment of the recipient *E. coli*. Transconjugants showing higher drug resistance have been described in the previous reports as well (Petroni et al., 2002; Sarkar et al., 2015b). The co-existence of ICEs with plasmids and class 1 integrons in clinical as well as environmental *V. cholerae* has been reported (Thungapathra et al., 2002; Pande et al., 2012). The involvement of plasmids carrying the ICEs was not tested in this study. We also observed that resistance to NA and AMP were not transferable, indicating that the resistance to these antimicrobials could be contributed by the chromosomal factors such as mutations and efflux pumps (Ghosh and Ramamurthy, 2011).

As shown in the PFGE analysis, the clonal relatedness of *V. cholerae* strains isolated during different years corresponded with the MDR profiles. ICE integrase-negative strains isolated in 2008, 2011, and 2012 were found to cluster together (cluster A). *V. cholerae* O1 strains harboring either ICE^{GEN} or ICE^{TET} were also grouped in different clusters (B and C, respectively). A similar observation was made with the outbreak strains of *V. cholerae* O1 in Kenya (Kiiru et al., 2009).

In conclusion, our findings revealed the existence of two types of ICEs in *V. cholerae* O1 strains from Kolkata. The ICE^{GEN} that contained conserved backbone genes was most commonly detected in *V. cholerae* O1 circulating around Kolkata. Features of the Kolkata *V. cholerae* O1 strains with ICE carrying the Tet^R encoding genes are unique and the sequence of the ICE^{TET} had several variations from other sequenced ICEs. Also the ICE^{TET} harboring *V. cholerae* O1 strains reappeared after 4 years of disappearance in Kolkata. Unique PFGE clusters of *V. cholerae* O1 harboring different ICEs are linked with the AMR patterns. The primer pair designed in this study may be useful in the detection of ICEs carrying the *tet*. The transmission potential of ICEs identified in this study was very high, as evidenced from the conjugation assay. Therefore, the impact of ICE regulation and interactions between bacteria prevailing in the same ecological niches should be explored in detail. Emergence of new types of ICEs may pose challenges in the existing cholera management strategies.

AUTHOR CONTRIBUTIONS

AG, TR, and KO conceived and designed the experiments. AS, DM, and GC performed the experiments. KO contributed reagents, materials, and analysis tools. TR and AM analyzed the data. AS and TR wrote the manuscript. All authors discussed the results, and reviewed and commented on the manuscript.

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