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Antibiotic resistance and its continuity in the environmental niche

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Antibiotic resistance and its continuity in the environmental niche

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Editorial: Antibiotic resistance and its continuity in the environmental niche

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Editorial on the Research Topic
Antibiotic resistance and its continuity in the environmental niche

Antibiotic resistance is a consequence of biased and exaggerated antibiotic treatments in both humans and animals and has recently been subject of wide-ranging community concern (1). Lack of clinical reasoning and consideration for presence or absence of epidemiological pressure selected representatives of the microbial community showing resistance plasmids looked at as "resistome." The "resistome" is continuously increasing due to changes in the ultrastructure and subsequently in the metabolism prompted by various factors, due to further introduction of new generation antibiotics and also concurrent influence of other environmental components. Highly performant and rapid laboratory methods are now utmost important for understanding the resistome from a One Health perspective, involving humans, animals and the environment and for its timely diagnosis (2).

Intensive farming technologies for food animals broadly imply the use of antibiotics as therapeutic means, thus trying to reduce the economic and health impact of infectious diseases by diminishing morbidity and mortality. Nevertheless, the spread of antibiotic resistant and MDR bacteria from animal farming to the broader environment may cause diseases in humans, livestock, and wildlife (3).

Research was conducted to clarify the transfer mechanisms of multi drug resistant (MDR) bacteria from farmed animals/food products to humans due to continuously increasing emergence of resistance. The variable potential for innate antibiotic resistance in the soil was also described based on geo-chemical conditions, while bacterio-plankton tolerance to antibiotics in heavy metal polluted areas was highlighted, suggesting the selective importance of pollution in maintaining and spreading antibiotic resistance (4).

Reciprocal relationships that exist between resistant and potentially pathogenic bacteria and their habitat or broader environment need to be precisely defined to allow the development and implementation of preventive and control measures with highest benefits for humans, animals and the environment. A solid connection can be established between the amounts of antimicrobials used and the increase of numbers of bacterial species resistant to them, above the presence of antimicrobial resistance in pristine sources before introduction of antimicrobials in medical and/or farming practice (5).

This Research Topic aimed at updating research results on antibiotic resistance, its emergence and persistence, horizontal transfer of antibiotic resistance genes, rapid diagnosis of multi antibiotic resistance, its prevention and control, and its connections to environmental factors (such as geography, climate, and climate change) as well as the influence of farming and sewage water management, wildlife and its conservation, and others, to have a comprehensive view of the importance of the often disregarded environmental niche in increasing virulence of pathogenic bacteria.

In this special e-collection there are 18 papers covering the above mentioned aspects.

The presence, persistence and technological influences on antimicrobial resistance in domestic animals were the most tackled subjects (10 of 18 papers, 55.55%). Numerous categories of animals from farmed species (pigs, bovine, chickens, and fish) companion animals (dogs, cats, pigeons, and horses), and also wildlife (foxes, seals, and Geoffrey's cats) inhabiting terrestrial and aquatic environments were monitored for the presence of multidrug resistant microbiome. Comprehensive studies of bacteriome resistance in various environments were also published.

Swine industry is a continuously developing economic sector, while there is a constant need and demand for meat and meet products by consumers. Nevertheless, pigs are hosts for numerous zoonotic pathogens, including ported bacteria, whose increasing antimicrobial resistance support their pathogenicity and aggressivenes and thus, their survival in the habitat. Therefore, ubiquitous bacteria such as *Bordetella bronchiseptica* and *Escherichia coli* need close monitoring for their antimicrobial resistance gene profile. The resistance profile found in *B. bronchiseptica* in pigs from China, one of the largest pork producers in the world, was highly variable, but including percentages as high as 83.98 to ampicillin, very commonly used in animal therapy. Over 90% of the isolates were positive to the five virulence factor encoding genes examined, representing a reason for major consumer health concern (Zhang et al.). Similarly, other researchers (Khine et al.) found *mcr* resistance genes to colistin, a last resort antibiotic to fight the infections with *Enterobacteriaceae* in MCRPE (*mcr* positive *E. coli*) isolates also showing MDR and connection to *E. coli* ETEC (enterotoxigenic) pathotype shared by human and animal hosts.

Further, it has been proven that *E. coli* strains originating from non-organic chickens, raised in low-income communities harbor antibiotic resistant genes found in multidrug-resistant and extended-spectrum beta-lactamase (ESBL) phenotypes, which could eventually colonize the human gut of bird contacts or consumers (Murray et al.). In dairy cow herds, mastitis is maybe the most economically impacting disease caused by a variety of agents, with a rapidly changing etiology, frequently involving antibiotic resistant agents, therefore the phenotypic and molecular analysis of *Candida krusei*, a yeast collected from mastitis cases provided valuable information for disease control, indicating that drug-resistance was relying on mutations of the ERG11 gene (Du et al.).

Farming aquatic animals became a widespread practice lately, following the trend of increased protein need for feeding the planet. Due to specific use of antibiotics for microbial disease control, fish farms not only represent a possible source for antimicrobial resistance for consumers but also impact on the environment health, by the location of the ponds either down- or upstream the rivers, thus showing an potentially enhanced multidirectional spread of this resistance. A study carried out in Southern Lithuania, envisaging the simultaneous presence of heavy metal pollution and antimicrobial resistance in the sediment of fish ponds, indicated that in spite of the heavy metal (Co, Cu, and As) levels which did not exceed the maximum allowable concentrations and antibiotic residues (oxytetracycline, florfenicol, and florfenicol amine) present in low amounts or below the detectable limit, the resistance determinants identified (aminoglycoside, β-lactam) create risks to human hosts by potential transfer (Lastauskiene et al.). Experiments aiming at investigating the horizontal transfer of antimicrobial and arsenic resistance genes have provided positive evidence of this between certain bacteria, such as *Rheinheimera* spp. and *E. coli*, suggesting a permanent monitoring of antibiotic/arsenic resistance profile of the bacteriome to reduce or avoid the spread of gene pollutants (Fu et al.).

Similarly, the identification of increase in the CTX-M type ESBL producing *E. coli* variants in the Seine river over time underlined that the aquatic environment exposed to numerous polluting sources, posing risks during recreational activities, is of broad community concern (Girlich et al.).

Several studies mention the MDR or pan-drug resistance in companion animals such as dogs, cats and horses. An extended research of clinical cases in the Iberian peninsula provides an overview of the bacteria most frequently found in dogs and cats and also their resistance profile to antimicrobials, indicating the highest antimicrobial resistance in *Enterococcus* spp. and *Pseudomonas* spp., while interestingly, *Klebsiella* spp., *Proteus* spp. or *Enterobacter* spp. seemed to be the most resistant of *Enterobacteriacea* (Li et al.), when compared to the otherwise MDR ESBL *E. coli*, as indicated by other researchers (Huang et al.). Nevertheless, *Pseudomonas spp*. of canine origin seemed

to also be resistant (72.7–100%) to antimicrobials linezolid (LZD) and tigecycline (TGC) efficient in fighting with pan-drug resistant bacteria isolated from humans (Kim and Kim).

A quite widespread category of companion or hobby birds are racing pigeons. Their close contact with humans during feeding, handling and competitions creates the premises for transfer of multidrug resistant bacteria or yeasts (*Staphylococcus aureus*, non-hemolytic *E. coli*, and *C. albicans*) and also the antimicrobial resistance genes to the latter (Chrobak-Chmiel et al.). Further, such birds could, through their interactions and lifestyle, close a loop of antimicrobial resistance in their closer or further environment.

As already mentioned (Li et al.), hospital environment could provide an appropriate environment for the persistence of antimicrobial resistance, not only through the patients seen but also on the contact surfaces. Such a study carried out in an equine hospital consulting local and international patients revealed the presence of multidrug resistant, host-versatile *Salmonella typhimurium* on human and patient contact surfaces, which led back to the significance of biosecurity measures implemented at all times to preserve patient and personnel safety (Soza-Ossandón et al.).

Wildlife, out of direct connection with antibiotic therapy, has been disregarded as a link in the antibiotic resistance transfer chain. Recent research has proven the presence and high incidence (66%) of enterococci in the feces of wild foxes (*Lycalopex gymnocercus*) and Geoffroy's cats (*Leopardus Geoffroyi*) from the Brazilian Pampa, with resistance percentages as high as 94 or 72.6 to rifampicin, one of the most potent broad spectrum antibiotics, and erythromycin, respectively. This phaenomenon was supported by the identified resistance genes (*tet*M/*tet*L and *msr*C/*erm*B) along with virulence genes (*gel*E, *ace*, agg, *esp*, and *cly*A), standing most probably for human interference in the pampa habitat (Oliveira de Araujo et al.).

The marine ecosystem is not spared of antimicrobial resistance, the investigation of marine mammals and costal environment providing valuable information on another direction of antimicrobial resistance spread. As such, the identification of 66.6% MDR of the total *E. coli* isolates from feces of rescued seals, identification of resistance genes in 16 of 39 of the isolates and virulence factors associated with adhesion and siderophores, augmenting the pathogenicity of these strains was relevant (Vale et al.).

A broader study comparing MRSA and MSSA from various animal sources and different environments disclosed clear differences between *mec*C-positive and *mec*C-negative types, with possible human origin of the *mec*C-MRSA including the typically human "immune evasion cluster" (IEC) (Gómez et al.).

A review of the perspectives on antimicrobial resistance at the level middle and low income countries, with special reference to the COVID period, brings forward a pertinent analysis of the antibiotic types toward which the resistance is augmented, the most frequent fields of activity and host species, representing important links within the antimicrobial resistance chain as well as multi-level and multi-actor mitigation strategies (Bandyopadhyay and Samanta).

The food chain represents another potential route for spreading the antimicrobial resistance from farm to fork. Further, the beneficial effects of lactic acid bacteria have been recognized and given attention since decades. Nevertheless, such strains could serve as vehicle for spreading antimicrobial resistance as indicated (Stefańska et al.) during a study which included probiotic feed additives/silage inoculants. Tests carried out on their antibiotic susceptibility/resistance indicated the resistance to aminoglycosides and tetracyclines mainly (26%). Therefore, the authors suggested as a safety criterion the preliminary resistance analysis of the strains of *Lactobacillus* and *Pediococcus* to ensure their appropriate effects. Moreover, the analysis for resistance genes in a non-pathogenic bacteria such as *Bifidobacterium animalis* subsp. *lactis* revealed the presence of *tet*(W) (tetracyclin resistance gene) in 41 of 44 examined strains, but being a part of the ancient resistome, is different from other species and possesses a very low transfer risk (Nøhr-Meldgaard et al.).

In conclusion, the data gathered in the studies and reviews mentioned before provide beneficial information, which, without being exhaustive, offer a valuable insight in the complex matter of antimicrobial resistance and its transmission chain, leaving room for the intriguing and still undiscovered interaction of humans, animals and environment with the aim of preserving One Health.

Author contributions

Both 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.

Publisher's note

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BRIEF RESEARCH REPORT
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Widespread Environmental Presence of Multidrug-Resistant *Salmonella* in an Equine Veterinary Hospital That Received Local and International Horses

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Salmonella enterica is a highly infectious microorganism responsible for many outbreaks reported in equine hospitals. Outbreaks are characterized by high morbidity and mortality rates, nosocomial transmission to other patients, zoonotic transmission to hospital personnel, and even closure of facilities. In this study, 545 samples (environmental and hospitalized patients) were collected monthly during a 1-year period from human and animal contact surfaces in an equine hospital that received local and international horses. A total of 22 *Salmonella* isolates were obtained from human contact surfaces (e.g., offices and pharmacy) and animal contact surfaces (e.g., stalls, surgery room, and waterers), and one isolate from a horse. Molecular serotyping revealed 18 isolates as *Salmonella* Typhimurium and three as *Salmonella* Infantis. Nineteen isolates were resistant to at least one antimicrobial class, and only two isolates were susceptible to all antimicrobials tested. In addition, we identified nine multidrug-resistant (MDR) isolates in *S*. Typhimurium, which displayed resistance to up to eight antimicrobials (i.e., amoxicillin/clavulanate, ampicillin, ciprofloxacin, chloramphenicol, streptomycin, gentamicin, trimethoprim/sulfamethoxazole, and tetracycline). Pulsed-field gel electrophoresis (PFGE) revealed the presence of three PFGE patterns permanently present in the environment of the hospital during our study. The persistent environmental presence of MDR *Salmonella* isolates, along with the fact that local and international horses are attended in this hospital, highlights the importance of improving biosecurity programs to prevent disease in horses and the hospital personnel and also for the global dissemination and acquisition of MDR *Salmonella*.

Keywords: *Salmonella enterica*, multidrug-resistant, equine hospital, hospital-acquired infections, biosecurity

INTRODUCTION

Salmonella enterica, a Gram-negative bacteria of the family Enterobacteriaceae, is an important zoonotic pathogen that causes an estimated of 93.8 human cases and 150,000 deaths every year worldwide (1). *Salmonella* is usually transmitted to humans as foodborne and through contact with infected animals (2). This pathogen is a microorganism responsible for gastrointestinal disease affecting equines (among other animals) of all ages (3). Clinical symptoms include diarrhea, fever, and dehydration, with severity ranging from a subclinical colonization to a severe systemic illness (4). As a highly contagious disease, it can be reported as sporadic cases or as an outbreak (5, 6). Previous studies have reported significant mortality (38–44%) (7, 8) associated with salmonellosis outbreaks in equine veterinary hospitals (EVHs). Also, hospitalization and associated use of health-care resources increase the susceptibility of horses to strains of *S. enterica* disseminated by asymptomatic animals (4, 5).

It has been reported that one of the main reasons for the increasing rate of salmonellosis outbreaks are multidrug-resistant (MDR) strains of *Salmonella* (9–13). Last year, the New York State Veterinary Diagnostic Laboratory reported the isolation of *Salmonella* Group C2 from four different horse farms, which had shown the same MDR profile (14). This is rather concerning if we consider that back in the early 2000s, a strain of an MDR–*Salmonella* Newport (G2) was responsible of a serious outbreak in a Large Animal Teaching Hospital (9, 15). It is still unclear when or how MDR–*Salmonella* emerged, being one of the main suspects in the non-therapeutic use of antibiotics (14).

Salmonellosis outbreaks in animal health facilities are full of challenges beside the sole medical treatment and control the outbreak *per se*; they also involve communication with owners and referring veterinarians of infected horses (10). On the other hand, the consequences are serious including hospital-acquired infections of patients and hospital personnel, the establishment of expensive infection control programs, and decrease in clients' trust and hospitals' revenues and may even lead to litigation procedures (11, 12). Infection control programs should be an integral part of every animal health facility (16, 17). Several studies have reported outbreak control measurements (7, 12, 18) and assessment of protocols of contamination, which have been adopted by many facilities (16, 19). To date, there are no reports of salmonellosis in veterinary hospitals in Chile, and therefore, scarce biosecurity protocols have been established. Hence, this study was performed to determine the presence, antimicrobial resistance, and subtypes of *Salmonella* in the environment and patients from an EVH without reported history of outbreaks or hospital-acquired infections.

MATERIALS AND METHODS

Description of the Setting and Location

The EVH is located at a thoroughbred horse racetrack at the center of the city of Santiago (Chile). It has an average flow of 100 incoming patients daily, providing equine health services to Thoroughbred, Arabian, Chilean rodeo, and Warmblood horses. This veterinary hospital has no records of outbreak or hospital-acquired infections due to *Salmonella* spp., and this information is remarkable in view of the lack of biosecurity measures or infection control programs (e.g., isolation of infected patients and protocols for cleaning and sanitation).

TABLE 1 | Results of *Salmonella* spp. on samples collected in the equine veterinary hospital during the study.

Sample origin	No. of samples	No. positive samples	% positive samples
Animal feces	53	1	1.88
Environmental/surgery (SA)[a]			
Stalls (1–4)	48	1	2.08
Surgery room floor	12	2	16.67
Bed	12	0	0
Pharmacy	12	1	8.33
Washing room	12	1	8.33
Dressing room	12	0	0
Personal entrance	12	0	0
Office	12	0	0
Induction/recovery room	12	0	0
Area Floor	12	0	0
Environmental/hospitalization (HA)[a]			
Stalls (5–10)	72	3	4.17
Floor	12	1	8.33
Environmental/proceeding (PA)[a]			
Pharmacy	12	1	8.33
Floor	12	1	8.33
Main office	12	2[b]	16.67
Environmental/equipment (EQ)[a]			
Twitches (3×)	36	1	2.78
Endoscope	12	1	8.33
Gastroscope	12	1	8.33
Pitchforks (2×)	24	2	8.33
Waterers (1×)	120	1	0.83
Environmental/exterior (EA)[a]			
Manure collection site	12	1	8.33
Total	545	21	3.85

[a] *Environmental samples were classified according to how the hospital was divided into four main areas, plus equipment (see* ***Figure 1*** *and Materials and Methods).*
[b] *Two different isolates were obtained from one sample taken on September 2015.*

Sampling Procedure

A total of 545 samples were obtained in a longitudinal study conducted from July 2015 to June 2016. With the corresponding consent from the Chief Director, we collected both environmental ($n = 61$, for details see **Table 1**) samples and patient fecal samples, from one to nine, depending on hospitalized horses at a given time (20, 21). Samples were conducted during the afternoon on the last Friday of every month. The hospital was divided into four areas: surgical area (SA), proceeding area (PA), hospitalization area (HA), exterior area (EA), and a fifth category for equipment (EQ), similarly as described by Alinovi et al. (18) (**Figure 1A**). In addition, the

surfaces sampled were classified into animal contact surfaces (direct contact of animals and humans) ($n = 396$) and human contact surfaces (direct contact of humans, but out of reach of animals) ($n = 96$), as previously described Alinovi et al. (20) (**Figure 1A**). The samples were obtained using a sterile gauze soaked in 90 ml of peptone water (Becton-Dickinson™, Franklin Lakes, NJ) and rubbed on the surface for 5 min. For patient samples, approximately 100 g of manure was collected and transferred into a sterile recipient. To avoid interference with the normal activities of the EVH, only one sample per hospitalized patient was collected on each sampling day. All the samples were maintained at 4°C during sampling and immediately transferred to the laboratory at Universidad Andres Bello (Santiago, Chile) for further analysis.

Bacterial Culture and Molecular Identification

Salmonella isolation was conducted as previously described (22). In brief, all samples were cultured in peptone water at 37°C overnight, and 100 µl and 1 ml were transferred into Rappaport–Vassiliadis (RV) (BD, Franklin Lakes, NJ) supplemented with novobiocin (20 mg/ml) and 100 µl of Tetrathionate (TT) (BD, Franklin Lakes, NJ) supplemented with iodine, respectively, and incubated at 42°C overnight. Finally, 100 µl of aliquot of each selective broth was streaked into an XLT-4 agar plate (BD, Franklin Lakes, NJ) and incubated at 37°C overnight. Four colonies of each agar plate were selected and transferred into Tryptic Soy Agar (TSA) (BD, Franklin Lakes, NJ). All presumed colonies of *Salmonella* spp. were confirmed by *invA*-PCR. Primers and PCR conditions used in this study have been previously described (23). Confirmed colonies were grown overnight in Trypticase Soy Broth (TSB) (BD, Franklin Lakes, NJ) and then immersed in a 20% solution of glycerol (Winkler, Santiago, Chile) and stored at −80°C.

Determination of Antimicrobial Susceptibility

The disk diffusion method of Kirby–Bauer was used to determine antimicrobial susceptibility (24). PCR-confirmed colonies were suspended in 5 ml of Mueller–Hinton (MH) broth (BD, Franklin Lakes, NJ) and incubated at 37°C overnight. Cultures were adjusted to MacFarland 0.5 (bioMérieux, France) (equivalent to 1.5×10^8 CFU/mL) and streaked on MH agar. An OXOID™ (Hampshire, UK) sensitivity disk dispenser was used, along with the antimicrobial disks, detailed as follows: amikacin (AMK; 30 µg), amoxicillin/clavulanate (AMC; 30 µg), ampicillin (AMP; 10 µg), cefoxitin (FOX; 30 µg), ceftriaxone (CTR; 30 µg), ciprofloxacin (CIP; 5 µg), chloramphenicol (CHL; 30 µg), streptomycin (STR; 300 µg), gentamicin (GEN; 10 µg), kanamycin (KAN; 30 µg), trimethoprim/sulfamethoxazole (SXT; 23.75 µg), and tetracycline (TET; 30 µg). The agar plates were incubated at 37°C overnight. *Escherichia coli* American Type Culture Collection (ATCC) 25922 was used as control. Interpretations were made based on the guidelines of Clinical Laboratory Standard Institute (25). The samples were classified according to Magiorakos's criteria as MDR when resistant to at least one agent in three or more antimicrobial classes (26).

Molecular Characterization of *Salmonella* Serotype

A previously described molecular method for serotype prediction was used (27, 28). Briefly, DNA extraction of the isolates was conducted using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany). The molecular scheme included an initial multiplex PCR, conducted to identify the serogroup of each isolate, followed by PCR-sequencing approaches to determine H1 and H2 antigens (27, 28). PCR products were sent to MACROGEN™ (Korea) for Sanger sequencing. Consensus sequences were obtained using CAP3 Sequence Assembly Program (http://doua.prabi.fr/software/cap3); the complementary reverse was obtained by using Bioinformatics.org. The results were analyzed using basic local alignment tool (BLAST) on the National Center for Biotechnology Information (NCBI).

Molecular Typing

Molecular typing of the isolates was conducted by pulsed-field gel electrophoresis (PFGE), using the CDC PulseNet standard protocol (29). For this, overnight cultures in brain hearth infusion broth (BHI, BD, Germany) were embedded in 1% of SeaKem® Gold Agarose (Lonza, Rockland, ME, USA). Upon lysis and washing, the plugs were digested with *Xba*I (Thermo Fisher Scientific Inc., Waltham, MA). The CHEF-DR® III System (Bio-Rad Laboratories, Hercules, CA) was used for the electrophoresis for 20 h. A standard, *Salmonella* Braenderup digested with *Xba*I was used. BioNumerics v 7.5 (Applied Maths, Sint-Martens-Latem, Belgium) (30) was used to analyze the PFGE images using unweighted pair group method with arithmetic mean (UPGMA) and the Dice correlation coefficient. PFGE was conducted at the Microbiology Unit of the Clinical Laboratory Services of Red Salud UC-CHRISTUS, Catholic University. The results were analyzed using Tenover guidelines as previously described (31).

RESULTS

Salmonella spp. Were Obtained Mostly From Environmental Samples in Human Contact Surfaces

A total of 545 samples (environmental, $n = 492$; patient, $n = 53$) were analyzed. Among these, 21 samples (3.85%) yielded positive for *Salmonella*, which were confirmed by *invA*-PCR (**Table 1** and **Supplementary Figure 1**). In 3/21 (14.2%) samples, *Salmonella* isolates were obtained from TT enrichments; in 10/21 (47.6%), *Salmonella* isolates were obtained from RV enrichments; and in the remaining 10/21 (47.6%) samples, *Salmonella* isolates were obtained from both enrichments conducted. On positive samples, one isolate was selected, except for one sample, in which two different colonies were obtained; therefore, a total of 22 *Salmonella* colonies were further characterized.

FIGURE 1 | Schematic diagram of the equine veterinary hospital. **(A)** Black circles show the animal contact surfaces, whereas the white circles show the human contact surfaces. **(B)** Locations of the different pulse types of *Salmonella enterica* found in this study (colors matching **Figure 2**). SA, surgery area; PA, proceeding area; HA, hospitalization area; EA, exterior area; S1–10, stalls; Sr, surgery room; I/Rr, induction/recovery room, SPh, surgery pharmacy; Wr, washing room; SOf, surgery office; En, entrance; Bth, bathroom; Dr, dressing room; MOf, main office; SYr, surgery yard; HYr, hospitalization yard; mcs, manure collection site.

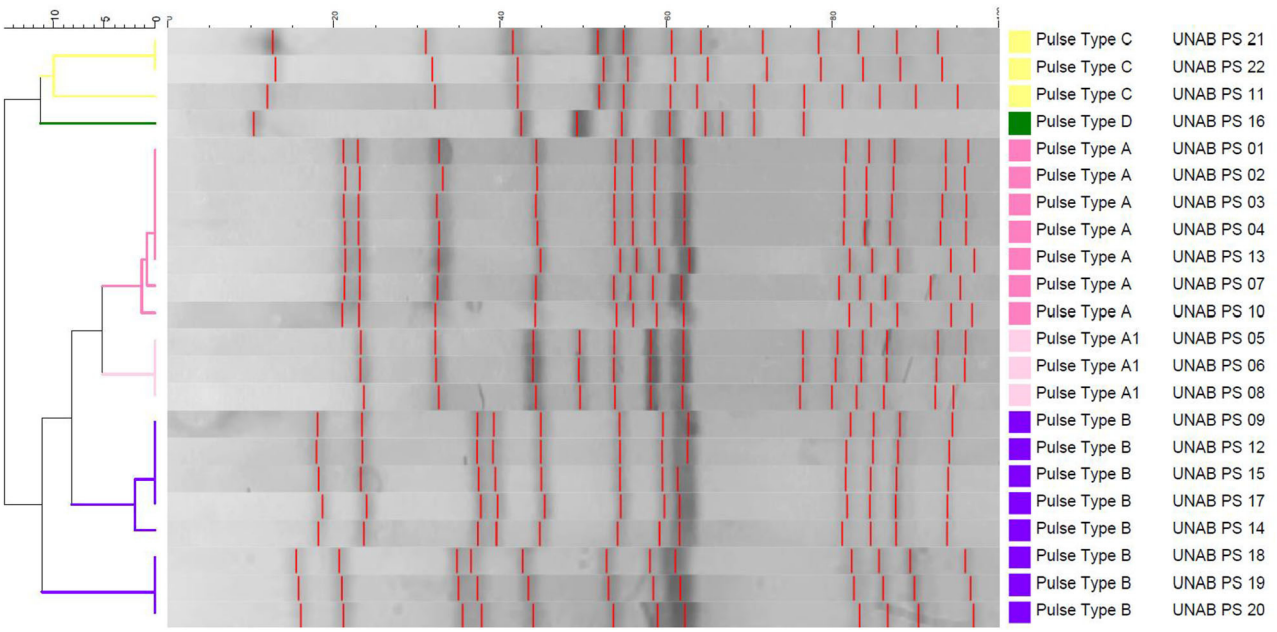

FIGURE 2 | Dendrogram representation of *Salmonella enterica* isolates clustered using unweighted pair group method with arithmetic mean (UPGMA) method. Five pulse types of *Salmonella* were identified at the right; colors have been assigned for each pulse type (A–D), matching **Figure 1B**.

From the 22 isolates, 1/22 (4.5%) was obtained from a sick Chilean rodeo patient, which died of peritonitis after colic surgery (no positive foreign patients were found), and the other 21 (21/23; 95.4%) were obtained from 20 environmental samples (i.e., stalls, surgery room floor, surgical pharmacy, washing room, hospitalization area floor, main office, pitchforks, endoscope, gastroscope, twitches, waterers, manure collection site, proceeding area floor, and pharmacy) (**Table 1**). Regarding the type of contact surface, 13/396 (3.28%) isolates were obtained from animal contact surfaces and 8/96 (9.38%) from human contact surfaces (**Table 2**). About the dates of isolation, two peaks were seen during the months of September 2015 and May 2016, where 9/22 and 8/22 isolates of *Salmonella* spp. were obtained, respectively (**Supplementary Figure 1**). A few isolates were also obtained during October 2015 ($n = 1$), December 2015 ($n = 1$), April 2016 ($n = 1$), and May 2016 ($n = 2$) (**Table 2**, **Supplementary Figure 1**).

Presence of Multidrug-Resistant *Salmonella* Isolates

Kirby–Bauer tests revealed six antimicrobial resistant profiles (**Table 2**). From the 22 *Salmonella* isolates, two were pan-susceptible, 10 isolates were resistant to AMP; one isolate was resistant to STR; six isolates were resistant to AMC, AMP, CHL, STR, and TET; one isolate was resistant to AMC, AMP, CTR, CHL, STR, and TET; and two isolates were resistant to AMC, AMP, CIP, CHL, STR, GEN, SXT, and TET. From these, 9/22 (40.1%) were classified as MDR, as these were resistant to one agent in three or more antimicrobial classes (26).

Predominance of *Salmonella* Serotype Typhimurium

All isolates were tested to predict the serogroup and serotype as described above. The molecular methods showed 19/22 (86.4%) of *Salmonella* isolates to O:4 (B) serogroup and three *Salmonella* isolates 3/22 (13.6%) to O:7 (C1) serogroup. Concerning flagellar antigens, DNA was amplified for both genes, *fli*C and *flj*B, in all *Salmonella* isolates. The BLAST algorithm of the FASTA consensus sequences of the PCR products allowed us to predict the serotype. All isolates belonging to O:4 (B) serogroup (20/22) yielded positive for serotype Typhimurium, whereas the isolates belonging to O:7 (C1) serogroup (2/22) were predicted as Infantis serotype (**Table 2**).

Five Different Pulsed-Field Gel Electrophoresis Types of *Salmonella* Were Identified

According to the PFGE, four PFGE patterns were identified in 19 *Salmonella typhimurium* isolates, and one PFGE type was found in three *S.* Infantis isolates. Among *S.* Typhimurium, seven isolates (1, 2, 3, 4, 7, 10, and 13) were indistinguishable from each other and classified as PFGE pattern A. In three isolates (5, 6, and 8), PFGE patterns were also indistinguishable from each other and related to PFGE pattern A, which was therefore classified as A1. All PFGE patterns A and A1 were detected only in the sampling of September 2015. Eight isolates (9, 12, 14, 15, 17, 18, 19, and 20) were indistinguishable from each other and different from all others, classified as PFGE pattern B; these isolates were obtained in samplings of September 2015 and in April and May 2016. One additional PFGE pattern D of isolate 16 was found in

TABLE 2 | Characteristics, serotypes, PFGE patterns, and antimicrobial resistance of *Salmonella* isolates.

Isolate ID (UAB)[a]	Isolation date	Source[b]	Area[b]	Serotype	PFGE pattern	Antibiotic resistance profiles[c]
PS-001	Sept 2015	Stall 1	Surgery[d]	Typhimurium	A	AMP
PS-002	Sept 2015	Stall 7	Hospitalization[d]	Typhimurium	A	AMP
PS-003	Sept 2015	Stall 10	Hospitalization[d]	Typhimurium	A	AMP
PS-004	Sept 2015	Yard	Hospitalization[d]	Typhimurium	A	AMP
PS-005	Sept 2015	Pitchfork	Equipment[d]	Typhimurium	A1	AMP
PS-006	Sept 2015	Manure collection site	Exterior[d]	Typhimurium	A1	AMP
PS-007	Sept 2015	Main office	Proceeding[e]	Typhimurium	A	Pan-susceptible
PS-008	Sept 2015	Waterers	Equipment[d]	Typhimurium	A1	STR
PS-009	Sept 2015	Main office	Proceeding[e]	Typhimurium	B	AMC–AMP–CHL–STR–TET
PS-010	Oct 2015	Stall 10	Hospitalization[d]	Typhimurium	A	AMP
PS-011	Dec 2015	Surgery room floor	Surgery[e]	Infantis	C	AMP
PS-012	Apr 2016	Pharmacy	Proceeding[e]	Typhimurium	B	AMC–AMP–CIP–CHL–STR–GEN–SXT–TET
PS-013	May 2016	Surgery room floor	Surgery[e]	Typhimurium	A	AMC–AMP–CHL–STR–TET
PS-014	May 2016	Twitch	Equipment[d]	Typhimurium	B	AMC–AMP–CTR–CHL–STR–TET
PS-015	May 2016	Endoscope	Equipment[d]	Typhimurium	B	AMC–AMP–CIP–CHL–STR–GEN–SXT–TET
PS-016	May 2016	Gastroscope	Equipment[d]	Typhimurium	D	Pan-susceptible
PS-017	May 2016	Proceeding area floor	Proceeding[d]	Typhimurium	B	AMC–AMP–CHL–STR–TET
PS-018	May 2016	Main office	Proceeding[e]	Typhimurium	B	AMC–AMP–CHL–STR–TET
PS-019	May 2016	Washing room	Surgery[e]	Typhimurium	B	AMC–AMP–CHL–STR–TET
PS-020	May 2016	Pharmacy	Surgery[e]	Typhimurium	B	AMC–AMP–CHL–STR–TET
PS-021	Jun 2016	Pitchfork	Equipment[d]	Infantis	C	AMP
PS-022	Jun 2016	Patient	Surgery[d]	Infantis	C	AMP

PFGE, pulsed-field gel electrophoresis.
[a] *All isolates with pre-fix UAB after Universidad Andres Bello laboratory.*
[b] *Sources and areas in the hospital where the samples were taken (see **Figure 1**).*
[c] *Amikacin (AMK), amoxicillin/clavulanate (AMC), ampicillin (AMP), cefoxitin (FOX), ceftriaxone (CTR), ciprofloxacin (CIP), chloramphenicol (CHL), streptomycin (STR), gentamicin (GEN), kanamycin (KAN), trimethoprim/sulfamethoxazole (SXT), and tetracycline (TET).*
[d] *Animal contact surfaces.*
[e] *Human contact surfaces.*

S. Typhimurium. Isolates 11, 21, and 22 were indistinguishable from each other and different from all others, classified as PFGE pattern C. Importantly, these isolates were classified as *S.* Infantis (**Table 2**).

DISCUSSION

This study examined the environmental presence of *Salmonella* in an equine hospital with no history of outbreak or hospital-acquired infections. Here, we identified two serotypes that were widely distributed. The major findings of this study are the following: (i) wide spatial distribution of *Salmonella* in the hospital, mainly in spring and autumn; (ii) MDR *Salmonella* Typhimurium accounted for most of the isolates; and (iii) multiple *Salmonella* PFGE patterns present in human contact surfaces highlight the need of developing biosecurity standard protocols.

Wide Spatial Distribution of *Salmonella* in the Hospital, Mainly in Spring and Autumn

In this study, we found a considerable presence of *Salmonella* in the EVH environment, compared with the equine's samples. The prevalence of *Salmonella* in equine subclinical shedders (1–2%) tends to increase under stress conditions owing to hospitalization to 9–13% (5, 6, 10). In the environmental samples, positivity was widespread to all sampled areas (including equipment), reaching 4.5%. A previous study conducted at a large animal hospital has shown the presence of *Salmonella* in several areas, accounting for a positivity rate of 3.9% during a post-outbreak period (32). Importantly, in our study, no outbreak or hospital-acquired infections were reported, before and/or during the study.

It has been shown that the peak incidence of salmonellosis in horses occurs in summer and autumn (5, 33), although there are some outbreak reports during spring (7). Here, we obtained *Salmonella* isolates in every season of the year, although the highest number of isolates was obtained during September 2015 and June 2016, spring and winter for the southern hemisphere, respectively (**Table 2**, **Supplementary Figure 2**). Our first peak, on September 2015, was an incoming Chilean rodeo patient suffering from severe acute diarrhea, which died within 24 h after being admitted to the EVH. As *Salmonella* was isolated from the stall of that patient, Stall 10 (**Figure 1**), it may have been introduced to the EVH by this patient, but further investigation is needed, which is beyond the scope of this study. Importantly, these isolates represented a closely related PFGE pattern. Nevertheless, neither official information nor patient

history could be collected to explain the second peak, in June 2016. Although it is uncertain about the origin of these isolates, shedding patients present during non-sampling periods could be a common source of dissemination (5). Other possible sources of contaminations, such as other animals (rodents), feed, or even environmental persistent strains (34), are also plausible and have to be considered.

Multidrug-Resistant *Salmonella* Typhimurium Accounted for Most Isolates

Reported outbreaks of *Salmonella* in EVHs have involved serotypes such as Typhimurium, Newport, Agona, Anatum (12, 35), Infantis (36), Heidelberg (37), and Oranienburg (38). Here, we found that 87% of the isolates were represented by *S.* Typhimurium. This serotype has been commonly isolated from horses, causing severe clinical signs, along with high morbidity and mortality rates (7, 8, 33). In Chile, only one outbreak of *S.* Typhimurium has been reported, which affected weanling foals with a morbidity rate of 87% and mortality rate of 13% (39). Regarding *Salmonella* Infantis, which is less commonly reported compared with *S.* Typhimurium, only three isolates were found. Nonetheless, there is a report of a serious outbreak in 1996, which caused important economic losses and even the closure of the facilities (36).

Antimicrobial resistance profiles, which include resistance to AMP (10 isolates), as the most common profile, followed by the profile AMC–AMP–CRO–CHL–STR–TE (six isolates), include antimicrobials in which resistance has already been described in other salmonellosis outbreaks (38), not only in equine hospitals but also in small animal shelters (13). Notably, we found that almost half of the isolates ($n = 10$) displayed an MDR phenotype, showing resistance to three or more antimicrobial classes (26), which is a major concern for the public health, the personnel at the hospital, and the treatment of hospitalized horses.

Multiple *Salmonella* Pulsed-Field Gel Electrophoresis Patterns Present in Human Contact Surfaces Highlight the Need of Developing Biosecurity Standards

We found five different PFGE patterns, which were present in all areas of the hospital, including human contact surfaces. Environmental presence of *Salmonella* indicates that personnel without animal contact at all (e.g., secretary) could also be at risk of infection. As pointed before, no information concerning hospital-acquired infections was reported during our study, neither from incoming patients nor from veterinary staff. In the environment, *Salmonella* could put into high risk the incoming susceptible patients, as young horses or immunocompromised individuals (33). This leads us to think that it may be a potential risk of an outbreak. There has been reports of $755,000 USD of estimated cost to control salmonellosis outbreaks in a large animal teaching hospital in Virginia (USA) (12), which lead us to the conclusion that biosecurity standard protocols must be implemented to prevent any undesirable event (17). There are many guidelines of biosecurity protocols (e.g., rubber boots, hand washing, and foot bath) (21, 40, 41) and also published articles in which salmonellosis outbreaks have been controlled (7, 12, 16, 18, 19). Although the implementation of biosecurity protocols is quite expensive, it is much less than controlling an outbreak itself, especially considering the fact that the EVH located at a thoroughbred racetrack, harbors nearly 1,500 horses together with hospital personnel (17).

CONCLUSIONS

This study has revealed the importance of implementing mitigation strategies and biosecurity protocols to control MDR *Salmonella* to ensure the safety of patients and hospital personnel. Also, this could set an example for other veterinary facilities to establish or recheck their functioning biosecurity protocols, especially in developing countries.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The University Andres Bello Bioethics Committee, Santiago, Chile. Written informed consent for participation was not obtained from the owners because fresh fecal samples were obtained from the floor.

AUTHOR CONTRIBUTIONS

PS-O designed the study, conducted the experiments, and wrote the manuscript. AM-S wrote the manuscript, analyzed data, and designed the study. DR and RT conducted the experiments. RR-N critically reviewed the manuscript. AA, GG-R, and CH-W analyzed the data. PG conducted the experiments. All authors contributed to the article 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/fvets.2020.00346/full#supplementary-material

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Characteristics of Extended-Spectrum β-Lactamase–Producing *Escherichia coli* From Dogs and Cats Admitted to a Veterinary Teaching Hospital in Taipei, Taiwan From 2014 to 2017

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Extended-spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to newer β-lactam antibiotics, including extended-spectrum cephalosporins and monobactams. The production of ESBL is primarily plasmid mediated, and such plasmids often comprise the genes that encode resistance to other classes of antimicrobials, such as aminoglycosides and fluoroquinolones. Therefore, ESBL-producing microorganisms leave clinicians with limited therapeutic options in both human and veterinary medicine. Compared with human medicine, information regarding ESBL-producing microorganisms is limited in veterinary medicine. We screened for ESBL-producing *Escherichia coli* in dogs and cats admitted to National Taiwan University Veterinary Hospital, Taipei, from 2014 to 2017 and further analyzed the genotypes and phylogenetic traits of these ESBL producers. Double disk tests specified by the Clinical and Laboratory Standards Institute were performed on 283 *E. coli* isolates and revealed a total of 65 *E. coli* (54 from dogs and 11 from cats) with the ESBL phenotype (22.8%). $bla_{\text{CTX-M-1 group}}$ and $bla_{\text{CTX-M-2group}}$ were the most commonly identified ESBL gene groups. $bla_{\text{CTX-M-55}}$ was the main ESBL gene within the $bla_{\text{CTX-M-1group}}$, whereas the $bla_{\text{CTX-M-2group}}$ contained only $bla_{\text{CTX-M-124}}$. The ESBL-producing *E. coli* were all resistant to ampicillin. The resistance rate to ceftiofur, doxycycline, enrofloxacin, and ciprofloxacin was 93.8, 73.8, 80, and 78.5%, respectively. Of the antibiotics tested, greater sensitivity to imipenem and gentamicin was noted. Multilocus sequence typing indicated that ST457, ST131, and ST648 were the most common sequence types. Our study identified eight ST131/O25b isolates, which is a global zoonotic clone of public health concern. The major ESBL genes

of these clones were $bla_{CTX-M-174}$ and $bla_{CTX-M-194}$. Because companion animals such as dogs and cats are in close contact with humans, the characterization of ESBL producers originating from them is crucial from the perspective of both public health and veterinary medicine.

Keywords: extended-spectrum-β-lactamases, *Escherichia coli*, CTX-M, multilocus sequence typing, multidrug resistance

INTRODUCTION

Escherichia coli, a type of Gram-negative bacteria is a ubiquitous inhabitant of the gastrointestinal tract of both humans and animals. This microorganism frequently causes urinary tract, skin, or soft tissue infections in cats and dogs (1). Commonly prescribed medications to treat *E. coli* infection in companion animals include ampicillin, amoxicillin-clavulanic acid, fluoroquinolones, or cephalosporins. However, the emergence of drug-resistant bacteria encountered in clinical practice decreases the therapeutic efficacy of these antimicrobial agents. One major mechanism of this drug resistance is the production of enzymes by microbes to inactivate antimicrobial agents. For example, β-lactam agents are widely used to treat bacterial infections in veterinary medicine, whereas extended-spectrum β-lactamases (ESBLs) are a group of enzymes that mediate resistance to most β-lactam antibiotics, including extended-spectrum cephalosporins and monobactams but excluding carbapenems and cephamycins (2). ESBLs are inhibited by clavulanic acid, sulbactam, and tazobactam; this fact is used as a criterion to classify β-lactamases and for ESBL diagnosis purposes (3). TEM, SHV, and CTX-M-group enzymes are examples of commonly encountered ESBLs (2). ESBL producers usually exhibit a multi-drug-resistant phenotype. In addition, the ESBL genes are mainly plasmid mediated, thus facilitating the transmission of drug-resistant genes to other bacteria. Such a situation poses a challenge for infection management in clinical practice. ESBLs have been previously documented primarily in human clinical cases (4). Because companion animals such as dogs and cats are in close contact with humans, they could contract ESBL-producing microorganisms from humans and then possibly transmit them back to humans, which represents a public health concern (5).

Information regarding the prevalence of ESBL producers or the genotypes of these clinical isolates from cats and dogs is limited in Taiwan. It is imperative to investigate related matters from both a veterinary medicine and public health perspective (6). The present study analyzed a collection of *E. coli* isolates obtained from National Taiwan University Veterinary Hospital (NTUVH), a university-based veterinary teaching hospital in Taipei, from 2014 to 2017 to determine the prevalence of ESBL-producing *E. coli*, assess their antimicrobial profile, and characterize the strains phylogenetically through multilocus sequence typing (MLST). The results obtained should provide insights into the role of ESBL-producing *E. coli* in companion animals. Some of the data herein have previously been reported at a conference (7).

MATERIALS AND METHODS

Sample Collection

NTUVH is a teaching hospital affiliated with the College of Bioresources and Agriculture at National Taiwan University located in Taipei, Taiwan. Between 2014 and 2017, 283 *E. coli* isolates obtained from dogs ($n = 224$) and cats ($n = 59$) that were admitted to NTUVH were screened for ESBL producers. These *E. coli* isolates were cultured from different sources of the animals and identified using a Vitek 2 Compact (Biomérieux, Marcy-l'Etoile, France) to the species level and stored at −80°C. Urine and pus samples from the uterus or wounds comprised almost 70% (47 and 22%, respectively) of the *E. coli* sources. These samples were collected from the animals to facilitate diagnosis and treatment. An ethical review was not required for this study.

ESBL Phenotype Testing

The ESBL producers of *E. coli* were tested using combination disk tests with cefotaxime and ceftazidime (30 μg), with and without clavulanic acid (10 μg), as specified by the Clinical and Laboratory Standards Institute (8). Briefly, the tested *E. coli* were plated on Muller–Hinton agar at a concentration of 0.5 McFarland standards and incubated at 35°C for 16–18 h. A difference of 5 mm or more in the inhibition zones for either cefotaxime or the ceftazidime–clavulanic acid combination vs. the corresponding cefotaxime or ceftazidime alone was defined as an ESBL-producing *E. coli*. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as the positive and negative controls, respectively.

Detection of *bla* Genes

The *E. coli* isolates that were phenotypically ESBL producers were analyzed using polymerase chain reaction (PCR) to detect their *bla* genes. Bacterial DNA was extracted using the boiling method (9). Briefly, bacterial strains were cultured overnight at 37°C on tryptic soy agar plates (Difco/Becton Dickinson, Franklin Lakes, NJ), and a loopful of cells was boiled in 200 μL of ddH_2O for 10 min. The supernatant was saved after centrifugation at 12,000 × g for 10 min and used as the source of template DNA for PCR. The primers used to amplify $bla_{CTX-M-1-group}$, $bla_{CTX-M-2-group}$, $bla_{CTX-M-8-group}$, $bla_{CTX-M-9-group}$, $bla_{CTX-M-25-group}$, bla_{SHV}, bla_{TEM}, and the expected PCR product sizes are listed in **Table 1**. The PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, annealing at 52–55°C (as specified in **Table 1**) for 30 s, and a 72°C extension for 1 min. Ten microliters of each PCR sample were loaded onto a 1.5% agarose gel and electrophoresed at 100 V for 30 min. The gels were then

TABLE 1 | Sequences of primers used in this study.

PCR target	Primer	Sequences (5′-3′)	Annealing Tm (°C)	Predicted PCR size (bp)	References
bla_{TEM}	TEM-F	TCGGGGAAATGTGCGCG	55	972	(10)
	TEM-R	TGCTTAATCAGTGAGGCACC			
bla_{SHV}	SHV-F	GCCTTTATCGGCCCTCACTCAA	54	819	(11)
	SHV-R	TCCCGCAGATAAATCACCACAATG			
$bla_{CTX-M-1-group}$	CTX-M-1-F	CCCATGGTTAAAAAATCACTGC	54	942	(12)
	CTX-M-1-R	CAGCGCTTTTGCCGTCTAAG			
$bla_{CTX-M-2-group}$	CTX-M-2-F	CGACGCTACCCCTGCTATT	52	552	(13)
	CTX-M-2-R	CCAGCGTCAGATTTTTCAGG			
$bla_{CTX-M-8-group}$	CTX-M-8-F	TCGCGTTAAGCGGATGATGC	52	666	(13)
	CTX-M-8-R	AACCCACGATGTGGGTAGC			
$bla_{CTX-M-9-group}$	CTX-M-9-F	ATGGTGACAAAGAGAGTGCAAC	55	876	(14)
	CTX-M-9-R	TTACAGCCCTTCGGCGATGATT			
$bla_{CTX-M-25-group}$	CTX-M-25-F	GCACGATGACATTCGGG	52	327	(13)
	CTX-M-25-R	AACCCACGATGTGGGTAGC			
adk	adk-F	ATTCTGCTTGGCGCTCCGGG	54	583	(15)
	adk-R	CCGTCAACTTTCGCGTATTT			
fumC	fumC-F	TCACAGGTCGCCAGCGCTTC	54	806	(15)
	fumC-R	GTACGCAGCGAAAAAGATTC			
gyrB	gyrB-F	TCGGCGACACGGATGACGGC	60	911	(15)
	gyrB-R	ATCAGGCCTTCACGCGCATC			
icd	icd-F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	54	878	(15)
	icd-R	GGACGCAGCAGGATCTGTT			
mdh	mdh-F	AGCGCGTTCTGTTCAAATGC	60	932	(15)
	mdh-R	CAGGTTCAGAACTCTCTCTGT			
purA	purA-F	CGCGCTGATGAAAGAGATGA	54	816	(15)
	purA-R	CATACGGTAAGCCACGCAGA			
recA	recA-F	CGCATTCGCTTTACCCTGACC	58	780	(15)
	recA-R	TCGTCGAAATCTACGGACCGGA			
pabB	O25pabBspe.F	TCCAGCAGGTGCTGGATCGT	65	347	(16)
	O25pabBspe.R	GCGAAATTTTTCGCCGTACTGT			
trpA	trpA.F	GCTACGAATCTCTGTTTGCC	65	427	(16)
	trpA2.R	GCAACGCGGCCTGGCGGAAG			

stained with a fluorescent nucleic acid dye (Biotium, Hayward, CA) and examined under ultraviolet illumination. The PCR products were then purified using a GeneJet PCR purification kit (Thermo Fisher Scientific, Waltham, MA) according to the protocol provided by the manufacturer and subjected to sequencing (Mission Biotech, Taipei, Taiwan). The DNA sequences were examined using the Beta-Lactamase DataBase (www.bldb.eu) (17).

Antibiotic Susceptibility Test

The ESBL-producing *E. coli* isolates were tested for susceptibility to antimicrobial agents used in clinical settings using the standard Kirby–Bauer disk diffusion method (8). The antimicrobial agents tested included β-lactams (amoxycillin/clavulanic acid, ampicillin, imipenem, and ceftiofur), tetracyclines (doxycycline), quinolones (enrofloxacin and ciprofloxacin), aminoglycosides (gentamicin), and sulfonamides (sulfamethoxazole/trimethoprim). The isolates were classified as susceptible, intermediate resistant, or resistant to the antimicrobial agents.

Genotyping and Phylogenetic Analysis

The ESBL-producing *E. coli* strains were genotyped using MLST (15). Internal fragments of *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* were amplified through a PCR by using the primers listed in **Table 1** and sequenced. They were then uploaded to the EnteroBase MLST website (http://enterobase.warwick.ac.uk/) for comparison. Phylogenetic analysis of the strains was performed using BioNumerics version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium).

E. coli ST131 O25b Detection

The PCR-based detection of *E. coli* ST131/O25b was based on the method described by Clermont et al. (16). The *trpA* and *pabB* primers and annealing temperature used are listed in **Table 1**. The PCR cycling conditions were as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 5 s, annealing

at 65°C for 10 s, and 72°C extension for 5 min. Ten microliters of each PCR sample was loaded onto 2.0% agarose gel and electrophoresed at 100 V for 30 min. The gels were then stained with a fluorescent nucleic acid dye (Biotium) and examined under ultraviolet illumination.

RESULTS

A total of 283 *E. coli* isolates (59 from cats and 224 from dogs) were obtained during our study period (2014–2017). **Table 2** lists the prevalence of ESBL-producing *E. coli* from dogs and cats. In total, 65 ESBL-producing *E. coli* isolates, 54 from dogs and 11 from cats, were acquired from our assay. The prevalence of ESBL-producing *E. coli* isolates was 24.1% (54/224) in dogs and 18.6% (11/59) in cats, and the total prevalence for both animals was 23.0% (65/283).

Table 3 lists the distribution of *bla* genes from the 65 ESBL-producing *E. coli* isolates. $bla_{\text{CTX-M-55}}$ of the $bla_{\text{CTX-M-1group}}$ was the most prevalent *bla* gene encountered. The $bla_{\text{CTX-M-2group}}$ contained only $bla_{\text{CTX-M-124}}$. The $bla_{\text{CTX-M-9group}}$ contained eight *bla* gene types, and $bla_{\text{CTX-M-214}}$ was the most frequently observed. $bla_{\text{TEM-215}}$ was the most common type encountered in the bla_{TEMgroup}. We only detected $bla_{\text{SHV-199}}$ in the bla_{SHV} group. We did not detect $bla_{\text{CTX-M-8group}}$ or $bla_{\text{CTX-M-25group}}$.

The sequence type (ST), *bla* genes, and the sampling sites of the ESBL-producing *E. coli* isolates from cats and dogs, respectively, are detailed in **Tables 4**, **5**. MLST analysis identified 20 STs in our ESBL-producing *E. coli* isolates. In total, 16 *E. coli* isolates had STs that did not match any ST in the MLST databank. Combining the data of cats and dogs revealed that the commonest ST was ST457 (13/65, 20.0%), followed by ST131 (10/65, 15.4%), ST648 (6/65, 9.2%), ST38 (3/65, 4.6%), and ST405 (2/65, 3.1%); the other STs were encountered once. ESBL-producing *E. coli* were isolated from several sites but were principally observed in aspirated urine (44/65, 67.7%). **Figure 1** reveals the minimal spanning tree of the 65 ESBL-producing *E. coli* STs according to the degree of allele sharing.

The ESBL-producing *E. coli* isolates from cats were all resistant to ampicillin, ceftiofur, enrofloxacin, and ciprofloxacin, whereas those from dogs were all resistant to ampicillin. All the ESBL-producing *E. coli* were susceptible to imipenem, and more than 50% of the isolates were susceptible to gentamicin. Overall, most strains exhibited a multidrug resistant phenotype (**Table 6**).

PCR detection to target *trpA* and *pabB* was performed on 10 *E. coli* ST131 isolates, and 8 isolates were identified as *E. coli* ST131/O25b clones (**Figure 2**). The ESBL-producing *E. coli*

TABLE 4 | Sequence type, *bla* genes, and sampling site of ESBL-producing *E. coli* in cats.

ST type	*bla* genes
131 (1)[a]	$bla^{b}_{\text{CTX-M-194}}$
405 (1)	$bla_{\text{CTX-M-194}}+bla^{c}_{\text{CTX-M-124}}$
457 (5)	$bla^{d}_{\text{CTX-M-55}}$, $bla^{d}_{\text{CTX-M-214}}$, $bla_{\text{CTX-M-55}}+bla_{\text{CTX-M-214}}+bla^{d}_{\text{TEM-230}}$, $bla_{\text{CTX-M-55}}+bla_{\text{CTX-M-198}}+bla^{d}_{\text{TEM-230}}$, $bla_{\text{CTX-M-55}}+bla_{\text{CTX-M-198}}+bla^{c}_{\text{SHV-199}}$
648 (3)	$bla^{d}_{\text{CTX-M-124}}$, $bla^{d}_{\text{CTX-M-198}}$, $bla_{\text{CTX-M-55}}+bla_{\text{CTX-M-223}}+bla^{e}_{\text{TEM-81}}$
Unknown (1)	$bla_{\text{CTX-M-55}}+bla_{\text{CTX-M-124}}+bla^{c}_{\text{CTX-M-214}}$

[a] *Numbers in parentheses indicate isolation numbers.*
[b] *From an esophageal feeding tube wound.*
[c] *From a neck abscess.*
[d] *From aspirated urine.*
[e] *From the abdominal cavity.*

TABLE 2 | Prevalence of ESBL-producing *E. coli* in dogs and cats.

	2014	2015	2016	2017	Total
Number of ESBL$^+$ cat	4	1	3	3	11
Number of ESBL$^+$ dog	18	14	15	7	54
Number of ESBL$^-$ dog/cat	99	56	34	29	218
Total number assayed	121	71	52	39	283
ESBL prevalence	18.2%	21.1%	34.6%	25.6%	23.0%

TABLE 3 | Distribution of *bla* genes in the 65 ESBL-producing *E. coli* isolates.

bla CTX-M-1 group	*bla* CTX-M-2 group	*bla* CTX-M-9 group	*bla* TEM group	*bla* SHV group	*bla* CTX-M-8 and CTX-M-25 group
$bla_{\text{CTX-M-55}}$ ($n = 24$)	$bla_{\text{CTX-M-124}}$ ($n = 12$)	$bla_{\text{CTX-M-24}}$ ($n = 1$)	$bla_{\text{TEM-81}}$ ($n = 1$)	$bla_{\text{SHV-199}}$ ($n = 4$)	None
$bla_{\text{CTX-M-69}}$ ($n = 3$)		$bla_{\text{CTX-M-67}}$ ($n = 1$)	$bla_{\text{TEM-215}}$ ($n = 16$)		
$bla_{\text{CTX-M-194}}$ ($n = 7$)		$bla_{\text{CTX-M-148}}$ ($n = 1$)	$bla_{\text{TEM-219}}$ ($n = 2$)		
$bla_{\text{CTX-M-199}}$ ($n = 1$)		$bla_{\text{CTX-M-174}}$ ($n = 4$)	$bla_{\text{TEM-226}}$ ($n = 1$)		
$bla_{\text{CTX-M-211}}$ ($n = 3$)		$bla_{\text{CTX-M-196}}$ ($n = 1$)	$bla_{\text{TEM-230}}$ ($n = 5$)		
		$bla_{\text{CTX-M-198}}$ ($n = 1$)			
		$bla_{\text{CTX-M-214}}$ ($n = 11$)			
		$bla_{\text{CTX-M-223}}$ ($n = 1$)			

TABLE 5 | Sequence type, *bla* genes, and sampling site of ESBL-producing *E. coli* in dogs.

ST type	*bla* genes
10 (1)[a]	$bla^{b}_{CTX-M-69}$
38 (3)	$bla^{c}_{TEM-215}$, $bla_{CTX-M-198}+bla^{c}_{TEM-219}$, $bla_{CTX-M-198}+bla^{c}_{TEM-215}$
69 (1)	$bla_{CTX-M-24}+bla^{d}_{TEM-215}$
73 (1)	$bla^{e}_{TEM-230}$
131 (9)	$bla^{2c,f}_{CTX-M-194}$, $bla^{g}_{CTX-M-214}$, $bla^{3c}_{CTX-M-174}$, $bla_{CTX-M-124}+bla^{c}_{CTX-M-194}$, $bla_{CTX-M-55}+bla_{CTX-M-67}+bla^{c}_{TEM-215}$
359 (1)	$bla_{CTX-M-214}+bla^{c}_{TEM-215}$
372 (1)	$bla^{c}_{CTX-M-198}$
405 (1)	$bla^{h}_{CTX-M-214}$
428 (1)	$bla_{CTX-M-55}+bla^{c}_{TEM-230}$
457 (8)	$bla^{3c,b}_{CTX-M-55}$, $bla^{c}_{CTX-M-69}$, $bla_{CTX-M-55}+bla^{c}_{CTX-M-214}$, $bla_{CTX-M-69}+bla^{d}_{SHV-199}$, $bla_{CTX-M-55}+bla_{CTX-M-214}+bla^{c}_{TEM-230}$
636 (1)	$bla^{d}_{CTX-M-55}$
648 (3)	$bla^{i}_{CTX-M-198}$, $bla_{CTX-M-55}+bla^{c}_{CTX-M-174}$, $bla_{CTX-M-55}+bla_{CTX-M-148}+bla_{TEM-215}+bla^{c}_{SHV-199}$
1674 (1)	$bla^{c}_{TEM-215}$
3429 (1)	$bla_{CTX-M-124}+bla^{c}_{CTX-M-198}$
5229 (1)	$bla^{c}_{TEM-215}$
5640 (1)	$bla_{CTX-M-194}+bla^{j}_{TEM-219}$
5685 (1)	$bla_{CTX-M-55}+bla^{k}_{CTX-M-124}$
5686 (1)	$bla^{l}_{CTX-M-55}$
5703 (1)	$bla^{c}_{TEM-215}$
5865 (1)	$bla^{c}_{CTX-M-55}$
Unknown (15)	$bla^{b}_{CTX-M-55}$, $bla^{c}_{CTX-M-198}$, $bla^{c}_{CTX-M-211}$, $bla^{2c,m}_{TEM-215}$, $bla_{CTX-M-198}+bla^{c}_{TEM-215}$, $bla_{CTX-M-211}+bla^{b,c}_{CTX-M-214}$, $bla_{CTX-M-55}+bla_{CTX-M-124}+bla^{2c}_{CTX-M-214}$, $bla_{CTX-M-124}+bla_{CTX-M-214}+bla^{c}_{TEM-226}$, $bla_{CTX-M-55}+bla_{CTX-M-196}+bla^{c}_{TEM-215}$, $bla_{CTX-M-124}+bla_{CTX-M-198}+bla^{c}_{TEM-215}$, $bla_{CTX-M-199}+bla_{CTX-M-124}+bla_{TEM-215}+bla^{n}_{SHV199}$

[a] *Numbers in parentheses indicate isolation numbers.*
[b] *From a wound.*
[c] *From aspirated urine.*
[d] *From pyometra.*
[e] *From an oronasal mass.*
[f] *Pus from paws.*
[g] *From an abscess.*
[h] *Pus from left caudal abdomen.*
[i] *From an ear infection.*
[j] *From tonsils.*
[k] *Pus from the esophageal tube.*
[l] *Pus from intestinal anastomosis.*
[m] *From a vaginal smear.*
[n] *From the gallbladder.*

possessed only the *trpA* specific DNA fragment, whereas the ESBL-producing *E. coli* ST131/O25b clones contained both the *trpA* and *pabB* DNA fragments. Among the 10 ESBL-producing *E. coli*, only one ST131/O25b clone was from a cat (*E. coli* 1942), whereas the others were from dogs. The two non-ST131/O25b clones were both from dogs.

DISCUSSION

The overall prevalence of ESBL-producing *E. coli* in dogs and cats was 23.0% in our study. A comparable prevalence was also reported in Japan, China, and Switzerland (18–20). However, this prevalence is considerably higher than that reported in France (3.7%) and the Netherlands (2%) (21, 22). The medication strategy employed by first-line veterinarians from different countries or regions is a potential explanation for this difference. High prevalence of ESBL-producing *E. coli* threatens the efficacy of third-generation cephalosporins, such as cefovecin, approved for use in veterinary medicine (23).

The *E. coli* isolates were obtained from several sample types in cats and dogs. The most common source of ESBL-producing *E. coli* in cats and dogs was from aspirated urine samples, with prevalence's of 54.5% (6/11) and 68.5% (37/54), respectively. This is unsurprising because urinary tract infection (UTI) is a common diagnosis in companion animals (24). Moreover, UTIs in cats and dogs usually involve a single agent: *E. coli* (25).

The $bla_{CTX-M-1\ group}$ was observed in 58.5% of the *bla* genes. This *bla* gene group is also commonly detected in Europe, the Middle East, and Asia (26). $bla_{CTX-M-55}$ was the major *bla* gene in the $bla_{CTX-M-1\ group}$ in our study. CTX-M-15 used to be common in human and animal isolates (27). CTX-M-55 was first identified in Thailand and is closely related to CTX-M-15 with only one amino acid substitution: Ala-77-Val (28). CTX-M-55 is a derivative of CTX-M-15. The presence of CTX-M-55 is

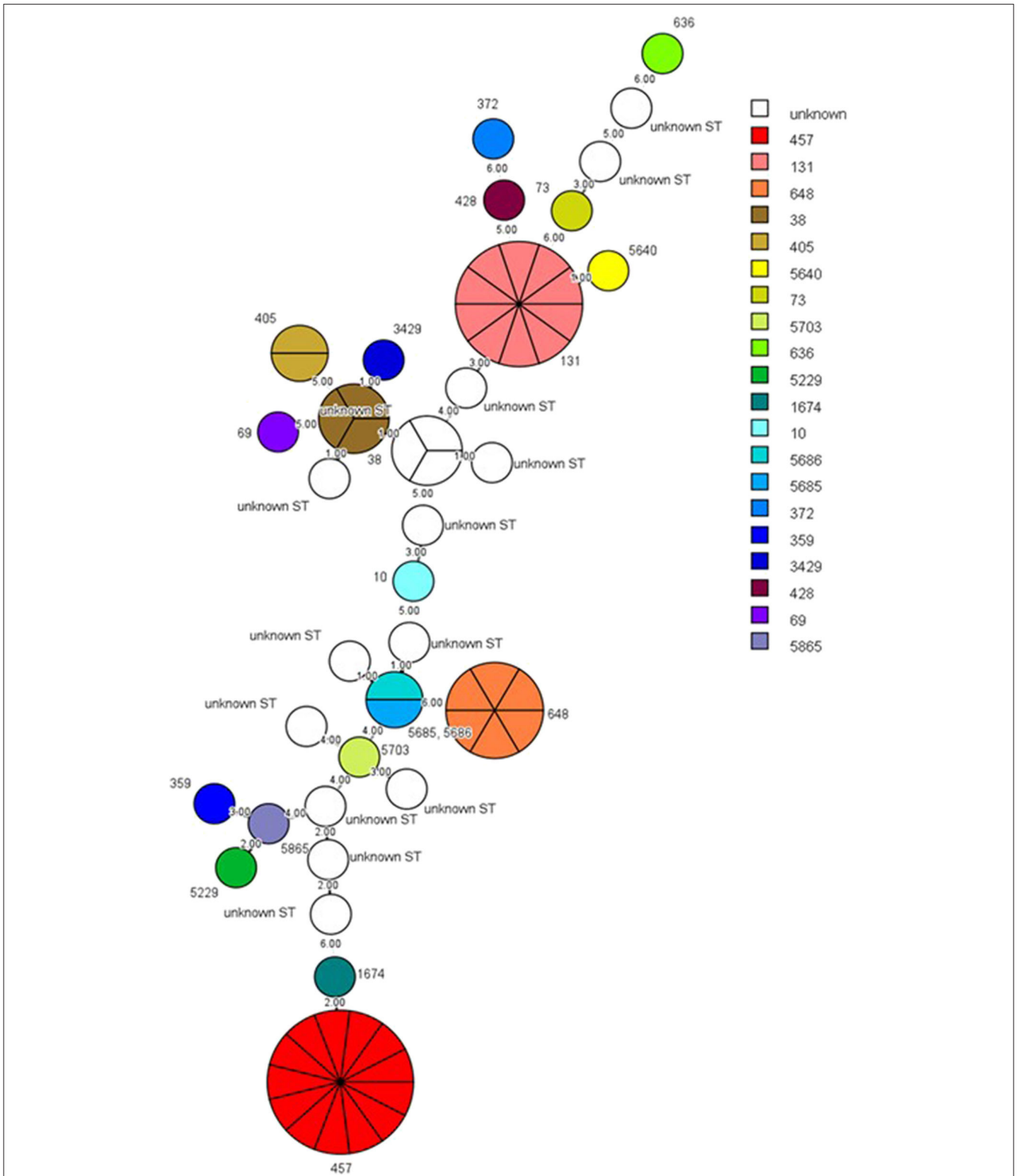

FIGURE 1 | Minimal spanning tree of ESBL-producing *E. coli*. Each circle indicates one ST, subdivided into one sector for each isolate, and bordered by the ST number. White circles or sectors without an ST number denote a lack of comparison standard in the current databank. The numbers on the connecting line between STs within the MSTree indicate the number of different alleles. Solid lines represent an allele difference of three or fewer, whereas dotted lines and faint lines indicate an allele difference of four or more. ESBL, extended-spectrum β-lactamases; MSTree, minimal spanning tree; ST, sequence type.

TABLE 6 | Antimicrobial susceptibility test of ESBL-producing *E. coli* from dogs and cats.

Antibiotic discs	Cat, $n = 11$ (%)			Dog, $n = 54$ (%)		
	Susceptible	Intermediate resistant	Resistant	Susceptible	Intermediate resistant	Resistant
Amoxycillin/clavulanic acid	4 (36.4)	1 (9.1)	6 (54.5)	21 (38.9)	14 (25.9)	19 (35.2)
Ampicillin	0 (0)	0 (0)	11 (100)	0 (0)	0 (0)	54 (100)
Imipenem	11 (100)	0 (0)	0 (0)	54 (100)	0 (0)	0 (0)
Ceftiofur	0 (0)	0 (0)	11 (100)	1 (1.9)	3 (5.6)	50 (92.6)
Doxycycline	1 (9.1)	1 (9.1)	9 (81.8)	9 (16.7)	6 (11.1)	39 (72.7)
Enrofloxacin	0 (0)	0 (0)	11 (100)	8 (14.8)	5 (9.3)	41 (75.9)
Ciprofloxacin	0 (0)	0 (0)	11 (100)	11 (20.4)	3 (5.6)	40 (74.1)
Gentamicin	8 (72.7)	0 (0)	3 (27.3)	32 (59.3)	0 (0)	22 (40.7)
Sulfamethoxazole/trimethoprim	3 (27.3)	2 (18.2)	6 (54.5)	25 (46.3)	2 (3.7)	27 (50.0)

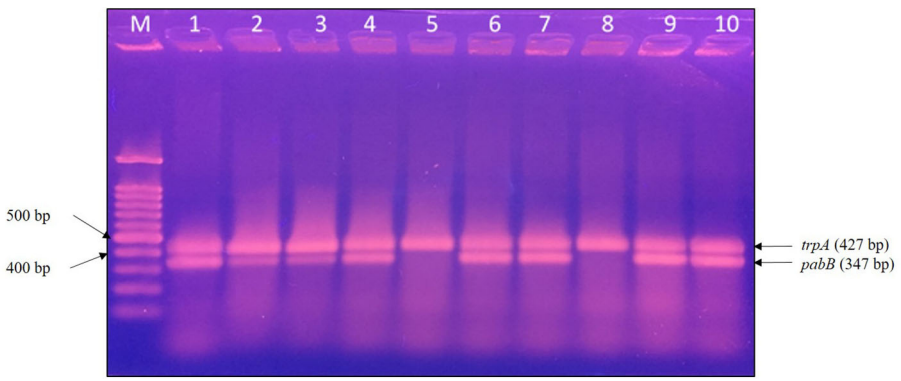

FIGURE 2 | PCR detection of *E. coli* ST131/O25b clone. The *trpA* band corresponds to the positive control in all isolates, whereas the *pabB* band corresponds to the allele-specific amplification obtained only for the ST131/O25b clone. Eight isolates were confirmed to be ST131/O25b clones. M, molecular weight marker, 100 bp DNA ladder; lane 1, *E. coli* 1372; lane 2, *E. coli* 1933; lane 3, *E. coli* 1942; lane 4, *E. coli* 1972; lane 5, *E. coli* 2279; lane 6, *E. coli* 2289; lane 7, *E. coli* 2532; lane 8, *E. coli* 2588; lane 9, *E. coli* 2624; and lane 10, *E. coli* 2670. PCR, polymerase chain reaction.

widely reported in food and pets in China, and its geographic distribution is primarily in Asian countries (29–31). Notably, CTX-M-55 has rarely been encountered outside Asia. However, the recent emergence of CTX-M-55 in companion animals in Switzerland may indicate the spreading of this enzyme due to international food or animal trade, which warrants further attention (18). A study in the United Kingdom also revealed a decreased prevalence of CTX-M-15 producers over some years in favor of new variants, particularly CTX-M-55 (32). CTX-M-124 was another frequently observed β-lactamase in our study. CTX-M-124 was first detected in wild birds (33); the transmission of CTX-M-124 to other animals from the migratory behavior of wild birds may explain, in part, the presence of CTX-M-124 in ESBL-producing *E. coli* from pets (34).

ST457, ST131, and ST648 are the three major STs of ESBL-producing *E. coli* detected in our study, with ST457 being the most prevalent. This ST has been associated with diseases in companion animals in other studies (21, 35). *E. coli* ST131 and ST648 with CTX-M have been reported worldwide in both human and animal samples. These two clones combine multidrug resistance and virulence; ST131, in particular, is a globally distributed uropathogenic *E. coli* lineage (36). *E. coli* ST131 O25b carrying CTX-M-15 is a globally spreading clone with a high virulence potential, making it a public health concern (37), whereas ST131 O25b with CTX-M-14 has predominated in Japan (38). By contrast, CTX-M-174 and CTX-M-194 were the two main β-lactamases in our *E. coli* ST131 O25b clones. An *E. coli* ST131 carrying CTX-M-174 was identified in humans in Korea (39). CTX-M-174 is a variant of CTX-M-14 with two amino acid substitutions (Glu-7-Leu and Asp-242-Gly). Regardless of the type of CTX-M present in our ST131 isolates, the presence of these clones in cats and dogs raises concerns about potential zoonotic risks. This finding also justifies the continued investigation of ESBL-producing *E. coli* to evaluate the persistence of these fast-spreading clones in companion animals in Taiwan. A study in Europe indicated that 1.6% of the diseased dogs and cats carried ESBL-producing *Enterobacteriaceae* but only 2 *E. coli* ST131 isolates were identified; therefore, companion animals may be a source of *bla* genes but may not be the major source of epidemic clones (40).

Previously, LeCuyer et al. (41) revealed a thought-provoking finding regarding uropathogenic *E. coli* in canines. They found that ST372 was the predominant ST in dogs, whereas ST372 was an infrequent human pathogen. The prevalence of ST372

observed in dogs was similar to that of ST131 in human uropathogenic *E. coli* and ST73 in feline *E. coli* that caused urinary tract infections. They therefore concluded that each host species may have a particular ST that comprises most of the *E. coli* uropathogens. A French study also reached a similar conclusion, identifying ST372 as the major pathogenic *E. coli* ST in dogs (42). Similar findings in two distinct geographic areas may indicate a dog-specific distribution of pathogenic *E. coli* clones instead of the effect of regional factors (42). In contrast to LeCuyer's and Valat's reports, ST372 was observed only once in our study. Different criteria for the screening of *E. coli* in the study design may have contributed to this discrepancy.

Some STs such as ST3429, ST5229, ST5640, ST5685, ST5686, ST5703, and ST5865, to the best of our knowledge, have not been reported before; therefore, the pathogenic potentials of these strains were unknown.

Imipenem reportedly remains relatively active against ESBL-producing bacteria (43), which is consistent with our results (**Table 6**). Nonetheless, the use of carbapenems in companion animals should be avoided, since the emergence of carbapenem resistance in companion animals has been reported (44).

The current study had some limitations. AmpC-β-lactamases, which also hydrolyze the third generation of cephalosporins, were not assayed for the *E. coli* isolates. In addition, resistant plasmids were not characterized using PCR-based replicon typing. Although the results obtained in this study originate from only one veterinary hospital, this university-based teaching hospital is the major referral hospital for local veterinary clinics in Taipei. We believe that the information regarding ESBL in cats and dogs reported herein could be helpful for infection management and prevention.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The purpose of collecting these samples from animals was for diagnosis and treatment. An ethical review process was not required for this study according to national/local guidelines.

AUTHOR CONTRIBUTIONS

Y-HH conducted the characterization of the phenotype and genotype of the ESBL-producing *E. coli* and drafted the manuscript. N-LK analyzed the ESBL-producing *E. coli* through MLST. K-SY conceived and coordinated this research plan. All authors have read and approved the final manuscript.

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REVIEW
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Antimicrobial Resistance in Agri-Food Chain and Companion Animals as a Re-emerging Menace in Post-COVID Epoch: Low-and Middle-Income Countries Perspective and Mitigation Strategies

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Antimicrobial resistance (AMR) leads to enormous financial losses from issues such as high morbidity, mortality, man-days lost, hospital length of stay, health-care, and social costs. In humans, over prescription of antimicrobials, which is presumably higher during COVID, has been identified as the major source of selection for antimicrobial resistant bacteria; however, use of antimicrobials in food and companion animals, fish, and vegetables, and the environmental resistance gene pool, also play important roles. The possibilities of unnecessary use of antibiotics as prophylaxis during and after COVID in livestock and companion animals exist in low-and middle-income countries. A considerable loss in gross domestic product (GDP) is also projected in low-and middle-income countries (LMICs) due to AMR by the year 2050, which is further going to be reduced due to economic slowdown in the post-COVID period. Veterinary hospitals dedicated to pets have cropped up, especially in urban areas of LMICs where use of antimicrobials has also been increased substantially. The inevitable preventive habit built up during COVID with the frequent use of hand sanitizer might trigger AMR due to the presence of cross-resistance with disinfectants. In LMICs, due to the rising demand for animal protein, industrial food animal production (IFAP) is slowly replacing the small-scale backyard farming system. The lack of stringent regulations and monitoring increased the non-therapeutic use of antimicrobials in industrial farms where the persistence of antimicrobial resistant bacteria has been associated with several factors other than antimicrobial use, such as co-resistance, cross-resistance, bacterial fitness, mixing of new and old animals, and vectors or reservoirs of bacterial infection. The present review describes types of antimicrobials used in agri-food chains and companion animals in LMICs with identification of the gap in data, updated categories of prevalent antimicrobial resistant bacteria, the role of animal farms as reservoirs of resistant bacteria, and mitigation strategies, with a special focus on the pivotal strategy needed in the post-COVID period.

Keywords: backyard, COVID, food animals, mitigation, industrial food animal production, antimicrobial resistance

INTRODUCTION

Human and animal populations are at risk of cross-transmission of zoonotic bacteria via direct contact due to close proximity with food animals, companion animals, live wildlife markets, environmental contamination, and the intake of contaminated animal origin food items. The situation becomes more complicated due to cross-transmission of antimicrobial resistance (AMR) determinants along with the infection. In humans, over prescription of antimicrobials is the major source of selection for antimicrobial resistant bacteria, but use of antimicrobials in food animals and, moreover, the environmental resistance gene pool ("resistome") also play important roles in this complex multi-factorial state of affairs. Recently, the bacteriostatic antimicrobial (azithromycin) was recommended in synergism with hydroxychloroquine against SARS-CoV2 in treatment protocols in several countries despite the dearth of precise clinical evidence (1, 2). The recent systematic review revealed use of antibiotics in 70% of COVID patients, mostly in Asian countries, although only 10% of them had a bacterial co-infection (3). Even the World Health Organization (WHO) warned against the overuse of antibiotics during the pandemic with the statement: "The COVID19 pandemic has led to an increased use of antibiotics, which ultimately will lead to higher bacterial resistance rates that will impact the burden of disease and deaths during the pandemic and beyond" (4). The Post-COVID epoch may add complexities to the AMR perspective, as antibiotics might be considered as a prophylactic measure among the community, especially in LMICs where antibiotics are easily available at the counter without prescriptions (5). The manufacturers of azithromycin are already facing difficulties to meet the ever-increasing demands (6). Prophylactic antimicrobial therapy in food and companion animals may witness a steep rise during and after the COVID episode, particularly in LMICs, even if it is not recommended in many countries (7). The situation becomes catastrophic as the companion animal practitioners prefer human antibiotics for their better quality and easy availability.

AMR leads to enormous financial losses associated with high morbidity, mortality, man-days lost, hospital length of stay (LOS), direct health-care costs, and the social costs of infection (8). About 700,000 deaths per year were attributed to AMR alone, which is more than the toll caused by malaria, acquired immunodeficiency syndrome (AIDS), and tuberculosis (9). The World Health Organization (WHO) identified eight pathogens relevant to AMR, including five bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, and *Mycobacterium tuberculosis*) (10). Among them, third-generation cephalosporin-resistant and carbapenem-resistant *Enterobacteriaceae* (CRE, e.g., *Escherichia coli* and *Klebsiella pneumoniae*) alone were reported to cause 6.4 million bloodstream infections and 50.1 million serious infections worldwide in a year (11). A recent estimate suggested 33,000 annual deaths due to AMR in the European Union and European Economic Area (12). Additional treatment costs and losses due to methicillin-resistant *Staphylococcus aureus* (MRSA) and third-generation cephalosporin-resistant and ESBL-producing *Enterobacteriaceae* ranged between 1,732 and 9,726 USD and 2.54–6.8 days per case, respectively (8). For the United States alone, average national health care expenditure was estimated at around 2.2 billion USD due to AMR (13).

Addressing AMR in developing countries was considered crucial by the United Nations to achieve sustainable development goals (SDGs) associated with poverty and hunger alleviation and the improvement of health and economic growth (14). In LMICs, the current rate of AMR-related infections is high and is projected to grow more rapidly than in developed countries. A substantial portion (40–60%) of human bacterial infections in Brazil, the Russian Federation, and India is associated with resistant bugs in comparison to developed countries (17%) (15). In LMICs, the direct and prominent effects of AMR include increased mortality, in addition to higher morbidity and economic losses (16). The recent projection about the financial vulnerability of LMICs revealed that an additional 19 million people are going to fall into great poverty by 2030 due to AMR producing direct impacts on labor productivity (neat GDP produced by 1 h of labor) and increased health care costs (17). A considerable loss in GDP is also projected in low-income countries due to AMR by the year 2050, which is further going to be reduced due to economic slowdown in a post-COVID scenario (18).

The present review describes types of antimicrobials used in food animals, companion animals, aquaculture, and vegetables in LMICs, categories of prevalent antimicrobial-resistant bacteria in LMICs, the role of industrial and backyard farms as a reservoir of resistant bacteria in LMICs, and mitigation strategies with special reference to a post-COVID scenario.

DEFINITIONS AND USES OF ANTIMICROBIALS

Antimicrobials (AM) are substances of natural, semisynthetic, or synthetic origin that kill or inhibit the growth of a microorganism but cause little or no damage to the host cells. Antibiotics (AB) are low molecular weight antimicrobials produced by a microorganism that at low concentrations inhibit or cause lysis of other microorganisms. WHO made a list of medically important antimicrobials (MIA) and classified them into three categories, critically important antimicrobials (CIA, highest priority CIAs and high priority CIAs), highly important antimicrobials (HIA), and important antimicrobials (IA), based on five criteria (19). Similarly, the World Organization for Animal Health (OIE) determined the degree of importance for classes of veterinary antimicrobial agents based on antimicrobial class, use in treatment of serious animal diseases, and availability of alternative antimicrobial agents (20). Different classes of important veterinary antimicrobials, mechanism of action, indication, and mechanism of resistance are described in **Tables 1**, **2**.

TABLE 1 | Characteristics of selected veterinary important antimicrobials.

Antimicrobials	Mechanism of action	Indications		WHO classification	OIE classification	Resistance mechanism
		Human	Animals			
Sulfonamides and Potentiated Sulfonamides Sulfachloropyridazine (sui & bov) Sulfadiazine (can and fel) Sulfadimethoxine (bov, can and fel) Sulfamethazine (bov, sui, can and fel) Sulfamethoxazole (can and fel) Sulfaquinoxaline (Calves, small ruminants and poultry) Ormetoprim + sulfadimethoxine (can and fel) Trimethorprim + sulfadiazine (equ, can and fel) Trimethoprim + sulfamethoxazole (equ, can and fel) Trimethoprim + sulfadoxine (bov)	Sulfonamide mimics paraamino benzoic acid (PABA) as a false substrate and trimethoprim/ormetoprim inhibits dihydrofolate reductase enzyme. Altogether, these compounds inhibit the synthesis of dihydrofolic acid, an important co-enzyme for many complex biochemical pathways in bacteria, including DNA synthesis.	sulfamethoxazole–trimethoprim combination (co-trimoxazole) indicated in UTI infections, prostatitis, chronic bronchitis and invasive salmonellosis	Bacterial (*Staphylococcus* spp., Corynebacterium, Nocardia asteroides, Stenotrophomonas maltophilia, and bacteria of the Enterobacteriaceae (Klebsiella, Proteus, Enterobacter, and *Escherichia coli*), Pasteurella) and protozoal (Histophilus, Toxoplasma, and coccidia.) infections Pneumonia, intestinal infection (coccidian), soft tissue infection, UTI Sulfaquinoxaline is indicated in coccidial enteritis.	HIA	VCIA	Efflux pumps and changes in the target enzymes
Penicillins Natural penicillins Penicillin G – (bov, sui,ovi and equ) Aminopenicillins Ampicillin (can, fel, equ) - AMC Ampicillin + sulbactam (can, fel, equ and ruminants) –A/S amoxicillin (bov, equ, can, fel) amoxicillin + clavulanate potassium (can and fel) Antistaphylococcal penicillins (e.g., oxacillin, oxacillin, cloxacillin, and dicloxacillin) Limited clinical use COX: Mastitis D/C: Can and fel Extended-spectrum penicillins (e.g. piperacillin) TI/TIC (can, fel and equ) CB (Can and fel) PI (Can and fel)	Penicillin and cephalosporins – β-lactam drugs inhibit bacterial cell wall synthesis by interfering the transpeptidation reaction.	Penicillins: Active against nonpenicillinase-producing *Staphylococcus, Streptococcus*, few Gram-negative bacteria - Arcanobacterium, Mannheimia haemolytica, Listeria monocytogenes, and Pasteurella, anaerobes *Fusobacterium, Peptococcus, Peptostreptococcus*, some strains of Bacteroides and Clostridium, *Bacillus anthracis* spirochetes (Leptospira, and Borrelia burgdorferi). Aminopenicillins are active against the bacteria resistant to Penicillin G – *Enterobacteriaceae, Pseudomonas*, *Bacteroides fragilis*, and penicillinase producing *Staphylococcus* spp., Antistaphylococcal penicillins penicillin G and the aminopenicillins resistant penicillinase producing *Staphylococcus* spp. Few other gram-positive and gram-negative bacteria and spirochetes. Extended-spectrum penicillins Gram-negative aerobic and anaerobic Bacteria; many strains of Enterobacteriaceae and Pseudomonas	Penicillin G – (Anthrax, BQ, HS in large animals) Aminopenicillins AMP: (UTI, pneumonia, wound) A/S: (acute infection – pneumonia, sepsis and infections caused by ESBL pathogens, prophylaxis in neutropaenic patients) AMX: (UTI, soft tissue infection, pneumonia) AMC: (skin, UTI, respiratory and wound infection) Antistaphylococcal penicillins COX: used in intramammary preparation for treating mastitis D/C: β-lactamase producing Staphylococcus Extended-spectrum penicillins TI/TCC, CB, PI, PIT Soft tissue/ bone infection, pneumonia (synergistic with aminoglycosides) Ampicillin-resistant bacteria, Pseudomonas aeruginosa, Clavulanate potentiates its action against Gram-negative bacteria and *Staphylococcus*	HIA	VCIA	Mediated by production of enzymes like β-lactamases that render the penicillins/cephalosporins by hydrolysis of β-lactam rings. *Staphylococcus* can become resistant by mutating the penicillin binding proteins (PBP2a) which have a reduced affinity to β-lactam drugs.

(Continued)

TABLE 1 | Continued

Antimicrobials	Mechanism of action	Indications		WHO classification	OIE classification	Resistance mechanism
		Human	Animals			
Cephalosporins First generation Cefacetrile (bov) Cefalexin (bov,cap, equ, ovi, sui, can, fel) Cefalothin (can, fel, equ) Cephapirin (bov) Cefazolin (bov, cap, ovi, can, can, fel) Cefalonium (bov, cap, ovi) Cefadroxil (can, fel, equ) Second generation Cefuroxime (bov) Cefaclor (can, fel) Cefoxitin (can, fel, bov and equ)		Active against most of the gram positive bacteria except *Enterococcus.* Greater activity against *Enterobacteriaceae* than penicillin. 2nd generation cephalosporins are more active against *Enterobacteriaceae* Cephamycin (cefoxitin) groups are also effective in anaerobic infection.	Skin, soft tissue, respiratory tract and urinary tract infections, wound, abscess Eft is useful in porcine respiratory disease complex (PRDC), bovine respiratory disease complex (BRDC) and bovine mastitis.	HIA	VCIA	Narrow-spectrum β-lactamases can neutralize early generation cephalosporins but not the higher generation cephalosporins. Extended-spectrum β-lactamases produced by some strains of Gram-negative bacteria can deactivate 3rd and 4th generation cephalosporins.
Third generation Cefoperazone (bov, cap, ovi) Ceftiofur (avi, bov cap, equ, ovi, lep, can, fel) Ceftriaxone (avi, bov, ovi, sui) Cefpodoxime (can, fel and equ) Cefotaxime (can, fel and equ) Fourth generation Cefquinome (bov, cap, equ, ovi, sui) Potentiated cephalosporins (CAZ/CTX with clavulinic acid, sulbactum or tazobactum)		3rd generation cephalosporins are more active against Gram-negative bacteria than the 1st and 2nd generations which are rendered ineffective by production of β-lactamase. CPZ and CAZ are useful in infections caused by *Pseudomonas* Eft is active against Pasteurella multocida, Mannheimia haemolytica, *Histophilus somnus*, *Fusobacterium* necrophorum, *Actinobacillus, Salmonella cholerasuis, Streptococcus* suis		CIA		
Tetracyclines Chlortetracycline (avi, bov, cap, equ, lep, ovi, sui, can, fel) Oxytetracycline (api, avi, bov, cam, cap, equ, lep, ovi, pis, sui, can, fel) Tetracycline (Api, avi, bov, cam, cap, equ, lep, ovi, pis, sui, can, fel) Doxycycline (avi, bov, cam, cap, equ, lep, ovi, pis, sui, can, fel) Minocylcine (can, fel)	Tetracycline binds to the 30S ribosomal subunit and interferes with the interaction of aminoacyl-tRNA with mRNA leading to bacterial protein synthesis inhibition.	Possibly, tetracycline has the broadest spectrum of activity being effective against mycoplasma, Rickettsia, chlamydia and blood protozoa apart from bacteria. Tetracyclines are indispensible drugs for treating Ehrlichiosis, anaplsmosis and as an adjunct therapy in theileriosis – both are endemic in many Asian and African countries. Use to treat infections caused by Pasteurella multocida, Mannheimia haemolytica, *Histophilus somnii*	Pneumonia including BRDC, PRDC, enteritis, abscess, skin and soft tissue infection. In pigs these drugs are useful in atropic rhinitis, *Mycoplasma* infection and pneumonic pasteurellosis. In foals tetracyclines are used to treat angular deformities, possibly for their anti-inflammatory, chondroprotective, and antiarthritic effects.	HIA	VCIA	Resistance is mediated by energy dependent efflux of the drugs and alteration of binding sites of tetracycline at the 30S ribosomal units.

(Continued)

TABLE 1 | Continued

Antimicrobials	Mechanism of action	Indications		WHO classification	OIE classification	Resistance mechanism
		Human	Animals			
Aminoglycosides Streptomycin (api, avi, bov, cap, equ, lep, ovi, pis, sui) Dihydrostreptomycin (avi, bov, cap, equ, lep, ovi, sui) Gentamicin (avi, bov, cam, cap, equ, lep, ovi, sui, can, fel) Amikacin (equ, bov, can and fel) Neomycin (api, avi, bov, cap, equ, lep, ovi, sui, can, fel) Kanamycin (api, avi, bov, cap, equ, lep, ovi, pis, sui, can, fel) Paromomycin (cap, ovi, lep) Apramycin (avi, bov, lep, ovi, sui)	Its irreversible attachment to 30S ribosomal subunit leads to interruption in mRNA translation process. This ultimately leads to premature termination or faulty protein synthesis due to misreading of genetic codes.	Effective against Gram-negative bacteria- *Enterobacteriaceae* and *Pseudomonas aeruginosa.* Efficacy against Gram-positive bacteria like *Staphylococcus* is limited. Anaerobic pathogens are inherently resistant.	Useful in septicaemias; digestive, respiratory and urinary tract infections. Few drugs have specific indication like apramycin in swine colibacillosis (pig scours). Paromomycin is useful in protozoal gastrointestinal infections.	CIA	VCIA	Anaerobes are inherently resistant as the drugs require oxygen for entry into the cell. Resistance mechanism involve alteration in the cell surface receptor to slow down or block the passage of the drugs, changes at the drug attachment sites (30S ribosome) and enzymatic degradation. Amikacin being unaffected by many of the hydrolyzing enzymes is more effective than other aminoglycosides in controlling infections caused by resistant bacteria.
Phenicol Florfenicol (avi, bov, cap, equ, lep, ovi, pis, sui, can, fel) Thiamphenicol (avi, bov, cap, ovi, pis, sui, can, fel)	Phenicols are bacteriostatic agents – phenicols interfere the peptidyltransferase enzyme activity at 50S ribosoma subunit leading to protein synthesis.	Effective against *Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni, Fusobacterium necrophorum, Bacteroides*, *Actinobacillus*, *Salmonella cholerasuis and Streptococcus suis, Aeromonas salmonicida*	Respiratory infections in poultry, BRDC, SRDC, foot rot, acute interdigital necrobacillosis and infectious pododermatitis	HIA	VCIA	Resistance mediated by a variety of mechanism *viz.,* efflux pumps, enzymatic modifications by rRNA methyltransferases, and chloramphenicol acetate esterases and inhibition of intracellular drug transport
Macrolides Azalide Azithromycin*(equ, sui, can, fel) Tulathromycin (bov, cap, lep, ovi, sui) Macrolides C14 Erythromycin (api, avi, bov, cap, equ, lep, ovi, pis, sui, can, fel) Macrolides C16 Spiramycin (api, bov, cap, equ, lep, ovi, pis, sui, can, fel) Tilmicosin (avi, bov, cap, lep, ovi, sui) Tylosin (api, avi, bov, cap, lep, ovi, sui, can, fel)	By binding to the 50S ribosomal subunit at 23sRNA site, macrolides inhibit the protein systhesis. Tilmicosin is effective in BRDC by reduced expression of PGE_2 and release of anti-inflammatory cytokines.	Gram-positive infections mainly, Mycoplasma, Rhodococcus, Chlamydophila Mycoplasma, Arcanobacterium, Erysipelothrix, Bordetella, and Bartonella Moraxella, Serpulina Lawsonia	Respiratory infection, hemorrhagic digestive diseases- swine dysentery and proliferative enteropathy (sui), liver abscess, pododermatitis (bov) Additionally, tylosin is effective in pink eye	CIA	VCIA	Resistance is mediated by *mef* gene governed drug efflux system, drug inactivating enzymes and modification of the drug binding sites at 50S ribosome (erm genes)

(Continued)

TABLE 1 | Continued

Antimicrobials	Mechanism of action	Indications		WHO classification	OIE classification	Resistance mechanism
		Human	Animals			
Lincosamides Clindamycin (can, fel) Lincomycin (api, avi, bov, cap, ovi, pis, sui, can, fel) Pirlimycin (bov)	Inhibit protein sysnthesis by binding with 50S ribosomal subunit.	Staphylococcus, Streptococcus, Actinomyces, Nocardia, Mycoplasma and Cornybacterium, Erysepelothrix, Leptospira, *Bacteroides fragilis*, *Fusobacterium* spp., *Peptostreptococcus* spp., and *Clostridium perfringens* Babesia Toxoplasma.	Gram-positive or anaerobic infections in oral cavity, skin, soft tissue, respiratory tract, protozoal infection Lincomycin is used in pyoderma in pets and mycoplasma infections in pigs and poultry and infectious arthritis and hemorrhagic enteritis in pigs			MRSP from dogs are usually resistant while community acquired MRSA are susceptible. Resistance driven 23sRNA methylation encoded by erm gene is the most common mechanism apart from drug efflux pumps (mef) and enzymatic modification of the drugs.
Quinolones Quinolone 1G Fluoroquinolones Danofloxacin (avi, bov, cap, lep, ovi, sui,) Difloxacin (avi, bov, lep, sui, equ, dog) Enrofloxacin (avi, bov, equ, lep, ovi, pis, sui, can, fel) Orbifloxacin (bov, sui, can, fel) Pradofloxacin (fel) Marbofloxacin (avi, bov, equ, lep, sui)	Quninoloes are bactericidal by inhibition of DNA replication and transcription. DNA gyrase encoded by *gyrA* and topoisomerase IV encoded by *ParC* and *ParD* are targets of this group of drugs.	Fluoroquinolones are broad-spectrum drugs; however, they are more active against the Gram-negative bacteria like *Enterobacteriaceae*. Gram-positive bacteria- *Staphylococcus* are variably susceptible. Marbofloxacin and pradifloxacin are more effective against Gram-positive bacteria. *Pseudomonas* is not invariably susceptible. Besides, this group is effective against *Pasteurella multocida, Mannheimia haemolytica, Histophilus somni* and other intracellular organisms - *Rickettsia* spp., *Chlamydia*, and *Mycobacterium* spp. and *Mycoplasma* spp	BRDC, septicemia, UTI, gastroenteritis, Enrofloxacin is effective against many Rickettsia but not against *Ehrlichia* CRD in poultry	CIA	VCIA	Decreased permeability, efflux pumps, altered targets, plasmid-mediated resistance were recorded. Mutation in the quinolone resistance determining region – gyrA, ParC and ParE is responsible for decreased affinity of quinolones or fluoroquinolones to gyrase and topoisomerase.
Peptides Bacitracin (avi, bov, lep, sui) Colistin	Bacitracin kills the bacteria by interfering with cell membrane function, suppressing cell wall formation and inhibiting protein synthesis in the presence of divalent cations, such as zinc. Colistin or polymixin interacts with LPS of Gram-negative bacteria leaving a porous cell-membrane and eventually cell-death.	Bacitracin is mainly effective against Gram-positive bacteria Colistin is useful in digestive diseases by Gram-negative infections	Bacitracin is useful in necrotic enteritis in poultry. With the concerns over selection of drug-resistant bacteria, use of colistin and Bacitracin Methylene Disalicylate (BMD) is under scrutiny and has been banned by various countries including India.	Colistin (CIA)	VHIA	Colistin-resistance is mediated by changes in the overall charges LPS of the bacterial cell membrane brought about by plasmid mediated gene *mcr* or alternation in two component signaling system. Resistance to bacitracin is rare.
Ionophores Lasalosid (avi, bov, lep, ovi) Maduramycin (avi) Monensin (avi, api, bov, cap) Salinomycin (avi, lep) Naracin (avi) Semiduramicin (avi)	Ionophores cause ion imbalance in bacterial cell making them energy deficient		Mainly used in treatment of coccidiosis In ruminants, it decreases methane production and better utilization of carbohydrate and protein utilization. Ionophores are also useful in liver abscess and rumen acidosis or bloat as they prevents propionic acid production.		VHIA	Bacteria may become temporarily ionophores-resistant by shedding out of cell membrane or by forming a glycoprotein armor (glycocalyx) around their body (Russell and Houlihan 2003).

(Continued)

TABLE 1 | Continued

Antimicrobials	Mechanism of action	Indications		WHO classification	OIE classification	Resistance mechanism
		Human	Animals			
Novobiocin	By binding to DNA gyrase, it blocks adenosine triphosphatase (ATPase) activity.	*S. aureus* and CoNS including MRSA	Mastitis and sepsis in fish		VIA	Mediated by a mutation in the target – gyrB (21)
Avilamycin	Avinamycin binds to the 50S ribosomal unit to prevent bacterial protein synthesis	Gram-positive bacteria like Clostridium perfringens	Necrotic enteritis in poultry, and enteric disease in pig and rabbits.		VIA	
Peuromutilin Tiamulin (Avi, Cao, Lep, Ovi, Sui)	It is a bacteriostatic antibiotic and inhibits the protein synthesis by binding to the 50S ribosomal subunit	Effective against gram-positive bacteria, mycoplasmas, and anaerobes, including Brachyspira hyodysenteriae.	It is also clinically effective in treatment of swine dysentery and mycoplasmal arthritis, respiratory diseases of pigs and poultry		VHIA	Chromosomal (mutations in the 23S rRNA and *rplC* genes) and plasmid-mediated (vga and cfr genes) resistance were reported (22)

AVI, avian; EQU, Equine; API, bee; LEP,Rabbit; BOV, bovine; OVI, Ovine; CAP, caprine; PIS, Fish; CAM, camel; SUI, Swine; Can, Canine; Fel, Feline; VCIA, Veterinary Critically Important Antimicrobial Agents; VHIA, Veterinary Highly Important Antimicrobial Agents; VIA, Veterinary Important Antimicrobial Agents; CIA, Critically Important Antimicrobials; HIA, Highly Important Antimicrobials.

USE OF ANTIMICROBIALS IN THE AGRI-FOOD CHAIN AND COMPANION ANIMALS

Vegetable Production

Some antibiotics are used to protect profitable fruits, vegetables, ornamental plants, and crops from bacterial diseases. The manure used in green houses and soils acts as an additional source of antimicrobial residue for fruits and vegetables which should be treated before direct use (manure composting or aerobic treatment). Although the studies could not detect any AMR-associated bacteria in vegetables as such (23), residues of tetracycline, virginiamycin, tylosin, monensin, and sulfamethazine could be detected in vegetables (24) and in greenhouse soil following manure application (25). The untreated irrigation water used for the production of vegetables, fruits, and crops was identified to contain AMR determinants (*tet*, *pAmpC*) in South Africa, which is an indirect indication, although the precise AMU data for vegetable production is not available in LMICs (26).

Food Animals

In food animals, antimicrobials are used for several purposes such as therapy, prophylaxis, metaphylaxis, and promotion of growth. Therapeutic usage of antimicrobials is difficult to discontinue, as it not only saves the animal's life but also decreases the zoonotic pathogen load in the environment and reduces methane production by livestock (monensin) (27). Nevertheless, the use of CIAs should be optimized and should only be allowed only in emergency or special infectious conditions, like higher generation or potentiated cephalosporins (cefoperazone, ceftiofur, ceftriaxone, cefquinome) in the treatment of septicemias, respiratory infections, and mastitis; aminoglycosides in septicaemias, severe digestive, respiratory, and urinary tract infections and in *Pseudomonas aeruginosa* infections; fluoroquinolones in the treatment of septicaemias, respiratory, and enteric diseases; and macrolides in *Mycoplasma* infections in pigs and poultry, *Lawsonia intracellularis* in pigs, and hepatic abscess in cattle due to *Fusobacterium necrophorum* [**Table 1**, (28)].

Use of colistin, the last resort antibiotic in human medicine, in poultry and pigs in China recently caused the emergence of colistin-resistant bacteria possessing the novel resistance gene *mcr-1* (29). Furthermore, other variants of *mcr* were found in animals and humans, viz., *mcr-2*, *mcr-3*, *mcr-4,* and *mcr-5* (30). China and India recently banned the use of colistin as a growth promoter in food animals (31, 32). Prophylaxis or prevention is the administration of antimicrobials to healthy animals considered to be at risk to prevent the future occurrence of infection, while the prophylactic use in healthy food animals, including poultry, is not yet scientifically validated (prohibited in Europe), except in blanket therapy. Prophylactic antibiotic use should not be a substitute for improper biosecurity and inadequate husbandry conditions of the farms. Metaphylaxis refers to the use of antimicrobials in the infected and healthy but at risk animals of the same herd to prevent the spread of the infection. It is preferred in large herds where separation of

TABLE 2 | Characteristics of selected veterinary important antifungals.

Antifungal	Mechanism of action	Indications	Resistance mechanism
Griseofulvin	Interaction of griseofulvin with mitotic spindles leads to cell cycle arrest and finally cell death.	Treatment of dermatophytosis Effective against *Microsporum* spp., *Trichophyton* spp., and *Epidermophyton*	Many are not responsive to griseofulvin due to the intrinsic resistance owing to the absence of energy dependent uptake of the drug is present in many fungus
Azole compounds Imidazole Clotrimazole (can, fel), Miconazole (can, fel) Ketoconazole (can, fel) Triazoles Fluconazole (avi,equ, can, fel) Voriconazole (avi, equ, can, fel)	Impairs the ergosterol synthesis by inhibition of lansosterol C14 demethylase enzyme (CYP51A/Erg11p)	Ketoconazole: Effective against *Candida*, *Malassezia pachydermatis*, *C. immitis*, *H. capsulatum*, and *B. dermatitidis* and most dermatophytes Useful in canine blastomycosis, histoplasmosis, cryptococcosis, and coccidioidomycosis Itraconazole and fluconazole are more effective than ketoconazole. Proven efficacy of itraconazole against Aspergillosis and in *Malassezia* dermatitis gives it an edge over ketoconazole. Voriconazole is also effective against Aspergillus and Fusarium Generally, miconazole used as topical agent (cream or spray) in canine dermatophytosis. Systemic use of clotrimazole is limited because of poor oral absorption. However, topical administration is effective in otitis externa caused by *Malassezia pachydermatitis.* Clotrimazole is effective in nasal aspergillosis and caniduria in small animals.	Increased biosynthesis of lanosterol C14α-demethylase, mutation at the target site (ERG11), efflux pump mediated drug expulsion and alternate pathways to replace ergosterol with other compounds are the major azole-resistance mechanisms.
Terbinafine (avi, can, fel)	Inhibits ergosterol biosynthesis by interacting squaline epoxidase enzyme	Dermatophytosis, topical forms are useful	Terbinafine resistance is uncommon; however, mutation of squalene epoxidase was recorded to mediate such resistance in clinical isolates of dermatophytes.
Polyene compounds Nystatin Natamycin Amphoterecin B	Binds with ergosterol of the fungal plasma membrane causing leakage of essential nutrients and cell death.	Nystatin as topical agent, oral and intestinal candidiasis Natamycin is useful in keratomycosis, nasal aspergillosis, guttural pouch mycosis and dermatophytosis in horses. Amphoterecin B: Histoplasma capsulatum, *Cryptococcus neoformans, Coccidioides immitis, Blastomyces dermatitidis, Candida* spp., and various species of Aspergillus.	Mutation in *ERG3* gene which is responsible for ergosterol biosynthesis leads to incorporation of other sterols in plasma membrance and polyene fails to act on them.

healthy animals from infected ones is difficult based on clinical signs and rectal temperature (33).

The growth promotion effect of antimicrobials in animals is doubtful as it is observed that satisfactory effects can be produced only during the early stages of animal production or in sub-optimal hygiene conditions (34). Many non-MIA, such as bacitracin, bambermycins, and carbadox, are currently used for growth promotion. Although following the immediate ban of antibiotics as growth promoters in Europe, a few clinical conditions, such as post-weaning scours, occurred in pigs with higher frequency (35). The meta-analysis showed that the restriction of antibiotics as growth promoters in animals reduced the occurrence of antimicrobial-resistant bacteria in animals and humans having close contact with the animals, but the analysis could not reveal the effect on the community (36). Other studies could not establish any strong evidence that the restriction of antibiotic use in animals reduced the occurrence of antimicrobial-resistant bacteria in the human population (37).

Use of MIA in animals for growth promotion, prophylaxis, and even metaphylaxis is considered as inappropriate antimicrobial usage (IAMU) by international agencies such as the World Health Organization (WHO), Food and Agricultural Organization (FAO), and World Organization for Animal Health (OIE). Recently, WHO recommended complete cessation of MIA use in healthy animals for prophylaxis or growth promotion (19). Among the MIA sold for animal production, tetracycline and penicillin constitute 32% and 6% of total sale on weight basis in the United States, 29% and 25% in European Union, 51% and 8% in Canada, 47% and 12% in Japan, and 9% and 9.8% in Australia, respectively. Cephalosporins are the MIA used with the lowest share (> 1%) among the sold antibiotics for animal production in the studied countries (38). Non-therapeutic antimicrobial use is

common among food animals, like prophylactic intramammary antimicrobial infusion in the form of penicillin or β-lactam in dairy animals, macrolides in beef feedlot cattle for respiratory illness, and tylosin in beef calves to prevent liver abscesses. Likewise, tylosin and tetracyclines are common antibiotics used as feed additives in 88% of the growing pigs in the USA (39). In the USA, about 74% of farm-animals that received antibiotics were in feed and 21% in the drinking water, and the sale of medically important antibiotics was three-time higher in the animal sector than in human beings (40). There is a considerable deficit of data on AMU in food animals, including poultry, from LMICs due to a lack of national-level surveys (41). The systematic study revealed the maximum use of tetracyclines followed by aminoglycosides, beta-lactams, macrolides, arsenicals, fluoroquinolones, ionophores, penicillins, polymyxins, polypeptides, and sulfonamides, but species-level consumption data from LMICs are largely unavailable (42). China exponentially increased the use of antimicrobials for animal production from 6 million kg in 2001 to 84.2 million kg in 2013, which is substantially higher than the United States and Europe (43). Tetracycline and penicillin constitute 33 and 20%, respectively, of total MIA sale for animal production in the Republic of Korea (38). Southeast Asia (SEA) is a group of rapidly developing LMIC (except Singapore, Brunei, and Laos) that shares a linked economy through export of aquaculture (Vietnam, Thailand, and Indonesia) and poultry (Thailand) products (44). The meta-analysis of the literature published from SEA (mostly Vietnam and Thailand) identified the use of amoxicillin in most of the farms, followed by enrofloxacin, norfloxacin, doxycycline, ampicillin, colistin, neomycin, gentamicin, tylosin, trimethoprim, florfenicol, erythromycin, chloramphenicol (although banned in Vietnam), sulfamethoxazole, and chlortetracycline for the production of pigs, chicken, and fish (45). The quantitative analysis revealed therapeutic use of 46 mg of antimicrobial compounds (penicillin, lincosamide, quinolone, and sulphonamides with trimethoprim) per kg of live pig and 52–276 mg per kg of live chicken in pig and poultry farms in Vietnam (23). For growth promotion, 286.6 mg and 77.4 mg of antimicrobials were used with feed to produce 1 kg of pork and chicken, respectively. The feeds of chickens and pigs in SEA mostly contained non-MIA groups of antibiotics, such as bacitracin (15–24% of feed formulations), enramycin (enduracidin), and florfenicol, except a single study from Vietnam which identified critically important antibiotics (colistin, amoxicillin, and neomycin) in chicken feed (25). Many of the antibiotics that are being used in food-animals for non-therapeutic purpose are not clinically relevant in human medicine but they may still confer cross or co-resistance to MIAs. Further, many of the antibiotics, like colistin (polymyxin), ardacin, avoparcin (glycopeptides), and virginiamycin (streptogramins), were classified as highest priority antimicrobials (polymyxin and glycopeptides) or highly important antimicrobial (streptogramins) (46). The global average annual consumption of antimicrobials per kilogram of animal produced was 45 mg/kg, 148 mg/kg, and 172 mg/kg for cattle, chicken, and pigs, respectively (24). The World Organization for Animal Health (OIE) estimated the amount of antimicrobial agents used in animals and detected an increase from 98.97 mg/kg in 2014 to 144.39 mg/kg in 2016 (47). Further increases in animal protein demand during and post the COVID period to boost immunity will increase meat production with higher intensity. Country-specific AMU surveillance data will allow for the scenario to be realistically predicted.

Companion Animals

Companion animals are not directly linked to the human food chain and possibly manage to escape the hunt for AMR drivers across the globe, although isolated studies on AMR bacteria surveillance have indicated the tip of the iceberg (48). With growing concern in modern society over pet welfare, more and more affluent families started treating pets almost like family members. Veterinary hospitals dedicated to pets have cropped up, especially in urban areas of LMICs. Keeping pace with the increasing pet-health care facilities, the use of antimicrobials has also increased substantially and multi-drug resistant bugs, like MRSA, methicillin-resistant *Staphylococcus intermedius* (49), carbapenem-resistant *Enterobacteriaceae* (CRE), and even colistin-resistance *E. coli,* are also being detected in China (50). The last group reported a steady increase in recovery of MDR and β-lactam- and fluroquinolone- resistant *E. coli* from pet animals in China over 5 years (2012–17), possibly due to increased β-lactam usage in pet clinics. The situation became catastrophic during the COVID period due to non-availability of qualified companion animal practitioners (associated with prolonged lockdown in few countries), over-prescription in telehealth consultations without laboratory-based diagnosis, easy accessibility of medicines from online pharmacies, and the preference for human antibiotics for their better quality and easy availability, especially during the break-down of the pharmaceutical supply chain. More elaborative studies are needed to address the issues in LMICs.

Aquaculture

In LMICs, open water aquaculture systems, such as ponds, are subjected to heavy pollution due to domestic and household activities, wastewater discharge, animal activities, and livestock manure fertilization. The pollution can significantly impact the microbial community and diversity of the pond water (51). Aquatic bodies are at the receiving end where untreated human and animal wastage manure are dropped and the aquatic bodies become ideal hosts for bacterial flora (52). Antimicrobial resistance genes (ARGs) are being exchanged across the bacterial species irrespective of their source and host specificity (53) (**Figure 1**). Antibiotics are applied *en masse* (metaphylactic) in fish as individual treatment is impractical, which also exposes the non-targets to such treatment (54). Fish generally excrete out most of the antibiotics into the water and sediment due to poor gut absorption and the waterbodies become storehouses for antibiotics, which exert selection pressure on the microflora (55). In the aquatic environment, transduction facilitates the lateral transfer of ARGs like β-lactamase (56) and humans can pick up resistances faster from aquatic sources than from terrestrial animals. Prophylactic use of antimicrobials in intensive/semi-intensive aquaculture is on the radar, since prolonged and

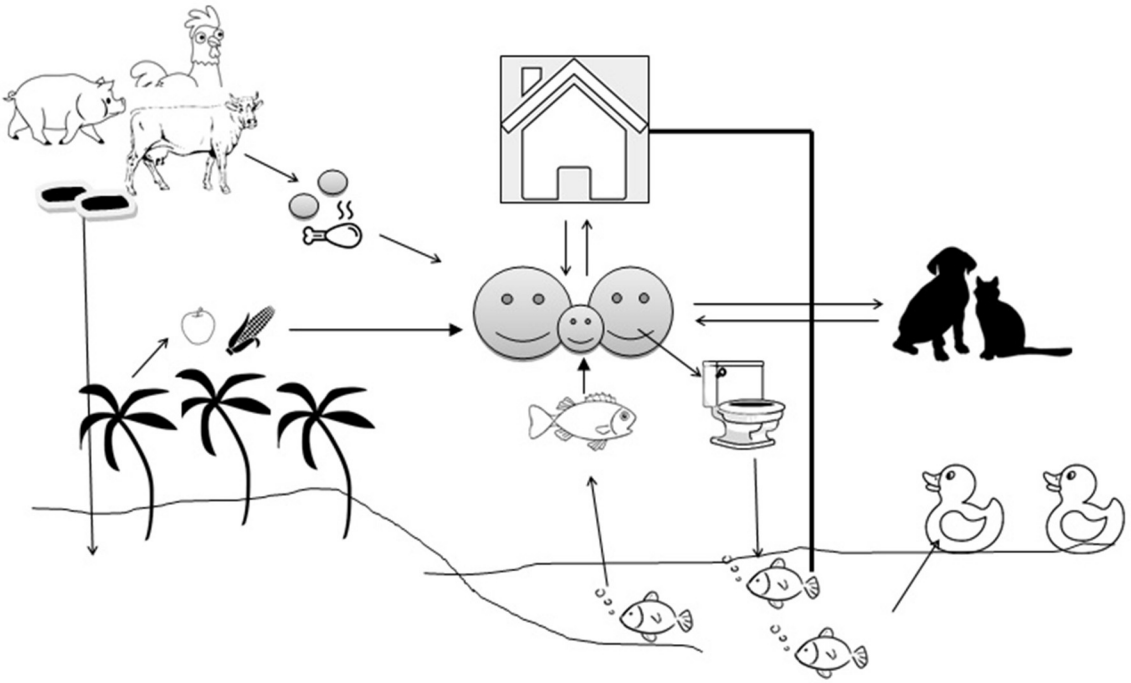

FIGURE 1 | Transmission of antimicrobial resistance genes between the environment and animal/human compartments. The arrows indicate the flow of antimicrobial resistance genes.

repeated use, even at a low concentration, is sufficient to exert selection pressure on the bacterial community to maintain their "resistome" and their spillover to human beings (57). Asian countries share the majority of global fish production, with China alone contributing to more than 60%. Little is known about the amount of AMU in aquaculture, particularly in LMICs due to a lack of strict monitoring, however, it varies depending upon geographical areas, climate, disease prevalence, and other socioeconomic factors. A single hatchery in Bangladesh experiences about 80 kg of antibiotics per cycle, a recent study reported (58). In Vietnam, about 72% of aquaculture farms used ∼3.3 g of antimicrobial per kg of fish/shrimp product in the form of pre-medicated feed (59). Metal-based antifouling compounds (biocides) which are in use in aquaculture to prevent or treat bacterial or parasitic diseases may confer co- or cross- resistance to many antimicrobials (60). Fish are the most affordable source of protein in LMICs, with low cholesterol and high fatty acids, as they are cheaper price than beef, chevon, or poultry meat. An increased protein demand to boost immunity during the COVID period, especially in infants, children, and the elderly, can be met by fish due to their easy digestibility. This increased demand will boost the commercial aquaculture farms, possibly encouraging the use of more antibiotics.

HOUSEHOLD USE OF ANTIMICROBIALS

Various scientific and medical bodies relied upon alcohol-based hand rub and handwashing with soap water as the most effective tools to combat the recent emergence of the COVID virus. Meanwhile, various commercial sanitizers, most of which contain medically important disinfectants, flooded the market with claims to effectively decimate the virus. The possible link between the use of disinfectants and the development of AMR received wide attention when the Food and Drug Administration (FDA) banned the use of triclosan in antibacterial soap (61). Sodium hypochlorite, commonly used in household cleaning or sewage decontamination and chlorination of water, has been on the news recently for its controversial use in purportedly eliminating coronavirus from the human body. Chlorination-induced oxidative injury was reported to increase resistance in *Pseudomonas aeruginosa* against ceftazidime, chloramphenicol, and ampicillin by 1.4–5.6-fold through overexpression of the MexEF-OprN efflux pump (62). Benzalkonium chloride (BAC) is a quaternary ammonium used as a preservative in eye, ear, or nasal drops. Lately, BAC was found to induce resistance even against last-resort antibiotics like polymyxin (63). There are reports of other quaternary ammonium compounds at sub-inhibitory concentrations affecting the susceptibility of *E. coli* strains to diverse antimicrobials, such as phenicol compounds, β lactams, and quinolones (64). There is conflicting evidence on the role of alcohol-based hand sanitizers (ABHS) in the spread and development of antibiotic-resistant bacteria. The repeated and prolonged use of ABHS may lead to the rise of alcohol-tolerant *Enterococcus faecium* in hospital environments due to mutations in carbohydrate uptake and regulation genes (65). Use of alcohol hand rubs was also reported to facilitate the growth of multidrug-resistant strains of *Acinetobacter baumannii* (65).

CATEGORIES OF ANTIMICROBIAL RESISTANT BACTERIA PREVALENT IN AGRI-FOOD CHAIN AND COMPANION ANIMALS IN LMICs

Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA)

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is an emerging pathogen that has been detected all over the world and, like many other bugs, its presence and spread are supported by the AMU and hygiene in addition to some other under-reported/poorly investigated factors like farm size, farming system, use of disinfectants, and in-feed zinc (66). Initially reported in animals, LA-MRSA is being increasingly reported in human beings; prolonged and frequent exposure of the farm-workers increased the risk of zoonotic transmission. Reservoir animals as asymptomatic carrier cannot be detected unless screened for MRSA, which is not under a routine surveillance program in any of the LMICs. Thus, we need to rely on the sporadic reports available from these countries. A number of studies from China demonstrated LA-MRSA infection as an occupational hazard for pig-farm workers (67), although such a possibility is very low (68). However, depending upon the prevailing farming style, nasal colonization of LA-MRSA in pig handlers may vary country wise (i.e., 5.5% in Malaysia, 15% in China, and 19.2% in Taiwan) (69). Unlike European and North American countries, which witnessed wide-spread detection of ST-398 clonal type of LA-MRSA, ST-9 predominates in the Asian continent. The majority of the LA-MRSA strains belong to SCCmec type IV and V and were frequently co-resistant to tetracycline and lincosamide, mediated by *tet* and *erm* genes, respectively (70), and glycopeptide-resistance remained a rare finding (71). MRSA needs special attention, as previously a SARS-CoV epidemic caused a significant rise in MRSA infection, especially in patients who required ventilation support (72). The recent episode of COVID19 may also produce a similar situation, as a large number of patients, especially with comorbid conditions, required ventilation support in the ICUs.

Cephalosporin Resistant Bacteria

Due to mutated or modified penicillin binding protein (PBP2a), MRSA are resistant to all β-lactam drugs, including cephalosporins. The Gram-negative bacteria (GNB), which are cephalosporin-resistant mainly through the production of β-lactamase or cephalosporinase- like AmpC type β-lactamase (ACBL) and extended-spectrum β-lactamase (ESBL), have become a cause of concern for their global spread, high infectivity, and associated mortality. Food and companion animals being the reservoir of ESBL or ACBL producers can put people in contact with them or consumers at significant risk. Due to abysmal public health infrastructure and poor hygiene, LMICs are overburdened with neonatal sepsis and healthcare-associated infections (73), with a heavy load of such drug-resistant pathogens in hospitals and communities (74). About 22% of healthy humans from Southeast Asia were found to harbor ESBL-producing bacteria in their gut-which is much higher than the global average (14%) (75). Increased contact with food animals is often assumed as an important underlying determinant for this higher ESBL colonization in Asian and African population (76). A number of studies from LMICs implicated food-animals as important reservoirs of ESBLs, however, their role in human infection cannot be confirmed. In contrast, chickens were reported to acquire ESBL resistance from water contaminated with human sewage through integrated farming, which is a common practice in many parts of Southeast Asia (77). In cows, ESBL-producers with multiple CTX-M variants were reported from mostly fecal sources and mastitic milk with a preponderance of CTXM-1 and CTXM-15 variants. Among other common types of the ESBLs in bovines from various LMICs were SHV-180, OXA-10, SHV-5 (Turkey), SHV-12 (Turkey and Egypt), SHV-180 (India), OXA-30 (Egypt), and TEM (India) (78). A number of studies conducted by our group revealed the presence of some ESBL variants in bovine mastitis wherein we could not trace any known ESBL genes by PCR screening – existence of a novel ESBL mechanism in this part cannot be ruled out (79, 80). Cattle manure is widely used as biological fertilizer, which increases the chance of environmental dissemination of ESBLs from a fecal source. Separate studies conducted by our group revealed the presence of ESBLs in broilers or birds kept exclusively for meat (81, 82) but not in backyard poultry reared by a large section of resource-poor farmers in India (83). Likewise, ESBLs and ACBLs were frequently detected in poultry from the developing countries of Asian (84, 85), African (86), and European (87) regions. Importantly, pigs, which form a favorite and affordable dish in many protein-hungry countries, were reported to carry ESBL determinants (88–90). The ESBL detection rate was much higher in chicken meat (∼93%) than meats from other animals, such as pork, beef (∼35%), and fish/shrimps (∼29%), in a study conducted on multiple species in Vietnam; possibly contamination with the caecal flora and poor disinfection measures led to this higher detection rate (91).

Carbapenem Resistant Bacteria

Carbapenems, due to their exceptional ability to withstand drug-resistance mechanisms like ESBLs, have remained an automatic choice of clinicians for treating refractory infections caused by ESBL-producing GNB. The emergence of carbapenem-resistance, which is often transmitted via mobile genetic elements, has far-reaching and rippling consequences, particularly in countries like India (92), China, and other Southeast Asian countries (50, 93) with heavy loads of ESBL infection in the healthcare setting. Carbapenem resistant *Enterobacteriaceae* (CRE) has serious repercussions in LMICs like many other bugs and infections, as it makes the poor vulnerable due to their lack of access to healthy environments, hygiene, and safety measures. Poor socio-economics put people at higher risk of contracting carbapenem-resistant pathogens (93) and, like many other bugs, this high priority pathogen was found to emanate amid poverty, violence, discrimination, and weak governance – key characteristics of LMICs (94). Fortunately, carbapenem is never chosen by veterinarians for treating food animals, possibly because of the cost and regulations putting a bar on its use. This is probably why

carbapenem resistance has been rarely reported among food animals, particularly from LMICs. However, animals can also be exposed to such drugs or bugs in the environment through contaminated and untreated wastewater discharges from hospitals; a lack of proper AMR surveillance in LMICs may be an important reason for the underreporting of CRE in food animals. In India, in two separate instances, bovine mastitis was reported to carry NDM-5 producing *E. coli* (95, 96). Further, two different studies – one in Algeria (97) and another in the Jiangsu province of China (98) - reported NDM-5 producing *E. coli* and NDM-5 producing *K. pneumoniae.* Again from China, recently, another study reported an NDM-5-producing *Escherichia coli* strain in poultry (99), indicating an insidious spread of CRE across food animals; however, proper surveillance is required to unveil it. Arguably, NDM-5 seems to predominate in animals - not only in food animals as discussed above, but also in pets as reported from three continents: Africa (100), Asia (50), and North America (101). Recent analysis of the COVID scenario indicated how resistant pathogens, such as carbepenem-resistant and cephalosporin-resistant *Enterobacteriaceae,* may complicate the SARS-CoV2 pneumonia (102). Overenthusiastic antibiotic therapy in managing COVID patients and chemoprophylaxis may promote these pathogens further.

Colistin Resistant Bacteria

Colistin, a near abandoned drug due to its nephrotoxicity, has once again begun gaining attention for its usefulness in treating extremely drug-resistant pathogens like carbapenem-resistant *Enterobacteriaceae* (103). Plasmid mediated colistin-resistance (*mcr*) was first reported from China, linked with the use of colistin in pig and poultry farming (29). Surprisingly, colistin was not a banned drug in veterinary practices even in many high-income countries (HICs) until the emergence of *mcr*. Till date, *mcr* and its different allelic variants have been reported from pigs, poultry (104), cattle (105), and companion animals (106), but not in human beings. Colistin-resistance was known to be mediated by chromosomal modification in the two component regulatory system or deletion of *mgr* (107); however, the plasmid-mediated colistin-resistance has struck the medical community for the possibility of its rapid spread. Since its initial detection in 2016 from China, plasmid-mediated colistin-resistance has been reported from different LMICs, such as Egypt (108), India (109), Vietnam (110), Brazil (111), and Argentina (106). Importantly, as the whole world is searching for an effective weapon against coronavirus, an *in silico* model predicted the ability of colistin to interfere with the function of novel coronavirus by interacting with the viral aminoacid residue pockets (Thr24-Asn28 and Asn119) through hydrogen bonds (112).

ROLE OF ANIMAL/POULTRY FARMS AS RESERVOIR OF AMR BACTERIA IN LMICs

The human population is expected to grow by 50% by the year 2050 (113), with consequential increases in the demand for food. In LMICs, consumer preference shifted toward animal protein from vegetables, consistent with enhanced income, urbanization, and demographic and lifestyle changes (114). While the global meat consumption is expected to rise by 76% between 2,000 and 2,050, the rise in LMICs is more than 200% due to an increased population with enhanced per capita consumption (115). Only in South-East Asian (SEA) countries is the demand for poultry projected to increase by 725% between 2,000 and 2,030 (116).

Industrial Food Animal Production (IFAP)

The Industrial Food Animal Production (IFAP) has witnessed a massive growth to meet the rising demand for animal protein. Because of intensive rearing, higher stocking density, zero-grazing, overdependence on MIA and non-MIA for therapy, prophylaxis and growth promotion, and poor waste management (117), IFAP is not without hazards, such as offensive odors, increasing risk of zoonoses, including AMR, and non-communicable disorders such as stress, hypertension, and cognitive impairment among animal handlers and people in the surrounding community (118). The only benefit of IFAP, as argued by the Brazilian Government, is less environmental degradation, such as reduced deforestation (119).

In LMICs, due to the rising demand and expansion of multi-national food production companies, the IFAP is slowing replacing the small-scale backyard/household rearing system (120). IFAP is preferred particularly in urban areas of countries like Ethiopia, Uganda, and Vietnam that experience a shortage of land and water (121). A lack of stringent regulations and monitoring increased the non-therapeutic use of antimicrobials in farms (mostly pig and poultry) in several LMICs, such as China (122), Vietnam (123), Ethiopia (124), Uganda (125), Kenya (126), Mexico (127), and Myanmar (128).

The persistence of antimicrobia- resistant bacteria in IFAP settings is associated with several factors, such as AMU, co-resistance, cross-resistance with heavy metals, bacterial fitness, mixing of new and old animals, vectors or reservoirs of bacterial infection, vertical and pseudo-vertical transmission, and cleaning and disinfection (129). Even animal transport vehicles and flies originated from IFAP play a major role in the transmission of AMR into the community (130).

Backyard Farming

Unlike the developed world, LMICs are largely dependent upon small-scale backyard farming and as a result are more environment-friendly; backyard farming is regarded as sustainable even after meeting the rising demand for animal protein. As the animals are kept in small flocks or herds and maintained in a free-range system with occasional supplementations of raw vegetables with minimum manpower, backyard farming poses a relatively low risk for zoonotic transmission (131). However, small-scale backyard farming (chicken and pigs) is converting rapidly into "medium- to large-scale" backyard faming by making agreements with different food companies ("contract farming").

In general, backyard farming is operated with minimal antimicrobial intervention, replaced instead by indigenous technical knowledge (ITK) or lower generation cheaper antibiotics (132). Sporadic studies in different LMICs (Tanzania, Ecuador, Vietnam, Ghana, Bangladesh, and Cambodia)

revealed the usage of antimicrobials like oxytetracycline, penicillin, erythromycin, enrofloxacin, and trimethoprim-sulfadozine by "medium- to large-scale" backyard pig and chicken farmers (77, 133), based on personal experience or communication without veterinary oversight. The lack of costly higher generation cephalosporin usage in backyard household poultry was reflected in the absence of extended spectrum beta-lactamase determinants in *Salmonella* and *E. coli* isolated from backyard layers in India (83, 134).

The use of AMUs by farmers in backyard farms is influenced by their capacity to detect the diseased animals, the farmer's expertise and attitude toward the disease-associated risk, and the cost-benefit analysis of treatment (135). The cheaper variety of the antimicrobials is always preferred, although sometimes it is unsafe due to compromising with the quality, especially in LMICs. The overall prevalence of low-quality medicine was estimated to be 13.6% in LMICs, and further, 12.4% of the antibiotics were substandard or falsified (136). The annual market for unregistered and poor quality veterinary drugs in Africa is estimated to be equal to the registered drug market (400 million US dollars) (137). The bacterial population exposed to the poor quality veterinary medicine is not wiped out completely due to sub-therapeutic dosages and the ineffective release of drugs, and the left over bacteria may remain as a resistant population with subsequent transmission (138).

MITIGATION STRATEGY

Substitution of AMU

AMU is the single most important driver for AMR; therefore, attempts are being made to slowly reduce or phase out antimicrobials in veterinary medicine. However, substitution of antimicrobials has short-term economic implications resulting from substantial loss of production and higher morbidity or mortality. In any case, if such measures compromise food security, that may have devastating impacts in poor, highly populous, and resource-deprived countries. An early warning system based on a local epidemiological database and regular health check-ups of the animals may allow us to detect the disease early and thereby prevent its spread to other animals in the herd or adjoining areas. Thus, widespread chemoprophylaxis that becomes indispensable in any outbreak may be avoided. This needs to be revisited in the face of the COVID outbreak, with several pets and wild animals having tested positive for SARS-CoV-2, like cats, dogs, tiger, lions, minks, ferrets, hamster, bats, and macaques. In addition, preventive non-antimicrobial strategies which include- timely vaccination, appropriate biosecurity measures, proper nutrition and housing may reduce the demand for preventive antimicrobial therapy. The adoption of herd-specific control measures to minimize the occurrence of diseases like mastitis may be helpful to promote the prudent use of antimicrobials (139).

Various alternative ways, like reducing meat consumption, capping the amount of antimicrobials per year per kilogram of animal product, and making antibiotics expensive by taxation, were proposed to cut down AMU in food animals (140). Some sort of economic shield in the form of insurance packages or incentives to safeguard the loss arising out of any infectious diseases in the farm may psychologically motivate farmers to use antibiotics more judiciously (135). Making the meat from antibiotic-treated animals more expensive and labeling such meat packages with its source (from antibiotic-treated animals) is another way to discourage consumers and to indirectly reduce AMU (141).

The alternative anti-infective strategies, such as nano-material based anti-infective particles, enzymes, antimicrobial peptides, quorum sensing quenchers, efflux pump inhibitors, clay, predatory bacteria, teat sealants, and antimicrobial photodynamic therapy, are in the pipeline to be evaluated at a field level. The supplementation of essential oils and spices as an alternative to antimicrobials was reported to have beneficial health effects in poultry (142, 143). However, the performance of essential oils still needs to be clinically tested in various conditions and they may not be equally effective against the Gram-negative pathogens because of inherent tolerance (144); the absence of maximum residue levels (MRL) data regarding those essential oils in food animals has to be checked.

Raising Awareness Among Farmers

FAO referred to farmers as "important frontline defenders" for the vital role they can play in stemming the spread of AMR by adopting good hygienic farm operations. Increasing awareness among farmers by imparting basic knowledge may help reduce the unnecessary and indiscriminate AMU in food animals (104); however, it can only be successful if adequate financial support and insurance packages are given to recuperate any loss in livestock farming (145). In most of the LMICs, small and marginal farmers often suffer huge economic losses due to disease outbreaks for meager investments on biosecurity and farm hygiene; these psychologically disadvantaged farmers can then be easily misled about the purported efficacy of antimicrobials for growth promotion and disease prevention by the unscrupulous push-sell of drug-marketing agencies. Inadequate veterinary healthcare facilities and limited drug regulations increase the magnitude of the problem in LMICs and, without reshaping these, efforts to bring sustainable change in farmers' behavior, knowledge, attitude, and practices may be futile (145). Changing farmers' behaviors or increasing their awareness for appropriate AMU in food animals requires multiple supportive measures, like incentives to the farmers raising livestock without antibiotics, subsidized insurance to make up for losses, the implementation of strict drug regulation, and the establishment of a strong network of veterinary healthcare facilities accessible to rural farmers in LMICs. The mobile veterinary clinic was introduced by the Government of India to reach out to the country's remote corners.

Implementation of Government Legislation

FAO underscored strong government legislation as the most important component in addressing the overuse, misuse, or abuse of antimicrobials that accounted for the rise in AMR. Such legislations are essential for defining the responsibilities and duties of all the stakeholders, and for the sustainability of the policies and technical objectives aimed at reducing AMR.

Drug regulatory agencies of European countries and the FDA implemented strong regulations by banning the prophylactic and growth-promoting use of antimicrobials in food animals and capping the limit of CIA/HIA in the veterinary sector. Many countries (Japan, the USA, Colombia, Denmark, the Netherlands, and Sweden) fixed national targets to reduce AMU in livestock. Nevertheless, any kind of regulatory endeavor is still at a primary stage in many of the LMICs (146); only a few could successfully implement such regulations or advisories given the prevailing socio-economic scenario, public administrative constraints, and absence of political commitment/goodwill in these countries. Therefore, emphasis on education, awareness, and training of all stakeholders, particularly the end users, might be more effective in LMICs (147).

Surveillance of AMU and AMR

Surveillance on AMR pathogens and AMU is undeniably the key driving force for controlling AMR, with WHO suggesting this point in the global action plan on antimicrobial resistance (GAP), which still remains the authentic source of information to fight against AMR. Many of the HICs (Norway, Japan, Denmark, Canada, the USA, Finland, the Netherlands, and France) have articulated national surveillance programs (NORM-VET, JVARM, DANMAP, CIPARS, NARMS, FINRES-VET, NethMap-MARAN, ONERBA- RESAPATH, and SWEDRES-SVARM) and the policies on AMU in animals are tailored based on the data generated from their networks. Such is not the case in LMICs, as most of them have no surveillance in place to monitor the antimicrobial consumption in animals. The government of India adopted the NAP on AMR and strongly recommended the need for a strong regulatory framework for restricting the AMU in animals. A Pan-India Network – ICAR-Indian Network for Fisheries and Animal Antibiotic Resistance (INFAAR) was initiated by the Indian Council of Agricultural Research in collaboration with FAO to cater to the objectives laid down in India's NAP on AMR. A lack of robust infrastructural support crippled by financial constraints remains the most pressing challenge for the establishment and proper functioning of a robust surveillance system on AMR/AMU in LMICs. On the contrary, Ashley et al. (148) proposed to tap the large amount of data generated by academic institutes and private laboratories to indirectly and passively monitor the problem in these areas for the time being.

Drug-Repurposing Strategy

The COVID19 panorama has been an eye-opener for scientists worldwide; when there are no or limited therapeutic choices, with a new drug or effective vaccine still a long way off, the only option left is to experiment with the existing modalities. The whole world is frantically searching for a solution through drugs such as hydroxychloroquine, azithromycin through ivermectin (149), famotidine (150), flavipiravir (151), and remdesivir (152). This is the same situation with novel antimicrobials as the plausibility of a new drug to hit the market in the near future is remote. Even if it comes to market, how long it will be effective for is not clear. The search for new antimicrobials is impeded by the huge investment requirement, time lag, and reluctance of pharma-leaders recently shifting their focus toward cheaper strategies like the repurposing of drugs which involves less time and investment. No such study has been conducted on repurposing of drugs in the veterinary sector. However, veterinary drugs were tried for repurposing; fenbendazole was found to be effective against non-small cell lung cancer cells (153) by microtubule destabilization and inhibition of glucose uptake. Likewise, isoxazoline was found to be promising in human vector-borne diseases. Many anthelmintic compounds of the salicylanilide family-niclosamide, oxyclozanide, rafoxanide, and closantel - demonstrated antibacterial properties against a wide range of pathogens -methicillin-, vancomycin-, linezolid-, or daptomycin-resistant *Staphylococcus aureus*, *Clostridium difficile, Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Helicobactor.* Avermectins were tested successfully against *Mycobacterium* and MRSA. Likewise, an antifungal property was reported for mebendazole. Trials with a few NSAIDs (Celecoxib, aspirin, ibuprofen, and tacrolimus) against a few bacterial and fungal pathogens turned out hopeful (154). A number of studies were conducted using anticancer drugs to repurpose as antibacterial, 5-fluorouracil and gallium nitrate were found to be effective against MDR *A. baumanni* and *Pseudomonas.* Tamoxifen, floxuridine, and streptozotocin exhibited appreciable antibacterial activity against *Staphylococcus* isolates.

However, the dose of the repurposed drugs is comparatively higher when used as an antimicrobial, so the pharmacokinetic profiles changes abruptly which necessitates a clinical revaluation and toxicity testing. LMICs, where the burden of infection is quite high, can provide ample scope for such trial and testing with funding support from international agencies.

Pivotal Mitigation Strategy to Be Focused in Post COVID Period

The pos- COVID scenario might be associated with a rise in AMR in human and animals due to more stress on antibiotic-dependent healthcare systems to combat secondary bacterial infections. Interruptions of antibiotic stewardship programs in hospitals and communities, prescription with antibiotics for COVID patients misdiagnosed with bacterial bronchitis, over-prescription in telehealth consultations, and easy accessibility of medicines from online pharmacy are just a few key factors associated with AMR identified during the pandemic (155). Enhancement of the immune system with an increased animal-based protein diet was promoted by governments and non-governmental organizations throughout the world during the pandemic. It will further increase the demand for animal protein, which may enhance the growth promotional use of antimicrobials in IFAPs.

Antimicrobial resistance is mostly dependent on the use of antibiotics in humans, agri-food chains, and companion animals, and the use of antibiotics is largely regulated by human instinct (156). The knowledge, attitude, and practice of the AMU by all types of prescribers and farmers varies a lot between developed countries and LMICs, which will be further modified with added complexities during a post-pandemic period. The qualitative

and quantitative survey should be established at a national level in LMICs to explore the behavioral basis of AMU during the post-COVID period. A national level monitoring system should be established for the quantification of AMU categorically in different species of animals, birds, fish, and agri-products to detect the risk factors for the emergence of any change in resistance pattern post the pandemic. The quantification of AMU will develop a benchmarking system with an immediate identification of the top-level user, although reduction of AMU is not always directly correlated with reduction of AMR, as it is a multi-factorial issue (as described earlier). More farm-level molecular epidemiological studies in livestock, poultry, and aquaculture to identify the reservoir of resistant bacteria, categorize the resistance determinants, establish the correlation between resistance determinants and true resistance against MIA, explore the environmental resistome, and explore the wildlife as carrier of resistant bacteria are in dire need. A holistic one health approach based intervention strategy incorporating all the local stakeholders of each LMIC is required to address the complex issue after identification of the major driver which may vary between the member countries and during the pre- and post-pandemic periods. The one health approach to address the AMR issue might be an easier process during the post-COVID period as several international collaborative groups were already created during the pandemic and, moreover, political will and subsequently more research and development investments by governments to address health-related issues is expected to save the human population.

AUTHOR CONTRIBUTIONS

SB and IS planned and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Multidrug Resistance and Virulence Factors of *Escherichia coli* Harboring Plasmid-Mediated Colistin Resistance: *mcr-1* and *mcr-3* Genes in Contracted Pig Farms in Thailand

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The presence of the plasmid-mediated colistin resistance encoding *mcr* gene family in the *Enterobacteriaceae* is one of the crucial global concerns. The use of colistin in livestock rearing is believed to be the cause of *mcr* gene spreading and is of impact to public health. The objective of this research was to detect the frequency and virulent genes of *mcr*-positive *Escherichia coli* (MCRPE) in fecal samples from healthy pigs in a contract farming system across Thailand. A total of 696 pooled samples were derived from 80 farms, located in 49 provinces across six regions of Thailand. The colistin-resistant *E. coli* were identified by MALDI-TOF mass spectrometry and antimicrobial susceptibility testing by broth microdilution. The antibiogram was determined using an automated susceptibility machine, and the genetic characteristics were investigated for *mcr-1–5* genes, phylogenetic group, replicon types, and virulent genes. In total, 31 of 696 samples were positive, with *E. coli* containing *mcr-1* or combination of *mcr-1* and *mcr-3* with incidence of 4.45 and 0.43%. Phylogenetic groups A and B1 and the IncF and IncFIB replicon types were predominantly found in the MCRPE located in the central area, with multidrug-resistant traits against 3–14 types of antimicrobials. Additionally, 19 of 31 isolates identified as enterotoxigenic *E. coli* were with the *stap* and *stb* (enterotoxin-encoding genes). In conclusion, a low carriage rate of *mcr*-positive *E. coli* was detected in the large-scale farming of healthy pigs. The association between multidrug-resistant MCRPE and their pathogenic potential should be of concern.

Keywords: colistin resistance, *Escherichia coli*, *mcr* genes, pigs, virulent factor

INTRODUCTION

Antimicrobial resistance (AMR) is an emerging concern for both human and animal sectors of the world. The inappropriate use of antimicrobials in clinical settings and, most importantly, in livestock farming imposes social and economic burdens on society (1). The diminishing number of active (effective) antimicrobial agents to treat sick farm animals is accompanied by the downfall

in food production and the likelihood of exposure of farmers to resistant bacteria. *Escherichia coli*, a commensal microbe, can accumulate resistance genes. It is widely used as a representative example for monitoring resistance genes, especially for horizontal gene transfer (2). Therefore, the assessment of mobile genetic elements from commensal *E. coli* could highlight the AMR transmission between hosts (3).

Colistin is a cationic antibiotic that has long been regarded as a last resort antibiotic for *Enterobacteriaceae* infections. However, the widespread use of colistin in animal production acts as a selective pressure for the spread of plasmid-mediated colistin resistance genes, which are in the *mcr* family. The first discovery of plasmid-mediated colistin resistance (*mcr-1* gene) in *E. coli* from China raised an enormous attention globally and was followed by the subsequent discovery of other *mcr* resistance genes, including *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5*, in different geographical areas (4). Recently, another four colistin resistance genes (*mcr-6*, *mcr-7*, *mcr-8*, and *mcr-9*) were identified mainly from members in the *Enterobacteriaceae* family (5–8). Among them, *mcr-1* is the most frequently detected in farmed animals and from *Enterobacteriaceae* infections in humans (9). These reports raised awareness upon colistin usage, especially in livestock animals.

In Thailand, over 80% of pig farming systems are contract farming between the primary producers and the agribusiness companies, for the latter to procure a certain pre-agreed quality and quantity of products at an economical price and is lesser from the primary producers. Antimicrobials including colistin are feed additives or prophylactic agents, including colistin, against bacterial infections in pig farms under veterinary prescription (10). Although there have been a few reports regarding a high prevalence (60–90%) of multidrug-resistant (MDR) *E. coli* in pigs in Thailand, the antimicrobials used on the farms have not always been clearly defined (11). Since colistin resistance is the crucial epidemiological data of public health concern, monitoring the prevalence of colistin-resistant *E. coli* and their characteristics is of high priority. The objective of this study was to characterize the antibiogram and virulent traits of *mcr*-positive *E. coli* (MCRPE) from the fecal samples of healthy pigs derived from the contract farming system across Thailand.

MATERIALS AND METHODS

Study Area and Animal Selection

Samples were collected from 80 farms, in 49 provinces across six regions of Thailand, comprised of 15, 5, 12, 7, 4, and 6 provinces from central, northern, northeastern, eastern, western, and southern Thailand, respectively. Farms were selected based on the available management data, including the antimicrobial usage, housing, vaccination, feed type, and production cycle. However, all historical data was allowed as inclusion criteria for farm selection only but not allowed to be included in the analysis. A total of 696 pooled fecal samples (5–10 samples per farm) were collected from individual 18- to 20-weeks-old fattening pigs with a normal clinical appearance and no recent history of enteric disease or therapeutic antimicrobial treatment.

Sample Collection and Bacterial Identification

At least 5 g of feces per pig was collected into a sterile container and kept at 4°C until processed. Then, the fecal samples were homogenized and mixed to get pooled fecal samples with a total mass of 25 g. Then, 5 g of well-mixed feces was collected and diluted 10-fold using sterile 0.85% (w/v) NaCl. Dilutions of 10^{-7}-10^{-8} were spread on eosin methylene blue agar (Oxoid, UK) plates containing 2 μg/ml colistin sulfate (Sigma-Aldrich, USA) to select for the presumptive colistin-resistant *E. coli*. The biohazard execution control was approved by the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC 1731021). One representative colony with typical *E. coli* morphology was picked and subcultured to get pure culture. The *E. coli* species was confirmed using matrix-assisted laser desorption ionization combined with time-of-flight analysis (MALDI Biotyper, Bruker, USA). The principle behind MALDI-TOF is based on mass spectrometry and "soft" ionization technique. Depending on the time of flight of each pathogen, the characteristic spectrum will be analyzed and displayed *via* the inbuilt software. Briefly, the bacterial colony sample was smeared as a thin film directly on a target plate and then coated with 1 μl polymeric matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and air-dried at room temperature. This matrix could penetrate the cell wall of microorganisms and able to extract proteins. The target plate was placed into the mass spectrometer and irradiated by a laser. Afterwards, the molecules vaporized and ionized at the same time into the vacuum and transported to the detection device. Lastly, the computerized database results compared with the reference library database were generated with interpretations (12).

Antimicrobial Susceptibility Determination and *mcr* Gene Detection

For colistin, the broth microdilution procedure was performed according to the Clinical and Laboratory Standards Institute (CLSI) recommendation (13). The plasmid-mediated colistin resistance genes (*mcr-1–5*) were detected by multiplex (m)PCR using GoTaq® Green Master Mix (Promega, USA) and the previously reported primers and PCR conditions (14). The *E. coli* strain CUP13 (15), which is positive for *mcr-1* and *mcr-3* (confirmed by Sanger sequencing), and ATCC25922 were used as positive and negative controls, respectively. Briefly, the thermocycling conditions were performed at 94°C for 15 min, followed by 25 cycles of 94°C for 30 s, 58°C for 90 s, and 72°C for 1 min, and then followed by 72°C for 10 min.

The minimal inhibitory concentration (MIC) of antimicrobial agents against the *E. coli* isolates was determined using the AST-GN 38 test kit in a Vitek2 compact automated susceptibility level detection apparatus (BioMérieux, France). The antimicrobial groups selected were synchronized with veterinary guidelines (16). Justification of the antibiotics chosen is for AMR monitoring and for the purpose of public health awareness such as the second generation of cephalosporin, aminoglycoside, fluoroquinolone, and carbapenem. *E. coli* ATCC 25922,

Pseudomonas aeruginosa ATCC 27853, and *Staphylococcus aureus* ATCC 25913 were used as the control strains. The antimicrobials selected were amikacin (AK), amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), cefalexin (CEX), cefpodoxime (CPD), cefovecin (INN), ceftiofur (XNL), chloramphenicol (C), enrofloxacin (ENR), gentamicin (GEN),

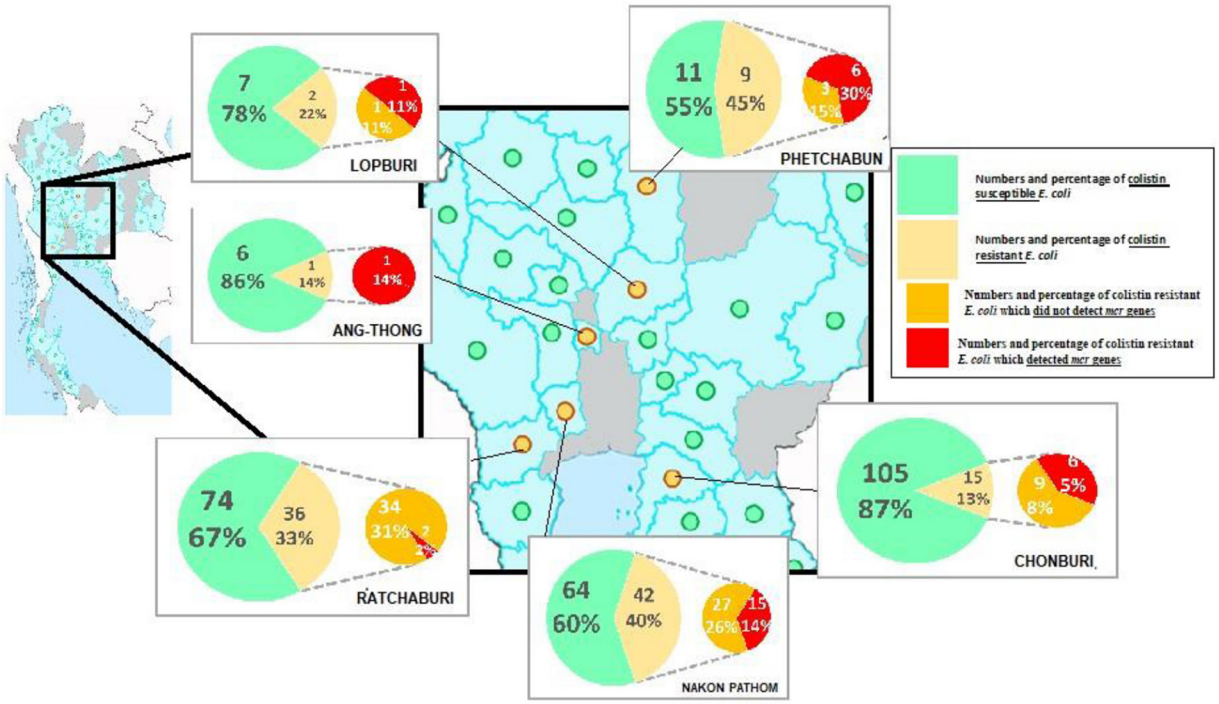

FIGURE 1 | Geographical distribution of either colistin resistant or susceptible *E. coli* from the surveyed contracted pig farms in Thailand.

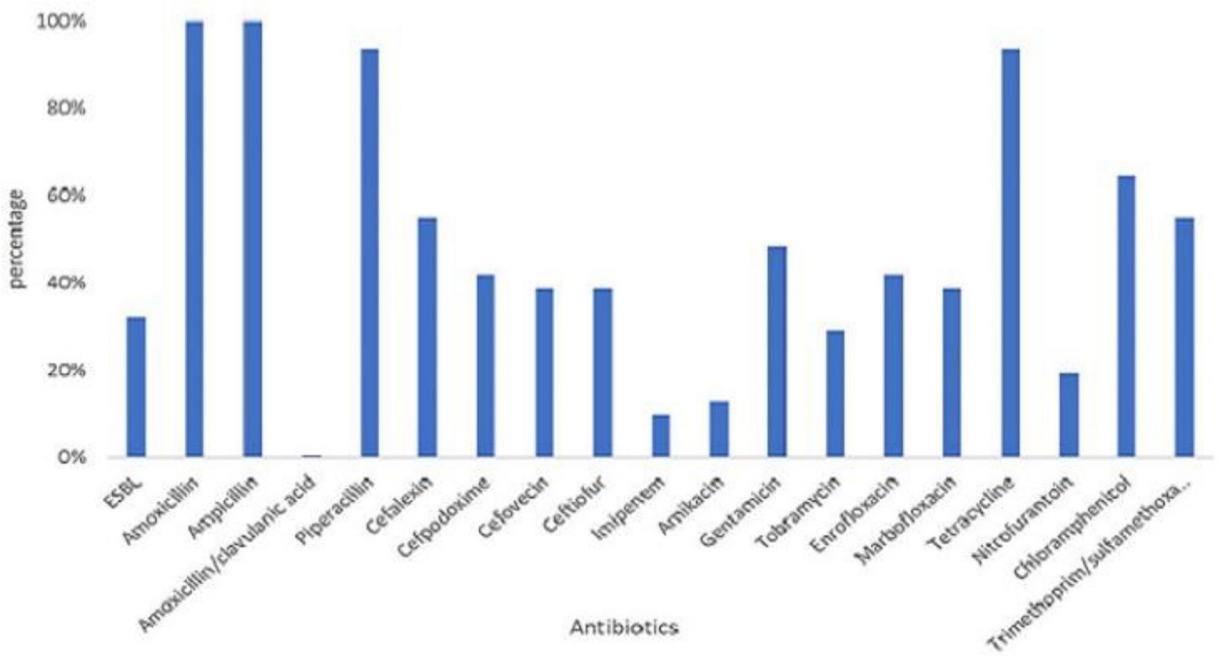

FIGURE 2 | Distribution of resistant rates against 18 antimicrobials and presence of extended- spectrum beta-lactamase (ESBL) characteristic among 105 colistin-resistant *E. coli* isolated from contracted pig farms in Thailand.

imipenem (IMP), marbofloxacin (MBR), nitrofurantoin (NIT), piperacillin (PIP), tetracycline (TET), tobramycin (TOB), and trimethoprim/sulfamethoxazole (SXT). The MIC interpretations will be reported according to Food and Drug Administration (FDA) (17), CLSI (13), and EUCAST values (18). The isolates that presented an extended-spectrum beta-lactamase (ESBL) phenotype were confirmed with a double disc synergy test and phenotypic disc confirmatory test as previously reported (19).

Phylogenetic Grouping

The MCRPE isolates were determined using an approved mPCR identification of their phylogenetic groups and subgroups (A, B1, B2, C, D, E, and F) as reported (20). Each reaction was performed in a 25-μl mixture containing 12.5 μl of GoTaq® Green Master Mix (supplied with *Taq* polymerase), 20 pmol of each primer, and 200 ng of genomic DNA. The *E. coli* ATCC 25922 and *E. fergusonii* CUVET427 (21) strains were used as the controls.

Plasmid Replicon Typing

The *Enterobacteriaceae* plasmid replicons IncF (IncFIA, IncFIB, IncFIC, and IncFrep), IncI1-Ig, IncN, IncP, IncW, IncHI1, IncHI2, IncL/M, IncT, IncA/C, IncK, IncB/O, IncX, and IncY were detected using five mPCR and three simplex PCR tests. The primers, PCR conditions, and thermal cycles were applied as previously reported (22). Briefly, PCR amplifications, except the F-simplex, were thermal cycled at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min, and then followed by 72°C for 5 min. The F-simplex PCR was performed with the same amplification program except at an annealing temperature of 52°C. Positive control samples were provided and used as reported (21).

Detection of Virulence Genes

The sets of mPCR and simplex PCRs were performed as previously reported (23), with the positive control strains taken from the previously sequenced enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) strains (24). Primers specific for the *StaP* (heat-stable toxin a subdivide p), *Stb* (heat-stable toxin b), *Stx2e* (Shiga toxin), *K88* (Fimbriae), *F4* (Fimbriae), and *Ltb* (heat-labile enterotoxin b subunit) genes were used. The PCR assays were prepared with GoTaq® Green Master Mix (Promega, USA) and thermocycled at 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 55°C for 45 s, and

TABLE 1 | Antibiograms of the 31 MCRPE isolates distributing in 26 pattern types.

Pattern	Profile	Number of resistant ABOs	Isolate(s)
A	AMX–AMP–PIP–CEX–CPD–INN–XNL–GEN–ENR–MBR–TET–C–NIT–SXT*	14	1
B	AMX–AMP–PIP–CEX–CPD–INN–XNL–IMP–AK–GEN–ENR–TET–C–SXT*	14	1
C	AMX–AMP–PIP–CEX–CPD–INN–XNL–GEN–TOB–ENR–MBR–TET–C*	13	3
D	AMX–AMP–PIP–CEX–GEN–TOB–ENR–MBR–TET–NIT–C–SXT	12	1
E	AMX–AMP–PIP–CEX–CPD–INN–XNL–IMP–AK–C–SXT*	11	2
F	AMX–AMP–PIP–CEX–CPD–INN–XNL–GEN–TOB–TET–NIT*	11	1
G	AMX–AMP–PIP–CEX–CPD–INN–XNL–TET–C–NIT–SXT*	11	1
H	AMX–AMP–PIP–CEX–CPD–INN–XNL–GEN–TOB–TET–C*	11	1
I	AMX–AMP–PIP–GEN–TOB–ENR–MBR–TET–C–SXT	10	2
J	AMX–AMP–PIP–CEX–CPD–INN–XNL–TET–C–SXT	10	1
K	AMX–AMP–PIP–CEX–CPD–INN–XNL–GEN–TET	9	1
L	AMX–AMP–PIP–CEX–ENR–MBR–TET–C–SXT	9	1
M	AMX–AMP–PIP–ENR–MBR–TET–NIT–C–SXT	9	1
N	AMX–AMP–PIP–CEX–ENR–MBR–TET–SXT	8	1
O	AMX–AMP–PIP–ENR–MBR–TET–C–SXT	8	1
P	AMX–AMP–PIP–GEN–ENR–MBR–TET–SXT	8	1
Q	AMX–AMP–PIP–GEN–TOB–TET–C–SXT	8	1
R	AMX–AMP–PIP–CEX–CPD–AK–TET	7	1
S	AMX–AMP–PIP–CEX–TET–C–SXT	7	1
T	AMX–AMP–PIP–TET–C–SXT	6	1
U	AMX–AMP–PIP–GEN–TET–NIT	6	1
V	AMX–AMP–PIP–TET–NIT	5	1
W	AMX–AMP–PIP–TET–C	5	2
X	AMX–AMP–GEN–TET	4	1
Y	AMX–AMP–PIP–TET	4	1
Z	AMX–AMP–TET	3	1

AMC, amoxicillin–clavulanic acid; AMP, ampicillin; AMX, amoxicillin; C, chloramphenicol; CEX, cephalexin; CPD, cefpodoxime; ENR, enrofloxacin; GEN, gentamicin; MBR, marbofloxacin; PIP, piperacillin; SXT, trimethoprim/sulfamethoxazole; INN, cefovecin; AK, amikacin; IMP, imipenem; TET, tetracycline; XNL, ceftiofur; TOB, tobramycin; NIT, nitrofurantoin.
**ESBL.*

72°C for 1.5 min increasing by 3 s each cycle, and then followed by 72°C for 10 min.

Data Analysis

The colistin resistance rates are presented as percentages divided by region and province in comparison of the rate with and without the *mcr* genes, and the antimicrobial resistance profiles are reported as the antibiogram patterns of *mcr*-positive *E. coli*. The patterns of virulence gene profiles among MCRPE isolates are presented in percentages. To define MDR and pathogenic traits among the colistin-resistant *E. coli*, the relation between AMR phenotypes and pathotype characteristics was analyzed using Fischer's exact test ($p \leq 0.05$).

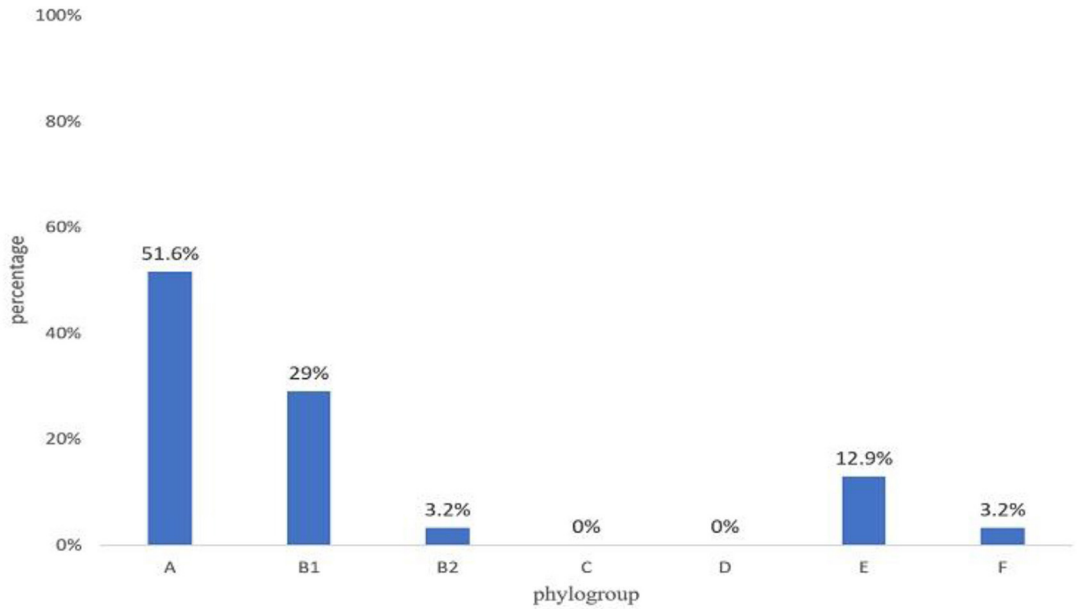

FIGURE 3 | The phylogroups detected among 31 MCRPE isolates in contracted pig farms in Thailand.

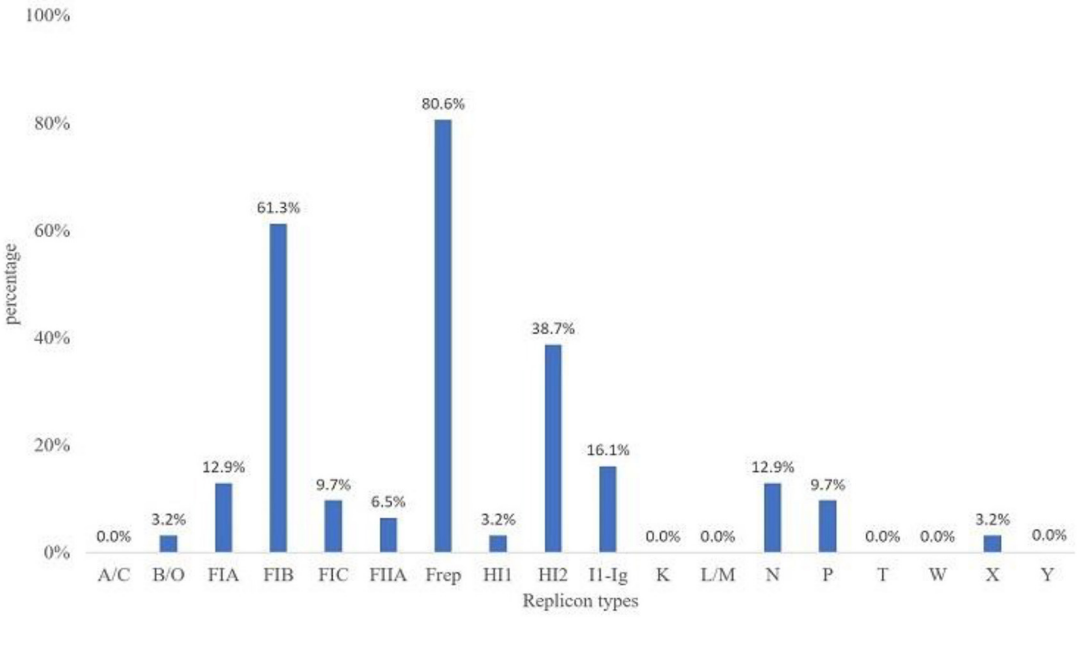

FIGURE 4 | Plasmid replicon types detected among 31 MCRPE isolates in contracted pig farms in Thailand.

RESULTS

Distribution of Colistin-Resistant *E. coli* Containing *mcr* Genes

A total of 105 colistin-resistant *E. coli* from the 696 samples were isolated using the eosin methylene blue (EMB) media. From the broth microdilution method, the MCRPE isolates had MIC values of 4 ($n = 17$) or 8 ($n = 14$) μg/ml. From the PCR detection, the *mcr-1* gene was found in 31 of these 105 colistin-resistant *E. coli* isolates, and among them, three isolates were found to also express *mcr-3*. The distributions of colistin-resistant *E. coli* were from central (5.4%) (Phetchabun, Nakhon Pathom, Ang-Thong, and Lopburi), western (0.4%) (Ratchaburi), and eastern (1.4%) (Chonburi) Thailand. The geographical distributions of *E. coli* with or without *mcr* genes are shown in **Figure 1**.

Antimicrobial Susceptibility Testing

All 31 MCRPE were multidrug resistant (**Figure 2**), with all being resistant to AMX, AMP, PIP, and TET, and over 50% were resistant to CEX, INN, XNL, GEN, ENR, C, and the SXT combination. No pan-drug resistance was detected among the MCRPE isolates. ESBL was found in 32.3% (10/31) *mcr-1* positive isolates. A total of 26 antibiogram patterns were recorded for 31 MCRPE isolates. Forty-eight percent (15/31) of these isolates were MDR with resistance to six antimicrobial groups (**Table 1**).

TABLE 2 | Presence of virulent profiles including toxin and antigenicity of the 31 MCRPEs.

Virulence genes	ESBL (%)	Pathotype(s)	Number	%
StaP–Stb–Stx2e	0	ETEC, EHEC	1	3.2
StaP–Stb–K88	3.2	ETEC	1	3.2
StaP–Stb	16.1	ETEC	13	41.9
StaP	3.2	ETEC	3	9.7
Ltb	0	ETEC	1	3.2
Negative	9.7	Non-pathogenic	12	38.7

ETEC, enterotoxigenic E. coli; EHEC, enterohemorrhagic E. coli; Stap, heat-stable toxin a subdivide p; Stb, heat-stable toxin b; Stx2e, Shiga toxin; K88, Fimbriae, F4; Ltb, heat-labile enterotoxin, b subunit.

Phylogenetic Grouping

Most isolates were from phylogenetic group A (51.6%), followed by group B1 (29%) and groups E (12.9%), B2 (3.2%), and F (3.2%) (**Figure 3**).

Plasmid Replicon Typing

The predominantly found plasmid replicons were of the IncF and IncFIB replicon types at 80.6 and 61.3%, respectively. Plasmid replicon types L/M, W, Y, A/C, T, and K were not detected in this study (**Figure 4**). The other replicon types were found at low prevalence rates among the MCRPE isolates, with IncX, IncB/O, and IncHI1 being present at the lowest percentages (3.2%).

Characterization of the Virulent Factors

The virulent genes representing ETEC or EHEC were found in 18 out of 31 (58.1%) MCRPE isolates (**Table 2**). The ETEC strains possessed the *StaP* and *Stb* enterotoxin-encoding genes as the most frequent pathotype, and one strain (from Phetchabun province) showed a hybrid ETEC–EHEC genotype.

Relation Analysis Between Antimicrobial Susceptibility and Pathogenicity

The association between antimicrobial susceptibility and pathogenicity of the 31 MCRPE isolates was analyzed by Fischer's exact test (**Table 3**). No association between pathogenicity and resistance to the six antibiotic groups was found (fluoroquinolones, sulfonamides, tetracyclines, nitrofurazones, phenicols, and aminoglycosides) ($p = 0.28$, 1.00, 1.00, 1.00, 1.00, and 0.15, respectively).

TABLE 3 | Relation analysis between MCRPE resistance to the other six antimicrobial groups and their pathogenicity.

Antimicrobial group	Pathogenicity	Resistant	Susceptible	*p*-value
Aminoglycosides	Non-pathogenic	9	4	0.15
	Pathogenic	7	12	
Fluoroquinolones	Non-pathogenic	7	6	0.28
	Pathogenic	6	13	
Tetracyclines	Non-pathogenic	11	2	1.00
	Pathogenic	17	2	
Nitrofurazones	Non-pathogenic	3	10	1.00
	Pathogenic	4	15	
Phenicols	Non-pathogenic	9	4	1.00
	Pathogenic	12	7	
Sulfonamides	Non-pathogenic	7	6	1.00
	Pathogenic	11	8	

Aminoglycosides: amikacin, gentamicin, and tobramycin; fluoroquinolones: enrofloxacin and marbofloxacin; tetracyclines: tetracycline; nitrofurazones: nitrofurantoin; phenicols: chloramphenicol; sulfonamides: trimetroprim/sulfamethoxazole; pathogenic: ETEC, ETEC–EHEC; non-pathogenic: negative for virulence genes.

DISCUSSION

This national-scale study of contract-farmed pigs in Thailand confirmed the existence of colistin-resistant *E. coli* containing *mcr* genes and that they showed diversity in their phylogenetic group, replicon type, antibiogram, ESBL trait, and pathogenic potential. All recruited contracted pig farms had strict historical data and management records that can be traced back as an essential inclusion criteria. The sample collection criteria were set up and executed by the farm workers under the authority of veterinarians. In this study, MALDI-TOF MS was used for the identification and confirmation of bacteria strains. This technique has emerged as a powerful technique for the identification of microorganisms with an overall 95% accuracy at the species level. The main advantage of MADLI-TOF is being able to identify bacterial species directly from the culture plates as fast as 1 to 15 min in a few simple steps (12).

According to mPCR, our results indicated the lower resistance rate of *mcr-1* (4.4% or 31/696) when compared with a previous report from healthy pigs in China (21%) (20). This study covered all parts of Thailand where high-intensity pig farming is done. Unfortunately, all the historical data could not be analyzed due to the company's policy. However, the positive areas were distributed in the western, central, and eastern parts within a radius of about 300 km. The distributions of colistin-resistant *E. coli* were higher (15–30%) in Nakhon Pathom, Ratchaburi, Chonburi, Lopburi, and Phetchabun provinces. These provinces reported to have a huge number of pig farms and total number of pigs. Colistin was legally use in pig feeds for prophylactic purposes in Thailand until March 2018. The high percentage of MCRPE isolates in certain provinces might come from prolonged cumulative selective pressure from their history of colistin usage in pig feeds. To the best of our knowledge, this is the first report of *mcr-1* gene in *E. coli* isolates from pigs in Thailand. Interestingly, three of the *mcr-1*-positive isolates also co-expressed *mcr-3*. These results could highlight the awareness of the distribution of *mcr* genes and for the national policy of livestock immigration. The *mcr-1* genes have been widely shown to be distributed in Asia, Europe, Africa, and America and primarily due to the consequence of long-term colistin application in animals (25). The *mcr-3* gene was first reported in China in 2017 (26) and the prevalence and spread of the *mcr-3* gene in Thailand should be carefully monitored from now on.

According to phylogenetic grouping, the majority of the isolates in our study were in phylogroups A or B1, predominantly related with commensal strains (27). On the other hand, for the virulent *E. coli* groups, phylogroup D was not detected in the current study and there was a low frequency of phylogroup B2. Several studies have reported that phylogroups B2 and D were associated with intestinal and extraintestinal pathogenic *E. coli* as well as MDR strains (28, 29). Nonetheless, even commensal *E. coli* from various phylogroups have been reported to harbor pathogenicity islands that can serve as integration sites for virulence and/or AMR determinants (30) and so may facilitate in converting commensal strains to pathogens.

With respect to plasmid replicon typing, the IncFIB and F plasmids were the most commonly found replicon types in this study. They are narrow host-range-type plasmids, which have been reported in worldwide members of the *Enterobacteriaceae* family, associated with various antimicrobial-resistant genes (31). The *mcr-1* and *mcr-3* genes were previously described on the IncI, IncHI2, and IncX4 plasmids (32). A variety of replicon types were found in the MCRPE isolates in this study, which suggest that the *mcr* genes can locate and/or transfer to different plasmid types. This is in accordance with a previous report that the *mcr-1* genes and ESBL could be co-transferred by more than one type of conjugative plasmid, which might alleviate their effective dissemination among bacteria (33).

The antibiogram profiles characterized among the MCRPE isolates revealed that MDR was a common phenotype in this study. *E. coli* resistance to beta-lactam and the tetracycline antibiotic groups was very common in Thailand, and aminoglycoside and fluoroquinolone resistance was found to be varied in farm management such as using antibiotic for prophylactic or treatment purposes (21). The MDR traits among *mcr-1*-positive *E. coli* have been reported frequently in pigs due to the usage of antibiotics in the production cycle (34). Interestingly, ESBLs were found at a high prevalence among the MCRPE isolates of this study, which might due to co-selection under selective pressure (33). Moreover, *E. coli* plasmids that harbor co-localization of *mcr-1* and $bla_{\mathrm{CTX-M}}$ genes and/or *mcr-1* and $bla_{\mathrm{NDM-5}}$ genes have been reported previously (35). Genomic characterization should be performed to resolve the reason for this apparent correlation.

The presence of the *Ltb*, *Stb*, *StaP*, *Stx2e*, and *K88* virulence genes in MCRPE isolates indicated that they also had the potential to cause an infection. Thus, healthy pigs could be an important reservoir of colistin-resistant ETEC. Interestingly, one MCRPE isolate was found to be an ETEC–EHEC hybrid strain. *E. coli* with highly virulent hybrid pathotype strains had been reported previously both in animals and human diarrhea patients (36). Since many of the virulence genes of *E. coli* are carried on mobile genetic elements, the genetic combination of these MGE resulted in the emergence of STEC/ETEC hybrid strains in multiple events (37). The recent finding of a clone of sequence type (ST) 95 showing extreme drug resistance with a high virulence potential underscores the need to monitor new and emerging trends in antibiotic resistance development in this important global lineage (38). On the other hand, aminoglycoside- and fluoroquinolone-resistant *E. coli* seemed to have a lower probability to act as an ETEC pathotype in this study. Pathogenic *E. coli* tends to be more susceptible to many antimicrobials (39). However, the mechanism is still not elucidated and clonal typing should be included for a more convincing analysis.

In conclusion, a low carriage rate of *mcr-1* and *mcr-3* co-positive *E. coli* was detected in large-scale contract pig farms in Thailand. The MCRPE isolates showed MDR *E. coli* and most of the isolates contained virulence genes representing an ETEC pathotype. These data provide an insight into the

occurrence of colistin resistance among *E. coli* in healthy pig carriages and their characteristics, in terms of virulence genes and antibiograms. However, genomic characterization of *mcr* genes found in Thailand is required for further study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because all fecal samples were submitted from veterinarians in pig industrial field to the veterinary diagnostic laboratory as the annual surveillance. We asked the permission to use these sort of samples which did not directly collect the feces by our team. However, the biohazard execution control was approved by the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC 1731021).

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/fvets.2020.582899/full#supplementary-material

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Multidrug Resistance in Enterococci Isolated From Wild Pampas Foxes (*Lycalopex gymnocercus*) and Geoffroy's Cats (*Leopardus geoffroyi*) in the Brazilian Pampa Biome

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Enterococci are ubiquitous microorganisms present in various environments and within the gastrointestinal tracts of humans and other animals. Notably, fecal enterococci are suitable indicators for monitoring antimicrobial resistance dissemination. Resistant bacterial strains recovered from the fecal samples of wild animals can highlight important aspects of environmental disturbances. In this report, we investigated antimicrobial susceptibility as well as resistance and virulence genes in fecal enterococci isolated from wild Pampas foxes (*Lycalopex gymnocercus*) ($n = 5$) and Geoffroy's cats (*Leopardus geoffroyi*) ($n = 4$) in the Brazilian Pampa biome. Enterococci were isolated from eight out of nine fecal samples and *Enterococcus faecalis* was identified in both animals. However, *E. faecium* and *E. durans* were only detected in Pampas foxes, while *E. hirae* was only detected in Geoffroy's cats. Antimicrobial susceptibility analysis showed resistance to rifampicin (94%), erythromycin (72.6%), ciprofloxacin/norfloxacin (40%), streptomycin (38%), and tetracycline (26%). The high frequency of multidrug-resistant enterococci (66%) isolated in this study is a matter of concern since these are wild animals with no history of therapeutic antibiotic exposure. The *tet*M/*tet*L and *msr*C/*erm*B genes were detected in most tetracycline- and erythromycin-resistant enterococci, respectively. The *gelE*, *ace*, *agg*, *esp*, and *clyA* virulence genes were also detected in enterococci. In conclusion, our data suggest that habitat fragmentation and anthropogenic activities in the Pampa biome may contribute to high frequencies of multidrug-resistant enterococci in the gut communities of wild Pampas foxes and Geoffroy's cats. To the best of the authors' knowledge, this is the first report of antimicrobial-resistant enterococci in the Pampa biome.

Keywords: *Enterococcus* spp., pampa biome, wildlife animals, Pampas fox, Geoffroy's cat, multidrug-resistance, virulence factors, antibiotic resistance genes

INTRODUCTION

Brazil hosts six terrestrial biomes, which include the Amazon, Atlantic Forest, Caatinga, Cerrado, Pampa, and Pantanal biomes. Notably, the Pampa biome covers 63% of Rio Grande do Sul State and extend to Uruguay and the central region of Argentina (1–3). The fauna of the Brazilian Pampa biome consists of 83 native mammal species, of which some are endemic and/or considered endangered species. Among the mammal species, Geoffroy's cat (*Leopardus geoffroyi*) (Felidae) and the Pampas fox (*Lycalopex gymnocercus*) (Canidae) are listed as species of "least concern" in the IUCN Red List of Threatened Species (4, 5). The main factors contributing to the decline of these species are habitat destruction and hunting (2, 6, 7). Farming activities have converted natural areas of the Brazilian Pampa into agricultural and grazing lands, with ∼48.7% of this biome now being used for plantation crops (1, 3).

This biome has been suffering constant disturbances due to anthropogenic impacts and the reduction of natural habitat has forced wild animals to live near human settlements, which has resulted in negative outcomes for wildlife conservation (8, 9). Pampas fox and Geoffroy's cat population density in Brazilian Pampa biome is 0.2 and 0.27 ind/km^2, respectively (10, 11). Studies of wild canids and felids from the Pampa biome have shown that these animals exhibit adaptability in foraging based on prey availability, which can lead them to establish secondary food sources on farms. They are known to consume domestic vertebrates, fruit, insects, and carrion as well as to get food into the farms trash (12–14). In the past year, various studies have been published regarding habitat degradation and its effects on the wildlife and environment of the Pampa biome; however, studies evaluating the impact of multidrug-resistant bacteria on the wildlife in this biome remain scarce.

Enterococci are ubiquitous microorganisms found in water, soil, plants, and gastrointestinal tracts of wild animals, domestic animals, and humans (15–19). This ubiquitous distribution has been associated with phenotypic plasticity since they can tolerate a wide range of temperature and pH and grow in the presence of 6.5% sodium chloride (NaCl) or 40% of bile salts (20). The genus *Enterococcus* comprises at least 50 species (21). Among these, *E. faecalis* is the predominant species in the gastrointestinal tracts of mammals, followed by *E. faecium*, *E. durans*, *E. hirae*, and *E. mundtii* (18).

Additionally, enterococci are considered opportunistic pathogens in susceptible hosts. They cause urinary tract, wound, and soft tissue infections as well as bacteremia (22, 23). Although enterococci are considered a common cause of nosocomial infections, they can also cause several diseases including bovine mastitis, endocarditis, septicemia, and diarrhea in dogs, cats, pigs, and rats (24). The treatment of enterococcal infections has been complicated by the emergence of antibiotic-resistant strains, which makes these infections an important public health concern. Resistance to different classes of antimicrobials is a hallmark of *Enterococcus* spp. since they are intrinsically resistant to β-lactams, cephalosporin, lincosamides, streptogramins, and aminoglycosides (25). Meanwhile, resistant strains are not restricted to clinically known species since such strains have been isolated from different environments, including wildlife (15, 17, 19, 24, 26–30). Due to their remarkable ability to adapt to the environment, ubiquity in gut and to acquire antibiotic resistance determinants, enterococci have been employed as sentinel organisms for resistance to antimicrobials with Gram-positive activity.

Resistant bacterial strains recovered from wild animals can highlight important aspects of microbial interactions and environmental disturbances in wildlife (31, 32). Wild animals can be considered sentinels for the emergence and spread of antimicrobial-resistant bacteria in the environment. Therefore, the present study evaluated the presence of resistant enterococci in wild mammals aiming to detect previously unstudied variation in antimicrobial resistance distribution patterns in these animals. Additionally, to date, relatively few reports on antimicrobial resistance strains have been produced based on samples from wild canids and felids when compared to the number of reports on domestic animals. This difference could largely be explained by the migratory habits of some wild species and the difficulty of obtaining samples from wildlife. To the best of the authors' knowledge, this is the first study of antimicrobial resistance profiles and virulence genes in fecal enterococci isolated from wild Pampas foxes and Geoffroy's cats in the Brazilian Pampa biome.

MATERIALS AND METHODS

Samples Collection

Rectal swabs were collected from wild Pampas foxes ($n = 5$) and Geoffroy's cats ($n = 4$) (**Figure 1**). The animals were captured in two sites from Brazilian Pampa Biome, Rio Grande do Sul, Brazil. The first site was located near to Candiota city (31°33′06.73″S; 53°40′40.63″W), proximal to Jaguarão river, and characterized by intense agricultural, mining activity and roads; in this site, five samples were obtained. The second site was located near Arroio Grande city (32°13′58.99″S; 53°05′11.75″W), characterized by forest fragments and agricultural activities; in this site, four samples were obtained (**Supplementary Table 1**).

The capture, manipulation, and samples collections were authorized by Brazilian Institute of Environment and Renewable Natural Resources, IBAMA, Brasília, Brazil, and Chico Mendes Institute for Biodiversity Conservation (ICMBio). The protocol was approved by the Information Authorization System in Biodiversity (SISBIO) number 0200 1.007 9 10 12006-32. The animals were captured with the assistance of Tomahawk traps and anaesthetized via intramuscular (100 mg/mL of ketamine hydrochloride and 20 mg/mL of xylazine hydrochloride).

Rectal swabs were collected by veterinarians, all animals were clinically healthy (e.g., heart and respiratory rates and body temperature) 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. After sample collection, the animals were returned to their habitats. All animals were in health conditions.

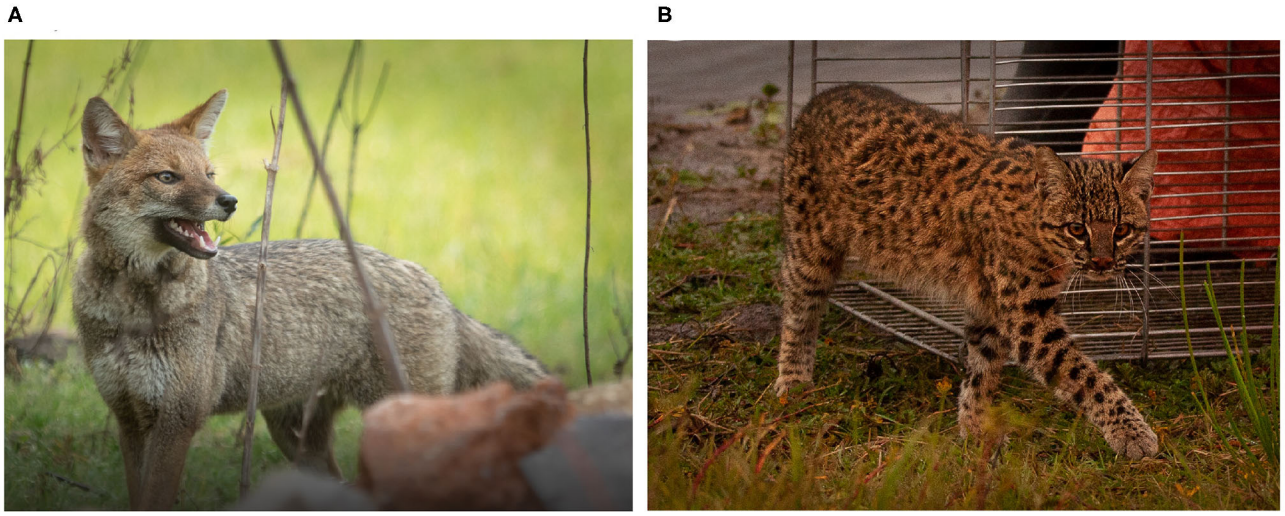

FIGURE 1 | Wild Pampas fox (*Lycalopex gymnocercus*) **(A)** and Geoffroy's cat (*Leopardus geoffroyi*) **(B)** during their capture in the Brazilian Pampa Biome. *Source*: Felipe Peters.

Isolation and Identification of Enterococci

Isolation of enterococci was performed as described previously (17). Rectal samples 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 1/1,000. From each dilution, 100 μL was inoculated in brain heart infusion (BHI) agar plates (Himedia, Mumbai, India) supplemented with 6.5% NaCl.

Since enterococci are present in high concentrations in fecal samples, typically between 10^5 and 10^7 CFU/g, we randomly selected 10 colonies from each fecal sample. Phenotypic criteria (size/volume, shape, color, Gram staining, catalase production), and bile esculin 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, USA) and 10% (v/v) glycerol (Neon Comercial Ltda).

Bacterial species identification was performed by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry method (MALDI-TOF) technique applied to *Enterococcus* (33). MALDI-TOF analysis was performed using a LT Bruker microflex mass spectrometer (Bruker Daltonik GmbH) and spectra were automatically identified using BrukerBioTyper™ 1.1 software. The identification by MALDI-TOF MS is based on the score value released by the equipment. A higher or similar 2.3 value indicates that the identifications of genus and species are reliable. 2.0–2.29 show that the genus is reliable and the species is probable. 1.7–1.99 values indicate that the identification of genus is probable.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of all strains was determined by Kirby-Bauer disk diffusion method, according to Clinical and Laboratory Standards Institute (34). Twelve antibiotics were tested: ampicillin 10 μg (AMP), vancomycin 30 μg (VAN), erythromycin 15 μg (ERY), tetracycline 30 μg (TET), ciprofloxacin 5 μg (CIP), norfloxacin 10 μg (NOR), nitrofurantoin 300 μg (NIT), chloramphenicol 30 μg (CHL), gentamicin 120 μg (GEN), linezolid 30 μg (LNZ), rifampicin 5 μg (RIF), and streptomycin 300 μg (STR). Reference strain *E. faecalis* ATCC 29212 was used as control.

Intermediate and resistant-strains were included in a single category as resistant-strains. Strains were classified as single (SR), double (DR) or multidrug-resistant (MDR) phenotype when showed resistance for one, two, and three or more antimicrobial classes, respectively (35).

Detection of Resistance and Virulence Genes

Genomic DNA was extracted by a physicochemical method as previously described (36). The presence of resistance and virulence genes commonly observed in clinical and environmental enterococci was tested by PCR (**Table 1**). The resistance-related genes evaluated were: *erm*B (which encodes a ribosomal methylase that mediates macrolides, lincosamides and type B streptogramins resistance); *msr*C (which encodes for a macrolide and streptogramin B efflux pump); *tet*M and *tet*S (which encodes for tetracycline resistance via a ribosomal protection protein mechanism); and *tet*L (which encodes for tetracycline resistance via efflux pumps proteins). As well the virulence genes tested were: *ace* (adhesin to collagen of *E. faecalis*); *cyl*A (cytolysin); *agg* (aggregation substance); *gel*E (gelatinase); and *esp* (enterococcal surface protein).

Amplifications were carried out in a total volume of 25 μL containing: 100 ng of template DNA, 1 X reaction buffer (Ludwig Biotechnology), 0.4 μM of each primer (Ludwig Biotechnology), 1.5 mM $MgCl_2$, 200 μM of dNTPs (Ludwig Biotechnology),

TABLE 1 | Primers used in the PCR reactions carried out for detection of resistance and virulence genes.

Gene	Nucleotide sequence (5′-3′)	AT[a] (°C)	Size (bp)[b]	References
Erythromycin				
*erm*B_F	GAAAAGGTACTCAACCAAATA	52	645	(37)
*erm*B_R	AGTAACGGTACTTAAATTGTTTAC			
*msr*C_F	AAGGAATCCTTCTCTCTCCG	52	342	(38)
*msr*C_R	GTAAACAAAATCGTTCCCG			
Tetracycline				
*tet*L_F	ACTCGTAATGGTGTAGTTGC	58	627	(26)
*tet*L_R	TGTAACTCCGATGTTTAACACG			
*tet*M_F	GTTAAATAGTGTTCTTGGAG	52	656	(39)
*tet*M_R	CTAAGATATGGCTCTAACAA			
*tet*S_F	TGGAACGCCAGAGAGGTATT	58	660	(39)
*tet*S_R	ACATAGACAAGCCGTTGACC			
Adhesion				
*ace*_F	AAAGTAGAATTAGATCACAC	56	320	(40)
*ace*_R	TCTATCACATTCGGTTGCG			
Cytolysin				
*cyl*A TE17	TGGATG'ATAGTGATAGGAAGT	56	517	(41)
*cyl*A TE18	TCTACAGTAAATCTTTCGTCA			
Biofilm				
esp 46	TTACCAAGATGGTTCTGTAGGCAC	60	1198	(42)
esp 47	CCAAGTATACTTAGCATCTTTTGG			
Gelatinase				
*gel*E_F	ACCCCGTATCATTGGTTT	50	402	(41)
*gel*E_R	ACGCATTGCTTTTCCATC			
Aggregation				
agg TE3	AAGAAAAAGAAGTAGACCAAC	62	1553	(41)
agg TE4	AAACGGCAAGACAAGTAAATA			

[a]*AT, annealing temperatures;* [b]*bp, base pair.*

1 U Taq DNA polymerase (Ludwig Biotechnology), and MilliQ water. PCR amplifications were performed in the conventional thermocycler (Applied Biosystems 2720 Thermal Cycler) according to the following program: 94°C for 5 min followed by 35 cycles of 94°C for 1 min, appropriate annealing temperature for each primer for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The DNA fragments amplified were analyzed in 1.5% (w/v) agarose gels stained with SYBR® Safe DNA Gel, and visualized on a photo-documenter.

RESULTS

In order to not overestimate the data referring to species distribution and antimicrobial susceptibility profile, strains isolated from the same animal with similar phenotypic and genotypic characteristics, which could indicate clonal strains, were grouped, generating a total of 50 strains, 30 from Pampas foxes and 20 from Geoffroy's cats. The number of isolates per wild animal ranged from 5 (samples PF3, PF4 and GC1) to 9 (sample GC3).

Isolation and Identification of Enterococci

Enterococci were isolated from eight out of nine fecal samples. Furthermore, 50 *Enterococcus* spp. strains were isolated and characterized of wild Pampas fox and Geoffroy's cat from the Brazilian Pampa biome, including *E. faecalis* (64%; $n = 32$), *E. faecium* (22%; $n = 11$), *E. hirae* (10%; $n = 5$), and *E. durans* (4%; $n = 2$).

The species distribution between wild Pampas foxes and Geoffroy's cats are shown on **Table 2**. Changes in the composition of *Enterococcus* species were detected in both animals. *E. faecalis* was the most frequent species in fecal samples of both animals; however, *E. faecium* and *E. durans* were isolated only in Pampas fox and *E. hirae* just in Geoffroy's cat.

Antimicrobial Susceptibility Profile

All enterococci isolated from wild canids and felids were tested for antimicrobial resistance, and almost all strains (98%, $n = 49$) were resistant to at least one evaluated antimicrobial agent (**Table 3**). Only one *E. hirae* isolated from Geoffroy's cat was susceptible to all antimicrobials tested. The highest frequency was found for rifampicin (94%; $n = 47$), followed by erythromycin (72%; $n = 36$), ciprofloxacin/norfloxacin (40%; $n = 20$), streptomycin (38%; $n = 19$), and tetracycline (26%; $n = 13$). Resistance to nitrofurantoin (18%; $n = 9$); gentamycin (14%, $n = 7$), and chloramphenicol (4%; $n = 2$), was noted in less frequency. No strains showed a resistance profile to ampicillin, linezolid and vancomycin.

The most remarkable result to emerge from the data is that a high frequency (66%; $n = 33$) of MDR strains isolated from wild canids and felids from Brazilian Pampa biome (**Table 3**). The percentages of double and MDR strains isolated from wild Pampas fox (30%; $n = 9$ and 63.33%; $n = 19$) were similar to wild Geoffroy's cat (20%; $n = 4$ and 70%; $n = 14$). Of the 33 MDR strains, 15 (45.45%) were resistant to four or more antimicrobials, it is important to highlight that one *E. faecalis* strain isolated from wild Pampas fox showed resistance to seven antimicrobials tested (ciprofloxacin; chloramphenicol; erythromycin; streptomycin; nitrofurantoin; rifampicin; tetracycline) (**Table 4**).

TABLE 2 | Distribution of *Enterococcus* species among wild Pampas fox and Geoffroy's cat.

		Number of species isolated				
		E. faecalis	***E. faecium***	***E. hirae***	***E. durans***	**Total**
Pampas fox	**PF1**	4	1	0	1	6
	PF2	2	5	0	0	7
	PF3	2	3	0	0	5
	PF4	2	2	0	1	5
	PF5	7	0	0	0	7
Geoffroy's cat	**GC1**	5	0	0	0	5
	GC2	0	0	0	0	0
	GC3	9	0	0	0	9
	GC4	1	0	5	0	6
	Total	32 (64)	11 (22)	5 (10)	2 (4)	50 (100)

Frequency of Antimicrobial Resistance and Virulence Related Genes

The resistance genes were investigated only in phenotypically resistant erythromycin and tetracycline strains (**Table 5**). Of the 36 erythromycin- resistant, four (11.11%) harbored *erm*B and nine (25%) *msr*C genes. Among the 13 tetracycline-resistant enterococci, *tet*L and *tet*M genes were found in 7 (53.85%) strains. None strain was positive to *tet*S gene.

All strains were tested for the presence of enterococci commonly associated virulence genes. The **Table 6** shows the results of *gelE, cylA, esp, ace,* and *agg* genes. The highest frequencies of virulence genes were found in *E. faecalis* and *E. faecium*. The *gelE* (62%; $n = 31$) and ace (48%; $n = 24$) showed elevated prevalence among these species. The *agg* gene (22%; $n = 11$) was recorded only on *E. faecalis* strains. Otherwise, *esp* and *cylA* genes were observed in just one *E. faecium* and *E. hirae* strains, respectively.

DISCUSSION

Isolation and Identification of Enterococci

Relatively few studies have reported enterococci isolated from wild canids and felids such as red foxes (43), Iberian wolves, and Iberian lynx (44, 45). The results of the present study corroborate with previous results showing that *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* are commonly encountered in the fecal samples of

TABLE 3 | Antimicrobial resistance profiles among enterococci isolated from fecal samples of wild Pampas fox and Geoffroy's cat.

Strains (n)	Number (%) of resistant strains[a]								Profiles[b]		
	ERY	**CIP/NOR**	**RIF**	**STR**	**GEN**	**NIT**	**CHL**	**TET**	**SR**	**DR**	**MDR**
Pampas fox											
E. faecalis (17)	13 (76.47)	7 (41.18)	16 (94.12)	7 (41.18)	4 (23.53)	3 (17.65)	1 (5.88)	2 (11.76)	1 (5.88)	5 (29.41)	11 (64.70)
E. faecium (11)	7 (63.64)	4 (36.36)	11 (100)	4 (36.36)	0	1 (9.09)	0	4 (36.36)	1 (9.09)	4 (36.36)	6 (54.55)
E. durans (2)	2 (100)	0	2 (100)	1 (50)	1 (50)	0	0	1 (50)	0	0	2 (100)
Subtotal (30)	22 (73.33)	11 (36.67)	29 (96.67)	12 (40)	5 (16.67)	4 (13.33)	1 (3.33)	7 (23.33)	2 (6.67)	9 (30)	19 (63.33)
Geoffroy's cat											
E. faecalis (15)	12 (80)	9 (60)	15 (100)	3 (20)	2 (13.33)	1 (6.67)	1 (6.67)	1 (6.67)	0	4 (26.67)	10 (66.67)
E. hirae (5)	2 (40)	0	3 (60)	4 (80)	0	4 (80)	0	5 (100)	1 (20)	0	4 (80)
Subtotal (20)	14 (70)	9 (45)	18 (90)	7 (35)	2 (10)	5 (25)	1(5)	6 (30)	1 (5)	4 (20)	14 (70)
Total (50)	36 (72)	20 (40)	47 (94)	19 (38)	7 (14)	9 (18)	2 (4)	13 (26)	3 (6)	13 (26)	33 (66)

[a] *Antimicrobials: ERY, erythromycin; CIP, ciprofloxacin; NOR, norfloxacin; RIF, rifampicin; STR, streptomycin; GEN, gentamicin; NIT, nitrofurantoin; CHL, chloramphenicol; TET, tetracycline.*
[b] *Profiles: SR, single-resistance; DR, double-resistance; MDR, multidrug-resistance.*

TABLE 4 | Antimicrobial resistance phenotypic profile of *Enterococcus* sp. isolated from fecal samples of wild Pampas fox and Geoffroy's cat.

Profile[a]	Antimicrobials[b]	Species	Number of resistances	
			PF[c]	GC[d]
SR	RIF	*E. faecalis*	1	
		E. faecium	1	
	TET	*E. hirae*		1
DR	ERY/RIF	*E. faecalis*	3	3
		E. faecium	2	
	STR/RIF	*E. faecium*	1	
	CIP-NOR/RIF	*E. faecalis*	1	1
		E. faecium	1	
	NIT/RIF	*E. faecalis*	1	
MDR	CIP-NOR/ERY/RIF	*E. faecalis*	3	4
		E. faecium	1	
	CIP/STR/RIF	*E. faecalis*	1	
	CIP/ERY/TET	*E. faecium*	1	
	CIP/CHL/RIF	*E. faecalis*		1
	ERY/STR/TET	*E.durans*	1	
	ERY/GEN/RIF	*E. faecalis*	1	
		E. durans	1	
	ERY/STR/RIF	*E. faecium*	1	
	STR/GEN/RIF	*E. faecalis*	1	
	CHL/ERY/RIF	*E. faecalis*		1
	CIP/ERY/GEN/RIF	*E. faecalis*		1
	CIP/STR/GEN/RIF	*E. faecalis*		2
	CIP/ERY/STR/RIF	*E. faecalis*	1	1
	STR/NIT/TET/NOR	*E. hirae*		1
	STR/NIT/TET/RIF	*E. hirae*		1
	ERY/STR/GEN/RIF	*E. faecalis*	1	
	ERY/STR/TET/RIF	*E. faecium*	1	
	ERY/STR/NIT/TET/RIF	*E. faecium*	1	
		E. faecalis	1	1
		E. hirae		2
	CIP/ERY/STR/GEN/RIF	*E. faecalis*	1	
	CIP/CHL/ERY/STR/NIT/TET/RIF	*E. faecalis*	1	

[a] *SR, single-resistance; DR, double-resistance; MDR, multidrug-resistance.*
[b] *Antimicrobials: ERY, erythromycin; CIP, ciprofloxacin; NOR, norfloxacin; RIF, rifampicin; STR, streptomycin; GEN, gentamicin; NIT, nitrofurantoin; CHL, chloramphenicol; TET, tetracycline.*
[c] *PF, Pampas fox (L. gymnocercus).*
[d] *GC, Geoffroy's cat (L. geoffroyi).*

wild and domestic canids and felids (31, 43–47). However, when we verified the distribution of enterococci in Pampas foxes and Geoffroy's cats, we observed a higher frequency of *E. faecalis* than those previously reported for wild red foxes, Iberian lynx, and Iberian wolves (44, 45). Moreover, our results are comparable to those of domestic canids and felids (31, 46, 47) since frequencies of *E. faecalis* (64.9%), *E. faecium* (18.2%), and *E. durans* (6.5%) were detected. This minor disagreement is supported by the fact that the distribution of enterococci may vary according to individual characteristics (e.g., species, age, and sex), habitat (e.g., seasonal variations and diet), and the geographic distribution of the animals (20).

Enterococcal species prevalence varied according to the host species studied. Although these species occupy the same area of the Biome, several types of foods are available to them. Geoffroy's cat and Pampas fox are considered generalist omnivores that opportunistically feed on a wide variety of foods. Pampas fox has a diet dominated by animal prey, mainly wild mammals, insects, while the Geoffroy cat feeds mainly on rodents and hares, and also remains of fish and frogs alongside reptiles and birds (48, 49). Thus, the distribution of *Enterococcus* species among hosts observed in the present study can be justified by the availability of the animals' food, since enterococcal species have been isolated from mammals, birds, fish, insects, and reptiles (20).

Notably, it was not possible to isolate enterococci from one of Geoffroy's cat fecal samples. Previously, Santestevan et al. (50) and Layton et al. (51) also sought to isolate enterococci from mammalian fecal samples and were unsuccessful.

Antimicrobial Susceptibility Profile

The results of this study are consistent with previous studies, which found high rates of resistance to erythromycin (65%), ciprofloxacin (59.5%), and tetracycline (36.5%) in fecal enterococci isolates from wild mammals, including wolves and foxes (31). Some reports have detected enterococci resistant to tetracycline and erythromycin in wild Iberian wolves, Iberian lynx, and red foxes in Portugal (43–45). Additionally, domestic canids and felids also harbored antimicrobial-resistant enterococci (47, 52, 53).

While MDR enterococci strains have previously been observed in enterococci isolated from wild mammals, their resistance levels were not as high as those detected here. In the present study, 66% of MDR was observed for wild canids and felids from the Brazilian Pampa biome. The high frequency of MDR strains may be associated with the proximity of these animals to human activities since they are sentinel species (i.e., indicators of danger to the environment). It is commonly known that wild canids and felids are indifferent to the presence of humans and often share the same environment. Our results are in line with those of Nowakiewicz et al. (54), who observed a high frequency of *E. faecalis* strains (44%) among wild mammalian carnivores in Poland. On the other hand, our data are six times higher than those detected by Dec et al. (30). According to Hu et al. (55), MDR bacteria are more commonly associated with environmental contamination than naturally occurring genes. Moreover, studies of wild foxes and carnivorous mammals revealed positive correlations with environmental pollution and the abundance of resistant bacteria in samples, thereby highlighting the selective pressures that

TABLE 5 | Distribution of erythromycin- and tetracycline-resistance genes in the enterococci isolated from wild Pampas Fox and Geoffroy's cat.

	Strains	Number (%) of strains positive for resistance genes						
		Erythromycin			Tetracycline			
		R*	*ermB*	*msrC*	R*	*tetM*	*tetL*	*tetS*
Pampa fox	*E. faecalis*	13	0	5 (38.46)	2	0	0	0
	E. faecium	7	0	3 (42.86)	4	0	0	0
	E. durans	2	1 (50)	1 (50)	1	1 (100)	1 (100)	0
	Subtotal	22	1 (4.55)	9 (40.91)	7	1 (14.29)	1 (14.29)	0
Geoffroy's cat	*E. faecalis*	12	1 (8.33)	0	1	1 (100)	1 (100)	0
	E. hirae	2	2 (100)	0	5	5 (100)	5 (100)	0
	Subtotal	14	3 (21.43)	0	6	6 (100)	6 (100)	0
	Total	36	4 (11.11)	9 (25)	13	7 (53.85)	7 (53.85)	0

**Resistant strains.*

TABLE 6 | Number (%) of virulence genes among enterococci isolated from wild Pampas Foxes and Geoffroy's cat.

Virulence genes	Pampas fox			Geoffroy's cat		Total (%)
	E. faecalis (*n* = 17)	*E. faecium* (*n* = 11)	*E. durans* (*n* = 2)	*E. faecalis* (*n* = 15)	*E. hirae* (*n* = 5)	
*gel*E	12 (70.59)	5 (45.45)	0	14 (93.33)	0	31 (62)
*cyl*A	0	0	0	0	1 (20)	1 (2)
esp	0	1 (9.09)	0	0	0	1 (2)
ace	12 (70.59)	7 (63.64)	0	5 (33.33)	0	24 (48)
agg	7 (41.18)	0	0	4 (26.67)	0	11 (22)

human activities and environmental disturbances exert on the microbial communities of wildlife (31, 54).

The elevated frequency of resistant and MDR enterococci observed in the fecal samples of wild Pampas foxes and Geoffroy's cats might be associated with anthropogenic activities. Agriculture and livestock are the main economic activities in the Brazilian Pampa and represents a source of food for billions of people and animals (mainly cattle and sheep). Since 1998, many drugs have been prohibited from being used as growth promoters in Brazil. In livestock, antimicrobials such as amoxicillin, erythromycin and tetracycline are used by veterinarians to treat bacterial infections (56). Despite bringing benefits to production, the use of antimicrobials in animals has fostered the emergence and spread of antimicrobial resistance. Antibiotics and/or antibiotic-resistant bacteria can be secreted with animal urine and feces and contaminate the environments (soils, surface waters, and ground waters) and species inhabiting these environments (57). In the presence of environmental concentrations of antibiotics, bacteria face a selective pressure leading to a gradual increase in the prevalence of resistance. The association of antibiotic resistance genes in mobile genetic elements is also an important factor for spreading and persistence of antimicrobial resistance in the environment (58). It is important to highlight that the impact created by the presence of antimicrobial agents in the environment and the frequency with which these resistance genes are transferred remains a subject of academic and practical debate. Our results suggest that the impacted environment occupied by Pampas foxes and Geoffroy's cats —with intense agricultural and livestock activities in the sampling area—possibly contributed to the selection of resistant bacteria in the environment and subsequent acquisition of resistant strains by these mammals. Despite anthropogenic activities, the presence of antibiotic-resistant strains in wild animals may also be associated with the environmental resistome, which is composed of genes that naturally occur in the environment (59). One example is the genes associated with the expression of efflux pumps, which protect cells against toxic molecules such as heavy metals, expelling them to the external environment and leading to antimicrobial resistance (60).

Frequency of Antibiotic Resistance Genes

The *erm*B and *msr*C genes, conferring resistance to macrolides, were present in 11.11 and 25% of isolates, respectively. The low frequency of *erm*B genes detected in the present study is congruent with the results obtained in previous studies conducted on *Enterococcus* strains isolated from wild animals (17, 18, 30, 50), as in regarding to *msr*C gene (28). Additionally, we detected the presence of the *msr*C gene not only in *E. faecium* but also in *E. durans* and *E. faecalis*. Although the *msr*C gene is considered an intrinsic gene to *E. faecium*, some studies have

noted the presence of this gene in other *Enterococcus* species such as *E. hirae* and *E. faecalis* (30, 38).

In the present study, *tet*L and *tet*M genes were detected in tetracycline-resistant enterococci strains. Previous findings of enterococci in wild animals such as Iberian wolves and Iberian lynx also harbored those genes in tetracycline-resistant strains (44, 45). Some erythromycin- and tetracycline-resistant strains did not amplify for the tested gene and may carry other antibiotic resistance genes such as *erm*A, C, D, E, F, G, Q, *msr*A/B, other *tet*-group genes, and the *poxt*A gene for tetracycline-resistance (61). Our results point to the notion that other reported genes could be associated with erythromycin-resistant enterococci isolated from Pampas foxes and Geoffroy's cats. Furthermore, whole-genome sequencing (WGS) of these enterococci might be useful in identifying additional mechanisms associated with resistance profiles.

Antibiotic resistance genes commonly reside on transmissible plasmids or on other mobile genetic elements, which allow the horizontal transfer of these genes between strains. The *tet*M, *tet*L, and *erm*B genes are carried out by mobile genetic elements, such as transposons (Tn*916*, Tn*1545*, and Tn*917*), conjugative transposons or plasmids (58). The association of these genes in mobile genetic elements might be an important factor for spreading of antimicrobial resistant enterococci in wild Pampas foxes and Geoffroy's cats.

Frequency of Virulence-Related Genes

The results of the present study suggest that enterococci obtained from wild Pampas foxes and Geoffroy's cats harbored virulence genes. Moreover, *E. faecalis* was the most common species to carry virulence factors. These results are congruent with previous studies highlighting *E. faecalis* as the most common enterococcal species associated with infections, which accounts for 80–90% of infections. The presence of virulence factors in clinical enterococci strains is associated with persistent and difficult-to-treat infections. However, some authors consider the occurrence of these genes in non-clinical strains as a common characteristic that increases their ability to colonize hosts, which improves the survival and proliferation of the strains. Since the ubiquity of enterococci across a wide range of environments was initiated by the establishment of these bacteria in either abiotic surfaces or live tissues, their colonization can be facilitated by the expression of virulence genes that likely contribute to the persistence of enterococci in the environment (20).

One limitation of our study is the low number of animals sampled, which is due to the difficulty of obtaining samples from wildlife. For example, a study conducted in an anthropogenic area of the Brazilian Pampa during a 1 year period, 12 Geoffroy's cat individuals were captured (62). Notably, capturing and handling wild animals requires specialized equipment, the consideration of animal welfare concerns (regardless of the reason for capture), and the efforts of experienced biologists and wildlife technicians to plan and study suitable capture methods. In light of these points, the number of animals evaluated in the present study should be well-considered. Despite its relatively small sample size, this study demonstrated the importance of conducting research related to the impact of human activities on the Brazilian Pampa biome.

In conclusion, this study observed the presence of resistant *Enterococcus* strains in wild Pampas foxes and Geoffroy's cats from the Brazilian Pampa biome. The presence of MDR enterococci in fecal samples from these wild animals suggests that habitat fragmentation and the impact of anthropogenic activities on the environment might contribute to the occurrence of resistant strains in the microbial gut communities of these animals. Furthermore, these animals may contribute to the spread of resistant strains between different ecosystems. To the best of our knowledge, this is the first study of resistant commensal enterococci recovered from wild animals in the Brazilian Pampa biome. We believe that our research will serve as a foundation for future studies on the Pampa biome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), and Chico Mendes Institute for Biodiversity Conservation (ICMBio). The protocol was approved by Information Authorization System in Biodiversity (SISBIO) no. 0200 1.007 9 10 12006-32.

AUTHOR CONTRIBUTIONS

GO, JF, and AG designed the study. FP and MF carried out the sampling work. GO, RH, MM, JF, and AG 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/fvets.2020.606377/full#supplementary-material

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Occurrence and Diversity of CTX-M-Producing *Escherichia coli* From the Seine River

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CTX-M-producing *Escherichia coli* are spreading since 1999 both in clinical and in community settings. Environmental samples such as rivers have also been pointed out as being vectors for ESBL producers. In this report, we have investigated the presence and the diversity of CTX-M-producing *E. coli* isolates in two samplings of the Seine River (next to Notre Dame), Paris France, performed in June 2016 and 2017. The total number of bacteria growing on the selective ChromID ESBL agar was 3.1×10^5 cfu/L (23.8% of all growing bacteria) in 2016, whereas it was 100-fold lower in 2017 (3×10^3 cfu/L; 8.3% of all growing bacteria). However, among them, the prevalence of ESBL-producing *E. coli* increased from <0.1 to 1.1% in one-year. ESBLs were exclusively of the CTX-M-type: CTX-M-1 (n = 5), CTX-M-15 (n = 7), CTX-M-14 (n = 1), and CTX-M-27 (n = 2). The isolates belonged to several multi locus sequence types, and a wide diversity of incompatibility groups of plasmids were identified in those *E. coli* isolates. The occurrence and diversity of *E. coli* isolates belonging to many clones and producing many CTX-M-variants have been identified in our study. The presence of these bacteria in rivers that are open again for recreational usage (swimming) is worrying as it may contribute to further dissemination of ESBL producers in the community.

Keywords: CTX-M-14, CTX-M-15, *Escherichia coli*, Seine river, plasmids

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INTRODUCTION

Escherichia coli is an ubiquitous human pathogen, most commonly involved in urinary tract infections and bacteremia in humans and animals (Rogers et al., 2011). Plasmid-mediated extended-spectrum β-lactamases (ESBLs) have become predominant in community-onset *E. coli* infection (Pitout et al., 2005). The first human CTX-M variant (previously named MEN-1) was initially reported in 1991 from a clinical *E. coli* isolate from France (Bernard et al., 1992). Since, CTX-M-producing *E. coli* have increasingly spread both in hospitals and in the community (Cantón et al., 2012) and represent now the most prevalent ESBLs worldwide. They are divided into five groups based on amino acid sequence: the CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 groups (Cantón et al., 2012).

The CTX-M-15 variant from the CTX-M-1 group, was first described in 2001 from several enterobacterial isolates from India on large-sized plasmids along with an IS*Ecp1* insertion sequence upstream of the $bla_{CTX-M-15}$ gene (Karim et al., 2001). Since 2008 (Coque et al., 2008), it has rapidly become the most prevalent ESBL worldwide in humans, especially linked to an *E. coli*

group B2, serogroup O25b, sequence type 131 (ST131). A collection from eight European countries demonstrated also the presence of ST131, including 6% of ESBL-producing *E. coli* isolates recovered from companion animals (Ewers et al., 2010). The water environment is conducive to the transfer of resistance genes between species, where ESBL producers from various sources get in contact with a broad range of potential recipients. A previous study, conducted in 2011, reported occurrence and diversity of ESBL-producing *Aeromonas* spp. in the Seine river (Girlich et al., 2011). However, at this date, no ESBL-producing *E. coli* isolates were identified in those samples, and the presence of CTX-M *E. coli* producers in rivers was still an exceptional event as shown by Kim et al. (2008) in Korea in 2008 or by Dhanji et al. (2011) in the United Kingdom in 2011. More recently, the emergence of ESBL-producing *E. coli* occurred in urban rivers. In Austria, CTX-M-producing *E. coli* were identified in the River Mur in the center of Graz, Austria's second largest city (Zarfel et al., 2017). In Guadeloupe, the predominance of CTX-M-producing *E. coli* has been reported from waste water treatment plant effluents (Guyomard-Rabenirina et al., 2017). In contrast to what was reported from European countries, the occurrence of CTX-M-*E. coli* producers was high in the Pearl River in China (Ye et al., 2017).

The aim of the present study was to investigate the presence of expanded-spectrum cephalosporin (ESC)-resistant *E. coli* isolates in the water of the Seine River, Paris, France, sampled in June of two consecutive years (2016 and 2017) at the same centrally located sampling spot (next to the Notre Dame). We report here, the isolation of CTX-M-type ESBL-producing *E. coli* isolates and the in-depth genomic characterization of 15 of them.

MATERIALS AND METHODS

Water Sampling, ESBL Detection, and Plasmids

Sampling of the Seine River water, Paris, France, was performed in June of two consecutive years (2016 and 2017) at the same centrally located sampling spot (next to the Notre Dame). Samples were collected *c.a.* 1 m from the shore and *c.a.* 20 cm below the water surface using a 1-L sterile plastic bottle connected to a rope. The bottle was immediately closed, transferred on ice to the bacteriology laboratory of the Bicêtre Hospital, Le Kremlin-Bicêtre, France, and directly processed upon arrival. Four hundred milliliters of water was filtrated through a nitrocellulose membrane (0.45 μm, Millipore), and the bacteria were resuspended from the membranes in 2 ml of sterile water. Aliquots (100 μl) were then plated on ChromID ESBL plates (bioMérieux, Marcy l'Etoile, France). Pink-colored colonies growing ChromID ESBL were identified by mass spectrometry (MALDI-TOF, Bruker, France), and the ESBL phenotype was evidenced by a double disk synergy test (Karim et al., 2001). Plasmids, extracted by the Kieser method were electroporated into *E. coli* Top10, as previously described (Girlich et al., 2011). In case electroporation did not work, mating out assay was performed as previously described (Girlich et al., 2011). Transformants or transconjugants were selected on cefotaxime (0.5 μg/ml) agar. Identification of replicon types of the plasmid incompatibility (Inc) groups was performed by PCR as previously described by Carattoli et al. (Carattoli, 2009). Using this typing scheme, 18 Inc groups may be identified: Hl1, Hl2, I1-Iγ, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIAs, F, K, and B/O.

Rapid Identification of ESBLs

NG-Test CTXM-Multi, a rapid Lateral Flow Immuno Assay (LFIA, NG-Biotech, Guipry, France) was used to detect all five CTX-M-groups, as previously described (Bernabeu et al., 2020). Briefly, one colony was resuspended in the extraction buffer, vortexed, and 100 μl was dropped on the LFIA strip. Results were eye read after 15 min of migration.

Genetic Analyses

Whole genome sequencing was performed on 15 selected ESBL-producing *E. coli* isolates using Illumina technology on a Nextseq 500 sequencer as previously described (Dabos et al., 2019). *De novo* assembly was performed by CLC Genomics Workbench v7.0.4 (Qiagen, Les Ulis, France) after quality trimming (Qs $\geq$ 20). The acquired antimicrobial resistance genes were identified using ResFinder (Bortolaia et al., 2020), incompatibility groups of plasmids were determined using Plasmid finder (Clausen et al., 2018), and the sequence type was obtained using the Multi Locus Sequence Typing (MLST) modules of the Center for Genomic Epidemiology with genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*[1] (Larsen et al., 2012).

RESULTS

Bacterial Counts and ESBLs

Total bacterial count on Mueller Hinton agar was 1.3×10^6 cfu/L of Seine water samples in 2016, whereas it was 3.5×10^4 cfu/L in 2017. Bacterial count growing on ChromID ESBL agar was 3.1×10^5 cfu/L (23.8% of all growing bacteria) in 2016, whereas it was 100-fold lower in 2017 (3×10^3 cfu/L; 8.3% of all growing bacteria). ESBL-producing *E. coli* isolates recovered were 11 per 100 ml and 4 per 500 ml of water, in 2016 and 2017, respectively. Among the total bacteria growing on ChromID ESBL agar, <0.1% (2.7×10^2 cfu/L) were ESBL-producing *E. coli* in the samples from June 2016, whereas 1.1% (33 cfu/L) ESBL-producing *E. coli* isolates were identified in May 2017. ESBLs produced by the *E. coli* isolates were exclusively CTX-M enzymes (**Table 1**). NG-Test CTX-M-gr1, a LFIA specific for group 1 CTX-M-β-lactamases gave positive results for 12/15 ESBL-producing *E. coli* isolates.

Resistome Analyses

WGS identified $bla_{CTX-M-1}$ (n = 5), $bla_{CTX-M-15}$ (n = 7), $bla_{CTX-M-14}$ (n = 1), $bla_{CTX-M-27}$ (n = 2), bla_{TEM-52} (n = 1) ESBL genes, bla_{DHA-1} (n = 1) cephalosporinase gene, and bla_{TEM-1} (n = 3) and bla_{OXA-1} (n = 2) penicillinase genes (**Table 1**). The results of the WGS were in accordance with those of the NG-Test CTX-M- MULTI LFIA, validating this

[1]http://genomicepidemiology.org/

TABLE 1 | Genetic characteristic of ESBL-producing *E. coli* isolates from the Seine river from 2016 and 2017.

Isolate	Acquired resistance determinants	Serogroup	ST	Inc groups	Inc group of pCTX-M[a]
S46[b]	**$bla_{CTX-M-15}$**, **bla_{OXA-1}**, **bla_{DHA-1}**, *aac(6′)Ib-cr*, *qnrB4*, *aadA5*, *catB3*, *mph(A)*, *sul1*, *tetB*, and *dfrA17*.	O101, H10-like	**617**	IncFIA + IncFIB + IncFII + Col 156 + ColMG + ColpVC	IncFIA + IncFIB
S47	**$bla_{CTX-M-15}$**, **bla_{OXA-1}**, *strA*, *aac(6′)Ib-cr*, *aac*(3)-*Iia*, *strB*, *aadA5*, *catB3*, *mph(A)*, *sul1*, *sul2*, *tetA*, and *dfrA17*.	O102-like, H6-like	**405**	IncFIA + IncFIB + IncFII + Col BS	IncFIA + IncFIB
S55	**$bla_{CTX-M-15}$**, *aadA5*, *mphA*, *sul1*, *tetA*, and *dfrA17*.	O?[c], H9-like	**410**	IncFIA + IncFIB + IncFII + Col 156	IncFIA + IncFIB
S56	**$bla_{CTX-M-1}$**, **bla_{TEM-52}**, *aadA17*, *aadA5*, *qnrS1*, *lnu(F)*, *sul2*, *dfrA1*, and *dfrA14*.	O8-like, H19-like	**162**	IncFIA + IncFIB + IncI1 + IncN + IncX1 + p0111	IncI1
S57	**$bla_{CTX-M-1}$**	O?, H1	**104**	IncI1 + IncX4 + Inc X1	IncI1
S58	**$bla_{CTX-M-14}$**	O25-like, H4	**131**	IncI1 + Col156 + IncFIA + IncFIB	ND[d]
S59	**$bla_{CTX-M-1}$**, *strB*, *aph(3′)-Ia*, *strA*, *sul2*, and *tetA*.	O80, H45-like	**4175**	IncFII + IncY + IncFIA + IncFIB + IncQ	ND
S61	**$bla_{CTX-M-1}$**, *aadA17*, *lnu(F)*, *sul1*, *sul2*, *tetA*, and *dfrA1*.	O9-like, H19-like	**162**	IncFIB + IncFIC	IncFIC
S65	**$bla_{CTX-M-27}$**	O25-like, H4	**131**	IncI1 + Col156 + IncFIA + IncFIB + IncFII	IncFIA + IncFIB
S66	**$bla_{CTX-M-15}$**, **bla_{TEM-1}**, *aac*(3)-*IId*, *aadA5*, *qnrS1*, *mphA*, *sul1*, *tetA*, and *dfrA17*.	O?, H8-like	**13**	IncB/OKZ + Col156 + IncFII	IncOKZ
S67	**$bla_{CTX-M-15}$**	O25-like, H4	**131**	IncFIA + IncFIB + IncFII	IncFIA + IncFIB
S17-1	**$bla_{CTX-M-15}$**, **bla_{TEM-1}**, *strA*, *aac*(3)-*IId*, *aadA5*, *strB*, *mphA*, *sul1*, *sul2*, *tetA*, and *dfrA17*.	O16, H5-like	**131**	IncFII + Col156 + IncFIB	ND
S17-2	**$bla_{CTX-M-27}$**	025-like, H4	**131**	IncFIA + IncFIB + IncFII + Col 156 + ColMG + ColpVC	IncFIA + IncFIB
S17-3	**$bla_{CTX-M-1}$**, **bla_{TEM-1}**, *aadB*, *aadA5*, *aadA1*, *floR*, *sul1*, *sul2*, and *dfrA17*.	O9-like, H25	**58**	IncI1 + IncFIB + IncFIC + IncFII	ND
S17-4	**$bla_{CTX-M-15}$**, *aadA5*, *qnrS1*, *mphA*, *sul1*, and *dfrA17*.	O6, H16-like	**4**	IncFII + IncFIB	ND

[a]*Acquired resistance determinants, in bold are β-lactamase genes.*
[b]*Samples numbered as "S#" are samples collected in 2016; samples numbered as "S17-#" are samples collected in 2017.*
[c]*O?, is a non-typable O serogroup by using NGS tools.*
[d]*ND, not determined. PCR amplification with previously reported primers of the most currently described Inc families remained negative (Carattoli, 2009).*

latest test for the rapid detection of the five groups of CTX-M-producing Enterobacterales, as previously reported (Bernabeu et al., 2020). As observed in other studies, group 1 CTX-M-producing *E. coli* isolates were dominant in our study (Zarfel et al., 2017; Hooban et al., 2020).

As commonly found in CTX-M-producers, most isolates were multidrug resistant, possessing aminoglycoside-modifying enzyme [e.g., *aac(6′)-Ib*, *aadA1*, *aadA5*, or *aph(3′)-Ia*], quinolone-resistance genes (*qnrS1*), tetracycline (*tetA*, *tetB*), chloramphenicol (*catB3*), and trimethoprim/sulfamethoxazole (*dfrA14*, *dfrA17*, *sul1*, and *sul2*). Those resistance gene are, for most of them, carried by a class 1 integron (**Table 1**; Cantón et al., 2012).

Clonal Relationship

Clonal relationship of these isolates was initially assessed by MLST and then by WGS SNP analysis. MLST analysis revealed a wide diversity of clonal groups with 10 different STs among the 15 isolates. Noticeably, only two STs were represented with at least two isolates being ST162 (n = 2) and ST131 (n = 5) (**Figure 1**). WGS-based phylogeny confirmed this diversity but also indicated that the five ST131 isolates can be divided into two subclones (**Figure 1**). It can be noticed that ESBL distribution did not follow the clonal relationship. For instance, in ST131 isolates, three types of ESBLs were identified: $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, and $bla_{CTX-M-27}$ genes. The wide diversity of clones may reflect the large spread of ESBLs in the community. Indeed, we did not identify a clonal spread of ESBL-producing *E. coli* but rather unrelated isolates that are present in the Seine River. Among the five ST131 *E. coli* isolates, four were genetically close (**Figure 1**). However, these isolates did not share the same resistome indicating that ST131 is widely distributed independent of the ESBL content as previously observed (Pitout and Finn, 2020). Moreover, the two closest ST131 (S65 and S17-2) possessing the $bla_{CTX-M-27}$ ESBL gene were recovered one-year apart indicating the persistence or continuing contamination by this clone.

Plasmid Analysis

The bla_{CTX-M} genes were located on large plasmids of different sizes (**Figure 2**) belonging to diverse incompatibility groups (**Table 1**). Several studies have shown that plasmids of the IncF family were the predominant group that carry the $bla_{CTX-M-15}$ gene, whereas the $bla_{CTX-M-14}$ gene is carried on a variety of plasmid types, including on IncF, especially in the Far-East, and on IncK, in Western Europe (Bevan et al., 2017). Horizontal transfer of antimicrobial resistance plasmids by conjugation in Enterobacterales occurs in the human gut, animals, and the environment (Bevan et al., 2017; Zarfel et al., 2017). As previously reported, the main ESBL types identified in companion animals were CTX-M-14 (26.8%), CTX-M-15 (24.4%), CTX-M-27 (19.5%), and CTX-M-55 (19.5%) (Kawamura et al., 2017), and the most prevalent STs were ST131 (n = 15, 35.7%), followed by ST38, ST10, and ST410 (Kawamura et al., 2017). For example, among those STs, ST10/CC10 corresponds to an international cluster already identified in humans, wildlife infections, domestic farm animals, companion animals, and commercial chicken meat (Nascimento et al., 2017).

DISCUSSION

We identified in this study the occurrence of different ESBL-producing *E. coli* isolates from the Seine River in Paris, France. A wide diversity of clones was identified here. The most prevalent, with 4 isolates out of 15, was ST131. This result is not surprising given its widespread occurrence, but the presence of different CTX-M-variants belonging to different groups of enzymes was unexpected, as ST131 is frequently associated to CTX-M-15. We also identified one ST410 isolate. This clone has recently attracted not only the light by its association with the spread of the carbapenemase OXA-181 (Patiño-Navarrete et al., 2020) but also for its isolation in animals (Yang et al., 2019). Of note, two isolates of ST162 were recovered in this study. This clone has been reported to be associated with the resistance gene in wild avian isolates (Oteo et al., 2018). However, it remains difficult to conclude on the original source of these isolates, which could be of avian/environmental or human sources. Hooban et al. (2020) reported that most of the ESBL producers identified in aquatic environments around the world between 2010 and 2017 expressed bla_{CTX-M} genes (n = 21 among 29 studies), followed by bla_{TEM} (n = 18), and bla_{SHV} (n = 11). Surprisingly, among eight Chinese studies, only three identified CTX-M-producing *E. coli*

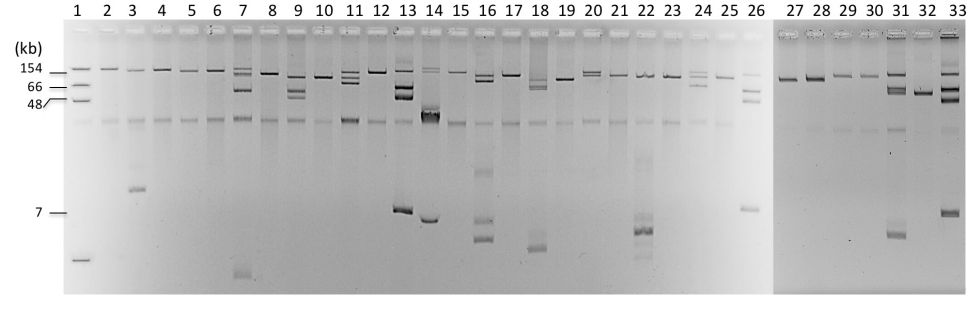

FIGURE 1 | Phylogenetic tree of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* from Seine River. The phylogeny was performed using CSIPhylogeny (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). Year of isolation is indicated by colored circles and broad-spectrum β-lactamases by colored pentagons.

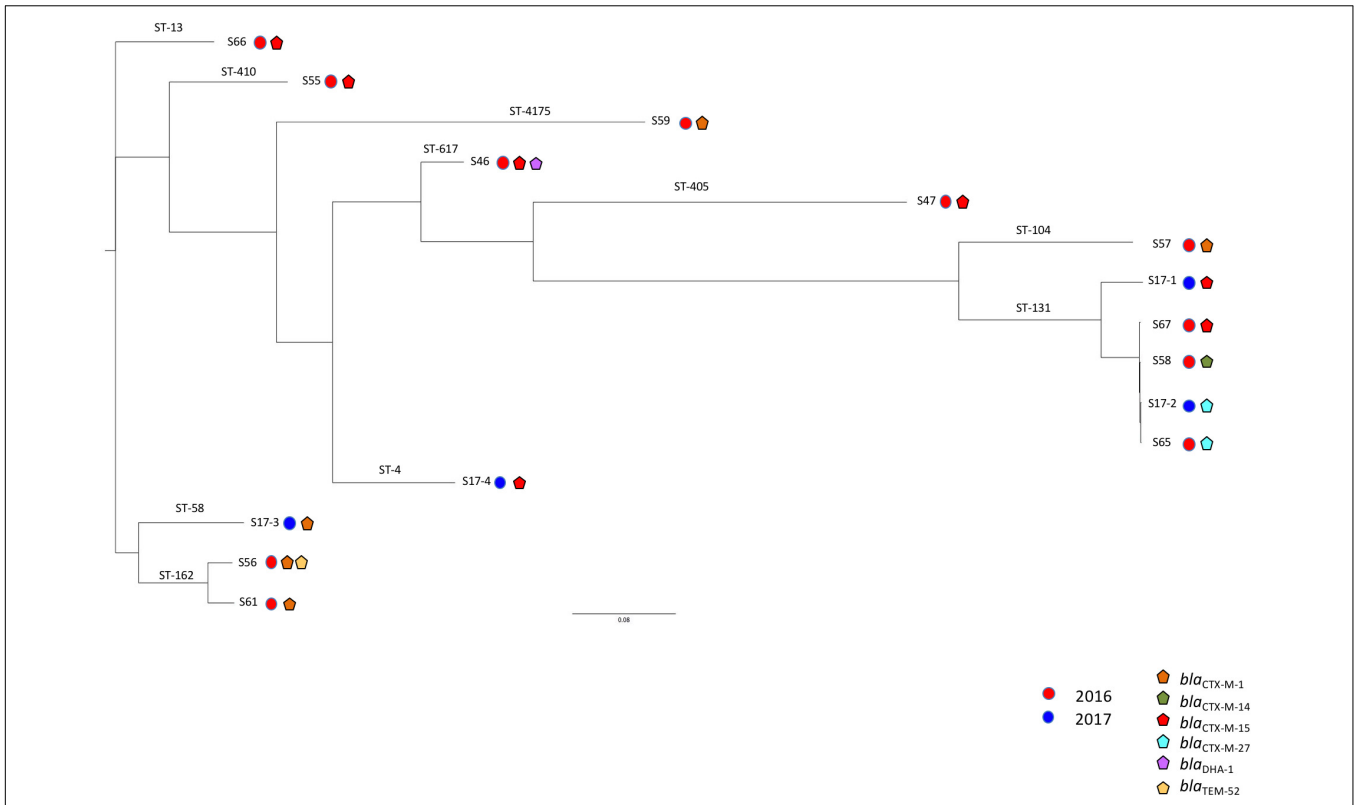

FIGURE 2 | Plasmid extractions from cultures of the different isolates and their transconjugants or transformants. Lanes 1, 3, 5, 7, 9, 11, 14, 16, 18, 20, 22, and 24 correspond to S46, S47, S55, S56, S57, S59, S61, S65, S66, 17.1, S17.2, S17.3, S58, S59, and S17.4; Lanes 2, 4, 6, 8, 10, 12, 15, 17, 19, 21, 23, 25, 28, 30, and 32 correspond to their respective *E. coli* transformants or transconjugants; Lanes 13, 26, and 33 correspond to *E. coli* 50192 harboring four plasmids: 7, 48, 66, and 154 kb.

isolates in rivers and lakes (Hooban et al., 2020). The prevalence of ESBL producers among waterborne thermo-tolerant coliforms ranged in amount from 11% (Ye et al., 2017) to 17% in Chinese rivers (Chen et al., 2010). Ye et al. (2017) identified only CTX-M-variants as ESBLs, with additional variants: i.e., CTX-M-55 and CTX-M-65 in addition to CTX-M-14 and CTX-M-15. Notably, a previous study, 6 years earlier, reported TEM (37.6%) and SHV (84.1%) as being the most common ESBL among clinical isolates from the same city of Chongqing in 2004 (Chen et al., 2010). In Brazil, four studies reported the presence of CTX-M but also of KPC-2 carbapenemase *K. pneumoniae* producers in rivers, lakes, and sea water (Hooban et al., 2020). The presence of bla_{CTX-M} genes in water is more and more frequent worldwide, most often associated with highly self-transferable plasmids. In all cases, it is likely that the transfer of these bacteria from the sewage to the rivers occurred. Most worrying, concomitant spread of carbapenemase genes has been witnessed in many countries including Switzerland, Spain, Portugal, Austria, United States, Brazil, India, and China (Hooban et al., 2020).

CONCLUSION

The epidemic dissemination of CTX-M-encoding genes is largely due to their localizations on mobile genetic elements, such as plasmids, transposons, and integrons, which allow these genes to easily spread among bacterial communities (Cantón et al., 2012). In 2016, the samples were collected a few days after floods that occurred between the end of May and the beginning of June 2016, thus explaining a high prevalence of ESBL-*E. coli* isolates in the Seine river that likely originated from animal feces that have been drained by the rains. However, the presence and diversity of those isolates one-year later is more worrying, as it indicates a persistent contamination of the Seine river with ESBLs-producing *E. coli* isolates. This is especially worrying given that the many rivers all over Europe open again for different recreational and sporting activities, including swimming.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI BioProject, NCBI Accession No. PRJNA662045.

AUTHOR CONTRIBUTIONS

TN: conception, data analysis, writing, and proof-reading. RB: data analysis and proof-reading. DG: experimental work, data

analysis, writing, and proof-reading. All authors contributed to the article and approved the submitted version.

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A Novel Mobile Element ICE*Rsp*D18B in *Rheinheimera* sp. D18 Contributes to Antibiotic and Arsenic Resistance

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Antibiotics and organoarsenical compounds are frequently used as feed additives in many countries. However, these compounds can cause serious antibiotic and arsenic (As) pollution in the environment, and the spread of antibiotic and As resistance genes from the environment. In this report, we characterized the 28.5 kb genomic island (GI), named as ICE*Rsp*D18B, as a novel chromosomal integrative and conjugative element (ICE) in multidrug-resistant *Rheinheimera* sp. D18. Notably, ICE*Rsp*D18B contains six antibiotic resistance genes (ARGs) and an arsenic tolerance operon, as well as genes encoding conjugative transfer proteins of a type IV secretion system, relaxase, site-specific integrase, and DNA replication or partitioning proteins. The transconjugant strain 25D18-B4 was generated using *Escherichia coli* 25DN as the recipient strain. ICE*Rsp*D18B was inserted into 3'-end of the *guaA* gene in 25D18-B4. In addition, 25D18-B4 had markedly higher minimum inhibitory concentrations for arsenic compounds and antibiotics when compared to the parental *E. coli* strain. These findings demonstrated that the integrative and conjugative element ICE*Rsp*D18B could mediate both antibiotic and arsenic resistance in *Rheinheimera* sp. D18 and the transconjugant 25D18-B4.

Keywords: antibiotic resistance, arsenic resistance, ICE*Rsp*D18B, integrative and conjugative element, *Rheinheimera*

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INTRODUCTION

In aquaculture systems, the indiscriminate use of chemical additives and antimicrobials (especially antibiotics) as preventative and curative measures for diseases has resulted in antimicrobial resistance among bacteria (Buschmann et al., 2012; Sun et al., 2016; Nakayama et al., 2017; Rico et al., 2017). Additionally, the transfer of antibiotic resistance elements from aquaculture facilities into the environment could have negative impacts on environmental biodiversity and human health as a result of further antimicrobial resistance development (Garcia-Aljaro et al., 2014; Xu et al., 2017). In addition to antibiotics, the metalloid arsenic (As) has been used as a feed additive, although it was ranked first on the priority list of

hazardous substances by the Agency for Toxic Substances and Disease Registry[1]; arsenic has a significant impact on the aquaculture environment because of its toxic, persistent, and accumulative properties in organisms, which have devastating effects on the diversity of aquatic animals and on the ecological balance of aquaculture systems (Miazek et al., 2015; Rahman and De Ley, 2017). Arsenic resistance genes, usually organized in *ars* operons, have been widely identified in bacteria (Fekih et al., 2018; Serrato-Gamino et al., 2018). Therefore, the aquaculture environment poses a potential risk for the dissemination of arsenic resistance genes as well as antibiotic resistance genes (ARGs) through mobile genetic elements (Abdelhamed et al., 2019).

Bacteria of the genus *Rheinheimera* are frequently isolated from freshwater and estuaries (Baek and Jeon, 2015; Chen et al., 2019); and saline and slightly alkaline lakes (Liu et al., 2012; Zhong et al., 2014). Currently, the genus comprises 27 species.[2] Comparative genomics analysis of *Rheinheimera* genomes revealed that the core genome is relatively small (Presta et al., 2017), which may be related to the different ecological niches colonized by members of this genus (Wang et al., 2018; Panda et al., 2020). It has been reported that many *Rheinheimera* strains are multidrug-resistant (Liu et al., 2012; Mengoni et al., 2014; Suarez et al., 2014; Kumar et al., 2015), and a series of ARGs in the genomes of *Rheinheimera* spp. have been uncovered, such as *acrD* in *Rheinheimera* sp. EpRS3, encoding an aminoglycoside efflux pump; *acrB* in *Rheinheimera* sp. KL1, encoding a multidrug resistance-nodulation-division efflux pump; and *tet*(B) in *Rheinheimera* sp. D18, encoding a tetracycline efflux major facilitator superfamily (MFS) transporter (O'Connor et al., 2015; Presta et al., 2017; Fu et al., 2020). In addition, bioinformatics analyses have predicted the widespread presence of arsenical resistance genes in *Rheinheimera*. However, the transferability of ARGs and arsenic resistance genes in *Rheinheimera* has not been well characterized.

Rheinheimera sp. D18 strain was previously isolated from mariculture environment in the Yellow Sea, which has been reported to be polluted by notable amounts of antibiotic residues (Du et al., 2017; Han et al., 2020) and arsenic (Jiang et al., 2015; Xiao et al., 2017), and D18 was found to have high-level resistance to tetracycline, florfenicol, amikacin, and sulfamethoxazole (Fu et al., 2020). In this study, the novel integrative and conjugative element ICE*Rsp*D18B was characterized in *Rheinheimera* sp. D18 genome. In addition to genes related to DNA replication/partitioning and conjugative transfer, ICE*Rsp*D18B was found to contain three repeated copies of a chloramphenicol/florfenicol efflux MFS transporter-encoding gene (*floR*), and several other ARGs. An arsenic tolerance operon was also identified in ICE*Rsp*D18B, indicating that ICE*Rsp*D18B mediates combined resistance to antibiotics and arsenic, and further analysis indicated that ICE*Rsp*D18B was transferable. This report characterized the first mobile genomic island (GI) ICE*Rsp*D18B that endows both antibiotic and arsenic resistance in the genus *Rheinheimera*, providing new insights into antibiotic and arsenic spread in the mariculture environment.

MATERIALS AND METHODS

Strains and Culture Conditions

Rheinheimera sp. D18 strain was previously isolated from maricultural environment (Fu et al., 2020). *Rheinheimera* sp. D18 was cultured in LB solid medium (tryptone 1%, yeast extract 0.5%, 1% sodium chloride, and agar 2%) at 28°C and was used as a donor in conjugation experiments. *Escherichia coli* strain 25DN was cultured at 37°C in LB medium and was used as recipient in conjugation experiments. Transconjugants from conjugation experiments were cultured on LB medium containing florfenicol (24 mg/l) and roxarsone (8 mM) at 37°C.

Identification of the Genomic Island

The *Rheinheimera* sp. D18 whole genome sequence has been deposited in GenBank (CP037745). The GIs were identified using Island Viewer 4 (Bertelli et al., 2017) and were further analyzed using ICEfinder (Liu et al., 2019). The genes in genomic island were annotated using the Prokaryotic Genome Annotation Pipeline on NCBI[3] and RASTtk server (Overbeek et al., 2014; Brettin et al., 2015). Insertion sequence transposases were detected using IS-Finder (Siguier et al., 2012).

Comparative Analysis of ICE*Rsp*D18B With Other Genetic Elements

Pairwise alignment of ICE*Rsp*D18B and other relevant genetic elements was performed using the BLAST search tool and ICEberg WU-BLAST search tool (Liu et al., 2019). Further alignment between two sequences was performed using BioXM 2.6 software.

Conjugation Experiments

To determine whether the antibiotic and arsenic resistance genes in ICE*Rsp*D18B could be horizontal transferred among bacteria, conjugation experiments were carried out as previously described with some modification (Fu et al., 2020). Transconjugants were selected on LB agar plates with florfenicol (24 mg/l), roxarsone (8 mM), X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid), and sodium azide. The donor (*Rheinheimera* sp. D18) and the recipient (*E. coli* 25DN) strains are inhibited and only the transconjugants would survive on the selective agar plates. ICE*Rsp*D18B and its insertion site in the transconjugant were demonstrated by PCR and direct DNA sequencing. The ability of ICE*Rsp*D18B to form a ring in *Rheinheimera* sp. D18 was also verified by PCR and DNA sequencing. All the primers used in this report are listed in **Supplementary Table S1**.

[1]https://www.atsdr.cdc.gov/spl/index.html
[2]http://www.bacterio.net/rheinheimera.html
[3]http://www.ncbi.nlm.nih.gov/genome/annotation_prok/

Metalloid Arsenic and Antibiotic Susceptibility Testing

The broth microdilution method was used (CLSI, 2017) to determine the MICs for roxarsone, sodium hexafluoroarsenate and different antibiotics, including amikacin, florfenicol, and sulfamethoxazole. *Escherichia coli* 25DN strain was also tested for MICs.

Data Analysis

All the experiments in this study were carried out in triplicate. The differences in MICs for the transconjugant strain and *E. coli* 25DN strain were analyzed using the Student's *t*-test ($p < 0.05$).

RESULTS

Structure of ICE*Rsp*D18B in the *Rheinheimera* sp. D18 Strain

A chromosomal GI in *Rheinheimera* sp. D18 was identified using Island Viewer 4 (**Figure 1**), while it was not predicted as a typical integrative and conjugative element (ICE) by ICEfinder software. This GI extends from position 2,629,186 to 2,657,721 in the chromosome of D18 and contains 28,536 bp. Gene annotation indicated that it contains 33 open reading frames (ORFs; **Supplementary Table S2**), among which six ORFs were predicted to be ARGs, including one sulfonamide resistance gene (*sul2*), two aminoglycoside resistance genes (*aph(3")-Ib* and *strB*), and three repeated copies of a chloramphenicol/florfenicol resistance gene (*floR*); and four ORFs were predicted to be arsenic resistance genes, forming the operon *arsRHCB*. The GI also contains three identical copies of a relaxase-encoding gene (E0Z06_RS12465, E0Z06_RS12485, and E0Z06_RS12505) related to a type IV secretion system; three conjugative transfer protein-encoding genes (*trbL*, *trbK*, and *trbJ*); four genes associated with DNA replication or partitioning (*repC*, *repA*, E0Z06_RS12575, and E0Z06_RS12520); and genes encoding a site-specific integrase (*int*) and its transcriptional regulator (E0Z06_RS12590). Sequence examination further indicated that the GI was bordered by a 20-bp direct repeat (DR; 5'-ACAATNGAGTGGGAATNNTT-3') at both ends and that it was inserted into the *guaA* gene (E0Z06_RS12600) in the chromosome of D18. These findings suggest that this GI might be an ICE-like genomic island, named as ICE*Rsp*D18B, and provide antibiotic and arsenic tolerance to *Rheinheimera* sp. D18, as we know, ICEs are now recognized as a large and diverse class of chromosomal mobile genetic elements in bacteria that can transfer between bacteria through conjugation (Baranowski et al., 2018; Partridge et al., 2018).

Pairwise Alignment of ICE*Rsp*D18B With Relevant DNA Sequences

The whole ICE*Rsp*D18B nucleotide sequence was analyzed using BLAST, and results revealed that this ICE*Rsp*D18B presents only in the *Rheinheimera* sp. D18 genome. GC content of ICE*Rsp*D18B is 58.28%, different from that of the overall GC content of *Rheinheimera* sp. D18 genome (44.39%), indicating that this genomic island ICE*Rsp*D18B was derived from other bacteria. Pairwise alignment of ICE*Rsp*D18B with other relevant DNA sequences was performed, and the sequence alignment results are shown in **Figure 2**. BLASTn analysis indicated that genes relating to conjugative transfer and DNA replication or partitioning (from E0Z06_RS12595 to E0Z06_RS12545) in ICE*Rsp*D18B were highly similar to genes in the *Klebsiella pneumoniae* NCTC9180 genome (GenBank accession number LR134202.1), and these genes were also predicted to be present in the *K. pneumoniae* NCTC9171 genome (GenBank accession number LR588410.1). A larger region that included the above genes and the arsenic operon (*arsRHCB*; from E0Z06_RS12595 to E0Z06_RS12510) in ICE*Rsp*D18B showed 99% identity with a genomic region of *K. pneumoniae* NCTC9171. In addition, the ICE*Rsp*D18B arsenic operon (*arsRHCB*) had 100% nucleotide sequence identity to the arsenic operon located in *Salmonella enterica* strain 20–56 plasmid 1 (GenBank accession number LR536427.1). Of particular note, there were three tandem repeats of a set of genes that includes IS*91*, *floR*, a relaxase-encoding gene, and a LysR family transcriptional regulator-encoding gene in ICE*Rsp*D18B, one or two set of these genes were also

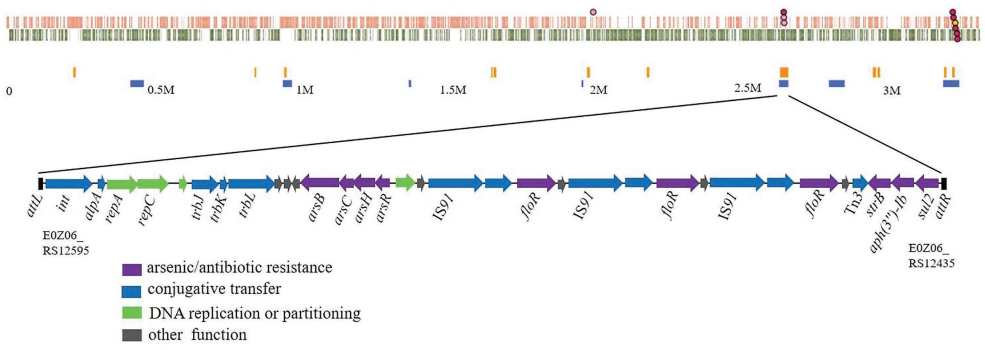

FIGURE 1 | Schematic view of a new identified genomic island (GI) and its position in *Rheinheimera* sp. D18. Top image, GIs predicted by Island Viewer 4 in *Rheinheimera* sp. D18. Putative genomic islands were predicted by IslandPath-DIMOB method (blue squares) or SIGI-HMM method (orange squares). Bottom image, gene arrangement in the genomic island named ICE*Rsp*D18B. ICE*Rsp*D18B (from E0Z06_RS12595 to E0Z06_RS12435) is bordered by a 20-bp DR (5'-ACAATNGAGTGGGAATNNTT-3') in the chromosome of D18. The diagram shows the predicted classification/function of each gene (represented by arrows) as follows: violet, arsenic, or antibiotic resistance; blue, conjugative transfer; green, DNA replication or partitioning; and gray, other functions.

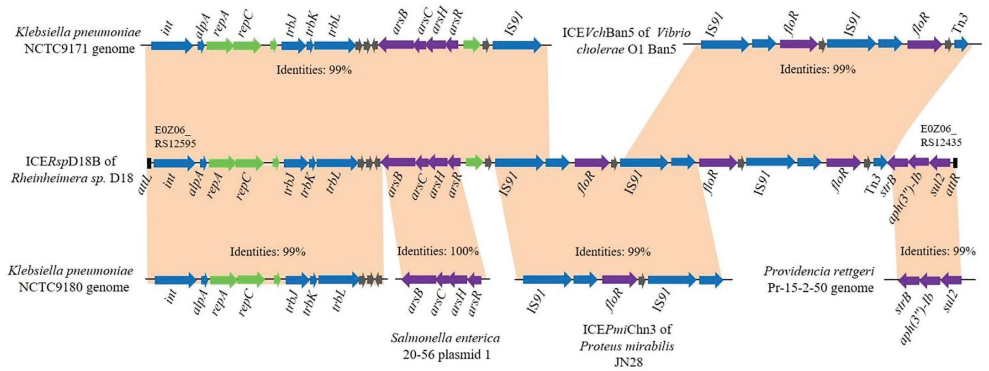

FIGURE 2 | Schematic representation of the potential sources of genes in ICE*Rsp*D18B. Pairwise alignment of ICE*Rsp*D18B of *Rheinheimera* sp. D18 with closely related DNA sequences from ICE*Vch*Ban5 of *Vibrio cholerae* O1 Ban5, ICE*Pmi*Chn3 of *Proteus mirabilis* JN28, plasmid 1 of *Salmonella enterica* 20–56, and the *Klebsiella pneumoniae* NCTC9171, *K. pneumoniae* NCTC9180, and *Providencia rettgeri* Pr-15-2-50 genomes. Genes are indicated by arrows, and colors represent the following predicted functions: violet, arsenic, or antibiotic resistance; blue, conjugative transfer; green, DNA replication or partitioning; and gray, other functions. Orange shading matches regions with high sequence identity.

predicted in ICE*Vch*Ban5 of *Vibrio cholerae* O1 Ban5 (GenBank accession number GQ463140) and ICE*Pmi*Chn3 of *Proteus mirabilis* JN28 (GenBank accession number KY437727). The structure of the remaining part of ICE*Rsp*D18B, including genes related to aminoglycoside and sulfonamide resistance, showed high similarity to genes in the *Providencia rettgeri* Pr-15-2-50 genome (GenBank accession number CP039844.1).

Transfer of ICE*Rsp*D18B to *Escherichia coli*

In order to determine whether the ICE-like chromosomal genomic island ICE*Rsp*D18B could be horizontally transferred, conjugation experiments between the donor strain D18 and the recipient strain *E. coli* 25DN (sodium azide-resistant) were performed. Florfenicol and roxarsone were used as the selective pressure, and the transconjugation frequency was about 2.76×10^{-7} colony-forming units/donor. One of the transconjugants was isolated and named 25D18-B4. To determine whether ICE*Rsp*D18B was inserted into the chromosome of *E. coli* 25D18-B4, PCR assays and DNA sequencing analysis were performed. The results demonstrated that genes *strB*, *floR*, and *arsB*, and the region between *repC* and *trbJ* in ICE*Rsp*D18B, were present in 25D18-B4 but not in strain 25DN (**Figures 3A,B**). Furthermore, these sequences had 100% identity with those of *Rheinheimera* sp. D18, revealing that ICE*Rsp*D18B had been transferred to 25D18-B4. Results also revealed that this ICE*Rsp*D18B had been excised from the chromosome and was present in a circular form in *Rheinheimera* sp. D18 (**Figure 3C**), which is considered to be the first step of conjugation.

Localization of ICE*Rsp*D18B in the Transconjugant 25D18-B4

The 3'-ends of tRNA/tmRNA genes are known attachment sites of ICEs (Williams, 2002; Liu and Zhu, 2010; Del Canto et al., 2011). However, the 3'-end of the guanosine monophosphate synthetase-encoding gene *guaA* has also been reported as an insertion site of genomic islands (Song et al., 2012). As bioinformatics analysis had indicated that ICE*Rsp*D18B was inserted into 3'-end of *guaA* in the *Rheinheimera* sp. D18 genome, we investigated its location in the transconjugant 25D18-B4 and whether integration was orientation-specific, using PCR and DNA sequencing. 25D18-B4 was analyzed by PCR using combinations of two primer pairs: Junction L-For/Junction L-Rev and Junction R-For/Junction R-Rev, with D18 and *E. coli* 25DN as controls (**Figure 4**). It should be noted that the sequence of the Junction L-For primer is also present in the *guaA* gene of D18, due to the high similarity of *guaA* in D18 and 25DN, and that Junction L fragments were amplified in both 25D18-B4 and D18 (**Figure 4B**). PCR results indicated that ICE*Rsp*D18B had been inserted into the 3'-end of *guaA* gene of the transconjugant 25D18-B4 strain, and DNA sequence analysis of PCR products confirmed that ICE*Rsp*D18B was inserted at this site.

Susceptibility of D18 and 25D18-B4 to Antibiotics and Arsenic

The susceptibility of transconjugant 25D18-B4 and *Rheinheimera* sp. D18 to metalloid arsenic and antibiotics was tested. As shown in **Table 1**, 25D18-B4 had acquired resistance to florfenicol (MIC, 92 mg/L), amikacin (MIC, 24 mg/L), sulfamethoxazole (MIC, 16 mg/L), sodium hexafluoroarsenate (MIC, 22 mM), and roxarsone (MIC, 14 mM). MIC testing revealed that the MICs for amikacin, florfenicol, sulfamethoxazole, sodium hexafluoroarsenate, and roxarsone in the transconjugant 25D18-B4 were higher than the MICs for the recipient strain 25DN (**Table 1**). The notable increase in antibiotic/arsenic resistance of 25D18-B4 suggested that ICE*Rsp*D18B genes involved in antibiotic and arsenic resistance had been horizontally transferred to the *E. coli* strain.

DISCUSSION

In this study, we reported the discovery and characterization of the ICE-like chromosomal genomic island ICE*Rsp*D18B in the genus *Rheinheimera*. BLASTn analysis indicated that

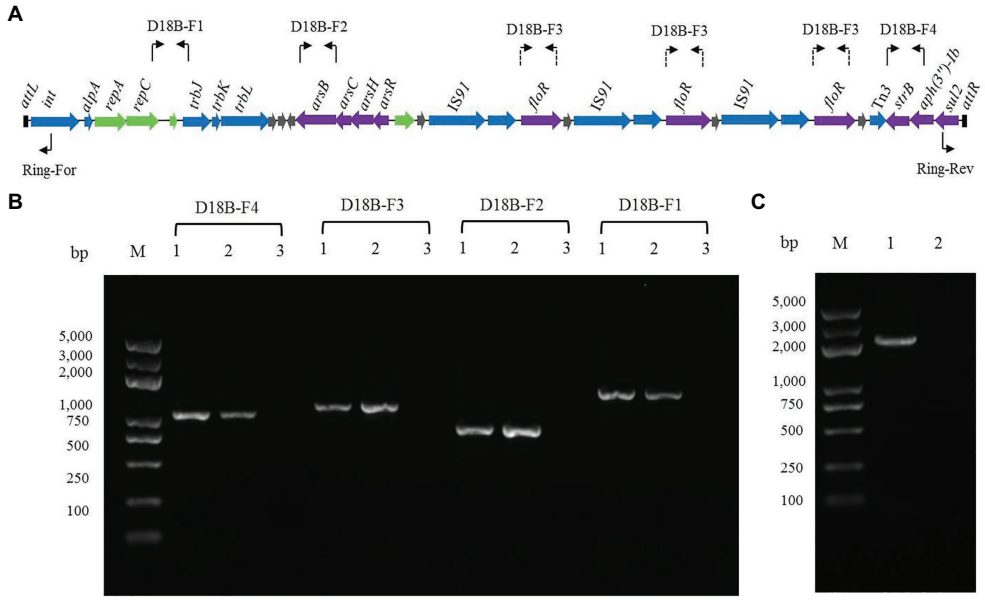

FIGURE 3 | Verification of the presence and the circular form of ICE*Rsp*D18B. **(A)** Primer positions in ICE*Rsp*D18B are indicated using bent arrows. As ICE*Rsp*D18B harbors three copies of *floR*, primers for determination of the presence of *floR* are shown in three locations by dashed arrows. **(B)** Four ICE*Rsp*D18B fragments were amplified by PCR using total DNA of transconjugant 25D18-B4 (lanes 1), strain D18 (lanes 2), strain 25DN (lanes 3) as templates. **(C)** Verification of the circular form of ICE*Rsp*D18B using the primer pair Ring-For/Rev. Total DNA of strain D18 (lane 1) or strain 25DN (lane 2) was used as template. M, molecular size markers.

only part sequence of ICER*sp*D18B exists in other species, and mainly derived from pathogenic bacteria such as *Vibrio cholerae*, *K. pneumoniae*, and *P. rettgeri* (**Figure 2**). Further alignment with ICEberg WU-BLAST search tool revealed that the overall nucleotide sequence of ICE*Rsp*D18B has low similarity to that of previously described ICEs, although a portion of ICE*Rsp*D18B showed high similarity to ICE*Vch*Ban5 of *Vibrio cholerae* O1 Ban5 and ICE*Pmi*Chn3 of *P. mirabilis* JN28 (**Figure 2**). Additionally, our conjugation experiments indicated that ICE*Rsp*D18B has the ability to transfer among bacteria. Hence, we speculate that ICE*Rsp*D18B was transferred horizontally from other unsequenced strains. Moreover, ICE*Rsp*D18B contains genes predicted to encode a site-specific integrase, relaxases associated with a type IV secretory pathway, conjugative transfer proteins, and DNA replication or partitioning encoding genes (**Supplementary Table S2**), further suggesting that ICE*Rsp*D18B is an ICE.

tRNA, tmRNA, and some small RNA genes are recognized as integration hotspots of genomic islands (Williams, 2002; Del Canto et al., 2011). However, the 3'-end of the *guaA* gene is also an insertion site of genomic islands (Song et al., 2012). Integrases in *guaA*-associated genomic islands are frequently phage P4 integrases, and genes encoding AlpA (the positive regulatory protein of P4 integrases) are located near the P4 integrase genes in these genomic islands (Song et al., 2012). The 8-bp consensus sequence 5'-GAGTGGGA-3' within the DR flanking these genomic islands was reported to be the cutting site of the P4 integrases (Song et al., 2012). In our study, bioinformatics analysis revealed that the site-specific integrase in ICE*Rsp*D18B belongs to the phage P4 integrases and that the AlpA-encoding gene *alpA* is next to the site-specific integrase-encoding gene *int* (**Supplementary Table S1**). Additionally, the 8-bp consensus sequence 5'-GAGTGGGA-3' was also found within the DR (5'-ACAATNGAGTGGGAATNNTT-3') of ICE*Rsp*D18B, and ICE*Rsp*D18B was confirmed to be inserted into the 3' end of *guaA* in the transconjugant 25D18-B4 (**Figure 4**). In addition, the circular, extrachromosomal form of ICE*Rsp*D18B was also observed in *Rheinheimera* sp. D18 using PCR (**Figure 3**). These data suggest that ICE*Rsp*D18B was first excised from the donor *Rheinheimera* sp. D18 chromosome, transferred *via* type IV secretory system-mediated conjugation and then inserted into 3'-end of *guaA* gene of the *E. coli* 25DN chromosome by site-specific recombination. These data also indicated that ICE*Rsp*D18B has the ability to transfer genes horizontally from *Rheinheimera* sp. D18 to other bacteria. Considering that ICE*Rsp*D18B is also located at the 3'-end of *guaA* in the *Rheinheimera* sp. D18 genome, our results further demonstrate that the 3'-end of *guaA* gene may be another integration hotspot of genomic islands.

Organoarsenic arsenical compounds (such as p-arsanilic acid and roxarsone) are widely used as feed additives in many countries, and the land application of poultry or swine litter could cause serious arsenic pollution in the environment (Liang et al., 2014; Xie and Cheng, 2019), potentially resulting in arsenic resistance among environmental bacteria and the dissemination of their arsenic resistance genes to other bacterial species. Arsenic resistance genes are usually organized in *ars* operons in bacteria, such as in *Pseudomonas putida*, which has two *arsRBCH* operons and which is highly resistant to

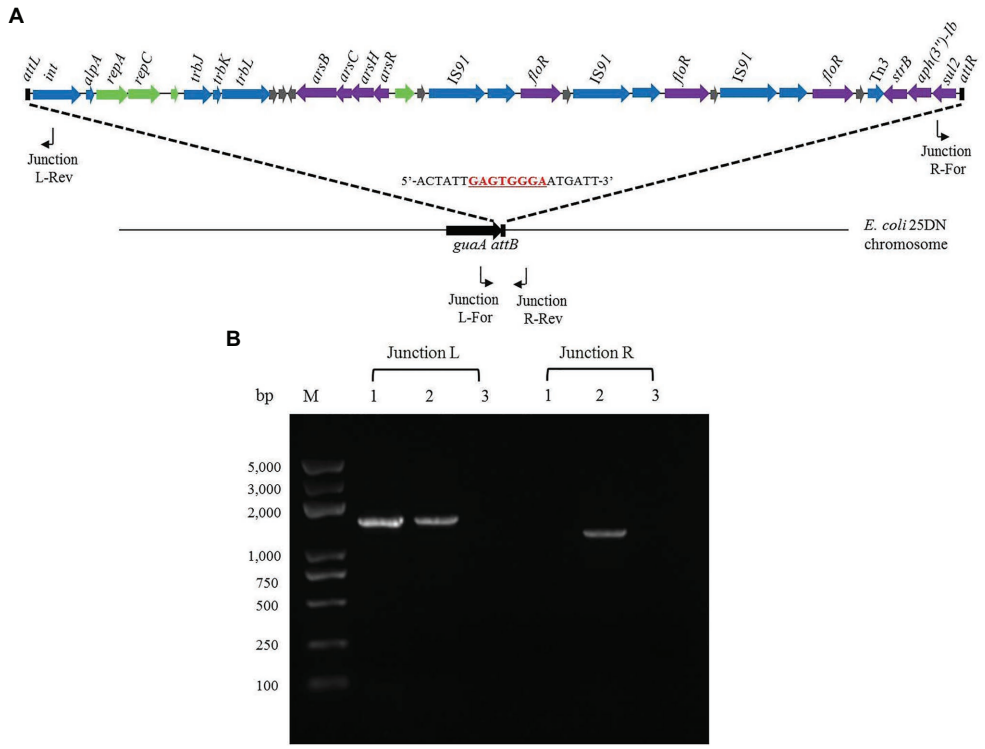

FIGURE 4 | Analysis of the insertion site of ICE*Rsp*D18B in transconjugant 25D18-B4. **(A)** PCR primer positions in ICE*Rsp*D18B and in the strain 25DN chromosome are indicated by bent arrows. The insertion site of ICE*Rsp*D18B in the strain 25DN chromosome is indicated by dashed lines, and the cutting site (5'-GAGTGGGA-3') of the integrase (Song et al., 2012) is underlined and marked in red. **(B)** Gel picture of the PCR products generated by the Junction L-For/Junction L-Rev and Junction R-For/Junction R-Rev primer pairs. Total DNA of strain D18 (lanes 1), transconjugant strain 25D18-B4 (lanes 2), and strain 25DN (lanes 3) was used as template. M, molecular size markers.

TABLE 1 | MICs of antibiotics and arsenic (As).

Strain	Amikacin*	Florfenicol	Sulfamethoxazole	Roxarsone#	Sodium hexafluoroarsenate#
D18	96	128	72	20	35
25DN	<2	<2	4	4	10
25D18-B4	24	92	16	14	22

Concentrations of the three antibiotics are given in mg/L.

#*Concentrations of roxarsone and sodium hexafluoroarsenate are given in mM.*

organoarsenicals and inorganic arsenic (Canovas et al., 2003; Villadangos et al., 2012). The *arsB* gene encodes an As(III) efflux permease, a*rsC* encodes an arsenate reductase for reduction of inorganic arsenate to As(III) and *arsR* encodes an As(III)-responsive transcriptional factor that controls expression of the operon (Yang et al., 2012). Arsenate [As(V)] is reduced to arsenite [As(III)] by the arsenate reductase ArsC prior to efflux, and then, arsenite is pumped out through ArsB (Shen et al., 2013). *arsH* encodes an organoarsenical oxidase that confers resistance to organoarsenic (Chen et al., 2015; Xie and Cheng, 2019). ICE*Rsp*D18B contains one *ars* gene cluster, which includes *arsBCHR* (**Figure 1**). The transconjugant 25D18-B4, which acquired ICE*Rsp*D18B, was found to have markedly higher MICs of roxarsone and sodium hexafluoroarsenate compared to those of the parental strain, *E. coli* 25DN (**Table 1**). These data suggest that ICE*Rsp*D18B can contribute to the dissemination of arsenic resistance genes among bacteria.

Sulfonamide, chloramphenicol/florfenicol, and aminoglycoside have been used widely to treat bacterial and protozoan infections in aquaculture systems (Dang et al., 2007; Hoa et al., 2008). ICE*Rsp*D18B also contains three copies of a chloramphenicol/florfenicol efflux MFS transporter-encoding gene (*floR*); one sulfonamide resistance gene (*sul2*); and two aminoglycoside resistance genes, *aph(3")-Ib*, and *strB*. *Escherichia coli* is an opportunistic bacterium that can cause a wide variety of intestinal and extraintestinal infections (Riley, 2014). In this study, ICE*Rsp*D18B was horizontal transferred to *E. coli* 25DN strain,

and generated the transconjugant 25D18-B4 strain. The transconjugant 25D18-B4 was found to have notably higher MICs of amikacin, florfenicol, and sulfamethoxazole when compared to the parental strain, *E. coli* 25DN (**Table 1**), suggesting that the ARGs in ICE*Rsp*D18B contribute to the antibiotic resistance profile of *Rheinheimera* sp. D18 as well as of *E. coli* 25D18-B4. These data suggest that the ICE-like genomic island ICE*Rsp*D18B has the ability to disseminate these ARGs, along with arsenic resistance genes, among bacteria in the environment.

In conclusion, the findings of this study demonstrate that ICE*Rsp*D18B is an ICE that increases host tolerance to arsenic and several antibiotics. Our results also reveal that this mobilizable ICE*Rsp*D18B could be horizontal transferred to *E. coli* 25DN strain, and the transconjugant 25D18-B4 also has resistance to arsenic and antibiotic. Continuous monitoring of the antibiotic/arsenic tolerance of bacteria detected in the aquaculture industry is recommended to reduce the spread of resistance genes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JF: executed the experiments and manuscript preparation and submission. CZ: resources, review and editing. PZ and GZ: data curation and investigation. YZ and QG: methodology. GC: designed the work and revised the manuscript. All authors contributed to the article 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.2020.616364/full#supplementary-material

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Antimicrobial Resistance in Bacteria Isolated From Cats and Dogs From the Iberian Peninsula

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Pet animals are assumed to be potential reservoirs in transferring antimicrobial resistance (AMR) to humans due to the extensively applied broad-spectrum antimicrobial agents and their close contact with humans. In this study, microbiological data and antimicrobial susceptibility results of dog (*n* = 5,086) and cat (*n* = 789) clinical samples from a private Laboratory of Diagnosis in Barcelona were analyzed. Samples came from different counties of the Iberian Peninsula during 2016–2018. In dogs, clinical samples were most commonly from otitis, and in cats from wounds, respiratory tract infections and conjunctivitis. In both pet groups, *Staphylococcus* spp. (31% in dogs vs 30% in cats), *Streptococcus* spp. (19% vs 17%), *Pseudomonas* spp. (16% vs 10%), *Escherichia coli* (8% vs 5.6%), and *Enterococcus* spp. (5.5% vs 6.8%) were shown as the most predominant bacteria. However, higher frequencies of *P. aeruginosa*, *P. canis*, and *S. pseudintermedius* were found in dogs, while *S. aureus* and *P. multocida* were more prevalent in cats. The antimicrobial susceptibility testing demonstrated that *Enterococcus* spp. and *Pseudomonas* spp. presented the highest levels of AMR in both dogs and cats. Within the Enterobacteriaceae, *E. coli* showed low levels of AMR compared to *Klebsiella*, *Proteus,* or *Enterobacter* spp. Respiratory tract infections caused by *K. pneumoniae* presented higher AMR in cats. By contrast, *Pasteurella* isolates from the respiratory tract were highly sensitive to all the antimicrobials in cats and dogs. Data from this study could be used to guide empirical antimicrobial selection in companion animal veterinary practices in the Iberian Peninsula.

Keywords: antimicrobial resistance, bacteria, cats, dogs, Iberian Peninsula

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INTRODUCTION

The emergence of antimicrobial resistance (AMR) has become a great concern worldwide, threatening the public healthcare system (Brinkac et al., 2017). Some studies assumed that food animals were the main contributors of human AMR by transferring resistant bacteria or genes through food chain (Witte, 1998; Fey et al., 2002; Smith et al., 2002; White et al., 2002; Angulo et al., 2009; McEwen and Fedorka-Cray, 2017). However, (Barber et al., 2016) established a new analytical model and assumed the non-foodborne transmission of AMR should be equally

emphasized. Thus, the companion animals, mostly dogs and cats, started to be considered a potential reservoirs of AMR due to their close contact with humans and being extensively treated by broad-spectrum antimicrobial agents (Guardabassi et al., 2004; Lloyd, 2007). If AMR can be transmitted to humans from companion animals, and if multi-drug resistant (MDR) bacteria exist among the household pets, the risk of antimicrobial treatment failure would highly increase in both animals and humans. Thus, understanding the prevalence of AMR among pets, mainly dogs and cats, is demanded from both veterinary and human medicine perspectives. However, due to the clinical cases are not always entirely recorded and monitored, the available data on pet-related AMR are very minimal.

In this study, we analyzed the clinical microbiological data on pet dogs and cats with data collected between 2016 and 2018 in the Iberian Peninsula, and found out the most prevalent bacterial infections and AMR profiles among the two companion animals.

MATERIALS AND METHODS

Data Source and Management

Retrospective records of 5,875 microbiological analyses of clinical specimens from dogs (n = 5,086) and cats (n = 789) between 2016 and 2018 were analyzed in the present study. The records were provided by the Veterinary Medicine Department of a large private Laboratory of Diagnosis in Barcelona. The lab records contained information about clinical cases submitted by veterinary clinics covered throughout the Spanish provinces, Portugal, and Andorra (**Figure 1**). Data were assessed for duplicates and missing information. Finally, only samples with complete records were analyzed. Repeat samples of the same case were not included. The following variables were extracted from the records: animal species, type/origin of sample, county of specimen, bacterial identification, and antimicrobial susceptibility testing.

The specimens were classified according to the sample origin as follows: otitis (n = 3,043), wounds (n = 1,142), respiratory tract infections (which included rhinitis, bronchitis, pneumonia, and pleuritic, n = 483), dermatitis (n = 341), abscesses (n = 218), conjunctivitis (n = 190), and others (which included reproductive tract infections, musculoskeletal infections, arthritis, and osteomyelitis, n = 458). Urine samples were not included in the study.

Microbiological Analysis and Antimicrobial Susceptibility Testing

Microbiological identification was performed using the MALDITOF mass spectrometeror the API® ID system (bioMérieux, Spain). All Gram-positive bacterial isolates were performed by the antimicrobial susceptibility test using the standard disk diffusion method according to Performance Standards for Antimicrobial Susceptibility Testing for bacteria isolated from animals (M31-A3, CLSI VET01, 2008) and from humans (M100-S24, CLSI, 2016) for drugs not licensed for veterinary use. The panel included the following antimicrobial classes: beta-lactams (amoxicillin-clavulanic acid, oxacillin, cefoxitin, penicillin, piperacillin, piperacillin/tazobactam, ampicillin, cephalexin, cephalotin, cefazolin, cefuroxime, ceftazidime, cefotaxime, cefovecin, cefotaxim, and cefepime), carbapenems (imipenem and meropenem), and aztreonam; fluoroquinolones (ciprofloxacin, enrofloxacin, and marbofloxacin); aminoglycosides (amikacin, gentamicin, tobramycin, and neomycin); macrolides (azithromycin and erythromycin); tetracyclines (doxycycline); clindamycin; polymyxin B; trimethoprim/sulphamethoxazole; chloramphenicol/florphenicol; fosfomycin; mupiracin; and glycopetides (vancomycin). For Gram negative bacteria, NM44 MicroScan (Beckman Coulter, Villepinte, France) system was performed for all the antimicrobials except for those antibiotics authorized for veterinary uses that are not included in the automatic scan panels (enrofloxacin, pradofloxacin, marbofloxacin, doxycycline, cephalexin, and cefovecin). The MicroScan is an automated bacterial identification and susceptibility testing system based on microbiology principles of true minimum inhibitory concentration (MIC) testing. Based on the lab readings, isolates were classified as Susceptible, Intermediate or Resistant. For statistical assessments, isolates that exhibited intermediate resistance were re-classified as resistant. The laboratory has the quality management system certificate ISO-9001 since 1998 and the accreditation from ENAC (National Accreditation Entity) according to criteria included in the ISO/IEC 17025 Standard defined in the Technical Annexes 511/LE1947 for Pharmaceutical Toxicology and Microbiology Testing.

Statistical Analysis

Descriptive and statistical analysis was performed using the SPSS Advanced Models TM 15.0 (SPSS Inc. 233 South Wacker Drive, 11th Floor Chicago, IL, United States 60.606-6412). The Chi-square (χ^2) or Fishers Exact tests were used to compare bacterial spp. and the AMR frequencies in both animal groups. Statistical significant was considered when $p < 0.05$.

RESULTS

Microbiological Diagnosis of Bacterial Infections

In dogs, most of the samples remitted to the lab were from cases related to otitis (55.3% dogs vs 29% cats, χ^2 = 187.2, and $p < 0.05$). In cats, samples from wounds (23% cats vs 19% dogs, χ^2 = 6.6, and p = 0.01), respiratory tract infections (24% vs 5.8%, χ^2 = 299, and $p < 0.05$), and conjunctivitis (6% vs 2.8% χ^2 = 21.6, and $p < 0.001$) were more frequently remitted (**Figure 2**).

Staphylococcus spp. (31–30%), *Streptococcus* spp. (19–17%) and *Pseudomonas* spp. (16–10%), followed by *Escherichia coli* (8.0–5.6%), and *Enterococcus* spp. (5.5–6.8%), were the most predominant bacteria isolated in both dogs and cats (**Table 1**). As a differential trait, dogs presented higher frequencies of *Pseudomonas aeruginosa* (92% vs 72%), *P. canis* (36.7% vs 6.5%), and *S. pseudintermedius* (17% vs 4.6%), while *S. aureus* (6% vs 1.5%) and *P. multocida* (63% vs 20.4%) were more prevalent in cats (**Table 1**).

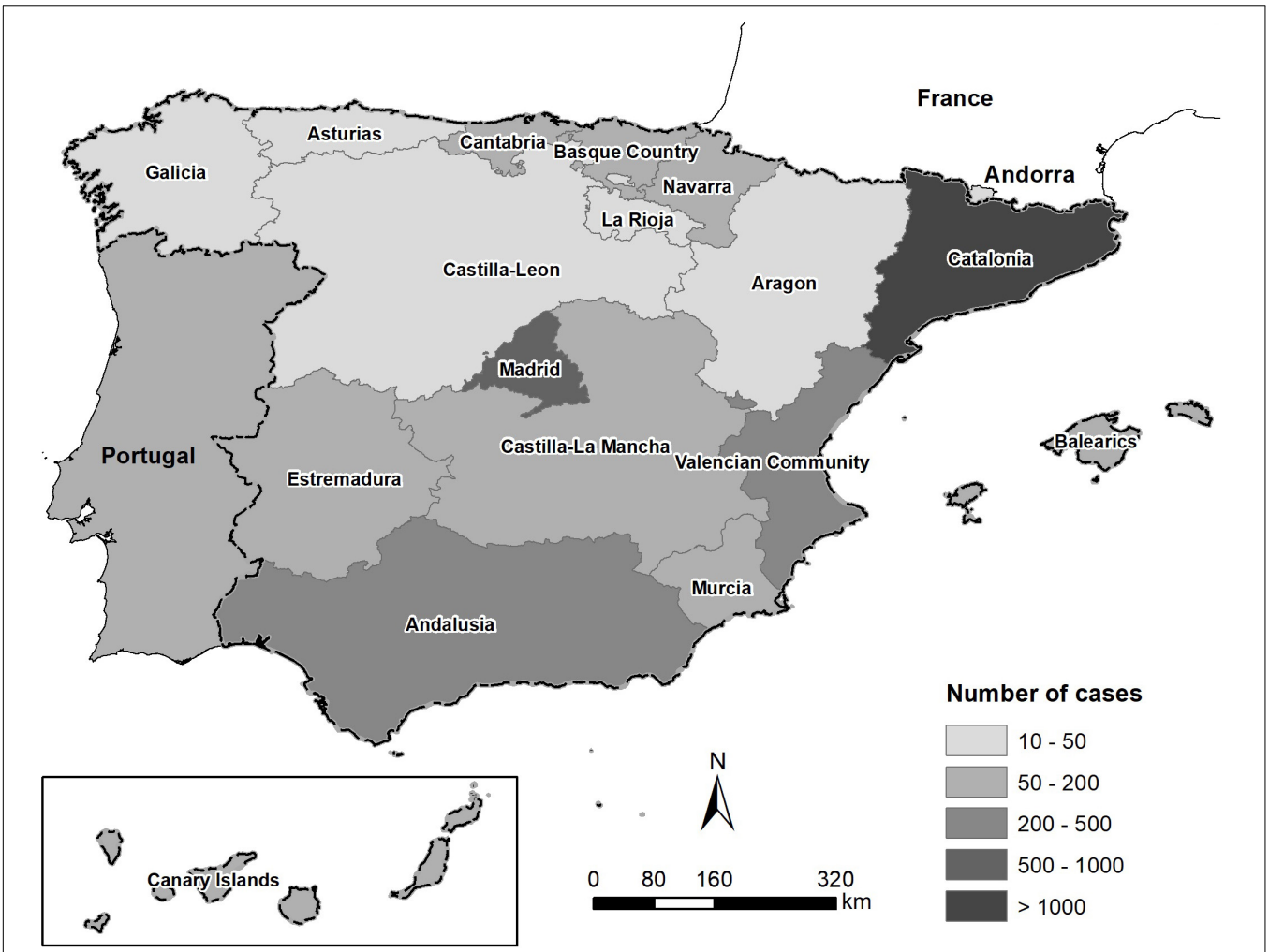

FIGURE 1 | Map of the origin and the number of clinical specimens from dogs and cats in the Iberian Peninsula between 2016 and 2018.

The distribution of pathogens for different sample categories showed that wounds and dermatitis presented similar patterns of distribution in dogs and cats, with *Staphylococcus, Streptococcus, Enterococcus*, and *E. coli* identified as the most frequently isolated agents (**Figure 3**). From otitis specimens, infections by *Staphylococcus* spp. were highly detected in both cats and dogs; meanwhile in cats, high frequencies of *P. aeruginosa* and *E. coli* were presented. On the other hand, dogs presented in general a larger bacterial diversity in samples from abscess, conjunctivitis and respiratory tract infections in comparison to cats. In this line, cats showed higher percentages of *Bordetella* spp. and *P. multocida* infections in conjunctivitis and respiratory specimens, respectively, (**Figure 3**).

Antimicrobial Susceptibility Testing

Comparisons of AMR levels between dogs and cats were only made for bacterial species, which were recorded for more than 20 different strains in the antibiotic sensitivity test. Thus, the following species were involved: *Staphylococcus* spp. (n = 1,572 isolates from dogs, n = 239 from cats), *Streptococcus* spp. (n = 969, n = 132), *Enterococcus* spp. (n = 281, n = 54), *Escherichia* spp. (n = 405, n = 44), *Enterobacter* spp. (n = 193, n = 22), *Klebsiella* spp. (n = 103, n = 23), *Pseudomonas* spp. (n = 825, n = 76), *Pasteurella* spp. (n = 49, n = 62), and *Corynebacterium* spp. (n = 194, n = 22). In addition, for the most relevant gram-negative bacteria species, minimal inhibitory concentration (CMI) values required to inhibit the growth of 50% (MIC50) and 90% (MIC90) of organisms were assessed for some antimicrobials (**Table 2**). Interestingly, the Enterobacteriaceae species presented high values of CMI90 for beta-lactams, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole in both animal groups. *Pseudomonas* spp. showed the highest CMI50 values for amoxicillin-clavulanate and cefoxitin (jointly with *Enterobacter* spp.), for ampicillin (jointly with *Klebsiella* spp.), and for cefotaxime and cefuroxime. Finally, *Proteus* spp. isolated from dogs presented a CMI90 value = 8 mg/L to imipenem, exceeding the resistant breakpoint (**Table 2**).

Among the Gram-positive bacteria, more than 80% of *Enterococcus* isolates presented resistance to oxacillin, cefoxitin, amikacin, clindamycin, polymyxin B, and fosfomycin from

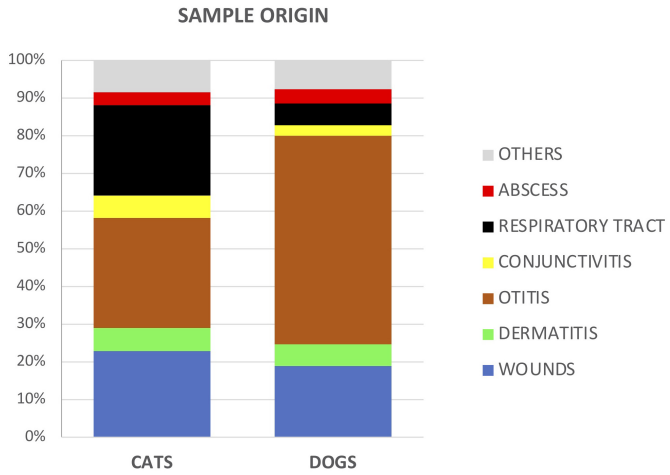

FIGURE 2 | Proportion of analyzed samples between cats and dogs according to the type or source of specimens.

both dogs and cats (**Figure 4**). Similar patterns but with lower frequencies were detected for *Staphylococcus, Streptococcus,* and *Corynebacterium* spp., principally in isolates from dog specimens. Besides, *Staphylococcus* spp. isolated from dogs presented higher levels of AMR to macrolides, tetracycline, trimethoprim/sulfamethoxazole and chloramphenicol compared to cat isolates. Of note, a significant higher frequency of imipenem and marbofloxacin *Corynebacterium* resistant isolates were found in dog cases (**Figure 4**).

Within the Enterobacteriaceae family, although *E. coli* was highly isolated from wounds, dermatitis, abscesses, and otitis in both dogs and cats, they presented low levels of AMR (with the exception of ampicillin where 50% of isolates were resistant), in comparison to other members of the family such as *Klebsiella*, *Proteus,* or *Enterobacter* spp. (**Figure 5**). More in detail, *Enterobacter* strains from dog specimens showed a higher level of AMR to β-lactams, imipenem, and mupirocin than cats. *K. pneumoniae* from cat respiratory tract infections presented an overall higher resistance to antimicrobials than from dogs, showing statistical differences for piperacillin and trimethoprim/sulfamethoxazole (**Figure 5**).

Finally, *Pseudomonas* spp. presented the highest levels of AMR in both dogs and cats, showing between 80 and 97% of resistance to penicillin and cephalosporin classes, including 3rd GC, 79–94% trimethoprim/sulfamethoxazole, 68–85% flophenicol, 55–62% chloramphenicol, and 69–78% fosfomycin. In general, isolates from dogs presented higher levels of resistance than the cat isolates (**Figure 5**).

Antimicrobial susceptibility in *Proteus* spp. (n = 205, n = 5), *Serratia* spp. (n = 104, n = 14), *Acinetobacter* spp. (n = 61, n = 18), and *Bordetella* spp. (n = 47, n = 15) was mainly done from dog isolates. (**Figure 6**) Interestingly, more than 80% of *Proteus* isolates were resistant to doxycycline and polymyxin B. *Acinetobacter* isolates presented a high resistance rate to cephalexin (66.1% of dog, 44.4% of cat, and $p < 0.05$), cefovecin (65.0%, 38.9%, and $p < 0.05$), ampicillin (63.8%, 44.4%), amoxicillin (59.0%, 22.2%, and $p < 0.05$), and cefuroxime (57.4%, 33.3%). Meanwhile, resistance to piperacillin, piperacilina/tazobactam, cefotaxime, ciprofloxacin, enrofloxacin, marbofloxacin, amikacin, tobramycin, and trimethoprim/sulfamethoxazole was also found in both pet groups but in a low proportion of isolates (<20%; **Figure 6**).

As regard *Pasteurella* isolates, they were detected principally from respiratory tract, most of the isolates were highly sensitive to all the antimicrobials in cats and dogs, showing low resistance frequencies only to cefuroxime (8.2%) and ampicillin (6.1%) in dogs, and cephalexin (6.5%) and cefovecin (4.8%) in cats (**Figure 6**).

DISCUSSION

This study provides data of the most frequently isolated bacteria from cat and dog infections and their associated AMR profiles based on a large number of clinical cases (N = 5875) within the Iberian Peninsula. This information can be a guide to clinicians, especially those working in this region, to make rational decisions on the use of antimicrobials, principally when empirical antimicrobial treatment is recurrent in companion animal veterinary medicine.

Most of the specimens submitted to the lab were from ears in both cats and dogs, and in cats, a large number of samples were also from respiratory tract infections and wounds. The distribution of pathogens showed that *Staphylococcus, Streptococcus, Pseudomonas, E. coli,* and *Enterococcus* were the most frequently isolated agents for different sample categories. In both cats and dogs, *Staphylococcus* spp. was commonly isolated from several sample sources including ears, skin, eyes, abscesses and wounds. This finding agrees with other studies conducted in Canada, Sweden, and South Africa (Windahl et al., 2015; Qekwana et al., 2017; Awosile et al., 2018) which confirms *Staphylococcus* spp. as an opportunistic pathogen of the integument and mucosae, causing otitis externa, pyoderma, and post-surgical complications in dogs.

Thirty-one and thirty percent of the studied samples were tested positive for *Staphylococcus* spp., respectively, in dogs and cats. In dogs, the identified species of *Staphylococcus* included *S. pseudintermedius* (17.4%), *S. intermedius* (7%), *S. schleiferi* (2%), *S. aureus* (1.5%), and *S. epidermidis* (0.6%), which presented a similar prevalence patterns as other studies reported in South Africa (Qekwana et al., 2017). The lower prevalence of *S. aureus* compared with *S. pseudintermedius* was in accordance with previously published works (Hanselman et al., 2009; Kawakami et al., 2010; Chanchaithong et al., 2014; Dos Santos et al., 2016). In cats, *S. aureus* was the most common isolated specie. The high rate of colonization with *S. pseudintermedius* and *S. aureus* found in dog and cat specimens could represent a public health concern, as has been described in many papers the potential transmission of *Staphylococcus* spp. from dogs to humans when exposing to carrier or infected dogs (Boost et al., 2007; Faires et al., 2009; Frank et al., 2009; Pantosti, 2012; Dos Santos et al., 2016).

The most common ear pathogens isolated from dogs are coagulase-positive staphylococci (*Staphylococcus pseudintermedius*) and *P. aeruginosa* (Cole et al., 1998). By contrast, a recent study conducted in France showed that the major causative agents of dog otitis were coagulase-positive staphylococci, *P. aeruginosa*, *P. mirabilis,* and streptococci (Bourély et al., 2019). In that study, the authors found that since 2003 resistance to fluoroquinolones has been decreased in both *P. aeruginosa* and *S. pseudintermedius* isolates, resulting

TABLE 1 | Frequencies of bacterial species identified in dog and cat specimens.

		DOGS (*N* = 5,086)		CATS (*N* = 789)
		***n* (%)**		***n* (%)**
Acineto bacter spp.		61 (1)		18 (2)
	A. baumannii	22 (36.1)	*A. lwoffii*	8 (44.4)
	A. lwoffii	14 (23)	*A. baumannii*	2 (11.1)
	A. haemolyticus	4 (6.6)	*A. haemolyticus*	1 (5.5)
	Others	2 (3.3)		
Bordetella spp.		47 (0.9)		15 (1.9)
	B. bronchiseptica	47 (100)	*B. bronchiseptica*	15 (100)
Candida spp.		30 (0.5)		7 (0.9)
	C. parapsilosis	5 (16.7)	*C. parapsilosis*	3 (42.9)
	C. albicans	2 (6.5)	*C. albicans*	2 (28.6)
	Others	4 (13.3)		
Coryne bacterium spp.		194 (3.8)		22 (2.8)
	C. amycolatum	7 (3.6)	*C. amycolatum*	2 (9.1)
	C. auriscanis	5 (2.6)		
	Others	2 (1)		
Entero bacter spp.		84 (1.6)		26 (3.3)
	E. cloacae	59 (70.2)	*E. cloacae*	22 (84.6)
	E. aerogenes	13 (15.5)	*E. aerogenes*	3 (11.5)
	E. gergoviae	8 (9.5)	*E. gergoviae*	1 (3.8)
Entero coccus spp.		281 (5.5)		54 (6.8)
	E. faecalis	92 (32.7)	*E. faecalis*	18 (69.2)
	E. faecium	8 (2.8)	*E. avium*	2 (7.7)
	E. canintestini	1 (0.4)	*E. faecium*	1 (3.8)
	E. durans	1 (0.4)	*E. hirae*	1 (3.8)
Escherichia spp.		405 (8)		44 (5.6)
	E. coli	400 (98.8)	*E. coli*	42 (95.5)
	E. vulneris	4 (1)		
Klebsiella spp.		103 (2)		23 (2.9)
	K. pneumoniae	73 (70.9)	*K. pneumoniae*	17 (73.9)
	K. oxytoca	28 (27.2)	*K. oxytoca*	6 (26.1)
	K. ornithinolytica	1 (1)		
Pasteurella spp.		49 (1)		62 (7.8)
	P. canis	18 (36.7)	*P. multocida*	39 (62.9)
	P. multocida	10 (20.4)	*P. canis*	4 (6.5)
	P. pneumotropica	3 (6.1)	Others	4 (6.5)
Proteus spp.		205 (4)		5 (0.6)
	P. mirabilis	198 (96.6)	*P. mirabilis*	5 (100)
	P. vulgaris	3 (1.5)		
Pseudo monas spp.		827 (16.3)		76 (9.6)
	P. aeruginosa	761 (92)	*P. aeruginosa*	55 (72.4)
	P. fluorescens	18 (2.2)	*P. fluorescens*	5 (6.6)
	Others	34 (4.1)	Others	16 (21.1)
Serratia spp.		104 (2)		14 (1.7)
	S. marcescens	96 (92.3)	*S. marcescens*	12 (85.7)
	S. liquefaciens	7 (6.7)	*S. liquefaciens*	2 (14.3)
Staphylo coccus spp.		1,581 (31)		239 (30.3)
	S. pseudintermedius	275 (17.4)	*S. aureus*	14 (5.9)
	S. intermedius	109 (6.9)	*S. epidermidis*	12 (5)
	S. schleiferi	30 (1.9)	*S. felis*	12 (5)
	S. aureus	23 (1.5)	*S. pseudintermedius*	11 (4.6)
	S. epidermidis	9 (0.6)	*S. schleiferi*	2 (0.8)
	Others	25 (1.6)	Others	16 (6.7)
Strepto coccus spp.		972 (19)		132 (16.7)
	S. canis	23 (2.4)	*S. canis*	2 (1.5)
	S. dysgalacticae	3 (0.3)		
	S. halichoeri	1 (0.1)		

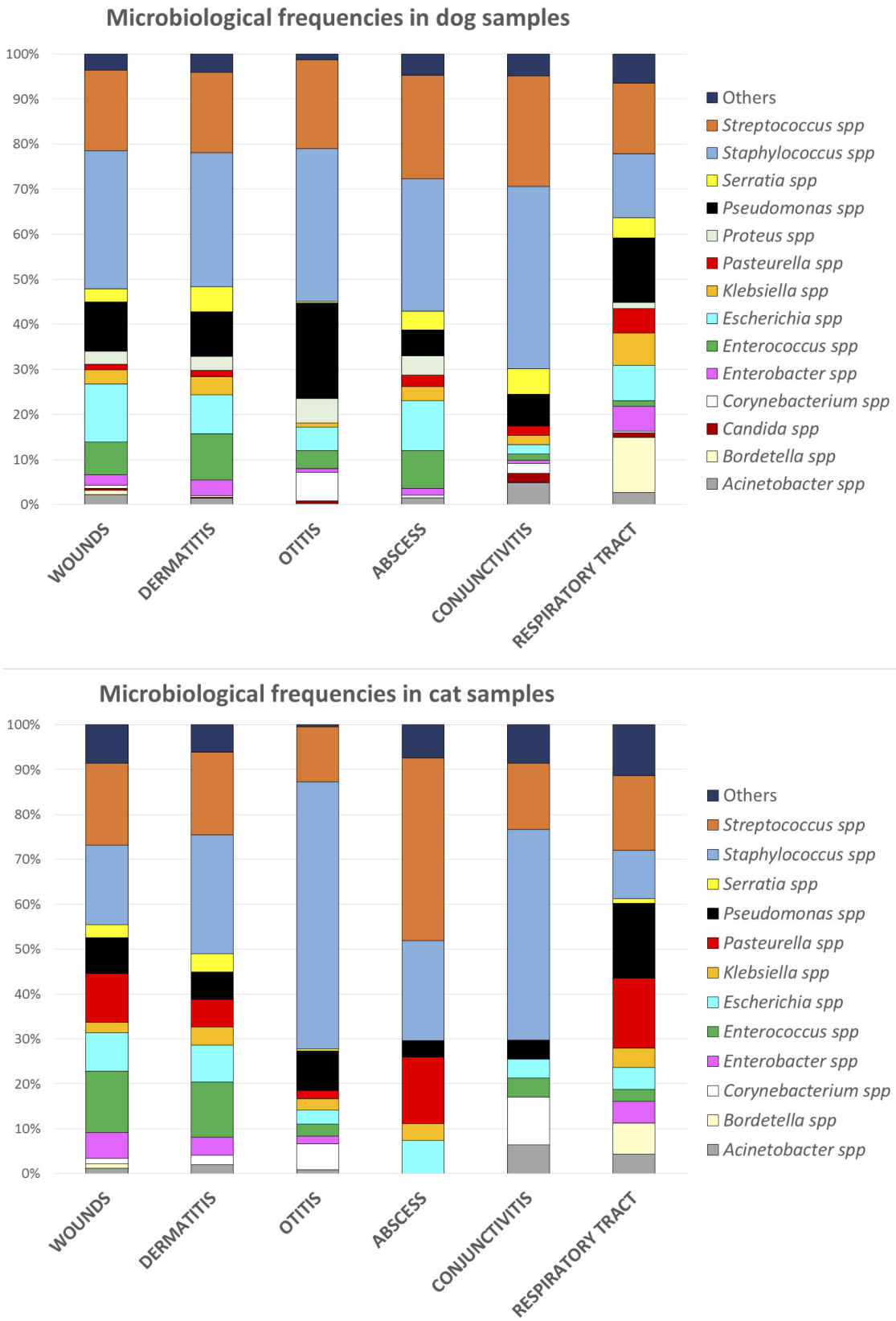

FIGURE 3 | Frequencies of bacterial species according to the origin of infections in dogs and cats.

for *P. aeruginosa*, 19.4% of isolates were resistant to both enrofloxacin and gentamicin (Bourély et al., 2019). In the present study, *S. pseudintermedius, P. aeruginosa,* and *E. coli.* were also frequently isolated from dog otitis specimens, and similar percentages of fluoroquinolones and gentamicin resistance were observed for *S. pseudintermedius* and *P. aeruginosa* isolates (<20%). Meanwhile, the *P. aeruginosa* isolates showed high levels of resistance to penicillin and cephalosporin classes (including 3rd GC), trimethoprim/sulfamethoxazole, phenicoles, and fosfomycin, both in dogs and cats. *Pseudomonas* spp. were intrinsically resistant to beta-lactams, combinations with β-lactamase inhibitors, chloramphenicol, erythromycin, and trimethoprim/sulfamethoxazole. In this study, high proportions of *Pseudomonas* were susceptible to the aminoglycosides (>95%). As well, the frequency of enrofloxacin resistance was low (27% in dogs and 20% in cats) compared to other studies conducted in Canada (Awosile et al., 2018). Enrofloxacin is commonly used systemically with concurrent topical treatment in cases of canine otitis caused by *P. aeruginosa* (Hariharan et al., 2006). These results suggest that aminoglycosides and fluoroquinolones have potential to be used as anti-pseudomonal drugs (Dowling, 1996). Our findings are also consistent with similar retrospective studies from Denmark, United States, and Canada (Petersen et al., 2002; Authier et al., 2006; Pedersen et al., 2007).

Enterococci are MDR from both intrinsic and acquired features. Specifically, *Enterococcus* spp. are naturally resistant to clindamycin, as well as to penicillin G and cephalothin, giving them a characteristic of AMR profile (Prescott et al., 2002; Delgado et al., 2007). Enterococci isolates of this study were principally isolated from wounds and dermatitis of companion animal specimens. More than 80% of *Enterococcus*

TABLE 2 | Minimal Inhibitory Concentrations (MIC, mg/L) values in Gram-negative bacteria isolated from dogs and cats.

	DOG SPECIMENS											
	***Acinetobacter* spp.**		***Pseudomonas* spp.**		***Escherichia* spp.**		***Klebsiella* spp.**		***Enterobacter* spp.**		***Proteus* spp.**	
	MIC_{50}	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**
AMC	4	>32	>32	>32	4	>32	4	>32	>32	>32	<2	16
AMK	<2	16	>2	16	<2	<8	<2	16	<2	<8	<2	4
AMP	16	>32	>32	>32	16	>32	>32	>32	16	>32	<2	>32
CAZ	4	16	4	8	<1	16	<1	16	<1	>64	<1	<1
CIP	<0.25	>4	<0.25	>4	<0.25	>4	<0.25	>4	<0.25	>4	<0.25	>4
CTX	8	32	16	>64	<1	8	<1	>64	<1	>64	<1	4
CXM	32	>64	>64	>64	4	>64	4	>64	4	>64	<1	16
FOX	>64	>64	>64	>64	<4	>64	>8	>64	>64	>64	<4	16
GEN	<1	8	<1	8	<1	>16	<1	>16	<1	8	<1	>16
IPM	<0.25	1	2	2	<0.25	<0.5	<0.25	<0.25	<0.5	2	2	8
SXT	<20	>320	160	>320	<20	>320	<20	>320	<20	>320	<20	>320
TZP	8	16	8	32	<4	8	<4	>128	<8	>128	<4	<4
	CAT SPECIMENS											
	***Acinetobacter* spp.**		***Pseudomonas* spp.**		***Escherichia* spp.**		***Klebsiella* spp.**		***Enterobacter* spp.**		***Proteus* spp.**	
	MIC_{50}	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**
AMC	4	16	>32	>32	4	>32	16	>32	>32	>32	8	8
AMK	<2	<8	<2	16	<2	8	<2	16	<2	16	<2	<2
AMP	4	>32	>32	>32	>16	>32	>32	>32	>32	>32	>32	>32
CAZ	4	4	16	>64	<1	>16	<1	>16	<1	>64	<1	<1
CIP	<0.25	<0.5	<0.5	>4	<0.25	>4	>2	>4	<0.25	>4	<0.25	2
CTX	8	16	16	>64	<1	>64	<1	>64	<1	>64	<1	<1
CXM	16	>64	>64	>64	4	>64	>16	>64	16	>64	<1	4
FOX	>64	>64	>64	>64	>4	8			>64	>64	<4	16
GEN	<1	<2	<1	8	<1	<2	<1	>16	<1	8	<1	>16
IPM	<0.25	<1	2	2	<0.25	<1	<0.25	<1	<0.25	<1	–	–
SXT	<20	<20	>320	>320	<20	>320	>320	>320	<20	>320	>320	>320
TZP	<8	16	8	>128	<4	64	16	>128	<8	>64	<4	<4

AMC, Amoxicillin-clavulanic; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; CXM, cefuroxime; FOX, cefoxitin; GEN, gentamicin; IPM, imipenem; SXT, trimethoprim/sulphamethoxazole; and TZP, piperacillin/tazobactam. CLSI (M100-S24): AMC ≥ 32/16, AMK ≥ 64, and AMP ≥ 16; CAZ ≥ 16, CIP ≥ 1, CTX ≥ 4, CXM ≥ 32, FOX ≥ 32, GEN ≥ 16, IPM ≥ 4, SXT ≥ 4/76, and TZP ≥ 128/4. CLSI (VET01): AMC ≥ 1, AMP > 8, AMK ≥ 16, CAZ ≥ 16, and GEN ≥ 8.

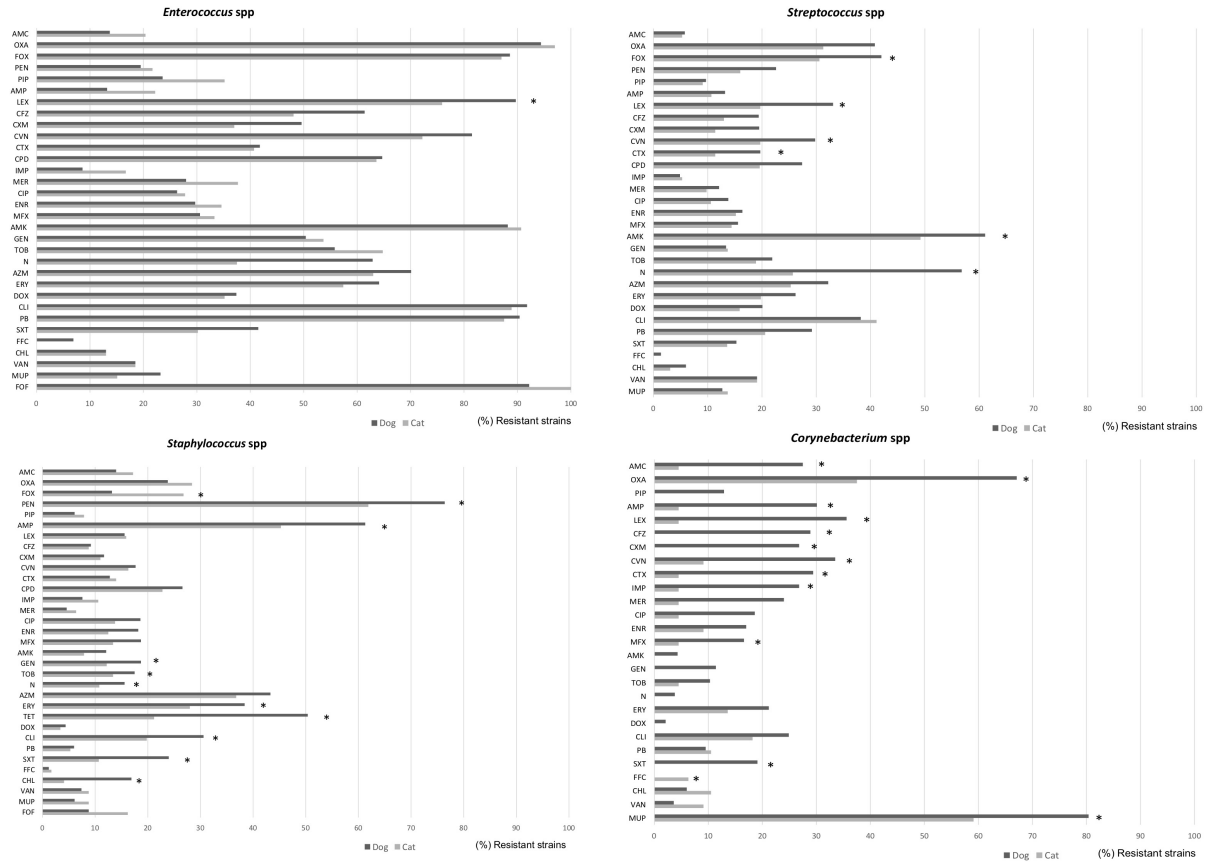

FIGURE 4 | Comparison of antimicrobial resistance frequencies in Gram negative bacteria isolated from dogs and cats. Statistical significance was calculated by Chi-square (χ^2) or Fishers Exact tests, $^*p < 0.05$. AMC, Amoxicillin-clavulanic; AMP, ampicillin; FOX, cefoxitin; LEX, cephalexin; CFZ, cefazolin; CEF, cephalotin; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; CVN, cefovecin; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; ENR, enrofloxacin; MFX, marbofloxacin; PRA, pradofloxacin; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; DOX, doxycycline; FOF, Fosfomycin; NIT, nitrofurantoin; and SXT, trimethoprim/sulfamethoxazole.

isolates showed resistance to cephalosporins, clindamycin and polymyxin B, and more than 50% of them were also resistant to aminoglycosides. These results are consistent with findings from Canada, United States, Portugal (Delgado et al., 2007; Jackson et al., 2009; Awosile et al., 2018), and Spain (unpublished data) where enterococcal isolates from urinary infections had similar levels of resistance to cephalosporins, clindamycin, and polymyxin B, but high levels of susceptibility to penicillin, ampicillin, and amoxicillin-clavulanate. Thus, oral ampicillin or amoxicillin which is commonly prescribed as a first line treatment for empirical therapy in enterococcal infections could be appropriate for the studied region. Nevertheless, the increased AMR to gentamicin observed in this study could compromise the effectivity of combined therapies with ampicillin or amoxicillin (Arias et al., 2010).

In this study, *Streptococcus* spp. were highly susceptible to several antimicrobials, including penicillin, ampicillin, amoxicillin-clavulanate, trimethoprim/sulfamethoxazole, fluoroquinolones, and allowing for several likely effective choices for empirical therapy. Similar susceptibility pattern of *Streptococcus* spp. has also been reported (Pedersen et al., 2007; Awosile et al., 2018). Nevertheless, our isolates from dogs presented the highest resistance percentage for amikacin and neomycin (>50%); this finding could compromise the bactericidal activity of therapies holding aminoglycosides for the empirical treatment of streptococcal infections in dogs of the studied region.

Among the Enterobacteriaceae family, *E. coli* and *Proteus* spp. were highly isolated from wounds, dermatitis, abscesses and otitis in dog specimens in this study. The reduced susceptibility patterns of these bacterial species was found to cephalosporins (1st generation, 30% for cephalexin) and to ampicillin (50%). *Proteus* isolates presented resistance to doxycycline and polymyxin B (>80%) as well. Ampicillin was used in the susceptibility test to predict activity of amoxicillin (Weese et al., 2019), and is a good first-line option for the treatment of sporadic bacterial cystitis associated to *E. coli* in cats and dogs (Weese et al., 2011, 2019). The use of this antimicrobial for empirical treatment of *E. coli* infections should be with caution due to the rapid development of resistance caused by beta-lactamase production (Boehmer et al., 2018). Nonetheless, our results

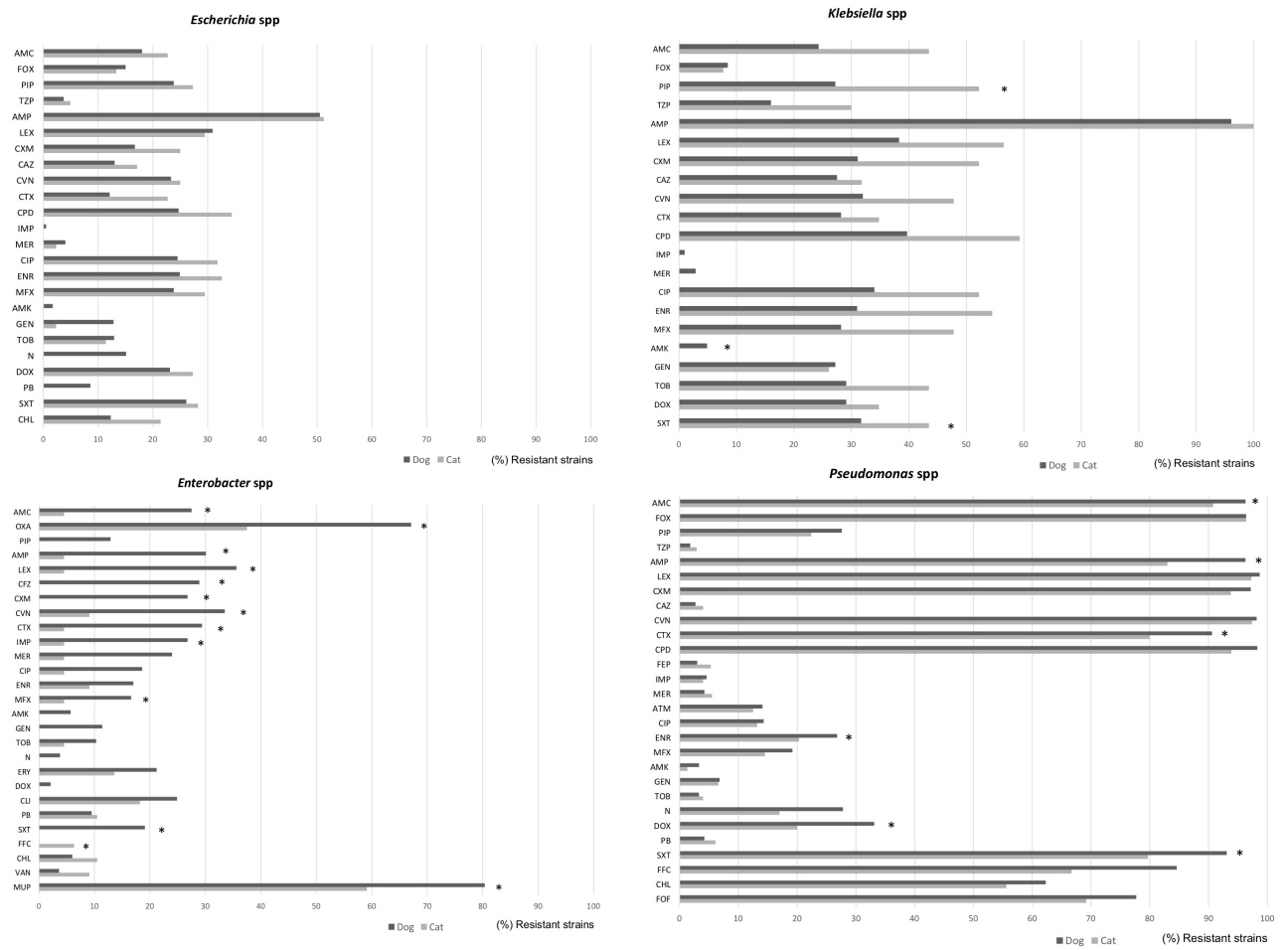

FIGURE 5 | Comparison of antimicrobial resistance frequencies in Gram positive bacteria isolated from dogs and cats. Statistical significance was calculated Chi-square (χ^2) or Fishers Exact tests, $^*p < 0.05$. AMC, Amoxicillin-clavulanic; AMP, ampicillin; LEX, cephalexin; CFZ, cefazolin; CXM, cefuroxime; CTX, cefotaxime; CVN, cefovecin; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; ENR, enrofloxacin; MFX, marbofloxacin; PRA, pradofloxacin; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; DOX, doxycycline; ERY, erythromycin; FOF, fosfomycin; NIT, nitrofurantoin; SXT, trimethoprim/sulfamethoxazole; and VAN, vancomycin.

support than other antimicrobials, also effective against *E. coli* and *Proteus* spp., such as amoxicillin-clavulanate, amikacin, and gentamicin could be included as empirical selection (Awosile et al., 2018).

In the present study, *E. coli* strains isolated from dogs and cats showed low levels of AMR (with the exception of ampicillin) in comparison with other members within the same family, i.e., *Klebsiella*, *Proteus,* or *Enterobacter* spp. Accordingly, *Enterobacter* strains from dog specimens showed higher levels of AMR for β-lactams, imipenem and mupirocin compared to cats. Moreover, *K. pneumoniae* from respiratory tract infections in cats presented in general higher resistance to antimicrobials than dog specimens, mainly for piperacillin, and trimethoprim/sulfamethoxazole.

On the other hand, high susceptibility to many antimicrobials has been observed for *Pasteurella* isolates from respiratory tract of cats and dogs. This is consistent with findings in other reports (Pedersen et al., 2007; Kroemer et al., 2014; Awosile et al., 2018). Clinically, doxycycline and amoxicillin-clavulanate are often used for the treatment of Pasteurella infections (Lappin et al., 2017). Since most of the isolates were highly sensitive to antimicrobials including fluoroquinolones, aminoglycosides, and trimethoprim/sulfamethoxazole which are reasoned to be used for the treatment of Pasteurella infections in cats and dogs.

The antimicrobial options for empirical therapy can be compromised in companion animals (Prescott et al., 2002; Jung et al., 2020) basically due to: (1) the increased incidence in the last years of antimicrobial-resistant bacteria such as MDR *Enterococcus* spp., *Enterobacter* spp., *P. aeruginosa* and *K. pneumoniae,* and (2) the extended AMR to other antimicrobial families (i.e., aminoglycosides, fluoroquinolones, and carbapenems). Of note, the results obtained from pets of this study are similar to those reported in human hospitals in Spain (ESTUDIO EPINE-EPPS, 2017). The most prevalent bacterial species found in human nosocomial and community infections are *E. coli* (19.5%), *S. aureus* (9%) and *P. aeruginosa* (8%),

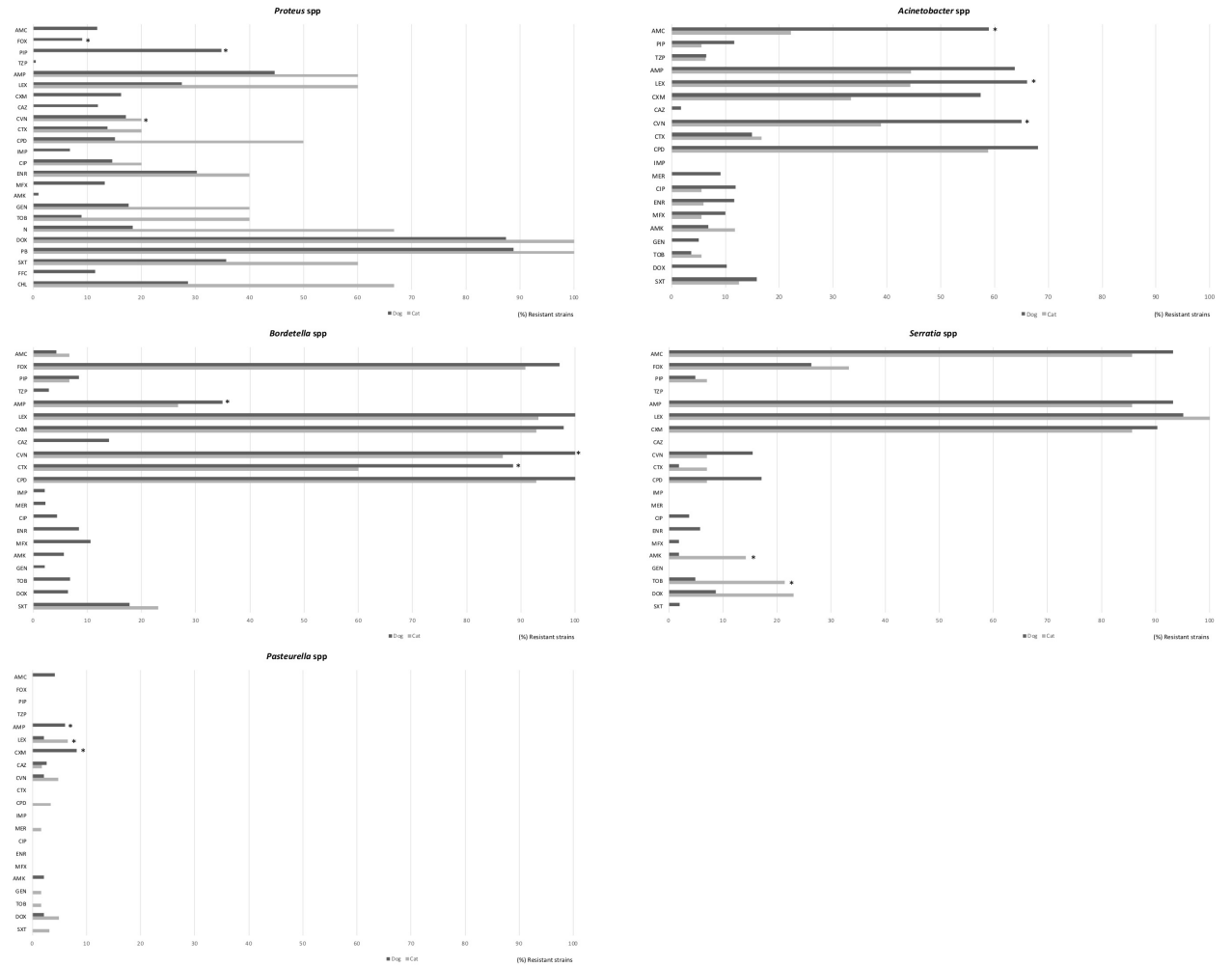

FIGURE 6 | Comparison of antimicrobial resistance frequencies in other bacteria spp. isolated from dogs and less representative from cats. Statistical significance was calculated by Chi-square ($\chi 2$) or Fishers Exact tests, $^{*}p < 0.05$. AMC, Amoxicillin-clavulanic; FOX, cefoxitin; PIP, piperacillin; TZP, piperacillin/tazobactam; AMP, ampicillin; LEX, cephalexin; CXM, cefuroxime; CAZ, ceftazidime; CVN, cefovecin; CTX, cefotaxime; CPD, cefpodoxime; IPM, imipenem; CIP, ciprofloxacin; ENR, enrofloxacin; MFX, marbofloxacin; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; N, Neomycin; DOX, doxycycline; PB, polymyxin B, SXT, trimethoprim/sulfamethoxazole; FFC, florfenicol, and CHL, chloramphenicol.

followed by *K. pneumoniae* (6.3%), *Enterococcus* spp. (5.8%), *P. mirabilis* (3.2%), and *Enterobacter* spp. (2.2%). Moreover, CMI90 results of Enterobacteriaceae isolated from dogs and cats of this study presented values for amoxicillin-clavulanate >32–16 mg/L, ceftazidime = 8 mg/L, cefotaxime = 4 mg/L, cefuroxime > 64 mg/L, cefoxitin > 32 mg/L, and piperacillin/tazobactam = 16–4 mg/L, which have been associated with a BLEE phenotype in *E. coli*, *K. pneumoniae,* and *E. cloacae* from human isolates (Canton, 2010). Finally, the presence of *Proteus* isolates from dogs with imipenem CMI90 > 4 mg/L is highly suspicious for carbapenemasa production. To prevent the selection of BLEEs and carbapenem- resistance profiles in both human and animal medicine, is very important to implement the One Health approach, and monitor the resistance patterns of these pathogenic bacteria in companion animals (ESTUDIO EPINE-EPPS, 2017; Nigg et al., 2019; Jung et al., 2020).

Some limitations have to be considered in the present study. Firstly, data on clinical history and antimicrobial usage were not available. Secondly, some cases might have been treated empirically prior to culture and susceptibility testing. Thirdly, the use of laboratory data may represent a bias toward resistance, since cultures from complicated cases tend to be requested more often than uncomplicated cases. Finally, isolates that exhibited intermediate resistance were classified as resistant, this could have biased the results to some extent toward overestimating the resistance levels among the tested strains.

Despite these limitations, the results of this study provides information on susceptibility patterns in major cat and dog bacterial isolates from the Iberian Peninsula. These results show *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *E. coli*, and *Enterococcus* spp. as the most predominant

bacteria in cats and dogs, and with the highest levels of AMR in *Enterococcus* spp. and *Pseudomonas* spp. Within the Enterobacteriaceae, *E. coli* presented low levels of AMR compared to *Klebsiella*, *Proteus* or *Enterobacter* spp. Since dogs and cats are supposed to act as reservoirs of AMR genes that may transfer to humans, data from this study combined with clinical judgment can be used as a guide for rationalizing antimicrobial treatment of companion animals, at least in the Iberian Peninsula. Finally, optimizing antimicrobial use in the vet clinics will benefit to limit the selection and spreading of resistant bacteria not only among our pets but also among the human population.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Authors declare no Institutional Animal Care and Use Committee (IACUC) or other approval declaration was needed.

AUTHOR CONTRIBUTIONS

YL and LD contributed to the analysis and the interpretation of the data and the writing of the manuscript. ID contributed to data collection. RF and RM-L contributed to data analysis. LD supervised the work. All authors contributed to the article and approved the submitted version.

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Market Chickens as a Source of Antibiotic-Resistant *Escherichia coli* in a Peri-Urban Community in Lima, Peru

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The widespread and poorly regulated use of antibiotics in animal production in low- and middle-income countries (LMICs) is increasingly associated with the emergence and dissemination of antibiotic resistance genes (ARGs) in retail animal products. Here, we compared *Escherichia coli* from chickens and humans with varying levels of exposure to chicken meat in a low-income community in the southern outskirts of Lima, Peru. We hypothesize that current practices in local poultry production result in highly resistant commensal bacteria in chickens that can potentially colonize the human gut. *E. coli* was isolated from cloacal swabs of non-organic (n = 41) and organic chickens (n = 20), as well as from stools of market chicken vendors (n = 23), non-vendors (n = 48), and babies (n = 60). 315 *E. coli* isolates from humans (n = 150) and chickens (n = 165) were identified, with chickens showing higher rates of multidrug-resistant and extended-spectrum beta-lactamase phenotypes. Non-organic chicken isolates were more resistant to most antibiotics tested than human isolates, while organic chicken isolates were susceptible to most antibiotics. Whole-genome sequencing of 118 isolates identified shared phylogroups between human and animal populations and 604 ARG hits across genomes. Resistance to florfenicol (an antibiotic commonly used as a growth promoter in poultry but not approved for human use) was higher in chicken vendors compared to other human groups. Isolates from non-organic chickens contained genes conferring resistance to clinically relevant antibiotics, including *mcr-1* for colistin resistance, *blaCTX-M* ESBLs, and *blaKPC-3* carbapenemase. Our findings suggest that *E. coli* strains from market chickens are a potential source of ARGs that can be transmitted to human commensals.

Keywords: AMR, genomics, LMIC, poultry, *Escherichia coli*, one health, WGS, Peru

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INTRODUCTION

Antimicrobial resistance (AMR) in human pathogens has become a major global health threat (O'Neill, 2014; World Health Organization [WHO], 2017b), with bacterial infections increasingly failing to first-line and "last-resort" antibiotic therapies. Decades of widespread antibiotic use in medicine and agriculture (Silbergeld et al., 2008) have resulted in the emergence and spread of various resistance determinants in microbial populations. In particular, the increasing demand for animal protein has led to a dramatic modernization of agriculture, including the regular use of antibiotics in feed to promote animal growth in addition to their therapeutic use. At low (sub-inhibitory) but constant dosages, antibiotics serve as growth promoters by reducing the levels of pathogenic strains and altering the microbiota to allow the host for more nutrient uptake (Evans and Wegener, 2003). This selective pressure has dramatically increased the rate of resistance to various drugs in the microbiota of farm animals, including commensals and pathogens alike (Woolhouse et al., 2015; Robinson et al., 2016; Liu et al., 2018; Nadimpalli et al., 2018; Van Boeckel et al., 2019). Resistant strains can be transmitted from animals to humans through meat consumption, direct animal contact, and exposure to environmental runoff (Hoelzer et al., 2017). Furthermore, horizontal gene transfer can enable the rapid exchange of resistance determinants between different bacterial lineages across hosts and environments (Marshall and Levy, 2011; Woolhouse et al., 2015). Because most antibiotic resistance genes (ARGs) are found in bacteria isolated from both humans and animals, the direction of transfer of most such genes and resistant organisms can be difficult to demonstrate.

We previously surveyed the antibiotic resistomes in the guts of healthy adults in a peri-urban community south of Lima and found high diversity and abundance of genes encoding resistance to amphenicol antibiotics (Pehrsson et al., 2016). A recent study in Cambodia compared *E. coli* isolates from humans, meat, and fish and found moderate levels of amphenicol resistance in human isolates (Nadimpalli et al., 2019). Although used widely until the 1980s, chloramphenicol is now rarely prescribed in human medicine in Peru and is banned from food animal production since 2013 (Diario Oficial El Peruano, 2013). However, florfenicol (a fluorinated thiamphenicol analog) is widely employed in broiler farming therapeutically and as a growth promoter and available in various commercial feed premixes (FAO, 2014). This has led to the hypothesis that amphenicol resistance in human commensals did not emerge from clinical use, but in food animal populations due to extensive veterinary use of chloramphenicol, florfenicol, and other related compounds. Chickens, most of which now are grown under local intensive farming systems, provide the primary source of animal protein for the Peruvian population (World Bank Group, 2017). Average per capita consumption was estimated at 49.5 kg in 2018, and up to 80.5 kg per person per year in the capital of Lima (Ministerio de Agricultura y Riego [MINAGRI], 2018).

We hypothesize that current practices in poultry production and handling in LMICs result in highly resistant chicken commensals that can potentially colonize the human gut. To test this, we assessed the distribution of resistant *E. coli* and associated ARGs in market chickens, chickens grown without antibiotics (organic chickens), and residents from a low-income, peri-urban community in Lima, with varying levels of exposure to poultry.

MATERIALS AND METHODS

Study Site

Local market stalls in Villa El Salvador (VES) and its neighboring district, San Juan de Miraflores (SJM) in southern Lima, were visited to purchase whole chickens. Human fecal samples were collected from the community surrounding the VES market (see **Supplementary Table 1**). These neighboring districts share similar demographic characteristics and contain various urban informal settlements (Instituto Nacional de Estadística e Informática [INEI], 2017). Informal housing arrangements, lack of running water, and inadequate sanitation in most households make these sites representative of peri-urban settlements in other LMICs, which are considered hotspots for AMR (Nadimpalli et al., 2020). We also collected laying hens' samples from an organic free-range farm in Vegueta (VEG), located approximately 150 km north of Lima.

Samples

Humans

Fecal samples were collected in March 2018 from three resident groups in the VES community: chicken vendors ($n = 23$) working in the markets where chickens were purchased, babies ($n = 60$) between 1 and 24 months old from an ongoing cohort study in the community, and non-vendor adults ($n = 48$). Fresh feces were collected by individuals and legal guardians as instructed. Fecal samples were swabbed and placed vials with Cary-Blair transport medium, stored at 4°C, and transferred to the laboratory for further processing. Ethical approval was obtained from Institutional Review Boards at Universidad Peruana Cayetano Heredia and Asociación Benéfica Prisma.

Market (Non-organic) Chickens

Forty-one recently slaughtered whole chickens were purchased in 14 market stalls of VES and SJM from March to April of 2018. Whole chickens and market stands were selected by convenience. We have no information on the exact rearing conditions or origin of these chickens. However, almost all of the chicken meat sold in Lima originates from conventional local production systems that heavily rely on routine antibiotic use as a standard industry practice. Chickens were taken to field laboratories for the collection of intestinal contents. Cloacal and intestinal swabs were put in sterile tubes with saline solution and transferred within 2 h to the laboratory for bacterial culture.

Organic Laying Hens

Cloacal swabs from 20 laying hens from the sole Certified Humane® (Humane Farm Animal Care [HFAC], 2018) organic free-range farm in Lima were obtained in May of 2019 to have a set of isolates originating from poultry raised without antibiotics as a comparison group to the market chickens. Cloacal swabs

were put in sterile tubes with Cary-Blair transport medium, stored at 4°C, and transferred to the laboratory for processing.

Culture and Isolation

Samples were streaked in CHROMagar Orientation Media (CHROMagar Microbiology, Paris, France) for rapid differentiation and presumptive identification of *E. coli*. Up to 3–5 dark pink to red colonies indicative of *E. coli* were re-streaked to MacConkey agar (Becton Dickinson, Heidelberg, Germany) for lactose fermentation confirmation and then selected for species confirmation with a conventional biochemical profiling panel (Garrity et al., 2005). Those confirmed as *E. coli* (n = 315) were included in the study and stored in Tryptic soy broth (TSB, Becton Dickinson) with glycerol at −20°C until DNA extraction.

Antibiotic Susceptibility Testing

Disk diffusion tests were performed with CLSI 2018 standards, using susceptible, intermediate, and resistant definitions for *Enterobacteriaceae* (Clinical and Laboratory Standards Institute (CLSI), 2018). A total of 18 antibiotics were used (see **Supplementary Table 2**). Extended-spectrum β-lactamase (ESBL) activity was detected using the cefotaxime-ceftazidime-cefepime-aztreonam with amoxicillin with clavulanic acid test, according to EUCAST standards (The European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2017). We interpreted florfenicol susceptibility using chloramphenicol's CLSI breakpoints as there are no approved cut-off values for *E. coli* (White et al., 2000; Clinical and Laboratory Standards Institute (CLSI), 2018). We did not report on colistin phenotypic resistance due to the lack of recommended cut-off values for colistin disk diffusion testing (Ezadi et al., 2018). A multidrug-resistant drug isolate was defined as expressing phenotypic resistance to three or more antibiotic classes (Magiorakos et al., 2012).

DNA Extraction and WGS

DNA was extracted from 1 ml TSB culture using the GeneJet Genomic DNA purification kit (Thermo Fisher Scientific, Waltham, MA, United States) following the manufacturer's instructions. DNA was eluted in 200 μl Tris-EDTA buffer and quantified using the Qubit dsDNA BR Kit (Thermo Fisher Scientific). We selected a subset of 118 isolates for WGS on the Illumina MiSeq platform. We randomized isolate selection within each study group to include representative drug susceptibility patterns. Libraries were prepared from 1 ng gDNA with the Nextera XT kit (Illumina, San Diego, CA, United States). Batches of 24 libraries were indexed and sequenced with MiSeq v3 sequencing kits to generate 300 bp paired-end reads and yield a mean of 84x genome coverage (minimum 17x, maximum 163x). Raw Illumina reads were uploaded to GenBank under BioProject PRJNA633873.

Genomic and Phylogenetic Analyses

Raw reads were assessed with FastQC v0.11.9, trimmed with Trimmomatic v0.36.6 (Bolger et al., 2014), assembled with SPAdes v.3.10.0 (Bankevich et al., 2012), and annotated with Prokka v1.5 (Seemann, 2014). MLST was determined from *de novo* assemblies using the CGE pipeline (Thomsen et al., 2016) based on the Enterobase scheme[1] accessed through PubMLST[2]. ARGs were annotated by querying assemblies against the CARD database (Alcock et al., 2020) at >90% identity. We clustered ARG-containing contigs with CD-HIT (Li and Godzik, 2006) at an 80% similarity threshold over the contig's length. Plasmid typing was done using the PlasmidFinder database (Carattoli et al., 2014) and BLAST (Ye et al., 2006) to identify assemblies containing an Inc reference gene, with a threshold of 90% identity and E-value <1e-35. Prokka-annotated assemblies were used as input for Roary v3.13.0 (Page et al., 2015) to determine the pangenome and perform a core gene alignment of all sequenced isolates using blastp identity threshold of 95%. Variable positions were extracted from an alignment of 2,233 core genes (2,252,390 bp) and used to build a maximum-likelihood phylogenetic tree with RAxML v8.2.4 (Stamatakis, 2014) with the general time-reversible (GTR) substitution model and gamma correction for rate heterogeneity. SNP-dists v0.7.0 was used to build a pairwise SNP distance matrix from the pangenome alignment. A published genome of *Escherichia fergusonii* (Manninger et al., 2016) was used to root the phylogenetic tree. CLC Genomics Workbench v20.0 (QIAGEN Bioinformatics) was used to visualize and annotate the tree.

Statistical Analysis

The proportion of resistant isolates was tabulated for each sample type. Comparisons of proportions were evaluated using the Chi-square test or Fisher's exact test as appropriate. Data management and statistical analysis were performed with a confidence level of 95% using STATA 16 (StataCorp, College Station, TX, United States) and R (v3.5.2).

RESULTS

Antimicrobial Susceptibility

Chickens

Escherichia coli isolates were obtained from market (non-organic) (n = 130) and organic (n = 35) chickens. Multidrug-resistant (MDR) rates were higher in non-organic animals (76.9 vs. 11.4%, $p < 0.001$, Chi-square test). Only the non-organic chicken isolates were ESBL producers (39.2%, n = 51), and presented resistance to at least five antibiotic families (46.2%, n = 60), including chloramphenicol (62.3%, n = 81), florfenicol (52.3%, n = 68), and meropenem (0.8%, n = 1). These isolates presented the highest resistance levels to almost every antimicrobial tested. In contrast, the organic chicken isolates were susceptible gentamicin, amoxicillin with clavulanic acid, cefotaxime, cefepime, ceftazidime, and cefoxitin (**Figure 1**). A comparison of resistance rates is detailed in **Table 1**.

[1]http://enterobase.warwick.ac.uk/
[2]https://pubmlst.org/

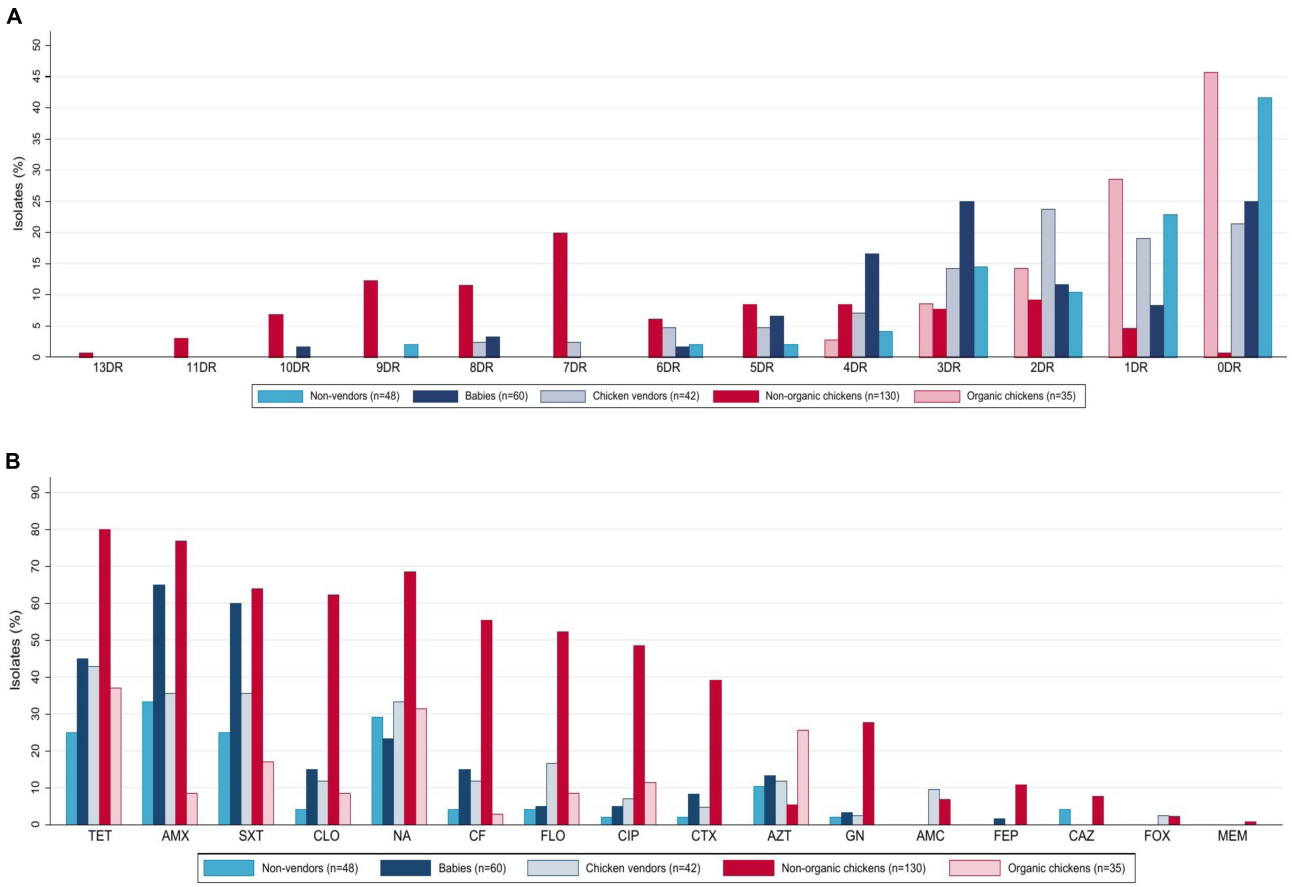

FIGURE 1 | Phenotypic antibiotic resistance of 315 *E. coli* isolates from humans and chickens in Lima, Peru. **(A)** Isolates were grouped by number of antibiotics to which they were resistant based on disk-diffusion assays. **(B)** Based on resistance to 16 antibiotics. **(A)** Percentage of resistances to different drugs, DR, drug resistance. **(B)** Resistance patterns to different antibiotics, TET, tetracycline; AMX, amoxicillin; SXT, trimethoprim/sulfamethoxazole; NA, nalidixic acid; CLO, chloramphenicol; CF, cefalotin; FLO, florfenicol; CIP, ciprofloxacin; CTX, cefotaxime; AZT, azithromycin; GN, gentamicin; AMC, Amoxicillin with Clavulanic Acid; FEP, cefepime; CAZ, ceftazidime; FOX, cefoxitin; MEM, meropenem.

Humans

Human isolates (n = 150) were obtained from babies aged 0–2 years (40%, n = 60), adult non-vendors (32%, n = 48), and chicken vendors in local markets (28%, n = 42). MDR isolates were more frequent in chicken vendors (38.1%, n = 16) compared to non-vendors (22.9%, n = 11). Isolates from chicken vendors presented higher resistance rates to florfenicol (16.7%, n = 7) compared to non-vendor adults (4.2%, p = 0.077, Fisher's exact test) and babies (5%, p = 0.087, Fisher's exact test). However, they were not more resistant to chloramphenicol (11.9 vs. 4.2%, p = 0.245, Fisher's exact test). *E. coli* isolates from babies presented high resistance levels to tetracycline (45%, n = 27), trimethoprim/sulfamethoxazole (60%, n = 36), amoxicillin (65%, n = 39), azithromycin (13.3%, n = 8), chloramphenicol (15%, n = 9), cefalotin (15%, n = 9), cefotaxime (8.3%, n = 5), and gentamicin (3.3%, n = 2).

Chickens Versus Humans

Overall, resistance rates were higher among chicken *E. coli* compared to human isolates (**Table 1**), including MDR (63 vs. 37.3%, $p < 0.001$, Chi-square test) and ESBL-producing *E. coli* (30.9 vs. 4.0%, $p < 0.001$, Chi-square test). Additionally, we found higher florfenicol resistance in 43.1% (n = 71) of chicken isolates and 16.7% (n = 7) of chicken vendors compared to other groups. Further resistance results are shown in **Table 1**.

Genomic Analysis

We selected a random subset of 118 isolates from babies (n = 19), adults (n = 22), chicken vendors (n = 23), non-organic chickens (n = 31), and organic chickens (n = 23) to further understand the flow of *E. coli* phylogroups and ARGs between animals and humans. The genomic dataset had a mean N50 of 102,136 bp (SD = 49,584 bp) and a mean total length of 4,490,970 bp (SD = 1,222,343 bp). Pangenome analysis using Roary identified a core genome (i.e., genes found in ≥99% of isolates) of 2,304 genes and an accessory genome (found in ≤15% of isolates) of 26,135 genes. To assess the genomic similarity between isolates, we built a maximum-likelihood phylogenetic tree from the pangenome alignment (**Figure 2**) and calculated all pairwise SNP distances (**Supplementary Figure 1**). We

TABLE 1 | Resistance profiles and bivariate analysis of *E. coli* isolates from chickens and humans.

Results	Humans							Chickens				Total (n=315)	p^e
	Total (n=150)	N (%)			p^a	p^b	p^c	Total (n=165)	N (%)		p^d		
		Non-vendor adults (n=48)	Babies (n=60)	Vendors (n=42)					Non-organic (n=130)	Organic (n=35)			
Multidrug-resistance													
Yes	56 (37.3)	11 (22.9)	29 (48.3)	16 (38.1)	0.007	0.305	0.117	104 (63)	100 (76.9)	4 (11.4)	<0.001	160 (50.8)	<0.001
ESBL													
Yes	6 (4)	1 (2.1)	4 (6.7)	1 (2.4)	0.379*	0.646*	1.000*	51 (30.9)	51 (39.2)	0 (0)	<0.001	57 (18.1)	<0.001
Amphenicols													
Cloramphenicol	16 (10.7)	2 (4.2)	9 (15)	5 (11.9)	0.107*	0.655	0.245*	84 (50.9)	81 (62.3)	3 (8.6)	<0.001	100 (31.8)	<0.001
Florfenicol	12 (8)	2 (4.2)	3 (5)	7 (16.7)	1.000*	0.087*	0.077*	71 (43.1)	68 (52.3)	3 (8.6)	<0.001	83 (26.4)	<0.001
Tetracyclines													
Tetracycline	57 (38)	12 (25)	27 (45)	18 (42.9)	0.032	0.830	0.073	117 (70.9)	104 (80)	13 (37.1)	<0.001	174 (55.2)	<0.001
Sulfonamides													
Trimethoprim/ sulfamethoxazole	63 (42)	12 (25)	36 (60)	15 (35.7)	<0.001	0.016	0.268	89 (53.9)	83 (63.9)	6 (17.1)	<0.001	152 (48.3)	0.034
Aminoglycosides													
Gentamicin	4 (2.7)	1 (2.1)	2 (3.3)	1 (2.4)	1.000*	1.000*	1.000*	36 (21.8)	36 (27.7)	0 (0)	<0.001	40 (12.7)	<0.001
Macrolides													
Azithromycin	18 (12)	5 (10.4)	8 (13.3)	5 (11.9)	0.643	0.831	1.000*	16 (9.7)	7 (5.4)	9 (25.7)	<0.001	34 (10.8)	0.511
Penicillins													
Amoxicillin	70 (46.7)	16 (33.3)	39 (65)	15 (35.7)	0.001	0.004	0.813	103 (62.4)	100 (76.9)	3 (8.6)	<0.001	173 (54.9)	0.005
Amoxicillin with Clavulanic Acid	4 (2.7)	0 (0)	0 (0)	4 (9.5)	N.A.	0.026*	0.044*	9 (5.5)	9 (6.9)	0 (0)	0.207*	13 (4.1)	0.214
Cephalosporins													
Cefalotin	16 (10.7)	2 (4.2)	9 (15)	5 (11.9)	0.107*	0.655	0.245*	73 (44.2)	72 (55.4)	1 (2.9)	<0.001	89 (28.3)	<0.001
Cefotaxime	8 (5.3)	1 (2.1)	5 (8.3)	2 (4.8)	0.223*	0.697*	0.597*	51 (30.9)	51 (39.2)	0 (0)	<0.001	59 (18.7)	<0.001
Cefepime	1 (0.7)	0(0)	1 (1.7)	0 (0)	1.000*	1.000*	N.A.	14 (8.5)	14 (10.8)	0 (0)	0.042*	15 (4.7)	0.001
Ceftazidime	2 (1.3)	2 (4.2)	0(0)	0(0)	0.195*	N.A.	0.497*	10 (6.1)	10 (7.7)	0 (0)	0.122*	12 (3.8)	0.029
Cefoxitin	1 (0.7)	0 (0)	0 (0)	1 (2.4)	N.A.	0.412*	0.467*	3 (1.8)	3 (2.3)	0 (0)	1.000*	4 (1.3)	0.624*
Carbapenems													
Meropenem	0 (0)	0 (0)	0 (0)	0 (0)	N.A.	N.A.	N.A.	1 (0.6)	1 (0.8)	0 (0)	1.000*	1 (0.3)	1.000*
Quinolones													
Nalidixic Acid	42 (28)	14 (29.2)	14 (23.3)	14 (33.3)	0.492	0.265	0.670	100 (60.6)	89 (68.5)	11 (31.4)	<0.001	142 (45.1)	<0.001
Ciprofloxacin	7 (4.7)	1 (2.1)	3 (5)	3 (7.1)	0.627*	0.688*	0.336	67 (40.6)	63 (48.5)	4 (11.4)	<0.001	74 (23.5)	<0.001

*P-values: Chi-square test and confidence level of 95%, *Fisher exact test and confidence level of 95%.*

p^a: babies and non-vendor adults, p^b: babies and chicken vendors, p^c: chicken vendors and non-vendor adults, p^d: non-organic and organic chickens, p^e: humans and chickens. ESBL, Detection of Extended Spectrum Beta-lactamases; N.A., not applicable.

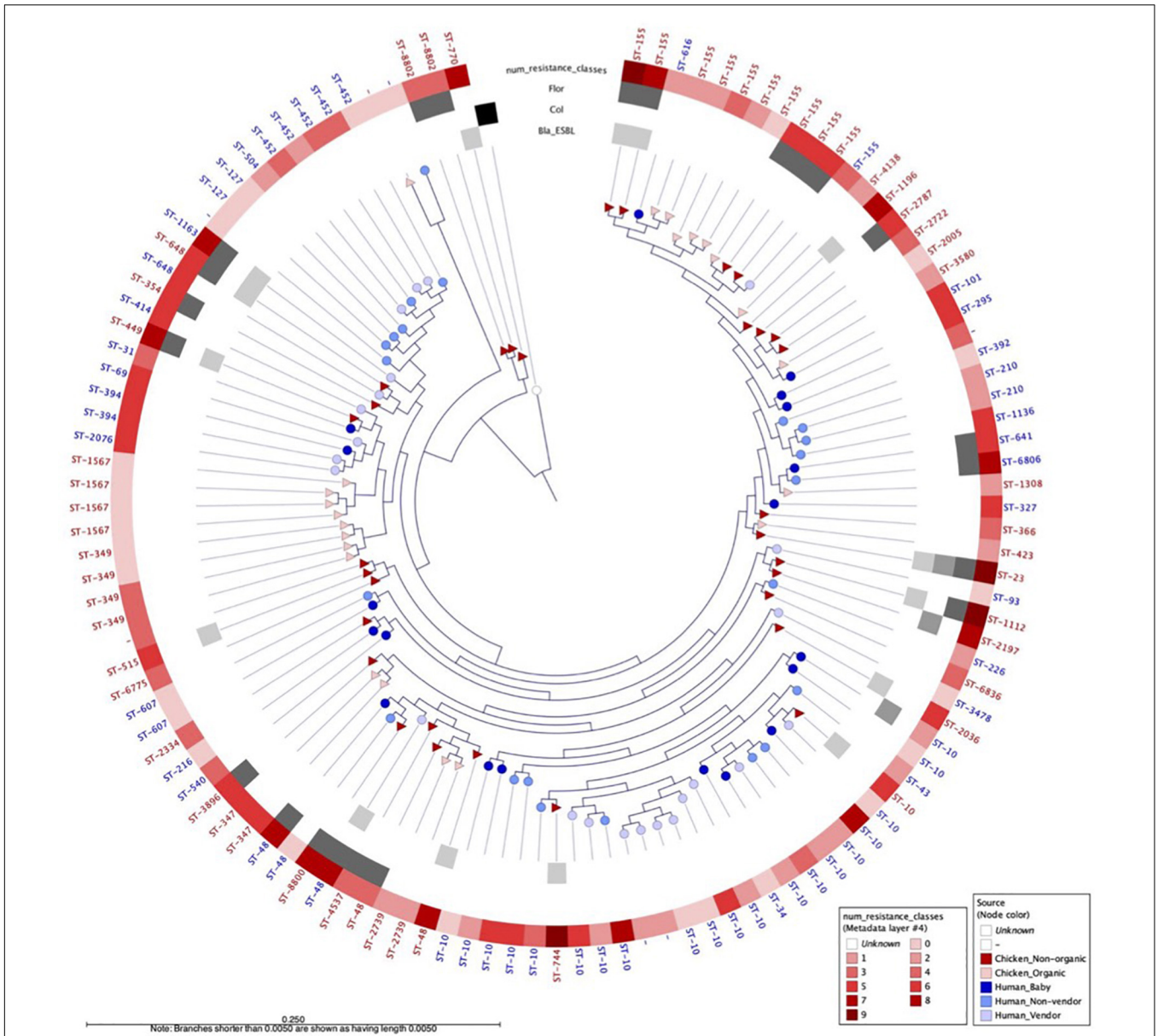

FIGURE 2 | Maximum likelihood phylogenetic tree built from the alignment of 2,233 *E. coli* core genes from 118 human and animal isolates, using *E. fergusonii* as outgroup. Nodes are shaped by host type (circle = human, triangle = chicken) and colored by sampling group. Outer gray circles indicate presence of ESBL, *mcr-1*, and *floR* genes. Outer red circles indicate the number of antibiotic classes to which the isolate is resistant. WGS-based sequence type (ST) is indicated for each isolate.

identified 58 sequence types (ST) and 14 clonal complexes in the dataset (**Figure 3**). ST-10 (n = 21), ST-155 (n = 11), ST-48 (n = 5), and ST-648 (n = 2) were assigned to isolates of both animal and human origin. Highly similar isolates (differing in less than 100 SNPs across their pangenomes) were only found within host groups. STs shared by humans and chickens were more distantly related: ST-155 isolates (differing in 951 SNPs) were found in organic chickens and babies; ST-10 (1,046 SNPs), ST-155 (1,141 SNPs), ST-48 (1,542 SNPs), and ST-648 (13,470 SNPs) were shared by chicken vendors, non-vendors and market chickens.

We identified 604 ARG hits and 81 unique ARGs in the dataset (**Figure 4**) with a mean of 5.1 genes (95%CI: 4.2–6.0) per isolate. Detected ARGs are associated with resistance to beta-lactams (n = 30), aminoglycosides (n = 18), trimethoprim (n = 7), amphenicols (n = 4), tetracyclines (n = 4), quinolones (n = 4), sulfonamides (n = 3), fosfomycins (n = 2), lincosamides (n = 2), macrolides (n = 1), glycopeptides (n = 1), polymyxins (n = 1), streptogramins (n = 1), and streptothricins (n = 1). Fifteen isolates (13 from market chickens and two from vendors) were positive for ESBLs; we found *blaCTX-M-55* in 73% (11/15) of them, in plasmid contigs that shared >96% sequence

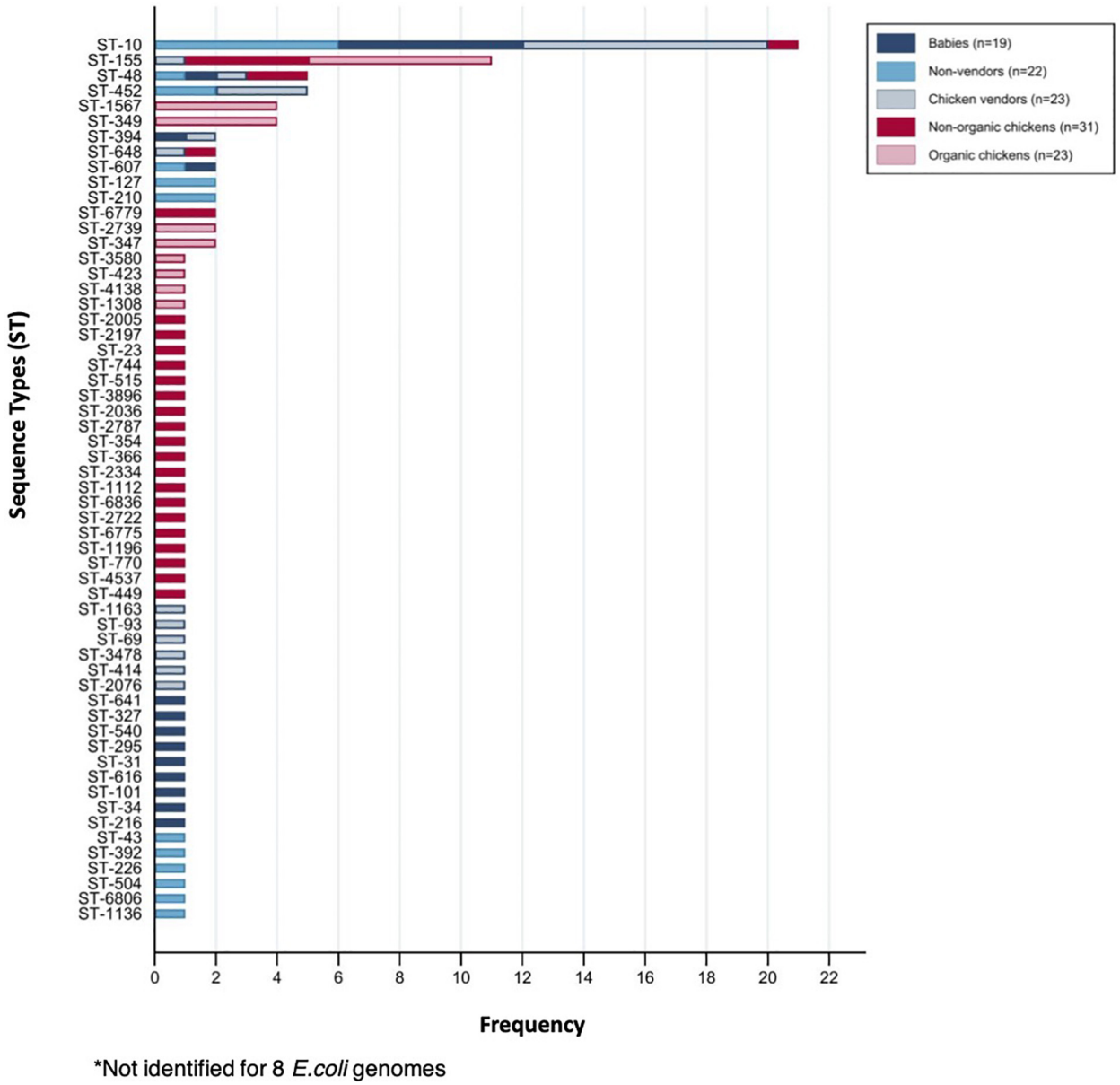

FIGURE 3 | Sequence types (ST) identified among sequenced *E. coli* isolates.

similarity between chickens and vendors. We found the *blaKPC-3* gene encoding carbapenem resistance in one market chicken isolate. Additionally, three isolates (two from market chickens and one from a baby) had the *mcr-1* colistin resistance gene (**Figure 2**). Forty-five plasmid replicon markers were identified in both humans and chickens (**Supplementary Data Set 1** and **Supplementary Figure 2**). The most frequent markers were IncFIB (AP001918) (42.4%), Col (pHAD28) (35.6%), and IncFII (28%). Some markers were found in only one host type, such as IncB/O/K/Z_4 ($p < 0.001$) and Col156 ($p = 0.003$) in humans, and IncHI1B ($p < 0.001$) in chickens (**Supplementary Figure 2**). We did not find significant differences in plasmid markers among CTX-M, mcr-1, and blaKPC-3 producers from chickens and humans (**Supplementary Table 3**). We identified the *floR* gene in 18.6% (22/118) of genomes, and their contigs clustered into eight unique (>80% identity) sequences that matched to plasmid replicons of the IncF family (**Figure 5**). They shared a common theme in which *floR* was often found along with other antibiotic resistance genes (*tetA*, *APH(6)-Id*, *sul2*) and proteins predicted to be involved in horizontal gene transfer and DNA recombination (transposases, resolvases, recombinases, relaxases). This suggests that *floR* has been transferred on multiple occasions to MDR plasmids commonly shared by animal and human hosts. The *cmlA1* and *catA1* genes (which encode resistance to chloramphenicol but not florfenicol) were found in 11 and four genomes, respectively. A summary of the genomic

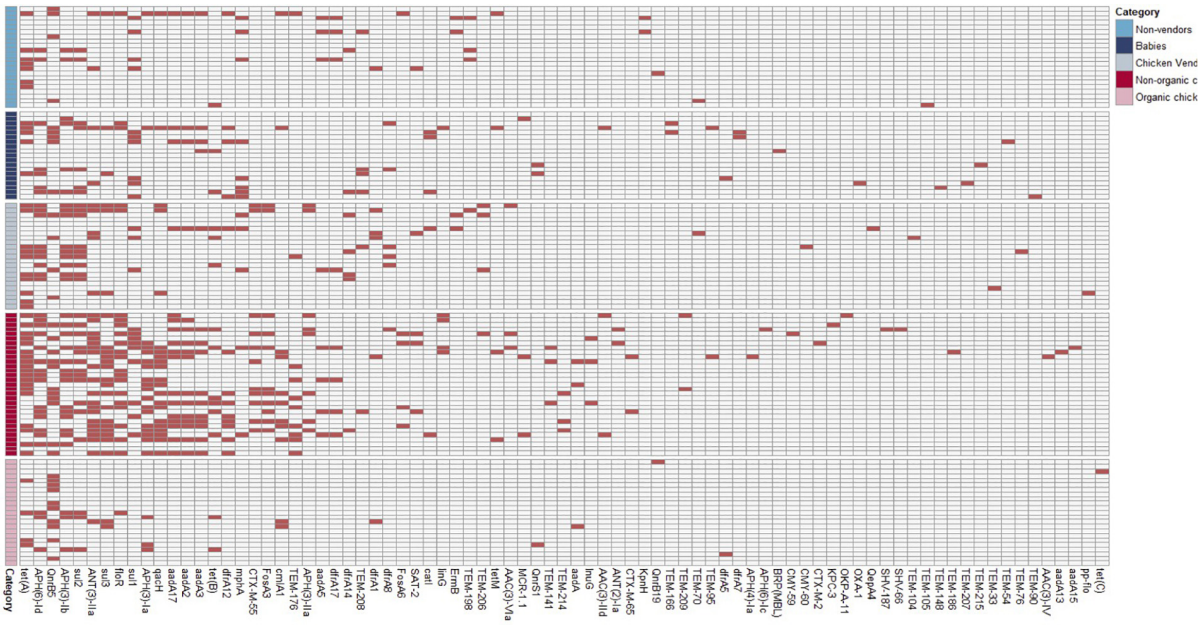

FIGURE 4 | Antimicrobial resistance genes detected among sequenced *E. coli*.

analysis is described in **Supplementary Data Set 2**. All 604 ARG hits are listed in **Supplementary Table 4**.

DISCUSSION

We compared the resistance rates, genotype distributions, and ARGs present in commensal *E. coli* isolates from human and chicken populations. 315 *E. coli* isolates from humans (n = 150) and chickens (n = 165) were identified, with chickens showing higher rates of MDR (63 vs. 37.3%) and ESBL (30.9 vs. 4%) phenotypes. Poultry production is one of the largest and most widespread industries in Peru, making use of large quantities of various antimicrobials critical for human medicine (Page and Gautier, 2012; World Health Organization [WHO], 2017a). Despite their importance for treatment and disease prevention, their extended and unregulated use as growth promoters increases selective pressure for MDR bacteria (Diarra and Malouin, 2014). Our results highlight the potential consequences of this practice in poultry production.

Given that many LMICs are now transitioning to industrial models of animal production, there is a concern that extensive animal exposure to antibiotics may result in the "spillover" of resistant bacteria and ARGs into humans. Although ARG transfer has been extensively studied in pathogenic organisms, the vast majority of transfer events occur silently among non-pathogenic bacteria in host-associated and environmental microbial communities (Smillie et al., 2011; Pehrsson et al., 2016; Wang et al., 2017). *E. coli* and members of the *Enterobacteriaceae* are well adapted to the gut environment, acquiring diverse functions and ARGs to colonize their hosts (Szmolka and Nagy, 2013). It is thus likely that ARGs can accumulate in commensal strains to enrich the human gut resistome, and later be mobilized into pathogenic strains to become multidrug-resistant (Penders et al., 2013).

Increased global consumer awareness of how animal meat is produced has increasingly lead to the establishment of organic and free-range farms (Holtcamp, 2011). This production model aims to stop the widespread use of antibiotics as prophylactics and growth promoters in chickens under the premise that it will reduce AMR rates in exposed bacteria due to an absence of this selective pressure (Tang et al., 2017). The lower rates of AMR found in organic chickens compared to conventionally raised ones support this assertion. Furthermore, organic chicken isolates were entirely susceptible to gentamicin, amoxicillin with clavulanic acid, cefotaxime, cefepime, ceftazidime, and cefoxitin; the first three, together with florfenicol, are frequently found as active ingredients in local commercially available premixes aimed toward infection prevention and enhancement of growth performance.

The number of peri-urban communities has increased dramatically in recent decades in Peru and other LMICs, on par with poorly regulated neighborhood markets. Despite regulatory authorities' supervision, many small markets function clandestinely for slaughtering to meet the consumers' demand for "fresh" goods. Such consumer preferences, combined with other external factors, result in the poultry industry trading around 80% of its chicken production live (De León, 2009). Consequently, poultry butchering and handling practices in market stalls and related environments (including households) pose a risk of exposure to fecal cross-contamination from the viscera, a possible transfer route of animal-derived *E. coli* into the human gut. Despite their close contact with chickens and regular manipulation of viscera, *E. coli* isolates from chicken vendors

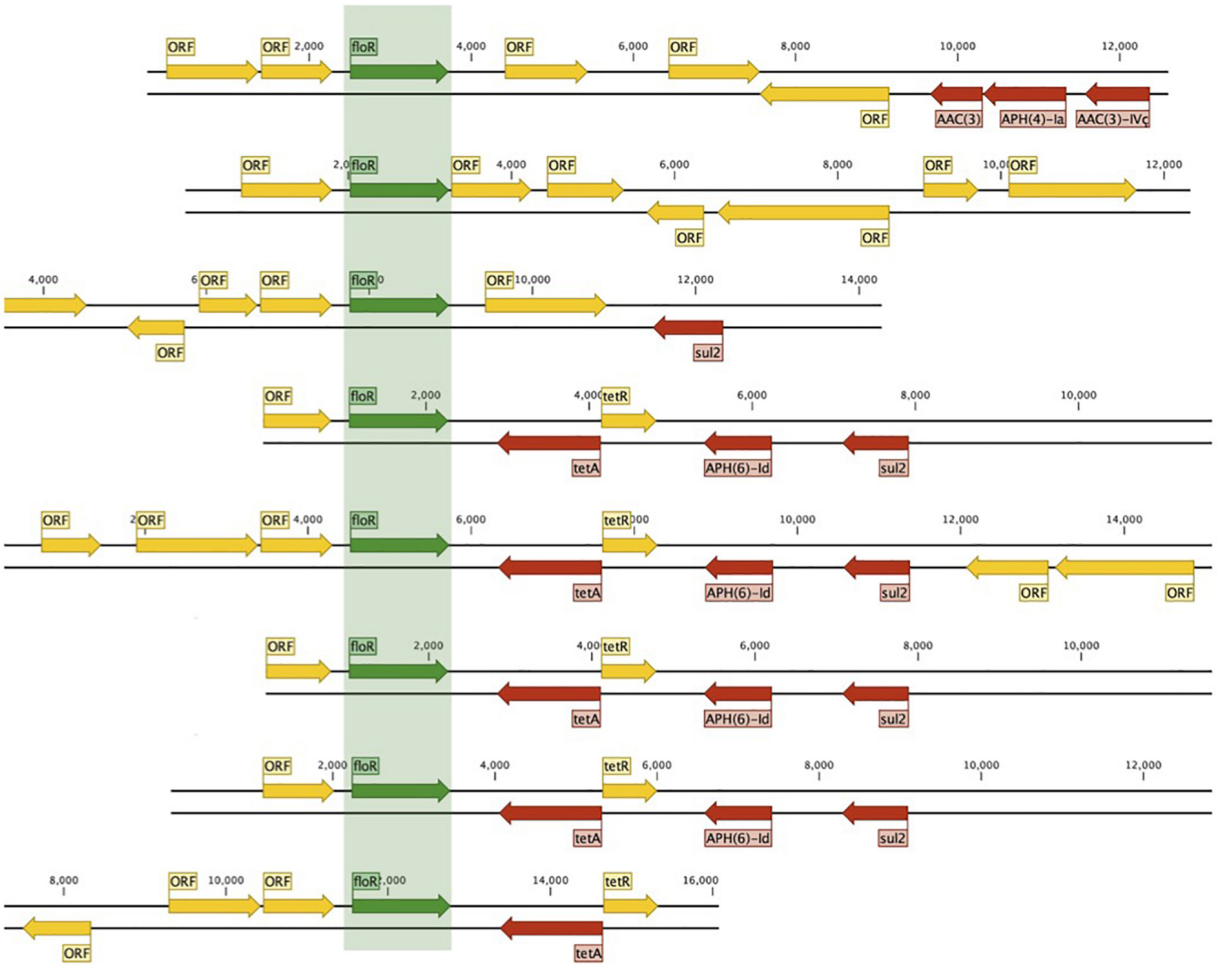

FIGURE 5 | Mobilization of *floR* in conjugative plasmids from animals and humans. Eight unique (80% ID clustering) *floR*-containing plasmids were found in 22/118 humans and chicken isolates.

did not fully match the resistance patterns observed in chicken isolates; this may be in part because the use of antibiotics to treat human infections also determine the resistance patterns of *E. coli* in the human gut. Shared STs (e.g., ST-10, ST-155, ST-48) were found in both chicken vendors and market chickens, coinciding with previous reports of globally successful STs linked to zoonotic transmission (Cohen Stuart et al., 2012; Yamaji et al., 2018; Falgenhauer et al., 2019; Hussain et al., 2019). However, shared STs differed in 900+ SNPs across their core genomes, which rules out a direct transmission between hosts and may reveal host-specific adaptations in *E. coli*.

Florfenicol, which is not approved for use in humans, was the only antibiotic tested for which resistance levels were significantly higher in chicken vendors than other human groups. We found florfenicol resistance in 43% of non-organic chicken isolates and 17% of chicken vendors. The *floR* gene was found in 17 *E. coli* genomes from chickens and five from humans and was associated with conjugative plasmids that were highly similar between humans and animal isolates (**Figure 5**). The high diversity of *floR*-carrying plasmids and the fact that they were identified in 15 different STs may reflect a strong selective pressure to maintain resistance to florfenicol in chicken *E. coli* populations. The *floR* gene confers resistance to florfenicol and chloramphenicol via an efflux pump mechanism (Bischoff et al., 2005; Braibant et al., 2005; Van Hoek et al., 2011) and is readily transferred among Gram-negative bacterial lineages via conjugative plasmids (Kruse and Sorum, 1994; Singer et al., 2004). We hypothesize that resistance to florfenicol in humans may occur via the colonization by *floR*-positive strains of animal origin or plasmid conjugation from animal strains into human commensals, both facilitated by improper handling of chicken meat by both vendors and consumers. This identifies *floR* as a potential marker of antibiotic resistance in humans that can be traced directly to antibiotic use in animals.

Resistance to last-resort drugs such as colistin and carbapenems is increasing worldwide (Peyclit et al., 2019). The *blaKPC-3* gene and phenotypic resistance to meropenem were observed in one market chicken isolate. The *bla*KPC-2 gene had been described in *Klebsiella pneumoniae* from Peruvian hospital settings (Horna et al., 2017; Roach et al., 2020) but

this is, to our knowledge, the first report of KPC-3 in Peru; its origins and spread into animal populations warrant further study. Three isolates harboring the colistin resistance *mcr-1* gene were found in humans and chickens. Colistin is used to treat human infections caused by carbapenem-resistant bacteria (Nation et al., 2017) and *mcr-1* has already been reported in local *E. coli* and *K. pneumoniae* clinical isolates (Ugarte-Silva et al., 2018; Deshpande et al., 2019). The import and trade of colistin in veterinary products was banned in Peru in 2019 (Diario Oficial El Peruano, 2019) but they were still in use in poultry farms at the time of sampling.

Despite our initial assumption that babies would present lower rates of resistance compared to adults, they had similar resistance profiles to chicken vendors and had higher rates of phenotypic resistance to amoxicillin and trimethoprim/sulfamethoxazole than adults. This supports the findings of a previous study in Peru that found older age protective against resistance (Kalter et al., 2010). Children are prone to play in soils and have a higher risk of colonization with enteropathogens via the fecal-oral route (Marquis et al., 1990; Lietzau et al., 2007; Fuhrimann et al., 2016). The effect may be exacerbated in this community, where water and adequate sanitation are not available in all households. Surveys collected during an ongoing cohort study in VES (unpublished data) indicate that the most commonly used antibiotics in this group were amoxicillin and amoxicillin with clavulanic acid, followed by trimethoprim/sulfamethoxazole and erythromycin, consumed between the first 2 months up to 2 years at a rate of 3.8 courses per child-year (Nadimpalli et al., 2020). Predictably, 63.9% of the baby isolates in our study exhibited resistance to amoxicillin and 52.5% to trimethoprim/sulfamethoxazole. Other antibiotics administered to this group but with no evidence of resistance were cephalexin, clarithromycin, azithromycin, ciprofloxacin, and furazolidone. Community-level education campaigns on antibiotic awareness, combined with behavior change interventions, could help limit the transmission of ARGs and resistant bacteria to babies.

Many reports have identified high levels of AMR in food animals and retail meats in the United States (Davis et al., 2018; Liu et al., 2018), China (Liu et al., 2017; Wu et al., 2018; He et al., 2019), and Europe (Gelbíčová et al., 2019; Mellor et al., 2019). Other studies have assessed ARG dissemination between isolates of human, animal, and environmental origin in LMICs (Nadimpalli et al., 2019; Subbiah et al., 2020). Our study is innovative because we compared animals raised with and without antibiotics, along with humans with varying levels of exposure to chicken meat, and used WGS to identify resistant isolates and ARGs among human and animal populations within the same community. However, it presents limitations: (i) We focused exclusively on *E. coli,* and our results do not account for the effects in other commensal species nor the transfer of mobile genetic elements (MGEs) between them; (ii) We included only one isolate for each subject, so we were unable to assess within-host *E. coli* diversity; (iii) The timeline for our collection of human stool samples and chicken intestinal and cloacal isolates do not overlap for much of the study; (iv) Illumina-based sequencing generated short reads that made it challenging to reconstruct full plasmid sequences. The use of long-read sequencing should vastly improve assemblies and provide new insights into the exchange and recombination of mobile genetic elements between hosts.

There are very few studies that can clearly link antibiotic use on farms with antibiotic resistance in humans, in part because of the lack of national antibiotic consumption surveys on farms and the high degree of HGT that occurs in enterobacterial genomes (Smillie et al., 2011; Partridge et al., 2018). WHO's 2017 Global Action Plan on AMR calls for strengthening national surveillance capacities (World Health Organization [WHO], 2017b). Surveillance data on antibiotic use and resistance rates in poultry may serve stakeholders to make evidence-based decisions and policies, as is the case with high-income countries. AMR surveillance studies conducted in South America are scarce compared to other LMICs (Bantar et al., 2000; García et al., 2012; Baker et al., 2017; Bazzo et al., 2018). As the antibiotic resistome expands through the accumulation of gene cassettes or novel plasmids, and with further ARG transfer from animals into commensal human strains, last-resort drugs such as colistin and carbapenems will become increasingly ineffective to combat pathogenic microorganisms.

This study highlights the potential dissemination of resistance genes in *Escherichia coli* from market chickens into human populations. Policy change is needed to curb the misuse of antibiotics in agriculture, which in the past has been successful at reducing the environmental burden of resistance without hurting the productivity of farmers (Aarestrup et al., 2010; Marshall and Levy, 2011). It is estimated that Peru will increase antimicrobial use in livestock by 160% from 2010 to 2030 (Van Boeckel et al., 2015). To offset this scenario, the National Multi-sectoral Action Plan to Combat Antimicrobial Resistance is set to provide a set of milestones involving regulations of antimicrobial use in food animals by 2021 (Ministerio de Salud [MINSA], 2019). We support the view that restricting non-therapeutic supplementation of antibiotics in animal feed and regulating the drug classes used to treat disease will help prevent the dissemination of AMR from animals into humans. Our research may serve as a baseline for future interventions aimed at limiting the spread of AMR in the environment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA633873/, PRJNA633873.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Comité Institucional de Ética en Investigacion, Universidad Peruana Cayetano Heredia. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PT, RG, DB, MM, and GS designed the study. MM, GS, AD-B, BA, LC, and MC collected the samples and conducted the experiments. CC-V, PT, GS, MH, MP, and AL analyzed the datasets. PT, GS, AD-B, and MM wrote the manuscript. RG, DB, AL, and MP reviewed the manuscript. All authors contributed to the article 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.2021.635871/full#supplementary-material

Supplementary Figure 1 | Pairwise SNP distances between all pairs of *E. coli* isolates.

Supplementary Figure 2 | Plasmid replicon marker sequences among sequenced *E. coli*.

Supplementary Table 1 | Location, type, and sample size of humans and chickens included in the study.

Supplementary Table 2 | Antibiotics used for resistance profiling.

Supplementary Table 3 | Plasmid markers and bivariate analysis of sequenced *E. coli* from chickens and humans. *Fisher exact test and confidence level of 95%.

Supplementary Table 4 | Antimicrobial resistance genes hits among 118 sequenced *E. coli*.

Supplementary Data Set 1 | Plasmid markers detection among 118 *E. coli* isolates.

Supplementary Data Set 2 | Genomic analysis of 118 *E. coli* isolates.

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frontiers in Veterinary Science

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Detection of bla_{OXA-1}, bla_{TEM-1}, and Virulence Factors in *E. coli* Isolated From Seals

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Marine mammals are frequently considered good sentinels for human, animal and environmental health due to their long lifespan, coastal habitat, and characteristics as top chain predators. Using a One Health approach, marine mammals can provide information that helps to enhance the understanding of the health of the marine and coastal environment. Antimicrobial resistance (AMR) is the quintessential One Health problem that poses a well-recognised threat to human, animal, and ecosystem health worldwide. Treated and untreated sewage, hospital waste and agricultural run-off are often responsible for the spread of AMR in marine and freshwater ecosystems. Rescued seals ($n = 25$) were used as sentinels to investigate the levels of AMR in the Irish coastal ecosystem. Faecal swabs were collected from these animals and bacterial isolates (*E. coli* and cefotaxime-resistant non-*E. coli*) from each swab were selected for further investigation. *E. coli* isolates were characterised in terms of phylogenetic group typing, AMR, and virulence factors. All *E. coli* isolates investigated in this study ($n = 39$) were ampicillin resistant while 26 (66.6%) were multi-drug resistant (MDR). Resistance genes bla_{OXA-1} and bla_{TEM-1} were detected in 16/39 and 6/39 isolates, respectively. Additionally, virulence factors associated with adhesion (*sfa*, *papA*, and *papC*) and siderophores (*fyuA* and *iutA*) were identified. An additional 19 faecal cefotaxime-resistant non-*E. coli* isolates were investigated for the presence of β-lactamase encoding genes. These isolates were identified as presumptive *Leclercia*, *Pantoea* and *Enterobacter*, however, none were positive for the presence of the genes investigated. To the authors knowledge this is the first study reporting the detection of bla_{OXA-1} and bla_{TEM-1} in phocid faecal *E. coli* in Europe. These results highlight the importance of marine mammals as sentinels for the presence and spread of AMR in the marine and coastal environment.

Keywords: antimicrobial resistance, β-lactamases, One Health, seals, virulence factors, *E. coli*

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INTRODUCTION

Located in the North Atlantic region, Ireland offers an important habitat for marine mammals including harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) in search of haul-out sites during breeding and moulting (1). Grey seals are known to migrate between countries, but harbour seals tend to travel less widely. Nonetheless, both species usually return to their breeding areas (2).

Marine mammals are frequently considered good sentinels for human and environmental health because their position at the top of the food chain, their long-life span and their coastal habitat can provide an early warning system for public health issues (3, 4). Using a One Health approach, marine mammals can be seen as an important source of information that helps to enhance our understanding of the health of the marine and coastal environment (3).

Antimicrobial resistance (AMR) is the quintessential One Health problem (5) that poses a well-recognised threat to human, animal and ecosystem health worldwide (6). Much of this problem has been associated with the misuse of antimicrobials in human, veterinary and agricultural settings (7) leading to the increased emergence of antimicrobial-resistant bacteria (ARB) in marine and fresh water ecosystems (8). Treated and untreated sewage, hospital waste and agricultural run-off are often responsible for the spread of AMR in these ecosystems (9–11). Studies have shown that natural environments, such as soils, sediments, and surface waters have complex microbiomes which include clinically important ARBs and antimicrobial-resistant genes (ARGs) (12). ARGs can be transferred into soils and leached to groundwater or carried by runoff and erosion to surface water (13). In addition, dense bacterial populations in treatment plants facilitate frequent genetic exchange through mobile genetic elements (MGE), such as plasmids, integrons, and transposons (8, 14). For example, the spread of β-lactamases, enzymes responsible for decreasing the efficacy of critically important β-lactam antimicrobials against Gram negative bacteria, is frequently due to MGEs (15). These enzymes are currently the most important mechanism of resistance in Gram negative pathogens with more than 2,600 enzymes described to date. The most frequently described enzymes in *E. coli* include CTX-M, TEM and SHV, Ambler class A enzymes, and OXA, Ambler class D enzymes (16). Also disseminated by MGEs, virulence factors including adherence factors, invasion factors, iron acquisition systems, capsules and toxins facilitate bacterial colonisation of the host (17).

An Irish technical report identified high levels of resistant *E. coli* in urban wastewater (18). More recently, Mahon et al. were the first in Europe to report the isolation of New Delhi metallo-beta-lactamase (NDM)-producing *Enterobacteriaceae* from both fresh water and seawater sampled on 2 Irish beaches located near an untreated human sewage ocean discharge (19). This finding raises concerns regarding the potential of sewage discharges to contribute to the spread of ARBs and ARGs in the environment, especially when recent studies have shown that resistant bacteria can be selected at extremely low antibiotic concentrations, similar to concentrations found in some aquatic and soil environments (20, 21). Although it is recognised that proximity to human activities shapes the AMR profile of the gastrointestinal microbiome of wild mammals, the presence of ARBs and ARGs in the intestinal microbiota of wild animals has not been thoroughly investigated (8, 22, 23). Additionally, the role played by wildlife colonised with ARB in the dissemination of ARGs worldwide needs to be addressed (24, 25).

In light of the recent findings of ARB in Irish coastal waters and the complexity of the factors governing dissemination of AMR in the environment, a pilot study was conducted to characterise the faecal *E. coli* populations of pinnipeds living in coastal waters surrounding Ireland, and investigate the presence of β-lactamase encoding genes and virulence factors. Furthermore, the presence of β-lactamase encoding genes was examined in cefotaxime-resistant non-*E. coli* isolates.

MATERIALS AND METHODS

Animals

In the summer breeding season of 2017, collection of faecal swabs from 23 harbour seals (*P. vitulina*) and two grey seals (*H. grypus*) was attempted at the premises of Seal Rescue Ireland (SRI), the only marine rehabilitation centre in the Republic of Ireland; however, three animals did not defecate during the visit. Convenience sampling was conducted on two occasions in July 2017, 22 days apart, to sample as many individual animals as possible (**Table 1**). Fourteen and eleven animals were sampled on the first and second sampling-days, respectively, which made up the total number of animals housed at SRI at the time (**Table 1**).

Sterile cotton swabs were used to collect freshly voided faeces from each animal's enclosure (individual pens with covered roof) without contacting the floor. Enclosures at SRI are cleaned daily; thorough washing and disinfection with bleach and Virkon© are performed before any new animal is moved into an enclosure.

Faecal swabs were kept refrigerated for a period no longer than 24 h before being processed in the laboratory.

At time of sampling, the age of animals ranged from 9 days to 10 months approximately. Samples were collected from animals between 24 h and 8 months after their arrival at the SRI facilities.

Faecal Swab Processing and *E. coli* Isolation

In the microbiology laboratory, the faecal swabs were placed into 20 mL sterile plastic tubes filled with 5 mL of buffered peptone water (BPW, Lab M) and vortexed for 10 s.

Aliquots of 0.1 mL of each initial suspension were plated onto the chromogenic selective medium Tryptone Bile X-glucuronide (TBX, Fisher Scientific) and TBX supplemented with cefotaxime (sc-202989 Cefotaxime Sodium Salt; 0.250 mg/L according to EUCAST epidemiological cut-off value (ECOFF) at the time of the study). TBX and TBX supplemented with cefotaxime were used to detect cefotaxime-susceptible and cefotaxime-resistant *E. coli* colonies (blue/green colonies), respectively. Sterile spreaders were used to evenly distribute the faecal suspension across the plates and then all plates were incubated at 37°C for a period of 20–24 h. Two to three colonies were isolated from each plate/sample if colonies differed phenotypically. *E. coli* ATCC 25922 and extended spectrum β-lactamase (ESBL)-producing isolate R5S (26) were used as negative and positive controls, respectively.

Antimicrobial Susceptibility Testing of *E. coli*

Thirty-nine *E. coli* isolates (selected from TBX and TBX supplemented with cefotaxime media) were

TABLE 1 | Sampling details including animal identification, sampling day and bacteria isolated from faeces; *E. coli* isolated from TBX supplemented with cefotaxime (REC), *E. coli* isolated from TBX (EC) and non- *E. coli* isolated from TBX supplemented with cefotaxime (RC) according to sampling-day (3rd of July and 25th of July).

Seal ID	Isolate ID	Sampling day	Bacterial species	Phylogenetic group typing	Resistance phenotype	Resistance genotype	Virulence factors	Previous antibiotic treatment	No. days between treatment and sampling
1	1EC1	1	*E. coli*	B1	AMP, PIP, ENR, TET		*sfa*	N.A.	N.A.
	1EC2	1	*E. coli*					N.A.	N.A.
2	2EC1	1	*E. coli*	B1	AMP			N.A.	N.A.
	2EC2	1	*E. coli*					N.A.	N.A.
3	3EC1	1	*E. coli*					N.A.	N.A.
	3EC2	1	*E. coli*	B1	AMP, PIP, ENR, TET	bla_{TEM-1}		Marbofloxacin	39
4	4EC1	1	*E. coli*	B1	AMP, CHL			N.A.	N.A.
	4EC2	1	*E. coli*					N.A.	N.A.
5	5EC1	1	*E. coli*	B1	AMP, CHL			N.A.	N.A.
	5EC2	1	*E. coli*					N.A.	N.A.
6	6EC1	2	*E. coli*	B1	AMP, PIP, TOB, SXT	bla_{TEM-1}	*fyuA, iutA, sfa, papC*	N.A.	N.A
	6EC2	1	*E. coli*	B1	AMP, PIP, TOB, STX	bla_{TEM-1}	*fyuA, iutA, papA*	N.A	N.A.
	6EC3	1	*E. coli*	B1	AMP, CEV, CHL			N.A.	N.A.
7	7EC1	1	*E. coli*	B1	AMP, CHL			N.A.	N.A.
	7EC2							N.A.	N.A.
9	9EC1	1	*E. coli*	A/C[a]	AMP			N.A.	N.A.
	9EC2	1	*E. coli*	B1	AMP, PIP, ENR, TET	bla_{TEM-1}		N.A.	N.A.
10	10EC1	1	*E. coli*	A	AMP			N.A.	N.A.
	10EC2	1	*E. coli*	B1	AMP			N.A.	N.A.
13	13EC1	1	*E. coli*	B1	AMP, PIP, ENR, TET	bla_{TEM-1}		N.A.	N.A.
	13EC2								
14	14EC1	1	*E. coli*	B2	AMP			N.A.	N.A.
	14EC2								
12	12REC1	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, sfa, papC*	N.A.	N.A.
	12REC2								
16	16REC1	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, papA*	Enrofloxacin Amoxicillin/ Clavulanate	202 192
	16REC2	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, AMK, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, papA*		
17	17REC1	2	*E. coli*	A/C[a]	AMP, AMC, PIP, LEX, CPD, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, sfa, papC, papA*	N.A.	N.A.
	17REC2	2	*E. coli*	A/C[a]	AMP, AMC, PIP, LEX, CPD, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, papC, papA*		
18	18REC1	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, papA*	Marbofloxacin	0
	18REC2	2	*E. coli*	A/C[a]	AMP, AMC, PIP, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, papA*		
19	19REC1	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, papC, papA*	Marbofloxacin	1
	19REC2								

(Continued)

TABLE 1 | Continued

Seal ID	Isolate ID	Sampling day	Bacterial species	Phylogenetic group typing	Resistance phenotype	Resistance genotype	Virulence factors	Previous antibiotic treatment	No. days between treatment and sampling
20	20REC1	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, papC, papA*	Marbofloxacin	1
	20REC2								
23	23REC1	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, papC, papA*	N.A.	N.A.
	23REC2								
24/25	24/25REC1	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, papC, papA*	Marbofloxacin	2
	24/25REC2	2	*E. coli*	A/C[a]	AMP, AMC, PIP, CEF, GEN, TOB, ENR, MAR, CHL, STX	bla_{OXA-1}	*fyuA, iutA, papC, papA*		
1–10 and 13–14	1RC1 1RC2 2RC1 2RC2 3RC1 3RC2 4RC2 5RC1 5RC2 6RC1 7RC1 7RC2 8RC1 8RC2 9RC2 10RC1 13RC2 13RC3 14RC3	1	Non-*E. coli*	N.A.	N.A.		N.A.	N.A.	N.A.

Characterisation of faecal E. coli isolated from seals according to their phylogenetic group typing, virulence factors, antimicrobial resistance genes (genotype), and antimicrobial susceptibility profile (phenotype) given by Vitek2. For the purpose of this study intermediate antimicrobial susceptibility was interpreted as resistant. Antimicrobials tested: AMP, Ampicillin; AMC, Amoxicillin/Clavulanic Acid; PIP, Piperacillin; LEX, Cefalexin; CPD, Cefpodoxime; CEV, Cefovecin; CEF, Ceftiofur; IPM, Imipenem; AMK, Amikacin; GEN, Gentamicin; TOB, Tobramycin; ENR, Enrofloxacin; MAR, Marbofloxacin; TET, Tetracycline; NIT, Nitrofurantoin; CHL, Chloramphenicol; PMB, Polymyxin B; SXT, Trimethoprim/Sulfamethoxazole. Additional information on antimicrobial use, number of days between antimicrobial treatment and sampling-time for the samples with antimicrobial resistance genes. N.A., not applicable.
[a]*Phylogenetic groups A and C could not be differentiated.*

grown on blood agar plates for 18 h at 37°C before testing for antimicrobial susceptibility by the VITEK 2 automated system (Biomerieux©), as recommended by the manufacturer (**Table 1**). Vitek 2 AST-GN65 cards (Biomerieux©) were used to investigate the susceptibility of the isolates to amoxicillin, ampicillin, amoxicillin and clavulanic acid, piperacillin, cefalotin, cefalexin, cefpodoxime, cefovecin, ceftiofur, imipenem, amikacin, gentamicin, tobramycin, enrofloxacin, marbofloxacin, tetracycline, nitrofurantoin, chloramphenicol, and trimethoprim/sulfamethoxazole. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. *E. coli* ATCC 25922 was used as control strain (27–31).

Rapid DNA extraction was performed on all isolates by the boiling method (32).

Investigation of Phylogenetic Group Typing and Virulence Factors of *E. coli*

The phylogenetic group of 33 *E. coli* was investigated using an adapted version of the Clermont method (**Table 2**) (33). Each isolate was assigned to a group (A, B1, B2, C, D, E, F) according to the presence or absence of genes *arpA*, *chuA*, *yjaA*, and the DNA fragment TSPE4.C2 (33). Positive controls were provided by the Galway University Hospital National Microbiology Reference Laboratory.

Briefly, all PCR reactions were performed in a final volume of 25 µl containing 1× master mix [2× Qiagen Multiplex PCR Master Mix, final primer concentrations of 0.2–0.7 µM as appropriate (**Table 2**), PCR grade water] and 1.5 µl of bacterial lysate. PCR reactions were performed as follows: denaturation 15 min at 95°C, followed by 30 cycles of 20 s at 94°C, 20 s at 60°C, and 30 s at 72°C with a final extension step of 5 min at 72°C. PCR

TABLE 2 | List of primers, target genes, primer sequences, annealing temperatures, and primer concentrations used for *E. coli* phylogenetic group typing in this study.

Primer ID	Target	Primer sequence (5′ - 3′)	Annealing T (°C)	Final concentration (μM)	PCR product (bp)	PCR reaction	References
chuA.1b	*chuA*	ATGGTACCGGACGAACCAAC	60	0.3	288	Quadruplex	(a)
chuA.2		TGCCGCCAGTACCAAAGACA		0.3			
yjaA.1b	*yjaA*	CAAACGTGAAGTGTCAGGAG		0.6	211		
yjaA.2b		AATGCGTTCCTCAACCTGTG		0.6			
TspE4C2.1b	TspE4.C2	CACTATTCGTAAGGTCATCC		0.7	152		
TspE4C2.2b		AGTTTATCGCTGCGGGTCGC		0.7			
AceK.f	*arpA*	AACGCTATTCGCCAGCTTGC		0.3	400		
ArpA1.r		TCTCCCCATACCGTACGCTA		0.3			
trpAgpC.1	*trpA*	AGTTTTATGCCCAGTGCGAG	59	0.2	219	Group C duplex	(b)
trpAgpC.2		TCTGCGCCGGTCACGCCC		0.2			
ArpAgpE.f	*arpA*	GATTCCATCTTGTCAAAATATGCC	57	0.2	301	Group E duplex	
ArpAgpE.r		GAAAAGAAAAAGAATTCCCAAGAG		0.2			
trpBA.f	*trpA*	CGGCGATAAAGACATCTTCAC	59/57	0.2	489	Internal control group C and E	(c)
trpBA.r		GCAACGCGGCCTGGCGGAAG		0.2			

Adapted from (a) Clermont et al. (33) and Tim Julian (Eawag, Switzerland), (b) Lescat et al. (34), and (c) Clermont et al. (35).

products were loaded on 2% agarose gels with SYBR® Safe DNA gel stain and run for 60 min at 100 V. DNA bands were visualised using a UV-transilluminator. For groups C and E, two further PCRs were performed using the previous protocol with 5× Q-Solution (Qiagen©) included in the master mix. PCR reactions were performed as follows: denaturation 15 min at 95°C, 30 cycles of 20 s at 94°C, 20 s at 59°C (group C) or 57°C (group E), respectively and 30 s at 72°C, with a final extension step of 5 min at 72°C.

Selected genes encoding virulence factors associated with adhesion (*afaE8*, *papA*, *papC*, and *sfa*), capsular antigen (*kpsMFII*), toxins (*CNF1*), and siderophores (*fyuA* and *iutA*) were also investigated as previously published (36–40). Positive PCR products were Sanger sequenced for identification of gene variants. The nucleotide sequence queries were loaded into the Virulence Factor Database (VFDB) (41).

Identification of Cefotaxime-Resistant Non-*E. coli*

Faecal samples collected on the first-sampling day did not yield any *E. coli* colonies that grew on medium supplemented with cefotaxime. However, 19 other colonies that were not *E. coli* were selected from this medium for further analysis (**Table 1**). Identification of these colonies was carried out by performing 16s rRNA PCR according to Marchesi et al. (42). PCR products (1,300 bp) were Sanger sequenced and the nucleotide sequence queries were loaded into the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST©). The highest query cover, identity and max score were used to determine the best fit for sequence alignment.

Investigation of β-Lactamase-Encoding Genes

Thirty-three *E. coli* isolates yielding a phenotype of resistance to ampicillin (**Table 1**) were tested to see if they contained β-lactamase-coding genes SHV, TEM, and OXA (Multiplex I) while 16 isolates with susceptibility reported as intermediate or resistant to 3rd generation cephalosporins (**Table 1**) were further investigated for the presence of ESBL (CTX-M) (Multiplex II) and plasmid-mediated AmpC (ACC, FOX, MOX, CMY, DHA, LAT, ACT, BIL, MIR) (Multiplex III) encoding genes (32). Additionally, 19 cefotaxime-resistant non-*E. coli* isolates were investigated for the presence of β-lactamases. Positive controls were provided by the Galway University Hospital National Microbiology Reference Laboratory.

Briefly, for multiplex PCRs I, II and III, reactions were carried out in 25 μl of reaction mix containing master mix (2× Qiagen Multiplex PCR Master Mix, 5× Q-Solution (Qiagen), primers at concentration of 0.2–0.5 μM as appropriate, PCR grade water) and 1.0 μl of bacterial lysate. PCR reactions were performed as follows: denaturation 15 min at 95°C, 30 cycles of 30 s at 94°C, 90 s at 60°C, and 90 s at 72°C with a final extension step of 10 min at 72°C.

Agarose gels ranging between 1.2 and 2% (according to the size of PCR product) were run at 100 V for 60 min. A UV-transilluminator was used to visualise the PCR products.

Positive PCR products were Sanger sequenced. The nucleotide sequence queries were loaded into the Comprehensive Antibiotic Resistance Database (CARD) (43).

RESULTS

Cefotaxime Resistant *E. coli*

Faecal samples from 22 seals were collected over two sampling days. Samples were plated onto TBX and TBX supplemented with cefotaxime. After overnight incubation, samples were examined for growth and the results are shown in **Table 1**. *E. coli* were not retrieved from faeces sampled from 2 animals, while there was no *E. coli* growth on TBX supplemented with cefotaxime on samples collected on day 1. *E. coli* was recovered from all faecal samples from day 2 plated on TBX supplemented with cefotaxime.

Antimicrobial Susceptibility Testing of *E. coli*

In total, 39 *E. coli* isolates were investigated in the present study; 23 from the faeces collected on sampling-day 1 and 16 from sampling-day 2. All isolates were ampicillin-resistant, 16 of them were also resistant to amoxicillin and clavulanic acid (**Table 1**), while 22 of the isolates were intermediately susceptible or resistant to fluoroquinolones. From day 1 *E. coli* isolates, 10 (43.5%) were multidrug resistant (MDR) showing resistance to 3–4 different antimicrobial classes (penicillins, cephalosporins, tetracyclines, and potentiated sulphonamides) (44). In contrast, all *E. coli* isolates from sampling-day 2 were MDR, displaying resistance to 4–6 different antimicrobial classes including penicillins, fluoroquinolones, amphenicols, and potentiated sulphonamides (**Table 1**).

Molecular Investigation of *E. coli*

From a total of 39 *E. coli* isolates, 33 were selected for analysis by PCR and sequencing. Twenty-two carried β-lactamase encoding genes; $bla_{\mathrm{TEM-1}}$ was detected in six *E. coli* isolated from four seals on Day 1 while $bla_{\mathrm{OXA-1}}$ was detected in 16 *E. coli* isolated from nine animals on Day 2. Of four seals shedding TEM-1 *E. coli*, two originated from county Galway and one had been treated at SRI with marbofloxacin, 39 days before sampling. Of nine seals shedding *E. coli* carrying $bla_{\mathrm{OXA-1}}$, five had been medicated with marbofloxacin (**Table 1**). Reasons for medication included wounds, otitis, and umbilical abscess.

Additionally, the presence of CTX-M and AmpC encoding genes was investigated in the 16 isolates from day 2 (cefotaxime-resistant *E. coli*) using multiplex II and multiplex III PCRs; however, none of these genes was detected.

E. coli isolates belonged to phylogenetic groups A ($n = 1$), B1 ($n = 13$), B2 ($n = 2$), and A/C ($n = 17$) with the all 16 isolates from Day 2 belonging to group A/C. For isolates characterised as A/C it was not possible to further determine their phylogenetic group.

To further characterise the *E. coli* isolates investigation of selected virulence factors was performed. From day 1 samples, 3 of 17 *E. coli* isolates carried at least one virulence factor associated with adhesion (*sfa*, *papA*, and *papC*) and/or siderophores (*fyuA* and *iutA*) while all *E. coli* (16) isolated on the second sampling day carried at least two virulence factors. Some isolates carried multiple virulence factors including isolate 17REC1 that carried five of the eight virulence factors investigated.

Investigation of Non-*E. coli*

Cefotaxime-resistant non-*E. coli* isolates were grown from samples collected on both sampling-days and 19 colonies recovered on the first sampling-day were selected for further investigation based on colony morphology.

16s rRNA PCR was used to amplify a specific region of the genome of each isolate. Despite the limitations of this method, sequence homology suggests that most of the isolates belong to the genera *Leclercia*, *Enterobacter*, *Pantoea*, and/or *Psychrobacter* (**Supplementary File**). Definitive species identification was not established. None of these isolates carried any of the β-lactamase encoding genes investigated (**Table 1**).

DISCUSSION

To the authors' knowledge this is the first study reporting the detection of $bla_{\mathrm{OXA-1}}$ and $bla_{\mathrm{TEM-1}}$ in phocid faecal *E. coli* in Europe. The presence of β-lactamase producing *E. coli* in the microbiota of wild seals, some of which had not been previously medicated with antimicrobials is a cause for concern and highlights their potential to serve as One Health sentinels when investigating AMR. There is also scope to explore zoonotic diseases including avian influenza and environmental contamination by heavy metals and domoic acid, among other things, using these species as sentinels.

All 39 *E. coli* isolated from seal faeces at the SRI marine rehabilitation centre were ampicillin resistant and 26 of 39 (66.6%) were MDR, which highlights the presence of MDR bacteria in the microbiome of marine mammals. Despite rigorous cleaning and disinfection protocols at the SRI centre, it cannot be proven that faecal isolates were not simply representative of the in-house flora of the centre however, the isolation of MDR *E. coli* from animals only recently arrived in the rescue centre strongly suggests that the organisms may have been present in the gastrointestinal tract of at least some of the seals before arrival at the centre. It is noteworthy that all *E. coli* (16) isolated on the second sampling-day were MDR which contrasts with 10 MDR *E. coli* of 23 *E. coli* recovered on the first sampling-day. Interestingly, the number of MDR *E. coli* isolates sampled on these two sampling-days differs considerably. The only major difference recorded between the 2 days was an outbreak of disease due to phocid herpes virus diagnosed soon after the second sampling-day. Whether this bears any relationship with the findings of this study is unclear. Research has shown that neurohormones released in the gut as a result of stressful events can increase the rate of horizontal gene transfer of genes encoding for AMR which can lead to an increase in shedding of resistant bacteria (45–47). Although little is known about the impact of acute viral infections on the composition and kinetics of the microbiome, these types of infections could be classified as systemic stressful events and therefore one could hypothesise that an increase in horizontal gene transfer may occur (48, 49). Fifty-six per cent of *E. coli* investigated showed resistance to fluoroquinolones, which may be associated with the use of marbofloxacin at the SRI, although not all isolates with resistance to fluoroquinolones came from animals with a history of marbofloxacin treatment.

In Ireland, a wide range of β-lactamases has been reported in bacteria isolated from humans, companion animals, production animals and wastewaters (26, 50–54). Karczmarczyk et al. (52) identified bla_{TEM} in 89.2% of the *E. coli* examined in their study while bla_{OXA} were detected in 1.35%. Additionally, Carroll et al. (55) identified bla_{TEM} in faecal *E. coli* sampled from one Irish herring gull and one Irish black-headed gull while another study identified $bla_{\mathrm{CTX-M}}$ group 1 in 4.5% of the samples collected from Irish gulls (55, 56). In line with this, isolates with either bla_{OXA} or bla_{TEM} were identified in the faecal samples collected, suggesting these genes are circulating in the marine environment also. ESBL-producing *E. coli* have been identified in more than 30 wild animal species (11) but none were identified in the *E. coli* or non-*E. coli* isolates investigated in this study. While this does

not rule out their presence, it may suggest that these genes are not as prevalent in the environment of these seals as the ones that were identified.

This study further characterised *E. coli* isolated from seals in terms of phylogenetic groups and virulence factors. Due to the lack of information in the literature, a subset of virulence factors was selected for investigation, based on data available for *E. coli* isolated from domestic animals. In this study, virulence factors associated with adhesion and siderophores were detected in many isolates. Despite the constraints of the small number of animals investigated and further bias by the selection criteria of *E. coli* (1, 2, or 3 colonies per sample according to colony phenotype), a difference between the number of *E. coli* carrying virulence genes on each sampling day is clear. Horizontal gene transfer is an essential mechanim for the spread of virulence determinants between different bacterial strains and species (57). Moreover, studies have shown that stress can induce the release of norepinephrine in the gut and this catecholamine can promote horizontal gene transfer by conjugation and influence the production of virulence factors including toxins and adhesins in *E. coli* (46, 58–61). It is possible that the differences reported between sampling day 1 and day 2 were triggered by the herpes virus infection that was subsequently diagnosed (48, 62).

Studies have shown that ecological niches and life events impact the phylogenetic group dynamics and diversity of *E. coli* (63, 64). In the present study, phylogenetic groups A, B1, B2, and A/C were detected on the first sampling day while on the second sampling day only A/C *E. coli* were detected. Further characterisation of A/C isolates was not possible due to non-specific DNA amplification. Differences in the antimicrobial susceptibility profile and virulence factors exclude the possibility of clonal spread of A/C *E. coli* on the second sampling day. These suprising findings, including the number of MDR isolates, number of *E. coli* carrying β-lactamases and virulence factors and phylogenetic diversity detected on two different sampling days suggest differences in the population sampled on these two occasions again pointing to the impact a natural herpesvirus infection could have had on the profile of the samples. These data highlight the importance of examining the resistome of sentinal species throughout time.

Phocid faecal cefotaxime-resistant non-*E. coli* isolates were homologous to members of the *Enterobacterales: Leclercia*, *Pantoea*, and *Enterobacter*. *Leclercia adecarboxylata* is an opportunistic pathogen associated with water affecting both immunocompromised and immunocompetent patients (65). Studies have reported *Leclercia adecarboxylata* susceptibility to cephalosporins and $bla_{\mathrm{SHV-12}}$ has been identified in *Leclercia adecarboxylata* clinical samples (66, 67). *Pantoea agglomerans* may cause infections in humans and is variably susceptible to antimicrobials while *Enterobacter ludwigii*, previously included in the *Enterobacter cloacae* complex, is a MDR bacterium that can carry β-lactamase encoding genes (68–71). Because all the above bacteria belong to the *Enterobacterales* order, distinction between species is complex. For more accurate identification, PCR protocols investigating genetic characteristics other than 16S rRNA would be required.

Despite the fact that animals sampled in this study represented all live stranded seals in Ireland housed at SRI during the period of the trial, the relatively small sample size is a limitation of this study. A larger population would have given a better idea of the magnitude of the problem, but it is clear that even with a small sample size, this study has pinpointed issues and has provided justification and a roadmap for future studies in this area. Stringent cleaning and disinfection and other infection control protocols in place at SRI and a rigorous sampling technique greatly reduced the possibility of cross-contamination between different enclosures/pens.

It is difficult to determine the exact origin of β-lactamase encoding genes circulating in the population of young Irish seals as they share their costal habitat with different species, but the fact that $bla_{\mathrm{TEM-1}}$ and b$la_{\mathrm{OXA-1}}$ were present in their faeces and MDR *E. coli* were frequently detected, is concerning. The presence of β-lactamases jeopardises the use of critically important antimicrobials including penicillins and cephalosporins and the findings of this study indicate the spread of AMR mechanisms to bacteria in coastal areas.

Marine mammals can act as reservoirs, vectors, and bioindicators of resistant bacteria and AMR genes in the environment (72, 73). Treated and untreated sewage, hospital waste, aquaculture discharges and agricultural runoff provide means to deliver antibiotics, pollutants and resistant bacteria to the aquatic environment, thus playing a major role in driving ARG transfer, ecology, and evolution (14). Additionally, seals can interact with other marine wildlife and birds and engage in the transfer of ARB between populations across large parts of the world. Future investigations should acknowledge the presence of these issues and seek to understand the movement of ARGs between populations and the extent to which global spread of ARGs in human populations is reflected in wild animal populations.

This study shows that some isolates of *E. coli* carried β-lactamase encoding genes ($bla_{\mathrm{OXA-1}}$ or $bla_{\mathrm{TEM-1}}$) as well as virulence factors associated with adhesion (*sfa*, *papA*, and *papC*) and/or siderophores (*fyuA* and *iutA*). While harbour seals have the potential to migrate to different locations, they tend to return to the same breeding grounds (2) and young pups similar to the ones sampled in this study do not tend to migrate long distances. The presence of MDR bacteria in the seal pups indicates that were probably acquired locally, however, it is also possible that the adult seals or other migratory wildlife in the area may have acquired these resistant bacteria elsewhere, brought them to Ireland and passed them to the pups. At this point, although it is difficult to identify the geographic source exactly, the data presented in this study clearly establishes the presence of MDR *E. coli* circulating in the Irish marine environment at the time of sampling.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the seals were not manipulated for the purpose of collecting faecal swabs as these were sampled from the floor. This study was part of a bigger project that got exemption from full ethical review according to the University College Dublin Animal Research Ethics Committee (AREC-E-17-24-Barry).

AUTHOR CONTRIBUTIONS

AV designed the study, conducted the experiments, and wrote the manuscript. LS and JC designed the study and conducted the experiments. FL and GB designed the study and critically reviewed the manuscript. All authors contributed to the manuscript 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/fvets.2021.583759/full#supplementary-material

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Genomic Analysis of *Staphylococcus aureus* of the Lineage CC130, Including *mecC*-Carrying MRSA and MSSA Isolates Recovered of Animal, Human, and Environmental Origins

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Most methicillin resistant *Staphylococcus aureus* (MRSA) isolates harboring *mecC* gene belong to clonal complex CC130. This lineage has traditionally been regarded as animal-associated as it lacks the human specific immune evasion cluster (IEC), and has been recovered from a broad range of animal hosts. Nevertheless, sporadic *mecC*-MRSA human infections have been reported, with evidence of zoonotic transmission in some cases. The objective of this study was to investigate the whole-genome sequences of 18 *S. aureus* CC130 isolates [13 methicillin-resistant (*mecC*-MRSA) and five methicillin-susceptible (MSSA)] from different sequences types, obtained from a variety of host species and origins (human, livestock, wild birds and mammals, and water), and from different geographic locations, in order to identify characteristic markers and genomic features. Antibiotic resistance genes found among MRSA-CC130 were those associated with the SCC*mec*XI element. Most MRSA-CC130 strains carried a similar virulence gene profile. Additionally, six MRSA-CC130 possessed *scn-sak* and one MSSA-ST130 had *lukMF'*. The MSSA-ST700 strains were most divergent in their resistance and virulence genes. The pan-genome analysis showed that 29 genes were present solely in MRSA-CC130 (associated with SCC*mec*XI) and 21 among MSSA-CC130 isolates (associated with phages). The SCC*mec*XI, PBP3, GdpP, and AcrB were identical at the amino acid level in all strains, but some differences were found in PBP1, PBP2, PBP4, and YjbH proteins. An examination of the host markers showed that the 3' region of the bacteriophage φ3 was nearly identical to the reference sequence. Truncated *hlb* gene was also found in *scn*-negative strains (two of them carrying *sak*-type gene). The *dtlB* gene of wild rabbit isolates included novel mutations. The *vwbp* gene was found in the three MSSA-ST700 strains from small ruminants and in one MSSA-ST130 from a red deer; these strains also carried a *scn*-type gene, different from the human and equine variants. Finally, a phylogenetic analysis showed that the

three MSSA-ST700 strains and the two MSSA-ST130 strains cluster separately from the remaining MRSA-CC130 strains with the *etD2* gene as marker for the main lineage. The presence of the human IEC cluster in some *mecC*-MRSA-CC130 strains suggests that these isolates may have had a human origin.

Keywords: MRSA, MSSA, whole genome sequencing, CC130, ST700, IEC, *etD2*

INTRODUCTION

Staphylococcus aureus is a common colonizer of the nasopharynx and skin of animals and humans; however, it is also a versatile opportunistic pathogen causing a wide variety of diseases from mild skin problems to life-threatening bacteraemias. The situation may be complicated when infections are caused by methicillin-resistant *S. aureus* (MRSA) isolates. Currently, the expression of *mecA* gene, as well as of other *mec* homolog genes, *mecC* and *mecB*, have been described in *S. aureus* conferring methicillin resistance (Becker et al., 2018).

The *mecC*-gene has been found in several MRSA lineages, mainly associated with animals, such as CC130, CC49, ST425, CC599, and CC1943. The ruminant associated CC130 is the most commonly found *mec*C lineage (Paterson et al., 2014a; Zarazaga et al., 2018). *mecC*-MRSA-CC130 was first described in cattle and in humans in the United Kingdom, Denmark, and Ireland (García-Álvarez et al., 2011; Shore et al., 2011). Since then, this lineage has been detected in diverse hosts in many European countries, with cattle and wildlife (including free grazing domesticated animals) being the most common hosts (Zarazaga et al., 2018). The prevalence of *mecC*-MRSA in people seems to be low (Paterson et al., 2014a,b; Lozano et al., 2020), however, the zoonotic transmission from livestock to people has been reported (Harrison et al., 2013), as well as its ability to cause disease (Petersen et al., 2013). This *mecC*-MRSA-CC130 lineage seems to be susceptible to many non-β-lactam agents and lacks major human virulence factors (Cuny et al., 2011; Monecke et al., 2013; Paterson et al., 2014a). However, they are carriers of a novel allele of exfoliative toxin gene (named *etd2*), which could explain the wide variety of hosts (Monecke et al., 2013). Adaptation of *S. aureus* to particular host species can be associated with mobile genetic elements (MGEs) or chromosomal mutations. In particular, the genes of the human specific immune evasion cluster (IEC) are considered to be a marker indicating some degree of human host adaptation. This IEC system is found in seven different configurations (types A–G) depending on the combination of five genes (*scn*, *chp*, *sak*, *sea*/*sep*); the *scn* gene (encodes a staphylococcal complement inhibitor) is included in all IEC types, and is often used as a marker of IEC-positive isolates, and is functionally essential (van Wamel et al., 2006). None of the *mecC*-MRSA reported strains harbored the *scn* gene (essential for the IEC system) (Lozano et al., 2020), with the exception of a few isolates belonging to ST1945, ST1581, and ST1583 previously described by our group from wildlife and extensively farmed domestic animals (Gómez et al., 2014, 2015; Ruiz-Ripa et al., 2019) and one ST1945 MRSA strain from a human sample (Harrison et al., 2017); it is worth noting that all these IEC-positive isolates were of type-E (carrying the *scn* and *sak* genes).

The methicillin-susceptible *S. aureus* (MSSA) isolates of the CC130 clonal complex are commonly found in cattle and are an important cause of disease (Monecke et al., 2016). The *mecA* gene has never been found in isolates belonging to the CC130 clonal complex and *S. aureus*-CC130 was initially described as a MSSA of animals from Europe and Africa (Smith et al., 2014). The ST700 lineage is part of CC130 by definition, as a single locus variant of ST130 (*tsi* allele different between them). MSSA-ST700 isolates are frequently found in Italian sheep populations (Azara et al., 2017; Vitale et al., 2018) and ST700 and some of its single locus variants (CC700) may be considered as a distinct, or separate, lineage due to its independent evolution and different epidemiology (Smith et al., 2014).

Studies of the intrinsic Penicillin-Binding-Proteins (PBPs) of *S. aureus* have shown that PBPs may contain mutations that affect β-lactam resistance, as highlighted by the case of a PBP4 capable of conferring high-level and broad-spectrum resistance to β-lactams, comparable to that provided by PBP2a (Chan et al., 2016).

In order to better understand the genetic characteristics of *S. aureus* CC130, the objective of this study was to analyze data from whole genome sequencing (WGS) of a collection of CC130 *S. aureus* strains (MRSA and MSSA) belonging to different sequences types, obtained from various host species, and from different geographic locations, in order to identify distinctive markers and genomic features of public health relevance.

MATERIALS AND METHODS

Strains Included in the Study

Eighteen *S. aureus* strains of the clonal complex CC130 were included in this study for genomic comparison. These strains were as follows: (1) 13 MRSA, carrying the *mecC* gene, and belonging to the sequence types ST130, ST1945, ST3061, ST1571, ST1581, and ST1583; (2) two MSSA-ST130; and (3) three MSSA-ST700 (as a possible divergent CC130 lineage). These 18 MRSA-CC130, MSSA-ST130, and MSSA-ST700 strains were studied by WGS, having been collected during previous studies from different host samples: animals from extensive farms [four red deer (*Cervus elaphus*), two sheep (*Ovis* sp.), and one goat (*Capra* sp.)] (Gharsa et al., 2015; Gómez et al., 2015), wildlife [four magpies (*Pica pica*), two wild rabbits (*Oryctolagus cuniculus*), one wood mouse (*Apodemus sylvaticus*), one white stork (*Ciconia ciconia*), and one cinereous vulture (*Aegypius monachus*)] (Gómez et al., 2014, 2016; Ruiz-Ripa et al., 2019),

the environment (one river water) (Gómez et al., 2017), and humans (one skin lesion of a cattle farmer)] (Benito et al., 2016). The characteristics of the included strains are indicated in **Table 1**.

Whole Genome Sequencing and Analysis of Sequences

Genomic DNA from each isolate was extracted with MasterPure™ DNA Purification Gram Positive (Cambio, United Kingdom). WGS was performed on an Illumina HiSeq 2000 using paired-end mode (100 bp). *De novo* assembly and initial annotation was carried out using bioinformatic tools at the Wellcome Trust Sanger Institute. Reordering of the contigs was performed by alignment against *S. aureus* LGA251 genome (GenBank accession number: NC_017349) using Mauve (Rissman et al., 2009). Predicted coding sequences were identified and annotated automatically using RAST (Aziz et al., 2008) and manually with Genious Prime (Biomatters, Auckland, New Zealand). The resistance and virulence genotypes as well as the presence of *rep* genes were studied using ResFinder, VirulenceFinder and PlasmidFinder, respectively[1]. *In silico* analysis of the presence of antimicrobial substances related genes was performed using some genome-mining tools as antiSMASH and BAGEL (de Jong et al., 2006; Blin et al., 2019). PHASTHER Search Tool was used to determine the presence of prophage sequences (Arndt et al., 2016). When the study required it, the sequences were compared using Clustal Omega[2].

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

[2]https://www.ebi.ac.uk/tools

The pan-genome was analyzed to estimate the core genome and the accessory or variable genome using Roary (Page et al., 2015) and BLAST-Ring-Image-Generator (BRIG) was employed to obtain a visual comparison with *S. aureus* LGA251 genome as reference (GenBank accession number: NC_017349) (Alikhan et al., 2011). Phylogenetic trees were generated using Geneious Prime with default settings.

RESULTS AND DISCUSSION

Whole Genome Sequencing Results

The genome data of the 13 MRSA-CC130, two MSSA-ST130 and three MSSA-ST700 strains analyzed in this study have been placed in the European nucleotide archive[3], and general sequence data, with the accession numbers are shown in **Supplementary Table 1**.

Antimicrobial and Heavy Metal Resistance and Virulence Genotype

The resistance genotype analysis showed that all MRSA-CC130 strains contained the *mecC* as well as the *blaZ*-SCC*mec*XI (β-lactamase), *arsB* (arsenite efflux pump), and *arsC* (arsenate reductase) genes, which are described as being part of SCC*mec*XI element (Shore et al., 2011). No other resistance genes were detected among MRSA-CC130 strains, which agrees with the fully susceptible phenotype for non-β-lactams previously found in these *mecC*-positive strains. Among MSSA strains, three out of the five showed resistance to at least one of the antimicrobial

[3]http://www.ebi.ac.uk/ena

TABLE 1 | Characteristics of the 18 *S. aureus* CC130 strains (13 MRSA and 5 MSSA).

Strain	Origin	Location (Region, country)	Molecular typing		Resistance Phenotype
			spa-type	Sequence-type (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*)	
C3817	Goat	Tunisia	t773	ST700 (6, 57, 45, 2, 7, 95, 52)	–
C3608	Sheep	Tunisia	t773	ST700 (6, 57, 45, 2, 7, 95, 52)	Tetracycline
C3630	Sheep	Tunisia	t7579	ST700 (6, 57, 45, 2, 7, 95, 52)	Tetracycline
C5802	River water	La Rioja, Spain	t843	ST130 (6, 57, 45, 2, 7, 58, 52)	Penicillin
C6771	Red Deer	Aragón, Spain	t1535	ST130 (6, 57, 45, 2, 7, 58, 52)	–
C7705	Red Deer	Cádiz, Spain	t1535	ST1945 (6, 57, 45, 2, 215, 58, 52)	Methicillin
C6595	Wood Mouse	Cádiz Spain	t1535	ST1945 (6, 57, 45, 2, 215, 58, 52)	Methicillin
C7708	Red Deer	Cádiz, Spain	t1535	ST1945 (6, 57, 45, 2, 215, 58, 52)	Methicillin
C7246	Farmer	La Rioja, Spain	t843	ST1945 (6, 57, 45, 2, 215, 58, 52)	Methicillin
C7925	White stork	Ciudad Real, Spain	t843	ST3061 (6, 57, 393, 2, 215, 58, 52	Methicillin
C7697	Red Deer	Cádiz, Spain	t843	ST1945 (6, 57, 45, 2, 215, 58, 52)	Methicillin
C8664	Magpie	Ciudad Real, Spain	t843	ST1583 (6, 57, 45, 2, 215, 58, 476)	Methicillin
C8666	Magpie	Ciudad Real, Spain	t843	ST1583 (6, 57, 45, 2, 215, 58, 476)	Methicillin
C8667	Magpie	Ciudad Real, Spain	t843	ST1583 (6, 57, 45, 2, 215, 58, 476)	Methicillin
C8671	Magpie	Ciudad Real, Spain	t843	ST1581 (417, 57, 45, 2, 215, 58, 476)	Methicillin
C8699	Cinereous vulture	Madrid, Spain	t843	ST1571 (6, 548, 45, 2, 215, 58, 52)	Methicillin
C8483	Rabbit	Aragón, Spain	t843	ST130 (6, 57, 45, 2, 7, 58, 52)	Methicillin
C8500	Rabbit	Aragón, Spain	t843	ST130 (6, 57, 45, 2, 7, 58, 52)	Methicillin

TABLE 2 | Antimicrobial resistance and virulence genes detected in the 18 *S. aureus* CC130 strains included in this study.

Strain	Resistance genotype (antimicrobials and heavy metals)	Virulence genotype and host adaptation markers[a]
C3817	No related genes	***lukMF'***, *lukED*, *hlgAB*, *hlgCB*, ***tst*-variant, *sec*, *sel***, *edinB*, *splA/B/E*, *aur*, ***vwbp*, *scn*-type**
C3608	*tet*(K)	***lukMF'***, *lukED*, *hlgAB*, *hlgCB*, ***tst*-variant, *sec*, *sel*,** *edinB*, *splA/B/E*, *aur*, ***vwbp*, *scn*-type**
C3630	*tet*(K)	***lukMF'***, *lukED*, *hlgAB*, *hlgCB*, ***tst*-variant, *sec*, *sel*,** *edinB*, *splA/B/E*, *aur*, ***vwbp*, *scn*-type**
C5802	*blaZ*	***lukMF'***, *lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B*, *aur*
C6771	No related genes	*lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*, ***vwbp*, *scn*-type, *sak*-type**
C7705	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	***scn*, *sak***, *lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C6595	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	***scn*, *sak***, *lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C7708	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	***scn*, *sak***, *lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C7246	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	***sak***, *lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C7925	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	*lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C7697	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	***scn*, *sak***, *lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C8664	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	***scn*, *sak***, *lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C8666	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	*lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*,*splA/B/E*, *aur*
C8667	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	*lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C8671	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	***scn*, *sak***, *lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*
C8699	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	*lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B/E*, *aur*, ***sak*-type**
C8483	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	*lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B*, *aur*
C8500	*blaZ*-SSC*mec*XI, *mecC*, *arsB*, *arsC*	*lukED*, *etD2*, *hlgAB*, *hlgCB*, *edinB*, *splA/B*, *aur*

[a] *In bold are highlighted the genes associated with: human immune evasion system, bovine leucocidin, toxic shock syndrome, enterotoxins, von Willebrand factor-binding protein, and staphylococcal complement inhibitor and staphylokinase-type genes (of about 45% of amino acid similarity).*

agents tested, one MSSA-ST130 strain for penicillin (with *blaZ* gene) and two MSSA-ST700 strains for tetracycline [with *tet*(K) gene] (**Table 2**).

A list of selected virulence and/or fitness genes are shown in **Table 2**. All the strains carried the genes: *lukED*, *hlgAB*, *hlgCB*, *edinB*, *splA*/*B*, and *aur*. Nevertheless, some differences were detected with respect to genes belonging to the IEC system, leucocidins, exfoliative toxins, allele variant of toxic shock syndrome toxin, enterotoxins, and immune evasion proteases. The three MSSA strains of lineage ST700 carried *sec* and *sel* genes, and also a variant of *tst* with an amino acid sequence closer to the *tst* gene found associated with bovine origin than with the one of human origin (Monecke et al., 2007); this combination of pyrogenic toxin superantigens is associated with the pathogenicity island SaPIbov (Fitzgerald et al., 2001), and has been previously described in strains from ruminants with the same ST (Luzzago et al., 2014). The ST700 strains were obtained from apparently healthy animals although a subclinical mastitis cannot be ruled out. All of them presented the *tst-variant*, *sec*, and *sel* virulence genes, as well as the *lukMF*' gene, previously found in isolates from cases of mastitis (Schlotter et al., 2012). All our CC130 strains, except those belonging to ST700, carried the *etD2* gene. The *lukMF*' genes, encoding a leucocidin strongly associated with ruminants (Monecke et al., 2007), were only detected in four MSSA strains obtained from sheep and goats (MSSA-ST700) and from river water (MSSA-ST130); these data support the association of this leucocidin with a ruminant origin, and also may suggests that the strain from river water could have a bovine origin.

On the other hand, the *lukED*, *hlgAB*, *hlgCB*, *edinB*, *splA*/*B*, and *aur* genes were present in the 18 strains. Usually, *S. aureus* has up to 6 types of toxin genes in the core genome (HlgAB, HlgCB, and LukAB) (Alonzo and Torres, 2014). The combination of LukED with *splA*/*splB* genes has been detected previously among other clonal complexes (Jamrozy et al., 2012), generally being found on the genomic island νSaβ, highly conserved in some lineages (McCarthy and Lindsay, 2013). Other genes, such as *aur* (immune evasion proteases), *edinB* (exfoliative toxin) or *splA*/*B*/*E* (immune evasion proteases), are found highly conserved in *S. aureus* (Sabat et al., 2008; Munro et al., 2010; Paharik et al., 2016). Nevertheless, the *splE* gene was absent in three of our strains, and some authors suggested the implication of this nuclease in clinical manifestations (Stach et al., 2018). The analysis of genes encoding bacteriocins revealed the presence of the gene encoding the bacteriocin lactococcin 972 (GenBank accession number: NC_004955) in all the analyzed strains; furthermore, this gene showed in all isolates an identical genetic environment, which corresponds to the one found in the reference sequence of *S. aureus* LGA251.

Comparison Between the Strains

The pan-genome study showed that a total of 2,318 genes were included in all strains, 539 were in two or more strains, and 345 were unique genes of specific strains. Circular genome comparison of MSSA and MRSA strains (LGA251 as reference) showed some differences between MRSA and MSSA strains (**Supplementary Figure 1**). It was determined that 29 genes were present in all 13 MRSA and in none of the MSSA strains (mainly associated with SCC*mec*XI mobile genetic element), and 21 genes in all 5 MSSA strains and not in the MRSA (mostly associated with phages) (**Supplementary Table 2**). It has been reported that the core genome is largely preserved within the same lineage (McCarthy et al., 2011). In addition, we analyzed the presence

of unique genes in *scn*-positive (n = 6) and *scn*-negative strains (n = 12). The *scn*-negative strains did not carry unique genes, however, *scn*-positive strains presented different genes encoding proteins associated with bacteriophages (including the human *scn*-IEC gene), that were not present among *scn*-negative strains (**Supplementary Table 3**).

SCC*mec*XI Element and Penicillin Binding Proteins (PBPs)

The structure of the SCC*mec*XI element in the 13 MRSA CC130 strains was compared with the one of MRSA strain M10/0061 (GenBank accession number: FR823292), used as reference. This structure seems to be highly conserved among the 13 MRSA-CC130 strains, pointing out the potential of this type of SCC*mec* to be transferred among S. *aureus*, due to the relatively small size of this mobile genetic element, approximately 30 Kb (Shore et al., 2011). In fact, it has been suggested that SCC*mec*XI could have originated in another species or genus, being distantly related to the other SCC*mec* elements and possibly SCC*mec*XI represent an ancestral form (Shore et al., 2011).

The results of the study of amino acid changes in PBPs and in other three proteins previously associated with β-lactam resistance (Ba et al., 2014, 2019) are shown in **Table 3**. Amino acid changes in our strains were included, as well as those of MRSA LGA251 using the corresponding sequences of MSSA ATCC 25923 (GenBank accession number: CP009361) as reference for all sequences, except for PBP2c. In the case of PBP2c, the sequence of MRSA LGA251 was used as reference. The

TABLE 3 | Identified amino acid changes in PBPs 1, 2, 2c, 3, 4, YjbH, GdpP, and AcrB proteins of the 18 *S. aureus* strains included in this study and also of MRSA LGA251 strain (MRSA LGA251 as used as reference strain for PBP2c and MSSA ATCC 25923 as reference strain for PBPs 1, 2, 3, 4, YjbH, GdpP, and AcrB protein analysis).

Strain	ST	PBP1	PBP2	PBP2c	PBP3	PBP4	YjbH	GdpP	AcrB
LGA 251	ST425	Wild	T439V, T691A, A705V	Wild	M1L, K504R, D563E	E398A	L95V	I456V, D561E	S52T, L198V, T282A, E456D, T577A, S861F
C3817	ST700	Wild	T439V	–	M1L, K160N, K504R, D563E	D28N, K349E, E398A	L95V	I456V, D561E	S52T, T282A, T577A
C3608	ST700	Wild	T439V	–	M1L, K160N, K504R, D563E	D28N, K349E, E398A	L95V	I456V, D561E	S52T, T282A, T577A
C3630	ST700	Wild	T439V	–	M1L, K160N, K504R, D563E	D28N, K349E, E398A	L95V	I456V, D561E	S52T, T282A, T577A
C5802	ST130	T371I	T439V	–	M1L, K160N, K504R, D563E	K349E, E398A	L95V	I456V, D561E	S52T, T282A, T577A
C6771	ST130	Wild	T439V	–	M1L, K160N, K504R, D563E	K349E, E398A	L95V	I456V, D561E	S52T, T282A, T577A
C7705	ST1945	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C6595	ST1945	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C7708	ST1945	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C7246	ST1945	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C7925	ST3061	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C7697	ST1945	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C8664	ST1583	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C8666	ST1583	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C8667	ST1583	Wild	T439M	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C8671	ST1581	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C8699	ST1571	Wild	T439V	Wild	M1L, K160N, K504R, D563E	K349E, E398A	L95V, A83P	I456V, D561E	S52T, T282A, T577A
C8483	ST130	Wild	T439V	Wild	M1L, K160N, K504R, D563E	A288T, K349E, E398A	L95V	I456V, D561E	S52T, T282A, T577A
C8500	ST130	Wild	T439V	Wild	M1L, K160N, K504R, D563E	A288T, K349E, E398A	L95V	I456V, D561E	S52T, T282A, T577A

PBP2c protein, encoded by the *mecC* gene, presented a 100% of amino acid similarity to PBP2c of MRSA LGA251. As regards the other PBPs, some amino acid changes were found, especially in PBP3 and PBP4. These amino acid changes seem to be repeated in the 18 strains, including MSSA ones, with some few exceptions (PBP1: T371I in a MSSA-ST130 water strain; PBP2: T439M in a MRSA-ST1583 magpie strain; PBP4: D28N in MSSA-ST700 strains, and A288T in two MRSA-ST130 rabbit strains). Nevertheless, differences were greater in the case of MRSA LGA251. It should be noted that previous studies indicate that mutations in PBP4 are related to increased MICs for β-lactams (Alexander et al., 2018), and a modified PBP1 had been previously associated with a reduced susceptibility in *S. lugdunensis,* but not in *S. aureus* (Kotsakis et al., 2012). Only one of the changes detected in this study, T371I in PBP1, was previously reported, combined in that case with other PBP mutations in a clinical MRSA ST1 strain lacking *mec* gene (Ba et al., 2014); in our case, the strain which harbored the T371I change was MSSA. In addition to PBPs, the study of GdpP, YjbH, and AcrB proteins, which could be implicated in β-Lactam resistance (Banerjee et al., 2010; Göhring et al., 2011; Ba et al., 2019), showed the same amino acid changes in all analyzed strains, with the exception of YjbH in which two changes (L95V, A83P) were detected in all strains, but in MSSA-ST700, MRSA-ST130 and MSSA-CC130 where only one change was found (L95V).

Host Adaptation, Prophages, and Other Mobile Genetic Elements

The presence of the *scn* gene in some of our *mec*C-positive MRSA-CC130 strains is a remarkable feature since *mecC*-MRSA, as well as CC130 strains in general, are considered animal-associated, and IEC system is considered a human adaptation marker. The 3' conserved region of the β-hemolytic bacteriophage φ3 (approximately 8,000 pb) of the *mecC*-positive strain C6595 (IEC type E, isolated of a wood mice) was compared with the IEC of the reference strain MRSA252 (GenBank accession number: BX571856, type A) (**Figure 1**), and no differences were observed apart from the different content in genes that give rise to the type of IEC. It can be assumed that these strains have an advantage in colonizing and/or infecting humans, as already was described in unusual IEC-positive MRSA livestock associated CC398 strains (Cuny et al., 2015; Pérez-Moreno et al., 2017; Ceballos et al., 2019).

The *hlb* gene was also analyzed, showing that it was truncated in all the strains that presented the IEC system. The truncated *hlb* gene was also found in other three *scn*-negative strains (C6771, C7246, and C8699), which showed an integrated phage of about 45 kb, that only contained phage-related genes; it should be highlighted that C6771 and C8699 isolates contained a *sak*-related gene with a 45% of similarity respect to the *sak* IEC virulence gene (GenBank accession number: NC_026016). The *dtlB* gene, present in the two MRSA-ST130 *mecC*-positive strains isolated from healthy wild rabbits (C8483 and C8500), showed the following amino acid changes with respect to the reference MRSA252 strain (GenBank accession number: BX571856): (a) C8483 (I227T, A382S, and *405Q); (b) C8500 (A382S, G401D, and K402R). None of the strains presented the mutations T113K, Y250H, or *405Y previously described (Viana et al., 2015), and only the A382S change present in both strains has not been previously reported (Viana et al., 2015; Holmes et al., 2016). The *vwbp* gene (SaPIbov5, GenBank accession number: JP5338 used as reference) was found in the three MSSA-ST700 strains from small ruminants and in the MSSA-ST130 strain from a red deer, indicating in this case an adaptation to the host (Viana et al., 2010).

Phage analysis showed 12 different intact prophages in genome with an average of 2 ± 2 prophage regions per genome. The strains C3608, C3630, C3817, and C6771 (3 MSSA-ST700 and one MSSA-ST130, that also carried the *vwbp* gene), showed an identical coding sequence contained in a phage described as a protein related to the expression of fibrinogen (*scn*-type gene), but different from human variant (47% similarity of amino acid sequence with WP_000702262 as reference) and from the new variant described and associated with the evasion of the equine immune system (45% similarity of amino acid sequence with WP_106096712 as reference) (**Supplementary Figure 2**).

Only three strains (C3608, C3630 and C5802, corresponding with two MSSA-ST700 and one MSSA-ST130), presented *rep* genes: rep_7, rep_{24}, rep_{US23}, rep_5. In addition, only rep_7 was detected showing a 100% nucleotide identity in two of the strains (C3608 and C3630). The gene rep_7 has been previously described

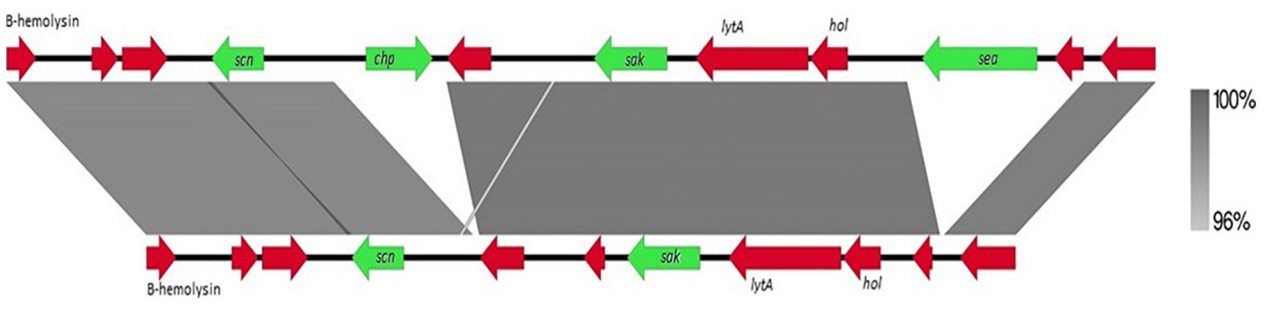

FIGURE 1 | Comparison of 3′ conserved region of β-hemolytic bacteriophage (Φ3) between reference strain MRSA-252 (above) and C6595 (below). The percentage of similarity is indicated (right). Arrows in green corresponding to the IEC genes, and in red other coding sequences.

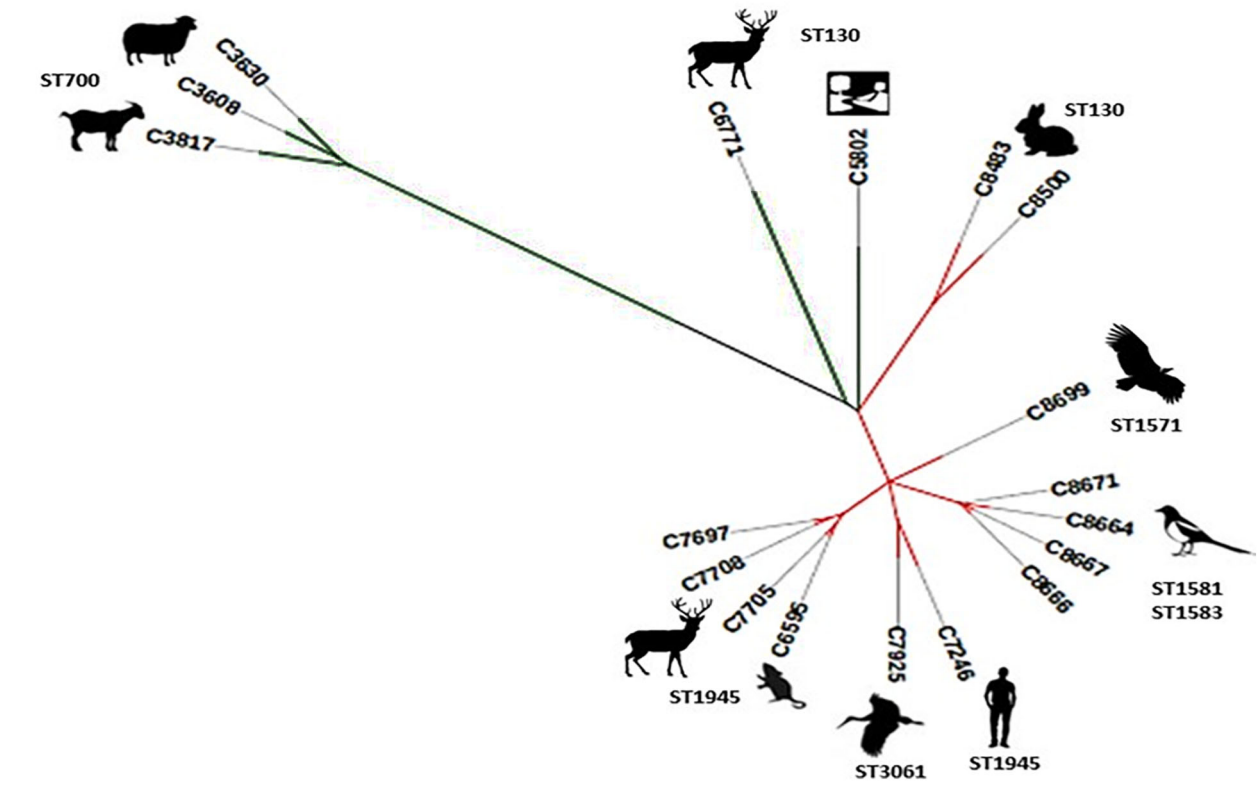

FIGURE 2 | Phylogenetic tree showing the phylogenetic distance between 18 strains CC130 included in this study. Green lines corresponding to MSSA and red lines to MRSA strains.

widely distributed in other CCs within the species *S. aureus* (Lozano et al., 2012).

Phylogenetic Analysis

The phylogenetic tree (**Figure 2**) showed that the three MSSA strains belonging to ST700, clearly constituted a separate clade from the remaining CC130 strains included in this study. Our ST700 MSSA strains were very different from the other 15 strains of the studied collection based on the results from all the analyses performed, supporting the consideration of ST700 as a lineage distinct from CC130 (Smith et al., 2014). The other 2 MSSA-ST130 strains also form a distinct clade in this collection.

Finally, the 13 MRSA-CC130 strains are grouped and the following associations can be seen: (1) C8483 and C8500 from rabbits from Aragon are clustered together indicating the possible animal-animal transfer; (2) C6595, C7697, C7705, and C7708, from red deer and small mammals from the same geographical area, highlighting C7705 and C6595 MRSA strains that were indistinguishable (by this analysis), which might suggest an interspecies transmission event; (3) C8666, C8667, C8664, and C8671, all isolated from magpies in the same location; (4) C7925 and C7246 that were isolated in a different geographical area, of different origins (stork and human, respectively), and with different STs; and (5) C8699 (from vulture) that is grouped individually.

CONCLUSION

Taking into account the relatively small number of strains included in this study, the comparison of fifteen strains CC130 from different animal origins, geographical locations and STs, demonstrated clear differences between isolates depending if they were *mecC*-positive or *mecC*-negative and between sequence types. Markedly divergent results from the three MSSA-ST700 isolates reinforce the idea of considering this lineage as distinctly separate from CC130. The *etD2* gene appears to be a genetic marker of CC130 lineage (MSSA and MRSA), which is missing from ST700 strains although further studies are required to confirm this. The presence of IEC system in some of the MRSA-*mecC* from animals opens questions about the origins and evolution of *mecC*-MRSA.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CT and MH conceived and designed the study. PG, LR-R, RF-F, HG, KB, and UH performed the initial sampling procedure and the initial characterization of isolates. PG performed laboratory work. PG, MZ, CT, and MH interpreted the results and contributed to producing the first draft of the manuscript. All authors have revised and agreed to 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.2021.655994/full#supplementary-material

Supplementary Figure 1 | Circular comparison of MSSA in Green-blue rings and MRSA in warm colors using LGA251 as reference.% GC content and GC Skew are represented in innermost circles (colors indicated in the coded legend).

Supplementary Figure 2 | (A) Amino acid comparative of *scn*-like found in 4 MSSA isolates of this study. **(B)** Amino acid comparative between one *scn*-like found in this study, one *scn*-equine (WP_106096712 as reference) and one *scn*-human (WP_000702262 as reference).

Supplementary Table 1 | Results of whole genome sequence and accession number of the strains included in this study.

Supplementary Table 2 | Result of unique genes in MRSA and in MSSA strains analyzed by Roary pipeline.

Supplementary Table 3 | Result of unique genes in *scn* positive and *scn* negative strains analyzed by Roary pipeline.

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BRIEF RESEARCH REPORT
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Pigeons as Carriers of Clinically Relevant Multidrug-Resistant Pathogens—A Clinical Case Report and Literature Review

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Pigeons are widespread bird species in urban regions (*Columba livia* forma *urbana*) and may carry pathogens with zoonotic potential. In recent years, more and more data indicate that these zoonotic pathogens are multidrug resistant. Our results confirmed that global trend. Three different multidrug-resistant pathogens were isolated from an oral cavity of a racing pigeon with lesions typical for pigeon pox virus infection. *Staphylococcus aureus* was recognized as methicillin resistant, thus resistant to all beta-lactams. Additionally, it was also resistant to many other classes of antibiotics, namely: aminoglycosides, tetracyclines, phenicols, lincosamides, and macrolides. *Escherichia coli* showed resistance to all antimicrobials tested, and it was classified as intermediate to amikacin. Moreover, *Candida albicans* resistant to clotrimazole, natamycin, flucytosine, and amphotericin and intermediate to ketoconazole, nystatin, and econazole was also isolated. This raises the question how pigeons acquire such highly resistant strains. Therefore, more data are needed concerning the resistance to antibiotics in strains from domestic and wild pigeons in Poland. Until the problem is fully understood, it will be challenging to implement adequate planning of any control measures and check their effectiveness.

Keywords: antimicrobial resistance, *Candida albicans*, *Escherichia coli*, MRSA, pigeon

INTRODUCTION

In pigeons, most staphylococcal infections are caused by *Staphylococcus aureus*; however, a few studies have indicated that after *S. aureus*, the most prevalent coagulase-positive staphylococci (CoPS) in pigeons are *Staphylococcus delphini* and *Staphylococcus intermedius* (1, 2), which inhabit the choanal slit (posterior nasal apertures) of healthy birds.

S. aureus is widely spread among humans and numerous animal species. It means that it can be easily transmitted between animals and humans. Since pigeons share common environment with humans, they may not only be the source of staphylococcal infection but may also pose a reservoir of bacteria-carrying resistance and virulence factor genes. Therefore, this might be of a great importance in the context of public health.

Extensive and often inappropriate use of antimicrobials causes a strong selective pressure that leads to the rapid increase in antimicrobial resistance in bacteria. Thus, the antibiotic use plays a crucial role in the emerging public health crisis of antimicrobial resistance. Increased number of multidrug-resistant bacteria has become a global problem. The World Health Organization (WHO) alarms that humanity is at risk of returning to the "pre-antibiotic era" (3). It should be noted that resistant bacteria may circulate among humans, animals, and the environment. Therefore, the "One World—One Health" concept created in 2004 becomes an especially important issue nowadays (4, 5).

Homing pigeons and fancy pigeons, which are bred for ornamental traits are very popular in Poland. Currently, homing pigeons are mainly used in racing competitions. Nowadays, there is a huge problem in Poland related to the frequent use of antimicrobials by breeders without consulting a veterinarian (6, 7). This directly contributes to the increase of drug resistance in bacteria occurring in pigeons.

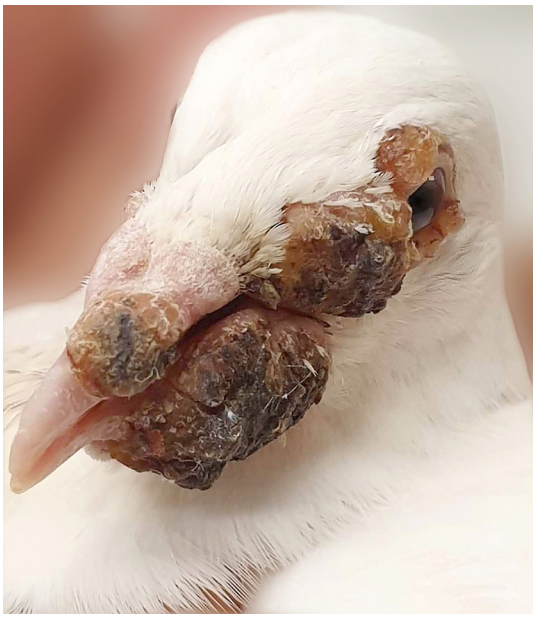

FIGURE 1 | Pox in pigeon from which multidrug-resistant *E. coli* and *S. aureus* strains were isolated: note typical yellow-to-brown nodules on and around beak and eyes.

METHODS

In August 2019, one racing pigeon from the affected pigeon loft was submitted to the veterinary clinic. Clinical examination revealed several dry, yellowish nodular lesions on the eyelids, as well as protuberant black pocks in the nostrils, cere region, and lower beak. Lesions were firmly attached to the skin. In addition, the abscess was found on the palate. Clinical examination allowed the recognition of pigeon pox virus infection based on the presence of typical cutaneous and mucosal diphtheritic lesions (**Figure 1**). The swab from oral cavity was collected for laboratory tests. Basing on the clinical changes, bacteriological as well as mycological examinations were performed. Collected swab was cultured on Columbia agar supplemented with 5% sheep blood (Graso Biotech, Poland), MacConkey agar (Graso Biotech, Poland), and Sabouraud agar (Biomerieux, France). Bacterial isolates were identified based on their phenotypic properties, such as: Gram stain characteristics, catalase and oxidase results, as well as on colony morphology on blood agar and MacConkey agar plates. For further identification of staphylococcal isolate, a tube coagulase test was performed. Additionally, a rapid agglutination test was used for the differentiation of *S. aureus* by the detection of clumping factor and protein A specific for this staphylococcal species (Microgen Staph, Graso Biotech, Poland). Moreover, for tested staphylococcal strain multiplex PCR assay based on the amplification of *nuc* gene was used. This method allows for differentiation of coagulase-positive staphylococci isolated from animals (8). Four reference strains from the Culture Collections of the University Göteborg *S. intermedius* CCUG 6520^T, *S. schleiferi* subsp. *coagulans* CCUG 37248^T, *S. delphini* CCUG 30107^T, and *S. pseudintermedius* CCUG 49543^T used in this study were obtained from the Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen. One strain of *S. aureus* ATCC 6538 belonged to the strain collection of the Warsaw University of Life Sciences. *Candida* species was identified based on the positive germ tube test and API Candida (Biomerieux, France). A disk-diffusion method was used to check antimicrobial susceptibility profiles of isolated microorganisms. *Escherichia coli* isolate was tested for susceptibility to amoxicillin with clavulanic acid (AMC; 30 μg), cefpodoxime (CPD; 10 μg), cephalothin (CF; 30 μg), gentamicin (GM; 10 μg), tetracycline (TE; 30 μg), doxycycline (D; 30 μg), sulfamethoxazole with trimethoprim (SXT; 23.75 μg/1.25 μg), florfenicol (FFC; 30 μg), enrofloxacin (ENO; 5 μg), ampicillin (AM; 10 μg), and amikacin (AN; 30 μg), while *S. aureus* isolate was tested for penicillin (P; 10 μg) instead of ampicillin, and it was additionally tested for susceptibility to clindamycin (CC; 2 μg) and erythromycin (E; 15 μg) (Becton Dickinson, USA). The presence of *mecA* gene was checked by PCR method according to Larsen et al. (9). *Candida albicans* isolate was tested for susceptibility to: clotrimazole (CTM; 1 0μg), natamycin (NAT; 10 μg), flucytosine (FY; 1 μg), amphotericin (AMB; 20 μg), ketoconazole (KCA; 10 μg), nystatin (NY; 100 units), and econazole (ECM; 10 μg) (Mast Group, UK). After incubation at 37°C for 24 h, the growth inhibition zones were measured and interpreted in accordance with CLSI guidelines (10, 11).

To evaluate the cumulative data concerning antimicrobial resistance in selected bacteria isolated from pigeons, comprehensive literature search was performed in the PubMed

database for studies published from 01.01.2000 to 01.07.2020. The database was searched for the following keywords: bacterial infection, antimicrobial resistance, and pigeon, giving a total of 35 search results. Manual revision and selection of data were based on information in the titles and/or abstracts. Selected publications had to contain extractable data in English on the number of bacterial strains isolated from clinical and/or non-clinical samples from feral and/or domestic pigeons. Moreover, they had to contain data on the resistance profile to the tested antibiotics separately for each tested strain. Considering the fact that among the publications that meet the above criteria, the most numerous were those relating to *E. coli,* 11 publications were selected for the final analysis. Selection of studies and extraction of data were done independently by the authors AG and EK and then compared and reviewed by the third author DCC. The extracted data was collected in a database created for this publication and analyzed for the percentage of strains resistant to particular classes or subclasses of antibiotics. The results obtained in the research on feral pigeons and homing pigeons were also compared.

RESULTS

In the present study, *S. aureus*, non-hemolytic *E. coli*, and *C. albicans* were isolated from oral cavity of racing pigeon. Disk-diffusion method revealed in *E. coli* isolate intermediate susceptibility to amikacin only. Furthermore, it was resistant to amoxicillin with clavulanic acid, cefpodoxime, cephalothin, gentamicin, tetracycline, doxycycline, sulfamethoxazole with trimethoprim, florfenicol, enrofloxacin, and ampicillin. Whereas, *S. aureus* isolate was resistant to all beta-lactam antibiotics tested and to amikacin, gentamicin, tetracycline, doxycycline, florfenicol, and clindamycin, erythromycin. Intermediate susceptibility was confirmed only to enrofloxacin. The detection of the *mecA* gene in isolated *S. aureus* strain correlated with the antimicrobial resistance phenotype indicating MRSA (methicillin-resistant *S. aureus*). Both bacterial isolates were resistant to at least three antimicrobial classes, thus could be classified as multidrug-resistant pathogens (12).

In mycological examination, *C. albicans* isolate was resistant to clotrimazole, natamycin, flucytosine, and amphotericin. Moreover, it was intermediately susceptible to ketoconazole, nystatin, and econazole.

According to our best knowledge, 10% florfenicol acquired from unknown source was administered orally despite the antibiogram result. The outcome of the disease has remained unknown.

DISCUSSION

The highlight of this case is the fact that three different pathogenic microorganisms were isolated from an affected racing pigeon, and all of them were multidrug resistant. Although, increasing resistance to antimicrobials in bacteria and fungi is a well-known fact, mistakes in antimicrobial therapy are still common (6, 7). Antibiotics are often administered "blindly," without previous microbiological examinations, and the drug selection is often random. Antimicrobial therapy must base on the results of antimicrobial susceptibility testing and on the prescription of a veterinarian. In many cases, the antibiotic use is unnecessary because the etiological agent of a disease is not of bacterial origin. Other common problems are wrong dosage of a drug, and too long or too short duration of the treatment. Therapy is often not continued as soon as the clinical symptoms subside. In case of animals taking part in competitive sport, including racing pigeons, before the sporting event, antibiotics are frequently given preventively to treat any possible disease, even if the animal shows no clinical symptoms. Among the domestic pigeon breeders even more irresponsible practices concerning antibiotic usage may occur. Antimicrobial cocktails (preparations consisting of antibiotics from different classes) are purchased from unknown sources and sometimes also shared between breeders. This cocktails can contain not only antimicrobials registered for pigeons or other animals but also antimicrobials registered for humans (13).

The resistance of the *E. coli* isolate to enrofloxacin and doxycycline, as well as the resistance of the *S. aureus* isolate to doxycycline and intermediate susceptibility to enrofloxacin, may be associated with an extensive use of those antimicrobials authorized for treatment of pigeons in Poland. Similar observations were described previously for pigeon pathogens by other research groups (7, 13, 14). However, the resistance to aminoglycosides, macrolides, and phenicols, which are not registered in Poland for use in pigeons, suggests the possible acquisition of resistance determinants from other bacteria, as well as an effect of selective pressure caused by unauthorized previous treatment with antibiotics from these classes. Moreover, we recognized MRSA in the racing pigeon in Poland by PCR with *mecA*-specific primers. Up to date, there is only one report concerning the presence of pigeon methicillin-resistant staphylococci in Poland, but this feature was not genetically

TABLE 1 | Cumulative results of antimicrobial resistance in *E. coli* isolated from pigeons, according to publications available in the PubMed database (18, 21–30).

Antimicrobial or antimicrobial Class		% of resistant strains
Beta-lactams	Penicillins	45
	Cephalosporins	18
	Cefamycins	17
	Penicillins with betalactamase inhibitors	8
Olaquindox		82
Tetracyclines		65
Lincosamides		42
Aminoglycosides		40
Phenicols		32
Fluoroquinolones		29
Macrolides		25
Sulfonamides		17
Nitrofurantoin		17
Tigecycline		3

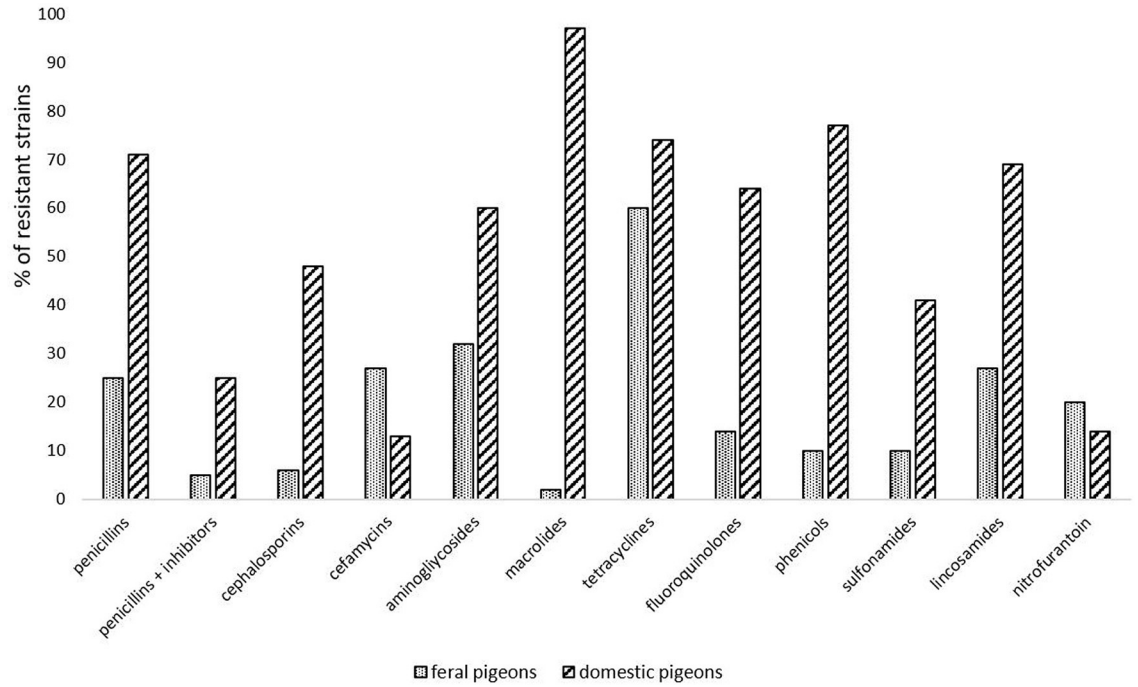

FIGURE 2 | Comparison of antimicrobial resistance in *E. coli* strains isolated from feral and domestic pigeons, according to publications available in the PubMed database (18, 21–30).

confirmed (14). Multidrug-resistant, biofilm-producing *S. aureus* strains were also isolated from pigeons with conjunctivitis in Iran (15). Moreover, in Italy it was shown that pigeons can be colonized by methicillin-resistant *S. aureus* (16).

In this study, we also found multidrug-resistant *E.coli* isolate. It was previously shown that pigeons are reservoir of multidrug-resistant *E. coli*, including ESBL-producing strains (17–19). Cunha et al. (20) found that feral pigeons carried ESBL-positive *E. coli* strains producing the enzymes CTX-M-2 and CTX-M-8 (20).

Cumulative data based on the analysis of available publications concerning antimicrobial resistance in *E. coli* isolated from pigeons has shown that the majority of them were resistant to tetracyclines. This may be due to the fact, that tetracyclines are registered for birds in many European countries, including Poland. Another class of antimicrobials registered for birds are fluoroquinolones and according to the cumulating data 29% of strains were reported as resistant to them. The highest percentage of strains was resistant to olaquindox; however, data on this antibiotic came only from one study from China (21) (**Table 1**). **Figure 2** compares the differences in resistance to different classes of antibiotics of *E. coli* strains isolated from feral and domestic pigeons. In general, *E. coli* strains obtained from domestic pigeons shown higher rate of resistance to all antimicrobials tested, except nitrofurantoin. However, it is worth noting that most studies on the prevalence of multidrug-resistant zoonotic pathogens concerned feral pigeons, and infectious agents were isolated from faeces of healthy birds. There is only limited data on the isolation of such pathogens from clinical samples, and they are mainly obtained from racing pigeons.

There is also literature data indicating the presence of multidrug-resistant yeasts in pigeons (31). Multiple studies showed the prevalence of yeasts belonging to the genus *Cryptococcus*, *Candida*, *Rhodotorula*, and *Trichosporon* in pigeon droppings (32–36). Moreover, many strains were resistant to the azole antifungal drugs (36). However, as it was described in the case of bacterial isolates, there is only limited data on the isolation of multidrug-resistant yeasts from clinical samples of pigeon origin.

The occurrence of methicillin-resistant staphylococci and other multidrug-resistant microorganisms in pigeons is alarming due to the fact that these pathogens can be transmitted to humans and other animal species. Pigeons may shed such microorganisms in a wide geographical area because the competition flights cover considerable distances (37). Moreover, these birds share the same environment with humans, domestic and wildlife animals, and act as carriers of many emerging pathogens. It is worth to mention that feral pigeons are known to be the source of human pathogens such as toxigenic *E. coli*, *Salmonella*, and *Enterococcus* (25, 30, 38–42).

The potential risk for public health posed by drastically increasing multidrug resistance of microorganisms isolated from pigeons must be highlighted. However, it must be also emphasized that veterinarians should inform pigeon breeders that multidrug resistance leads to higher morbidity, mortality, and increased treatment costs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DC-C, AG, and EK contributed to conception and design of the study. BD and KA performed the initial sampling procedure and the collection of isolates. DC-C, EK, and MJB conducted the experiments. DC-C, AG, EK, and MR analyzed the data. DC-C, AG, and EK wrote the draft of the manuscript. MB, MR, and MS critically reviewed sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
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The Impact of Intensive Fish Farming on Pond Sediment Microbiome and Antibiotic Resistance Gene Composition

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Aquaculture is a fast-growing animal food sector, and freshwater fish farming is particularly common in Central and Eastern Europe. As the biodiversity of fishery ponds is changed toward fulfilling the industrial needs, precautions should be taken to keep the system sustainable and protect the adjacent environment from possible damage. Due to risk of infectious diseases, antibiotics are used in aquaculture production systems. The constant exposure to antimicrobials can contribute to the rise of antibiotic resistance in aquaculture products and the adjacent ecosystems, with possibility of dissemination to the wider environment as well as between animals and humans. Even though previous studies have found antibiotic resistance genes in the sediments and water of farming ponds, the tendency and direction of spreading is not clear yet. The objective of this project was to evaluate the influence of intensive fish farming on the condition of water bodies used for the aquaculture and the environment, concentrating on the impact of the aquaculture on the surrounding water ecosystems as well as the possibility of transferring the pollutants and antibiotic resistance genes to both environment and the human hosts. Combined measurement of antibiotic and heavy metal contamination, toxicity assessment, microorganism diversity, and the detection of common antibiotic resistance genes was performed in the sediments of one fishery farm ponds as well as sampling points upstream and downstream. All the tested sediment samples did not show significantly elevated heavy metal concentrations and no substantial veterinary antibiotic pollution. From the antibiotic resistance genes tested, the presence of aminoglycoside and β-lactam resistance determinants as well as the presence of integrons could be of concern for the possibility of transfer to humans. However, despite the lack of heavy metal and antibiotic pollution, the sediments showed toxicity, the cause of which should be explored more.

Keywords: antibiotic resistance genes, fish farming, heavy metals, sediment microbiomes, sediment toxicity

INTRODUCTION

According to the report of Food Agriculture Organization of the United Nations 2020 aquaculture is one of the most important food sectors which, increased by annual 3.1% during 1961–2017 and exceeded annual world population growth (1.6%) almost double for the same period. Fish ponds are rich in dissolved organic materials due to the intensive feeding and fecal waste. Ponds continuously accumulate sediments after the formation of their basins with the influence of water regime (filling and discharging). These sediments are formed from biological remains originating in the ponds and its catchment area as well as soil particles and other non-biological materials that were transported to the pond. The most common type of sediments that is found in ponds is organogenic sediments (1). The intensity and combination of these processes are very variable depending on the different geological and geomorphological settings, hydrological regimes, and atmospheric conditions, as well as human activities (2, 3). The composition of sediments of aquaculture ponds could also be influenced by changes in the biodiversity, as the aquaculture is directed toward fulfilling the industrial needs. The monoculture of highly-productive industrial aquatic organisms is introduced and sustained by intensive rearing system, differing greatly from wild aquatic ecosystems.

Another factor that should be considered in the sediments of the fishery ponds is the presence of heavy metals. Heavy metals which enter aquatic environment typically bond with bottom sediments and, thus over time, can reach high concentrations. In these circumstances heavy metals can become a potential risk to human health through the food chain (4).

Heavy metal toxicity is of great ecological concern, due to their stability, bioaccumulation and non-biodegradability. The accumulation of the heavy metals can lead to the changes in microbial community composition and activation and accumulation of heavy metals resistance genes, that are often closely related to antibiotic resistance genes (5–9). It has been previously observed, that the co-selection of heavy metal and antibiotic resistance genes (ARGs) are happening in the environment (5), thus increasing the concern of the accumulation and spread of potentially hazardous ARGs from the environment to humans.

A more straightforward influence on accumulation and spread of ARGs in aquaculture is the use of antimicrobial substances for animal treatment. In the Eurozone, the use of veterinary drugs is regulated through EU Council Regulations (10, 11), which describe procedures for establishing maximum residue limits for veterinary medicinal products in foodstuffs of animal origin. In Lithuania, only two broad-spectrum antibiotics florfenicol and oxytetracycline are authorized for aquaculture use (http://vetlt1.vet.lt/vr/). Florfenicol is a structural analog of chloramphenicol similar to thiamphenicol, but is more active against some bacteria than chloramphenicol (12). Oxytetracycline is a tetracycline broad-spectrum antibiotic with bacteriostatic action, used to treat systemic bacterial infections of fish (13, 14). Among the 11 major aquaculture producing countries, about 73% applied oxytetracycline and florfenicol (15). The introduced antibiotics not consumed with the feed or excreted by the fed animals enter the water where they can persist or even concentrate in the sediments. The residual amounts of antibiotics in the environment have the potential to cause considerable impact on human health and ecosystems (16). However, these antibiotics are still not included in the (updated) Watch List of the Water Framework Directive (17). The antibiotic pollution problem deepens as farm animal manure can also be used to increase productivity of fishery ponds (18), thus introducing antibiotics used for animal treatment.

The analysis of sediments composition of long-running aquaculture farming is important for determination of the impact of anthropogenic activity and dynamics of pond ecosystems. The comparison of the sediments in the fishery ponds as well as upstream and downstream could show the impact of the intense aquaculture on the surrounding water ecosystems as well as the possibility of transferring the ARGs to the human hosts. We have chosen to analyze the sediments of Simnas fishery farm in Southern part of Lithuania, comparing them with samples in the inflow point located upstream from the fishery farm (chosen as an area untouched by antropogenic activity) and the outflowing river, carrying surplus water from the ponds. The aim of this study was to investigate the pollution of heavy metals and residual antibiotic in fishery ponds and the inflow and outflow points. Together with sediment composition analysis we explored the toxicity and determined the diversity and changes in microbiota composition as well as the presence of ARGs.

MATERIALS AND METHODS

Sediment Sample Collection

Sediment samples were collected during September 2019, from 20 sites in or near Simnas fishery ponds, located in Southern part of Lithuania. The sampling areas cover the inflow (Kalesnykai pond, C1) and outflow (Dovine river, E2) points of Simnas fishery ponds and 18 sample points directly in the fishing ponds (B1–B8 main fishery pond, BE1 exit from the main pond, U1–U8 unused ponds, S1–S2 nurseries). Sediment samples were collected from surface sediment layer using Kajak corer, registered and placed in plastic bags. The exact locations of the sampling points were recorded using the GNSS (Global Navigation Satellite System) device. In addition, altitudes of the main pond were registered and bathymetry was produced (**Figure 1**). ArcMap 10.8.1 software was used for mapping and geo-spatial analysis.

Determination of Heavy Metal Concentration

Sediment samples were dried at 110°C to the constant mass, then the particles of the 125 μm size were separated and concentrations of HM (Heavy metals) were analyzed using X-ray fluorescence spectrometer NITON XL2 Analyzer (2009). The overall accuracy of chemical elements analyzed is between 10 and 20% for different chemical elements.

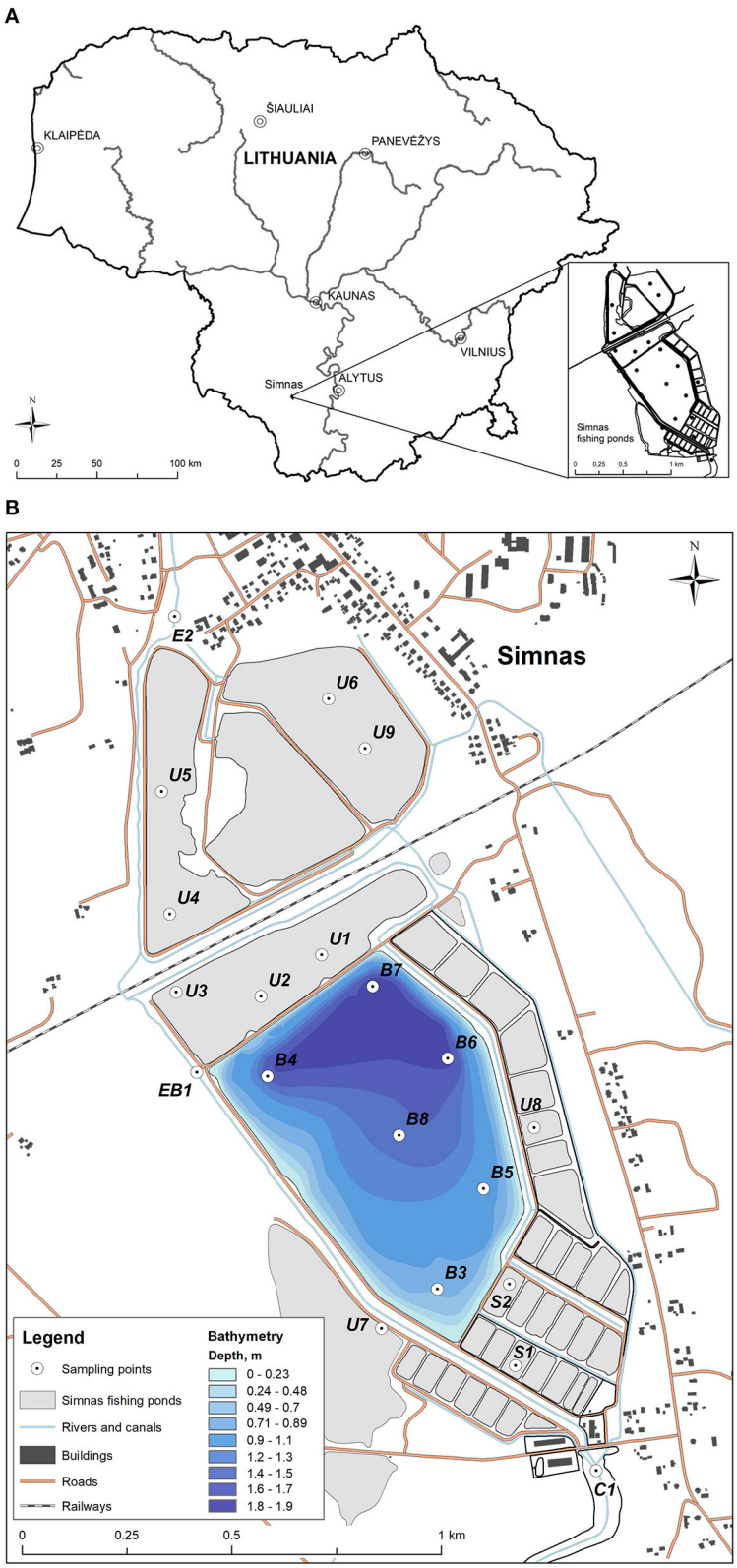

FIGURE 1 | The sampling points in Simnas fishing ponds. **(A)** The location of Simnas fishery ponds in Lithuania, **(B)** The map of Simnas fishery ponds. Sample collection points are indicated and named in the figure, as well as the legend for the bathymetry measurements.

Detection of Antibiotic Residues by HPLC-MS

Antibiotic residues were extracted from the sediment samples located in the main fishery pond and the exit areas, based on the method described previously (19). The sediment extracts were cleaned-up and concentrated using solid-phase extraction SAX cartridges (Merck, Germany) and HLB cartridges (Merck, Germany) in a tandem arrangement. The samples were eluted with 10 ml of methanol. Finally, the eluted samples were evaporated to dryness and dissolved in 1 ml of an aqueous 40% methanol solution (v/v). The resulting sediment extraction samples were analyzed using high-performance liquid chromatography-mass spectrometry (HPLC-MS) system (Shimadzu, Japan) equipped with a photodiode array (PDA) detector (Shimadzu, Japan) and mass spectrometer (LCMS-2020; Shimadzu, Japan) with an electrospray ionization (ESI) source. The chromatographic separation was conducted using a YMC Pack Pro column (3 × 150 mm; YMC, Japan) at 40°C and a mobile phase that consisted of 0.1% formic acid water solution (solvent A) and acetonitrile (solvent B) delivered in the 5–95% gradient elution mode. Mass scans were measured from m/z 50 up to m/z 1,200 at a 350°C interface temperature, 250°C desolvation line (DL) temperature, ±4,500 V interface voltage, and neutral DL/Qarray, using N_2 as nebulizing and drying gas. Mass spectrometry data were acquired in both positive and negative ionization modes. The data were analyzed using LabSolutions liquid chromatography-mass spectrometry (LCMS) software.

Sediment Toxicity Bioassay

The acute luminescent bacteria test was performed in compliance with ISO 11348-3:2007 using the *Aliivibrio fischeri* strain NRRL B-11177. The composition of bacterial culture growth medium and growth conditions were presented earlier (20). Biomass and suspension of marine *A. fischeri* for luminescence measurement was prepared as previously described (21). Sediment suspensions (solid-phase), aqueous elutriates and respective serial dilutions were prepared as described earlier (22). The exposure experiment started after addition of 20 μl bacteria suspension to each well containing 80 μl of prepared samples (sediment suspensions or elutriate supernatants at different concentrations), control (2% w/v NaCl) and reference chemical (3.5-dichlorphenol). The effect of elutriate supernatants and sediment suspensions was determined after 1 and 30 min, respectively, using microplate reader Tecan Infinite M200 (Tecan Group Ltd., Männedorf, Switzerland) at 20°C. Three independent measurements were conducted in duplicate. The level of luminescence inhibition in exposed groups was expressed as percentage relative to control according to formula: INH (%) = $100 - BL_S/BL_C \times 100$; where BL_S bacterial luminescence after exposure to samples; and BL_C bacterial luminescence in control after respective incubation time. Median effective concentration (EC_{50} in mg dry weight/ml) of sediment suspensions was obtained using Regtox software (version EV7.0.5, Eric Vindimian, Paris, France). Since it was not possible to derive EC_{50} values for sediment elutriates it was replaced by the inhibition value after 1 min exposure to undiluted sediment elutriates corresponding to 75 mg dw sed./ml as suggested earlier (22). Solid-phase EC_{50} values were converted to toxic units (TU) values as follows: $TU = 100/EC_{50}$. Samples were classified using Persoone et al. (23) classification system. Toxicity classes were determined according TU values estimated for solid-phase and percentage effect (PE) for sediments elutriates. No acute toxicity if $PE < 20$; slight acute toxicity if $PE < 50$; acute toxicity if $1 \leq TU < 10$; high acute toxicity if $10 \leq TU < 100$; very high acute toxicity if $TU \geq 100$.

DNA Extraction

Genomic DNA was isolated from sediment samples using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, USA) according to the manufacturer's recommendations. The concentration of extracted DNA was evaluated using a biophotometer (Eppendorf, Germany). Four DNA extractions were carried out for each sample. DNA was stored at −80 °C until further analysis. PCR inhibition was tested using primers Frrs/Rrrs (**Supplementary Table 1**).

Microbiome Analysis

Sequencing

The composition of the bacterial community was determined by next-generation sequencing (NGS) by scanning the amplicons of the bacterial 16S rRNA gene. The V3–V4 16S rRNA regions were chosen for sequencing because they are capable to detect both bacterial and archaea taxons with high resolution (24, 25). NGS was performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) on Illumina paired-end platform to generate 250 base pairs (bp) length paired-end raw reads.

16S rRNA Data Analysis

The reads were demultiplexed. Barcode and primer linker sequences were removed using "cutadapt" tool (26). The following steps were performed in QIIME2 (version 2020.11) (27). Data were denoised using read quality scores, low-quality part at the end of reads was trimmed (227 bp were left in forward and 224 bp in reverse reads), paired-end reads were merged and chimeras were removed using the pipeline that includes DADA2 algorithm (28). The result of DADA2 pipeline was amplicon sequence variants (ASV). Phylogenetic trees were created using MAFFT sequence alignment (29) and FastTree tree generation (30). Taxonomic, alpha and beta diversity analyses were based on ASV's. Taxonomic annotation was assigned by using pre-fitted Scikit-learn (31) based taxonomy classifier trained on full 16S gene (available at https://data.qiime2.org/2020.11/common/gg-13-8-99-nb-classifier.qza) based on Greengenes database (v13_8) at 99% threshold (32) *via* QIIME 2's "q2-feature-classifier" plugin (33). Core alpha and beta diversity metrics were generated with rarefaction depth equal to the lowest feature count of a single sample (34). Jaccard index was used as a measure of beta diversity.

Antibiotic Resistance Gene Detection

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, previously found in the environmental samples were also screened. The presence of genes encoding antibiotic resistance determinants was assessed by PCR at the same conditions as described earlier (35). The genes tested and specific primers used are described in **Supplementary Table 1**. Together with ARGs detection, the presence of genes conferring resistance to heavy metals (As, Co, Cu, Pb, Cr) was also tested, the genes and primers are presented in **Supplementary Table 1**. PCR amplifying 16S rDNA fragment was used in parallel as amplification control.

RESULTS

Heavy Metal Content Analysis

The concentrations of heavy metals that were detected in fishing ponds were similar to the geochemical composition of the bottom sediments of other Lithuanian lakes, with the exception of individual ponds located on both sides of the railway. The bottom sediments of these ponds were contaminated with Co and Cr which are common pollutants of railways (**Figure 2**). Co concentrations were up to 4 times (varies from 69.2 to 191.2 ± 23 mg/kg) higher than the maximum allowable concentrations (MAC) stated for lake bottom sediments. Concentrations of Cr did not exceed the MAC, but higher concentrations were also detected among both sides of the railway (varies from 18.8. to 53.9 ± 8.2 mg/kg).

An increase of As and Cu concentrations was also observed. Concentrations of these elements in the bottom sediments of the main fishing pond did not exceed the MAC, but a higher accumulation of these elements was observed in the northern part (concentrations of As varies from 6.1 to 13.7 ± 2.3 mg/kg, Cu—from 11.3 to 26.8 ± 6.4 mg/kg), due to the relief of the bottom of the pond (**Figure 2**), which descends from south to north. In this part, optimal conditions are formed for the sedimentation processes of bottom sediments (accumulation of sediments). In this way chemical elements are not removed or redistributed to other parts of the pond together with bottom sediments.

An analysis of the distribution of concentrations of chemical elements in different fisheries ponds showed that no influx of high concentrations of hazardous heavy metals could be observed during fish rearing activities. Only slight increase of As and Cu concentrations could be related with the activity of fishing ponds. The main sources of the pollution were the railway line crossing the territory of the fishery ponds and the nearby city of Simnas.

Determination of the Antibiotic Residues in Sediments

Eight sediment samples (B3-B8, collected from the main fishery pond, and exit points BE1 and E2, located at the exit from the main fishery pond and the exit from the whole Simnas fishery farming, respectively) were analyzed for the presence of veterinary antibiotic residues by HPLC-MS. The characteristic molecular ions indicating oxytetracycline, florfenicol, and florfenicol amine (**Supplementary Figures 1A–C**) were not detected in any sediment sample tested, including sample no. B5 (**Supplementary Figures 2**, **3**) which was chosen for representation. The limits of detection were 5.3, 9.2, and 15 ng/g in dry sediments for oxytetracycline, florfenicol, and florfenicol amine, respectively. Limits of detection were defined as the sample concentrations at a signal-to-noise ratio (S/N) of 3. Our findings indicate that the concentrations of oxytetracycline, florfenicol, and florfenicol amine in the sediment samples collected from Simnas fishery ponds were very low or below the detection limit.

Sediment Toxicity

Solid-phase test results indicated that all tested sediment samples caused acute toxicity to *A. fischeri* (**Table 1**). Interestingly, the most toxic solid-phase of sediment sample was from Kalesnykai pond (C1), which was analyzed here as a clean entry point. The least toxic solid-phase of sample was collected from Exit site (E2). As it was expected due to complexity the solid-phase was more toxic than sediment elutriates. In case of undiluted aquatic sediment elutriates, the most part of analyzed samples did not inhibit the luminescence of *A. fischeri* bacteria, but enhanced light production (**Table 2**). Only one sample, which was collected in the main fishing pond (B7) caused slight acute toxicity.

Microbiome Analysis

The number of species identified in one sample varied from 1,949 to 3,619 species for samples from ponds, 2,673 for samples at the entrances to the ponds (C1), as well as 3,619 and 3,450 for samples at the exit points (BE1, E2).

Sediment sample analysis showed that 10 phyla with highest average relative abundance of identified microorganisms were *Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Chloroflexi, Acidobacteria, Nitrospirae, Verrucomicrobia,* and *Chlorobi* (**Figure 3**, left). *Proteobacteria* phylum microorganisms predominate in all samples (mean 55.8%, SD = 5.4%). The highest relative abundance of *Proteobacteria* was identified in the U7 sample (72.4%) and the lowest in the U8 sample (47.6%). *Actinobacteria* phylum microorganisms also make up a large part of the microorganism communities (mean 11.4%, SD = 4.1%). The highest number of these microorganisms was detected at the S2 point (15.6%) and the lowest at the B7 point (5.2%). *Bacteroidetes* abundance varies between 3.1 and 15.2%, *Firmicutes*—1.3–12.4%, *Chloroflexi*—1.1–7.6%, *Acidobacteria*—0.9–6.7% and form a significant proportion of bacterial communities. *Nitrospirae*, *Verrucomicrobia,* and *Chlorobi* were less frequently detected, with a <5% in relative abundance. It is important to note that *Cyanobacteria* are particularly characteristic of U2 (14.0%), U8 (31.2%), and S2 (16.3%) samples. At the remaining points, the cyanobacterial content did not exceed 5%.

The analysis of the relative abundance of the classes (**Figure 3**, right) revealed that dominant microorganisms in the samples belong to *alpha-proteobacteria* (3.7–23.0%), *beta-proteobacteria* (13.9–22.8%), *gamma-proteobacteria* (7.2–19.7%), and *delta-proteobacteria* (9.2–23.8%) classes. The abundance of the *Acidimicrobiia* class varies from 1.0% (B7) to 5.4% (S2). Small amount of *Thermoleophilia* (0.8–5.9%) was detected in all the samples, as well as *Bacilli* class microorganisms (0.6–11.1%).

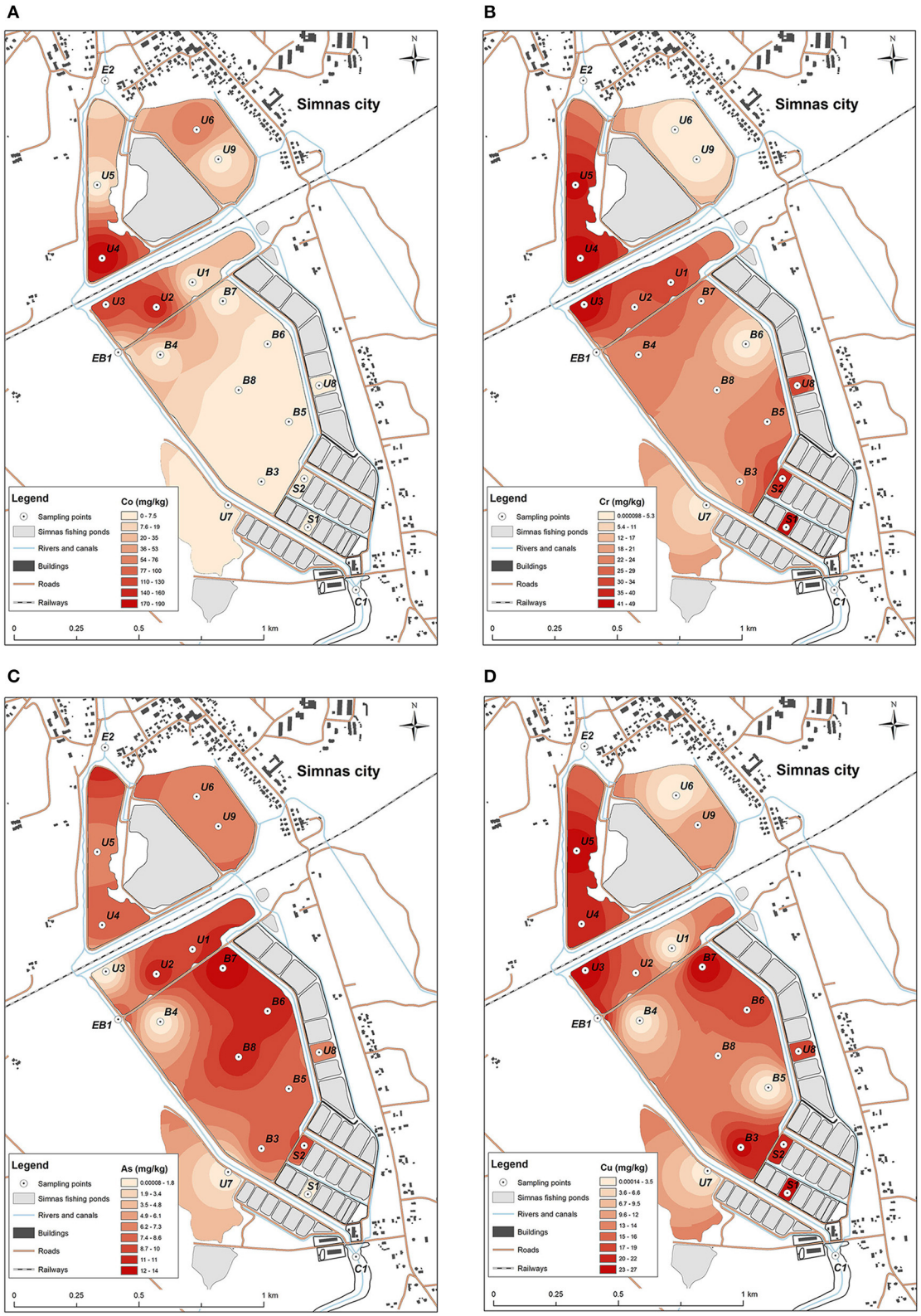

FIGURE 2 | Distribution of heavy metals concentrations: **(A)** Co, with the highest concentration detected in U4, U3, and U2 sediment samples, **(B)** Cr, most abundant in small nursery ponds S1, S2, and U4, U5, U3, U8, **(C)** As, mostly detected in the sediments from the main pond at B8, B7, B6, and U2 sampling point from the unused pond, **(D)** Cu, prevalent in all the testes ponds, highest concentrations determined in U4, U5, U3, B3, B7, U8, S1 and S2 locations.

TABLE 1 | Solid-phase EC_{50} (mg sediment dry weight/ml), 30 min, determined using *A. fischeri* luminescence inhibition test.

Sample No.	EC_{50}, mg dry weight/ml, 30 min	Confidence interval, 95%	TU (toxic unit)	Classification according Persoone et al. (23)
C1	18.71	12.21–25.77	5.35	acute toxicity
B3	38.05	37.90–38.30	2.63	acute toxicity
B4	21.94	21.81–22.01	4.56	acute toxicity
B5	52.53	50.29–54.40	1.90	acute toxicity
B6	45.11	38.52–49.78	2.22	acute toxicity
B7	25.63	23.40–28.72	3.90	acute toxicity
B8	51.60	47.50–54.33	1.94	acute toxicity
E2	62.94	62.94–62.94	1.59	acute toxicity

TABLE 2 | Inhibition of *A. fischeri* luminescence caused by undiluted sediment elutriates corresponding to 75 mg sediment dry weight/ml, after 1 min incubation.

Sample No.	Inhibition average, %	Standard deviation	Classification according Persoone et al. (23)
C1	−66.15	0.18	No acute toxicity
B3	−7.70	3.48	No acute toxicity
B4	−3.52	10.50	No acute toxicity
B5	19.59	22.33	No acute toxicity
B6	15.80	3.09	No acute toxicity
B7	38.23	4.56	Slight acute toxicity
B8	16.60	5.37	No acute toxicity
E2	−54.94	33.33	No acute toxicity

The microbiome beta-diversity analysis results clearly indicated the differences between the microbiota composition of all pond sediments and entrance point, treated as a clean area (**Figure 4**). In the PC plot, the samples from the exits of the ponds were situated in the middle of the other sample points, and the points from the main pond (orange) formed a separate cluster. In this plot, the differences of the clean (taken upstream the ponds) and the remaining specimens were evident.

Members of *Archaea* domain were found in the sediments as well. Most of them belonged to phylum *Parvarchaeota*, *Crenarchaeota*, and *Euryarchaeota* (**Figure 5**). *Euryarchaeota* was the predominant phylum of *Archaea* found in all the specimens, with abundance varying from 0.1 to 1.1%. Highest abundance of *Archaea* (2.0%) was discovered in U1 sample (*Parvarchaeota* 0.1%, *Crenarchaeota* 0.1%, *Euryarchaeota* 1.8%).

Antibiotic Resistance Gene Detection

The ARG detection results are presented in **Figure 6**. Tetracyclin resistance genes were quite common in the sediment samples, the most common one being *tetM*, detected in more than a half of the samples. However, *tetM* was also found in entrance point sample (C1), indicating the spread of tetracyclin ARGs might not be related to fishery pond treatment. The screening for β-lactamase ARGs, revealed an extended spectrum β-lactamase (ESBL) *tem* gene, which was found in the samples both main pond and in the unused ponds, and one case of the *shv* ESBL gene. *shv* gene was detected in the sample B7 located in the deepest part of the pond where accumulation of sediments could occur (**Figures 1**, **6**). Apart from ESBLs, the only genes of known clinical relevance were the ones coding for aminoglycoside modifying enzymes. *The aph(3′)-Ia, aac(6′)-Ib, aac(3)-Iab, ant(3″)-Ia and ant(6)-I*, genes, coding for a range of aminoglycoside resistance were detected, three of them in the entrance point of the fishery ponds C1. Macrolide resistance gene *ermC* was present in the majority of samples, while *ermA* and *ermB* were also detected. We have also tested for the most common heavy metal resistance genes (**Supplementary Table 1**), however, only one instance of *chrB* gene, coding for a regulator of Cr resistance operon, was detected in U3 sample (not shown), which represents one of the most Cr polluted areas in the ponds (**Figure 2**).

Since an important trait of both heavy metal resistance genes and ARGs is their ability to be transferred between the organism thus spreading the trait, we also tested the sediment samples for the presence of integrons. Only integrase genes belonging to class I were found in the sediment samples, spread evenly between main ponds and unused ponds and also in the C1 sample, indicating the presence of mobile elements in the fishery ponds as well as the adjacent areas.

DISCUSSION

In this study, we aimed to evaluate the condition of Simnas fishery ponds, that have been used for aquaculture since 1964. Only one main pond is currently used for fish farming, and several smaller ones are still used as nurseries, therefore we had an opportunity to see the differences in fishery pond sediments composition under intense use vs. unused for several years.

Most of the tested heavy metal concentration mostly did not exceed the MAC, only Co was detected up to 4-fold higher concentrations than MAC. The highest concentrations of Co and Cr was apparently due to railway line passing the fishery ponds territory. However, increased concentrations of Cr were also observed in small ponds used as nurseries (S1 and S2). Increased concentrations of Cu and As were mostly dependent on the descending relief of the ponds, concentrations increasing where the sediments collect. Concentrations of heavy metals in sediments of Simnas fishing ponds were similar to other already investigated lakes in Lithuania, situated in a moderate anthropogenic environment (36, 37) and were much higher than the background concentrations of natural and semi natural lakes (38). Though the concentration in the fishery pond samples did not exceed the MAC (39, 40), the observed heavy metal concentration could indicate the increase of over time is ongoing.

The presence of heavy metals in the environment is known to be connected with ARG co-selection (9). Even present in low levels, heavy metals and antibiotics could enhance the selection of bacteria carrying ARGs (41). Oxytetracycline accumulation in sediments has been reported at concentration levels of a few to hundreds of μg/kg in different water bodies (42, 43), reaching maximum concentration of hundreds μg/kg d.w. was found in sediments sampled near fish farms in Italy (44, 45). However

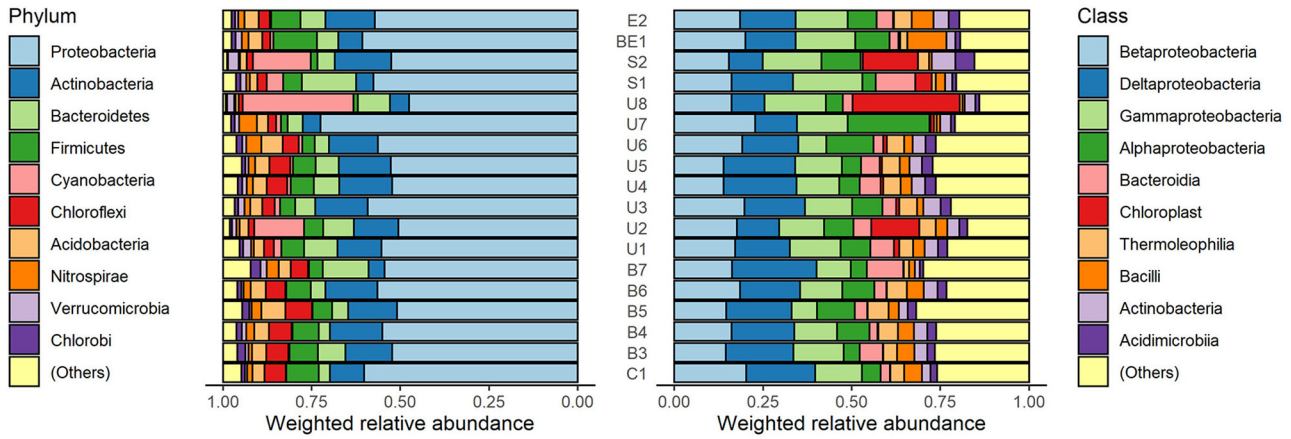

FIGURE 3 | Weighted relative abundance of micro-organisms in phylum **(left)** and class **(right)** level in sediment specimens. Both phylum and class named "(Others)" represent relative abundance of the remaining phyla or classes besides the top 10 ones.

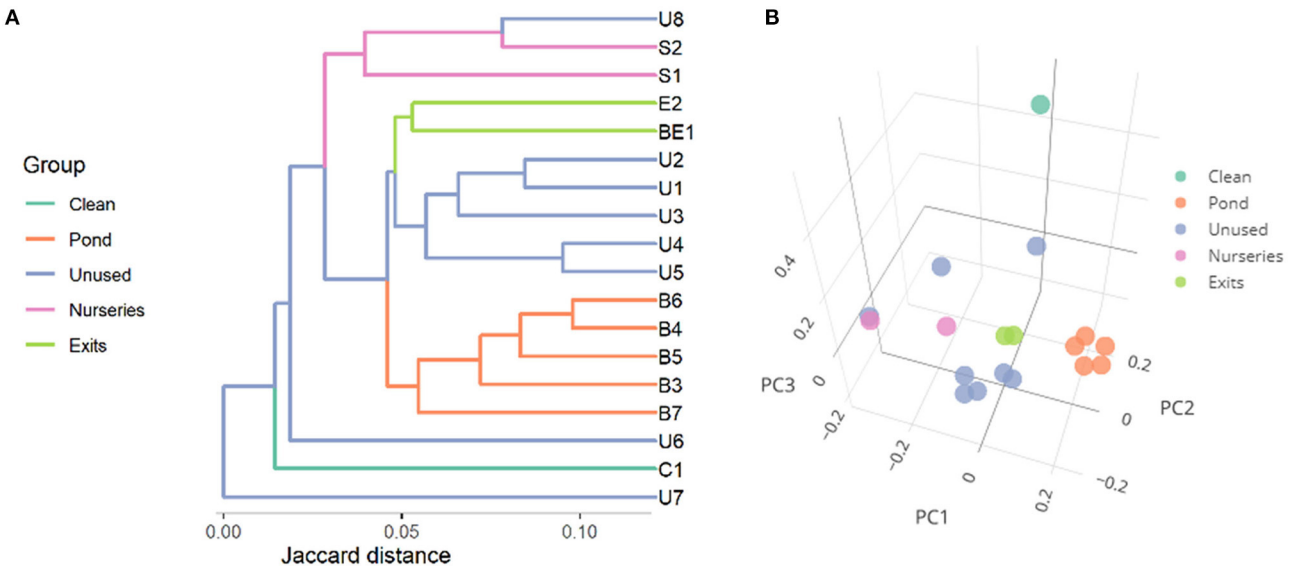

FIGURE 4 | Microbial similarity (beta-diversity) of specimens expressed as Jaccard distances and represented as **(A)** a dendrogram and **(B)** principal coordinates plot. The first three principal coordinates (PC) explain, respectively, 10.8, 8.4, and 7.1% of variance.

florfenicol and its metabolite florfenicol amine can be detected in surface water but not in sediments of aquaculture systems (45). Our testing for the residues of two antibiotics, that are allowed for use in veterinary setting in Lithuania, also did not detect their presence in the main pond sediments.

Our results show only a minor accumulation of heavy metals and no substantial pollution with antibiotics, hopefully indicating no additional pressure on ARG co-selection. However, one case of Cr resistance gene was observed in the area of one of the highest Cr concentrations, indicating further increase of heavy metal concentration could push the microorganisms toward obtaining heavy metal resistance genes, which could be followed by co-selection of ARGs.

The sediment microbiome analysis revealed that *Proteobacteria* are the most abundant phylum found in all the sediment samples. Sediments from fishing ponds are commonly characterized by high concentrations of organic and inorganic substances. These substances settle to the bottom of ponds together with fish feces and uneaten feed and cause eutrophication of water bodies and depletion of oxygen. Our findings are in agreement with other authors indicating that *Proteobacteria* is the most frequent phylum found in water bodies and dominate between fishery ponds microorganisms (46, 47). *Proteobacteria* in aquaculture are known as organic-degrading microorganisms (47). Liu et al. found that *Proteobacteria* predominated in both water and sediment samples, regardless

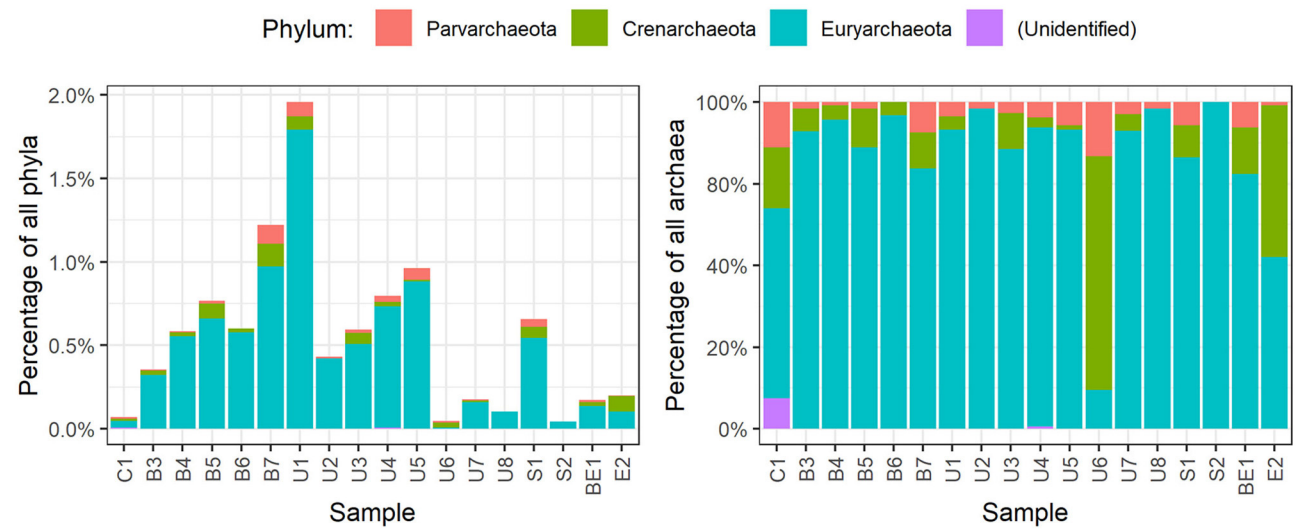

FIGURE 5 | Distribution of archaea phyla in each specimen of sediments.

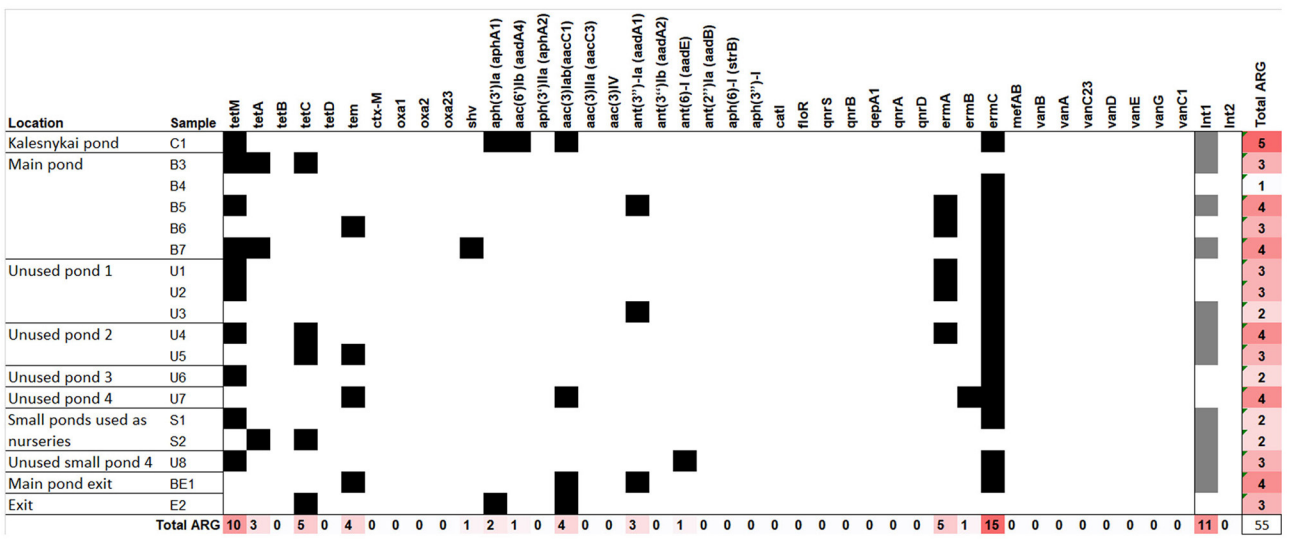

FIGURE 6 | The antibiotic resistance genes detected in the total DNA of fishery pond sediments. Black squares denote the gene was detected. The same samples were tested for the presence of class I and II integrase genes, the presence of the genes is indicated by gray squares.

of the species farmed in the ponds and the aquaculture pattern. meanwhile, discovered that the use of different fish feeds also did not affect the dominance of *Proteobacteria*. The second type of bacteria in terms of the highest abundance is *Actinobacteria* (mean 11.4%, SD = 4.1%). These bacteria are also commonly found in water bodies (48). In addition, the abundance of *Actinobacteria* and *Firmicutes* is known to be positively correlated with sediment pH (46, 49). *Firmicutes*-type bacteria were characteristic of all studied groups of the samples, but most of them were detected in the BE1 sample (12.4%). Meanwhile, *Chloroflexi* microorganisms abundant in all samples play an important role in sediment carbon metabolism (50). An equally important process is the oxidation of fish-toxic nitrites to fewer toxic nitrates (51). *Nitrospirae*-type microorganisms are known to be able to catalyze these oxidation reactions and were found in all samples.

Analysis of the class structure in the samples revealed that the dominant type of *Proteobacteria* consists of microorganisms of the classes *Alfaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria*. High abundance of *Gammaproteobacteria* is associated with an environment enriched with organic substances (46). *Deltaproteobacteria* also can be used as a bioindicator of organic compound contamination. *Betaproteobacteria*, are known as nitrifying

bacteria, capable to oxidize potentially toxic ammonia to non-toxic nitrates. This process is particularly important in aquaculture ponds, where ammonia can reach concentrations harmful to fish (46). It is important to note that very few human pathogenic genera were identified in this study. Highest abundance of family *Listeriaceae* were found in E2 (2.29%) and B3 (2.50%) samples. In C1, B4, U6, U7, BE1 samples number didn't exceed 0.5% and *Listeriaceae* were not detected in the rest of the sampling points. *Listeria monocytogenes* is reported to be predominant in temperate aquaculture. *Listeria monocytogenes* can be found in lightly preserved or raw aquatic food products and become the cause of human disease (52). Other genera with the members exhibiting potential pathogenicity detected in our research are *Bacillus* and *Pseudomonas*, but their relative abundance in sediment samples is low.

The *Archaea* community analysis revealed the predominance of *Archaea* belonging to phylum *Euryarchaeota, Crenarchaeota*, and *Parvarchaeota.* The knowledge of uncultivated archaea, previously known as extreme environment microorganisms, revealed that they can be found in various environments from extreme to ordinary (53). They are an important part of the ecosystem capable of cycling of carbon, nitrogen, sulfur, and others playing the important role in the biogeochemical cycle of those elements (54). Many *Archaea* species are capable to fix carbon from inorganic sources and can affect the dynamics and balance of greenhouse effect related gases. Moreover, *Archaea* are the microorganisms capable to fulfill various metabolic strategies using organic and/or inorganic electron donors and acceptors (55).

ARG were not abundant in the fishery pond sediments, most of the ARGs found were the ones conferring resistance to tetracyclines, which could indicate the history of oxytetracycline use. However, the presence of tetracycline resistance genes has been previously observed also in pristine environments (56), therefore the connection with veterinary antibiotic use need to be analyzed further. A variety of aminoglycosides ARGs were detected, which confer resistance to various aminoglycosides, even the ones used in clinical setting (such as gentamicin, amikacin, tobramicin), which could be a reason for concern. However, the presence of three aminoglycoside genes in C1 sample, which was upstream from the fishery ponds, indicated the ARGs could be present as components of naturally inhabiting microorganisms. Our preliminary data indicate, aminoglycoside ARGs can be found also in the water bodies higher upstream from the fishery ponds, which would further confirm them being a part of the natural microbiota. From β-lactam ARGs *tem* was the most common, which has been observed elsewhere (57, 58), and one sediment sample (B7, located in the deepest part of main pond) also had *shv*. The presence of *shv* in fishery farming samples has also been reported previously (57). However, finding ESBL gene in the environment could always be considered a hazard due to the possibility of transferring it to humans by means of fish produce. Interestingly, *tetM*, *ermC* were also often found in the soil samples from Lithuanian farmland (59), indicating the spread of such ARGs in Lithuania or a natural habitat of the microorganism bearing them. The presence of integrons was also checked. More than a half of tested sediment samples contained integrons, as detected by the presence of integrase (class I) genes, indicated that the discovered ARGs could indeed be mobilized and transferred between the species, including human pathogens.

Even though the heavy metal concentrations did not exceed the MAC and residues of antibiotics were not detected, the toxicity of the samples has been observed. Inhibition of bacteria luminescence could be caused by mixtures of various components, which are at MAC or lower concentrations. Effects of elutriates and solid-phase reflect toxicity of water soluble compounds and whole sediments containing adsorbed chemicals, respectively. The determined toxicity of elutriates was lower than of solid-phase, such differences in toxicity have been observed earlier (22, 60). The solid-phase EC_{50} values (19–63 mg/ml) were similar to sediment toxicity results observed for contaminated river (22) and freshwater aquaculture (61). More than two orders of magnitude lower solide-phase EC_{50} values were determined for sediments of Atlantic coast of Spain (ranged of 0.051–20.23 mg/ml) and was highly affected by sulfide concentrations (60).

Altogether, no elevated heavy metal concentrations and no substantial veterinary antibiotic pollution was detected in Simnas fishery ponds. From the ARGs tested, the presence of aminoglycoside and β-lactam resistance determinants as well as the presence of integrons could be of concern. However, despite the lack of heavy metal and antibiotic pollution, the toxicity of the sediments and its cause should be explored more, as other compounds causing it could be affecting the health of fish population and consequently humans.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI Bioproject PRJNA715198.

AUTHOR CONTRIBUTIONS

EL: sample preparation, total genomic DNA extraction, preparation of the manuscript, acquiring of funding, and interpretation of the metagenome analysis results. VV: sample collection and determination of the heavy metal concentrations. JS: antibiotic detection. VK: toxicity analysis. VG and JK: bioinformatic analysis of the metagenome data. MR: supervision and acquiring funding. JA: supervision of ARG detection, interpretation of antibiotic resistance results, funding acquisition, and preparation of manuscript. All authors contributed to the article 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/fvets.2021.673756/full#supplementary-material

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Isolation, Antimicrobial Resistance Phenotypes, and Virulence Genes of *Bordetella bronchiseptica* From Pigs in China, 2018–2020

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Bordetella bronchiseptica is a leading cause of respiratory diseases in pigs. However, epidemiological data of *B. bronchiseptica* in pigs particularly in China, the largest pig rearing country in the world is still limited. We isolated 181 *B. bronchiseptica* strains from 4259 lung samples of dead pigs with respiratory diseases in 14 provinces in China from 2018 to 2020. The average isolation rate of this 3-year period was 4.25% (181/4259). Antimicrobial susceptibility testing performed by disc diffusion method revealed that most of the *B. bronchiseptica* isolates in this study were resistant to ampicillin (83.98%), while a proportion of isolates were resistant to cefotaxime (30.39%%), chloramphenicol (12.71%), gentamicin (11.60%), florfenicol (11.60%), tetracycline (8.84%), amoxicillin (8.29%), tobramycin (6.63%), ceftriaxone (4.97%), and cefepime (0.55%). There were no isolates with resistant phenotypes to imipenem, meropenem, polymyxin B, ciprofloxacin, enrofloxacin, and amikacin. In addition, ∼13.18% of the isolates showed phenotypes of multidrug resistance. Detection of antimicrobial resistance genes (ARGs) by PCR showed that 16.57% of the *B. bronchiseptica* isolates in this study was positive to *aac(3)-IV*, while 3.87%, 2.21%, 1.10%, 0.55%, 0.55%, and 0.55% of the isolates were positive to *aac6'-Ib*, *rmtA*, bla_{TEM}, bla_{SHV}, *oqxB*, and *tetA*, respectively. Detection of virulence factors encoding genes (VFGs) by conventional PCR showed that over 90% of the pig *B. bronchiseptica* isolates in this study were positive to the five VFGs examined (*fhaB*, 97.24%; *prn*, 91.16%; *cyaA*, 98.34%; *dnt*, 98.34%; *betA*, 92.82%). These results demonstrate *B. bronchiseptica* as an important pathogen associated with pig respiratory disorders in China. The present work contributes to the current understanding of the prevalence, antimicrobial resistance and virulence genes of *B. bronchiseptica* in pigs.

Keywords: *Bordetella bronchiseptica*, isolation, antimicrobial resistance, virulence factors encoding genes, pigs

INTRODUCTION

Bordetella bronchiseptica is an aerobic, motile, gram-negative rod, or coccobacillus belonging to genus *Bordetella*. It is an important pathogenic bacterium in agriculture and in veterinary medicine (1). In veterinary medicine, *B. bronchiseptica* is a leading cause of many respiratory infections including rhinitis, tracheitis, bronchitis, and pneumonia in a wide spectrum of animals (2). It can also enhance respiratory colonization of *Streptococcus suis* and *Haemophilus parasuis*, promote disease caused by *S. suis*, and interact with porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV) to increase severity of respiratory disease (3). While rarely to be reported, *B. bronchiseptica* is also potentially involved in infections in humans, and human cases are frequently associated with direct contact with infected animals such as swine, dog, rabbit and/or cat (4–6). Similar to the other members belonging to genus *Bordetella*, many *B. bronchiseptica* produces several important virulence factors, including filamentous hemagglutinin, and protein toxins, adenylate cyclase toxin, pertussis toxin, dermonecrotic toxin as well as type III secretion system (T3SS) and effector proteins, contributing to its pathogenesis (7, 8).

In swine, *B. bronchiseptica* is proposed as a main causative agent of porcine respiratory disease complex (PRDC) and atrophic rhinitis; both of which are economically-important diseases in pig industry (9, 10). Continuously monitoring the prevalence, antimicrobial resistance (AMR) and virulence profiles of *B. bronchiseptica* in pigs are beneficial for the prevention and control of swine bordetellosis. However, the relevant data are still limited. China is the largest pig-farming and pork consuming country in the world. Although the outbreak of African Swine Fever in August 2018 caused a huge loss of pigs in China, there are still more than 406 million pigs rearing in China in 2020 (11). To understand the current epidemiological and microbiological characteristics such as the antimicrobial resistance profiles of *B. bronchiseptica* isolates from pigs in China, we performed bacterial isolation of *B. bronchiseptica* strains from lung samples of dead pigs with a history of respiratory disorders in China from 2018 to 2020 in this study. These isolates were characterized by testing the antimicrobial susceptibility and detecting the antimicrobial resistance genes (ARGs) as well as virulence encoding genes (VFGs).

MATERIALS AND METHODS

Study Design, Sample Collection, and Ethic Statement

Study design was shown in **Figure 1A**. From 2018 to 2020, a total of 4259 lung samples (3022 samples in 2018, 841 samples in 2019, 396 samples in 2020) from 14 provinces (Guangdong, Henan, Hubei, Shandong, Fujian, Hebei, Zhejiang, Hunan, Anhui, Sichuan, Shanxi, Inner Mongolia, Xinjiang, Guizhou) in China were used for *B. bronchiseptica* isolation and identification (**Figure 1B**). All of the clinical samples used in this study were submitted by veterinarians/or the farm owners to the Veterinary Diagnostic Laboratory of Huazhong Agricultural University (Wuhan, China) for routine testing.

Bacterial Isolation and Identification

Collected samples (∼10 grams per sample) were cut into pieces and lysed in sterile 0.9% normal saline by using a TissueLyser II (QIAGEN, Venlo, Netherlands). Thereafter, tissue homogenates of each sample were streak-plated onto one tryptic soy agar (TSA; Becton, Dickinson and Company, MD, USA) containing 10 μg/ml nicotinamide adenine dinucleotide (NAD; Sigma, St. Louis, MO) and 10% new-born bovine serum. The agar plates were incubated at 37°C for 24∼48 h. Isolates growing on the plates were then purified and cultured following the standard methods used for bacterial identification (12). On each of the agar plates, five colonies with similar morphological characteristics to *B. bronchiseptica* [small circular glistening or rough colonies with 0.5 to 1.0 mm in diameter after 48 h of incubation in air at 37°C (4)] were selected for biochemical test. Presumptive isolates of *B. bronchiseptica* were finally confirmed using polymerase chain reaction (PCR) assay amplifying the species-specific gene *fla* with the primers listed in **Table 1** (26). Considering *B. bronchiseptica* possesses only one serotype (27), we therefore chose one colony confirmed by both PCR and biochemical tests (positive for *fla* and displaying similar biochemical characteristics to *B. bronchiseptica*) to represent *B. bronchiseptica* strain recovered for its corresponding sample.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the *B. bronchiseptica* isolates was tested by using Disk diffusion method following the Clinical and Laboratory Standards Institute (CLSI) antimicrobial susceptibility testing standards (28). Briefly, purified overnight-cultured colonies of *B. bronchiseptica* were picked up from TSA plates and resuspended in sterile 0.9% normal saline to 0.5 McFarland standard. The suspension was then prepared by swabbing on Mueller-Hinton (MH) agar (Sigma-Aldrich, 102 St. Louis, MO) using sterile swabs. After dry for ∼5 min, disks containing specific antibiotics (Hangzhou Microbial Reagent, Hangzhou, China) were dispensed onto the plates. All plates were finally incubated overnight at an incubation temperature of 37°C. A total of 16 types of antibiotics including amikacin [AMK; 30 μg], gentamicin [GEN; 10 μg], tobramycin [TOB; 10 μg], ceftriaxone [CRO; 30 μg], cefotaxime [CTX; 30 μg], cefepime [CPM; 30 μg], imipenem [IPM; 10 μg], meropenem [MRP; 10 μg], enrofloxacin [ENR; 10 μg], ciprofloxacin [CIP; 5 μg], chloramphenicol [CHL; 30 μg], florfenicol [FLO; 30 μg], amoxicillin [AMX; 20 μg], ampicillin [AMP; 10 μg], tetracycline [TET; 30 μg], and polymyxin B [PMB; 300 IU] were tested. The zone diameter values were measured and the results were interpreted according to CLSI document (28). As clinic breakpoints specific to *B. bronchiseptica* are limited available (2), we thereby used breakpoints to *Enterobacteriaceae* published in CLSI document M100 for result-interpretation in this study. Breakpoints used are listed in **Table 2**. *Escherichia coli* ATCC®* 25922 was used as quality control.

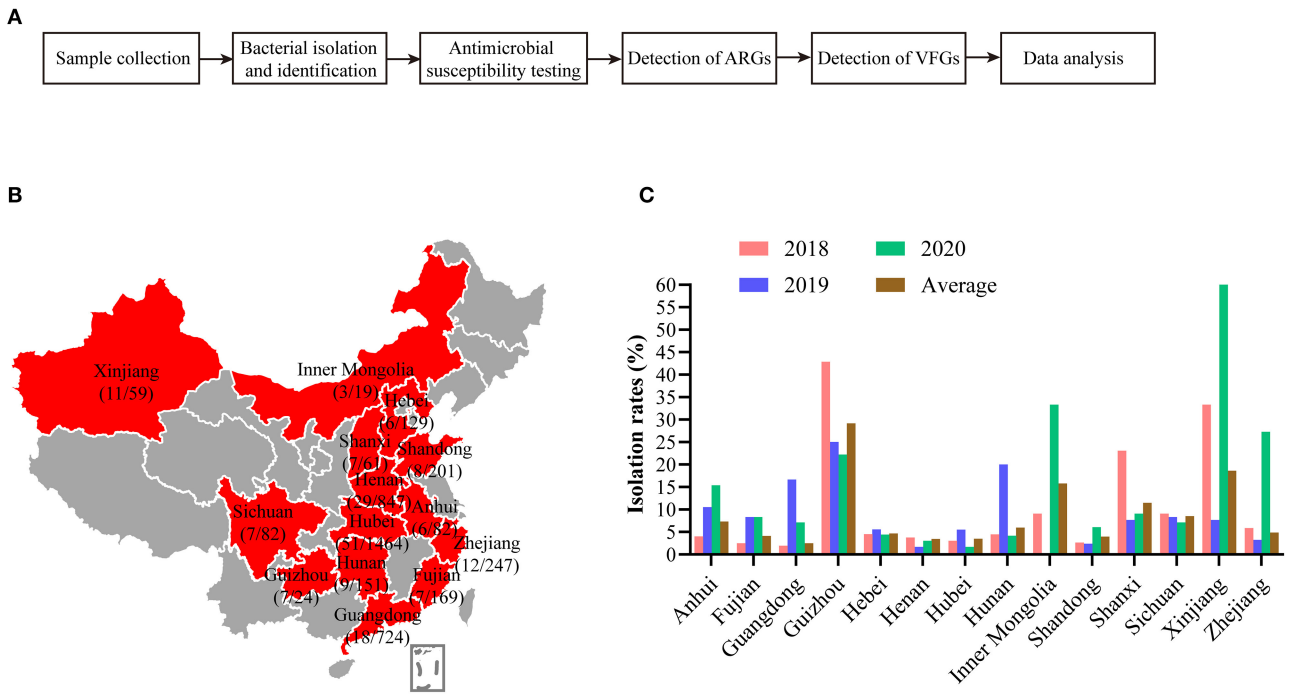

FIGURE 1 | Study design and isolation of *B. bronchiseptica* from swine lung samples in different regions in China from 2018 to 2020. **(A)** Shows study design of this work; **(B)** shows the geographic sites of sample collection. Numbers of *B. bronchiseptica* from the samples as well as numbers of samples collected from each of the provinces are shown; **(C)** shows the isolation rates of *B. bronchiseptica* from swine lung samples in different regions in China from 2018 to 2020.

Detection of Antimicrobial Resistance Genes

PCR assays were performed to detect the presence of putative genes conferring resistance to aminoglycosides [*aac(3)-IV*, *aac6'-Ib*, *rmtA*], β-lactams (bla_{VIM}, bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *MOX*), quinolones (*qnrS*, *oqxA*, *oqxB*), phenicols (*floR*, *catA1*, *catB1*), tetracyclines (*tetA*, *tetB*), and polymyxins (*mcr-1*) in each of the *B. bronchiseptica* isolates with the primers listed in **Table 1**. PCR assays were performed in a 20-μL reaction mixture comprised of 2-μL bacterial DNA, each of the forward and reverse primers 1-μL, 2×Taq Master Mix (Dye Plus) 10-μL, DMSO 2-μL, and ddH_2O 4-μL. The cycling conditions were 94°C for 5 min, followed by 35 cycles consisting of denaturation for 30 s at 94°C, annealing for 30 s at 52∼63°C, and extension for 30 s at 72°C, and a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on a 1% agarose gel. Genomic DNAs extracted from our previously sequenced multidrug resistant *E. coli* strain RXD033 (GenBank accession no. SQQZ00000000) (29) and drug-sensitive bacterium *Pasteurella multocida* strain HND05 (GenBank accession no. PPWG00000000) (30) were used as positive and negative controls, respectively.

Detection of Virulence Factors Encoding Genes

The presence of five well-characterized VFGs, including the filamentous haemagglutinin encoding gene *fhaB*, the pertactin encoding gene *prn*, the adenylate cyclase-haemolysin toxin encoding gene *cyaA*, the dermonecrotic toxin encoding gene *dnt*, and the Bordetella type-III secretion system effector A encoding gene *bteA* in each of the isolates were examined by PCR with primers listed in **Table 1**, as described previously (25). PCR assays were performed in a 20-μL reaction mixture comprised of 2-μL bacterial DNA, each of the forward and reverse primers 1-μL, 2×Taq Master Mix (Dye Plus) 10-μL, DMSO 2-μL, and ddH_2O 4-μL. The cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. Our laboratory stored *B. bronchiseptica* strain HH0809 (31) and the sterile ddH_2O were included as the positive and negative controls, respectively. PCR products were analyzed by electrophoresis on a 1% agarose gel.

Statistical Analysis

We used SAS version 9.0 (SAS Institute Inc.) software to perform statistical analyses in this study, as described previously (26). Univariate association between variables and isolation rates of *B. bronchiseptica* was determined by using univariate ordinary logistic regression analysis. $P < 0.05$ was considered to be significant.

RESULTS

B. bronchiseptica Isolation and Identification

From 2018 to 2020, we isolated a total of 181 *B. bronchiseptica* strains (4.25%) from 4259 lung samples of dead pigs with

TABLE 1 | Primers used in the present study.

Primers	Sequences (5′-3′)	Product size (bp)	Annealing temperature (°C)	Description	References
Bacterial species identification genes					
Fla1	TGGCGCCTGCCCTATC	237	56	*B. bronchiseptica* identification	(13)
Fla2	AGGCTCCCAAGAGAGAAA				
Antimicrobial resistance genes					
SHV1	CCCTGTTAGCCACCCTGCCG	829	62	Detection of bla_{SHV}	(14)
SHV2	CGTTGCCAGTGCTCGATCAGC				
CTXM1	GCTGTTGTTAGGAAGTGTGCCGC	798	61	Detection of bla_{CTX-M}	(14)
CTXM2	GCCGCCGACGCTAATACATC				
TEM1	GTATTCAACATTTCCGTGTCG	854	56	Detection of bla_{TEM}	(14)
TEM2	CCAATGCTTAATCAGTGAGGC				
VIM-1	GATGGTGTTTGGTCGCATA	390	57	Detection of bla_{VIM}	(15)
VIM-2	CGAATGCGCAGCACCAG				
NDM-1	GGTTTGGCGATCTGGTTTTC	621	56	Detection of bla_{NDM-1}	(15)
NDM-2	CGGAATGGCTCATCACGATC				
MOX-1	GCTGCTCAAGGAGCACAGGAT	520	59	Detection of *OMX*	(16)
MOX-2	CACATTGACATAGGTGTGGTGC				
AAC-1	GTTACACCGGACCTTGGA	674	55	Detection of *aac(3)-IV*	(17)
AAC-2	AACGGCATTGAGCGTCAG				
Aac6-1	TTGCGATGCTCTATGAGTGGCTA	482	58	Detection of *aac6′-Ib*	(18)
Aac6-2	CTCGAATGCCTGGCGTGTTT				
RmtA-1	ATGAGCTTTGACGATGCCCTA	756	53	Detection of *rmtA*	(19)
RmtA-2	TCACTTATTCCTTTTTATCATG				
QnrS1	CGACGTGCTAACTTGCGTGATA	537	58	Detection of *qnrS*	(20)
QnrS2	TACCCAGTGCTTCGAGAATCAG				
OqxA-1	GATCAGTCAGTGGGATAGTTT	670	52	Detection of *oqxA*	(21)
OqxA-2	TACTCGGCGTTAACTGATTA				
OqxB-1	TTCTCCCCCGGCGGGAAGTAC	512	61	Detection of *oqxB*	(22)
OqxB-2	CTCGGCCATTTTGGCGCGTA				
TetA-1	GTAATTCTGAGCACTGTCGC	937	56	Detection of *tetA*	(23)
TetA-2	CTGCCTGGACAACATTGCTT				
TetB-1	CTCAGTATTCCAAGCCTTTG	416	44	Detection of *tetB*	(23)
TetB-2	CTAAGCACTTGTCTCCTGTT				
FloR-1	CACGTTGAGCCTCTATAT	868	52	Detection of *floR*	(23)
FloR-2	ATGCAGAAGTAGAACGCG				
CatA11	CCACCGTTGATATATCCC	623	55	Detection of *catA1*	(17)
CatA12	CCTGCCACTCATCGCAGT				
CatA21	TTTGCCCTTTATCGTCAGC	486	55	Detection of *catA2*	This study
CatA22	GCGGTCACCTTCCTGCT				
Mcr-1	CGGTCAGTCCGTTTGTTC	309	58	Detection of *mcr-1*	(24)
Mcr-2	CTTGGTCGGTCTGTAGGG				
Virulence factors encoding genes					
FhaB-1	GCGCAGAACATCACCAATG	475	59	Filamentous haemagglutinin encoding gene *fhaB*	(25)
FhaB-2	TGAAATACTCCATGGCGGAC				
Prn-1	GACCTCGCTCAGTCGATC	555	59	Pertactin encoding gene *prn*	
Prn-2	GAAGACATTCATGCGGAACAG				
CyaA-1	CTACGAGCAGTTCGAGTTTC	377	59	Adenylate cyclase-haemolysin toxin encoding gene *cyaA*	
CyaA-2	TATTCATGTCGCCGTCGTA				
Dnt-1	TGATCCTGCAGTGGTTGATC	491	59	Dermonecrotic toxin encoding gene *dnt*	
Dnt-2	ATCGGCATACGCCAGATC				
BteA-1	TGTTGAGCAACAACGTCAATC	474	59	Bordetella type-III secretion system effector A encoding gene *bteA*	
BteA-2	TATGCAGGTCTTCGAGGTTC				

TABLE 2 | Zone Diameter Breakpoints (mm) used in the present study.

Antibiotics		Amikacin	Gentamicin	Tobramycin	Ceftriaxone	Cefotaxime	Cefepime	Imipenem	Meropenem
Z.*	R	≤14	≤12	≤12	≤13	≤14	≤14	≤13	≤19
D.	I	15∼22	13∼14	13∼14	14∼20	15∼22	15∼17	14∼15	20∼22
B.	S	≥23	≥15	≥15	≥21	≥23	≥18	≥15	≥23
Antibiotics		**Enrofloxacin**	**Ciprofloxacin**	**Chloramphenicol**	**Florfenicol**	**Amoxicillin**	**Ampicillin**	**Tetracycline**	**Polymyxin B**
Z.	R	≤15	≤15	≤12	≤12	≤17	≤19	≤14	≤8
D.	I	16∼20	16∼20	13∼17	13∼17	18∼20	20∼22	15∼18	8∼11
B.	S	≥ 21	≥ 21	≥ 18	≥18	≥ 21	≥ 23	≥19	≥12

**Zone Diameter Breakpoints (Z.D.B.) were defined as sensitive (S), intermediately resistant (I), or resistant (R) with reference to CLSI (CLSI document M100, 28th Edition).*

respiratory diseases. The isolation rates of *B. bronchiseptica* over the 3 years were 3.51, 5.47, and 7.32%, respectively. Rates of isolation across different provinces in China ranged from 2.49 to 29.17% (**Figures 1B,C**). Biochemical tests revealed that *B. bronchiseptica* isolates could not ferment fructose, glucose, mannitol, maltose, rhamnose, and lactose; the methyl red (MR), voges-proskauer (VP), and indole reactions were negative. It is positive testes for oxidase and catalase.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) revealed that 9.39% ($n = 17$) of the *B. bronchiseptica* isolates recovered in this study were susceptible to all of the 16 types of the antibiotics tested while the remaining 90.61% ($n = 164$) of the isolates were resistant to at least one type of the antibiotics. All of the *B. bronchiseptica* isolates recovered in this study were susceptible to imipenem (100%, $n = 181$), meropenem (100%, $n = 181$), and polymyxin B (100%, $n = 181$); more than 80% of the *B. bronchiseptica* isolates were susceptible to ciprofloxacin (99.45%, $n = 180$), cefepime (97.79%, $n = 177$), enrofloxacin (97.79%, $n = 177$), tobramycin (92.27%, $n = 167$), gentamicin (86.74%, $n = 157$), florfenicol (86.74%, $n = 157$), chloramphenicol (86.19%, $n = 156$), tetracycline (85.08%, $n = 154$), amikacin (83.43%, $n = 151$), and amoxicillin (83.43%, $n = 151$) (**Figure 2A**). Approximately 55.25% ($n = 100$) of the *B. bronchiseptica* isolates were susceptible to ceftriaxone, while only 14.36% ($n = 26$) and 10.50% ($n = 19$) of the *B. bronchiseptica* isolates were susceptible to cefotaxime and ampicillin, respectively (**Figure 2A**). Among the 164-drug resistant *B. bronchiseptica* isolates, resistance rates to 1 type, 2 types, 3 types, 4 types, 5 types, 6 types, and 7 types of drugs were 53.05% ($n = 87$), 23.17% ($n = 38$), 7.32% ($n = 12$), 6.10% ($n = 10$), 4.88% ($n = 8$), 3.66% ($n = 6$), and 1.22% ($n = 2$), respectively (**Figure 2B**). Approximately 50.00% ($n = 82$), 26.83% ($n = 44$), 17.07% ($n = 28$), 9.76% ($n = 16$), and 4.88% ($n = 8$) of the isolates were resistant to at least 2 types, 3 types, 4 types, 5 types, and 6 types of the antibiotics tested, respectively (**Figures 2B,C**).

The tested antibiotics in the present study could be divided into eight classes: aminoglycosides (AMK, GEN, TOB), broad-spectrum-cephalosporins (CRO, CTX, CPM), carbapenems (IPM, MRP), fluoroquinolones (ENR, CIP), phenicols (CHL, FLO), penicillins (AMX, AMP), tetracyclines (TET), and polymyxins (PMB). Most of the *B. bronchiseptica* isolates (86.19%, $n = 156$) in this study were resistant to less than three classes of the antibiotics. Among these isolates, 55.77% ($n = 87$) and 32.05% ($n = 50$) of them were resistant to one and two classes of drugs, respectively (**Figure 3A**). Approximately 13.18% ($n = 25$) of the isolates were resistant to more than three classes of the antibiotics. According to the international expert proposal for interim standard definitions for acquired resistance (32), these 25 *B. bronchiseptica* isolates could be defined as multidrug resistant (MDR) strains. Among these MDR strains, proportions of isolates resistance to three-, four-, and five-classes of drugs were 64.00% ($n = 20$), 28.00% ($n = 7$), and 8.00% ($n = 2$), respectively (**Figure 3A**). Most MDR-strains possessed a phenotype of co-resistance to aminoglycosides, broad-spectrum-cephalosporins, and penicillins (37.93%, $n = 11$) (**Figure 3B**).

Detection of Antimicrobial Resistance Genes

Detection of ARGs showed that 16.57% ($n = 30$) of the *B. bronchiseptica* isolates in this study was positive to *aac(3)-IV*, while 3.87% ($n = 7$), 2.21% ($n = 4$), 1.10% ($n = 2$), 0.55% ($n = 1$), 0.55% ($n = 1$), and 0.55% ($n = 1$) of the isolates were positive to *aac6'-Ib*, *rmtA*, bla_{TEM}, bla_{SHV}, *oqxB*, and *tetA*, respectively (**Figure 4**). All isolates were negative to the other ARGs detected (bla_{VIM}, $bla_{\mathrm{NDM-1}}$, $bla_{\mathrm{CTX-M}}$, *MOX*, *qnrS*, *oqxA*, *tetB*, and *mcr-1*).

Detection of Virulence Factors Encoding Genes

Screening of VFGs revealed that 98.90% ($n = 179$) of the *B. bronchiseptica* isolates in this study was positive to at least one of the five VFGs detected while the remaining 1.10% ($n = 2$) ones were negative to all VFGs. The detection rates of *fhaB*, *prn*, *cyaA*, *dnt*, and *betA* were 97.24% ($n = 176$), 91.16% ($n = 165$), 98.34% ($n = 178$), 98.34% ($n = 178$), and 92.82% ($n = 168$), respectively (**Figures 5A,B**). Among the VFG-positive isolates, 84.36% ($n = 151$) of the isolates contained *fhaB*, *prn*, *cyaA*, *dnt*, and *betA*, simultaneously (**Figure 5C**). The remaining isolates harbored "*fhaB+prn+cyaA+dnt*" (6.15%, $n = 11$), "*fhaB+cyaA+dnt+betA*" (7.26%, $n = 13$), "*prn+cyaA+dnt+betA*" (1.68%, $n = 3$), and "*fhaB+dnt+betA*" (0.56%, $n = 1$), respectively (**Figure 5C**).

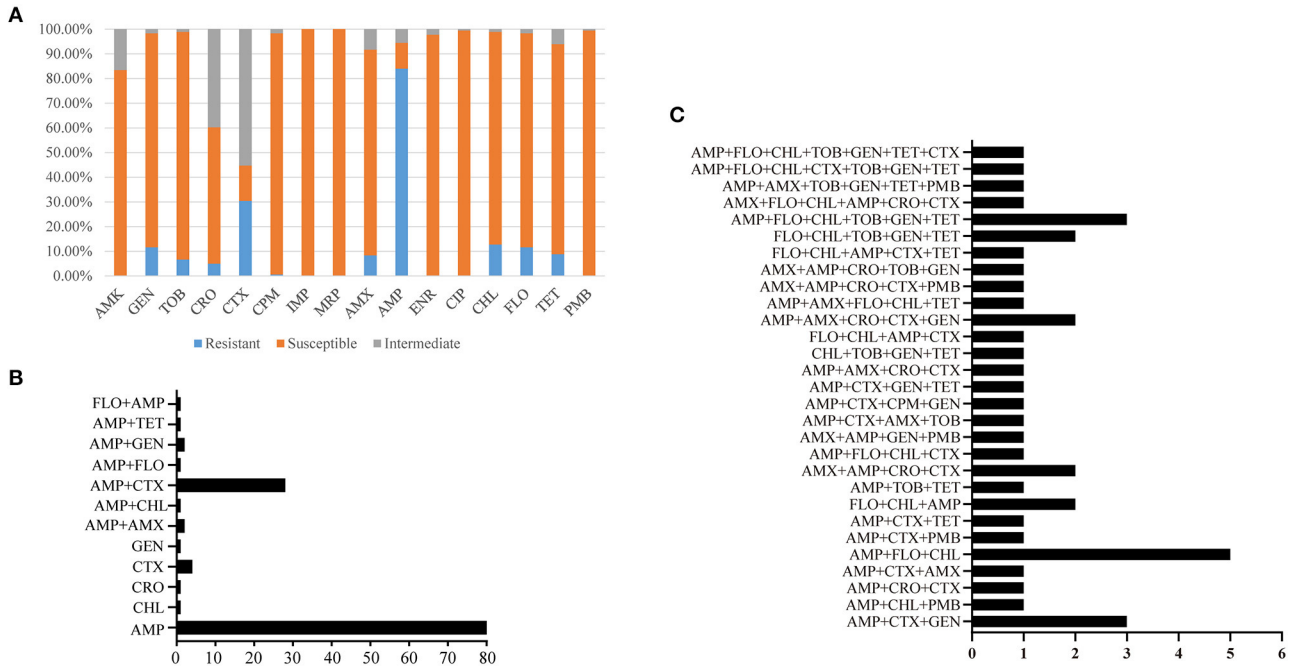

FIGURE 2 | Resistance phenotypes of *B. bronchiseptica* from pigs in China. **(A)** Shows percent isolates susceptible or resistant to the 16 kinds of antibiotics tested; **(B,C)** display the number of isolates with different resistance patterns. In **(B,C)**, X axes show the number of *B. bronchiseptica* strains while Y axes indicate different resistance patterns. AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CRO, ceftriaxone; CTX, cefotaxime; CPM, cefepime; IPM, imipenem; MRP, meropenem; ENR, enrofloxacin; CIP, ciprofloxacin; CHL, chloramphenicol; FLO, florfenicol; AMX, amoxicillin; AMP, ampicillin; TET, tetracycline; PMB, polymyxin B.

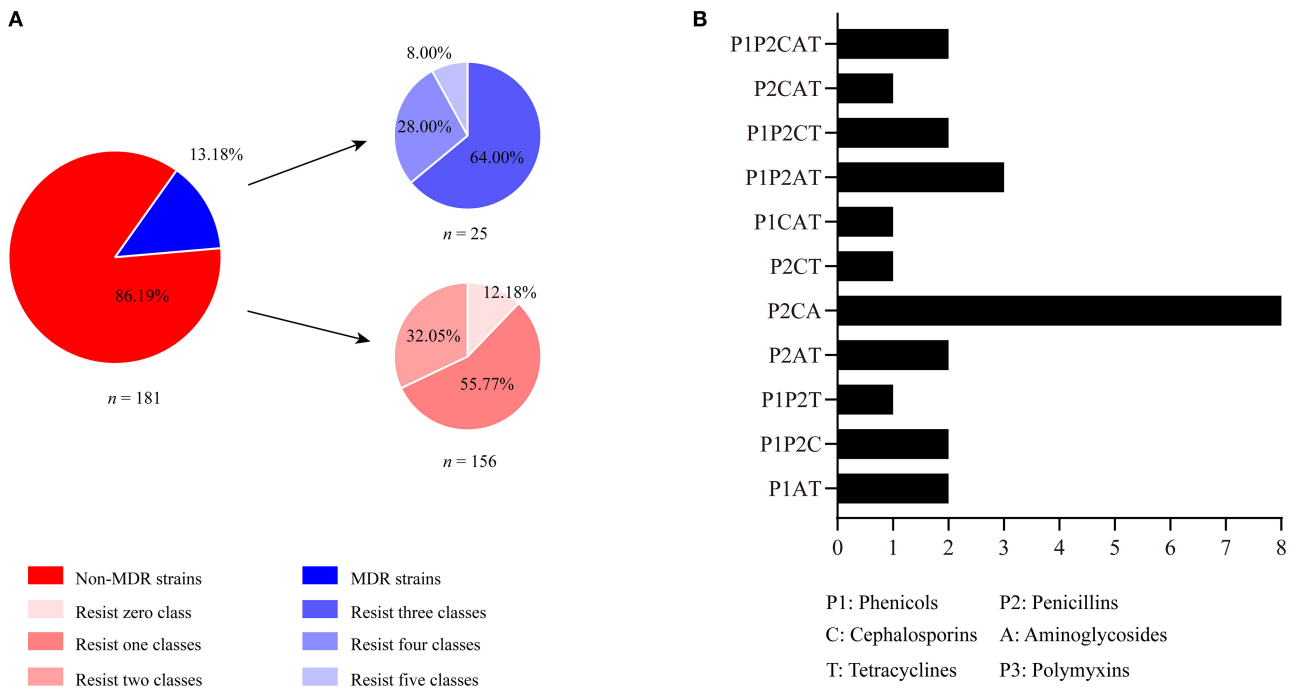

FIGURE 3 | Distribution of multidrug resistant (MDR) strains and non-MDR strains *of B. bronchiseptica* from pigs in China. **(A)** Shows the percentages of MDR and non-MDR strains as well as percent strains resisting 0, 1, 2, 3, 4, and 5 classes of drugs; **(B)** displays the number of isolates resistance to different groups of drug classes. In **(B)**, X axis shows the number of *B. bronchiseptica* strains while Y axis indicates different resistance patterns.

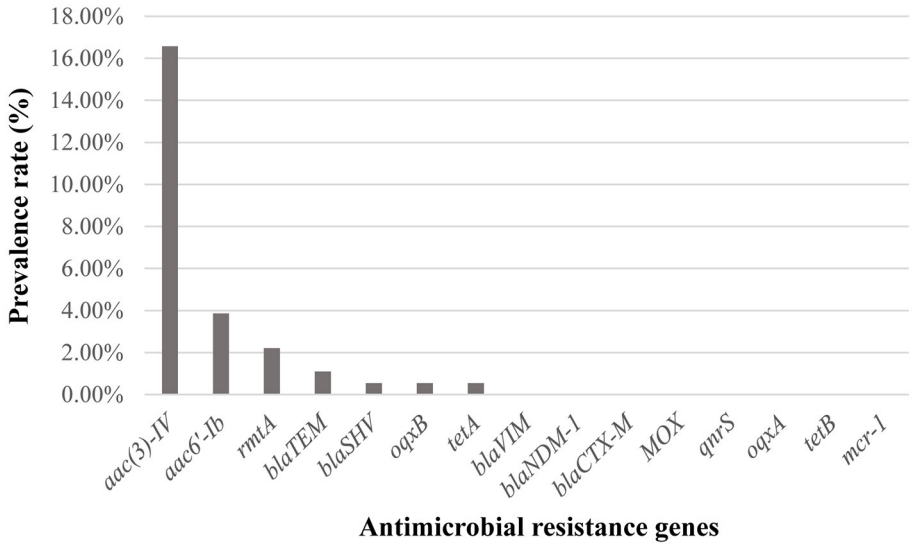

FIGURE 4 | Distribution of antimicrobial resistance genes (ARGs) among *B. bronchiseptica* isolates in this study.

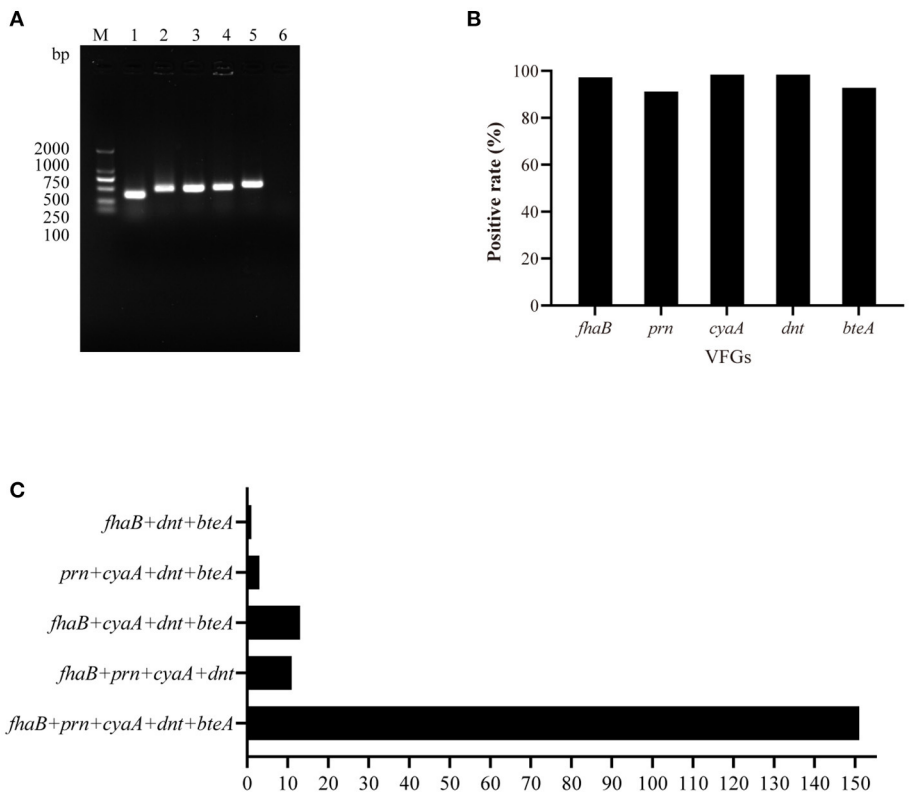

FIGURE 5 | PCR detection of virulence factors encoding genes (VFGs) among *B. bronchiseptica* isolates in this study. **(A)** Shows agarose gel analysis on the PCR products on the five VFGs *cyaA* (band 1, 377 bp), *betA* (band 2, 474 bp), *fhaB* (band 3, 475 bp), *dnt* (band 4, 491 bp), and *prn* (band 5, 555 bp); **(B)** shows the detection rates of the five VFGs while **(C)** shows the number of strains containing different groups of VFGs. In **(C)**, X axis shows the number of *B. bronchiseptica* strains while Y axis indicates different groups of VFGs.

DISCUSSION

Although *B. bronchiseptica* is a well-known leading cause of pig respiratory disorders and an important causative agent of PRDC, there is not too much report on the epidemiology of *B. bronchiseptica* in pigs round the world, particularly in China, the largest pig rearing and production country. In this study, we described the isolation and characterization of *B. bronchiseptica* in pigs in China from 2018 to 2020. The average isolation rate of this 3-year period was 4.25% (181/4259), which is much lower than that reported in pigs with clinical respiratory disease in China from 2003 to 2008 (4.25 vs. 18.6%, $P < 0.05$) (26). The average isolation rates of *B. bronchiseptica* in pigs in different regions from 2018 to 2020 were also much lower than those reported in the same regions from 2003 to 2008 (Hubei: 3.48 vs. 18.0%, $P < 0.05$; Henan: 3.42 vs. 19.6%, $P < 0.05$; Fujian: 4.14 vs. 18.4%, $P < 0.05$; Hunan: 5.96 vs. 19.2%, $P < 0.05$; Anhui: 7.32 vs. 18.0%, $P < 0.05$; Shandong: 3.98 vs. 20.7%, $P < 0.05$) (26). The significant decreasing average isolation rate of *B. bronchiseptica* from 2018 to 2020 compared to that from 2003 to 2008 might be owing to China's continuously efforts to promote transformation and upgrading of pig industry as well as improve the level of disease prevention and control in pig farms. In addition, the outbreak of African Swine Fever in 2018 and its continuous circulation in pigs in China also accelerates the improvement and enhancement of biosecurity on pig farms in recent years (33), which may also be beneficial for the control of *B. bronchiseptica* and the other pathogens.

Administration of antimicrobials is still one of the most effective way to control *B. bronchiseptica* and the other bacteria, but the emergence of drug-resistant bacteria may lead to the failure of using antibiotics in clinic (34–36). Therefore, monitoring the drug resistance profile of clinical microbiology is an important aspect in many epidemiological studies (25, 37, 38). In this study, we characterized the resistance phenotypes of *B. bronchiseptica* from pigs in China from 2018 to 2020. The results revealed that all isolates were susceptible to imipenem (100%), meropenem (100%), and polymyxin B (100%). All of these three types of antibiotics are proposed to be the last-resort antibiotics for the treatment of infections caused by gram-negative bacteria (29), and they are not approved to be used in veterinary medicine in China. In addition, the majority of the isolates were sensitive to ciprofloxacin (99.45%), cefepime (97.79%), enrofloxacin (97.79%), tobramycin (92.27%), gentamicin (86.74%), florfenicol (86.74%), chloramphenicol (86.19%), tetracycline (85.08%), amikacin (83.43%), and amoxicillin (83.43%). These results are in agreement with the results of previous studies in China (25, 39), as well as in other countries such as Germany and Korea (2, 40–42), suggesting these antibiotics might be suitable candidates for treating *B. bronchiseptica* infections when necessary. A high level of resistance was found for ampicillin (83.98%), followed by resistance for cefotaxime (30.39%). These findings are also in agreement with those from the other articles (2, 25, 39), and in particular, *B. bronchiseptica* is documented to be commonly resistant to ampicillin (2). Therefore, these drugs are not recommended to be used in clinic settings. It should be also reminded that several *B. bronchiseptica* isolates from pigs in China displayed a level of multidrug resistance, particularly co-resistance to aminoglycosides, broad-spectrum-cephalosporins, and penicillins. Continues studies should be taken to monitor the prevalence and change-trend of these MDR-isolates in clinic, as some antibiotics belonging to aminoglycosides, broad-spectrum-cephalosporins, and penicillins are commonly used for treating *B. bronchiseptica* infections in veterinary medicine (2, 35).

Virulence factors (VFs) play an important role in the pathogenesis of bacteria (43). For *B. bronchiseptica*, important VFs include filamentous haemagglutinin (FHA), pertactin (PRN), adenylate cyclase-haemolysin toxin, dermonecrotic toxin (DNT), and types III secretion system (44–48), and the expression of these VFs facilitates the invasion of *B. bronchiseptica* in hosts (49). In the present study, we examined five genes encoding these VFs, including *fhaB* which encodes filamentous haemagglutinin; *prn* which encodes pertactin; *cyaA* which encodes adenylate cyclase-haemolysin toxin; *dnt* which encodes DNT; and *bteA* which encodes the T3SS effector A. Surprisingly, over 90% of the pig *B. bronchiseptica* isolates in this study were positive to these five VFGs examined (*fhaB*, 97.24%; *prn*, 91.16%; *cyaA*, 98.34%; *dnt*, 98.34%; *betA*, 92.82%). Importantly, approximately 84.36% of the isolates contained these five kinds of VFGs simultaneously. These results are also in agreement with those reported in *B. bronchiseptica* isolates from rabbits in China (25), suggesting carrying of these VFGs are broad characteristics of *B. bronchiseptica*. Laboratory studies have shown that FHA, and PRN expressed in *E. coli* and *Salmonella* enterica, as well as adenylate cyclase-haemolysin toxin expressed in *B. bronchiseptica* provide protection against fatal infections with *B. bronchiseptica* in mouse models (5, 50, 51).

Despite the findings, this work has several limitations that should be noted. First, all samples used for bacterial isolation were submitted by pig farms from different provinces in China. This way of sample collection may have some influences on the isolation rate. However, the outbreak of African Swine Fever since 2018 and its continuous circulation in pigs in China, and more recently, the worldwide pandemic of the novel coronavirus disease since the late 2019 (COVID-19) made it very difficult for us to collect samples initiatively. Second, the results of antimicrobial susceptibility testing in this study were interpreted by using breakpoints to *Enterobacteriaceae* published in CLSI document M100, and this is because clinic breakpoints specific to *B. bronchiseptica* are limited available (2). Third, a very few published epidemiological studies of swine *B. bronchiseptica* in China are available to date [On March 18, 2021, we searched PubMed with key words "(((*Bordetella bronchiseptica*) AND (Prevalence)) AND (Pigs)) AND (China)" for reports published, with no language restrictions. Our search identified two articles (26, 39) of relevance to this study. All of them were published by our group in 2011], therefore, we only compared the results we obtained from this study to those reported in our previously published two studies in 2011 (26, 39). However, the results from this work could still help understand the current epidemiological and microbiological characteristics of *B. bronchiseptica* in pigs in China.

In summary, we reported the isolation, antimicrobial resistance phenotypes, the detection of ARGs and VFGs of *B. bronchiseptica* from pigs in China from 2018 to 2020 in this study. Our results showed that *B. bronchiseptica* remains an important pathogen associated with pig respiratory disorders in China. While most of the isolates were still susceptible to ciprofloxacin, cefepime, enrofloxacin, tobramycin, gentamicin, florfenicol, chloramphenicol, tetracycline, amikacin, and amoxicillin, MDR-isolates were still determined. These isolates should receive more attentions and further studies are necessary to monitor the prevalence of drug-resistant *B. bronchiseptica*. In addition, our results also revealed that several VFGs, including *fhaB*, *prn*, *cyaA*, *dnt*, and *betA* displayed a high level of detection rate.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

YZ, ZP, and BW delineated the study conception and design. ZP and BW supervised the study. YZ, HY, LG, MZ, FW, WS, LH, LW, WL, and XT collected the bacterial isolates and performed laboratory tests as well as analyzed the data. ZP and YZ wrote the manuscript and approved the final version for publication. ZP, BW, and WL participated in the manuscript discussion and revision. All authors have read and approved the final version of the manuscript.

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Antimicrobial Susceptibility of Lactic Acid Bacteria Strains of Potential Use as Feed Additives - The Basic Safety and Usefulness Criterion

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The spread of resistance to antibiotics is a major health concern worldwide due to the increasing rate of isolation of multidrug resistant pathogens hampering the treatment of infections. The food chain has been recognized as one of the key routes of antibiotic resistant bacteria transmission between animals and humans. Considering that lactic acid bacteria (LAB) could act as a reservoir of transferable antibiotic resistance genes, LAB strains intended to be used as feed additives should be monitored for their safety. Sixty-five LAB strains which might be potentially used as probiotic feed additives or silage inoculants, were assessed for susceptibility to eight clinically relevant antimicrobials by a minimum inhibitory concentration determination. Among antimicrobial resistant strains, a prevalence of selected genes associated with the acquired resistance was investigated. Nineteen LAB strains displayed phenotypic resistance to one antibiotic, and 15 strains were resistant to more than one of the tested antibiotics. The resistance to aminoglycosides and tetracyclines were the most prevalent and were found in 37 and 26% of the studied strains, respectively. Phenotypic resistance to other antimicrobials was found in single strains. Determinants related to resistance phenotypes were detected in 15 strains as follows, the *aph(3″)-IIIa* gene in 9 strains, the *lnu*(A) gene in three strains, the *str*(A)-*str*(B), *erm*(B), *msr*(C), and *tet*(M) genes in two strains and the *tet*(K) gene in one strain. The nucleotide sequences of the detected genes revealed homology to the sequences of the transmissible resistance genes found in lactic acid bacteria as well as pathogenic bacteria. Our study highlights that LAB may be a reservoir of antimicrobial resistance determinants, thus, the first and key step in considering the usefulness of LAB strains as feed additives should be an assessment of their antibiotic resistance. This safety criterion should always precede more complex studies, such as an assessment of adaptability of a strain or its beneficial effect on a host. These results would help in the selection of the best LAB strains for use as feed additives. Importantly, presented data can be useful for revising the current microbiological cut-off values within the genus *Lactobacillus* and *Pediococcus*.

Keywords: acquired resistance genes, antimicrobial susceptibility testing, food additives, minimum inhibitory concentration, lactic acid bacteria, probiotics, reservoir of resistance determinants

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INTRODUCTION

Lactic acid bacteria (LAB) strains are important industrial microorganisms, and they have a long history of safe use as feed additives. They are commonly used as probiotics, animal growth biopromoter, as well as bacterial inoculants for forage ensiling to improve not only the quality but also safety of feed (1, 2). Many LAB species are part of the resident microbiota of the gastrointestinal and genitourinary tracts of humans and animals, where they are thought to exert many health-associated beneficial effects (2). Moreover, they have ability to inhibit other microorganisms, including pathogens that cause foodborne diseases or food spoilage (3).

Among the different genera belonging to the LAB group, mainly *Lactobacillus* spp. and *Pediococcus* spp. have been register as gut biota stabilizers and silage additives (4). The interest in the application of pediococci in animal husbandry is gradually increasing due to the improvement of the characteristics and growth abilities of animals that can be achieved with their use (5). They were shown to be effective as probiotics for broiler chickens, laying hens, piglets, fish, crustaceans, and as silage additives (4). Moreover, many strains produce bacteriocins or bacteriocin-like substances that have well-recognized pathogen inhibitory activities (5). Although *Enterococcus* spp. strains as human probiotics remain controversial, in a point of view of the opportunistic and nosocomial infections caused by these bacteria, they are used as silage additives and probiotics for stabilizing the microbial communities of the gastrointestinal tract of animals (4, 6).

Increasing awareness of probiotics and their therapeutic and prophylactic properties constantly encourages the search for new LAB strains, with beneficial health properties and safe for animal consumption. A wide variety of LAB is used in animal nutrition, either directly or as a source of feed additives. Most LAB species are granted the GRAS status (Generally Regarded As Safe) provided by the US Food and Drug Administration (FDA) and within Europe "QPS status (Qualified Presumption of Safety)" notified by European Food Safety Authority (EFSA), The Panel on Biological Hazards (BIOHAZ), which means that they are considered safe for human and animal consumption and for the environment (7).

Despite that LAB species are widely used and recognized as safe food and feed additives, the rare cases of serious infections in humans caused by LAB have been described in the literature, including bacteremia (8–11), endocarditis (12, 13), pleuropneumonia (8, 14), meningitis (15), and urinary tracts infections (16). The infections occur mainly in patients with serious underlying illnesses, the immunocompromised ones, premature newborns, or elderly individuals. In case of *Lactobacillus* spp. most of the reported clinical cases are related to *Lactobacillus rhamnosus*. Infections associated with *Lactococcus* spp. are mainly concerned to *Lactobacillus lactis* subsp. *lactis* and *Lactobacillus garvieae*, while infections caused by *Pediococcus* spp. and *Leuconostoc* spp. have rarely been described (17, 18). Little is known about the role of LAB in animal infections, although the genus *Lactococcus* may be associated with bovine mastitis and infections in fish and birds (19), up to date there are no reports of *Lactobacillus* and *Pediococcus* infections in animals.

The second serious concern is acquired resistance to antimicrobials of human and veterinary importance among LAB strains (20). There has been increasing attention to this phenomenon since LAB are considered as a reservoir of resistance genes that can be transferred to pathogenic bacteria, leading to the spread of antibiotic resistance among pathogens and complicating the treatment of infection caused by these bacteria (19). Therefore, caution is needed in selecting and monitoring potentially probiotic strains, and antimicrobial resistance (AMR) is regarded as a crucial safety issue during assessing and approving LAB as feed additives (21). The safety assessment of microbial feed additives is governed under specific EU regulatory frameworks in accordance with Regulation (WE) No 1831/2003 and Commission Regulation (EC) No 429/2008. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) provides the scientific opinion on the efficacy of feed additives and their safety to target animals, the consumers of products derived from animals treated with the additives, and to the environment. In line with the FEEDAP recommendation, any bacterial strain carrying an acquired gene conferring AMR or strains with the unknown genetic nature of a demonstrated resistance to antimicrobial agents should not be used as a feed additive due to the greatest risk of horizontal spread (21).

The aim of the present study was an AMR safety assessment of selected LAB strains intended for use as feed additives by phenotypic screening of resistance to clinically relevant antimicrobials. The identification of resistance determinants in the resistant LAB strains was also performed in order to exclude the presence of potentially transferable AMR genes.

MATERIALS AND METHODS

Bacterial Strains

The study provides a safety assessment of 65 LAB strains potentially useful as probiotics and other feed additives. Fifty-seven *Lactobacillus* strains [*Lactobacillus plantarum* ($n = 26$), *Lactobacillus fermentum* ($n = 7$), *Lactobacillus casei* ($n = 3$), *L. rhamnosus* ($n = 3$), *Lactobacillus reuteri* ($n = 3$), *Lactobacillus brevis* ($n = 3$), *Lactobacillus buchneri* ($n = 2$), *Lactobacillus salivarius* ($n = 2$), *Lactobacillus agilis* ($n = 2$), *Lactobacillus acidophilus* ($n = 1$), *Lactobacillus johnsonii* ($n = 1$), *Lactobacillus diolivorans* ($n = 1$), *Lactobacillus delbrueckii* ($n = 1$), *Lactobacillus paracasei* ($n = 1$), *Lactobacillus farraginis* ($n = 1$)], six *Pediococcus* strains [*Pediococcus pentosaceus* ($n = 5$), *Pediococcus acidilactici* ($n = 1$)], and two *Enterococcus* strains [one *Enterococcus durans* strain and one *Enterococcus faecium* strain] were selected for this study (**Supplementary Table 1**). A total of 47 strains are available at the culture collections: 42 strains at the Collection of Industrial Microbial Cultures (KKP), located at the prof. Waclaw Dabrowski Institute of Agricultural and Food Biotechnology (IAFB) in Warsaw (Poland), four strains at the Polish Collection of Microorganisms (PCM), located at the Institute of Immunology and Experimantal Therapy in Wroclaw (Poland) and one strain from American Type Culture Collection

(ATCC). The rest 18 strains were isolated from fermented or fresh vegetables and fruits ($n = 14$) or probiotic drinks ($n = 4$). The isolates were identified by nucleotide sequence analysis of the gene encoding 16S rRNA. LAB strains belonging to the *L. plantarum* phylogenetic group (*L. plantarum, Lactobacillus pentosus,* and *Lactobacillus paraplantarum*) were differentiated by multiplex PCR using species-specific primers amplified the fragment of the *recA* gene encoding the recombinase A (22). The strains isolated from the same sources were typed by RAPD-PCR (Random Amplified Polymorphic DNA) with primers RP and PRIMO2 (23) in order to confirm their intraspecies diversity (data not shown). All strains were stored in a liquid nitrogen atmosphere in MRS (deMan- Rogosa-Sharpe) broth (Oxoid) supplemented with glycerol (15% v/v). Before the antibiotic susceptibility assay, LAB strains were cultivated in MRS agar (Oxoid) at 37°C for 24–48 h in 5% CO_2. After incubation, the colonies were suspended in 0.85% NaCl solution to prepare the inoculum for the broth microdilution test.

Phenotypic Antimicrobial Resistance

The following antimicrobials, used in therapy of common infections, were tested: gentamicin (0.125–64 mg/L), kanamycin (0.5–256 mg/L), streptomycin (0.5–256 mg/L), tetracycline (0.125–64 mg/L), chloramphenicol (0.06–32 mg/L), ampicillin (0.015–8 mg/L), erythromycin (0.015–8 mg/L), and clindamycin (0.015–8 mg/L). Gentamicin, kanamycin, erythromycin, clindamycin originated from the European Pharmacopoeia (EP) Reference Standards, while streptomycin, tetracycline, chloramphenicol, and ampicillin from Sigma-Aldrich. LSM broth (IsoSensitest broth (90%) and MRS broth (10%), adjusted to pH 6.7) and the microdilution method according to Klare et al. (24) were used. The lowest concentration of each antibiotics that inhibits the visible growth of bacteria (MIC, Minimum Inhibitory Concentration) was determined after 48 h of incubation at 37°C and in the presence of 5% CO_2. Susceptibility of strains was established in accordance with the microbiological cut-off values defined by the EFSA Panel on Additives and Products or Substances used in Animal Feed (21). The accuracy of antimicrobial susceptibility testing was monitored by parallel use of the reference strains, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 as a quality control. The study was performed in triplicate. The differences of MICs for independent sample never exceed 1 order of dilution.

Genetic Determinants of Antimicrobial Resistance

All LAB strains phenotypically resistant to the tested antimicrobial agents were examined by PCR for the presence of selected AMR genes. The following genes were detected: *bla* gene (ampicillin-resistant strains); the *erm*(A), *erm*(B), *erm*(C), *msr* genes, and the *lnu*(A) gene (erythromycin and/or clindamycin-resistant strains); genes encoding ribosomal protection proteins (universal primer set and subsequently, specific primers for *tet*(W) and *tet*(M) genes for positive strains) and the *tet*(K) and *tet*(L) genes encoding a tetracycline efflux pump (tetracycline-resistant strains); the *cat* gene (chloramphenicol-resistant strains); the *aph*(3″)-*IIIa* gene (kanamycin-resistant strains); the *ant*(6), *str*(A)/*str*(B) and *aad*(A) genes (streptomycin-resistant strains); the *aac*(6′)-*aph*(2″) gene (aminoglycosides-resistant strains). In case of the detection of resistance genes, the cut-off values given in the previous EFSA guidance (25) were additionally used for a results analysis.

The characteristics of the primers used in the study and appropriate references (26–36) are shown in **Supplementary Table 2**. The primer set for *msr*(C) detection was designed using the PCR Primer Design Tool (https://eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design) and checked using an Oligo Analysis Tool (https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis). PCR reactions were performed in a total volume of 25 μL containing 1 μL of each primer (10 pmol/μL), 12.5 μL of DreamTaq PCR Master Mix (2×) (ThermoFisher Scientific) or JumpStart REDTaq ReadyMix Reaction Mix (2×) (Sigma-Aldrich) and 50 ng of DNA template. A template bacterial genomic DNA was purified using GenElute™ Bacterial Genomic DNA Kits (Sigma-Aldrich) following the manufacturer's instruction for Gram-positive bacteria cells (pre-incubation with lysozyme). The amount and quality of DNA was determined using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

PCR products were separated by electrophoresis on a 1% agarose gel (Sigma-Aldrich), stained with ethidium bromide, in TBE buffer (100 V). The O'RangeRuler™ 200bp DNA Ladder, GeneRuler™ 100 bp DNA Ladder or GeneRuler™ 100 bp Plus DNA Ladder (ThermoFisher Scientific) were used as size standard markers. Additionally, PCR products were purified and sequenced (Genomed S.A.). The obtained DNA sequences were analyzed using BLASTn (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with sequences available in GenBank (National Center for Biotechnology Information) and CARD database (The Comprehensive Antibiotic Resistance Database, https://card.mcmaster.ca) (**Supplementary Table 3**).

Nucleotide Sequence of AMR Genes

The nucleotide sequences of the *msr*(C), *erm*(B), *lnu*(A), *aph*(3″)-*IIIa, str*(B), *tet*(M), and *tet*(K) genes described in this study are shown in **Supplementary Table 4**.

RESULTS

Phenotypic Antimicrobial Resistance

Each strain was able to grow on LSM medium without antibiotic (growth positive control). The MICs of antibiotics for studied strains are presented in **Table 1** and **Supplementary Table 5**. The MIC ranges for particularly antibiotics were varied and were within the used concentration ranges of tested antibiotics: for gentamicin <0.125–32 mg/L, for kanamycin 4–≥256 mg/L, for streptomycin <0.5–≥256 mg/L, for tetracycline 0.25–32 mg/L, for chloramphenicol 1–8 mg/L, for ampicillin <0.015–≥8 mg/L, for erythromycin <0.015–≥8 mg/L, and for clindamycin <0.015–≥8 mg/L (**Table 2**). Only 31 strains (17 *L. plantarum*, four *L. fermentum* and *L. casei*, three *L. reuteri* and one

TABLE 1 | Distribution of MICs of tested antibiotics among phenotypically resistant LAB strains ($n = 34$).

Number	Strain	MIC (mg/L)[a]							
		GM[b]	K	TE	CH	A	E	CL	S
	Microbiological cut-off values (mg/L) proposed by EFSA for obligate heterofermentative *Lactobacillus*								
		16	64(32)[c]	8	4	2	1	4(1)	64
1	*L. buchneri* KKP 2047p	4	**128**	16[d]	4	1	0,25	0,5	64
2	*L. diolivorans* KKP 2036p	8	**128**	**16**	2	2	0,25	0,125	**128**
3	*L. fermentum* KKP 2020	2	32	**16**	4	0,5	0,25	0,25	32
4	*L. fermentum* KKP 830	8	64	**16**	2	1	0,25	0,25	64
5	*L. fermentum* Sieger	16	**128**	4	4	0,125	0,125	0,03	32
6	*L. brevis* Pap3/4	2	64	**16**	4	0,25	0,125	0,25	64
7	*L. brevis* Pat1	0,5	16	**16**	4	2	0,5	≤0,015	8
8	*L. brevis* Solaris	1	16	**16**	4	1	0,25	0,5	16
9	*L. farraginis* E/J	0,5	8	**16**	4	0,125	0,03	0,03	8
	Microbiological cut-off values (mg/L) proposed by EFSA – facultative heterofermentative *Lactobacillus*								
		16	64	8	4	4	1	4(1)	64
10	*L. agilis* KKP 1834	**32**	**≥256**	0,25	4	1	**≥8**	1	**≥256**
11	*L. salivarius* KKP 1828	16	**128**	1	4	0,25	0,125	0,125	**128**
12	*L. salivarius* KKP 1835	8	**128**	**16**	4	2	0,125	0,03	**128**
	Microbiological cut-off values (mg/L) proposed by EFSA for *Lactobacillus rhamnosus*								
		16	64	8	4	4	1	4(1)	32
13	*L. rhamnosus* KKP 849	4	**128**	1	**8**	1	0,5	0,5	32
14	*L. rhamnosus* B/J	**32**	**128**	1	4	0,5	0,125	0,5	32
	Microbiological cut-off values (mg/L) proposed by EFSA for *Lactobacillus plantarum/pentosus*								
		16	64	32	8	2	1	4(2)	n.r.
15	*L. plantarum* KKP 804	4	64	32	4	**≥8**	0,25	4	n.r.
16	KKP 815	8	**128**	16	8	1	0,25	2	n.r.
17	KKP 835	8	**≥256**	16	8	2	0,25	1	n.r.
18	KKP 870	16	**≥256**	32	8	2	0,25	4	n.r.
19	KKP 872	16	**≥256**	16	8	2	0,25	4	n.r.
20	KKP 2021p	4	**128**	16	8	1	0.25	4	n.r.
21	KKP 1821	4	**128**	16	4	1	0,25	0,5	n.r.
22	KKP 1822	8	**128**	16	8	1	0,25	0,5	n.r.
23	ATTC 8287	8	**128**	16	8	2	0,5	2	n.r.
	Microbiological cut-off values (mg/L) proposed by EFSA for obligate homofermentative *Lactobacillus*								
		16	16	4	4	2(1)	1	4(1)	16
24	*L. delbrueckii* PCM 490	4	**32**	2	2	0,06	0,06	0,06	8
	Microbiological cut-off values (mg/L) proposed by EFSA for *Lactobacillus acidophilus* group								
		16	64	4	4	1	1	4(1)	16
25	*L. acidophilus* PCM 2499	4	16	**32**	2	0,25	1	0,125	**32**
26	*L. johnsonii* KKP 878	4	64	**16**	**8**	0,125	0,25	0,5	**32**
	Microbiological cut-off values (mg/L) proposed by EFSA for *Pediococcus* spp.								
		16	64	8	4	4	1	1	64
27	*P. pentosaceus* KapA	4	**128**	**16**	4	2	0,25	0,03	**128**
28	*P. pentosaceus* Pom7	4	64	**16**	2	1	0,25	0,03	64
29	*P. pentosaceus* AG	16	**128**	**16**	4	2	0,5	0,03	**128**
30	*P. pentosaceus* MA	16	**≥256**	**16**	4	2	0,25	0,03	64
31	*P. pentosaceus* WN1	8	64	**16**	4	1	0,5	0,03	**128**
32	*P. acidilactici* KKP 1839	4	**128**	**16**	4	2	0,25	0,03	**128**
	Microbiological cut-off values (mg/L) proposed by EFSA for *Enterococcus* spp.								
		32	1024	4	16	2	4	4	128
33	*E. durans* KKP 1586	16	64	0,5	8	0,25	**≥8**	**≥8**	128
34	*E. faecium* TR2	32	128	**32**	2	0,125	**≥8**	4	128

[a]MICs higher than EFSA cut-off values in bold; [b]GM, gentamicin; K, kanamycin; TE, tetracycline; CH, chloramphenicol; A, ampicillin; E, erythromycin; CL, clindamycin; S, streptomycin; [c]the previous EFSA proposed cut-off values (2012) are given in brackets; [d]L. buchneri the cut-off for tetracycline is 128; KKP - strains from the Culture Collection of Industrial Microorganisms; PCM - strains from The Polish Collection of Microorganisms; n.r., not required.

TABLE 2 | Distribution of the MIC, MIC_{50}, and MIC_{90} values of eight antibiotics among studied LAB species ($n = 65$).

Antibiotic	MIC values (mg/L)																
	0,015	0,003	0,06	0,0125	0,25	0,5	1	2	4	8	16	32	64	128	256	MIC_{50}	MIC_{90}
Gentamicin				1		3	8	10	19	16	7	3				4	16
Kanamycin									2	1	8	14	21	16	5	64	128
Streptomycin[a]						1			2	4	6	11	6	9	1	32	128
Tetracycline					2	2	8	3	5	3	37	7				16	32
Erythromycin	1	2	4	20	30	5	2			3						0,25	1
Clindamycin	6	13	5	7	9	14	4	3	5	1						0,25	2
Ampicillin	1	1	3	14	10	5	17	14	1	1						0,5	2
Chloramphenicol							2	13	34	18						4	8

[a]*27 L. plantarum strains were not tested.*

L. buchneri, *L. agilis* and *L. rhamnosus*) out of 65 strains were susceptible to all antibiotics as the microbiological cut-off values were below the proposed by the FEEDAP Panel breakpoints (21). Nineteen LAB strains were resistant to one of the investigated antibiotics (i.e., 11 strains to kanamycin, seven to tetracycline and one to ampicillin), whereas 15 strains displayed resistance to more than one of the investigated antibiotics (i.e., 8 strains to two antibiotics, 6 strains to three and one strain to four antibiotics) (**Tables 1**, **3**). The resistance to aminoglycosides was the most prevalent (37%), since 21 strains (32%) were resistant to kanamycin, 10 to streptomycin (15%) and two to gentamicin (3%). The *Lactobacillus agilis* KKP 1834 strain was highly resistant to all aminoglycosides tested, as the MIC values were twice higher than the corresponding breakpoints proposed by the EFSA. The tetracycline resistance was the second common antibiotic resistance found in the studied LAB strains, and was reported in 17 resistant strains (26%). The resistance to erythromycin or chloramphenicol was reported in two strains, while single strains were phenotypically resistant to clindamycin or ampicillin. The MIC, MIC_{50}, and MIC_{90} values of tested antibiotics for all studied strains are shown in **Tables 1**, **2**.

Distribution of AMR Genes

To identify determinants responsible for the displayed resistance phenotypes, the strains were screened by PCR for the presence of selected AMR genes. Acquired AMR genes were only found in 15 strains (**Table 3**). When investigating 17 tetracycline-resistant strains, the *tet*(M) gene encoding ribosomal protection proteins were found in two strains (*L. salivarius* KKP 1835 with tetracycline MIC value of 16 mg/L and *E. faecium* TR2 with MIC value of 32 mg/L). *L. acidophilus* 2499 strain displaying the MIC value of tetracycline three times higher than the breakpoint (32 vs. 4 mg/L), was positive for the *tet*(K) gene. The *erm*(B) gene was detected in *L. plantarum* KKP 2021p (the MIC value of clindamycin was 4 mg/L, but the strain was susceptible to erythromycin, MIC = 0.25 mg/L) and in *E. durans* KKP 1586 (erythromycin and clindamycin MIC values were 8 mg/L and higher than 8 mg/L, respectively). In addition, two *L. plantarum* strains resistant to clindamycin (870 and 872, with MIC value 4 mg/L) and *E. faecium* TR2, susceptible to clindamycin, carried the *lnu*(A) gene. Two strains were positive for the *msr*(C) gene, *L.agilis* KKP 1834 and *E. faecium* TR2 strains (erythromycin MIC value was 8 mg/L). The *aph(3″)-IIIa* gene was detected in 9 strains belonging to the species: *L. plantarum* ($n = 3$), *L. fermentum* ($n = 3$), *L. buchneri* ($n = 1$), *L. diolivorans* ($n = 1$), and *L. agilis* ($n = 1$). Two strains, *L. acidophilus* 2499 and *L. salivarius* 1835, with streptomycin MIC values 32 and 128 mg/L, respectively, were positive for *str*(A)/*str*(B) genes.

The selected PCR amplicons were sequenced, and the obtained sequences of the tested AMR genes (**Supplementary Table 4**) indicates the homology to the DNA sequences detected in other LAB, as well as in pathogens (**Supplementary Table 3**). The PCR product for *msr*(A)/*msr*(B) genes, encoding for a macrolide efflux protein and conferring resistance to macrolides and streptogramins B, were identified as the *msr*(C) gene by sequencing (**Supplementary Table 3**). No specific primers targeting the *msr*(C) gene were found in the available literature, thus we designed a primer set to detect this gene without the need for sequencing of the PCR product. For both strains, *L. agilis* KKP 1834 and *E. faecium* TR2, the specific product of 354-bp with newly designed primer set was obtained. In the case of ampicillin resistant strains, a product of ∼297 bp obtained with primers specific for the *bla* gene was found in one strain (*L. plantarum* 804). However, the presence of this gene is questionable as the chromatograms obtained by sequencing were unreadable despite the repetition.

DISCUSSION

It is generally accepted that starter cultures or feed additives contain strains isolated from target raw materials, in accordance with their intended use. The source of probiotic strains used in animals are often the gastrointestinal tract or feces of the same or different animal species (37). Natural microbiota isolated from the host usually more easily and quickly adapts and could be more effective as a probiotic compared to strains from other sources. Nevertheless, numerous studies indicate high prevalence of drug resistance in strains isolated from various animals, including pigs, ruminants, companion animals, poultry, or even wild animals (38–41) as well as

TABLE 3 | Correlation between resistance phenotype and genotype among studied LAB species ($n = 40$).

Strains	Resistance phenotype[a]	Resistance genotype
L. fermentum KKP 2020	TE	n.d.
L. fermentum KKP 830		n.d.
L. brevis Pat1		n.d.
L. brevis Solaris		n.d.
L. brevis Pap3/4		n.d.
L. farraginis E/J		n.d.
P. pentosaceus Pom7		n.d.
L. buchneri KKP 2047p	K	*aph*(3″)-*IIIa*
L. fermentum Sieger		n.d.
L. plantarum KKP 815		n.d.
L. plantarum KKP 835		*aph*(3″)-*IIIa*
L. plantarum KKP 870		*aph*(3″)-*IIIa*
L. plantarum KKP 872		n.d.
L. plantarum KKP 2021p		n.d.
L. plantarum KKP 1821		n.d.
L. plantarum KKP 1822		*aph*(3″)-*IIIa*
L. plantarum ATCC 8287		n.d.
L. delbrueckii PCM 490		n.d.
P. pentosaceus MA	K – TE	n.d.
L. salivarius KKP 1828	K – S	n.d.
L. rhamnosus KKP 849	K – CH	n.d.
L. plantarum KKP 804	A	n.d.
L. rhamnosus B/J	GM – K	n.d.
L. acidophilus PCM 2499	TE – S	*tet*(K), *str*(A)/*str*(B)
P. pentosaceus WN1		n.d.
E. durans KKP 1586	E – CL	*erm*(B)
E. faecium TR2	TE – E	*tet*(M), *msr*(C), *lnu*(A)
L. diolivorans KKP 2036p	TE – K – S	*aph*(3″)-*IIIa*
L. salivarius KKP 1835		*tet*(M), *str*(A)/*str*(B)
P. pentosaceus KapA		n.d.
P. pentosaceus AG		n.d.
P. acidilactici KKP 1839		n.d.
L. johnsonii KKP 878	TE – CH – S	n.d.
L. agilis KKP 1834	GM – K – S – E	*aph*(3″)-*IIIa*, *msr*(C)
***L. fermentum* KKP 811, KKP 830, KKP 843**	K	*aph*(3″)-*IIIa*
***L. plantarum* KKP 870, KKP 872**	CL	*lnu*(A)
***L. plantarum* KKP 2021p**	CL	*erm*(B)

[a]*GM, gentamicin; K, kanamycin; TE, tetracycline; CH, chloramphenicol; A, ampicillin; E, erythromycin; CL, clindamycin; S, streptomycin; n.d., tested resistance genes not detected. The strains carrying a resistance gene but phenotypically resistant only in line to cut-off values adopted in previous EFSA guideline (2012) are in bold.*

from food of animal origin (30, 42). The intensive and irresponsible (especially non-therapeutic) use of antimicrobial agents in animal husbandry and veterinary practice contributes to developing of resistance of gut microbiota and potentially beneficial LAB to antibiotics, including tetracycline, enrofloxacin, ampicillin and MLS antibiotics (macrolides, lincosamides and streptogramins) (20, 40, 41, 43, 44). Such strains considered as a reservoir of AMR genes for other commensal bacteria, as well as pathogenic and opportunistically pathogenic species through horizontal gene transfer (20, 45). This poses a threat not only to animals, but resistant strains can also be widely distributed through the food chain. Hence, the use of LAB strains isolated from non-intestinal sources has become increasingly attractive and justified. The alternative sources from which beneficial LAB can be isolated are fruits, vegetables and juices, cereals, silages, sourdough, fermented foods and beverages, as well as raw materials and ingredients used to make non-fermented and fermented foods (37, 46). The strains selected from various "unconventional" sources meet the criteria for probiotic strains, such as resistance to low pH and high bile concentrations, adherence capacity to epithelial intestinal cells, and strong antimicrobial activity against pathogenic microorganisms, including bacteriocin-like activity (37). The strains deposited in different Microbial Culture Collections can also be screened to find beneficial LAB strains, although this does not appear to be a common practice. The advantage of strains from the Collections with the status of International Deposit, however, may be their widespread availability. In the present study we used LAB strains from both sources, isolated from animal origin and strains from alternative sources. Most of the strains are deposited in the Microbial Culture Collections.

Recently, the taxonomy of genus *Lactobacillus* changed significantly. The genus *Lactobacillus* was one of the most taxonomically complex and extremely heterogeneous and composed 261 genera (as of March 2020) (47). In 2020, based on polyphasic approach (phylogenomic analysis), Zheng et al. (47) reclassified the genus *Lactobacillus* into 25 genera, including 23 new one. The emended genus of *Lactobacillus* currently consists of 38 species well adapted to vertebrates' or invertebrates' hosts. The general term lactobacilli are further used to designate bacteria classified to the family *Lactobacillaceae* until 2020. In our work, we use the names of the former *Lactobacillus* classification to avoid any confusion and for maintenance of compliment with the nomenclature used in EFSA guidance for microbiological cut-off values. It should be highlighted that the complexity of this phylogenetic group of microorganisms make it difficult to generalize about this genus and contributes to many difficulties in antimicrobial susceptibility testing of these bacteria, regarding the appropriate medium or establish the cut-off values.

LAB species differ significantly in their growth requirements. The M45 (3rd ed.) CLSI (Clinical Laboratory Standards Institute) procedure proposes the use of cation-adjusted Mueller-Hinton broth (CAMHB) supplemented with 2.5 or 5% lysed horse blood (LHB) as a conventional susceptibility test medium, however, some lactobacilli exhibited weak growth in this medium (24, 48). In this study, we used the LSM broth proposed by Klare et al. (24) and in line with ISO/IDF standard procedure, which is more accurate and reproducible for lactobacilli and pediococci (24, 48). To distinguish strains with phenotypic resistance from susceptible one, the MIC-off value proposed by the EFSA FEEDAP were used (21). The standard procedures of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI provide the same breakpoints for all

lactobacilli species, while the EFSA's guidelines refer to different groups within LABs, which is relevant considering the great differences in AMR among lactobacilli species. Some species of *Lactobacillus* are intrinsically resistant to certain antibiotics (e.g., *L. plantarum*/*L. pentosus* to streptomycin), while other lactobacilli have variable activity against these antimicrobials (49). Moreover, the breakpoint values are best established for clinically important microorganisms. In the case of lactobacilli, which are infrequently associated with a clinical infection, the collected data are limited, and the guidelines of CLSI and EUCAST provide breakpoints for only four of antibiotics testing (ampicillin, clindamycin, chloramphenicol, and erythromycin).

Antimicrobial susceptibility is a key criterion that must be met when microorganisms are intentionally introduced into the food chain. Numerous data indicate that LAB exhibit highly variable sensitivity to antimicrobial agents. In our study, a total of 65 strains intended for use as a feed or silage additives were tested for their susceptibility to eight selected antimicrobials. Thirty-four tested strains were resistant to at least one antimicrobial agent according to a current EFSA guidance (21).

The high susceptibility of LAB strains to ampicillin (98.5%) was observed in our study, which is in line with a number of previous data (29, 50, 51). However, it should be noted that higher resistance for this antibiotic was also noted in lactobacilli, mainly in isolates from poultry and fermented dairy products (40, 41, 52). The resistance to β-lactam antibiotics is related to the presence of the *bla* gene whilst we not confirmed by sequencing the presence of this gene in ampicillin-resistant *L. plantarum* strain. The absence of genes associated with β-lactam resistance among strains with relatively high MIC values was observed by others (40, 41).

High susceptibility among tested LAB strains has been also noted in case of chloramphenicol (96.9%). This is consistent with many published data (40, 50, 51, 53), although resistance to chloramphenicol in lactobacilli strains isolated from various fermented products has also been reported (52, 54). The genotypic resistance to this antibiotic class is usually associated with the presence of *cat* gene (55) and the occurrence of this gene was noted among some of LAB strains, including *L. salivarius*, *L. johnsonii*, *L. crispatus*, *L. reuteri*, *L. plantarum*, *L. ingluviei*, and *P. acidilactici* (40, 41, 54). Interestingly, the *cat* gene was not detected in chloramphenicol-resistant *L. rhamnosus* and *L. johnsonii* strains in this study (MIC = 8 mg/L while the cut-off values is 4 mg/L). According to the literature data, the resistance to chloramphenicol may not be related only to the presence of specific genes encoding antibiotic-modifying enzymes, but may also result from diminished expression of many genes, including efflux pumps and oxidative stress-related genes as well as genes encoding outer membrane proteins (56). This phenomenon may be a cause of phenotype and genotype inconsistency observed also in the tested strains.

The occurrence of tetracycline resistance was found in 26.2% of LAB strains in this study. In other studies conducted in Poland, the percentage of tetracycline-resistant lactobacilli was significantly higher (40, 41, 53), however, it is not surprising considering that these strains were isolated from poultry. The *tet* genes are often found in isolates of animal origin (38, 39), while in lactobacilli strains isolated from fermented food the resistance to tetracyclines is less frequent, like our findings (29, 52). The prevalence of the *tet* genes which confers resistance to teracyclines was not significant among tested LAB strains. The *tet*(M) gene encoded the ribosomal protection protein was found in *L. salivarius* and *E. faecium* strains whilst *tet*(K) encoded the energy-dependent efflux protein was presented in *L. acidophilus*. Similarly, the *tet*(M) gene was noted in *E. faecium* and *L. salivarius* isolates from fermented food in India (57). Nawaz et al. (29) detected this gene in *L. plantarum*, *L. salivarius*, *L. animalis*, and *L. brevis* strains isolated from fermented food. This gene was also widespread in *L. salivarius*, *L. agilis*, and *L. crispatus* strains isolated from chickens, turkeys, and pigeons in Poland (40, 41, 53). Generally, the *tet*(M) gene is one of the most widespread tetracycline resistance determinants in lactobacilli (55). The *tet*(K) gene has so far been detected in strains of *L. fermentum*, *L. buchneri*, and *P. pentosaceus* from fermented food (51, 57) or *L. plantarum*, *L. salivarius*, and *L. reuteri* isolates from meat pork and poultry in Italy (42). Interestingly, to the best of our knowledge, it seems that *tet*(K) has not been previously described in *L. acidophilus*. Among the LAB strains tested, we observed the highest prevalence of phenotypic tetracycline resistance in obligate heterofermentative lactobacilli (64% strains) and pediococci (100% strains) (MIC = 16 mg/L), but *tet* resistance genes were not detected in any of the strains. Similar results were reported by other authors (40, 41, 58). This contradiction between the phenotypic resistance and the absence of the *tet* genes indicates that tetracycline resistance in these bacteria is likely to be intrinsic and the current microbiological cut-off values for tetracycline should be reevaluated. We propose the MIC = 16 mg/L as cut-off value for categorization of susceptible and resistant strains within obligate heterofermentative *Lactobacillus* spp. and *Pediococcus* spp. The pediococci resistance to tetracyclines was considered as intrinsic also by other authors, who failed to detect the *tet* genes in strains with MIC values ≥16 mg/L (32, 58–60). The high resistance to tetracycline that may be naturally conditioned was also discussed in lactobacilli species (50, 61). The intrinsic resistance to tetracyclines is related to the complex regulatory network that modulate the uptake, as well as intracellular accumulation of these antibiotics. The mutations affect to expression and function of activator or repressor of pumps and porins (62). The regulation of intrinsic tetracycline resistance is better characterized in Gram-negative bacteria. The available data about this resistance in Gram-positive species are still poorly understood.

The low rates of resistance to erythromycin (4.6%) and clindamycin (1.5%) were observed in tested LAB strains, although other reports showed the high prevalence of resistance to these antimicrobials among lactobacilli strains (40, 41, 43, 44, 52). The *erm*(B) gene encoding the ribosomal RNA methylase was detected in *L. plantarum* and *E. durans*. The presence of the *erm* genes is related to exhibit of MLS_B resistance phenotype (macrolides-lincosamides-streptogramins B), however, only *E. durans* 1586 was resistant to erythromycin and clindamycin, whereby *L. plantarum* 2021p was susceptible to both antimicrobials. It is also worth highlighted that the recommendation for clindamycin has been revised and the

current cut-off value for all lactobacilli is MIC = 4 mg/L (21). According to the previous guidance (25), this strain would be considered phenotypically resistant to clindamycin, however still susceptible to erythromycin. The presence of the *erm* genes in strains with phenotypic susceptibility to MLS or only erythromycin was previously reported by others (40, 43, 44) and may be related to defective expression of this gene (43, 44). The relatively high occurrence of *erm*(B) was noted for different *Lactobacillus* and *Enterococcus* strains isolated from fermented food (29, 57). The *erm*(B) gene was detected in different lactobacilli (*L. plantarum*, *L. jonsonii*, *L. salivarius*, *L. reuteri*, *L. crispatus, L. amylovorus*, *L. gallinarum*) isolated from broilers (43, 44), from swine and poultry meat products (42) or from wine (59). Moreover, in our study two erythromycin-resistant strains, *L. agilis* 1834 and *E. faecium* TR2, carried the *msr*(C) gene. To the best of our knowledge, this is the first study which reports the presence of this gene in *L. agilis*. The *msr*(C) gene was initially considered as characteristic for *E. faecium* (63), then it was found in other *Enterococcus* species, including *E. durans*, *E. lactis*, and *Enterococcus casseliflavus*, and also *P. pentosaceus* and *L. fermentum* strains (57). The ever frequently occurrence of *msr*(C) in different LAB species may be associated with increasingly widespread transfer of this gene between these bacteria. Moreover, two *L. plantarum* (870, 872) and one *E. faecium* TR2 strains, phenotypically susceptible to clindamycin with MIC = 4 mg/L, carried the *lnu*(A) gene which encoding lincosamide *O*-nucleotidyltransferase. This gene was found in *L. salivarius*, *L. johnsonii*, *L. crispatus*, *L. reuteri*, *L. agilis*, and *L. ingluviei* (40, 53). Similarly, to our results, also Dec et al. (53) noted the *lnu*(A) gene in lactobacilli strains susceptible to clindamycin. However, the reason of this relationship remains unknown. In the other hand, the presence of *lnu*(A) gene in strains with the clindamycin MIC of 4 mg/L may suggest that the previous cut-off values (25) were more suitable to distinguish between a susceptible and a resistant strain. Interestingly, it seems that according to available data *lnu*(A) has not been described so far in *L. plantarum* and *E. faecium* species.

In the current study, we observed a high resistance of LAB strains to kanamycin (32.3%) and streptomycin (15.4%), while gentamicin resistance was much less prevalent (3.1%). Similarly, more frequent occurrence of resistance to streptomycin than to gentamicin was recorded for lactobacilli from chickens and turkeys in Poland (40, 41). However, the higher resistance to gentamicin was also reported previously (52). The widespread occurrence of kanamycin-resistant lactobacilli strains of various species has been noted by others (29, 51, 53). It is generally known that some lactobacilli species display resistance to aminoglycosides. Of the genes that determine resistance to aminoglycosides, the most prevalent was *aph*(3″)-*IIIa*, encoding the kinase APH(3″)-IIIa, which confer resistance to kanamycin. This gene was found in 6 kanamycin-resistant strains with the MIC value in the range from 128 to ≥256 mg/L, including *L. plantarum* (835, 870, 1822), *L. buchneri* 2047p, *L. diolivorans* 2036p, and *L. agilis* 1834. The *aph*(3″)-*IIIa* gene has been previously detected in *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains from yogurts (64) and *L. plantarum* isolated from wine (59). Surprisingly, the presence of *aph*(3″)-*IIIa* was also noted in this study in three *L. fermentum* strains (811, 830, 843) with MIC = 64 mg/L, classified as susceptible to kanamycin. Similarly, to our results, the presence of aminoglycoside resistance genes in phenotypically susceptible lactobacilli have been observed previously (40). Moreover, the *str*(A)/*str*(B) genes, encoding the streptomycin kinases APH(3″)-Ib and APH(6′)-Id, respectively, were noted in *L. acidophilus* 2499 and *L. salivarius* 1835. Interestingly, both these strains had MIC values on-fold higher than the cut-off value for streptomycin (32 mg/L for *L. acidophilus* 2499 and 128 mg/L for *L. salivarius* 1835). It should be highlighted, that the *str*(A) and *str*(B) genes are most frequently linked (65). In this study, we used the primer set which can detect both these genes, whereby a primer forward is complementary to the final part of the *str*(A) gene. Therefore, the partial sequence of *str*(B) is the main PCR product. The possible occurrence of *str*(A) should be confirmed by additional sequencing of longer fragments of this gene or using a specific primer set. It should be mentioned that the vast majority of phenotypically aminoglycoside-resistant strains did not contain any of the known genes that determine this resistance. This phenomenon has been described in other reports (53) and it was suggested that resistance to aminoglycosides, such as kanamycin and streptomycin, is innate in pediococci and some lactobacilli species, including *L. fermentum* (32, 50). The intrinsic aminoglycoside resistance may be associated with the low level of transmembrane potential or its absence that leads to the impaired uptake of these antibiotics. Moreover, the chromosomal mutations which impact to transmembrane electrical potential, were described in Gram-positive bacteria, while in Gram-negative bacteria the variable efflux systems were identified (32, 66). Furthermore, a high spontaneous mutation rate to resistance to kanamycin and streptomycin in lactobacilli has been reported (67).

In our study, the phenotypic and genotypic resistance do not correspond in many cases since the strains had the MIC values higher that the microbiological cut-off values but did not have the corresponding resistance genes. These findings are consistent with the results reported in other studies regarding AMR of LAB (31, 40–42, 60). The simple explanation could be a mutation and mismatches at the primer annealing site that prevents detection of the target resistance gene (68). The phenotype-genotype discrepancies observed in our study could be also explained by the fact that other resistance genes may exist that were not investigated by us; however the number of the known resistance genes continues to increase. The presence of novel, unknown or unusual resistance determinants should also be considered. Moreover, the resistance might be also acquired through some mutations, for example a high spontaneous mutation rate to resistance to aminoglycosides in lactobacilli has been reported (67). Finally, some LAB species could be intrinsically resistant to certain antimicrobials due to inherent structural and functional features which aid their survival in an environment, but are independent of antibiotic selective pressure and are not spread through horizontal gene transfer. Generally, the regulation of intrinsic resistance is better characterized in Gram-negative

bacteria. The available data about AMR in LAB species, are still poorly understood and the further studies should certainly be carried out to clarify this phenomenon (60).

The recent studies have shown the potential of whole genome sequencing (WGS) for define the accurate genotype and link it to the observed phenotypes (55). WGS analysis for AMR allows detection of a much higher number of resistance markers, including the complete set of resistance genes present in isolates as well as the mutations and mobile genetic elements associated with resistance (69). Nevertheless, WGS analysis is still quite expensive as a technique and creates vast amounts of data and requires specialized bioinformatics expertise. Most authors still rely on phenotypic characterization of isolates and PCR-based detection of AMR genes.

The transfer of AMR genes between different LAB species and other bacteria has been well-documented and demonstrated by *in vitro* studies with a filter mating technique, as well as by *in vivo* models of animal rumen and alfalfa plant (29, 70). Moreover, it was shown that AMR genes may be transfer from lactobacilli to *E. faecalis*, which is an inhabitant of the animal and human gut, but also a potential pathogen (70, 71). Although the transferability of the detected resistance markers was not analyzed in our study and specific mobile genetic elements in tested LAB strains were not identified, the nucleotide sequences of the identified AMR genes showed high similarity or even identity to the AMR genes associated with mobile genetic elements, such as transposons and plasmids, described in LAB and other bacteria, even distantly related, and in some cases pathogenic (**Supplementary Table 3**). This suggests possible acquisition of detected AMR genes from other bacteria. Furthermore, it can be predicted that detected genes are located on mobile genetic elements. Thus, it is important to consider the possibility of further transfer of the detected AMR genes to other bacteria in the gut via horizontal transfer, which poses a serious health risk to animals and humans.

Despite the improved awareness and understanding of AMR of LAB, and the possibility of its spread through the food chain, this safety criterion is not always taking into consideration by researchers (72–74). The results of the current study highlight that the AMR assessment of LAB strains should be the first and key step in considering their applicability and should precede other studies regarding the beneficial effects of the strains, their usefulness or adaptation criteria.

CONCLUSION

Concluding, the presence of acquired AMR genes in the tested LAB strains, including genes that were not previously described in this bacterial group, like those found in pathogenic bacteria, confirms that LAB are capable of acquiring resistance determinants via horizontal gene transfer. Importantly, many studies show that such genes can be transferred in both directions. While conjugation is the most common way of dissemination of AMR genes, transformation and transduction may also play an important role in this process, even greater than previously thought (45). Therefore, all strains in this study carrying the acquired AMR genes cannot be considered as safe and should not be used as feed or silage additives. On the other hand, the susceptibility of most of the tested strains to the antibiotics recommended by EFSA make them safe for direct use in agriculture and animal husbandry and thus, worth further exploration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

IS contributed to conception and design of the study. IS, KJ-P, and MG contributed with resources to the study and performed the collection of isolates. IS, EK, and KJ-P conducted the experiments. IS, EK, and MR analyzed the data. IS and EK wrote the draft of the manuscript. MB and MR critically reviewed sections of 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/fvets.2021.687071/full#supplementary-material

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The Tetracycline Resistance Gene, *tet*(W) in *Bifidobacterium animalis* subsp. *lactis* Follows Phylogeny and Differs From *tet*(W) in Other Species

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The tetracycline resistance gene *tet*(W) encodes a ribosomal protection protein that confers a low level of tetracycline resistance in the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis.* With the aim of assessing its phylogenetic origin and potential mobility, we have performed phylogenetic and *in silico* genome analysis of *tet*(W) and its flanking genes. *tet*(W) was found in 41 out of 44 examined *B. animalis* subsp. *lactis* strains. In 38 strains, *tet*(W) was flanked by an IS5-like element and an open reading frame encoding a hypothetical protein, which exhibited a similar GC content (51–53%). These genes were positioned in the same genomic context within the examined genomes. Phylogenetically, the *B. animalis* subsp. *lactis tet*(W) cluster in a clade separate from *tet*(W) of other species and genera. This is not the case for *tet*(W) encoded by other bifidobacteria and other species where *tet*(W) is often found in association with transferable elements or in different genomic regions. An IS5-like element identical to the one flanking the *B. animalis* subsp. *lactis tet*(W) has been found in a human gut related bacterium, but it was not associated with any *tet*(W) genes. This suggests that the IS5-like element is not associated with genetic mobility. *tet*(W) and the IS5 element have previously been shown to be co-transcribed, indicating that co-localization may be associated with *tet*(W) expression. Here, we present a method where phylogenetic and *in silico* genome analysis can be used to determine whether antibiotic resistance genes should be considered innate (intrinsic) or acquired. We find that *B. animalis* subsp. *lactis encoded tet*(W) is part of the ancient resistome and thereby possess a negligible risk of transfer.

Keywords: antimicrobial, antibiotic, resistance evolution, non-pathogenic bacteria, ribosomal protection, intrinsic resistance

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INTRODUCTION

Antibiotic resistance genes are widely spread among bacteria and they pose a serious threat to human health as they can compromise our ability to treat bacterial infections (World Health Organisation (WHO), 2017). Although the extensive use of antibiotics to treat infections in both humans and animals is considered to be the main reason for the development and spread of resistance genes (Levy and Bonnie, 2004; WHO, 2011), they have been present long

before the introduction of antibiotics to the clinic (Martínez, 2008; Allen et al., 2010). Antibiotics are naturally produced by environmental microorganisms and the producers often have "self-resistance" encoded by antibiotic resistance genes located in the antibiotic biosynthesis gene clusters (Martínez, 2008). Some antibiotic resistance genes show homology to housekeeping genes such as those involved in protein synthesis suggesting that they may have evolved from such functions and this could explain their prevalence among bacteria (Martínez, 2008; Allen et al., 2010). Antibiotic resistance genes have mainly been studied in clinically relevant bacteria and often in relation to horizontally transferable elements (Shrivastava et al., 2018). In contrast, less attention has been paid to antibiotic resistance in non-pathogenic bacteria (Klare et al., 2007; Agersø et al., 2019; Campedelli et al., 2019), e.g., bacteria ingested via the food chain.

When products contain viable, non-pathogenic bacteria, e.g., fermented food, probiotics or feed additives, it is a requirement from legal authorities [e.g., European Food Safety Authority (EFSA)] that these bacteria do not possess acquired genes encoding resistance toward antimicrobials, which are considered as highly or critically important for treatment of humans and/or animals by the World Health Organization (WHO) (WHO, 2011; EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018). However, some bacteria are intrinsically resistant to some of the antimicrobials (Peterson and Kaur, 2018). Impermeability of the outer membrane provides resistance to vancomycin for *Escherichia coli* and other Gram-negative bacteria (Arthur and Courvalin, 1993). *Bacillus licheniformis* and *Bacillus paralicheniformis* are resistant (or reduced in susceptibility) to erythromycin, chloramphenicol and streptomycin due to putative intrinsic resistance genes (Agersø et al., 2019).

Thus, homology to a known antibiotic resistance gene does not in itself indicate whether a putative resistance gene is acquired or intrinsic. Therefore, analysis of the genetic context and comparison to other genomes within the same species/subspecies are needed, although exact guidance on this is not provided by EFSA (EFSA panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2018).

Tetracyclines are broad spectrum antibiotics, which have been used for treatment of infections in humans and animals since the early 1950s and resistance toward tetracyclines is widespread. The *tet*(W) tetracycline resistance gene encodes a protection protein that attaches to the ribosome and causes an alteration of the ribosomal conformation to which tetracycline cannot bind and therefore protein synthesis can proceed (Chopra and Roberts, 2001; Connell et al., 2003). Genes with more than 80% identity to *tet*(W) have been found in 19 different genera belonging to both Gram-positive and Gram-negative bacteria and thus, it is the most widely spread tetracycline resistance gene class (Chopra and Roberts, 2001). The first *tet*(W) gene was reported in *Butyrivibrio fibrisolvens* located on a Tn *B1230*-like transposable element, which has spread to several different genera due to the broad host range of the element (Scott et al., 1997; Barbosa et al., 1999). Transfer of *tet*(W) in association with mobile genetic elements has also been reported to occur at low frequencies in *Bifidobacterium longum* strain F8 (Kazimierczak et al., 2006), *Arcanobacterium pyogenes* (Billington et al., 2002) and *Streptococcus suis* (Palmieri et al., 2011).

Several bifidobacterial species carry *tet*(W) genes, including *B. longum*, *B. thermophilum* and *B. bifidum* (Ammor et al., 2008). *tet*(W) is widespread and confers a low level of tetracycline resistance in *B. animalis* subsp. *lactis* that varies over three two-fold dilutions between different strains (Gueimonde et al., 2010), which has been suggested to be caused by genetic diversity in the *mia*A gene encoding for a tRNA dimethylallyltransferase (Milani et al., 2013). Furthermore, bile exposure have been shown to induce *tet*(W) expression (Gueimonde et al., 2010). The widespread nature of *tet*(W) suggest that it confers a selective advantage, perhaps a physiological function such as improving translation under the stress conditions of the gut. Although unsuccessful transfer studies are often not published, several studies on transferability of *tet*(W) from *B. animalis* subsp. *lactis* to other bacterial species and genera are published and all were unsuccessful (Gueimonde et al., 2010; Naghizadeh Raeisi et al., 2018; Polit et al., 2018). Bifidobacteria are Gram-positive, anaerobic, non-motile and non-spore-forming bacteria, which are commonly found in the gastrointestinal tract of various animals and humans, the human oral cavity and sewage (Milani et al., 2014). Members of the *Bifidobacterium* genus are among the first microbes to colonize the human gastrointestinal tract of newborns. Multiple health beneficial effects including reduction of diarrhea, colorectal cancer prevention and inhibition of pathogen growth and adherence have been reported for *Bifidobacterium* spp. (Turroni et al., 2012; O'Callaghan and van Sinderen, 2016). Therefore, many *Bifidobacterium* spp. are widely used in probiotic products (Garrigues et al., 2010). *B. animalis* including *B. animalis* subsp. *lactis* have had Qualified Presumption of Safety (QPS) status by EFSA since the establishment of the QPS concept in 2007 (Barlow et al., 2007; Koutsoumanis et al., 2020) and specific strains have acquired the Generally Recognized as Safe (GRAS) status from the Food and Drug Administration (FDA) in the United States (O'Callaghan and van Sinderen, 2016).

The aim of this study was to assess the phylogenetic relationship of *tet*(W) in *B. animalis* subsp. *lactis* through phylogenetic analysis, analysis of the genetic context surrounding the gene and core genome analysis. The study will serve as evidence to further establish that *tet*(W) in *B. animalis* subsp. *lactis* is innate; it originates from the ancestral host and has retained the same genomic position ever since. This supports the common perception that *tet*(W) should be considered an intrinsic and non-transferable gene in *B. animalis* subsp. *lactis*.

MATERIALS AND METHODS

Bacterial Genomes, Subspecies Identification and Genome Quality

All publicly available genome sequences of *B. animalis* subsp. *lactis* (50 strains including the type strain DSM 10140)

and *B. animalis* subsp. *animalis* (8 strains including the type strain ATCC 25527) were downloaded from the NCBI microbe genome database on the 21st of November 2019 (Sayers et al., 2019).

Subspecies identification was either obtained from previously published articles (Lugli et al., 2019) or performed by employing the *rpo*A and 16S ribosomal DNA sequence. A >98% identity to the type strain genes was used as threshold and the genes should furthermore be different from the type strain of a related subspecies, in this case *B. animalis* subsp. *animalis*, as shown through a phylogenetic tree (data not shown).

The sequence quality was assessed and sequences with an average coverage of ≥30× and a contig number below 120 were considered acceptable for phylogenetic analysis. The quality of the genomes was also evaluated by checking that the length of the sequenced genome corresponds with the expected length of the genome, based on the type strain (Milani et al., 2014).

Other bifidobacterial species, which have been shown to harbor *tet*(W) (Ammor et al., 2008; Wang et al., 2017) were also downloaded from the NCBI microbe genome database on the 21st of November 2019 and included *B. longum* (14 strains, type strain NCTC11818), *B. thermophilum* (6 strains, type strain DSM 20212), *B. bifidum* (11 strains, type strain ATCC 29521), *B. pseudolongum* (4 strains, type strain DSM 20099), *B. pseudocatenulatum* (3 strains, type strain DSM 20438) and *B. breve* (41 strains, type strain NCTC 11815). All *tet*(W) sequences from other genera where the gene have been described (Scott et al., 1997; Chopra and Roberts, 2001; Flórez et al., 2006; Kazimierczak et al., 2006; Ammor et al., 2008; Palmieri et al., 2011; Schröder et al., 2012) and shared identity to the *tet*(W) gene found in *B. animalis* subsp. *lactis* were also downloaded from NCBI on the 21st of November 2019.

Screening for *tet*(W), Genome Annotation and Examination of Sequences Flanking *tet*(W)

ResFinder (Zankari et al., 2012), with a 80% identity threshold, was used to search for the presence of *tet*(W) in the examined genomes and the Rapid Annotation using Subsystems Technology (RAST) server with default settings was used to annotate the genomes. The annotated genomes were downloaded in GenBank format from the RAST server (Aziz et al., 2008; Overbeek et al., 2014) and imported to CLC Genomics Workbench 20 (Qiagen Bioinformatics, Aarhus, Denmark), where the presence of *tet*(W), its flanking genes and presence of mobile genetic elements was examined. *tet*(W) nucleotide and protein sequences was extracted from the annotated genomes for further phylogenetic analysis. GC content of *tet*(W) and other genes was assessed by employing the DNA/RNA GC Content Calculator at ENDMEMO (Endmemo, 2020).

ISFinder

The blastN tool available at ISFinder (Siguier et al., 2006) with default settings was used to determine the identity of the mobile genetic protein next to *tet*(W) in *B. animalis* subsp. *lactis* and its sequence was used to search for its presence in other genomic regions in the *B. animalis* subsp. *lactis* genomes, which was performed in CLC Genomics Workbench 20 (Qiagen Bioinformatics, Aarhus, Denmark).

tet(W) Nucleotide and Amino Acid Phylogenetic Analysis

The phylogenetic analysis of *tet*(W) included both the nucleotide and protein sequences from *B. animalis* subsp. *lactis* (**Supplementary Table 1**) and *tet*(W) genes found in other bifidobacterial species and other genera where the presence of *tet*(W) previously have been published (**Table 1**) (Scott et al., 1997; Chopra and Roberts, 2001; Flórez et al., 2006; Kazimierczak et al., 2006; Ammor et al., 2008; Palmieri et al., 2011; Schröder et al., 2012). The nucleotide and protein *tet*(W) sequences was either extracted from the annotated genomes or from NCBI (Sayers et al., 2019).

ClustalX2 (Larkin et al., 2007) was used to perform a pairwise multiple alignment of the *tet*(W) sequences (Higgins and Sharp, 1988) and BioEdit (Hall, 1999) was used to remove gaps and unpaired ends. The nucleotide phylogeny was built by evolutionary analysis by the Maximum Likelihood method and Tamura-Nei model by MEGA X (Tamura and Nei, 1993; Kumar et al., 2018) and the amino acid phylogeny was built by evolutionary analysis by Maximum Likelihood method and JTT matrix-based model also by MEGA X (Jones et al., 1992; Kumar et al., 2018). Number of single nucleotide polymorphisms (SNPs) and single amino acid polymorphisms (SAPs) was obtained from the multiple alignment output from MEGA X that was used to build the phylogenetic relationships.

Core Genome Phylogeny

The genomes, either fully assembled or contigs were annotated by Prokka, which annotates genomes through the use of different tools including Prodigal (coding sequences), RNAmmer (Ribosomal RNA genes), Aragorn (Transfer RNA genes), SignalP (Signal leader peptides) and Infernal (Non-coding RNA) (Seemann, 2014). Prokka annotation is a requirement for using Roary, since the .gff file (file containing sequences and annotations) provided by Prokka is used by Roary to create a multi-FASTA alignment of all the core genes (Page et al., 2015). Roary was set to perform nucleotide alignment using MAFFT and a Blastp percentage identity at 80% (Katoh, 2002). FastTree was used to produce an approximately maximum-likelihood phylogenetic tree from the core gene alignment file, which was visualized by MEGA X (Price et al., 2009, 2010; Kumar et al., 2018).

RESULTS AND DISCUSSION

Assessment of Genome Quality

A total of 50 publicly available *B. animalis* subsp. *lactis* strains including the type strain DSM 10140 were downloaded from NCBI and consisted either of contigs or assembled genomes (**Supplementary Table 1**). The sequence quality

TABLE 1 | *tet*(W) encoded by Gram-positive and Gram-negative bacteria.

Strains	Nucleotide identity (%) to *B. animalis* subsp. *lactis* DSM 10140 *tet*(W)	Accession number	Mobile genetic elements	Horizontal transfer confirmed	References
Gram-positive bacteria					
Arcanobacterium pyogenes					
BBR1	91.79%	AY049983	Integrase, putative mobilization protein, mobilization protein	Yes (18)	Chopra and Roberts, 2001; Billington et al., 2002
Bifidobacterium bifidum					
L22	98.01%	EU434755	No MGE		Ammor et al., 2008
Bifidobacterium breve					
12L	98.01%	NZ_CP006711	Integrase		NCBI database
139W423	99.74%	CP021556	Transposase, integrase and mobile element protein		Bottacini et al., 2018
Iw01	98.06%	CP034192	No MGE		Wang et al., 2019
Bifidobacterium longum					
BG7	98.85%	CP010453	Transposase, mobile element protein and phage infection protein		Kwon et al., 2015
BXY01	99.74%	CP008885	Transposases and mobile element proteins		NCBI database
H66	98.06%	DQ060146	No MGE		Flórez et al., 2006
F8	99.37%	DQ294299	Tandem repeat flanking a transposase	Yes (17)	Kazimierczak et al., 2006
L42	98.06%	EU434756	Transposase		Ammor et al., 2008
B93	97.96%	EU434749	NA		Ammor et al., 2008
B94	97.96%	EU434750	NA		Ammor et al., 2008
E111	98.01%	EU434751	NA		Ammor et al., 2008
LMG 13197	99.69%	EU434752	NA		Ammor et al., 2008
Bifidobacterium thermophilum					
DSM 20210 (type strain)	99.69%	NZ_JDUB00000000	No MGE		Sun et al., 2015
DSM 20212	99.74%	NZ_JHWM00000000	No MGE		NCBI database
LMG 21813	99.69%	EU434753	No MGE		Ammor et al., 2008
RBL67	99.74%	CP004346	No MGE		Rbl et al., 2013
Bifidobacterium pseudocatenulatum					
DSM 20438 (type strain)	99.38%	NZ_AP012330	No MGE		Morita et al., 2015
12	98.01%	CP025199	No MGE		NCBI database
Bifidobacterium pseudolongum					
DSM 20092	98.06%	CP017695	Mobile element protein, transposase		NCBI database
Clostridium difficile					
CD5	98.85%	AM749838	No MGE		Spigaglia et al., 2008
Corynebacterium					
DSM 45100, pJA144188	99.69%	NC_014167	Plasmid		Schröder et al., 2012
Lactobacillus reuteri					
PA-16	99.74%	FJ489649	Transposase		Egervärn et al., 2009
ATCC 55730, pLR581	99.63%	EU583804	Plasmid		Egervärn et al., 2010
Roseburia sp.					
A2-183	98.01%	AJ421625	Putative mobilization protein		Flórez et al., 2006; Kazimierczak et al., 2006
Streptococcus suis					
SsCA-1	98.85%	FN396364	Protein with putative involvement DNA transfer		Chopra and Roberts, 2001; Palmieri et al., 2011

(Continued)

TABLE 1 | Continued

Strains	Nucleotide identity (%) to *B. animalis* subsp. *lactis* DSM 10140 *tet*(W)	Accession number	Mobile genetic elements	Horizontal transfer confirmed	References
Phi-SsUD	99.69%	FN997652	Genetic element with typical phage organization	Yes (19)	Palmieri et al., 2011
GZ1	99.74%	CP000837	No MGE		Palmieri et al., 2011
Trueperella pyogenes					
TP3	98.33%	CP033904	IS21 family transposase, conjugal transfer protein TrbL		Feßler and Schwarz, 2017
Gram-negative bacteria					
Butyrivibrio fibrosolvens					
Tn 1230	98.06%	AJ222769	Tn1230 transposon	Yes (16)	Scott et al., 1997; Chopra and Roberts, 2001
JK51	98.01%	AJ427421	No MGE		Chopra and Roberts, 2001; Kazimierczak et al., 2006
Megasphaera elsdenii					
2–9	No significant similarity found	AY196917	NA		Chopra and Roberts, 2001; Stanton and Humphrey, 2003
7–11	No significant similarity found	AY196919	NA		Chopra and Roberts, 2001; Stanton and Humphrey, 2003
4–13	No significant similarity found	AY196918	NA		Chopra and Roberts, 2001; Stanton and Humphrey, 2003
25–50	98.01%	AY485125	NA		Stanton and Humphrey, 2003
Mitsuokella multiacidus					
P208-58	98.06%	AJ427422	No MGE		Chopra and Roberts, 2001; Flórez et al., 2006; Kazimierczak et al., 2006
Selenomonas ruminantium					
FB322	99.58%	DQ294295	No MGE		Kazimierczak et al., 2006

NA, whole genome sequence was not available, the flanking sequences could therefore not be examined. Accession number provided are either nucleotide or genome accession number.

was assessed and sequences with an average coverage of ≥30 fold and a contig number below 120 were considered acceptable. On this basis, six strains (B420, DS1_2, BI-04, IDCC4301, CF3_2, AD011) were excluded from the study. The genomes of CNCM I-2994 (Chervaux et al., 2011) and AD011 (Kim et al., 2009) had both been sequenced by Sanger shotgun sequencing and consist of complete genomes. However, AD011 has previously been shown to exhibit a poor sequence quality and was therefore excluded (Garrigues et al., 2010), CNCM I-2994 was not excluded from the study. A total of 44 genome sequences were therefore acceptable for further phylogenetic analysis.

The *B. animalis* subsp. *lactis* genomes exhibited a size of 1.91–2.08 Mb with a GC content of 60.0–60.6% (**Supplementary Table 1**), which is in agreement with data for the type strain of the subspecies (Milani et al., 2014).

Subspecies identification was either obtained from previously published articles (Lugli et al., 2019) or performed by analysis of the *rpo*A and 16S ribosomal DNA sequence.

Diversity of the *B. animalis* subsp. *lactis* Genomes

The majority of the *B. animalis* subsp. *lactis* strains originated from human feces, but also from food samples, dietary supplements and domestic pigs, chimpanzees, rabbits, vervet monkeys, a barbary macaque, three different dog breeds and one strain, the genomic unique ATCC 27673 (Loquasto et al., 2013) originated from sewage (**Supplementary Table 1**). Species within the bifidobacterial genera are commonly found in the gastrointestinal tract of various animals, the human oral cavity and sewage (Milani et al., 2014) and the strains in this study therefore represent the most common habitats of bifidobacteria.

Since *B. animalis* subsp. *lactis* is included in a wide range of probiotics, it cannot be excluded that the strains isolated from human feces, domestic pigs and dogs originate from ingested products such as probiotics. However, the strain collection also include strains such as Bl12 that has been isolated from a healthy patient, which has not ingested probiotic

products (Milani et al., 2013) and rabbits and monkeys have with high likelihood not been exposed to probiotics and these strains are therefore expected to be diverse from the industrially exploited strains. The genome sizes of the different strains also vary, which also indicate that the strains are diverse (**Supplementary Table 1**). Most of the strains are isolated or submitted to NCBI between year 2006–2018, which reflect the increased focus on probiotics in the last decades (Gogineni, 2013), while the type strain DSM 10140 originates from 1997 (**Supplementary Table 1**). However, the submission date of the genome sequences to NCBI does not necessarily reflect the time of isolation as some strains are isolated even earlier.

B. animalis subsp. *lactis* has previously been shown to be a strict monophyletic bifidobacterial taxon that has recently evolved (Milani et al., 2013), however, some diversity is observed between the strains within the subspecies based on the presence of truly unique genes in some of the strains (Lugli et al., 2019). The strains with the highest number of truly unique genes are also included in this study. It is therefore concluded that the strains included in the current study represent the diversity within the subspecies.

The *tet*(W) Gene and its Genomic Location in *B. animalis* subsp. *lactis*

A 1920 bp *tet*(W) gene flanked by genes annotated as mobile element protein (966 bp), with inverted repeats at both ends of 50 bp and a hypothetical protein (HP) of unknown function (183 bp) was found in the majority of the studied *B. animalis* subsp. *lactis* strains (38 out of 44). These genes exhibit similar GC content (51.01–53.23%), which is lower than the flanking genes in the genetic region (52.46–62.25%) (**Figure 1**) and the average of the genome (60.0–60.6%) (**Supplementary Table 1**). *tet*(W) genes found in non-bifidobacterial and bifidobacterial species exhibit a GC content of 52.19–53.18%, indicating that *tet*(W) genes generally exhibit a GC content around 53%.

The three strains originating from dogs (2007B, 2010B, 2011B) did not encode *tet*(W), the mobile element protein or the HP (**Figure 1** and **Supplementary Table 1**). Two strains (DS28_2, LMG P-17502_2) only encoded the *tet*(W) gene, while LMG P-17502 encoded *tet*(W) and the mobile element protein (**Figure 1**). UBBLa 70 exhibited a large deletion in the *tet*(W), with only 117 bp remaining and two strains (ATCC 27673, 1528B) encoded a truncated version of the mobile element protein. This indicate that the three genes have been present originally in *B. animalis* subsp. *lactis* but have been subject to deletion in some strains. Despite these differences, the presence of *tet*(W), the putative mobile element protein and the HP are highly conserved within *B. animalis* subsp. *lactis* strains. This conservation was even observed in the strains that are more genomic unique which include ATCC 27673 and 1528B, and the Bl12 strain and the strains isolated from monkeys and rabbits. This suggest that the genetic organization surrounding *tet*(W) is not only present in the industrially exploited *B. animalis* subsp. *lactis* strains.

The *tet*(W), the mobile element protein and the HP genes were positioned in the same genomic context in the majority of the examined strains, however, in a few strains, alterations downstream (DS28_2, LMG P-17502_1, LMG P-17502_2, 2007B, 2010B, 2011B) and upstream (2011B) (**Figure 1**) of the three genes were observed. These were the same strains that exhibited complete or partial deletions of the *tet*(W), the mobile element protein and HP genes.

The genomic position of *tet*(W) was also reported by Rozman et al. (2020). They suggest that *tet*(W) and its flanking genes from the HP before the IS element to the HP after isochorismate pyruvate-lyase (**Figure 1**), based on nucleotide bias and codon usage bias, is part of a putative genomic island that has co-evolved together with *B. animalis* subsp. *lactis* and originate from an ancestral host (Guo et al., 2012; Bertelli et al., 2017). The codon usage bias corresponds with the gene GC content being lower in these genes compared to the rest of the genome. Genomic islands are defined as clusters of genes in bacterial genomes of probable horizontal origin and they often provide adaptive traits that has the ability to enhance the fitness of bacteria within a specific niche (Dobrindt et al., 2004). The putative genomic island in *B. animalis* subsp. *lactis* encodes for genes involved in cell metabolism and gene regulation and has not been found in other bacteria (Rozman et al., 2020). This could suggest that the putative genomic island including *tet*(W) encodes for important *B. animalis* subsp. *lactis* niche factors, which enable it to survive and compete for nutrients in the gut and has been part of the genome of *B. animalis* subsp. *lactis* long before the antibiotic era.

The *tet*(W), the mobile element protein and the HP gene were absent in all eight *B. animalis* subsp. *animalis* strains included in the study (**Supplementary Table 1**), which otherwise exhibited almost identical gene organization in the genomic region including the genes part of the putative genomic island (**Figure 1**). This could suggest that the *tet*(W), the mobile element protein and HP genes have been inserted in an ancestor of the *B. animalis* subsp. *lactis* close to subspecies differentiation and most likely lost by the three dog originating strains (2007B, 2010B, 2011B) not carrying *tet*(W).

Identification of the Putative Mobile Element Protein Flanking *tet*(W)

The presence of a putative mobile element protein next to *tet*(W) has previously been reported (Ammor et al., 2008; Gueimonde et al., 2010; Rozman et al., 2020). The sequence encodes a putative DDE transposase gene that is flanked by inverted repeats upstream and downstream of 50 bp, which collectively belong to the insertion sequence (IS) 5-like element ISBian1 family that originate from *B. animalis* according to ISFinder (Siguier et al., 2006).

DDE transposases are able to catalyze the movement of IS elements and transposons by introducing nicks at each end of the elements (Frost et al., 2005) and are able to move within a genome or horizontally if they are part of mobile genetic element vectors such as plasmids, conjugative transposon and phages (Vandecraen et al., 2017). However, several studies have been unsuccessful in transferring *tet*(W) from *B. animalis* subsp. *lactis* to other species and genera (Gueimonde et al., 2010;

Naghizadeh Raeisi et al., 2018; Polit et al., 2018), A BLASTp analysis showed that the IS5-like element ISBian1 family with 99.07% identity was found in the human ileum isolated *Angelaksiella massiliensis* (Mailhe et al., 2017) and the IS5 element was not associated with *tet*(W) in this species. The IS5 element was not found in other bifidobacterial species besides *B. animalis* subsp. *lactis*. The IS5 element was not found in other positions within the *B. animalis* subsp. *lactis* genomes and the inverted repeats flanking the transposase was only flanking the transposase next to *tet*(W). This indicates that the IS element is stably positioned next to *tet*(W) and does not mobilize within the *B. animalis* subsp. *lactis* genome, which is in accordance with the stable nature of the *B. animalis* subsp. *lactis* genome (Morovic et al., 2018).

Besides IS elements involvement in mobilization, IS5 elements are mainly able to modulate the expression of neighboring genes through co-transcription from the transposase promoter located in the terminal inverted repeat if inserted into non-coding regions (Schnetz and Rak, 1992; Luque et al., 2006; Vandecraen et al., 2017). The IS5 element flanking *tet*(W) in *B. animalis* subsp. *lactis* is positioned in a non-coding region meaning it does not cause deletion of other genes (**Figure 1**) and has previously been shown to be co-transcribed with *tet*(W) (Gueimonde et al., 2010). This indicates that the IS5 element potentially is involved in modulating the expression of *tet*(W) rather than mobilization.

tet(W) Encoded by Gram-Positive and Gram-Negative Bacteria

All previously published *tet*(W) genes were included in the analysis. Direct submissions at NCBI also include other *tet*(W) genes, however, none of these exhibited 100% identity to the subspecies *B. animalis* subsp. lactis *tet*(W) and we did not find any variants not represented in the analysis (data not shown). The published *tet*(W) genes are therefore a good presentation of *tet*(W).

tet(W) is one of the most widely spread resistance genes and is both found in Gram-positive and -negative bacteria (Chopra and Roberts, 2001). Despite the wide spread nature of *tet*(W), it was not found to be encoded by all the strains within the examined Gram-positive and -negative species, showing that *tet*(W) has been acquired by a few strains or lost as compared with *B. animalis* subsp. *lactis* where it is a general genetic feature of the subspecies. For both the Gram-positive and -negative bacteria other than *B. animalis* subsp. *lactis, tet*(W) was often found to be flanked by mobile genetic elements (**Table 1**) and in some strains *tet*(W) was positioned in a genomic region with several mobile genetic elements, e.g., *B. longum* BG7 and *A. pyogenes* BBR1. Transfer of *tet*(W) has been reported for *B. longum* strain F8 (Kazimierczak et al., 2006), *A. pyogenes* (Billington et al., 2002), *S. suis* (Palmieri et al., 2011) and *B. fibrosolvens* (Scott et al., 1997). Within species, the *tet*(W) genes in the examined Gram-positive and -negative bacteria were positioned in different

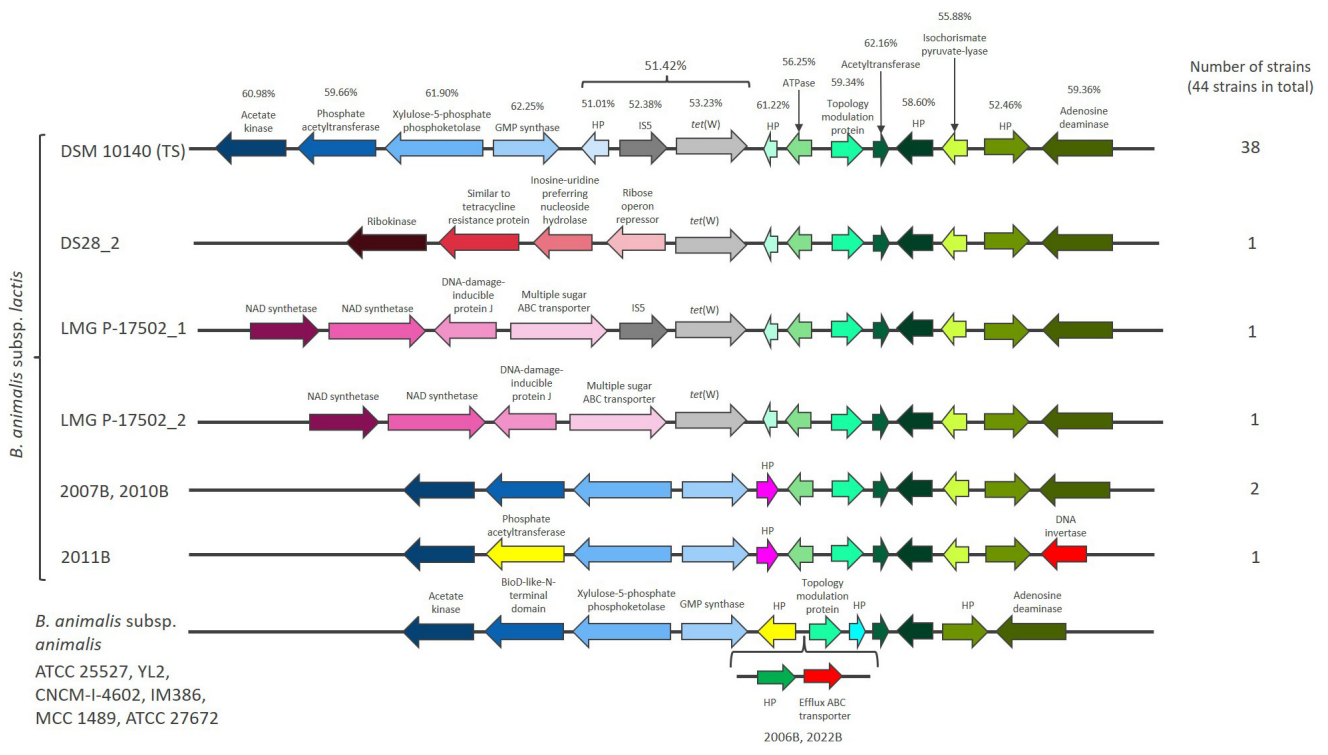

FIGURE 1 | The chromosomal region flanking *tet*(W) in *Bifidobacterium animalis* subsp. *lactis* and the same region in *Bifidobacterium animalis* subsp. *animalis*. Hypothetical proteins are designated HP. GC content (%) is provided for the genes found in the *B. animalis* subsp. *lactis* type strains (TS) DSM 10140. Genes that are present in the majority of the examined *B. animalis* subsp. *lactis* strains (represented by DSM 10140) has the same color in all the shown strains [blue colors downstream of *tet*(W) and green colors upstream of *tet*(W)].

TABLE 2 | Clades in the nucleotide and protein phylogenetic trees based on number of SNPs and SAPs.

Clades	SNPs	SAPs	Species
I	0–1	0–1	*Bifidobacterium animalis* subsp. *lactis*
II	12	5	*Bifidobacterium pseudocatenulatum*
III	11–13	5–7	*Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium thermophilum, Streptococcus suis, Corynebacterium, Lactobacillus reuteri*
IV	15	6	*Selenomonas ruminantium*
V	19	8	*Bifidobacterium longum*
VI	26–29	15	*Bifidobacterium longum, Clostridium difficile*
VII	38	20	*Trueperella pyogenes*
VIII	44–46	21–23	*Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium pseudolongum, Bifidobacterium pseudocatenulatum, Butyrivibrio fibrosolvens, Mitsuokella multicidus*, *Megasphaera elsdenii*, *Roseburia* sp.
IX	13	6	*Bifidobacterium longum, Bifidobacterium thermophilum*
X	28	13	*Streptococcus suis*
XI	161	69	*Arcanobacterium pyogenes*

genomic regions. Together, this indicates that *tet*(W) probably has been acquired independently in the examined bacteria in **Table 1**.

The observation that *tet*(W) is generally present in *B. animalis* subsp. *lactis* strains and is positioned in the same genomic region indicates that *tet*(W) is conserved and thereby an innate part of the subspecies, while *tet*(W) only has been acquired by a few strains within the examined Gram-positive and -negative bacterial species.

tet(W) Encoded by *B. animalis* subsp. *lactis* Is Distinct From *tet*(W) Encoded by Other Bacteria

A phylogenetic analysis was conducted of the *tet*(W) gene (**Supplementary Figure 1**) and protein (**Figure 2**) present in *B. animalis* subsp. *lactis* (**Supplementary Table 1**) and in the examined Gram-positive and -negative bacteria (**Table 1**).

The *tet*(W) genes encoded by the *M. elsdenii* strains (2–9, 7–11, 4–13) was shorter (1474–1476 bp) and exhibited a GC content (54.61–55.22%) higher compared to the other examined *tet*(W) genes and was therefore excluded from the phylogenetic analysis. The *tet*(W) gene of the remaining *M. elsdenii* strain (25–50) was found to be more similar to the other *tet*(W) genes and therefore included in the analysis.

Generally, the phylogenetic trees showed a high similarity between the different *tet*(W) genes and proteins, which is in agreement with previous observations (Aminov and Mackie, 2007), with the number of SNPs ranging from 1 to 46 and single amino acid polymorphisms (SAPs) ranging from 1 to 23 in the coding region compared to the *tet*(W) genes encoded by *B. animalis* subsp. *lactis*. The *tet*(W) gene encoded by *A. pyogenes* differed the most from *B. animalis* subsp. *lactis tet*(W) (161 SNPs and 69 SAPs). None of the SNPs lead to a premature stop codon. Based on the number of SNPs and SAPs (**Table 2**), clades were formed in the phylogenetic trees (**Figure 2** and **Supplementary Figure 1**), which follows the phylogeny for *B. animalis* subsp. *lactis* but not the other examined Gram-positive and -negative bacteria.

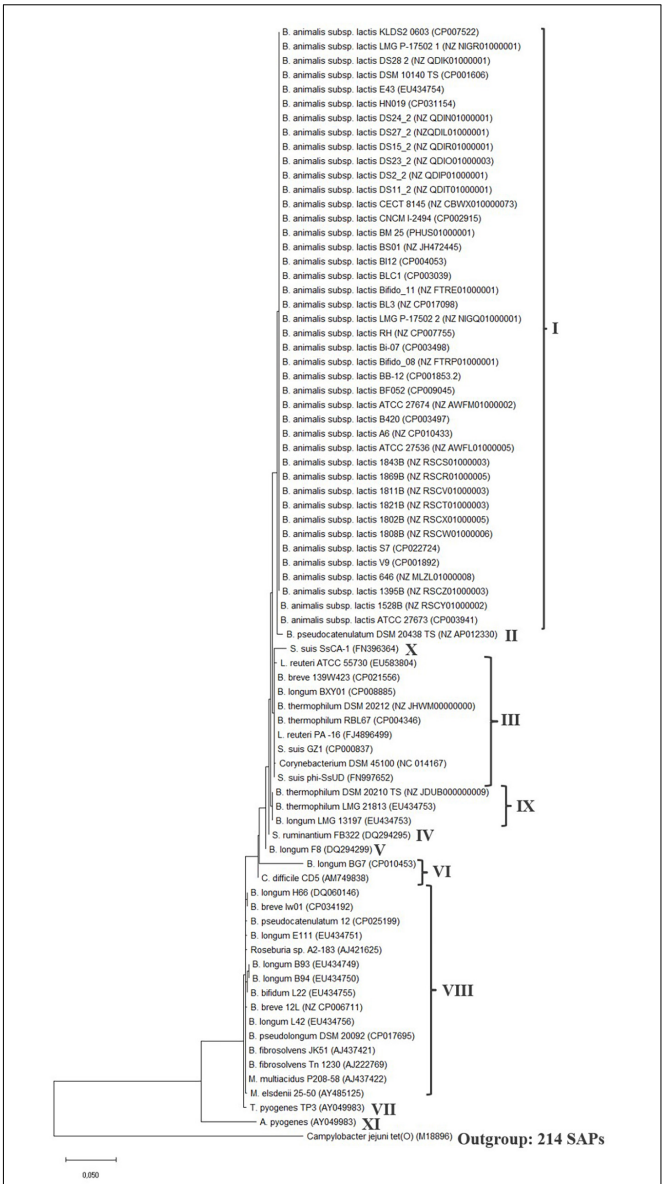

FIGURE 2 | *tet*(W) protein phylogenetic tree. The tree was built by evolutionary analysis by maximum likelihood method and JTT matrix-based model (Jones et al., 1992; Kumar et al., 2018). The branch lengths are measured in the number of substitutions per site. Strain name and genome or *tet*(W) gene accession number is provided for the sequences. Type strains (TS) are included for the species, when the type strain encodes *tet*(W). Clades are defined by the number of SAPs, which can be seen in **Table 2**. The phylogenetic tree was rooted with the ribosomal protection gene *tet*(O) from *Campylobacter jejuni* (M18896) as an outgroup and similar results was obtained with the *Streptococcal* ribosomal protection gene *tet*(M) (X04388) (data not shown) (Levy et al., 1999).

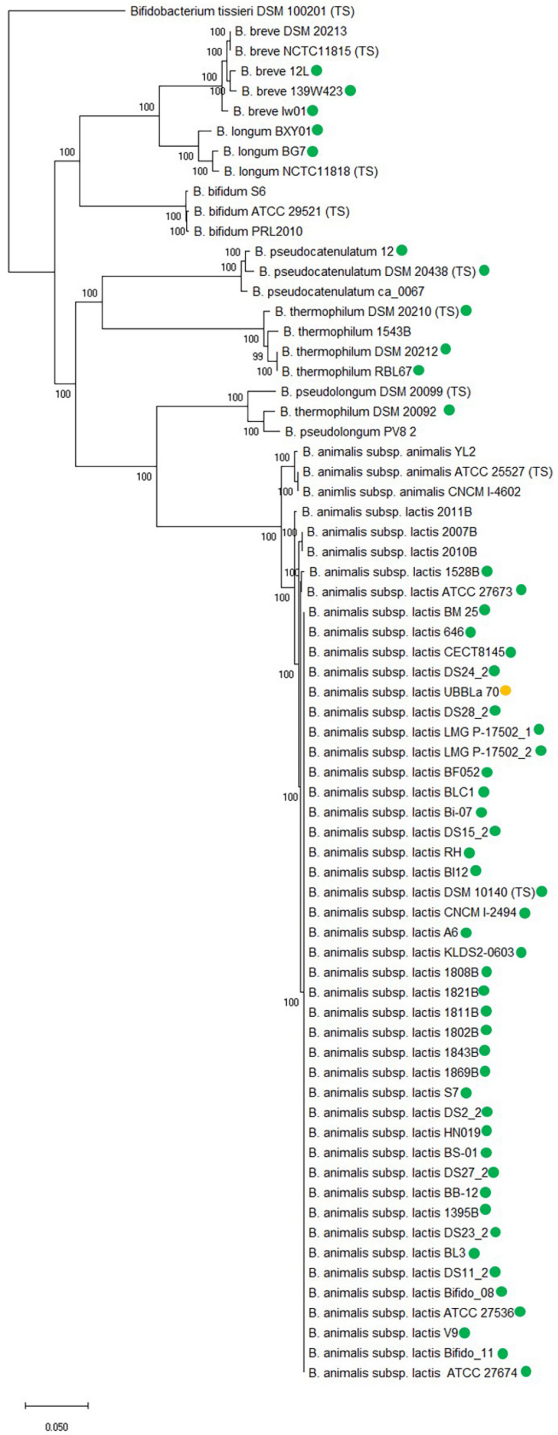

FIGURE 3 | Core genome phylogenetic tree based on 250 core genes which include *B. animalis* subsp. *lactis* strains and other related *Bifidobacterium* species. Type strain has been included for each species, designated TS and strains both with and without *tet*(W) are included for each species, except for *B. animalis* subsp. *animalis* and *B. bifidum*. *tet*(W) positive strains are marked with a green circle. *B. animalis* subsp. *lactis* UBBLa 70 exhibit a *tet*(W) gene with large deletions and is marked with a yellow circle. The tree is rooted with the *Bifidobacterium tissieri* type strain DSM 100201 as an outgroup (Lugli et al., 2018). Bootstrap percentages are shown at node points.

The phylogenetic analysis showed that the *tet*(W) genes (**Supplementary Figure 1**) and proteins (**Figure 2**) from the *B. animalis* subsp. *lactis* strains share a high degree of homology and forms a separate clade.

The *tet*(W) gene and protein in the *B. pseudocatenulatum* type strain DSM 20438 (Genome GC content 56.40%) was located nearest the *B. animalis* subsp. *lactis tet*(W) genes and proteins in the phylogenetic trees and exhibited 12 SNPs and 5 SAPs compared to the *tet*(W) genes and proteins encoded by *B. animalis* subsp. *lactis*. The *tet*(W) gene encoded by *B. pseudocatenulatum* DSM 20438 and *B. animalis* subsp. *lactis* both exhibit a high identity to *tet*(W) from *S. suis* (FN396364). The *tet*(W) gene encoded by *B. pseudocatenulatum* strain 12 exhibited 45 SNPs and 22 SAPs and was located in another clade than the DSM 20438 *tet*(W) gene, indicating that the *tet*(W) encoded by the two *B. pseudocatenulatum* strains differ. *tet*(W) has been shown to be present in 33–41% of *B. pseudocatenulatum* isolates from human (Aires et al., 2007; Wang et al., 2017), no mobile genetic elements including IS5 elements was found in the flanking regions of *tet*(W) in the two examined strains (**Table 1**) and transfer of *tet*(W) from *B. pseudocatenulatum* have so far not been shown to occur (Wang et al., 2017). An examination of the flanking sequences of *tet*(W) in *B. pseudocatenulatum* type strain DSM 20438 revealed that the downstream genes were organized similarly as the genes downstream of *tet*(W) in the majority of the studied *B. animalis* subsp. *lactis* strains (**Figure 1**), except that six hypothetical proteins was present between *tet*(W) and the GMP synthase gene and no IS5-like element was present (**Supplementary Figure 2**). These genes were also present in *B. pseudocatenulatum* strain 12 but in another genetic location than *tet*(W), and in a *B. pseudocatenulatum* strain (ca_0067, NZ_RCXS00000000) that did not encode *tet*(W). This indicates that the presence of these genes is independent of the presence of *tet*(W) and are shared genes between *B. animalis* subsp. *lactis* and *B. pseudocatenulatum*.

The *tet*(W) genes present in the examined Gram-positive and -negative bacteria including the two *B. pseudocatenulatum* strains, were scattered over different clades in the phylogenetic tree indicating that the *tet*(W) genes encoded by these bacteria are diverse, does not follow the phylogeny of the specific species and thereby support the acquired nature of these *tet*(W) genes.

tet(W) Encoded by *B. animalis* subsp. *lactis* Follows the Phylogeny of the Subspecies

A core genome phylogenetic analysis was conducted with the examined B. animalis subsp. lactis strains (**Supplementary Table 1**), the bifidobacterial species from **Table 1** and B. animalis subsp. animalis strains from **Supplementary Table 1** (**Figure 3**). For each species, strains were included that both did and did not encode *tet*(W), except for *B. animalis* subsp. *animalis* and *B. bifidum*.

The core genome phylogenetic analysis showed that the bifidobacterial species separated from each other in individual clades and both strains with and without *tet*(W) clustered

together within species, showing that the core genome analysis was able to separate at species and subspecies level.

The fact that the *tet*(W) gene encoded by the examined *B. animalis* subsp. *lactis* strains formed a separate clade in the gene and protein phylogenetic analysis (**Supplementary Figure 1** and **Figure 2**) similar to the one formed in the core genome phylogenetic tree shows that the phylogeny of *tet*(W) follows the phylogenetic relationship of the subspecies, indicates that *tet*(W) originates from an ancestral host. This is further supported by the gene being positioned in the same genomic context in the examined strains. For the other examined bifidobacterial species, the *tet*(W) genes does not follow the phylogeny of the species, indicating that the *tet*(W) gene has been acquired at different timepoints, which is in line with them being flanked by different mobile genetic elements and positioned in different genomic contexts. This indicates that *tet*(W) present in *B. animalis* subsp. lactis is distinct from *tet*(W) found in other bifidobacterial species and other genera.

CONCLUSION

The paper presents a method where *in silico* genome analysis together with phylogenetic analysis can be used to determine whether a gene is innate and thereby not considered a safety concern.

A phylogenetic analysis of *tet*(W) in *B. animalis* subsp. *lactis*, a widely used probiotic bacterium, was performed and shows that *tet*(W) in this specific subspecies is present in the majority of the strains (41 out of 44), positioned in the same genomic region and is different on the amino acid level from *tet*(W) genes found in other species. *tet*(W) is flanked by an IS5-like element, which is known to be present in other human gut related bacteria, however, the IS5-like element was not associated with *tet*(W) in these bacteria. Previously results show that *tet*(W) is co-transcribed with the IS5 transposase in *B. animalis* subsp. *lactis*, indicating that the expression of *tet*(W) is regulated by the IS5 transposase. Together with the previous unsuccessful attempts to transfer *tet*(W), our data suggest that *tet*(W) is non-transferable and that the flanking IS5 element is not involved in mobilization of *tet*(W). The phylogenetic analysis showed that *tet(*W) follows the phylogenetic relationship of the subspecies and is distinct from *tet*(W) found in other genera and bifidobacterial species.

We conclude that *tet*(W) in *B. animalis* subsp. *lactis* originates from an ancestral host and is therefore an innate part of the subspecies and should be considered as innate (intrinsic) in this subspecies. There is therefore a negligible risk that *tet*(W) from *B. animalis* subsp. *lactis* will add to the pool of mobile resistance genes and thus potentially cause treatment failures in humans and animals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

KN-M wrote the manuscript, made figures, tables, performed the analysis and was involved in developing the concept and the method. CS was involved in developing the concept, guiding the analysis, discussion, and review and editing. HI was involved in developing the concept, discussion, and review and editing. YA was involved in conceiving the idea, developing and guiding the concept, analysis, design, discussion, and review and editing. All authors have read and approved the submitted 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.2021.658943/full#supplementary-material

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Efficacy of Tigecycline and Linezolid Against Pan-Drug-Resistant Bacteria Isolated From Companion Dogs in South Korea

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The emergence of multidrug-resistant bacteria in companion animals is an increasing concern in view of the concept of One Health. The antimicrobials linezolid (LZD) and tigecycline (TGC) are effective against multidrug-resistant bacteria isolated from humans; however, thus far, no previous study has evaluated the efficacy of these drugs against bacteria isolated from companion animals. This study aimed to evaluate the efficacy of LZD and TGC against bacteria that were isolated from companion dogs and showed resistance to all classes of antimicrobial agents. Clinical samples (auditory channel, eye, skin, and urine) were collected from dogs that visited the Veterinary Medical Teaching Hospital of Konkuk University (Seoul, South Korea) from October 2017 to September 2020. In total, 392 bacterial isolates were obtained, of which 85 were resistant to all classes of antimicrobial agents tested and were, therefore, considered potentially pan-drug resistant (PDR). The susceptibility of isolates to LZD and TGC was determined by the disk diffusion method and interpreted using the Clinical Laboratory Standards Institute guidelines. In total, 95.6% (43/45) and 97.8% (44/45) of gram-positive isolates were susceptible to LZD and TGC, respectively, whereas 82.5% (33/40) of gram-negative isolates were sensitive to TGC. In conclusion, both agents showed favorable efficacy, with the susceptibility rates for all potential PDR bacteria, except *Pseudomonas* spp., ranging from 72.7 to 100%. Thus, these drugs may serve as excellent antimicrobial options for veterinary medicine in the future.

Keywords: linezolid, tigecycline, antimicrobial resistance, pan-drug-resistant, companion animals

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INTRODUCTION

The emergence of multidrug-resistant (MDR) bacteria, which are resistant to three or more categories of antimicrobials, in companion animals is highly concerning. Particularly, resistance to antimicrobials is growing among bacteria such as *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, and *Escherichia coli* (1), which cause infections in dogs. The transmission of such bacteria can be either direct or indirect among dogs, owners, and veterinary staff. Indeed, practicing veterinarians are far more likely to experience nasal colonization with *S. aureus* than the general population (2). Notably, under the One Health concept, companion animals have been documented to be reservoirs of some high-risk MDR clones of Enterobacteriaceae (3), which are likely to be acquired from their human owners. Overuse of antimicrobials in veterinary clinics may amplify

antimicrobial resistance and result in a subsequent spread of resistant microorganisms to animal owners. Therefore, the appropriate use of antimicrobials to prevent pain, illness, or death should be adopted for infection management in animals to improve the public health of both humans and animals.

Previous studies that analyzed the temporal trends of antimicrobial resistance in small collections of bacterial isolates from infected companion animals have provided evidence for a significant increase in antimicrobial resistance, particularly to agents frequently used in clinical settings, such as cephalosporins, ampicillins, and quinolones (4).

Linezolid (LZD) is a member of the oxazolidinone class of synthetic antibacterial agents that inhibit bacterial protein synthesis through a unique mechanism. In contrast to other inhibitors of protein synthesis, oxazolidinones act early in translation by preventing the formation of a functional initiation complex (5). Discovered in 1987 at E.I. DuPont de Nemours and Co., Inc., DuP-721 was the first well-characterized oxazolidinone (5) that exhibited strong activity against MDR gram-positive pathogens (6). In fact, it is currently used for the treatment of clinical methicillin-susceptible and methicillin-resistant *S. aureus* infections and for managing infections caused by vancomycin-resistant enterococci (7) in humans.

Tigecycline (TGC) is a glycylcycline antibiotic that is effective against a variety of gram-positive and gram-negative bacteria (8). TGC is currently one of the most potent antimicrobial agents for treating infections caused by MDR bacteria in humans (8). This drug has demonstrated *in vitro* activity against important resistant organisms, including methicillin-resistant *S. aureus*, penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant enterococcal species, in addition to extended-spectrum beta-lactamase-producing *E. coli* and *Klebsiella pneumoniae* (9). A previous study has shown that more than 90% of Enterobacteriaceae isolates are susceptible to this drug (10).

Nevertheless, LZD and TGC have not been used as first- or second-line treatment options in companion animals owing to concerns that the overuse and abuse of antimicrobials in animals would limit treatment options for human bacterial infections, in view of the One Health concept. Moreover, no previous study has evaluated the efficacies of LZD and TGC against bacteria originating from companion animals.

The present study aimed to evaluate the efficacies of LZD and TGC against potential pan-drug-resistant (PDR) bacteria (i.e., resistant to all classes of antimicrobial agents) isolated from dogs. Antimicrobial stewardship and related policies are beyond the scope of the current work.

MATERIALS AND METHODS

Sampling

A total of 359 clinical samples were collected from different lesions in dogs that visited the Veterinary Medical Teaching Hospital of Konkuk University (Seoul, South Korea) from October 2017 to September 2020. The samples were immediately placed into a transport medium (ESwab, Copan, Brescia, Italy). The sampling sites included the auditory channels, eyes/conjunctiva, gastrointestinal tract, skin/mucosa, blood, and urogenital tract (**Table 1**). Auditory channel, eye, and skin samples were routinely collected using sterile cotton swabs, and urine samples were collected by cystocentesis. In addition, we collected at least 2 g of feces, which were cubed to ∼½ to ¾ inch on a single side using a fecal loop. Peripheral blood was collected by venipuncture of the jugular vein to identify systemic infections. Furthermore, cerebrospinal fluid was obtained at the junction between lumbar vertebrae 5 and 6 using a conventional lumbar tapping method, and samples from the peritoneal walls were collected with sterile cotton swabs. All samples were immediately transported to the NosVet Laboratory (Gyeonggi-do, South Korea) and analyzed within 3–4 h.

TABLE 1 | Distribution of sampling sites and isolates.

Sites	No. of samples	No. of isolates
Urogenital tract	101	99
Auditory channel	92	80
Skin/mucosa	79	105
Eye/conjunctiva	33	30
Gastrointestinal tract	10	25
Tooth	7	7
Joint effusion	7	0
Blood	6	5
Respiratory tract	5	17
Other sites*	19	24
Total	359	392

Other sites include cerebrospinal fluid, tissue of various types, synovial capsule, foreign body, mass, bone (Supplementary File 1**).*

The animal study and the protocol was reviewed and approved by the Institutional Animal Care and Use Committee (KU20218). Written informed consent was obtained from the owners for the participation of their animals in this study.

Bacterial Isolates

In total, 392 isolates were obtained from the dogs by directly inoculating blood agar plates with the clinical samples using cotton swabs, followed by incubation of the agar plates at 37°C for up to 24 h. Morphologically identical colonies were picked and sub-cultured onto blood agar plates, and species were identified using a matrix-assisted laser desorption/ionization mass spectrometer (ASTA, Gyeonggi-do, South Korea). Bacterial stock solutions were stored at −20°C.

Antibiotic Susceptibility Testing

Commercial antimicrobial disk diffusion tests were performed by NosVet, Inc., according to the Clinical Laboratory Standards Institute (CLSI) guidelines (VET08). Susceptibility to 21 antibiotics from 10 classes, namely, amikacin (AK), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), azithromycin (AZM), cefixime (CFM), cefotaxime (CTX), cefpodoxime (CPD), ceftazidime (CAZ), cephalexin (CL), cephazolin (KZ), ciprofloxacin (CIP), clindamycin (DA), doxycycline (DO), enrofloxacin (ENR), erythromycin (E), gentamicin (CN), lincomycin (MY), ofloxacin (OFX),

spiramycin (SP), sulfamethoxazole/trimethoprim (SXT), and tetracycline (TE), was determined using a Vitek® AST-P601 card (bioMérieux, Marcy l'Étoile, France).

Two additional antibiotics, namely, LZD and TGC (Thermo Fisher Scientific, Waltham, MA, USA), which are not included in the CLSI VET08 guidelines, were tested against potential PDR strains. Susceptibility to LZD and TGC was determined by the disk diffusion method and interpreted based on the CLSI guidelines. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were used when information was missing in the CLSI guidelines. Briefly, bacteria were inoculated from stock solutions onto Mueller–Hinton agar plates and incubated at 37°C for 24 h. Colonies were suspended in normal saline, and the turbidity was adjusted to a 0.5 McFarland standard equivalent ($\sim 10^8$ colony-forming units per milliliter). Sterile cotton swabs were dipped into inoculation broth and subsequently streaked over Mueller–Hinton agar plates. Antibiotic disks of 30 μg of LZD and 15 μg of TGC were then placed on these plates, followed by incubation of the plates at 37°C for 24 h. Diameters of the inhibition zones were used to categorize bacteria as susceptible, intermediate resistant, and resistant according to the CLSI and EUCAST guidelines.

Statistical Analysis

Descriptive statistics were used for the analyses of signalment, clinical data, and laboratory findings. All statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). An exact chi-square test was used to compare the efficacies of LZD, TGC, and the 21 other antibiotics against potential PDR bacteria from dogs. Differences with *P*-values of <0.05 were considered statistically significant.

RESULTS

Based on the antibiotic sensitivity evaluation conducted by NosVet, Inc., 211 bacterial isolates were classified as extensively drug resistant (XDR), as previously described (11). Of these 211 isolates, 57 were resistant to eight classes and 69 were resistant to nine classes, while 85 were resistant to all ten classes of antimicrobial agents tested and were thus considered potential PDR bacteria (11). **Table 2** presents the taxonomic distribution of the 85 isolates.

Gram-Positive Isolates

According to the tests conducted by NosVet, Inc., 163 (65%) of 249 gram-positive strains showed sensitivity to AK, 131 (52.6%) were sensitive to AMC, 90 (36.1%) were sensitive to AMP, 110 (44.2%) were sensitive to AZM, 5 (2.0%) were sensitive to CFM, 89 (35.7%) were sensitive to CTX, 88 (35.3%) were sensitive to CPD, 11 (4.4%) were sensitive to CAZ, 97 (39.0%) were sensitive to CL, 114 (45.8%) were sensitive to KZ, 20 (8.0%) were sensitive to CIP, 93 (37.3%) were sensitive to DA, 124 (49.8%) were sensitive to DO, 133 (53.4%) were sensitive to ENR, 99 (39.8%) were sensitive to E, 88 (35.3%) were sensitive to CN, 8 (3.2%) were sensitive to MY, 125 (50.2%) were sensitive to OFX, 87 (34.9%) were sensitive to SP, 84 (33.7%) were sensitive to SXT, and 85 (34.1%) were sensitive to TE. Among these strains, 215 (86.3%) were MDR, 125 (50.2%) were XDR, and 45 (18.0%) were potential PDR bacteria (**Supplementary File 1**).

TABLE 2 | Species distribution of potential pan-drug-resistant isolates tested in this study.

Gram	Species	No. of isolates
Negative	*Escherichia coli*	12
	Klebsiella pneumoniae	11
	Proteus mirabilis	10
	Pseudomonas aeruginosa	3
	Citrobacter freundii	1
	Enterobacter aerogenes	1
	Enterobacter cloacae complex	1
	Pasteurellaceae bacterium	1
Positive	*Staphylococcus pseudintermedius*	26
	Enterococcus faecium	6
	Enterococcus faecalis	5
	Staphylococcus schleiferi	3
	Corynebacterium auriscanis	2
	Rothia nasimurium	1
	Staphylococcus epidermidis	1
	Streptococcus canis	1
Total		85

Among the potential PDR gram-positive bacteria, 95.6% (43/45) and 97.8% (44/45) of isolates were susceptible to LZD (**Figure 1**) and TGC (**Figure 2**), respectively. Superiority of LZD and TGC over the other antibiotics was statistically analyzed, and the result is presented in **Tables 3**, **4**. Overall, the potential PDR bacteria were significantly more susceptible to these two agents than to the other 21 agents ($P < 0.05$). The average diameter of the zone of inhibition for LZD was 31 mm, which fell within the susceptibility zone diameter of gram-positive bacteria for LZD. The average diameter of the zone of inhibition was 24.5 mm for TGC, which exceeded the zone of resistance size.

Staphylococcus spp.

One hundred sixty *Staphylococcus* spp. isolates showed sensitivity to AK (93.7%), followed by AMC (53.1%) and DA (52.4%). Of these isolates, 30 (18.7%) were potential PDR strains, and all of them were susceptible to LZD and TGC, with an average diameter of the zone of inhibition of 31.7 mm.

Enterococcus spp.

Forty-two *Enterococcus* spp. isolates showed sensitivity to AMP (17.5%), followed by AMC (16.8%). Of these isolates, 11 (26.2%) were potential PDR strains. The sensitivity of potential PDR *Enterococcus faecium* and *Enterococcus faecalis* isolates to LZD was 83.3% (5/6) and 80.0% (4/5), respectively, and the sensitivity to TGC was 83.3% (5/6) and 100% (5/5), respectively.

Uncommonly Encountered Species

One *Rothia nasimurium*, one *Streptococcus canis*, and two potential PDR *Corynebacterium auriscanis* isolates were susceptible to LZD and TGC.

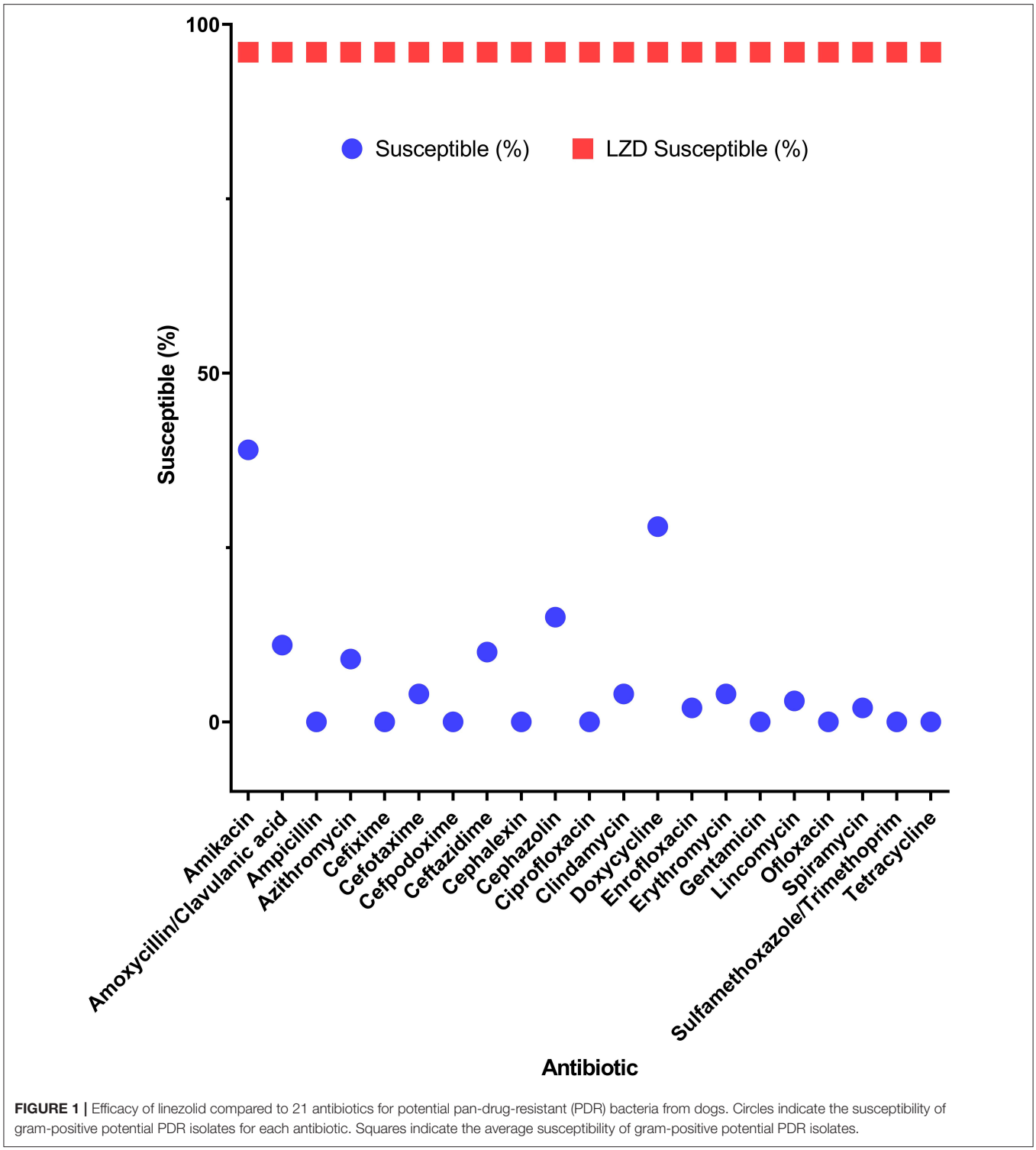

FIGURE 1 | Efficacy of linezolid compared to 21 antibiotics for potential pan-drug-resistant (PDR) bacteria from dogs. Circles indicate the susceptibility of gram-positive potential PDR isolates for each antibiotic. Squares indicate the average susceptibility of gram-positive potential PDR isolates.

Gram-Negative Isolates

According to the tests conducted by NosVet, Inc., 105 (73.4%) of the 143 gram-negative strains showed sensitivity to AK, 68 (47.6%) were sensitive to AMC, 33 (23.1%) were sensitive to AMP, 37 (25.9%) were sensitive to AZM, 56 (39.2%) were sensitive to CFM, 61 (42.7%) were sensitive to CTX, 59 (41.3%) were sensitive to CPD, 83 (58.0%) were sensitive to CAZ, 55 (38.5%) were sensitive to CL, 49 (34.3%) were sensitive to KZ, 17 (11.9%) showed sensitivity to CIP, 4 (2.8%) were sensitive to DA, 58 (40.6%) were sensitive to DO, 64 (44.8%) were

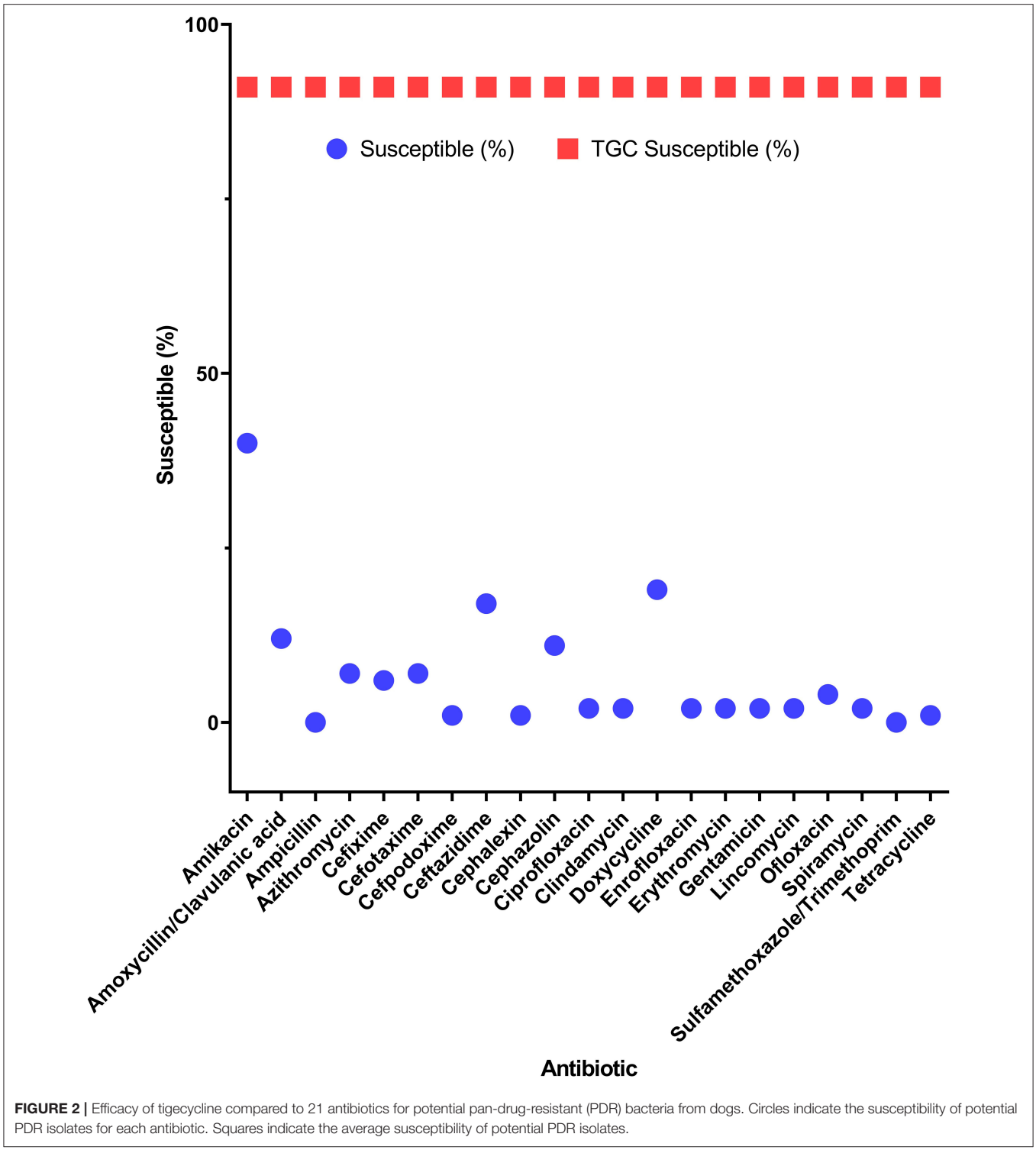

FIGURE 2 | Efficacy of tigecycline compared to 21 antibiotics for potential pan-drug-resistant (PDR) bacteria from dogs. Circles indicate the susceptibility of potential PDR isolates for each antibiotic. Squares indicate the average susceptibility of potential PDR isolates.

sensitive to ENR, 11 (7.7%) were sensitive to E, 79 (55.2%) were sensitive to CN, 1 (0.7%) showed sensitivity to MY, 71 (49.7%) were sensitive to OFX, 1 (0.7%) showed sensitivity to SP, 53 (37.1%) were sensitive to SXT, and 50 (35.0%) were sensitive to TE. Among these strains, 130 (90.9%) were MDR, 87 (60.8%) were XDR, and 40 (27.9%) were potential PDR bacteria (**Supplementary File 1**).

Of note, 82.5% (33/40) of the potential PDR gram-negative isolates were sensitive to TGC. The average inhibition zone diameter was 18.5 mm for positive isolates, which slightly

TABLE 3 | Superiority of linezolid efficacy over 21 different antibiotics to eradicate potential pan-drug-resistant bacteria from dogs.

Antibiotic	Susceptible (%)	Linezolid *P-value*
Amikacin	39.1%	<0.001
Amoxycillin/Clavulanic acid	10.9%	<0.001
Ampicillin	0.0%	<0.001
Azithromycin	8.7%	<0.001
Cefixime	0.0%	<0.001
Cefotaxime	4.3%	<0.001
Cefpodoxime	0.0%	<0.001
Ceftazidime	9.7%	<0.001
Cephalexin	0.0%	<0.001
Cephazolin	15.2%	<0.001
Ciprofloxacin	0.0%	<0.001
Clindamycin	4.3%	<0.001
Doxycycline	28.3%	<0.001
Enrofloxacin	2.2%	<0.001
Erythromycin	4.3%	<0.001
Gentamicin	0.0%	<0.001
Lincomycin	3.2%	<0.001
Ofloxacin	0.0%	<0.001
Spiramycin	2.2%	<0.001
Sulfamethoxazole/Trimethoprim	0.0%	<0.001
Tetracycline	0.0%	<0.001
Linezolid	95.7%	

TABLE 4 | Superiority of tigecycline efficacy over 21 different antibiotics to eradicate potential pan-drug-resistant bacteria from dogs.

Antibiotic	Susceptible (%)	Tigecycline *P-value*
Amikacin	40.0%	<0.001
Amoxycillin/Clavulanic acid	11.8%	<0.001
Ampicillin	0.0%	<0.001
Azithromycin	7.1%	<0.001
Cefixime	5.7%	<0.001
Cefotaxime	7.1%	<0.001
Cefpodoxime	1.2%	<0.001
Ceftazidime	17.1%	<0.001
Cephalexin	1.2%	<0.001
Cephazolin	10.6%	<0.001
Ciprofloxacin	1.9%	< 0.001
Clindamycin	2.4%	<0.001
Doxycycline	18.8%	<0.001
Enrofloxacin	2.4%	<0.001
Erythromycin	2.4%	<0.001
Gentamicin	2.4%	<0.001
Lincomycin	1.9%	<0.001
Ofloxacin	3.5%	<0.001
Spiramycin	1.5%	<0.001
Sulfamethoxazole/Trimethoprim	0.0%	<0.001
Tetracycline	1.2%	<0.001
Tigecycline	90.6%	

exceeded the resistance cutoff. Among Enterobacteriaceae, 100% (12/12) of the *E. coli* strains and 72.7% (8/11) of the *K. pneumoniae* strains, as well as single *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae* complex isolates, were susceptible to TGC. The overall sensitivity to TGC was 88.5% (23/26).

Escherichia coli

Fifty-two *E. coli* isolates showed sensitivities of 27.3% to AK and 21.0% to CN. Of these, 12 (23.0%) were potential PDR strains, as recommended by the CLSI VET08 guidelines. The average diameter of the zone of inhibition for TGC was 18.6 mm, which fell within the susceptibility zone (≥18 mm).

Klebsiella pneumoniae

Nineteen isolates of *K. pneumoniae* showed <10% sensitivity to the 21 antibiotics, and 11 (57.8%) were potential PDR strains, which was very high compared to that in other species. Furthermore, *K. pneumoniae* showed the highest resistance rate (27.3%; 3/11) to TGC among the bacteria tested in this study. The inhibition zone diameters of the resistant isolates were in the range of 13–17 mm, which was within the resistance zone (≥18 mm).

Proteus mirabilis

Among the 31 *P. mirabilis* isolates, 10 were classified as potential PDR strains, and only one of these (10%; 1/10) was classified as being resistant to TGC.

Pseudomonas aeruginosa

P. aeruginosa isolates were less sensitive to the 21 antibiotics tested than those of other species. Only three of the 13 isolates were classified as potential PDR strains, and all three isolates (100%) were resistant to TGC, with an inhibition zone diameter of 0 mm.

Uncommonly Encountered Species

One (100%) isolate of each of the following species was susceptible to TGC: *C. freundii*, *E. aerogenes*, *E. cloacae* complex, and an unclassified species of Pasteurellaceae.

DISCUSSION

To the best of our knowledge, no studies reported the susceptibility of *S. aureus*, isolated from animals, to LZD and TGC; however, some reports indicated that all human *S. aureus* isolates show susceptibility to these drugs (12, 13), consistent with our isolates from dog samples.

S. pseudintermedius, the most common opportunistic pathogen in dogs (14), exhibits resistance to commonly used antimicrobials (15). It is the most common pathogen causing recurrent skin infections in dogs because of allergies, endocrine diseases, or other immunocompromising factors including old age and cancer (15). The possibility of *S. pseudintermedius* transmission from animals to humans was evaluated in four clinical human cases, among which two dog owners and their

dogs carried identical *S. pseudintermedius* strains (16). Although some strains can acquire methicillin resistance and cause severe refractory infections, even potential PDR strains are susceptible to LZD and TGC.

R. nasimurium and *S. pseudintermedius* can exhibit increased pathogenicity through synergistic effects (17); if these bacteria are potential PDR, then the associated fatality rate can significantly increase.

E. faecalis is the most frequently encountered enterococcal species in the anus and tonsils of dogs, followed by *E. faecium* (18). These two species are considered the third and fourth most prevalent nosocomial human pathogens worldwide, respectively (18), necessitating their control in both humans and animals. Although there is scarce evidence for susceptibility testing of *Enterococcus* spp. in animal samples, the sensitivity of *E. faecalis* and *E. faecium* isolates from human samples to LZD was 94.3 and 93.5%, respectively (12). In another study, the sensitivity of *Enterococcus* spp. isolates to TGC was 100% (201/201) (19), consistent with our results.

Oxazolidinones, including LZD, are excluded from gram-negative bacteria-related infection treatment because they enhance pump activity against LZD and expel the antibiotic from the cytoplasm, resulting in lower LZD accumulation levels in *E. coli*, *C. freundii*, and *E. aerogenes* than in *S. aureus* and *E. faecium* (20).

E. coli is frequently encountered and causes severe infections in both humans and dogs (21). The possible transmission of virulent and/or resistant *E. coli* strains between animals and humans through numerous pathways is highly concerning (21). *E. coli* represents a main reservoir of resistance genes probably responsible for treatment failure in both human and veterinary medicine (22). Indeed, an increasing number of resistance genes have been identified in *E. coli* during the past decades, mostly acquired through horizontal gene transfer (23). In the enterobacterial gene pool, *E. coli* acts as a donor and recipient of resistance genes from other bacteria (24). Hence, the broad-spectrum resistance of this species is quite likely, considering that most recommended antimicrobials do not effectively inhibit its growth. Therefore, TGC may broaden antimicrobial treatment choices for refractory *E. coli* infections.

K. pneumoniae is an important nosocomial agent that spreads easily (25) and causes community-onset infections in companion animals and humans. It is the second most common Enterobacteriaceae species causing urinary tract infections in dogs; strains are frequently MDR, posing important therapeutic limitations (26, 27). In previous studies, 84.6% (55/60) and 87.6% (340/388) of human *K. pneumoniae* isolates showed TGC susceptibility (19, 28). Our results showed a lower susceptibility TGC rate (73.7%), indicating the stronger resistance of isolates of animal origins to TGC. The resistance gene appears to have originated from the chromosome of a *Pseudomonas* species and may have been transferred to plasmids by adjacent site-specific integrases. Although the gene appears to be rare in human clinical isolates, the transferability of the gene cluster and its broad-spectrum substrate make further dissemination of this mobile TGC resistance determinant possible (29). The rapid development of TGC resistance necessitates further expansion of other treatment options.

Human *Proteus* spp. and *P. aeruginosa* isolates exhibit strong resistance to TGC. Nevertheless, we evaluated their susceptibility to TGC because strains of the same species may have different antimicrobial susceptibility profiles depending on their host of origin (30). *P. mirabilis* is the epitome of an opportunistic nosocomial pathogen in humans and animals (31), causing urinary tract infections (32) and chronic otitis externa (33) in companion animals. Moreover, *P. mirabilis* has low susceptibility to TGC (34). Moreover, a novel TGC resistance gene, *tet*, has recently been identified in *Proteus* species isolated from animals (35). Fortunately, isolates from the current study, collected over 3 years, showed sensitivity to TGC, suggesting that TGC-resistant *P. mirabilis* has not yet been disseminated in Korea.

P. aeruginosa is a clinically important opportunistic pathogen causing serious acute and chronic infections (36). It is ubiquitous in the environment and can persist in water and soil despite minimal nutrients, tolerating a broad spectrum of humidity and temperature conditions (37). *P. aeruginosa* is one of the pathogens that most frequently acquire or develop multidrug resistance (37). The exceptional array of intrinsic and acquired drug resistance mechanisms employed by *P. aeruginosa* renders the antibiotic-based treatment of these infections difficult. One important resistance mechanism is mediated by the resistance–nodulation–cell division family of efflux pumps (38). In one study, all (15/15) human *P. aeruginosa* isolates were resistant to TGC (28). Similarly, our results showed that TGC is not suitable for treating *P. aeruginosa* infections.

C. auriscanis was first discovered in a dog with ear infection (39) and typically acts as an opportunist in mixed infections associated with bacterial otitis externa, which can be resolved by treating and controlling other causative agents, but it may have pathological significance when occurring alone (40). *C. auriscanis* is often resistant to beta-lactam antibiotics; therefore, other antimicrobials may be necessary if skin lesions are not resolved after antimicrobial therapy (41).

S. canis is considered part of the healthy microbiota of the skin and mucosa of dogs but may be responsible for opportunistic infections. In dogs, *S. canis* is isolated from skin infections, urogenital and respiratory tract infections, otitis externa, septicemia, necrotizing fasciitis, and streptococcal toxic shock syndrome (42). Only one strain was tested in this study and found to be susceptible to LZD and TGC, indicating they are possible treatment options for *S. canis* infections.

E. aerogenes and *E. cloacae* complexes are members of the intestinal microbiota and are commonly MDR. Fortunately, both isolates were susceptible to TGC in this study, indicating the potential application of TGC for treating urinary tract infections, accounting for 38% (8/21) of animal cases, and wound infections, accounting for 19% (4/21) (43). *C. freundii* is intrinsically resistant to AMP, AMP/sulbactam, and cephalosporins and causes sepsis in dogs (44). A single isolate of this species was tested in this study and was susceptible to TGC; therefore, this antibiotic may be considered for treating *C. freundii* infections.

This study has several limitations. First, for certain bacteria (e.g., *C. auriscanis*, Pasteurellaceae, *P. mirabilis*, *P. aeruginosa*, and *R. nasimurium*), there are no susceptibility or resistance criteria. Second, many countries prohibit LZD and TGC use in animal settings to preserve treatment options for human infections. However, these agents may be employed in the future under well-organized antimicrobial stewardship frameworks and policies, and our results provide foundational knowledge for using LZD and TGC in veterinary medicine.

In conclusion, we evaluated the efficacies of TGC and LZD against potential PDR bacteria that are frequently isolated from companion dogs, and the results showed resistance of the organisms to all other antimicrobial classes recommended by the veterinary CLSI guidelines. Both agents showed favorable efficacy, with susceptibility rates of all potential PDR bacteria, except *P. aeruginosa*, ranging from 72.7 to 100%. Thus, TGC and LZD may serve as promising antimicrobial options for veterinary medicine in the future. For application in patients, *in vivo* pharmacokinetic and pharmacological studies are needed. To avoid exacerbating bacterial antibiotic resistance, legal regulations for TGC and LZD are needed to prevent their misuse.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (KU20218). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

D-HK and J-HK conceptualized and designed the study, analyzed the data, drafted and edited the manuscript, and approved the final submission. All authors contributed to the article 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/fvets.2021.693506/full#supplementary-material

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The A756T Mutation of the *ERG11* Gene Associated With Resistance to Itraconazole in *Candida Krusei* Isolated From Mycotic Mastitis of Cows

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Candida krusei (*C. krusei*) has been recently recognized as an important pathogen involved in mycotic mastitis of cows. The phenotypic and molecular characteristics of 15 *C. krusei* clinical isolates collected from cows with clinical mastitis in three herds of Yinchuan, Ningxia, were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry. In addition to sequencing analysis, the *ERG11* gene that encodes 14α-demethylases, the expression of the *ERG11* gene, and efflux transporters *ABC1* and *ABC2* in itraconazole-susceptible (S), itraconazole-susceptible dose dependent (SDD), and itraconazole-resistant (R) *C. krusei* isolates was also quantified by a quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay. Sequencing analysis revealed three synonymous codon substitutions of the *ERG11* gene including T939C, A756T, and T642C in these *C. krusei* clinical isolates. Among them, T642C and T939C mutations were detected in itraconazole-resistant and -susceptible *C. krusei* isolates, but the A756T substitution was found only in itraconazole-resistant isolates. Importantly, the expression of the *ERG11* gene in itraconazole-resistant isolates was significantly higher compared with itraconazole-SDD and itraconazole-susceptible isolates ($p = 0.052$ and $p = 0.012$, respectively), as determined by the qRT-PCR assay. Interestingly, the expression of the *ABC2* gene was also significantly higher in itraconazole-resistant isolates relative to the itraconazole-SDD and itraconazole-susceptible strains. Notably, the expression of *ERG11* was positively associated with resistance to itraconazole ($p = 0.4177$ in SDD compared with S, $p = 0.0107$ in SDD with R, and $p = 0.0035$ in S with R, respectively). These data demonstrated that mutations of the *ERG11* gene were involved in drug resistance in *C. krusei.* The A756T synonymous codon substitution of the *ERG11* gene was correlated with an increased expression of drug-resistant genes including *ERG11* and *ABC2* in itraconazole-resistant *C. krusei* isolates examined in this study.

Keywords: *Candida krusei*, resistance, mycotic mastitis, cows, *ERG11*, *ABC1*, *ABC2*

INTRODUCTION

Cow mastitis has a major negative impact on dairy industries, causing significant economic losses to farmers. Pathogenically, a wide variety of microorganisms have been identified as causative agents of cow mastitis, mainly bacteria and fungi (1). In cases of fungal infection of the mammary gland, yeasts of *Candida* genus are the most reported fungal pathogens in cow mastitis (2).

Historically, the mycotic mastitis caused by fungi of the *Candida* genus was first described by Fleischer as early as 1930 (3). Although the infection of *C. albicans* was considered as the most common cause of mastitis, cases of cow mastitis caused by the infection of non-albicans *Candida* spp. (NAC), such as *C. krusei*, *C. parapsilosis*, *C. glabrata*, and *C. tropicalis*, have increased significantly during the last decade (4). Among these NACs, *C. krusei* ranked as the fifth most common cause of cow mycotic mastitis (5, 6). *C. krusei* was reported as the causative agent of bovine mastitis and bronchopneumonia in Canada, Mexico, Japan, the United Kingdom, Turkey, Poland, and Algeria (7, 8). Our previous investigation also suggested that *C. krusei* was one of the most important pathogens in mycotic mastitis in dairy farms of the Yinchuan region in Ningxia, China. This result was in accordance with the report of Erbaş et al. (9).

Candida krusei has been regarded as a multidrug-resistant fungal pathogen because of its intrinsic resistance to fluconazole (FLC) (10, 11), with more than 96% of human clinical and veterinary isolates being fluconazole-resistant (12). Azole is one of the most common antifungal drugs in agricultural practices (13), including itraconazole, ketoconazole, and tetraconazole. Although it has been reported that multiple mechanisms are involved in drug resistance in *Candida* spp., mechanisms involved in alterations of target enzymes and upregulation of multidrug resistance (MDR)-related proteins are the common mechanisms of *Candida* resistant to azoles. In this regard, 14α-lanosterol demethylase (14-DM) is the target enzyme of azoles, which is responsible for the production of an ergosterol precursor and is encoded by the *ERG11* gene. In *C. albicans* and *C. parapsilosis*, the efflux pump genes *CDR1*, *CDR2*, and *MDR1* are also associated with azole resistance (14). Nowadays, although transporter genes *ABC1* and *ABC2* were involved in drug resistance in *C. krusei* (15, 16), increased lines of evidence suggested that changes in the expression of activity of target enzyme and upregulation of *MDR* were the main mechanisms of drug resistance in *C. krusei* (11, 16). To date, the study on mechanisms of *Candida* in azole resistance has mainly focused on *C. albicans, C. glabrata*, and *C. tropicalis*, but studies on azole resistance mechanisms in *C. krusei*, especially the *C. krusei* isolates from cow mastitis resistant to itraconazole, are limited, and the involvement of *ERG11*, *ABC1*, and *ABC2* genes in the drug resistance of *C. krusei* has not been determined (17).

In the present study, we evaluated the profile of the susceptibility of *C. krusei* to itraconazole and investigated the potential alterations of the *ERG11* gene and the differential expression of *ERG11, ABC1*, and *ABC2* genes of 15 clinical *C. krusei* isolates that were isolated from cow mastitis in Yinchuan, Ningxia, China.

MATERIALS AND METHODS

Isolation and Identification of Fungal Pathogens

This study was submitted to and approved by the Ethic Committee of Animal Study in Ningxia University. A total of 465 quarter-milk samples were collected from the cows with clinical or subclinical mastitis, which originated from three herds in Yinchuan, Ningxia, China. Clinical or subclinical mastitis was defined by swelling, reduced milk flow, and abnormal milk appearance (watery to viscous with clots varying from gray-white to yellowish). Additionally, other signs of infection such as fever, inappetence, ataxia, and depression were also considered. These cows have been treated with antibiotics before sample collection. The isolates of *C. krusei* were identified by using *Candida* chromogenic medium (CHROMagar, France) (18) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) with score values of >2.000 (VITEK® MS, BioMerieux, France) (19) and stored in liquid nitrogen.

Antimicrobial Susceptibility Tests

The susceptibility assay was conducted using the Clinical and Laboratory Standard Institute (CLSI) broth microdilution (BMD) method. The CLSI BMD method was performed in a 96-well polystyrene microtiter plate in accordance with CLSI M27-A3 and M27-S4 guidelines (20, 21). The ranges of concentrations of tested drugs were as follows: 5-flucytosine (0.03–64 μg/ml), amphotericin B (0.008–16 μg/ml), fluconazole (0.03–64 μg/ml), itraconazole (0.03–64 μg/ml), and ketoconazole (0.03–64 μg/ml) (15, 22). The antifungal drugs were all purchased from Meilun Biotechnologies (Dalian, China). The *C. krusei* NCCLS reference strain ATCC 6258 served as quality control to ensure the test (3, 23); 1×10^3 CFU/ml working suspension of the *C. krusei* isolates was added into the each well. Results were recorded as resistant, susceptible dose dependent, and sensitive as shown in **Table 1**.

PCR Amplification and Sequencing Alignment of the *ERG11* Gene

Candida krusei isolates were subcultured twice on Sabouraud agar at 37°C for 18–24 h to revive and ensure the purity of cultures. A single colony was then transferred to 20 ml of liquid YPD (yeast extract 1%, dextrose 2%, and peptone 2%) broth and cultured at 35°C in a shaking incubator (120 rpm) exponential growth phase. The bacteria cells were collected by centrifugation at 3,000 rpm for 20 min, and the bacteria pellet was used for total genomic DNA preparation using a DNI5-A new Plant Genomic DNA Rapid Extraction Kit (Aidlab Biotechnologies, Beijing, China) according to the manufacturer's instruction. The isolated DNA was used as a template for amplification of the *ERG11* gene. The primer set of *ERG11* was designed by Primer 5.0 and synthesized at Sangon Biotech (Shanghai, China), based on the available sequence information of the *C. krusei ERG11* gene (Gene accession number DQ903905) at the National Center for Biotechnology Information (NCBI) (**Table 2**). The PCR amplification was conducted in 25 μl volume containing

TABLE 1 | Minimal inhibitory concentration (MIC) and susceptibility profile of *C. krusei* clinical isolates ($n = 15$).

Name	Susceptibility profile				
	ITR	AMB	FLC	5-FC	KET
ATCC 6258	0.06/S	0.5/S	8/S	4/S	0.125/S
CK1	4/R	2/SDD	64/R	8/SDD	1/R
CK2	8/R	4/R	64/R	32/R	1/R
CK3	0.25/SDD	2/SDD	16/SDD	32/R	1/R
CK4	0.25/SDD	2/SDD	64/R	8/SDD	0.25/SDD
CK5	8/R	4/R	64/R	32/R	1/R
CK6	0.5/SDD	2/SDD	64/R	32/R	1/R
CK7	0.06/S	2/SDD	16/SDD	8/SDD	0.25/SDD
CK8	0.25/SDD	2/SDD	64/R	32/R	1/R
CK9	4/R	2/SDD	64/R	32/R	1/R
CK10	0.25/SDD	2/SDD	32/SDD	32/R	1/R
CK11	0.25/SDD	2/SDD	64/R	16/SDD	0.5/SDD
CK12	4/R	4/R	64/R	32/R	1/R
CK13	0.5/SDD	2/SDD	64/R	32/R	1/R
CK14	0.06/S	2/SDD	16/SDD	32/R	0.5/SDD
CK15	0.25/SDD	2/SDD	64/R	16/SDD	1/R

CK, Candida krusei; S, susceptible; SDD, susceptible dose dependent; R, resistant; 5-FC, 5-flucytosine, MIC break point: S, ≤4 μg/ml; SDD, 8–16 μg/ml; R, ≥32 μg/ml; AMB, amphotericin B, MIC break point: S, ≤1 μg/ml; SDD, 2 μg/ml; R, >2 μg/ml; FLC, fluconazole, MIC break point: S, ≤8 μg/ml; SDD, 16–32 μg/ml; R, ≥64 μg/ml; ITR, itraconazole, MIC break point: S, ≤0.125 μg/ml; SDD, 0.25–0.5 μg/ml; R, ≥1 μg/ml; KET, ketoconazole, MIC break point: S, ≤0.125 μg/ml; SDD, 0.25–0.5 μg/ml; R, ≥1 μg/ml.

1 μl of genomic DNA (200 ng/μl), 0.5 μl of specific forward and reverse primers (50 μmol/L), and 12.5 μl of 2× Phanta Max Master Mix (Vazyme Biotech, Nanjing, China). The PCR parameters were set as denaturation for 3 min at 95°C, followed by 35 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 1 min, and a final step of elongation (72°C for 5 min). The resultant PCR product was cleaned by gel purification in 1.5% agarose prior to being cloned into the pTOPO-TA Vector using a CV16-Zero Background pTOPO-Blunt Cloning Kit with Blue/white selection (Aidlab Biotechnologies, Beijing, China). The white colonies were analyzed for clones containing the DNA fragment of gene of interest. Eight to fifteen plasmids from clones generated from an identical PCR product were further sequenced for the *ERG11* gene (Sangon Biotech, Shanghai, China). The sequences were then aligned with the online published sequence of the *ERG11* gene of *C. krusei* strain (Gene Accession Number DQ903905) to determine gene mutation (17).

Quantitative Real-Time PCR Analysis

For quantitative real-time PCR (qRT-PCR) analysis, total RNA was extracted from *C. krusei* cultures with RNAiso Reagent (TaKaRa, Dalian, China) and reverse transcribed to cDNA with HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech, Nanjing, China) according to the manufacturer's instruction. For the *ERG11* target gene and GAPDH reference gene, primer pairs were designed with the Primer 5.0 program and synthesized by Sangon, Shanghai, China (**Table 2**). qRT-PCR was conducted with a 20-μl volume containing the following reagents: 10 μl of 2× ChamQ Universal SYBR qPCR Master Mix (Vazyme), 1 μl of total RNA sample, 0.5 μl of each primer pair at a concentration of 10 μmol/L, and 8 μl of distilled water. Each reaction was run in triplicate. Samples were subjected to an initial step at 95°C for 3 min, followed by 40 cycles, each of which consisted of 10 s at 95°C and 30 s at 60°C. Melting curves were recorded every 5 s. The fluorescence data were collected and analyzed with the QuantStudio Design Analysis Software 1.3.1. A $2^{-\Delta\Delta Ct}$ algorithm was employed to analyze the relative expression levels of drug-resistant genes at resistant, susceptible dose-dependent, and sensitive strains.

Statistical Analysis

Statistical analysis was performed with the GraphPad Prism program (GraphPad 8.0.1 Software Inc., San Diego, CA). The two-tailed Student's *t*-test was used to analyze significant differences between gene expression displayed by the distinct *C. krusei* strains; $p < 0.05$ was considered statistically significant.

RESULTS

Resistance of *C. krusei* Isolates to Antifungal Agents

A total of 15 *C. krusei* isolates (designated as CK1–CK15) were isolated from clinical samples from April 2018 to October 2019 in three herds at Yinchuan, Ningxia, China. Drug sensitivity testing was performed according to the broth microdilution method M27-A2 (NCCLS 2002), and the result showed that among 15 *C. krusei* isolates, 73.4, 73.4, and 66.7% were resistant to fluconazole (FLC), ketoconazole (KET), and 5-flucytosine (5-FC), respectively. However, 20 and 33.3% of the isolates were susceptible to amphotericin B (AMB) and itraconazole (ITR), respectively. Interestingly, isolates CK2, CK5, CK6, CK8, CK9, CK12, and CK13 showed resistance to both 5-FC and FLC. The

TABLE 2 | Primers used in this study.

Gene	Primer sequence[a] (5′-3′)[a]	Annealing temperature	Accession number	PCR product size (bp)
Sequencing primers				
ERG11	F: ATGTCCGTCATCAAGGCAAT	60°C	DQ903905	1,587
	R: CTAGTTCTTTTGTCTTCCCTCCC			
Real-time PCR primers				
ABC1	F: GATAACCATTTCCCACATTTGAGT	60°C	DQ903907.1	139
	R: CATATGTTGCCATGTACACTTCTG			
ABC2	F: CCTTTTGTTCAGTGCCAGATTG	60°C	AF250037.1	133
	R: GTAACCAGGGACACCAGCAA			
ERG11	F: AGCAACAACAATGTCCGTCA	60°C	DQ903905	108
	R: TTTGTCTTCCCTCCCACTTG			
GAPDH	F: GTGCCAAAAAAGTTATCATC	60°C	CP039612.1	112
	R: AGTTCTACCACCTCTCCAGT			

[a]*F, forward; R, reverse.*

TABLE 3 | Results of antimicrobial susceptibility tests of *C. krusei* isolates ($n = 15$).

Antibiotic	Resistant, % (no.)	Susceptible dose dependent, SDD, % (no.)	Susceptible, % (no.)
ITR	33.3 ($n = 5$)	53.3 ($n = 8$)	13.3 ($n = 2$)
AMB	20 ($n = 3$)	80 ($n = 12$)	0
FLC	73.4 ($n = 13$)	26.6 ($n = 4$)	0
5-FC	66.7 ($n = 10$)	33.3 ($n = 5$)	0
KET	73.4 ($n = 13$)	26.6 ($n = 4$)	0

rates of resistance to azole of these *C. krusei* isolates are listed in **Table 3**. Moreover, isolates CK2, CK5, and CK12 showed multidrug resistance to AMB, 5-FC, FLC, KET, and ITR. In contrast, the reference strain ATCC 6258 was susceptible to all of the five antifungal agents. Overall, among these 15 isolates, 5 were isolates resistant to ITR, 8 belonged to susceptible dose-dependent isolates, and 2 were isolates susceptible to ITR (**Tables 1**, **3**).

Mutational Analysis in *ERG11* of *C. krusei* Isolates

The *ERG11* gene fragment was amplified from ATCC 6258 and all 15 *C. krusei* isolates. The PCR product of the open frame of the *ERG11* gene was 1,587 bp, which encodes 529 amino acids. Sequencing analysis identified four different mutations, three synonymous mutations (C642T, A756T, and T939C), and one missense mutation (C44T) in these 15 *C. krusei* isolates (**Table 4**). Synonymous mutations C642T and T939C were presented in all sequenced isolates, but the synonymous mutation A756T was found in the *ERG11* gene of *C. krusei* isolates resistant to itraconazole (**Table 4**). One missense mutation was also found at 44 bp (C→ T) of the *ERG11* gene (**Table 4**), which resulted in an amino acid alteration from alanine to valine. However, such a missense mutation was also found in the reference strain ATCC 6258 and all *C. krusei* strains, which indicated that the C44T missense mutation might not be associated with drug resistance to azoles in *C. krusei*.

Increased *ERG11* Gene Transcript in Itraconazole-Resistant *C. krusei*

In order to examine whether an alteration of *ERG11* gene expression was correlated with the drug resistance of *C. krusei* clinical isolates, the transcript of the *ERG11* gene was accessed by a qRT-PCR assay. In comparison with the reference strain ATCC 6258, the relative *ERG11* gene expression of field isolates in five itraconazole-resistant isolates was significantly upregulated ($p < 0.01$), while only two itraconazole-susceptible dose-dependent isolates showed a significantly upregulated *ERG11* gene expression ($p < 0.01$, **Figure 1A**). The result showed that the transcript of the *ERG11* gene in itraconazole-resistant isolates was significantly more abundant than itraconazole-susceptible strains ($p = 0.0012$, **Figure 1B**) and itraconazole-susceptible dose-dependent (SDD) strains ($p = 0.0052$, **Figure 1B**). However, there was no significant difference between itraconazole-susceptible dose-dependent isolates and itraconazole-susceptible isolates ($p = 0.2562$, **Figure 1B**).

Increased *ABC2* Gene Transcript in Itraconazole-Resistant *C. krusei*

ABC transporters are involved in drug resistance; next, we therefore sought to examine the alteration of ABC transporters in *C. krusei* isolates. Interestingly, unlike the *ERG11* gene, none of field isolates showed an upregulated *ABC1* gene expression as compared with that of the reference strain

TABLE 4 | *ERG11* gene point mutations in *C. krusei* clinical isolates.

Information of strains		ERG11 gene mutation sites			
Name	**ITR susceptibility category**	**44**	**642**	**756**	**939**
DQ903905		T	T	A	T
ATCC 6258	S	C	C	—	—
CK1	R	C	—	T	C
CK2	R	—	—	T	C
CK3	SDD	C	—	—	—
CK4	SDD	—	—	—	C
CK5	R	—	C	T	C
CK6	SDD	C	—	—	—
CK7	S	C	—	—	—
CK8	SDD	—	—	—	C
CK9	R	—	—	T	—
CK10	SDD	C	C	—	—
CK11	SDD	—	—	—	—
CK12	R	—	—	T	C
CK13	SDD	C	—	—	—
CK14	S	—	C	—	C
CK15	SDD	C	—	—	—

The numbering of the nucleotides shown in this table starts with 1 for the A of the ATG start codon. DQ903905, GenBank Accession no. of C. krusei whose ERG11 sequence published online is used to align with C. krusei clinical isolates in this study.

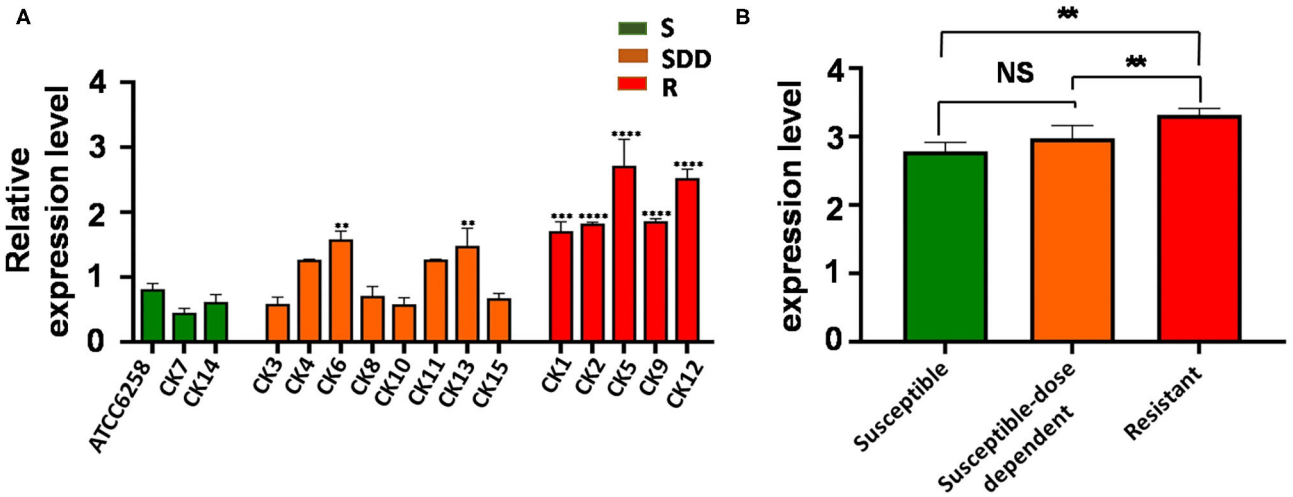

FIGURE 1 | *ERG11* relative gene expression levels in three groups of *C. krusei* clinical isolates. **(A)** Relative levels of *ERG11* mRNA in all the *C. krusei* clinical isolates. *ERG11* gene expression levels were quantified and normalized relative to the reference gene, GAPDH; S, itraconazole-susceptible; SDD, itraconazole-susceptible dose dependent; R, itraconazole-resistant. Asterisks indicate that the difference between field isolates with the reference strain ATCC 6258 is significant compared to the reference strain ATCC 6258: $^{**}p < 0.01$ in CK6, CK13 with ATCC6258; $^{***}p < 0.001$ in CK1 with ATCC6258; and $^{****}p < 0.0001$ in CK2, CK5, CK9, CK12 with ATCC6258. **(B)** Log10+3 fold increase of gene expression levels in three groups (NS, no significance in SDD compared with S; $^{*}p < 0.05$ in R with SDD; $^{**}p < 0.01$ in R with S).

ATCC 6258 (**Figure 2A**). The results of qRT-PCR for *ABC1* genes showed that the expression of *ABC1* gene mRNA was not significantly different between itraconazole-resistant isolates, itraconazole-susceptible dose-dependent isolates, and itraconazole-susceptible strains ($p = 0.3844$, $p = 0.9997$, and $p = 0.2996$, respectively, **Figure 2B**). Similar to that seen in the *ERG11* gene, the relative *ABC2* gene expression was extremely significantly upregulated in four itraconazole-resistant isolates ($p < 0.01$) and significant in one itraconazole-resistant isolate ($p < 0.05$), as compared with the reference strain ATCC 6258, whereas only two itraconazole-susceptible dose-dependent isolates showed an extremely significant upregulation of *ABC2* gene expression compared to the reference strain ATCC 6258 ($p < 0.01$, **Figure 3A**). Intriguingly, the transcript

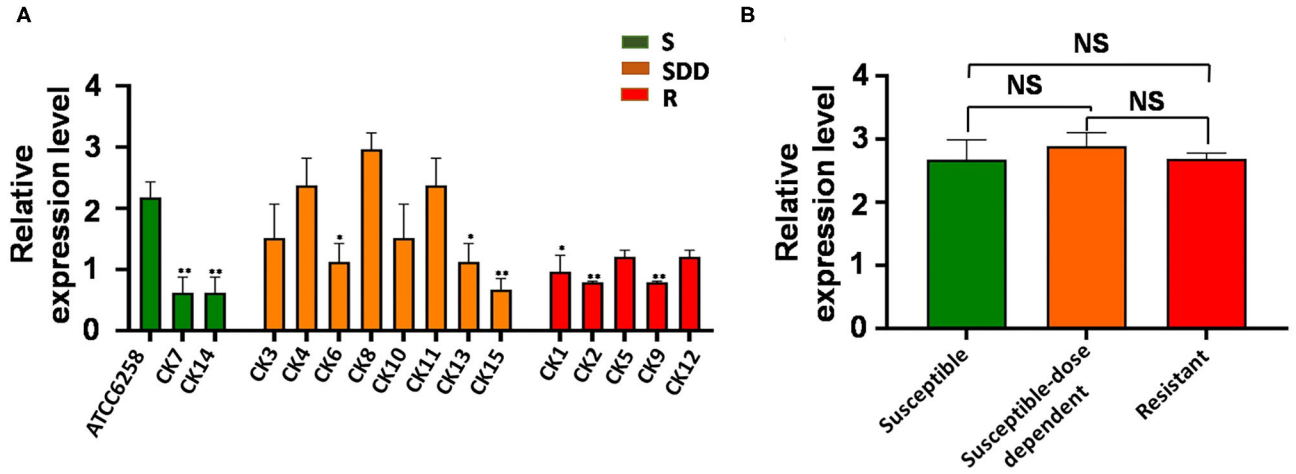

FIGURE 2 | *ABC1* relative gene expression levels in three groups of *C. krusei* clinical isolates. **(A)** Relative levels of *ABC1* mRNA in all the *C. krusei* clinical isolates. *ABC1* gene expression levels were quantified and normalized relative to the reference gene, GAPDH; S, itraconazole-susceptible; SDD, itraconazole-susceptible dose dependent; R, itraconazole-resistant. Asterisks indicate that the difference between field isolates with the reference strain ATCC 6258 is significant compared to the reference strain ATCC 6258: $*p < 0.05$ in CK1, CK6, CK13 with ATCC6258 and $**p < 0.01$ in CK2, CK7, CK9, CK14, CK15 with ATCC6258. **(B)** Log10+3 fold increase of gene expression levels in three groups (NS, no significance in SDD compared with S; $*p < 0.05$ in R with SDD; $**p < 0.01$ in R with S).

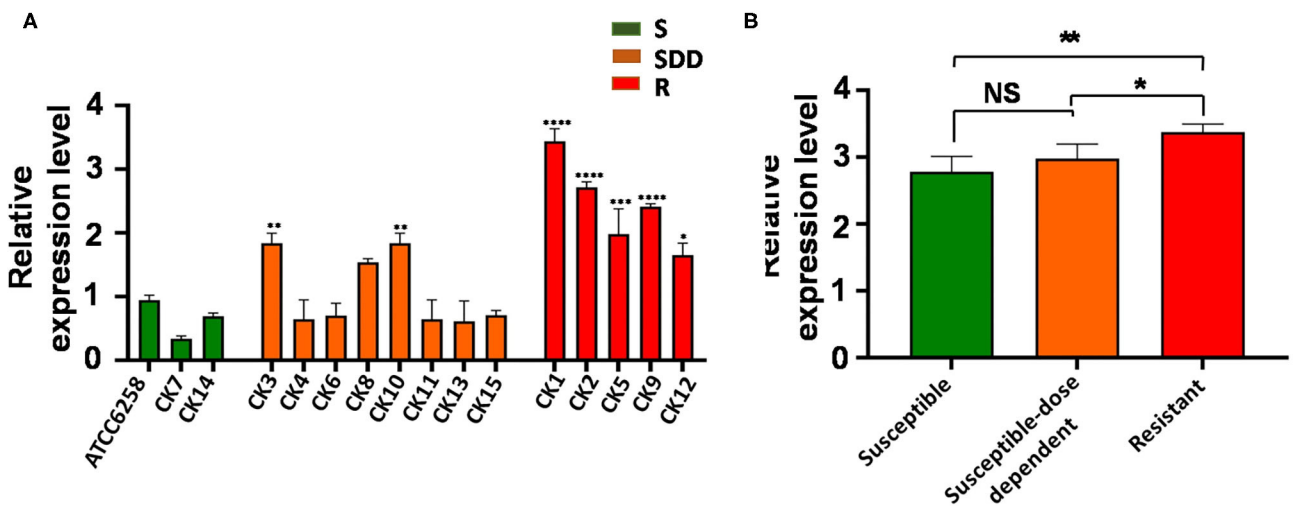

FIGURE 3 | *ABC2* relative gene expression levels in three groups of *C. krusei* clinical isolates. **(A)** Relative levels of *ABC2* mRNA in all the *C. krusei* clinical isolates. *ABC2* gene expression levels were quantified and normalized relative to the reference gene, GAPDH; S, itraconazole-susceptible; SDD, itraconazole-susceptible dose dependent; R, itraconazole-resistant. Asterisks indicate that the difference between field isolates with the reference strain ATCC 6258 is significant compared to the reference strain ATCC 6258: $*p < 0.05$ in CK12 with ATCC6258; $**p < 0.01$ in CK3, CK10 with ATCC6258; $***p < 0.001$ in CK5 with ATCC6258; and $****p < 0.0001$ in CK1, CK2, CK9 with ATCC6258. **(B)** Log10+3 fold increase of gene expression levels in three groups (NS, no significance in SDD compared with S; $*p < 0.05$ in R with SDD; $**p < 0.01$ in R with S).

of the *ABC2* gene in itraconazole-resistant isolates was more abundant relative to itraconazole-susceptible strains ($p = 0.0035$) and itraconazole-susceptible dose-dependent strains ($p = 0.0107$, **Figure 3B**). No significant difference was found in the expression of the *ABC2* gene was determined between the itraconazole-susceptible dose-dependent group and itraconazole-susceptible *C. krusei* isolates ($p = 0.4177$, **Figure 3B**).

DISCUSSION

Our previous study demonstrated that *C. krusei* was a predominant pathogen isolated from mycotic mastitis of cows in Yinchuan, Ningxia, China (12), suggesting that it may be an important fungal pathogen of mycotic mastitis of cows in this area. The crucial roles of drug-resistant genes *ERG11*, *ABC1*, and *ABC2* in FLC-resistant clinical isolates of *C. krusei*

from human have been well-established (15, 24). However, the pathogenic molecular mechanism of *C. krusei* isolated from cow mastitis remains unclear. In the present report, we evaluated the susceptibility profiles and mutations in the *ERG11* gene in 15 clinical *C. krusei* isolates. The expression of drug-resistant genes *ERG11, ABC1*, and *ABC2* between isolates susceptible, susceptible dose-dependent, and resistant to ITR was also analyzed. We identified three synonymous mutations and one missense mutation in the *ERG11* gene in these clinical *C. krusei* isolates, as previously described in human *C. krusei*. Furthermore, the A756T was only presented in ITR-resistant strains, suggesting that it might be correlated with drug resistance in *C. krusei*, while mutations T642C and T939C were presented in all these 15 *C. krusei* isolates. The expression of drug-resistant *ERG11* and *ABC2* was also significantly higher in ITR-resistant *C. krusei* isolates compared to ITR-susceptible and susceptible dose-dependent isolates, suggesting a correlation of mutations of the *ERG11* gene with the resistance to antifungal agents in *C. krusei.*

In the present study, based on the CLSI BMD method's susceptibility to ITR, the 15 *C. krusei* clinical isolates from cows with clinical mastitis could be divided into three groups: ITR-susceptible (2 isolates), ITR-susceptible dose-dependent (8 isolates), and ITR-resistant (5 isolates). The antifungal testing showed that 13.3, 53.3, and 33.3% were susceptible, susceptible dose-dependent, and resistant to itraconazole among these *C. krusei* clinical isolates, respectively. Notably, 13 of the 15 *C. krusei* isolates (73.4%) were also resistant to FLC and KET, 10 of the 15 *C. krusei* isolates (66.7%) were resistant to flucytosine, and 3 of the 15 isolates (20%) were resistant to amphotericin B. This finding was consistent with a study by Namvar et al. (25), but was different from reports by others (8, 9, 26). Of interest, the rate of resistance to antifungal agents in these *C. krusei* isolates was lower than our previous findings (12), which might be attributed to the reduction in the use of antifungal drugs during breeding. Consistent with our previous studies, *C. krusei* isolates were double-resistant and multidrug-resistant to antifungal drugs. It is strongly recommended that ketoconazole and other azole antifungal agents should not be used in the treatment of *C. krusei* infection in dairy cows in Ningxia, China, owing to high drug resistance.

It is worth noting that CK2, CK5, and CK12 showed resistance to amphotericin B, but such cases are rare (27). However, several lines of studies evidenced an increased minimum inhibitory concentration (MIC) of amphotericin B in *C. krusei* isolates. In *Candida* spp., resistance to amphotericin B was found to be associated with a decreased ergosterol content of cell membrane (28–30). In addition, an inactivation of *ERG3* could substitute 14α-methylfecosterol with ergosterol, thus reducing ergosterol levels, which, in turn, resulted in deficient ergosterol to counter the function of amphotericin B (31). Moreover, several other mutations in the ergosterol biosynthetic genes such as *ERG2*, *ERG5*, *ERG6*, and *ERG24* can also result in *C. albicans* and *C. glabrata* resistant to amphotericin B (32–34). However, such mutations have not been reported in *C. krusei* and further studies are needed in future studies.

A number of studies have focused on *ERG11* gene mutation in *Candida* species (35–37). In order to fully understand *ERG11* gene mutation of *C. krusei*, the whole open reading frame of the *ERG11* gene was amplified for sequencing analysis, and three synonymous mutations and one missense mutation were identified in this study. C44T has one missense mutation and was found in all 15 isolates and the reference strain, suggesting that it had no impact on the itraconazole resistance of *C. krusei*. Molecularly, the C44T mutation resulted in the alteration of alanine to valine in the 15th amino acid of 14α-lanosterol demethylase (14-DM); this mutation might occur outside the active site of the *ERG11* gene, which might not affect the mutual interaction of azole and 14-DM, or a single missense mutation might not be sufficient to change the affinity of the 14-DM to azole (15). Moreover, synonymous mutations (C642T, A756T, and T939C) in the 15 isolates were consistent with a previous report (24). Synonymous mutations can affect transcription, splicing, mRNA transport, and translation, any of which could change phenotype, rendering the synonymous mutation non-silent (38). He et al. (15) also reported that C642T, T1389C, and G1536C mutations occurred in all the experimental strains. In addition, the T1389C mutation was also reported by Ricardo et al. (16). Tavakoli et al. (35) revealed a heterozygous polymorphism at position T939C in the *ERG11* coding region and speculated that this polymorphism might play a key role in the transcriptional regulation of genes and be involved in the processes of ergosterol biosynthesis. Of note, several previously reported mutations, including the T418C missense mutation (16), and C51T, T1389C, and G1536C synonymous mutations (15), have not been found in this study; thus, limited clinical isolates (15) were analyzed. Mechanistically, previous studies on *C. albicans* and *C. tropicalis* have demonstrated that the missense mutation was associated with resistance to azole, which was partly through changing the conformation of the target enzyme 14-DM, which, in turn, decreased its drug affinity and influenced the enzyme's function in ergosterol biosynthesis (39, 40). The resistance mechanism of these resistant strains may be due to one or multiple mutations in these genes, which needs further investigation.

In addition, the expression of *ABC2* and *ERG11* genes was significantly upregulated in *C. krusei* veterinary clinical isolates resistant to itraconazole. Previous studies demonstrated that resistance to azole was also due to the increased expression of *ERG11*. This results in insufficient azole activity owing to the overproduction of the target enzyme (41). Although *ERG11* overexpression has been reported in *C. krusei*, the mechanism behind the overexpression remains unclear (15, 35). Another mechanism of resistance to azole is via the decreased intracellular accumulation of azole. This can be due to efflux pump activity or changes in the cell membrane. In this regard, drug efflux pumps belong to either the ATP-binding cassette (ABC) family of transporters or the major facilitator super family (MFS) class. These proteins can pump out fungicidal compounds across the cell membrane, and their overexpression results in multidrug resistance phenotype in pathogenic fungus (17). In contrast to members of the MFS class that are actuated by electrochemical proton-motive force, ABC family members depend on the hydrolysis of ATP for energy (42, 43). Indeed, along with the decrease of susceptibility to itraconazole, the *ABC2* gene mRNA

expression in the isolates appeared to increase. Although it was considered that Abc1p played an important role in the innate FLC resistance of *C. krusei* (16, 17), the *ABC2* gene could be activated slower than the *ABC1* gene in the presence of voriconazole (16). Given voriconazole tolerance, the Abc1p efflux pump is supposed to be more efficient in expelling drug and plays a late role in the development of resistance, and the accumulation of itraconazole in drug-susceptible *C. krusei* was higher than that of resistant strains (44). Interestingly, Venkateswarlu et al. found that two isolates highly resistant to fluconazole showed a different sensitivity to itraconazole, suggesting that itraconazole and fluconazole in *C. krusei* may have different resistance mechanisms (44). The authors stated that *C. krusei* resistant to itraconazole was due to decreased drug accumulation in cells and speculated that there might exist more efflux pumps contributing to itraconazole resistance of *C. krusei*, which could be well-explained in this study. The overexpression of both *ERG11* and *ABC2* has been reported to be involved in itraconazole resistance in *C. krusei* (15, 35). However, an unusual transient or stable resistance of *C. krusei* to voriconazole has also emerged. Overexpression of *ABC2* and *ERG11*, as observed in itraconazole resistance, imparts a transient resistance to voriconazole, while a more stable resistance was observed due to the overexpression of *ABC1* and point mutation in *ERG11* (16). Taking this into account, we speculate that the resistance mechanisms of itraconazole and voriconazole in *C. krusei* clinical isolates may be different. Moreover, other genes encoding ATP-dependent efflux transporters may occur in *C. krusei*, such as a CgSNQ2 homologous gene that was verified as an azole-associated resistance gene in *C. glabrata* (45). Although the *C. krusei* genome has been sequenced, it is not completely annotated yet; thus, other transporter genes were not assessed. Our mRNA expression data showed that Abc2p may play a more important role in itraconazole resistance of *C. krusei*, instead of Abc1p.

This study has enriched our knowledge in the veterinary clinical *C. krusei* resistance gene expression and mutation data by comparing the difference between the veterinary clinical and the human clinical *C. krusei*, and further deepened our understanding of the resistance mechanism of *C. krusei* in veterinary clinics. The limitation of this study is that the sample size was small and no drug susceptibility test and resistance mechanism research related to echinocandin has been included, which require further investigations.

In conclusion, in this study, we found that *C. krusei* veterinary clinical isolates exhibited a different susceptibility to antifungal agents. Mechanistically, the A756T mutation in the *ERG11* gene resulted in an upregulation of drug-resistant genes *ERG11* and *ABC2*, substantially enhancing the resistance to itraconazole of *C. krusei*. Although we have identified four point mutations in the *ERG11* gene associated with itraconazole resistance and have already described their role on the itraconazole resistance of *C. krusei*, it is necessary to confirm the effect of these mutations by site-directed mutagenesis of the *C. krusei* strain in the future. Nevertheless, this study may thus provide an insight into the mechanism of the resistance of *C. krusei* to antifungal agents, which warrants for further investigation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Animal Research of Ningxia University.

AUTHOR CONTRIBUTIONS

JD, YW, and XZ conceived and designed the experiments. JD and JF analyzed the data and drafted the manuscript. JD, WM, JF, and XL performed experiments and acquired data. XL and XZ interpreted data and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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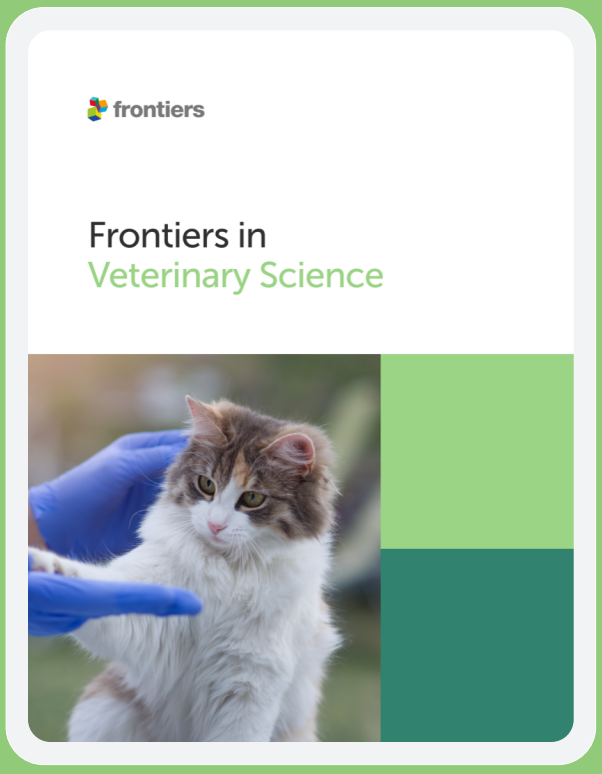

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