ANTIMICROBIAL RESISTANCE IN ZOONOTIC BACTERIA IN DEVELOPING COUNTRIES: THE ROLE OF FOOD ANIMAL PRODUCTION IN PUBLIC HEALTH

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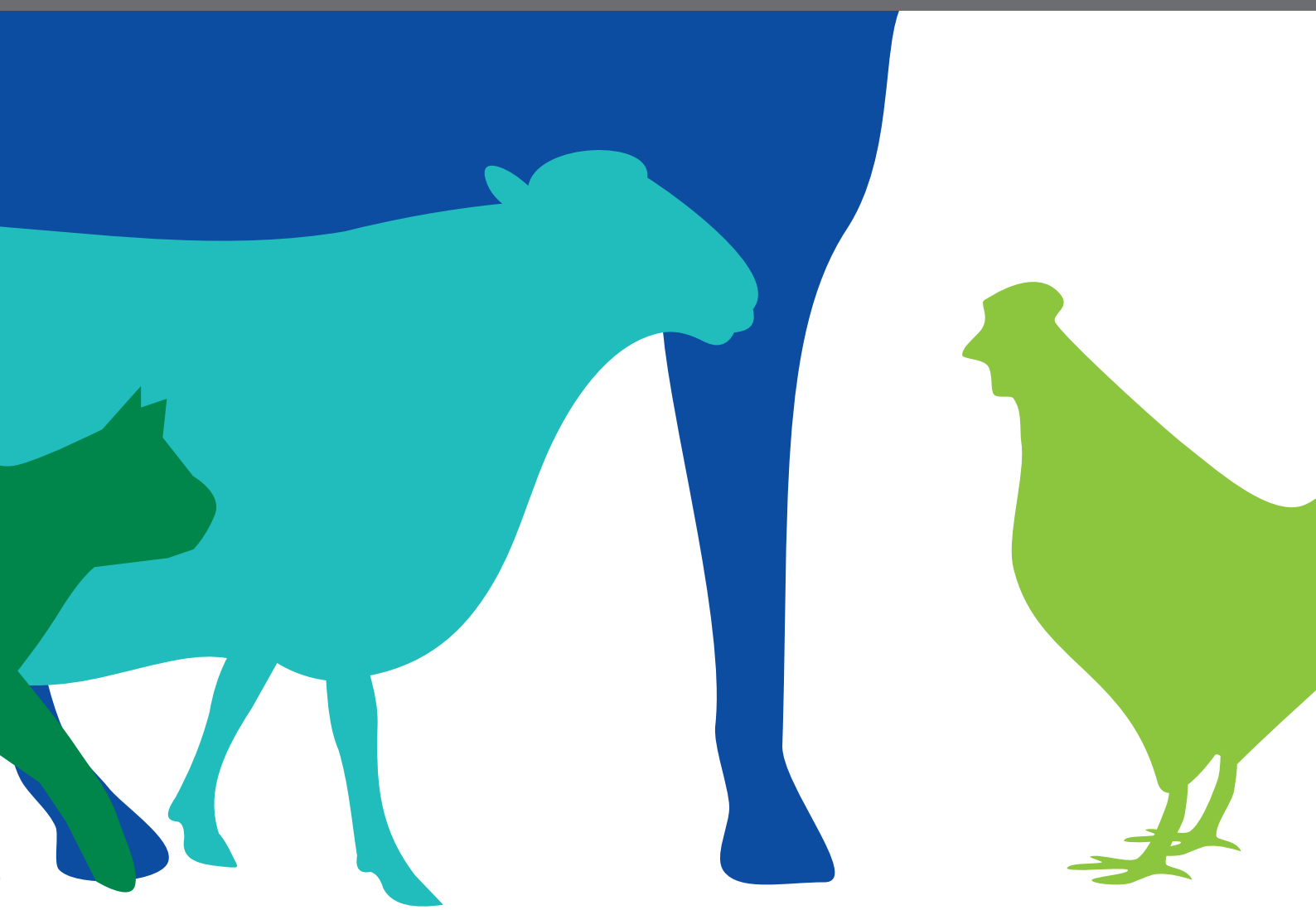
PUBLISHED IN: Frontiers in Veterinary Science



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ISSN 1664-8714
ISBN 978-2-88971-123-9
DOI 10.3389/978-2-88971-123-9

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ANTIMICROBIAL RESISTANCE IN ZOONOTIC BACTERIA IN DEVELOPING COUNTRIES: THE ROLE OF FOOD ANIMAL PRODUCTION IN PUBLIC HEALTH

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Citation: Arriagada, G., Leon-Felix, J., Vignoli, R., Gutkind, G., eds. (2021). Antimicrobial Resistance in Zoonotic Bacteria in Developing Countries: The Role of Food Animal Production in Public Health. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88971-123-9

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frontiers in Veterinary Science

EDITORIAL
published: 02 June 2021
doi: 10.3389/fvets.2021.685281

Editorial: Antimicrobial Resistance in Zoonotic Bacteria in Developing Countries: The Role of Food Animal Production in Public Health

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Keywords: antimicrobial resistance, zoonotic bacteria, food animal, public health, developing countries

Editorial on the Research Topic

Antimicrobial Resistance in Zoonotic Bacteria in Developing Countries: The Role of Food Animal Production in Public Health

The control of zoonotic bacteria and combatting antimicrobial resistance are two approaches particularly relevant of the "One Health" concept (https://www.who.int/news-room/q-a-detail/one-health). Zoonotic bacteria such as *Salmonella*, *Escherichia coli*, *Campylobacter*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Brucella* are top priority for antimicrobial resistance (1).

The first group of papers in this Research Topic explores the prevalence and incidence of resistant bacteria in Latin America and the Caribbean (LAC).

Mota et al. evaluated the frequency of phenotypic antimicrobial resistance and the presence of related genes in Shiga-toxin producing *E. coli* (STEC) and *L. monocytogenes* isolated from human, food and animal sources in Uruguay. Their results indicated that 8.8% of STEC and 6% of *L. monocytogenes* were phenotypically resistant to at least one of the tested antibiotics. All phenotypically resistant *L. monocytogenes*, harbored *fosX*, *lin*, *norB*, *lde*, *mdrL*, and *fepA* resistance genes. The high load of resistance genes found, even in the susceptible isolates, indicates these two pathogens contribute significantly to the burden of antimicrobial resistance in Uruguay.

Ortega-Paredes et al. evaluated the antimicrobial resistance and prevalence of *ESBL/AmpC* and *mcr* genes from animal, food, and human components in third generation cephalosporin-resistant *E. coli* by PCR and Sanger sequencing. The high 3GC-R *E. coli* prevalence points out the risk of transmission to humans via the food chain. Implication of poultry products on the prevalence of the genes studied in 3GC-R *E. coli* should be investigated in antimicrobial resistance surveillance.

On the other hand, Mejía et al. showed, through a genomic epidemiology study that *Salmonella* Infantis ST32 is a relevant problem, with potential dissemination from poultry production farms and the food chain to the general public. They suggested Ecuadorian isolates were linked to a common ancestor, in which the participation of two pF219-like plasmids was found as responsible for distinguishing highly virulent strains.

Meanwhile, Coppola et al. conducted an investigation to detect *E. coli* isolates displaying resistance to oxyimino-cephalosporins, quinolones, and colistin in feces from livestock in Uruguay. Their results showed that the most frequently detected resistance gene recovered from animal isolates was *qnrB19*. Regarding plasmid mediated quinolone resistance genes, *qnrS1* was the

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Edited and reviewed by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *24 March 2021*
Accepted: *11 May 2021*
Published: *02 June 2021*

Citation:
León-Félix J, Gutkind G, Arriagada G and Vignoli R (2021) Editorial: Antimicrobial Resistance in Zoonotic Bacteria in Developing Countries: The Role of Food Animal Production in Public Health. Front. Vet. Sci. 8:685281. doi: 10.3389/fvets.2021.685281

second in prevalence followed by *qnrE1*, found in chickens and calves. Different β-lactamase genes were detected as responsible for oxyimino-cephalosporins resistance. This work highlights that transferable resistance genes to the three antibiotics considered critical to human health were present in feces from farm animals in Uruguay, most also reported previously in microorganisms of human origin from Uruguay.

Moreno et al. described available information to identify research and/or information gaps regarding themes of interest for antimicrobial resistance (AMR) in water in LAC. The most relevant research gaps identified are in resistance transfer, AMR surveillance, evaluating health impact of AMR, improving water treatment for AMR removal, and concluded that AMR environmental situation is driven by few countries, and therefore, research is needed in other LAC countries to better represent the region.

A second group of articles corresponded to phenotypic and genotypic characterization of resistant zoonotic bacteria.

Galarce et al. characterized antimicrobial resistance of 54 STEC isolates sampled from cattle and swine in central Chile, their findings indicate that all the isolates exhibited phenotypical resistance to cefalexin and a great proportion to colistin. The resistance genes detected were *dfrA1* and *tetA* (100%) followed by *tetB* (94.4%), *blaTEM-1* (90.7%), *aac*(6)-Ib (88.9%), *blaAmpC* (81.5%), *cat1* (61.1%), and *aac*(3)-IIa (11.1%).

Pavez-Muñoz et al. characterized antimicrobial resistance of STEC isolated from backyard production systems in central Chile, where 100% of the evaluated isolates were resistant to cephalexin and 50% to chloramphenicol, where a *stx1* type gene was present in all isolates. Several factors were identified at the farm level as responsible for determining the use of antibiotics, such as difficulties for clear disease definition and the close contact between different species. This study also constitutes the first report of resistant STEC strains circulating in the low complexity backyard production systems in Chile.

Guo et al. characterized the genome of *mcr1* positive *E. coli* isolated from pigs with post-weaning diarrhea in China by whole genome sequencing. This study found that 455 *E. coli* isolates recovered from fecal samples or small intestine contents, most were *E. coli* enterotoxigénica (ETEC), followed by atypical enteropathogenic *E. coli* (aEPEC). In the five colistin resistant isolates, three were categorized as ETEC/STEC hybrids, O3:H45, ST4214 and the remaining two as aEPEC O4:H11 ST29 and O103:H2 ST20 respectively. All displayed multiple antibiotic resistance genes, including *mcr1.1* gene and, in three cases, also *mcr3.1* presence.

Torres et al. focused their research on a strong biofilm producer strain of *S. aureus* (Sa1FB) associated with subclinical bovine mastitis in Colombia. The major differences with a reference strain were found in the number of mobile genetic elements, that could increase mutations, pathogenesis, and adaptability to new hosts, representing a risk for transmission of resistant *S. aureus* to people by milk consumption obtained from infected animals.

Umair et al. quantified antibiotics use in two large corporate dairy farms from Pakistan, reporting that the amount of antibiotic used was considerably higher than in similar studies around the world. Most used antibiotic classes were aminoglycosides, penicillin and tetracyclines, and 43% of the active principles used were critically important antimicrobials for human medicine, constituting the first study of its kind in Pakistan.

In conclusion, this eBook provides new insights about the frequency of antimicrobial resistance and other pathogenic elements of various strains of *Salmonella*, *E. coli* (mainly STEC), *L. monocytogenes*, *S. aureus*, in cattle, poultry, swine, dairy farms, and other sources, such as water samples, mainly in Latin America and the Caribbean. The evidence presented here highlights that even no coordinated studies have been sustainability undertaken. Antimicrobial resistance burden is very high, with implications not only on human health, but also potentially compromising production systems sustainably in the next future. Coordinated local, regional, and global actions regarding the use of antimicrobials in the production of food from animal origin are necessary and implementing precise diagnostic strategies would allow establishing clear and forceful guidelines that lead to the efficient use of antibiotics.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 10 July 2020
doi: 10.3389/fvets.2020.00367

OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 27 February 2020
Accepted: 27 May 2020
Published: 10 July 2020

Phenotypic and Genotypic Antimicrobial Resistance in Non-O157 Shiga Toxin-Producing *Escherichia coli* Isolated From Cattle and Swine in Chile

Nicolás Galarce [1*†], ***Fernando Sánchez*** [1†], ***Verónica Fuenzalida*** [1†], ***Romina Ramos*** [1†], ***Beatriz Escobar*** [1†], ***Lisette Lapierre*** [1†], ***Esteban Paredes-Osses*** [2†], ***Gabriel Arriagada*** [3†], ***Raúl Alegría-Morán*** [1,4†], ***Nilton Lincopán*** [5†], ***Danny Fuentes-Castillo*** [6†], ***Alejandra Vera-Leiva*** [7†], ***Gerardo González-Rocha*** [7,8†], ***Helia Bello-Toledo*** [7,8†] ***and Consuelo Borie*** [1†]

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Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic pathogen that causes bloody diarrhea and hemolytic-uremic syndrome in humans, and a major cause of foodborne disease. Despite antibiotic treatment of STEC infections in humans is not recommended, the presence of antimicrobial-resistant bacteria in animals and food constitutes a risk to public health, as the pool of genes from which pathogenic bacteria can acquire antibiotic resistance has increased. Additionally, in Chile there is no information on the antimicrobial resistance of this pathogen in livestock. Thus, the aim of this study was to characterize the phenotypic and genotypic antimicrobial resistance of STEC strains isolated from cattle and swine in the Metropolitan region, Chile, to contribute relevant data to antimicrobial resistance surveillance programs at national and international level. We assessed the minimal inhibitory concentration of 18 antimicrobials, and the distribution of 12 antimicrobial resistance genes and class 1 and 2 integrons in 54 STEC strains. All strains were phenotypically resistant to at least one antimicrobial drug, with a 100% of resistance to cefalexin, followed by colistin (81.5%), chloramphenicol (14.8%), ampicillin and enrofloxacin (5.6% each), doxycycline (3.7%), and cefovecin (1.9%). Most detected antibiotic resistance genes were *dfr*A1 and *tet*A (100%), followed by *tet*B (94.4%), $bla_{\text{TEM}-1}$ (90.7%), *aac(6)-Ib* (88.9%), bla_{AmpC} (81.5%), *cat*1 (61.1%), and *aac(3)-IIa* (11.1%). Integrons were detected only in strains of swine origin. Therefore, this study provides further evidence that non-O157 STEC strains present in livestock in the Metropolitan region of Chile exhibit phenotypic and genotypic

resistance against antimicrobials that are critical for human and veterinary medicine, representing a major threat for public health. Additionally, these strains could have a competitive advantage in the presence of antimicrobial selective pressure, leading to an increase in food contamination. This study highlights the need for coordinated local and global actions regarding the use of antimicrobials in animal food production.

Keywords: antimicrobial resistance, Shiga toxin, *Escherichia coli*, drug resistance, cattle, swine

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic pathotype of *E. coli* recognized as an important cause of food-borne illness worldwide. Several animal species are reservoirs of STEC strains, mainly cattle with a reported prevalence of up to 70.1% in beef cattle (1) and up to 68.7% in swine (2). STEC can cause severe gastroenteritis, hemorrhagic colitis, and life-threatening hemolytic-uremic syndrome (HUS) in children (3, 4), and extrarenal manifestations in adults and the elderly, such as thrombotic thrombocytopenic purpura (5). Among these different illnesses caused by STEC infection, HUS is the most severe, as it has a 2% mortality rate during the acute phase (5), and is considered the main cause of acute renal failure in children, with about 30% of them developing chronic kidney disease (6).

Global incidence of STEC infections in people was estimated in a previous study, which showed that this pathogen is responsible for 2,801,000 acute infections annually, with 3,890 HUS cases and 230 deaths (7). In this context, and according to official data, the incidence of HUS in Chile is 3.2/100,000 in children under 4 years, with a mortality rate of 3–5% (8, 9).

The O157 serogroup is the most frequently associated with outbreaks and sporadic cases of HUS in people (10, 11), although other serogroups such as O26, O45, O103, O111, O121, and O145, have also been associated with severe disease (11, 12). In addition, the economic costs associated with STEC infections also have a high impact. In this context, it has been estimated that average economic losses in the United States reach US$ 896/case and US$ 101 million for non-O157:H7 STEC infections, and that combined economic losses for public health and food agriculture are estimated at US$ 993 million per year (13).

Antibiotic treatment of STEC infections in humans is not recommended, as there is evidence that treatment may worsen the disease by inducing toxin-related tissue damage and symptoms in patients (14). However, toxin production depends on the type and concentration of the drug used (15). During the O104:H4 outbreak in Germany, patients treated with azithromycin at the acute phase showed decreased STEC carriage periods (16), while no patients treated with azithromycin for long-term STEC shedding developed HUS (17). Although antibiotic therapy is not recommended for STEC infections, multidrug-resistant (MDR) strains constitute a public health concern, both for human and veterinary medicine, as these strains contribute to the resistance gene reservoir that can be easily exchanged among different bacterial species either in the host or in the environment (18).

It is widely accepted that extensive use of antimicrobials in animal production systems is a major driver of multi-drug resistance in bacteria (19). Furthermore, long-term subtherapeutic exposure to antibiotics can result in mutation enrichment and/or acquisition of mobile genetic elements such as plasmids, transposons, and integrons that can confer a phenotype of increased resistance to these compounds (20). The presence of antibiotic-resistant bacteria in animals and food, regardless of their pathogenicity, constitutes a public health risk as the genetic pool from which bacterial pathogens can acquire antibiotic resistance has increased in the environment (21).

STEC strains resistant to β-lactams, aminoglycosides, phenicols, and tetracyclines, among others, have been isolated from livestock worldwide, together with their resistance-encoding genes and integrons (22, 23). These studies indicate variable antimicrobial resistance (AMR) levels in the STEC isolates according to geographic area, possibly due to control policies in the use of these compounds in animal husbandry. However, international trade of animals and their products can enable the transmission of strains and/or their resistance genetic determinants among countries. In addition, new resistance patterns have emerged in *E. coli* strains, being colistin resistance one of the most important threats to public health worldwide (24).

As part of a larger study, cattle and swine were screened for STEC as previously published (25), recovering culturable STEC strains at a frequency of 17% in cattle and 1% in swine. The aim of this study was to characterize the phenotypic and genotypic AMR of the isolated strains, to assess the potential impact in public health and contribute updated data to national and international AMR surveillance programs.

MATERIALS AND METHODS

Bacterial Strains

During 2018, samples from intestinal content of cattle and swine ($n = 300$, each) at four abattoirs located in the Región Metropolitana were obtained. From these samples, 54 STEC strains were isolated from cattle ($n = 51$) and swine ($n = 3$) (25). Strains were stored in trypticase soy broth (Oxoid, Basingstoke, UK) mixed with glycerol (1:1, v/v) at −80°C. Sampling, processing, bacterial identification and characterization were detailed in a previous study (25).

Phenotypic Antimicrobial Resistance

AMR of all isolated strains was quantified by a minimal inhibitory concentration (MIC) test using the VITEK2

system (bioMérieux, Marcy-l'Étoile, France) and the AST-GN98 card according to the manufacturer's instructions, and clinical cut-off values were applied according to the Clinical and Laboratory Standards Institute guidelines (26). The cards included aminoglycosides (amikacin and gentamicin), β-lactams (amoxicillin-clavulanic acid, ampicillin, cefalexin, cefovecin, cefpodoxime, ceftazidime, ceftiofur, and imipenem), folate synthesis inhibitors (trimethoprim-sulfamethoxazole), nitrofurans (nitrofurantoin), phenicols (chloramphenicol), quinolones (ciprofloxacin, enrofloxacin, and marbofloxacin), tetracyclines (doxycycline), and also cefepime, cefotaxime, ceftazidime alone, and in combination with clavulanic acid for the detection of extended-spectrum β-lactamase (ESBL). Colistin resistance was determined with the broth microdilution method (27–29), analyzing eight antibiotic concentrations (32–0.25 μg/mL). *E. coli* ATCC 25922 was used as quality control and *E. coli* NCTC 13846 as positive control. MDR was confirmed if an isolated strain presented resistance to three or more antibiotics of different classes (30). Intermediate strains were classified as resistant.

Genotypic Antimicrobial Resistance

The presence of 12 AMR genes in all STEC strains was assessed by PCR in a LifeECO® Thermocycler (Hangzhou Allsheng Instruments Co, Hangzhou, China). For DNA extraction, an inoculum of each strain plated on MacConkey agar plates (Oxoid, Basingstoke, UK) and incubated at 37°C for 18–24 h was resuspended in sterile plastic tubes containing 500 µl of sterile nuclease-free water and boiled for 15 min at 100°C. Subsequently, tubes were centrifuged at 26,480 g for 5 min at room temperature. In parallel, plasmid DNA was obtained using the E.Z.N.A.® Plasmid DNA Mini Kit II (Omega Bio-Tek, Norcross, GA, USA), following manufacturer's instructions. Concentration and quality of the obtained DNA was measured in a NANO-400 micro-spectrophotometer (Hangzhou Allsheng Instruments Co). Samples with a 260/280 nm absorbance ratio close to the optimal range (1.8–2.0) were kept at −20°C for further analyses (31). The genes analyzed included $bla_{\text{TEM}-1}$, $bla_{\text{CTX}-\text{M}}$, chromosomal bla_{AmpC} and bla_{NDM1} for β-lactams; *aac(6)-Ib* and *aac(3)-IIa* for aminoglycosides; *tet*A and *tet*B for tetracyclines; *cml*A and *cat*1 for phenicols; and *dfr*A1 for folate synthesis inhibitors (32–39). To detect the presence of colistin resistance genes, eight types of *mcr* genes were analyzed (*mcr*1-*mcr*8) following previous protocols (40, 41). Additionally, class 1 and class 2 integrons were detected by conventional PCR (42). All PCR reactions were performed in duplicate. Gene selection was based on their distribution in *E. coli* and their clinical impact in both animal and public health, under the concept of One Health (24, 43, 44). Strains belonging to our collection, whose PCR products for the detection of the aforementioned genes were sequenced and their nucleotide identity corroborated by comparison to sequences deposited at GenBank® (National Center for Biotechnology Information, Bethesda, MD, USA) (data not published), were used as positive controls. **Table 1** summarizes all primers used for molecular detection of AMR genes.

Statistical Analysis

For the phenotypic AMR characterization, multiple correspondence analysis (MCA) was used to evaluate the proximal relationships of the resistant/susceptible conditions among the different antibiotics tested. MCA is a non-parametric technique for assessing the pattern of relationships among several categorical variables by identifying a reduced number of orthogonal dimensions that capture most variability present in the original variables (45). The same statistical analyses were performed to assess the relationship of the presence or absence of AMR genes among the isolates. In all cases, MCA analyses were limited to the derivation of two dimensions as a preliminary analysis indicated that these captured a substantial amount of the total variance, and were performed only with variables that presented variability (i.e., antibiotics, genes). The relationships among the antibiotics' resistant/sensitive condition, and among the presence/absence condition of resistance genes were graphically assessed by the construction of two-dimensional correspondence maps. All MCA-related analyses were performed using IMB© SPSS© Statistics v.26 (IBM Corp., Armonk, NY).

RESULTS

Phenotypic Antimicrobial Resistance Characterization

All 54 strains analyzed were phenotypically resistant to at least one antibiotic, all being resistant to cefalexin (100%, $n = 54$), followed by colistin (81.5%, $n = 44$), chloramphenicol (14.8%, $n = 8$), ampicillin and enrofloxacin (5.6%, $n = 3$), doxycycline (3.7%, $n = 2$), and cefovecin (1.9%, $n = 1$). A 14.8% of the strains were MDR. No ESBL production was detected in any strain, nor resistance to amoxicillin-clavulanic acid, cefpodoxime, ceftazidime, ceftiofur, imipenem, amikacin, gentamicin, ciprofloxacin, marbofloxacin, nitrofurantoin, or trimethoprim-sulfamethoxazole. **Table 2** shows the MIC_{50} and MIC_{90} of the STEC strains for the analyzed antibiotics. All strains isolated from cattle were resistant to cefalexin (100%, $n = 51$), followed by colistin (80.4%, $n = 41$), chloramphenicol (11.8%, $n = 6$), ampicillin (3.9%, $n = 2$), and cefovecin (2%, $n = 1$). Additionally, five strains (9.8%) were MDR. On the other hand, all strains isolated from swine were resistant to cefalexin, enrofloxacin, and colistin ($n = 3$), followed by doxycycline and chloramphenicol ($n = 2$), and ampicillin ($n = 1$). All strains of swine origin were MDR ($n = 3$). **Table 3** shows the different phenotypic resistance profiles in the STEC strains analyzed, being the cefalexin-colistin resistant phenotype the most frequently detected (66.7%, $n = 36$).

Genotypic Antimicrobial Resistance Characterization

As for the AMR genes, the most detected were *dfr*A1 and *tet*A (100%, $n = 54$), followed by *tet*B (94.4%, $n = 51$), $bla_{\text{TEM}-1}$ (90.7%, $n = 49$), *aac(6)-Ib* (88.9%, $n = 48$), bla_{AmpC} (81.5%, $n = 44$), *cat*1 (61.1%, $n = 33$), and *aac(3)-IIa* (11.1%, $n = 6$). No strains harboring $bla_{\text{CTX}-\text{M}}$, bla_{NDM1}, *cml*A, and *mcr*1-8 genes were detected. Both classes of integrons were detected in 5.5% (*n*

TABLE 1 | Oligonucleotide sequences for antimicrobial resistance genes and integrons, expected product size, and references.

Gene	Primers (5′-3′)	Expected product size (bp)	References
bla_{TEM-1}	F: ATCAGCAATAAACCAGC R: CCCCGAAGAACGTTTTC	516	(33)
bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGTKATGGC R: TGGGTRAARTARGTSACCAGAAYCAGCGG	593	(36)
bla^{*}_{AmpC}	F: TTCTATCAAMACTGGCARCC R: CCYGTTTTATGTACCCAYGA	500	(35)
bla_{NDM1}	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	621	(39)
aac(6)-Ib	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	482	(37)
aac(3)-IIa	F: CGGAAGGCAATAACGGAG R: TCGAACAGGTAGCACTGAG	740	(34)
*tet*A	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	577	(32)
*tet*B	F: CCTCAGCTTCTCAACGCGTG R: GCACCTTGCTGATGACTCTT	634	(32)
*cml*A	F: CCGCCACGGTGTTGTTGTTATC R: CACCTTGCCTGCCCATCATTAG	698	(38)
*cat*1	F: AGTTGCTCAATGTACCTATAACC R: TTGTAATTCATTAAGCATTCTGCC	547	(34)
*dfr*A1	F: AAGAATGGAGTTATCGGGAATG R: GGGTAAAAACTGGCCTAAAATTG	391	(34)
*mcr*1	F: AGTCCGTTTGTTCTTGTGGC R: AGATCCTTGGTCTCGGCTTG	320	(40)
*mcr*2	F: CAAGTGTGTTGGTCGCAGTT R: TCTAGCCCGACAAGCATACC	715	(40)
*mcr*3	F: AAATAAAAATTGTTCCGCTTATG R: AATGGAGATCCCCGTTTTT	929	(40)
*mcr*4	F: TCACTTTCATCACTGCGTTG R: TTGGTCCATGACTACCAATG	1,116	(40)
*mcr*5	F: ATGCGGTTGTCTGCATTTATC R: TCATTGTGGTTGTCCTTTTCTG	1,644	(40)
*mcr*6	F: GTCCGGTCAATCCCTATCTGT R: ATCACGGGATTGACATAGCTAC	566	(41)
*mcr*7	F: TGCTCAAGCCCTTCTTTTCGT R: TTCATCTGCGCCACCTCGT	892	(41)
*mcr*8	F: AACCGCCAGAGCACAGAATT R: TTCCCCCAGCGATTCTCCAT	667	(41)
intI1	F: GGGTCAAGGATCTGGATTTCG R: ACATGGGTGTAAATCATCGTC	483	(42)
intI2	F: CACGGATATGCGACAAAAAGGT R: GTAGCAAACGAGTGACGAAATG	788	(42)

**Chromosomally encoded bla_{AmpC}.*

= 3) of the strains. Among the strains isolated from cattle, the most frequently detected genes were *dfr*A1 and *tet*A (100%, n = 51), followed by *tet*B (94.1%, $n = 48$), *aac(6)-Ib* (92.2%, $n = 47$), bla_{TEM-1} (90.2%, $n = 46$), bla_{AmpC} (80.4%, $n = 41$), *cat*1 (58.8%, $n = 30$), and *aac(3)-IIa* (11.8%, $n = 6$). On the other hand, all strains isolated from swine harbored *cat*1, *dfr*A1, bla_{TEM-1}, *tet*A, *tet*B, bla_{AmpC}, class 1, and class 2 integrons ($n = 3$), followed by *aac(6)-Ib* ($n = 1$). **Table 4** shows all the genotypic resistance profiles detected according to origin, being the *dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tet*A/*tet*B/*cat*1/bla_{AmpC} profile the most frequently detected (33.3%, $n = 18$).

Statistical Analysis

MCA for phenotypic AMR characterization included only ampicillin, cefovecin, enrofloxacin, doxycycline, chloramphenicol, and colistin, as there were both resistant and sensitive isolates for each of these antibiotics. The two derived dimensions accounted for 63.73% of total variable variance (first dimension = 38.66%; second dimension = 25.07%). The correspondence map indicated that the first dimension was dominated by isolates resistant to chloramphenicol, enrofloxacin and doxycycline, mostly due to swine isolates; while the second dimension was mainly explained by isolates resistant to cefovecin

TABLE 2 | MICs of selected antimicrobials against STEC strains isolated from cattle and swine.

Antimicrobial class	Antimicrobial	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Range (μg/mL)
Aminoglycosides	AMK	≤2	≤2	≤2
	GEN	≤1	≤1	≤1
β-lactams	AMC	≤2	≤4	≤2–8
	AMP	≤4	≤8	≤2–≥32
	LEX	8	≤16	8–16
	CFO	≤0.5	≤1	≤0.5–≥8
	CPD	≤0.25	≤0.5	≤0.25–1
	CAZ	≤0.12	≤0.25	≤0.12–0.25
	CFT	≤1	≤1	≤1
	IPM	≤0.25	≤0.25	≤0.25
Folate synthesis inhibitors	SXT	≤20	≤20	≤20
Nitrofurans	NIT	≤16	≤16	≤16
Phenicols	CHL	≤8	≤16	4-≥64
Polymyxins	CST	≤4	≤8	1–16
Quinolones	CIP	≤0.06	≤0.06	≤0.06
	ENR	≤0.12	≤0.12	≤0.12–1
	MRB	≤0.5	≤0.5	≤0.5–1
Tetracyclines	DOX	≤1	≤1	≤0.5–≥16

MIC_{50} and MIC_{90} are those concentrations required to inhibit growth of 50 and 90% of the isolates, respectively. Amikacin, AMK; amoxicillin-clavulanic acid, AMC; ampicillin, AMP; cefalexin, LEX; cefovecin, CFO; cefpodoxime, CPD; ceftazidime, CAZ; ceftiofur, CFT; chloramphenicol, CHL; ciprofloxacin, CIP; colistin, CST; doxycycline, DOX; enrofloxacin, ENR; gentamicin, GEN; imipenem, IPM; marbofloxacin, MRB; nitrofurantoin, NIT; trimethoprim-sulfamethoxazole, SXT.

TABLE 3 | Phenotypic resistance profiles detected in STEC strains isolated from cattle and swine.

Resistance profile	Origin	Number of strains (%)	Strain ID
LEX	Cattle	7 (12.9%)	1, 2, 3, 4, 5, 42, 48
LEX/CST	Cattle	36 (66.7%)	6, 7, 8, 12, 14, 15, 16, 17, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 37, 38, 44, 45, 46, 47, 49, 55, 57, 58, 60, 61, 63, 64
LEX/CHL/CST	Cattle	3 (5.6%)	9, 36, 40
LEX/CHL	Cattle	3 (5.6%)	13, 19, 39
LEX/AMP/CFO/CST	Cattle	1 (1.9%)	18
LEX/AMP/CST	Cattle	1 (1.9%)	20
LEX/ENR/CST	Swine	1 (1.9%)	67
LEX/AMP/ENR/DOX/CHL/CST	Swine	1 (1.9%)	68
LEX/ENR/DOX/CHL/CST	Swine	1 (1.9%)	69

LEX, cefalexin; CST, colistin; CHL, chloramphenicol; AMP, ampicillin; CFO, cefovecin; ENR, enrofloxacin; DOX, doxycycline.

and, in a lesser extent, to ampicillin (**Figure 1**). In parallel, MCA to assess the relationship pattern for the presence/absence condition among genes included bla_{TEM-1}, bla_{AmpC}, *aac(3)-IIa*, *aac(6)-Ib*, *tet*B, *cat*1, *intI1*, and *intI2* genes. The resulting model indicated that the two dimensions accounted for 52.78% of the total variance of the original variables (first dimension = 30.83%; second dimension = 21.95%). Dimension 1 was largely dominated by the presence of *intI1* and *intI2* genes, which belong to swine isolates. Dimension 2 was mostly driven by the presence of *aac(3)-IIa* gene, but also by the presence of *aac(6)-Ib*, bla_{TEM-1}, bla_{AmpC} and *cat*1 genes. These five genes belonged to bovine isolates (**Figure 2**).

DISCUSSION

Gram-negative pathogens like STEC represent a major challenge in Latin America, where MDR, fluoroquinolone-resistant and ESBL-producing strains have spread (46). STEC strains resistant to β-lactams, aminoglycosides, phenicols, and tetracyclines, have been isolated from livestock and humans worldwide (23, 47). However, studies focusing on the AMR of STEC strains isolated from animals in Latin America are scarce. In this context, Ferreira et al. (48) determined the antimicrobial susceptibility of 90 STEC strains isolated from sheep in Brazil, registering 25.5% of resistance to streptomycin, 22.2% to amoxicillin-clavulanic acid, and 19% to nalidixic acid. Furthermore, 6.7% of the strains showed MDR, mainly to gentamicin, streptomycin and tetracycline. The same year, Krüger et al. (49) analyzed the antimicrobial susceptibility of 29 STEC strains of various origins, including 21 strains isolated from cattle in Argentina. Of these 21 strains, only two exhibited resistance against at least one of the drugs analyzed, registering a 9.5% of resistance to ampicillin, amoxicillin-clavulanic acid, cephalothin, and tetracycline, and 4.8% to trimethoprim-sulfamethoxazole, chloramphenicol, and florfenicol. Furthermore, the authors reported the presence of the bla_{TEM} gene in the two resistant strains, and of *tet*B, *str*A, *aad*A1, *tet*A, *dfr*A1, *sul*1, *sul*2, *flor*R genes in only one of them. More recently, Amézquita-López et al. (47) evaluated the antimicrobial susceptibility of 59 STEC strains isolated from various domestic animals, including cattle and sheep, in Mexico. Of these strains, 78.0% exhibited resistance to cephalothin, 50.8% to chloramphenicol, 37.3% to kanamycin, 25.4% to ampicillin, 6.8% to amikacin and tetracycline, 3.4% to amoxicillin-clavulanic acid, and 1.7% to cefoperazone, gentamicin, and imipenem. In the other hand, and as far as we know, the present research describes for the first time the phenotypic and genotypic AMR of STEC strains isolated from livestock in Chile, including the characterization of colistin resistance and integron presence.

Antibiotics are usually not prescribed for the treatment of human STEC infections. However, monitoring AMR patterns of intestinal STEC from animal reservoirs, provides valuable information regarding the transmission of resistant strains to humans and of their genetic AMR determinants to other enteric pathogens (22). While most studies have focused on the O157 serogroup (23) AMR in non-O157 STEC strains has increased compared to the former serogroup. In this context, Buvens et al. reported a higher AMR in non-O157 STEC strains than in O157 strains, for ampicillin (23.5 vs. 5.2%), nalidixic acid (10.7 vs. 0%), streptomycin (58 vs. 26%), kanamycin (20 vs.

TABLE 4 | Genotypic resistance profiles detected in STEC strains isolated from cattle and swine.

Resistance profile	Origin	Number of strains (%)	Strain ID
*dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tetA*/*tetB*	Cattle	5 (9.3%)	1, 2, 4, 14, 15
*dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tetA*/*tetB*/*cat1*	Cattle	1 (1.9%)	3
*dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tetA*/*tetB*/*cat1*/bla_{AmpC}	Cattle	18 (33.3%)	5, 6, 7, 12, 17, 18, 19, 22, 23, 26, 28, 33, 44, 46, 49, 55, 61, 63
*dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tetA*/*tetB*/*cat1*/bla_{AmpC}/*aac(3)-IIa*	Cattle	5 (9.3%)	8, 13, 24 25, 27
*dfr*A1/*aac*(6)-Ib/*tetA*/*tetB*	Cattle	1 (1.9%)	9
*dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tetA*/*tetB*/bla_{AmpC}	Cattle	12 (22.2%)	16, 20, 29, 30, 31, 32, 35, 36, 37, 38, 39, 40
*dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tetA*	Cattle	1 (1.9%)	34
*dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tetA*/*tetB*/bla_{AmpC}/*aac(3)-IIa*	Cattle	1 (1.9%)	42
*dfr*A1/*aac*(6)-Ib/*tetA*	Cattle	1 (1.9%)	45
*dfr*A1/bla_{TEM-1}/*tetA*/*tetB*/bla_{AmpC}/*cat1*	Cattle	2 (3.7%)	47, 64
*dfr*A1/*aac(6)-Ib*/*tetA*/*tetB*/bla_{AmpC}/*cat1*	Cattle	1 (1.9%)	48
*dfr*A1/*tetA*/*tetB*/bla_{AmpC}/*cat1*	Cattle	1 (1.9%)	57
*dfr*A1/*tetA*/*tetB*/*cat1*	Cattle	1 (1.9%)	58
*dfr*A1/*aac(6)-Ib*/bla_{TEM-1}/*tetA*/*cat1*/bla_{AmpC}	Cattle	1 (1.9%)	60
*dfr*A1/bla_{TEM-1}/*tetA*/*tetB*/*cat1*/*intI1*/*intI2*	Swine	2 (3.7%)	67, 69
*dfr*A1/aac(6)-Ib/bla_{TEM-1}/*tetA*/*tetB*/*cat1*/*intI1*/*intI2*	Swine	1 (1.9%)	68

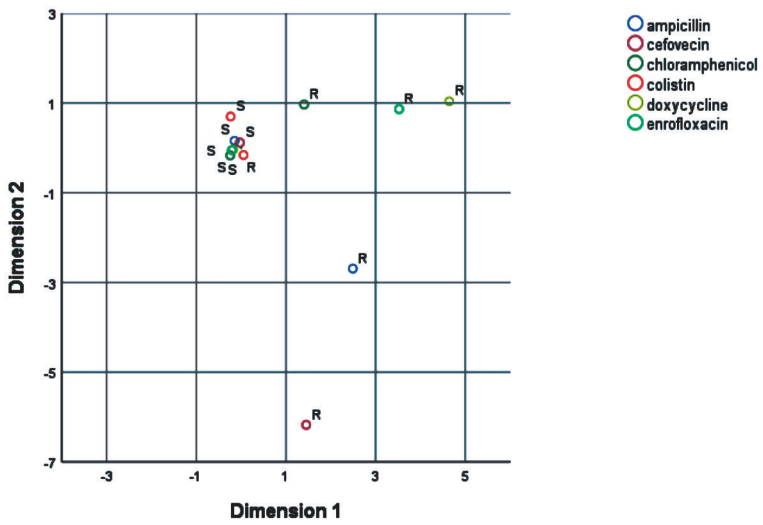

FIGURE 1 | Two-dimension correspondence map for phenotypic AMR characterization (S, sensitive; R, resistant).

5%), tetracycline (44 vs. 15%), sulphonamides (59 vs. 22%), and trimethoprim (24 vs. 4%) (50). More recently, a cattle study in Spain reported higher AMR and MDR levels in STEC strains of serogroups O111, O104, O91, and O26 than in serogroup O157 (51). Additionally, AMR acquisition could confer competitive advantages, allowing non-O157 STEC strains to preferentially colonize livestock over other bacterial enteropathogens when there is a selective antimicrobial pressure (18).

Regarding phenotypic AMR in the STEC strains analyzed, our results show that resistance against β-lactams was the most frequent, including cefalexin (100%), followed by polymyxins with an 80.4% of resistance against colistin; phenicols with an 11.8% against chloramphenicol; fluoroquinolones with an 5.6% against enrofloxacin; and tetracyclines with an 3.7% against doxycycline. In this context, Colello et al. reported an 86% of resistance to tetracycline, streptomycin, and chloramphenicol, 71% to trimethoprim/sulfamethoxazole, sulfisoxazole, and ampicillin, and 57% to nalidixic acid in STEC strains isolated from cattle, swine, food and farm environment in Argentina (22), showing higher levels of AMR than those registered here.

Furthermore, in our study the MCA for phenotypic AMR characterization suggests that isolates resistant to doxycycline also present resistance to enrofloxacin, and in a lesser extent to chloramphenicol. If resistance to these antibiotics is present, it is unlikely that the isolates are also resistant to cefovecin or ampicillin. However, these results may be due to the high resistance exhibited by all the three strains of swine origin, so they

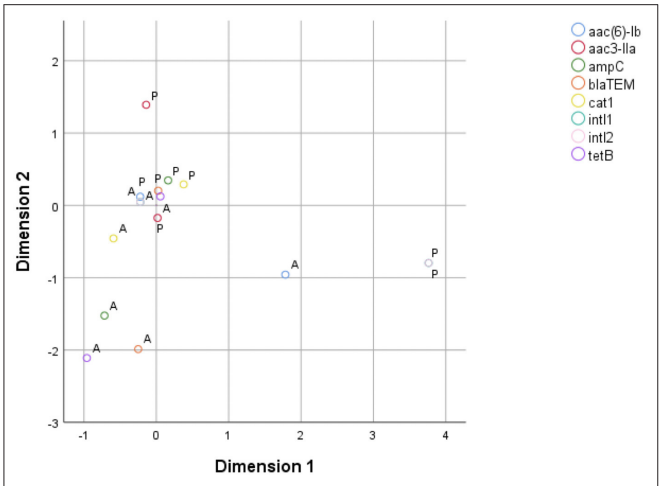

FIGURE 2 | Two-dimension correspondence map for resistance genes (A, absence; P, presence).

must be interpreted with caution. In addition, when an isolate is colistin resistant, it is probably sensitive to most of the other antibiotics tested in this study.

Although in Chile there are no official AMR monitoring plans in *E. coli* strains isolated from animals, some studies describe the antimicrobial susceptibility of these isolates in cattle and pigs. In this context, San Martín et al. (52) described the AMR of 50 *E. coli* strains isolated from dairy cattle and 72 strains isolated from beef cattle. Here, strains isolated from the former presented the highest levels of AMR, with 84% of resistance to oxytetracycline, 54% to enrofloxacin, ciprofloxacin and ceftiofur, and a 56% of MDR, being oxytetracycline/enrofloxacin/ciprofloxacin/ceftiofur the most frequently detected phenotypic resistance profile (46%). In contrast, in strains isolated from beef cattle, the highest resistance was to sulfamethoxazole/trimethoprim (10%), followed by oxytetracycline (4%) and ceftiofur (3%), with an 1.4% of MDR, where the most frequent resistance profile corresponded to sulfamethoxazole/trimethoprim (4%). In a more recent study, Hervé-Claude et al. (53) evaluated AMR in 88 *E. coli* strains isolated from calves, where 87.5% were resistant to at least one antimicrobial, 16% showed MDR, and the most frequent resistance profile corresponded to oxytetracycline/sulfamethoxazole/trimethoprim (9.1%). On the other hand, Lapierre et al. (54) evaluated the AMR of 87 strains of *E. coli* isolated from swine, registering 77% of resistance to tetracycline, 74% to streptomycin, and 38% to sulfamethoxazole/trimethoprim (38%), with a 74.7% of MDR, being tetracycline/streptomycin the most frequent resistance profile (33.3%). Good practices in antimicrobial use in Chile, as well as a correct implementation of current policies for antimicrobial use in livestock, could explain the low levels of AMR detected here, compared to previous studies.

β-lactam resistance in STEC strains is well-documented internationally. In this context, Kennedy et al. (18) reported a 53% of resistance to ampicillin, 31% to cephalothin, 16% to ceftiofur, and 6% to cefpodoxime, in non-O157 STEC strains isolated from cattle at farms and abattoirs in Ireland, and an 82% of those strains were MDR. In Latin America, a 100% and a 50% of resistance to ampicillin in non-O157 STEC strains isolated from cattle and swine was reported in Argentina, respectively (22). β-lactams are used in human and veterinary medicine, and are considered of critical importance (3rd and 4th generation cephalosporins, carbapenems, antipseudomonal penicillins, and aminopenicillins with or without β-lactamase inhibitors) and of highly importance (1st and 2nd generation cephalosporins, amidinopenicillins, anti-staphylococcal, and narrow spectrum penicillins) in human medicine, and of critical importance (3rd and 4th generation cephalosporins, penicillins), and of highly importance (1st and 2nd generation cephalosporins) in veterinary medicine (55, 56). Several genes provide resistance against β-lactams by encoding β-lactamases, including bla_{TEM}, bla_{NDM1}, and bla_{AmpC}, among others (57). In this study, only two of these genes were detected in STEC strains isolated from cattle, $bla_{\text{TEM}-1}$ (90.7%) and bla_{AmpC} (81.5%). Similar to our results, Colello et al. detected the $bla_{\text{TEM}-1}$ gene in 80% of STEC strains isolated from cattle and swine in Argentina, and also the bla_{AmpC} gene in an 81.5%, which encodes for a type C β-lactamases (22). Nevertheless, our results are higher than those reported by Kennedy et al., where 43 and 13% of the strains isolated at abattoirs and farms, respectively, harbored the bla_{AmpC} gene (18). The high rate of chromosomal bla_{AmpC} detected here was expected, as most of *E. coli* strains harbor this gene (58). Although in *E. coli* its expression is constitutive at a low level, overproduction of AmpC due to mutations in the promoter/attenuator leads to resistance to cephalosporins, penicillins, β-lactam-β-lactamase inhibitor combinations and/or aztreonam (58). Furthermore, AmpC production in combination with porin defects may also lead to carbapenem-resistance (59). According to the phenotypic antimicrobial susceptibility registered here, we could infer that these strains are not de-repressed mutants, and maintain their AmpC production at negligible levels. On the other hand, the high rate of $bla_{\text{TEM}-1}$ detection could explain the resistance of all strains to cefalexin. Conversely, and despite the high rate of detection, only three STEC strains were resistant to ampicillin. This discordant phenotype could be explained by the presence of deficiencies in outer membrane porins, such as OmpC and OmpF. In this context, Choi and Lee (60) analyzed how porins of *E. coli* affect the resistance to several antibiotics, including β-lactams. Thus, they registered an increase in β-lactams resistance in *omp*F mutants, while *omp*C mutants showed variable changes in the MIC to these compounds. More specifically, *omp*F mutants exhibited a 2-fold increase in the MIC of ampicillin, but an 8-fold in the MIC of cefoxitin, while *omp*A and *omp*C mutants did not alter the MIC of the former. Furthermore, triple mutants of the *omp*A, *omp*C, and *omp*F genes showed an 8-fold increase in the MIC of cefoxitin, 4-fold in the MIC of cefalotin, but a decrease in the MIC of ampicillin. These authors pointed out that transport of β-lactams by OmpC and OmpF is the most important factor in bacterial susceptibility to most of these antibiotics, and that this transport could be more important in bacterial susceptibility to ampicillin than to other β-lactams. In the case of the five isolates that did not harbor the $bla_{\text{TEM}-1}$ gene,

their phenotypic resistance against cefalexin could be explained by the presence of other non-ESBL encoding genes, such as bla_{TEM-2} (57). In this study, we detected only one strain (strain 18) resistant to cefalexin, cefovecin, and ampicillin, but sensitive to amoxicillin-clavulanic acid and negative for ESBL, which harbored both bla_{TEM-1} and bla_{AmpC} genes. The amoxicillin-clavulanic acid MIC of this strain was 8 μg/mL, a value that corresponds to the upper limit to be considered sensitive (26). This phenotype could be explained by a low production of AmpC that could confer resistance to at least one expanded-spectrum cephalosporin, but the MIC may not be high enough to classify the strain as resistant (61). However, further studies are needed to elucidate the role of AmpC in this discordant phenotype, using combinations of antibiotic substrates (such as cloxacillin) and inhibitors (boronic acid) or the cefoxitin-cloxacillin double disk synergy test (61). Apart from that, in this study we did not detect ESBL-producing strains nor the ESBL encoding gene bla_{CTX-M}. ESBL is a group of enzymes with the ability to hydrolyze and cause resistance to oxyimino-cephalosporins and monobactams, but not to cephamycins or carbapenems, and that are inhibited by β-lactamase inhibitors (57). This group includes TEM, SHV, OXA, and CTX types (57). CTX-M ESBLs have increased its prevalence in the last decade in *E. coli* strains isolated from humans and animals (62, 63) and are the most common type of ESBL worldwide (64). Similarly, we did not detect any strain resistant to carbapenems nor harboring the bla_{NDM-1} gene. NDM-1 is capable to hydrolyze penicillins, cephalosporins, carbapenems, but not aztreonam, and its encoding gene is usually located in conjugative plasmids, representing a significant threat to public health worldwide (65). NDM-1 harboring *E. coli* strains have been isolated worldwide, including Chile, since its discovery in 2008 (66, 67). Nevertheless, to date there are no reports of its detection in *E. coli* strains isolated from animals in Chile.

Regarding polymyxins, we detected an 81.5% of colistin resistance. Colistin resistance was associated only with point mutations on chromosomal genes, until a plasmid-mediated colistin resistance gene, *mcr*-1, was identified in Chinese clinical and swine-isolated *E. coli* strains in late 2015 (68). Just 3 months after this finding, it was described that this gene was present in most continents and mainly in *E. coli* strains isolated from animals, environment, foodstuff, and infected and asymptomatic human carriers (69). To date, 10 different *mcr* genes have been reported, some of them even with variants (24, 70, 71). Food-producing animals have been highlighted as potential reservoirs of *mcr*-harboring strains, and together with the fact that colistin is currently being used as the last resort against carbapenem-resistant Gram-negative bacteria in humans, this phenomenon poses a major threat to public health. To date, in Chile there is only one report of a human clinical isolate of colistin-resistant *E. coli* harboring the *mcr*-1 gene (72), and no reports of strains isolated from animal reservoirs harboring this gene. In Chile, the use of colistin is approved only for therapeutic purposes in cattle, poultry, and swine (73). According to the MIC values determined here, it is evident that most of the strains were phenotypically resistant to colistin, but it was not possible to associate these high levels of colistin resistance (MIC_{50} >4 μL/mL) with the presence of any of the *mcr* genes assessed. Similarly, Luo et al. detected a 47.5% of colistin resistant clinical isolates of *E. coli* in China that did not harbor any mobile *mcr* genes (74). This phenotypic resistance in absence of colistin-encoding mobile elements may be due to chromosomal mutations in the *mgr*B, *pho*PQ, and *pmr*AB genes, which would confer lipid A modifications (74).

Phenicol resistance is mainly due to the presence of chloramphenicol acetyltransferases encoded by *cat* genes that inactivate chloramphenicol but no other related compounds such as florfenicol; and to a lesser extent due to efflux pumps encoded by *cml* genes, among others (75). These genes can be detected in a wide variety of Gram-negative bacteria, including STEC, and are often associated with mobile elements such as plasmids, that can be transferred between bacteria of different species and genera (22, 75). Chloramphenicol resistance levels were low in the non-O157 STEC strains examined here (14.8%), but lower than results reported in México (47) and Argentina (22), where 60 and 80% of STEC strains isolated from cattle and swine were resistant to this drug, respectively. Contrary to the phenotypic resistance observed, we detected the *cat*1 gene in 61.1% of STEC strains, detection similar to that reported in Argentina where 40% of the STEC strains isolated from cattle and swine harbored this gene (22).

Resistance to quinolones is a major concern worldwide, as these antimicrobials are critically important for human and veterinary medicine (55, 56). Here, we registered only three isolates resistant to enrofloxacin, but sensitive to ciprofloxacin, similar to that reported in STEC strains isolated from cattle in South Africa (76), where 7.4% of the strains were resistant to enrofloxacin and 12.6% to ciprofloxacin. This different susceptibility to ciprofloxacin and enrofloxacin could be due to the presence of efflux pumps, as different members of this antimicrobial family show selective affinity for these (77). Conversely, mutations in topoisomerase genes would generate non-selective resistance to quinolones (58). Our findings suggest a restricted use of these drugs in livestock, probably due to national policies that do not encourage the use of quinolones as the first line of treatment, unless there is no other therapeutic alternative available. National policies also require that when quinolones are used as secondary treatment, their selection is based on the results of a susceptibility analysis (78).

Tetracyclines are broad-spectrum antibiotics that inhibit peptide elongation (75), and are considered of critical importance and high importance for veterinary and human medicine, respectively (55, 56). Tetracycline resistance occurs most frequently by the acquisition of genes that code for efflux pumps, ribosomal protection proteins, or by enzymatic inactivation. Many of the genes involved in these mechanisms are associated with mobile elements, and most of them encode resistance efflux proteins (75). Here, we detected tetracycline resistance in only a 3.7% of the isolated STEC strains, but *tet*A and *tet*B genes were detected in 100 and 94.4% of the strains, respectively. Tetracycline resistance levels reported here are lower than those reported previously in Ireland, where 82% of the non-O157 STEC strains isolated from cattle were resistant to tetracycline, while the *tet*A gene was detected in 60% of these strains (18). More recently, Colello et al. registered a 100% of tetracycline resistance in STEC strains isolated from cattle and swine, but the

presence of *tet*A and *tet*B genes in a 20 and 40%, respectively (22). This contradiction between phenotypic resistance and low detection of *tet* genes could be explained by the existence of other 43 tetracycline resistance genes (75), that could be present in those strains.

Of the detected AMR genes, *aac(6)-Ib*, $bla_{\text{TEM}-1}$, bla_{ampC}, and *cat*1 were located closed together in the correspondence map for resistance genes, suggesting that their presence was correlated among bovine isolates. In other words, when one of these four genes are present in an isolate, the others are likely to be present as well. The presence of the *aac(3)-IIa* gene seems to be less correlated to the presence of genes *aac(6)-Ib*, $bla_{\text{TEM}-1}$, bla_{AmpC}, and *cat*1. Additionally, the presence of genes *intI1* and *intI2* in all swine isolates explains their importance in the MCA, but interpretations must be made with caution due to the small number of swine samples in this study.

An interesting observation was the high detection levels for certain resistance genes with few or none STEC strains showing the associated phenotypic resistance, such as *tet*A (100%) and *tet*B (94.4%) vs. a 3.7% of resistance against doxycycline; *dfr*A1 (100%), *aac(3)-IIa* (11.1%), and *aac(6)-Ib* (88.9%) with no strains resistant to trimethoprim and aminoglycosides. One possible explanation to this is the lack of promoters or mutations in these regions, thus preventing gene expression (79). According to these authors, the accumulation and retention of deleterious mutations in resistance genes is higher in bacterial populations growing in absence of antimicrobial selection pressure than in bacterial populations under intense antimicrobial pressure (79). Nevertheless, other authors have shown that some of these inactivated resistance genes could be re-expressed due to genetic modifications or exposure to a selected drug, allowing the rapid reappearance of resistant phenotypes in previously antibiotic-susceptible strains (80). This fact highlights the need to detect AMR genes not only in phenotypically resistant isolates, but in all strains that could pose a risk to public health. Over time, random mutations should accumulate in gene sequences that encode resistance to rarely used drugs, because there would be fewer selection events resulting from the use of these antimicrobials (79). If so, we can hypothesize that the use of aminoglycosides, phenicols, tetracyclines, and trimethoprim used to be frequent in Chilean cattle and swine production. Nevertheless, current amount of sales of these compounds for therapeutic use in terrestrial productive animals in Chile is not one of the largest, being surpassed by macrolides, pleuromutilin, and penicillins (81). In Chile, the use of chloramphenicol as a growth promoter is prohibited since 1996 (82), and the use of any kind of antimicrobials for this purpose since 2006 (83). Probably, strains adapted to selective pressure by these antimicrobials became dominant in STEC populations, and now with the reduction in the use of these drugs, some resistance genes mutated and became pseudogenes. Moreover, AMR carries a fitness cost that can reduce bacterial growth rate, competitive ability, or virulence. This high cost could generate selection against resistance, being a relevant factor in the evolutionary dynamics of resistance, especially when bacteria encounter an antibiotic-free environment (84). Taken together, this evidence could explain the high detection levels of bacteria that harbor AMR genes without the associated resistant phenotype.

Regarding detection of class 1 and class 2 integrons, here we detected them in a 5.6% each, and only in strains isolated from swine. Integrons are natural mobile capture systems and assembly platforms that allow bacteria to incorporate gene cassettes and further convert them into functional proteins through proper expression, playing an essential role in the spread of a wide range of resistance genes among different bacterial populations (85). Kennedy et al. detected the presence of class 1 integrons in 21% of non-O157 STEC strains isolated from cattle, while no class 2 integrons were detected (18). More recently, class 1 integrons were detected in 0.8% of STEC strains analyzed (22). The high MDR observed here in the STEC strains isolated from swine could be due to combined presence of class 1 and class 2 integrons.

Some authors have also demonstrated an association between AMR and virulence in STEC strains. Thus, Mora et al. reported higher resistance levels in non-O157 STEC strains isolated from humans, cattle, sheep, and food in Spain that harbored the *eae* gene (86). This gene codes for intimin, it is involved in the attachment/effacing lesions of intestinal epithelia and it is often found in strains related to HUS (6). Later, Buvens et al. reported that non-O157 STEC strains isolated from humans, animals, food, and the environment in Belgium, which harbored the *eae* gene, presented higher resistance against streptomycin, kanamycin, and tetracycline than intimin negative non-O157 STEC strains (50). In this study, only one non-O157 strain harbored the *eae* gene (strain 7), but showed phenotypic and genotypic resistance like the other strains.

STEC strains do not only represent a major risk for public health due to the number of infections in humans and their sequels, but also due to the severe economic losses of the food industry due to the withdrawal of contaminated food products. Presence of STEC strains with phenotypic and/or genotypic resistance is especially relevant when it comes to establishing new antibiotic-based therapies for early-stage STEC infections in humans, which can help prevent serious sequelae (23). In addition, official control of STEC presence in food is progressing worldwide, through the introduction of a discussion paper and project document on "Control of Shiga toxin-producing *Escherichia coli* (STEC) in beef, unpasteurized milk and cheese produced from unpasteurized milk, leafy greens, and sprouts," presented by Chile, the United States of America, and Uruguay at the 50th Session of the Codex Committee on Food Hygiene (87). This joint strategy suggests that, in the short term, not only the presence of this pathogen must be of mandatory surveillance, but also its AMR determinants.

Finally, our results show that non-O157 STEC strains present in the animal component of the animal-human interface in the Metropolitan region of Chile exhibit phenotypic and genotypic resistance against critical and important antimicrobials for human and veterinary use, representing a major threat for public health. Furthermore, these strains could have a competitive advantage in the presence of antimicrobial selective pressure, leading to an increase in food

contamination. This study highlights the need for coordinated local and global actions concerning antimicrobial use in food animal production.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because they are part of a whole-genome sequencing study, not yet published. Requests to access the datasets should be directed to Dr. Nicolás Galarce (ngalarce@ug.uchile.cl).

ETHICS STATEMENT

The animal study was reviewed and approved by the Comité Institucional de Cuidado y Uso de Animales of the Universidad de Chile (permit code 17083-VET-UCH) for obtaining rectal samples from cats and dogs. Ethical approval for samples of pigs and cattle from abattoirs was not required according to national/local legislation.

AUTHOR CONTRIBUTIONS

NG and CB contributed to the conception and design of the study. NG, CB, LL, NL, EP-O, GG-R, and HB-T contributed with resources to the study. FS, BE, VF, RR, DF-C, and AV-L performed the laboratory analyses. RA-M and GA performed the statistical analysis. NG wrote the first draft of the manuscript. RA-M and GA wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) grant number 11170363.

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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.

Citation: Galarce N, Sánchez F, Fuenzalida V, Ramos R, Escobar B, Lapierre L, Paredes-Osses E, Arriagada G, Alegría-Morán R, Lincopán N, Fuentes-Castillo D, Vera-Leiva A, González-Rocha G, Bello-Toledo H and Borie C (2020) Phenotypic and Genotypic Antimicrobial Resistance in Non-O157 Shiga Toxin-Producing Escherichia coli Isolated From Cattle and Swine in Chile. Front. Vet. Sci. 7:367. doi: 10.3389/fvets.2020.00367

frontiers in Veterinary Science

SYSTEMATIC REVIEW
published: 21 August 2020
doi: 10.3389/fvets.2020.00546

Antimicrobial Resistance in Water in Latin America and the Caribbean: Available Research and Gaps

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Introduction: Antimicrobial resistance (AMR) is a public health concern that has gained increasing global awareness, and it is estimated that there will be 10 million deaths annually by 2050. The importance of the role of the environment in disseminating clinically relevant AMR is a concern. Although research on AMR in Latin America and the Caribbean (LAC) has been conducted, these data have not been analyzed together to better understand which areas in AMR have been more studied, and which require more attention.

Objective: Determine the state of knowledge and identify the information gaps for AMR in water in LAC through an exploratory review that identifies the scientific articles that have addressed the topic.

Method: The process of selecting scientific articles from databases consisted of the four phases of an exploratory review focusing on eight themes of interest.

Results: The selection process identified 289 studies that were published between 1973 and October 2017, and these studies were included in the analysis. Most of the research was performed from 2008 to 2017. Brazil was the main contributor to the study of AMR in the region while no research was identified in AMR in water in eight of 18 of LAC countries. The most researched topics in water are phenotypic detection of AMR (theme VIII), detection of antimicrobial resistance genes (ARG) (theme V), and degradation of AMR (theme III). Limited research was identified on insects, agricultural products, aquatic organisms, livestock, and wastewater other than hospital wastewater. Research on emerging pests and diseases with a potential impact on the production of AMR (theme VII), impact of the use of antimicrobials on agricultural production (theme IV), and negative effects of AMR on wildlife (theme II) was scarce.

Conclusions: We suggest to focus research efforts and resources to study themes I, II, IV, VI, and VII, for which there is little research in LAC, without hindering the valuable research conducted on themes III, V, and VIII. The AMR environmental situation is mainly driven by a few countries that are not representative of the LAC region, and therefore, research is needed in other LAC countries besides Brazil.

Keywords: agriculture, antibiotics, health effect, heavy metal, livestock, environment, co-selection, antibiotic resistance genes

OPEN ACCESS

Edited by:
Josefina Leon-Felix, Consejo Nacional de Ciencia y Tecnología (CONACYT), Mexico

Reviewed by:
Fernando Esperón, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Spain
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *11 March 2020*
Accepted: *13 July 2020*
Published: *21 August 2020*

Citation:
Moreno-Switt AI, Rivera D, Caipo ML, Nowell DC and Adell AD (2020) Antimicrobial Resistance in Water in Latin America and the Caribbean: Available Research and Gaps. Front. Vet. Sci. 7:546. doi: 10.3389/fvets.2020.00546

INTRODUCTION

Antibiotics are crucial therapeutic tools to treat infectious diseases in human and veterinary medicine. Since the introduction of antibiotics, millions of human lives have been saved, highlighting the impact of these drugs for humanity (1). In agriculture, antibiotics have played a crucial role for the modernization of food production, which has supported global food security and accessibility of food (2). However, the overuse of antibiotics has led to a major threat for human and animal health, which is the global emergence of antimicrobial resistance (AMR) (2). Antibiotics are largely used in food-producing animals as therapeutic, metaphylactic (to treat healthy animals in the same flock), or prophylactic treatments; to eradicate disease; or to promote growth (2). It is well-recognized that antibiotic exposure is a selective pressure for AMR, in which pathogenic and commensal or native environmental bacteria could acquire AMR (3). A susceptible bacterium can become multidrug resistant in one conjugation event through the transfer of plasmids carrying clusters of antibiotic resistance genes (4). Currently, AMR is one of the most important public health problems, and it is estimated that there will be 10 million deaths annually by 2050; there is also an estimated economic impact of 100 trillion USD if no new interventions are developed and available to combat AMR (5, 6).

Currently, there is worldwide recognition of the role of the environment as an important source and dissemination route of antibiotic resistance (3). The use of antibiotics in food production (e.g., to treat sick animals or plants) and in human activities (e.g., discharge from pharmaceuticals) releases AMR into the environment (7). However, current evidence raises a debate on the major contributor to AMR in the environment and the risk to human health from environmental AMR. An improved understanding of this is extremely relevant in terms of the implementation of mitigation strategies to control AMR dissemination in the environment. Major sources of these contaminants can originate from agricultural, livestock, and human activities, such as application of fertilizer with an animal origin, use of treated wastewater for irrigation, and direct excretion of animal, household, or hospital waste into the environment (8). Sources such as sewage, wastewater treatment plants, and water bodies are recognized as important vectors of AMR, and they play a role in bacterial transmission between hosts (humans, food animals, and wildlife). However, the human health impact that is attributed to the dissemination of environmental AMR is not fully understood and even less understood is the ecological impact of AMR dissemination in the environment (3).

Worldwide, most of the studies have investigated AMR in zoonotic and foodborne pathogens, such as *Salmonella* spp. and *Campylobacter* spp. Studies that have evaluated the links between the use of antimicrobials in animal husbandry and the health impact to humans have been reported in several countries (7). Recently, studies have diversified to integrate the abundance and diversity of AMR in soil and water and their association with spatial and temporal changes (9, 10). The current ability to use cutting-edge technologies, such as high-throughput qPCR or whole genome sequencing to determine the environmental resistome, is starting to advance our current knowledge in this area (9). The human risk of the environmental resistome depends on multiple factors, which varies according to the microorganism, resistance mechanism, location within the genome of the resistance genes (e.g., plasmid-encoded), and environmental factors (e.g., presence of heavy metals) (7).

For AMR in water, most studies have investigated water source categories such as aquatic environment dedicated to aquaculture (fish, shrimp), effluents from wastewater treatment plants, and surface and irrigation water (11–14). However, currently, the role of the aquatic environments with fresh or marine water contamination by AMR is poorly understood. In addition, the main sources of AMR in water are not clear. A review reported the following bias in current studies (8): little data are available to assess the magnitude of the effects of environmental antibiotics and antimicrobial-resistant bacteria and genes. Therefore, although plausible mechanisms for these effects exist, the level of exposure of humans, wildlife, agricultural systems, and natural ecosystems to antimicrobial resistant bacteria and genes in the environment and its consequences has not been elucidated. Consequently, intervention strategies to combat environmental dissemination of AMR are difficult to design and implement (3). Only a few countries have publicly accessible AMR surveillance systems or are active in AMR research in Latin America and the Caribbean (LAC). Although research on AMR in water in LAC has been conducted, most identified research corresponded to individual studies that focused on the description of resistant isolates or resistant genes, mainly in human pathogens, but there has been no analysis that has combined these individual studies. The main objective of the present scoping review is to analyze peer review studies to examine the available information and identify research and/or information gaps regarding eight themes of interest for AMR in water in LAC. The knowledge resulting from this scoping review will provide information to focus research efforts and funding in areas with no or little research in the LAC region.

METHODS

The methods used in this scoping review are consistent with the JBI Reviewer's Manual 2017 guidelines (15). The framework for conducting a scoping review is described in Levac et al. (16) and Khalil et al. (17) and consists of the following processes: (i) identify the research question; (ii) identify relevant studies; (iii) select the studies; (iv) present the data by charting results in a tabular and narrative format; (v) collate the results to identify the implications of the study findings for policy, practice, or research; and (vi) consult with stakeholders (optional). Steps 1 through 3 were specified and documented in advance in a protocol (18). Only relevant information regarding steps 1 through 5 and modifications of the Moreno-Switt et al. protocol (18) will be presented in this section to allow a more fluent and clearer reading of this study. Refer to the Moreno-Switt et al. protocol (18) for further and specific details regarding the process for conducting this scoping review.

Literature Search Strategy and Selection of Studies

A search for published AMR studies in LAC was based on the following eight themes of interest regarding AMR in water: (I) Livestock and aquatic production systems as sources of antibiotic resistance in environmental water; (II) Negative or unexpected effects of AMR and antibiotics on terrestrial and aquatic wildlife living organisms; (III) Degradation of AMR in the environment; (IV) Impact of the use of antimicrobials in agricultural production; (V) Transmission of AMR genes from both humans and animals through water and detection of microorganisms harboring resistant genes; (VI) Crossed AMR between antibiotics and heavy metals; (VII) Emerging pests and diseases with potential impact in the production of AMR; and (VIII) Detection of microorganisms with phenotypic evaluation of AMR in water. Refer to the Moreno Switt et al. protocol (18) for further details on the rationale and description of each theme.

A search for articles published on any of the themes of interest was performed using the electronic databases PubMed, Scopus, and Web of Science from July to October 2017. The process is summarized in **Figure 1**, and it consisted of four phases of a scoping review (15): (i) *Identification*: detection of articles with search criteria consisting of a combination of keywords; (ii) *Screening*: examination of the titles and abstract of articles to determine whether these continue in the selection process or were excluded; (iii) *Eligibility*: revision of the full text to assess suitability; and (iv) *Inclusion*: data extraction and final classification of the selected articles in at least one theme of interest.

The search for articles was based on specific search criteria. The articles that were identified using these search algorithms were subject to a further selection process where the articles were classified into each of the target themes based on specific inclusion criteria or removed if the article met any of the exclusion criteria (18).

Data Extraction

Data on the following variables were extracted and recorded from each article as described in the Moreno-Switt et al. protocol (18): (i) Title; (ii) First author; (iii) Publication year; (iv) Country where samples were collected or the research was executed; (v) Identification number of the paper (Pubmed ID, Doi, or Web of Science accession number); (vi) Matrix of interest from which samples were taken and analyzed; (vii) Antibiotic, biocide, heavy metal or resistant gene evaluated in the study; (viii) Objectives of the study; (ix) Main findings or conclusions; and (x) Gaps identified. The data for each variable was extracted from the selected articles and recorded in an excel spreadsheet. When an article referred to more than one theme or country, the variable was counted and considered as an article. The counts were recorded and analyzed as described in **Figure 2**.

The number of articles identified in this scoping review were classified by decades and countries to identify the variation on

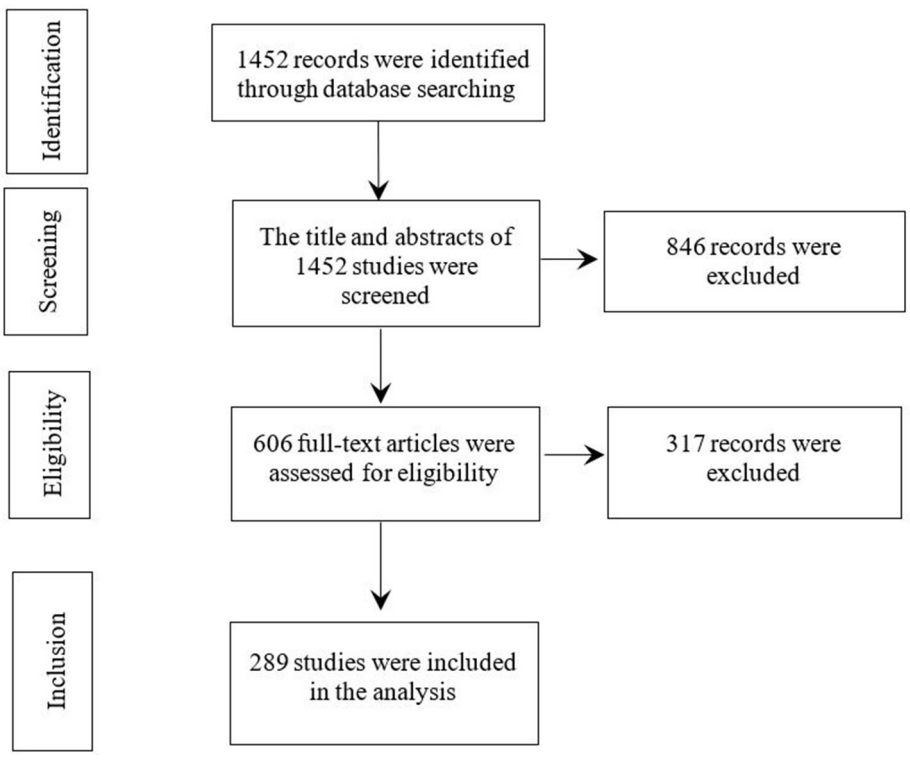

FIGURE 1 | Flow of information through the different phases of a scoping review regarding AMR in water in Latin America and the Caribbean.

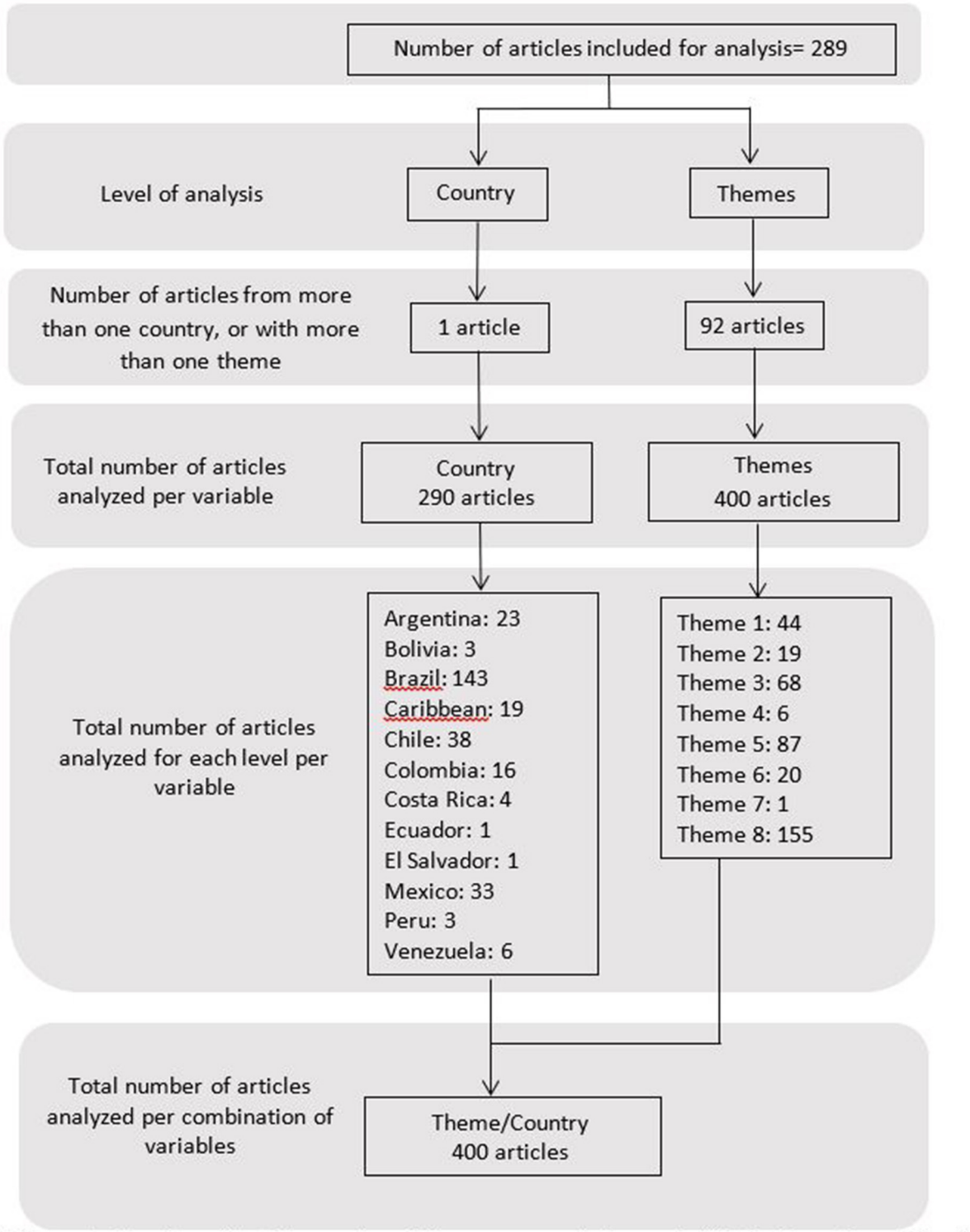

FIGURE 2 | Number of articles analyzed by country and theme, individually and combined.

the number of published studies in any of the themes of interest based on year of publication and country were the samples were collected or the study was conducted. Furthermore, an analysis based on the gross domestic expenditure on research and development (GERD) as a percentage of the gross domestic product (GDP) of each country was conducted. The original

database was downloaded from the UNESCO website (19). Modification of the original database consisted in including only those LAC counties that had GERD data reported in at least 1 year between 2010 and 2015, a mean value for these 6 years was calculated and presented as **Figure 3**.

Initially, attempts were made to contact authors for clarification, but because of the lack of responses, this approach was not implemented throughout the entire study. Analyses of the articles were included in the present scoping review for LAC and the gaps were identified in each of the themes. The quality of each included article was not evaluated because the aim of this scoping review was to identify the available research on AMR in the region's water regardless of the quality of the research.

RESULTS

Available Research on AMR in Water in LAC

Literature Search Strategy and Selection of Studies

The initial search identified 1,452 potentially relevant studies (**Figure 1**). After reviewing the titles and abstracts, 606 studies were considered for further screening because they met the inclusion criteria and none of the exclusion criteria (18), of which 317 were not eligible because at least one of the exclusion criteria was met after revising the full text. After examination of the title, abstract and full text, 1,163 studies were removed, and 289 studies were included for further analysis. The studies identified and included in this scoping review were published between 1973 and October 2017. **Supplemental Table 1** provides a summary of the data that were extracted by publication year, country, and theme, with their respective references.

Available Research Information by Country

In this section, 289 articles were included for all the LAC countries when analyzing the information regardless of the theme studied. However, as one study was conducted in Peru and El Salvador (20), the number of articles analyzed for the various countries increased to 290 (**Figure 2**). As shown in **Figure 4**, Brazil has the most articles included in this scoping review with 143 of 290 articles (49.3%), followed by Chile with 38 articles (13.1%), Mexico with 33 articles (11.3%), Argentina with 23 articles (7.9%), Caribbean countries with 19 (6.6%), Colombia with 16 articles (5.5%), Venezuela with six articles (2.1%), Costa Rica with four articles (1.4%), Bolivia and Peru with three articles (1%) each, and El Salvador and Ecuador with one article (0.3%) each.

Available Research Information by Decade and Theme of Interest

The time frame for studies conducted in LAC that were included for any of the themes of interest was 44 years, from 1973 to October 2017 (**Figure 5A**). During that time frame, 400 studies were identified that analyzed various themes. The difference in the article count between variables is detailed in **Figure 2**, and it was caused because some articles referred to more than one theme. For example, Cordano and Virgilio studied themes IV, V, VI, and VIII, contributing five articles to the total count for the various themes (21). Since 1973, the number of selected studies that were conducted in LAC and that related to at least one of the themes of interest has significantly increased over time (**Figure 5A**). In the 1970s and 1980s, seven of 400 studies (1.75%) were published; in the 1990s, 31/400 (7.75%) were published; in the 2000s, 93/400 (23.2%) were published; and between 2010 and October 2017, 270/400 (67.5%) were published.

When analyzing the information by theme (**Figure 5B**), themes VIII (155/400 articles; 38.8%), V (88/400 articles; 22%), III (68/400 articles; 17%), and I (45/400 articles; 11.25%) were identified as the most studied, while themes II (19/400; 4.7%), VI (20/400; 5%), IV (6/400; 1.5%), and VII (1/400; 0.25%) were the least studied (in descending order based on the number of studies that were identified in this scoping review). **Figure 5C** shows the number of articles included by theme of interest and by publication decade. Themes that show a consistent increase in the number of articles published over time were themes I, II, III, V, and VIII, while themes IV and VI maintained a similarly low number of published articles. Theme VIII was the most studied theme in LAC (155 of 400 articles; 38.8%).

Gaps Identified in the Selected Articles

The gaps that were most frequently reported in the identified articles focused on conducting more research in the following areas: (1) resistance transfer; (2) AMR surveillance; (3) evaluating the health impact of AMR; and (4) improving water treatment for AMR removal. Further analysis of these four gaps is presented in the discussion section.

DISCUSSION

The main objective of this scoping review was to examine the available information to identify research and/or information gaps regarding eight themes of interest for AMR in water in LAC.

Available Research Information by Country, Decade, and/or Theme

There were considerable differences in the number of articles identified by LAC countries, with Brazil having noticeably more articles published that were related to the themes of interest than other LAC countries. One of the many explanations for this difference is the gross domestic expenditure on research and development (GERD) as a percentage of the gross domestic product (GDP) of each country (19). Brazil was shown to have the highest GERD as a percentage of GDP in LAC counties (**Figure 3**). Chile is in the sixth position regarding GERD, and it is the second country in LAC to conduct research on any of the themes of interest, indicating that AMR is of interest to researchers in that country. In addition, Ecuador and Uruguay have a similar GERD compared to Chile, but there was little or no research on any of the AMR themes of interest identified in Ecuador and Uruguay, while Chile was the second country in LAC with more articles related to the themes of interest published. Little to no research was identified for Panama, Nicaragua, Paraguay, Peru, El Salvador, and Guatemala, which could be because of the low level of GERD and/or other country priorities.

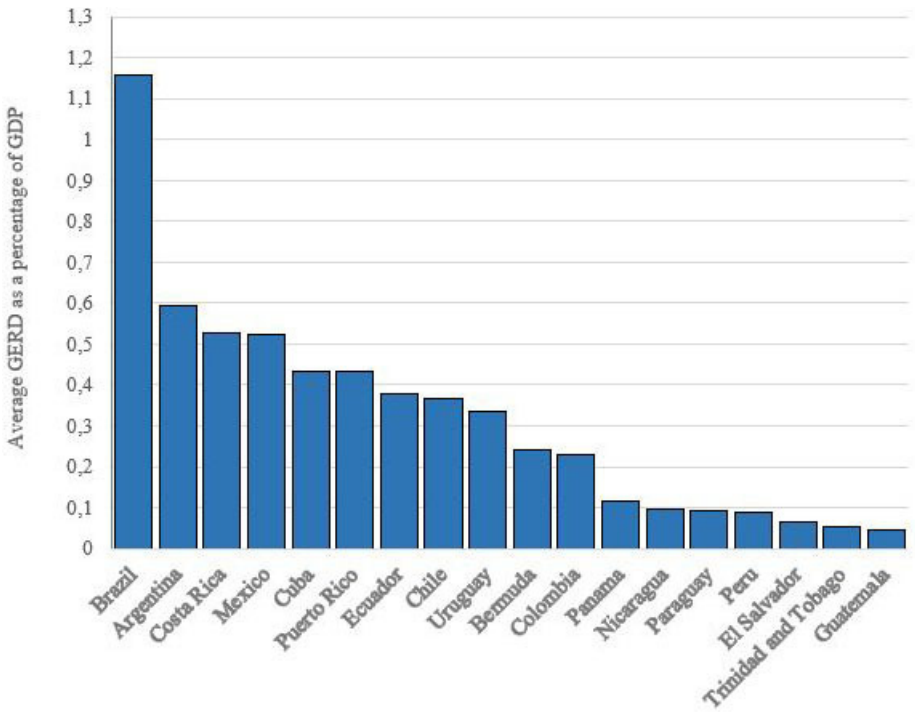

FIGURE 3 | Average of the gross domestic expenditure on research and development (GERD) as a percentage of the gross domestic product (GDP) for 2010–2015. Data were extracted from UNESCO (2018), and data for countries that are not shown in the figure were not reported.

FIGURE 4 | Map of the distribution of published articles included in this scoping review (number of articles included for the country [percentage of included articles at LAC level]). Map created using MAP tool in Microsoft Excel, Powered by Bing ©DSAT for MSFT, GeoNames, Microsoft, Navteq.

When analyzing the data by decade, although the number of studies conducted in LAC that were related to any of the themes of interest has considerably increased since 1973, the selected articles could be underrepresented because LAC researchers often publish in journals that are not indexed in major citation databases and they might not be identified in this scoping review (22). Furthermore, the results for the 2010s could also be underestimated because this period only included papers that

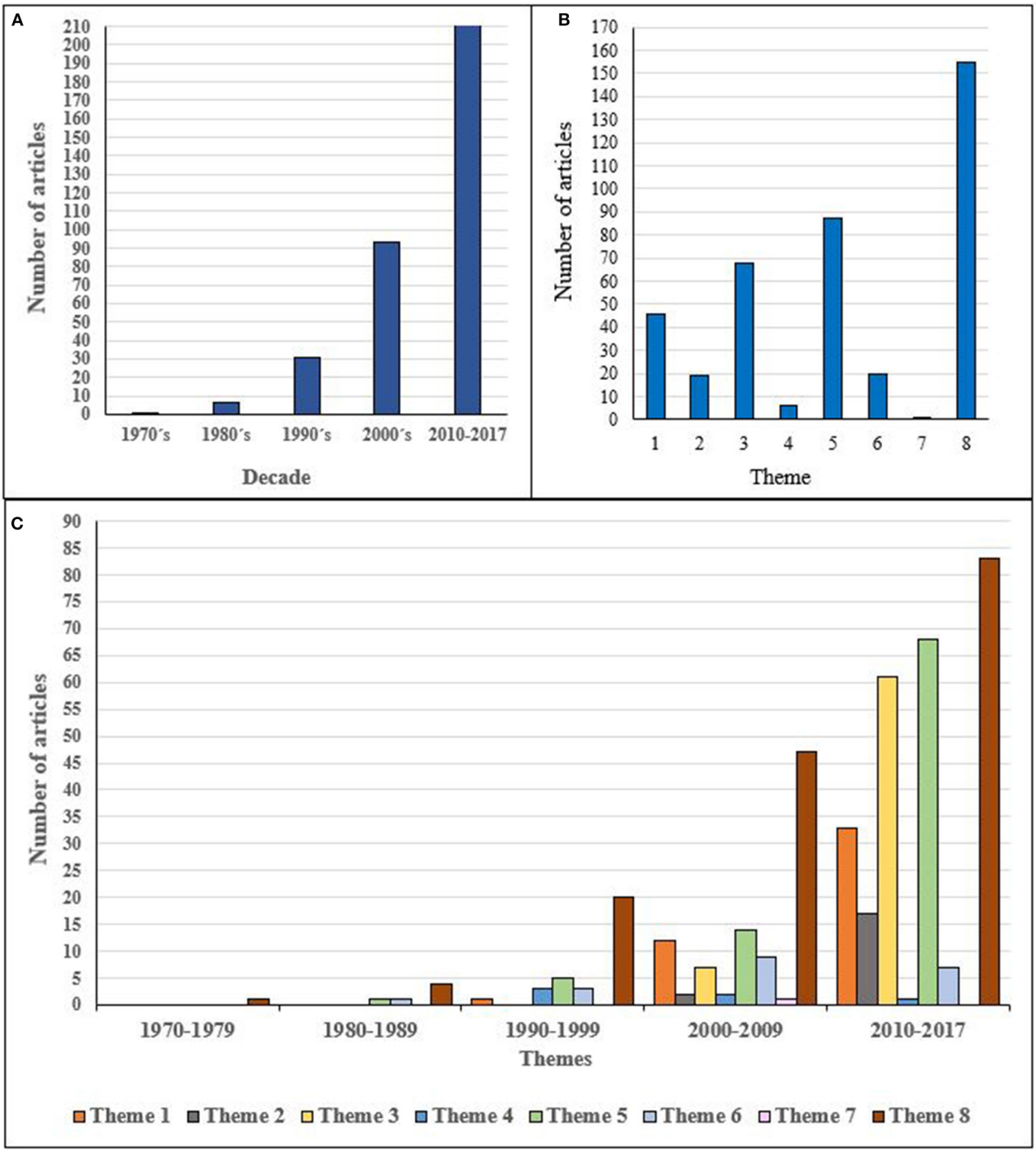

FIGURE 5 | (A) Number of articles for all themes published per decade; **(B)** Number of articles for each theme identified in the selected articles; **(C)** Themes published per decade. Themes: (1) Livestock and aquatic production systems as sources of antibiotic resistance in environmental water; (2) Negative or unexpected effects of AMR and antibiotics on terrestrial and aquatic wildlife living organisms; (3) Degradation of AMR in the environment; (4) Impact of the use of antimicrobials in agricultural production; (5) Transmission of antimicrobial resistance genes from both humans and animals through water and detection of microorganisms harboring detection genes; (6) Crossed antimicrobial resistance between antibiotics and heavy metals; (7) Emerging pests and diseases with potential impact in the production of AMR; (8) Detection of microorganisms with phenotypic evaluation of AMR in water.

were published up to October 2017. However, these data show that there were as many articles published from 2010 to 2017 as there were in previous decades (1980–2009), indicating a notable advancement in AMR research in water in the region. The results presented here follow a similar increasing trend compared to the publication records in Latin America that were reported by Van Noorden (22). The increase in the quantity of research that was identified in the last two decades could be because of the expanding economies in South America (22).

While this is a noticeable increase in the publication of articles regardless of the theme of interest that was studied, there are variations in the publications when the theme is considered. Research related to theme VIII was identified as the most studied theme between 1990 and 2017, and it was the earliest theme with published research. These studies relate to the phenotypic evaluation of AMR in water, focusing mostly on cultivable microorganisms that were pathogenic (e.g., *Vibrio cholerae*, *Shigella*), zoonotic (e.g., *Salmonella*, *Campylobacter*), commensal (e.g., *Escherichia coli*, *Enterococcus*), and environmental (e.g., *Aeromonas*, *Pseudomonas*, *Bacillus*). Furthermore, these studies characterized the antibiotic resistance profile using Kirby Bauer or minimum inhibitory concentration (MIC) upon isolation of microorganisms. While this is relevant information, most of the non-culturable bacteria are not included in these studies and might harbor antimicrobial resistance that has not been evaluated (23). This was the only theme in which a specific search algorithm was not applied (18). Caution is advised when interpreting the data for this theme, because the selection of articles for theme VIII was based on the search criteria that were used in all the other themes. Therefore, the data collected for theme VIII might be underestimated.

Theme V was the second most studied theme and it included articles that aimed to detect an antibiotic or heavy metal-resistant gene in bacterial microorganisms, or that evaluated the dissemination of these genes through plasmids, integrases, transposons, or other mobile genetic elements. The rapid development and current ability to use molecular cutting-edge technologies, such as high-throughput qPCR or whole genome sequencing (9), can explain the increase in publications on this theme. We expect that the number of studies that evaluate this topic will increase in the near future because genomics and metagenomics are methodologies that are becoming more accessible and inexpensive (24), providing relevant information that will cover many of the research gaps that were identified in this scoping review.

Theme III is related to reduction of AMR in water, and it was identified as the third most evaluated theme. Although the interest in theme III had a slow and late start, the number of articles published has been increasing since the year 2000, with the greatest increase in the 2010s. This can be explained by the global awareness that AMR in the environment is an actual concern, and therefore, methods to remove contaminants that are related to AMR (antibiotics, resistance genes, and resistant bacteria) in water are more frequently evaluated because it is crucial to reduce the AMR load in the environment. This theme is also interesting because when analyzing the data in detail, most of the selected studies corresponded to reduction of antimicrobials (antibiotics, antimicrobials, and/or anti-tuberculosis drugs). Few studies focused on reducing heavy metals and resistant bacteria harboring resistant genes or bacteria that were phenotypically identified as resistant to heavy metals or antibiotics. The number of studies that evaluated the reduction in antimicrobials, anti-tuberculosis drugs, and heavy metals could be underestimated because the search algorithms focused only on antibiotics. Only one of the selected studies evaluated the reduction of antimicrobial genes that were released by killing bacteria using the evaluated methods (25). Therefore, there is an urgent need to evaluate the reduction of heavy metals, resistant bacteria, and resistant genes in environmental water because all of them are associated with the AMR overall problem as the main sources of contamination (26).

Theme I relates to livestock and aquatic production systems as sources of antibiotic resistance in environmental water. The studies identified for this theme focused on terrestrial productive animals including cattle, dairy buffaloes, poultry, and swine, and on aquatic organisms such as fish, prawns, bivalves, algae, and aquatic amphibians. These studies evaluated the presence of antibiotic-resistant bacteria using phenotypic and genetic analysis. In most cases, bacterial studies correspond to the matrix evaluated. For example, in studies conducted on poultry, the target bacteria were mainly *Salmonella* or *Campylobacter*, and in those conducted on aquatic organisms, the target bacteria were mainly *Vibrio*, *Aeromonas*, and *Pseudomonas*. For this theme, none of the papers studied the link between the use of antimicrobials in animal production and generation of AMR in the environment, specifically in water. Instead, the papers focused on research that evaluated the presence of resistant microorganisms in feces of productive animals or the environment. In this scoping review, articles related to the evaluation of AMR in feces or productive animal premises were included under the assumption that the AMR detected in feces and premises could be disseminated at some point into environmental water. Articles that evaluated AMR in animal tissues were excluded because it was assumed that the presence of AMR microorganisms or genes from those tissues were not linked directly to environmental water. This agrees with global trends in which most of the studies investigated a link between animal production and human health, and mostly focused on foodborne and zoonotic pathogens, but without looking at the impact on the environment (7).

Theme II narrates the negative or unexpected effects of AMR and antibiotics on terrestrial and aquatic wildlife living organisms. Studies included in this category evaluated the impact of antibiotics or resistant bacteria on the health of only aquatic organisms (shellfish larvae, fish, shrimp, bivalves, crustaceans, amphibians, and algae), insects (Diptera), and the microbial community from the sediment in a water source. Studies related to terrestrial animals that consisted of evaluating the presence of AMR in feces or tissue without reporting negative health effects, were excluded. The health effects reported in the articles that were included for this theme could be categorized into the following two types of studies: (i) those that evaluated the health effects of antibiotics using *in vivo* models with outcomes such as survival rates, growth, cellular, and metabolic changes, among others;

and (ii) those that assessed the health effects of antimicrobial resistant bacteria and its relationship with outcomes such as disease, mortality, lesions, and outbreaks. These findings suggest that further research is needed to understand the health impact of AMR, especially on wildlife and terrestrial animals, which is currently lacking.

The number of published articles in themes I and II have also increased over time, although not as dramatically as for themes III, V, and VIII. From a global perspective, the link between the use of antimicrobials in production animals and the impact of AMR on the environment is controversial, and the problem caused by the existing link between antimicrobial drug use on farms and human health risks associated with antimicrobial resistant infections must be solved (7). Thus, more research regarding these topics should be conducted in the near future.

Themes with a similarly low number of published articles are themes IV and VI. The number of articles identified for theme IV was low and the publication rate has slowly decreased by decade since the 1990s, suggesting that this topic is not a priority for LAC countries. Theme IV corresponds to the impact of using antimicrobials in agricultural production. Each of the included studies investigated different produce (tomatoes, carrots, lettuce, and legumes). Moreover, these articles focused on the following three main groups of microorganisms: (i) foodborne pathogens (*Salmonella* and *E. coli*); (ii) phytopathogen (*Erwinia carotovora*); and (iii) soil associated bacteria (*Rhizobia* stains). The most frequent methodology was based on phenotypic evaluation of antibiotic resistance, and only one study focused on detection of bacteria harboring resistance genes for antibiotics. None of these studies focused on heavy metal resistance or on evaluating antimicrobial use in agriculture and the presence of antimicrobial resistant bacteria, focusing instead on detection of AMR in produce. Although the LAC region is considered to be an important agricultural producer for both local and international consumption, few studies were identified for this theme (27). A similar trend was identified for theme VI, which corresponded to evaluating cross-resistance between antibiotics and heavy metals. The articles selected for this theme consisted mostly of studies that detected microorganisms that were resistant to both antibiotics and heavy metals, but there was no specific research to identify and find an association between them. There was a reduction in the number of articles published in the 2010s. There are 2 years left before the beginning of the 2020 decade, and therefore, the number of papers for themes IV and VI could reach the same level as in the 2000s.

Theme VII, which is related to emerging pests and diseases with a potential impact on the production of AMR, was the least studied, with only one study identified (28). In this study, both pathogenic and environmental resistant bacteria were isolated from Mexican fruit flies, providing evidence that insects could play a role in disseminating AMR (28). More research on this theme must be conducted in LAC to provide information about the role insects play in disseminating AMR in the environment.

The trends identified in this scoping review could be explained by the worldwide relevance of the AMR problem and by the development of research infrastructure (laboratories and equipment), knowledge (universities, training courses, post graduate research, and training), and regional interest in the development and innovation (I+D) of research (e.g., creation of national commissions for scientific and technological Research in LAC) (22). Furthermore, the development and improvement of detection methods (cutting-edge technologies) such as whole genome sequencing (WGS) allow for more advanced research on AMR such as elucidation of the genetic mechanisms that underlie resistance in different multi-drug-resistant organisms and to characterize the relationship between antibiotic resistance determinants that are found in different human and environmental reservoirs (29). The FDA is using WGS to monitor pathogen occurrence and persistence in the environment and the food industry and to monitor for potentially emerging pathogens or development of antibiotic resistance (30, 31). Thus, an increase in research in theme V is expected.

Gaps Identified in the Selected Articles

Resistance Transfer

Several of the included studies agreed that more research should be conducted to evaluate resistance transfer, and two of these studies referred to the transmission among and between animals (32), especially those related to *Campylobacter jejuni* and animals besides broiler chickens and humans (33). The findings of this scoping review are consistent with this gap because most of the studies identified in theme I were conducted on poultry. Other studies referred to the need for research related to the resistance transfer into the environment. Research on AMR transfer in wastewater (34), hospitals, hospital effluents, natural environments (35–37), and irrigation canal sediments and vegetable surfaces (38) was reported as essential. Many of the articles included in this scoping review were conducted in wastewater, especially hospital sewage, and within the themes that were related to AMR transfer (themes V and VI). Few studies conducted on AMR in produce were identified and included, which is consistent with the knowledge gap reported by Roe et al. (38). Several articles reported that there is a need to study the mechanisms and molecular basis of AMR transfer and spread to and among microorganisms (39, 40), especially to those of clinical relevance for animals and/or humans (41–43). Because research on theme V has been increasing significantly in the last decade, in addition to molecular methods becoming more accessible and affordable, more research is expected to be conducted to fill this gap.

AMR Surveillance

Several of the selected articles in this scoping review frequently reported the need for more AMR surveillance and monitoring to evaluate undesirable consequences on the surrounding environments (44), the local biota, and population health (45, 46). In addition, it was reported that surveillance to better understand the emergence and spread of AMR in microbiota in hatcheries and salmon farms is also needed (44, 47). This agrees with the small number of articles that were identified for theme II in this scoping review. Several studies suggested the needed for continuous surveillance and monitoring for the emergence and spread of AMR (48, 49). Among the studies conducted on aquiculture, Miranda et al. (50) indicated that a surveillance

program is necessary to monitor the continuing evolution of distribution of tetracycline genes in fish farm environment outside Japan, Europe, and North America. This gap and the few articles that were identified in the themes of interest in this scoping review emphasize the need for more research in this area in LAC. The same author indicated that surveillance to document the spread of antibacterial resistance in microbiota associated with scallop larvae and rearing hatcheries in Chile is also required (44), especially for florfenicol resistance (47). Aizawa et al. (51) pointed out the need to evaluate the changes in the epidemiology and frequency of extended spectrum beta-lactamases (ESBLs) in different ecosystems and animal hosts. This is in agreement with Palhares et al. (52), who stressed the need for detection of antibiotic resistance in microorganisms in basins that are characterized by intensive animal production. The few articles conducted in superficial water and related to themes I and VIII that were identified in this scoping review focused on aquiculture water rather than water in proximity to terrestrial animal production, which also highlights the need for more research on this topic. Based on the information provided by some of the selected studies, continuous surveillance and monitoring using molecular techniques are critical to better understand of the emergence and spread of AMR in aquatic and terrestrial environment and their microbiota.

Evaluation of the Health Impact of AMR

Gaps related to evaluation of the AMR health impact in aquatic organisms were reported most frequently. Expanding knowledge about the consequences of sub-lethal effects and chronic exposure to antimicrobials and their metabolites in aquatic invertebrates, fish, other aquatic species, and aquatic environment was identified as an important necessity (53, 54). Peltzer et al. (55) pointed out the need for additional risk assessments to show bioaccumulation of antibiotics in tissue and organs of amphibian larvae and consequently ecological impairments in water and aquatic system sediments. Several authors pointed out the need for toxicological studies to evaluate and better understand the health risk that these bacteria may present (54, 56, 57). However, toxicological evaluation was not included in this scoping review because the focus was on AMR in the water. In addition, the aquatic organisms that were mainly studied corresponded to articles related to theme II, especially fish and aquatic invertebrates. Therefore, gaps related to aquatic organisms were expected. Controversially to the reports on fish, no studies conducted on wildlife or terrestrial productive species, including outbreaks occurring on terrestrial animals related to AMR, were identified in this review. Therefore, more studies are needed to expand the knowledge about the effect of AMR in these animal species.

Water Treatment

The theme that has the greatest increase over time has been the removal of AMR (theme III) and antibiotics from water. However, numerous gaps related to this area were identified in the selected studies, highlighting the necessity for more research to design more efficient ways of treating different wastewater (58) and aquiculture systems (59) to ensure the removal of AMR. More effective sewage treatment to remove pathogens (60, 61), suitable wastewater purification procedures before their discharge into receiving waters (62), and approaches that reduce environmental microbial contamination by hospitals (63) in developing countries (64) were identified as important measures that require implementation. To achieve efficient AMR removal from the water, re-evaluation of the criteria, laws, and regulations used to analyze the microbial quality of drinking water (65) and sewage treatment beyond hospitals (66) is required. Based on our knowledge, there are no water treatment regulations or laws that consider the removal of AMR from water. To create adequate policies, the environments that are potential sources of resistance traits that pose a threat to human populations need to be determined (67). In addition, greater use of massively paralleled deep sequencing approaches for community analysis to identify novel genes and indicators to assess water quality are also required (68). In summary, removal of AMR from water requires education and training, molecular methods to easily detect AMR in water, and adequate regulations and laws about AMR in water treatment plants especially in wastewater, aquiculture farms, and hospitals.

Recommendations Related to AMR in Water

Among the selected studies, several pointed out that to reduce the AMR problem, responsible prescription and use of antibiotics on productive animals, aquiculture farming, and humans is required (33, 69–71). Surveillance to establish accurate measures that eventually safeguard the effectiveness of last-resort antibiotics is also crucial to control AMR (72). This might be more important in developing countries because environmental risk associated with the use and emission of pharmaceuticals into the environment in developing countries might be higher than in developed countries, although more research in needed to test the validity of this hypothesis (73). Education and training for professionals about adequate drug prescribing is essential (52, 74) as well as education for farmers and the general public on the harm done by improperly using antimicrobials (52) are also control measures that could be implemented to reduce the AMR problem. In addition, regulations could be implemented on the sale of aquatic invertebrates (such as scallops) when antibiotics (such as florfenicol) has been used (75). Improving water treatment to address the AMR phenomenon, especially water associated with aquaculture systems and wastewater treatments, was another control measure that was reported to be required (59, 76, 77). These suggestions are in agreement with the WHO Global action plan on antimicrobial resistance, and therefore, are measures that urgently require implementation.

CONCLUSIONS AND KEY MESSAGES

Most of the research conducted in LAC that was identified in this scoping review was conducted in the present decade with Brazil as the main contributor to the study of AMR in the region, followed by Chile, Mexico, and Argentina. Although at least one scientific publication was identified for most of the LAC countries

many countries (eight of 18 LAC countries evaluated) have not conducted research regarding AMR in water. This is a growing concern because the studies on the environmental situation for AMR is mainly driven by Brazil, Chile, Mexico, and Argentina, which represent almost 75% of the total area of the LAC region.

Overall, the most investigated themes were themes VIII (phenotypical selection of AMR in water) and V (molecular detection of resistance genes and plasmids in water). However, there is a rapid increase in research on theme III (degradation of AMR in water).

For theme III, most of the studies that were identified in this systematic review evaluated methods to remove either resistant microorganism or antibiotics. However, none of these studies evaluated the environmental impact of reducing resistant genes, microorganisms harboring resistant genes, or antibiotics or their residues from water. This means that no study was conducted to establish a threshold level of AMR in the environment to reach a significant beneficial impact in both humans and animal health. More studies should be conducted in these areas.

Although research on all the themes of interest was identified in at least one LAC country, there has been little research on themes I, II, IV, VI, and VII that was identified in this systematic review. For example, the only study that isolated resistant microorganisms from an insect was conducted in Mexico. Therefore, we suggest that research efforts and resources should be focused on studying these themes, without hindering the valuable research conducted on themes III, V, and VIII.

More research is needed in other LAC countries besides Brazil because studies from this country might be influencing the AMR reality of the entire LAC region. There is an urgent need for research from countries such as Uruguay, Paraguay, Guatemala, Honduras, Nicaragua, and Panama because no published articles were identified in these countries for any of the themes of interest. In addition, more research should be conducted on those countries with few publications identified such as Ecuador, El Salvador, Bolivia, Venezuela, and the Caribbean.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AM-S, DR, MC, DN, and AA contributed to the conception and design of the study, analyzed the results, contributed to the manuscript revision, and read and approved the submitted version. AM-S, DR, and AA followed the steps for a scoping review and collected the data for each one of the themes analyzed in this study. AA wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

FAO, Regional Office for Latin America and the Caribbean funded this project. We recognize the sources of funding: FONDECYT 11160116 to AA. This funding source had no role in the study design, data collection, data analysis, interpretation of data, the writing of the report, or the decision to submit the manuscript for publication. This research was also supported, and the publication fee was covered by the ANID Millennium Science Initiative/Millennium Initiative for Collaborative Research on Bacterial Resistance, MICROB-R, NCN17_081.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00546/full#supplementary-material

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 August 2020
doi: 10.3389/fvets.2020.00503

Genome Characterization of *mcr-1*–Positive *Escherichia coli* Isolated From Pigs With Postweaning Diarrhea in China

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Diarrheagenic *Escherichia coli* is the causative agent of diarrhea in infants and animals worldwide. Many isolated strains recovered from pigs with postweaning diarrhea are multidrug resistance (MDR), and hybrids of *E. coli* are potentially more virulent, as enterotoxigenic *E. coli* (ETEC)/Shiga-toxigenic *E. coli* (STEC) hybrids. Here, we used whole-genome sequencing to analyze clinical isolates of the five colistin-resistant *E. coli*. The *E. coli* CAU15104, CAU15134, and CAU16060 belonged to ETEC/STEC hybrids, displaying the same serotype O3:H45 and sequence type ST4214. The *E. coli* CAU16175 and CAU16177 belonged to atypical enteropathogenic *E. coli* (aEPEC), display O4:H11 and O103:H2, ST29, and ST20, respectively. The *E. coli* CAU16175 carries six plasmids. An IncHI2-type plasmid, pCAU16175_1, harbors an IS*26*-enriched MDR region, which includes 16 antimicrobial-resistant genes. An IncFII-type plasmid, pCAU16175_3, harbors *mcr-1.1*, *tet*(M), and bla_{TEM-1B}, whereas *mcr-1.1* is located within a Tn*2* derivative. Our findings indicate that the ETEC/STEC strains of the O3:H45 serotype as well as the aEPEC strains of the O4:H11 and O103:H2 serotypes are associated with postweaning diarrhea in swine and that some of diarrheagenic *E. coli* contains IS*26*-enriched MDR region and the *mcr-1* gene located within a Tn*2* derivative on IncFII plasmid.

Keywords: *Escherichia coli*, whole-genome sequencing, multidrug resistance, *mcr-1*, Tn*2*, swine

OPEN ACCESS

Edited by:
Rafael Vignoli,
University of the Republic, Uruguay

Reviewed by:
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University of Buenos Aires, Argentina
Nilton Lincopan,
University of São Paulo, Brazil

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *01 April 2020*
Accepted: *02 July 2020*
Published: *25 August 2020*

Citation:
Guo L, Wang J, Wang S, Su J, Wang X and Zhu Y (2020) Genome Characterization of mcr-1–Positive Escherichia coli Isolated From Pigs With Postweaning Diarrhea in China. Front. Vet. Sci. 7:503. doi: 10.3389/fvets.2020.00503

INTRODUCTION

Diarrheagenic *Escherichia coli* (DEC) is a leading cause of infectious diarrhea in humans and animals around the world (1, 2). Diarrheagenic *E. coli* has six well-described pathotypes: enteropathogenic *E. coli* (EPEC), which is subdivided into typical EPEC (tEPEC) and atypical EPEC (aEPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli*, and enterohemorrhagic *E. coli*, which is subgroup of Shiga-toxigenic *E. coli* (STEC) (3, 4). All of these DECs possess diverse virulence factors, which are encoded by virulence genes and are responsible for their pathogenicity (5). Enterotoxigenic *E. coli* strains typically produce one or two toxins, heat-labile enterotoxin (LT) encoded by *ltc*, and heat-stable enterotoxin (ST) encoded by *st* (6). In pigs, STEC strains are characterized by producing the Shiga-like toxin variant Stx2e encoded by *stx2e* (7). Enteropathogenic *E. coli* strains are defined as forming the attaching and effacing (A/E) lesions mediated by genes located on the locus of enterocyte effacement (LEE) pathogenicity island (including *eae*) in the intestinal epithelium but not produce Shiga-like toxin

(Stx) (8). Moreover, *bfpA*, which encodes the major subunit of bundle forming pili, is used to subdivide EPEC into tEPEC and aEPEC. Hence, aEPEC strains are defined by eae^{+}, $bfpA^{-}$, and stx^{-} (9). Three pathotypes (ETEC, STEC, EPEC) are major DEC causing postweaning diarrhea (PWD) in pigs, and many hybrids of *E. coli* (ETEC/STEC, ETEC/STEC/EPEC) are present during PWD (6, 10). The recent data from The Global Enteric Multicenter Study showed that tEPEC and ETEC associated with a higher risk of fatal outcomes in children younger than 24 months with moderate to severe diarrhea (11). Atypical EPEC has outbreaks linked to diarrhea in children (12).

The occurrence of antimicrobial resistance and prevalence of multidrug resistance (MDR) Gram-negative Enterobacteriaceae has been increasing worldwide between humans and animals (13). In the cases of PWD, antimicrobial treatment has been widely used and caused severe drug resistance in DEC (1, 6, 14). In our previous study, among the 171 *E. coli* isolates, 94.15% of the strains were MDR, with antimicrobial resistance rates ranging from 2.34% for meropenem to 90.05% for nalidixic acid (6). Among the ETEC strains that cause PWD, resistance to apramycin, neomycin, trimethoprim sulfonamide, and colistin has been increasingly observed (1). Multidrug resistance strains may spread from animals to humans, causing antibiotics ineffective and increasing mortality and morbidity in developing countries (15).

Colistin as the last resort treatment against MDR bacterial infections may have been challenged by the mobile colistin-resistant gene (*mcr-1*), which has received widespread attention in different species of Enterobacteriaceae found in animals and humans around the world since it was first reported (16–19). So far, nine allelic variants of *mcr-1* (*mcr-2* to *mcr-10*) have been detected (20, 21). In addition, they have large number of variants, such as *mcr-1* (*mcr-1.1* to *mcr-1.22*), *mcr-2* (*mcr-2.1* to *mcr-2.3*), *mcr-3* (*mcr-3.1* to *mcr-3.30*), *mcr-4* (*mcr-4.1* to *mcr-4.6*), *mcr-5* (*mcr-5.1* to *mcr-5.4*), and *mcr-8* (*mcr-8.1* and *mcr-8.2*) (22). Our previous study found that the resistance rate to colistin was 20.47% in 171 *E. coli* isolates (6), and a recent article reported that direct sample testing rates of *mcr-1* were higher than the rates of *mcr-1*–positive *E. coli* (64.6 vs. 49.2%) (23).

Insertion sequences (ISs) and transposons (Tns), arguably most numerous autonomous transposable elements, are crucial to shape their host genomes, particularly important in the bacterial antimicrobial resistance (24). IS*26* and Tn*2*, which is a member of the IS*6* family and Tn*3* family, respectively, play a key role in the dissemination of antimicrobial-resistant genes in Gram-negative bacteria (25, 26). IS*26* is often existed in MDR Gram-negative bacteria, which usually carry large regions containing several antimicrobial-resistant genes that are flanked by and interspersed with copies of IS*26* (25). Tn*2* is the most abundant in commensal *E. coli*. (27). IS*Swi1*-m2 in pNJST258C2 is a derivative of Tn*2* that includes IS*26* compared with Tn*2* (28). IS*Apl1*, which is an IS initially identified in *Actinobacillus pleuropneumoniae*, is a member of the IS*30* family and is considered to be an essential element in the mobilization of *mcr-1* (29, 30).

In the present study, we used whole-genome sequencing (WGS) to analyze virulence and resistant genes of the five clinical colistin-resistant *E. coli* isolates recovered from pigs with PWD and to characterize the complete sequence of a Tn*2* derivative carrying *mcr-1.1* on IncFII plasmid, pCAU16175_3, from a swine aEPEC isolate.

Abbreviations: DEC, diarrheagenic *Escherichia coli*; WGS, whole-genome sequencing; MDR, multidrug resistance; ETEC, enterotoxigenic *E. coli*; STEC, Shiga-toxigenic *E. coli*; aEPEC, atypical enteropathogenic *E. coli*; tEPEC, typical enteropathogenic *E. coli*; PWD, postweaning diarrhea; IS, insertion sequence; Tn, transposon; PCR, polymerase chain reaction; DR, direct repeat.

MATERIALS AND METHODS

Bacterial Isolation and Identification

A total of 455 *E. coli* strains were obtained from feces samples or small intestinal content from pigs with diarrhea in China between 2014 and 2016 and identified with polymerase chain reaction (PCR) amplification of the *uspA* gene (6). Of these, 171 *E. coli* isolates were further screened by antimicrobial susceptibility testing, and the resistance rate of colistin was 20.47% (6). From these 35 colistin-resistant *E. coli*, we observed ST4214 was a major clone (6/35, 17.14%) based on the multilocus sequence typing and caused severe damage to IPEC-J2 cells (6). Then, ST4214 *E. coli* CAU15014, CAU15134, and CAU16060 were selected from different swine farms (one isolate per farm), and two MDR aEPEC isolates (*E. coli* CAU16175 and CAU16177) were randomly chosen for WGS.

Antimicrobial Susceptibility Testing

The susceptibility of the five isolates to 18 antimicrobials were tested by determining the minimum inhibitory concentration (MIC) using the US Clinical and Laboratory Standards Institute (CLSI) broth micro method (31). The results of MIC for ampicillin (AMP), co-amoxiclav (AMC), cefazolin (CZ), kanamycin (KAN), gentamicin (GEN), amikacin (AMK), tetracycline (TE), trimethoprim-sulfamethoxazole (SXT), ciprofloxacin (CIP), nalidixic acid (NAL), chloramphenicol (CHL), and nitrofurantoin (NIT) were interpreted according to guidelines of CLSI 2016 M100-S26 (31). The results of MIC for ceftiofur (EFT), enrofloxacin (ENR), and florfenicol (FFC) were interpreted according to CLSI VET01-A4 (32). In addition, the results with MIC values were defined resistant: streptomycin (STR) $\geq$64 μg/mL (33), olaquindox (OLA) $\geq$64 μg/mL (34), and polymyxin B (PB) >2 μg/mL (35). *Escherichia coli* ATCC 25922 was used as the quality control. According to the MIC determined for each antimicrobial, the isolates were defined as "susceptible," "intermediate," or "resistant."

Whole-Genome Sequencing

Bacterial isolates were recultured from stock; DNA was extracted using a TIANamp Bacteria DNA kit (Tiangen Biotech Inc., Beijing, China). We used WGS by the Illumina Hiseq platform to get draft genome of the five *E. coli* isolates and further used WGS by the Oxford Nanopore Technologies MinION platform to get complete genome of the *E. coli* CAU16175.

The complete genome assembly was constructed from the two sequence data sets using Unicycler (Shanghai Majorbio Biopharm Technology Co., Ltd., Shanghai, China). The serotype, multilocus sequence type, plasmid type, antimicrobial-resistant gene, and virulence gene detection were performed using the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk). Insertion sequence typing was carried out using the ISFinder database (https://www-is.biotoul.fr/). The complete genome sequences were initially annotated with Rapid Annotation using the Subsystem Technology server (http://rast.nmpdr.org) and curated manually using the BLAST server (https://blast.ncbi.nlm.nih.gov/Blast). The obtained plasmid sequences were aligned with homologous plasmid sequences from NCBI using the BRIG tool (36).

Conjugation Assay

The conjugation experiment was carried out using the *E. coli* CAU16175 as the donor and the *E. coli* J53 (resistant to sodium azide) as the recipient. The transconjugant was screened on BHI agar plates containing sodium azide (150 mg/L) and colistin (4 mg/L). The presence of *mcr-1* in the transconjugant was confirmed by PCR, and the MICs of antimicrobial agents for the transconjugant were determined using the agar dilution method. The conjugation experiment was repeated three times, and the conjugation frequencies were calculated as the number of transconjugants per recipient. The transconjugant strains were distinguished from donor occurring natural mutants of sodium azide–resistant *E. coli* by verifying *eae*, which is marker gene for *E. coli* CAU16175 donor.

GenBank Accession Number

The draft genome sequences of the *E. coli* CAU15104, CAU15134, CAU16060, and CAU16177 isolates have been deposited at GenBank under SRA accession no. SRR10828049, SRR10828048, SRR10828047, and SRR10828046, respectively. The complete genome sequences of the *E. coli* CAU16175 have been deposited at GenBank under SRA accession no. SRR10813965.

RESULTS

Antimicrobial Susceptibility Testing of the Five *E. coli* Isolates

Antimicrobial susceptibility testing of the five isolates showed that they were MDR exhibiting resistant to at least three different classes of antimicrobials (**Table 1**). All the isolates showed MDR to KAN, STR, TE, SXT, NAL, and PB.

Characterization of the *E. coli* Isolates From the Draft Genome Sequences

The clinical isolates of the *E. coli* CAU15104, CAU15134, and CAU16060 belonged to ETEC/STEC (*ltcA*$^+$, *stb*$^+$, and *stx*$^+$) hybrid strains, display the same serotype O3:H45 and sequence type ST4214, which are identical sequence type with the strains swine19 (LVMV00000000), swine22 (LVMY00000000), swine54 (LVMV00000000), and swine67 (LVOR00000000) from the NCBI database, all carrying *fedA*, *fedF*, *iha*, *stb*, *ltcA*, *sepA*, *stx2A*, and *stx2B* virulence genes and antimicrobial-resistant

TABLE 1 | Antimicrobial susceptibility profile of the five *E. coli* isolates.

Strain	Antibiotics															
	AMP	AMC	EFT	CZ	KAN	GEN	STR	AMK	TE	SXT	CIP	ENR	NAL	CHL	FFC	NIT	OLA	PB
CAU15104	S	R	R	R	R	S	R	I	R	R	R	R	R	R	S	S	I	R
CAU15134	R	S	S	S	R	S	R	S	R	R	S	S	R	S	I	I	S	R
CAU16060	R	R	I	S	R	S	R	S	R	R	I	R	R	R	S	R	I	R
CAU16175	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	S	I	R
CAU16177	S	R	R	S	R	R	R	S	R	R	S	I	R	R	S	R	I	R

AMP, ampicillin; AMC, co-amoxiclav; EFT, ceftiofur; CZ, cefazolin; KAN, kanamycin; GEN, gentamicin; STR, streptomycin; AMK, amikacin; TE, tetracycline; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; ENR, enrofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FFC, florfenicol; NIT, nitrofurantoin; OLA, olaquindox; PB, polymyxin B.

genes conferring resistant to KAN (*aph(3′)-Ia*), STR (*aadA2*, *strA*, and *strB*), TE [*tet*(A) and *tet*(M)], SXT (*dfrA12*, *sul1*, *sul2*, and *sul3*) and PB (*mcr-1.1*) (**Tables 1**, **2**). Besides, the *E. coli* CAU15104, CAU15134, and CAU16060 carry *cmlA1* and *floR*, which are associated to phenicol antibiotic (**Table 2**). The *E. coli* CAU15104 and CAU15134 carry *qnrS2* (associated to quinolone antibiotic) and *aac(6′)Ib-cr* (associated to quinolone and aminoglycoside antibiotic). The *E. coli* CAU15134 and CAU16060 carry *dfrA1* (associated to SXT), *mcr-3.1* (associated to PB), *oqxA*, and *oqxB*, which are efflux pump conferring antibiotic resistance, and exhibit resistant to AMP associated to $bla_{\mathrm{TEM-1B}}$.

The clinical isolates of the *E. coli* CAU16175 and CAU16177, belonged to aEPEC (eae^{+}, $bfpA^{-}$, and stx^{-}), display different serotype and sequence type, O4:H11 and O103:H2, ST29 and ST20, respectively. They both carry *cif*, *eae*, *espA*, *espB*, *espJ*, *gad*, *iss*, *nleB*, and *tir* virulence genes and antimicrobial-resistant genes conferring resistance to beta-lactam antibiotic ($bla_{\mathrm{OXA-1}}$), KAN (*aph(3′)-Ia*), GEN (*aac(3)-IVa*), STR (*aadA1* or *aadA2* or *strA* or *strB*), TE [*tet*(A) and/or *tet*(M)], SXT (*dfrA12*, *sul1*, *sul2*, *sul3*, and/or *dfrA14*), and PB (*mcr-1.1* and/or *mcr-3.1*) (**Tables 1**, **2**). In addition, the *E. coli* CAU16175 and CAU16177 both carry *aac(6′)Ib-cr* (associated to quinolone and aminoglycoside antibiotic), *aph(4)-Ia* (associated to aminoglycoside antibiotic),

TABLE 2 | Overview of the molecular typing, antimicrobial resistance genes and virulence genes of the five *E. coli* isolates from the whole-genome sequencing result by the Illumina Hiseq platform (CAU16175 Illumina/Nanopore) and four ST4214 *E. coli* strains from the NCBI database.

Strain	Collection date	Collection province	Serotype	MLST	Plasmid type	Antimicrobial resistance gene	Virulence gene	GenBank accession no.
CAU15104	2015	Shandong	O3:H45	ST4214	IncF, IncI1	*aac(6′)Ib-cr*, *aadA1*, *aadA2*, *aph(3′)-Ia*, $bla_{\mathrm{CMY-2}}$, *cmlA1*, *dfrA12*, *erm*(B), *floR, mcr-1.1*, *mph*(A), *qnrS2*, *strA*, *strB*, *sul1*, *sul2*, *sul3*, *tet*(A), *tet*(M)	*fedA*, *fedF*, *iha*, *ltcA*, *sepA*, *stb*, *stx2A*, *stx2B*	WVUR00000000
CAU15134	2015	Liaoning	O3:H45	ST4214	IncF, IncI1, IncHI2	*aac(6′)Ib-cr*, *aadA2*, *aph(3′)-Ia*, $bla_{\mathrm{TEM-1B}}$, *cmlA1*, *dfrA1*, *dfrA12*, *floR*, *mcr-1.1*, *mcr-3.1*, *oqxA*, *oqxB*, *qnrS2*, *strA*, *strB*, *sul1*, *sul2*, *sul3*, *tet*(A), *tet*(M)	*astA*, *fedA*, *fedF*, *gad*, *iha*, *ltcA*, *sepA*, *sta1*, *stb*, *stx2A*, *stx2B*	WVUQ00000000
CAU16060	2016	Shandong	O3:H45	ST4214	IncF, IncHI2	*aadA2*, *aph(3′)-Ia*, $bla_{\mathrm{TEM-1B}}$, *cmlA1*, *dfrA1*, *dfrA12*, *floR*, *mcr-1.1*, *mcr-3.1*, *oqxA*, *oqxB*, *strA*, *strB*, *sul1*, *sul2*, *sul3*, *tet*(A), *tet*(M)	*fedA*, *fedF*, *gad*, *iha*, *ltcA*, *sepA*, *sta1*, *stb*, *stx2A*, *stx2B*	WVUP00000000
CAU16175	2016	Hunan	O4:H11	ST29	IncHI2, IncFIB, IncFII	*aac(3)-IVa*, *aac(6′)Ib-cr*, *aadA1*, *aadA2*, *aph(3′)-Ia*, *aph(4)-Ia*, *ARR-3*, $bla_{\mathrm{OXA-1}}$, $bla_{\mathrm{TEM-1B}}$, *catB3*, *cmlA1*, *dfrA12*, *floR*, *mcr-1.1*, *sul1*, *sul2*, *sul3*, *tet*(A), *tet*(M)	*astA*, *cif*, *eae*, *efa1*, *espA*, *espB*, *espJ*, *gad*, *iha*, *iss*, *katP*, *lpfA*, *nleB*, *nleC*, *perA*, *toxB*, *tir*	CP047378-CP047384
CAU16177	2016	Hunan	O103:H2	ST20	IncF, IncHI2	*aac(3)-IVa*, *aac(6′)Ib-cr*, *aph(3′)-Ia*, *aph(4)-Ia*, *ARR-3*, $bla_{\mathrm{OXA-1}}$, $bla_{\mathrm{OXA-10}}$, *catB3*, *cmlA1*, *dfrA12*, *dfrA14*, *floR*, *mcr-1.1*, *mcr-3.1*, *oqxA*, *oqxB*, *qnrS1*, *strA*, *strB*, *sul1*, *sul2*, *sul3*, *tet*(A)	*cif*, *eae*, *espA*, *espB*, *espF*, *espJ*, *gad*, *iss*, *nleA*, *nleB*, *tir*	WVUO00000000
swine19	2014	Jiangsu	O130:H45	ST4214	IncA/C	*aadA2*, *strA*, *strB*, *mcr-1.1*, *sul3*, *sul1*, *tet*(A), *cmlA1*, $bla_{\mathrm{TEM-1B}}$, *oqxB*, *oqxA*	*astA*, *fedA*, *fedF*, *iha*, *sta1*, *stb*, *stx2A*, *stx2B*	LVMV00000000
swine22	2014	Jiangsu	O130:H45	ST4214	IncA/C	*aadA2*, *strA*, *strB*, *mcr-1.1*, *sul3*, *sul1*, *tet*(A), *cmlA1*, $bla_{\mathrm{TEM-1B}}$, *oqxB*, *oqxA*	*astA*, *fedA*, *fedF*, *iha*, *sta1*, *stb*, *stx2A*, *stx2B*	LVMY00000000
swine54	2012	Jiangsu	O3:H45	ST4214	IncA/C	*aph(3′)-Ia*, *strA*, *strB*, *aadA2*, *mcr-1.1*, *sul3*, *sul2*, *sul1*, *dfrA12*, *tet*(A), *tet*(M), *cmlA1*, *floR*, $bla_{\mathrm{TEM-1B}}$, *oqxA*, *oqxB*	*astA*, *fedA*, *fedF*, *gad*, *ltcA*, *iha*, *sepA*, *sta1*, *stb*, *stx2A*, *stx2B*	LVOE00000000
swine67	2012	Jiangsu	O3:H45	ST4214	IncA/C	*aadA2*, *aph(3′)-Ia*, *strA*, *strB*, *sul3*, *sul2, sul1*, *oqxB*, *oqxA*, *qnrS2*, *dfrA12*, *tet*(A), *tet*(M), *mcr-1.1*, *cmlA1*, *floR*, $bla_{\mathrm{TEM-1B}}$, *aac(6′)Ib-cr*	*astA*, *fedA*, *fedF*, *ltcA*, *iha*, *stb*, *sepA*, *stx2A*, *stx2B*	LVOR00000000

TABLE 3 | Genome summary for ST29 aEPEC isolate CAU16175.

	Sequence length (bp)	GC%	Plasmid rep type(s)	Antimicrobial resistance gene	Virulence region or gene	GenBank accession no.
Chromosome	5615389	50.66	NA	*mdf*(A)	LEE, non-LEE T3SS effectors	CP047378
pCAU16175_1	190219	47.10	IncHI2	*aac(6')-Ib-cr*, *sul1*, *sul2*, *sul3*, bla_{OXA-1}, *tet*(A), *ARR-3*, *aac(3)-IVa*, *aadA1*, *aadA2b*, *aph(3')-Ia*, *aph(4)-Ia*, *catB3*, *cmlA1*, *floR*, *dfrA12*		CP047379
pCAU16175_2	161176	47.91	IncFIB (AP001918)		*katP*, *sepA*, *perA*	CP047380
pCAU16175_3	76633	51.35	IncFII (29)	*tet*(M), *mcr-1.1*, bla_{TEM-1B}		CP047381
pCAU16175_4	10675	49.23	—			CP047382
pCAU16175_5	7098	50.52	—		*celb*	CP047383
pCAU16175_6	6988	47.58	—			CP047384

ARR-3 (associated to rifamycin antibiotic), *catB3*, *cmlA1*, and *floR* (associated to phenicol antibiotic) (**Table 2**). Interestingly, we observed that the *mcr-1.1* gene and the bla_{TEM-1B} gene existed in the same scaffold of the *E. coli* CAU16175 draft genome. Besides, partial sequences of this scaffold including *mcr-1.1* and bla_{TEM-1B} are highly homologous with Tn*2* (KT002541).

Complete Genome of the *E. coli* CAU16175

The 6.07-Mb complete genome of *E. coli* CAU16175 has a total GC content of 50.47% with a single chromosome and six plasmids. The 5.62 Mb chromosome has a GC content of 50.66%. The six plasmids sequences of *E. coli* CAU16175 range in length from 6.99 to 190.22 kb with a GC content from 47.10 to 51.35% (**Table 3**). The chromosome harbors LEE T3SS effectors, non-LEE T3SS effectors, and *mdf*(A) antimicrobial-resistant gene. The pCAU16175_1 (IncHI2 type) harbors an IS*26*-enriched MDR region, which includes bla_{OXA-1} and 15 additional antimicrobial-resistant genes. The pCAU16175_2 (IncFIB type) harbors *katP*, *sepA*, and *perA* virulence genes. The pCAU16175_3 (IncFII type) harbors *mcr-1.1*, *tet*(M), and bla_{TEM-1B} antimicrobial-resistant genes. The pCAU16175_5 harbors *celb* virulence gene (**Table 3**).

Conjugation Assay

The susceptibility testing of polymyxin B and E showed that the MIC values for the transconjugants (J53-*mcr-1*) increased to 8 and 16 mg/L, respectively (**Table 4**). The conjugation frequency was 2.1×10^{-8} transconjugants per recipient.

TABLE 4 | Minimum inhibitory concentration (mg/L) for CAU16175, J53, and J53-*mcr-1*.

	CAU16175	J53	J53-*mcr-1*
TE	32	1	1
GEN	>32	0.5	0.5
AMK	16	16	2
KAN	>128	8	2
AMP	>64	4	4
CFZ	>16	1	1
CIP	>16	<0.125	<0.125
CHL	32	1	2
RFP	<1	<1	<1
PB	**4**	**1**	**8**
PE	**16**	**2**	**16**
STR	>256	32	2
NaN_3	<75	300	300
EFT	16	<1	<1
FFC	32	8	4
AMC	>16	4	4

TE, tetracycline; GEN, gentamicin; AMK, amikacin; KAN, kanamycin; AMP, ampicillin; CFZ, cefazolin; CIP, ciprofloxacin; CHL, chloramphenicol; RFP, rifapentine; PB, polymyxin B; PE, polymyxin E; STR, streptomycin; EFT, ceftiofur; FFC, florfenicol; AMC, co-amoxiclav. Highlight the colistin resistance was transferred to J53.

Genetic Characterization of the pCAU16175_1 Harboring the MDR Region

The 190.22-kb plasmid pCAU16175_1 was blasted against the GenBank nucleotide collection (nr/nt) database. An overall nucleotide sequence identity (99.66–99.86%) with query coverages of 90–99% to pSH16G4498 (MH522423), pSH16G2457 (MH522421), pHNYJC8 (KY019259), pHNLDF400 (KY019258), pHXY0908 (KM877269), and other 25 IncHI2-type plasmids were observed (**Supplementary Table 1**). We chose the five most similar plasmids for comparison analysis; these plasmids have MDR regions and many IS*6* family insert sequences (IS*26*/IS*15DI*/IS*15DIV*/IS*1006*/IS*Ec59*) (**Figure 1**). The pCAU16175_1 shows almost exactly the same sequence in MDR region with pSH16G4498 and pSH16G2457, which both existed in *Salmonella typhimurium* recovered from humans in China (**Supplementary Table 1**). In the pCAU16175_1, the MDR region locates at nucleotide location 121,521–178,916 (56,625 bp). The structure of the MDR region comprises twelve IS*6* family insert sequence [including six copies IS*26*, three copies IS*15DI* (3 bp differ with IS*26*), one copy IS*15DIV* (1 bp differ with IS*26*), one copy IS*1006*, and one copy IS*Ec59*] that flank containing different antimicrobial-resistant genes. The MDR region contains *floR*, *sul2*, *aph(4)-Ia*, *aac(3)-IVa*, *aac(6')-Ib-cr*, bla_{OXA-1}, *catB3*, *ARR-3*, *sul1* (two copies), *dfrA12*, *aph(3')-Ia*, *sul3*, *aadA1*, *cmlA1*, *aadA2b*, and *tet*(A). The MDR region is also interspersed with a number of different mobile elements

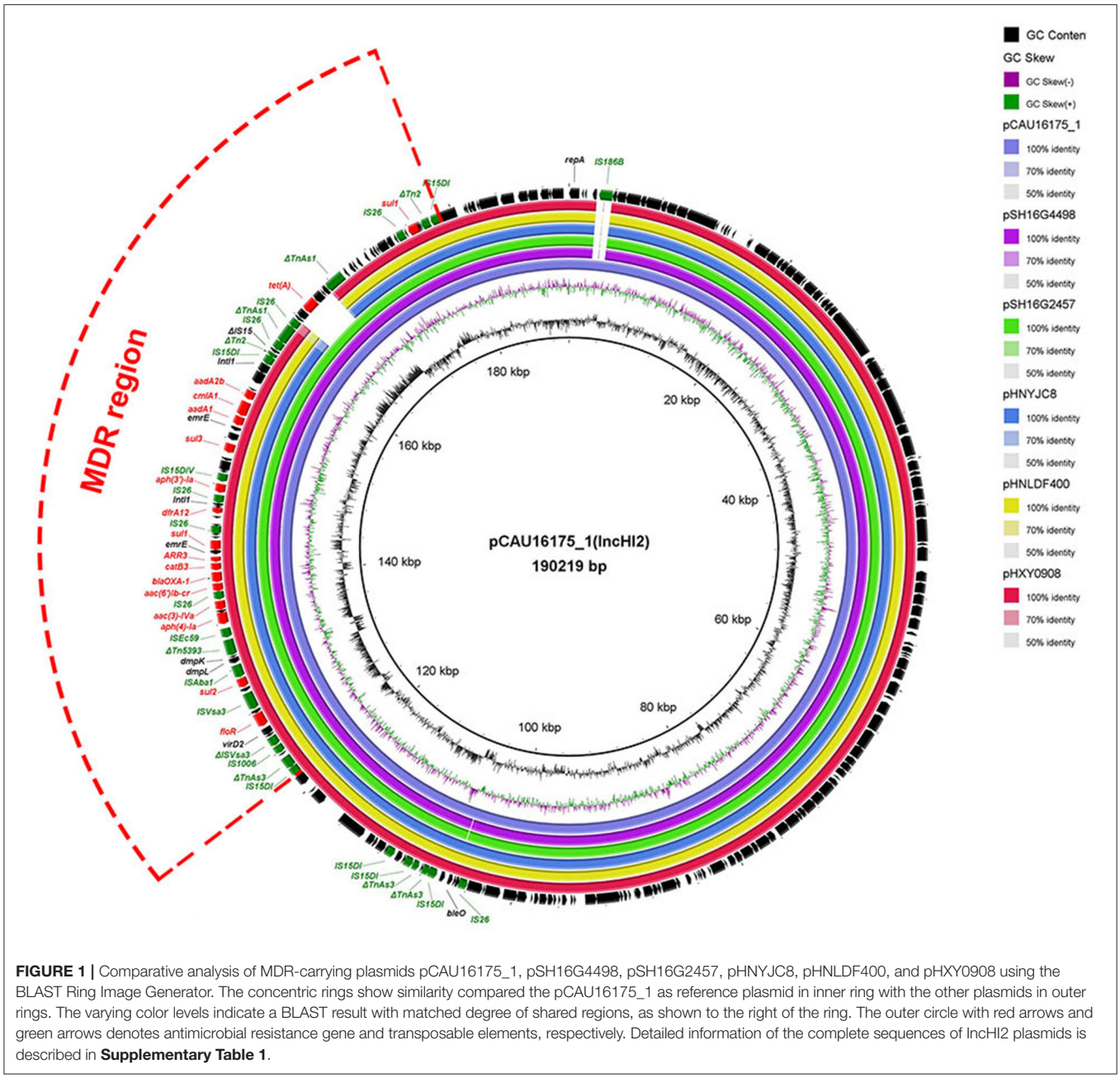

FIGURE 1 | Comparative analysis of MDR-carrying plasmids pCAU16175_1, pSH16G4498, pSH16G2457, pHNYJC8, pHNLDF400, and pHXY0908 using the BLAST Ring Image Generator. The concentric rings show similarity compared the pCAU16175_1 as reference plasmid in inner ring with the other plasmids in outer rings. The varying color levels indicate a BLAST result with matched degree of shared regions, as shown to the right of the ring. The outer circle with red arrows and green arrows denotes antimicrobial resistance gene and transposable elements, respectively. Detailed information of the complete sequences of IncHI2 plasmids is described in **Supplementary Table 1**.

including ΔTn*As3*, ΔIS*Vsa3*, IS*Vsa3*, IS*Aba1*, ΔTn*5393*, ΔTn*2*, ΔIS*15*, and ΔTn*As1*.

Genetic Characterization of the pCAU16175_3 Harboring the *mcr-1.1* Gene

The 76.63-kb plasmid pCAU16175_3 was blasted against the GenBank Nucleotide collection (nr/nt) database. An overall nucleotide sequence identity (94.46–97.88%) with query coverages of 80–89% to pEC1515-3 (CP021847), pEC974-3 (CP021843), pH1038-142 (KJ484634), pFORC_081_1 (CP029058), plasmid R1 (KY749247), and other 27 IncFII-type plasmids were observed (**Supplementary Table 2**). We chose five most similar IncFII-type plasmids and reported IncFII-type plasmid-*mcr-1* pKP81-BE (KU994859) for comparison analysis (**Figure 2**). Although the pCAU16175_3 demonstrates highly sequence homology with the other five IncFII-type plasmids, the *mcr-1.1* gene only exists in pCAU16175_3 and pKP81-BE. Besides the *mcr-1.1* gene, the pCAU16175_3 also carries the antimicrobial-resistant gene $bla_{\mathrm{TEM-1B}}$ and *tet*(M), which are close to the *mcr-1.1* gene.

Noticeably, the nucleotide location of the pCAU16175_3 from 2,079 to 12,231 has high homology with the Tn*2* derivative (**Figure 3A**). This structure is a unique identification compared with the NCBI database and was confirmed by conventional PCR

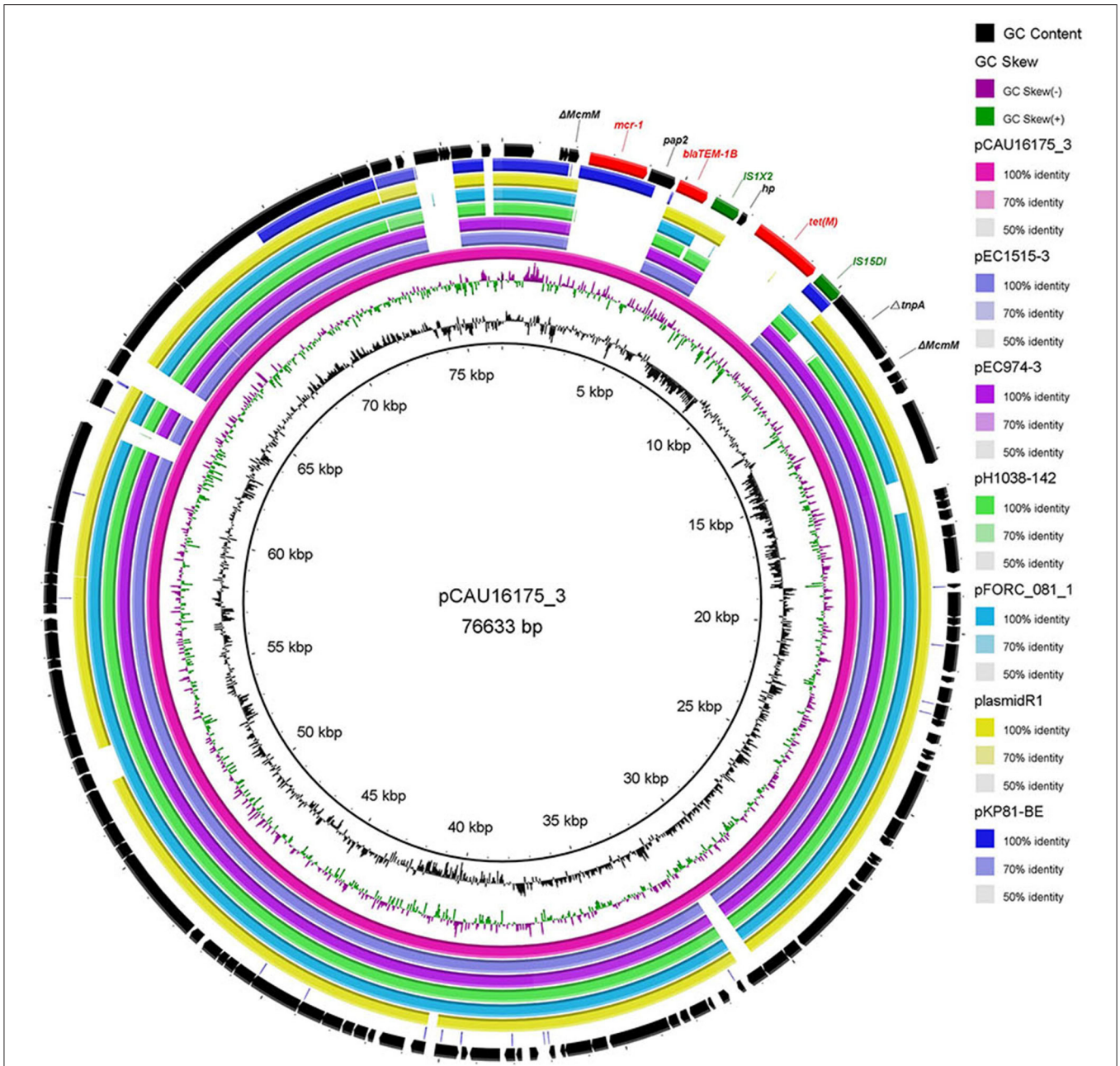

FIGURE 2 | Comparative analysis of seven IncFII-type plasmids pCAU16175_3, pEC1515-3, pEC974-3, pH1038-142, pFORC_081_1, plasmid R1, and pKP81-BE (carrying *mcr-1*) using the BLAST Ring Image Generator. The concentric rings show similarity compared the pCAU16175_3 as reference plasmid in inner ring with the other plasmids in the outer rings. The varying color levels indicate a BLAST result with matched degree of shared regions, as shown to the right of the ring. The outer circle with red arrows and green arrows denotes antimicrobial resistance gene and transposable elements, respectively. Detailed information of the complete sequences of IncFII plasmids is described in **Supplementary Table 2**.

(**Supplementary Figure 1A**). The genetic content of the structure analysis showed that the structure is located within *mcmM* (encoded microcin M). A five-nucleotide direct duplication (TATTT) was identified flanking the structure. Unlike the Tn*2*, this structure harbors *mcr-1–pap2*-ΔIS*Apl1* and IS*1X2-hp-tet*(M)-IS*15DI*, deletes the resolvase (*tnpR*), and truncates the transposase (*tnpA*). Besides, the striking features of the insertion sites of IS*Apl1* were found, which includes high AT content and 2-bp direct repeats (DRs) (AA) (**Figure 3B**). From the result of conjugation experiment and PCR, the whole structure gene did not occur transposition (**Table 4, Supplementary Figure 1B**). The *mcr-1* gene was transferred to J53 without the *pap2* gene,

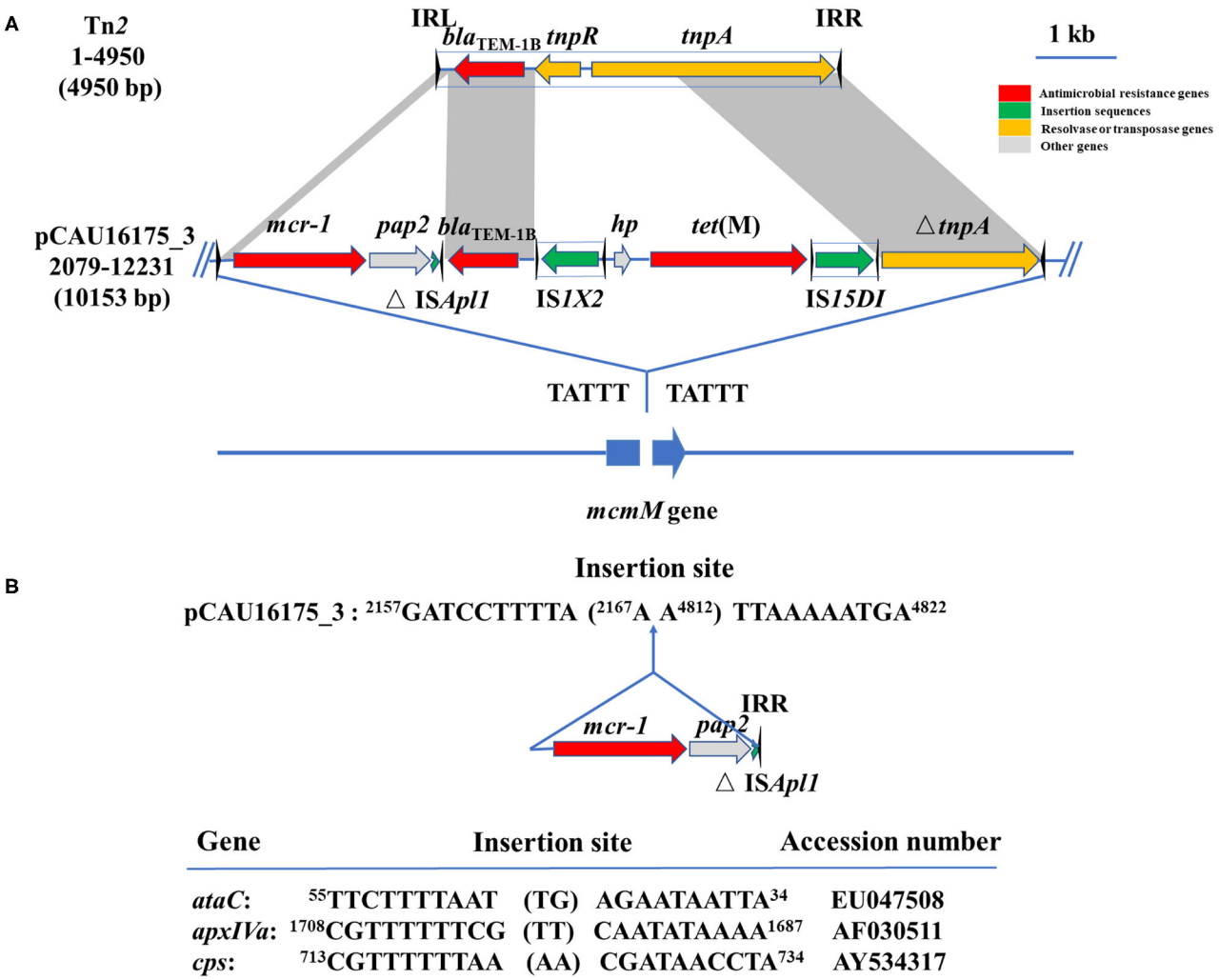

FIGURE 3 | Genetic features of pCAU16175_3 (nucleotide location 2079-12231). **(A)** Linear sequence comparison of pCAU16175_3 (nucleotide location 2079-12231) with Tn*2*. Arrows indicate the positions and directions of the genes, Δ indicates a truncated gene. Regions with >99% homology are indicated by gray shading. Inverted repeat nucleotide sequences (IRL: left IR; IRR: right IR) of IS are marked by triangles. Antimicrobial resistance genes, insertion sequences, resolvase or transposase genes, and other genes are indicated by red, green, khaki, and gray, respectively. **(B)** DNA sequence of insertion sites of *mcr-1-pap2*-Δ*ISApl1* in pCAU16175_3 and IS*Apl1* in *ataC*, *apxIVA*, and *cps* gene (30). Shown sequences represent 10 base pairs upstream and downstream of insertion site, and 2-base-pair direct repeats in brackets. Numbers represent positions in the indicated sequences deposited in GenBank.

whereas colistin resistance was transferred to J53. IS*15DI* was also transferred to J53.

DISCUSSION

Postweaning diarrhea caused by DEC is an economically important disease for the swine industry around the world. In our previous study, we collected 455 *E. coli* isolates recovered from feces samples or small intestinal content from pigs with diarrhea in China between 2014 and 2016 to know the *E. coli* pathotype and the antimicrobial susceptibility of the isolates (6). Most of the isolates belonged to ETEC, followed by aEPEC, which is similar to the report in Spain; that is, most cases of PWD are significantly associated with ETEC (67%) and aEPEC (21.7%) (37). Our previous study showed that 95.91% of 171 *E. coli* isolates were MDR exhibiting resistance to at least three different classes of antimicrobials, and 20.47% *E. coli* isolates were resistant to colistin. It has been revealed that MDR isolates that existed in swine industry are associated with the widespread use of antibiotics (38, 39). Overuse of colistin is considered to contribute to the emergence and spread of *mcr-1* (40). The 36 *mcr-1*–positive *E. coli* isolates recovered from pigs with PWD showed MDR (29). In this study, we used WGS to analyze the five MDR (including colistin resistance) of *E. coli* isolates from pigs with PWD and found that they demonstrate different serotype and sequence type and carry different antimicrobial-resistant genes and virulence genes.

The clinical isolates of the *E. coli* CAU15104, CAU15134, and CAU16060 belonged to ETEC/STEC, displaying the same

serotype O3:H45 and sequence type ST4214. As far as we know, there are no reports about this clone strains, and only four have WGS of these clone strains. The *E. coli* swine19, swine22, swine54, and swine67 also belong to ETEC/STEC and display the same sequence type ST4214. The *E. coli* swine19 and swine22 display the same serotype O130:H45. The *E. coli* swine54 and swine67 display the same serotype O3:H45, similar to our collected the *E. coli* CAU15104, CAU15134, and CAU16060. Five ETEC/STEC isolates recovered from pigs with enteric colibacillosis in Spain display the serotype O141:H4 and sequence type ST10 (37). In the present study, all of the ST4214 ETEC/STEC we collected were MDR (at least three different classes of antimicrobials) and showed MDR to PB, TE, and NAL, carried 17–19 antimicrobial-resistant genes and 8–11 virulence genes. Besides, all the ST4214 ETEC/STEC harbored aminoglycoside-resistant genes (*aadA2* and *aph(3′)-Ia*), phenicol-resistant genes (*cmlA1* and *floR*), tetracycline-resistant genes [*tet*(A) and *tet*(M)], trimethoprim-resistant gene (*dfrA12*), sulfonamide-resistant genes (*sul1*, *sul2*, and *sul3*), streptomycin-resistant genes (*strA* and *strB*), and colistin-resistant genes (*mcr-1.1*). The ST4214 ETEC/STEC also harbored fimbrial adhesin genes (*fedA* and *fedF*), adherence gene (*iha*), heat-labile enterotoxin (LT) gene (*ltcA*), heat-stabile enterotoxin (ST) gene (*stb*), *Shigella* extracellular protein gene (*sepA*), and Shiga toxin 2 variant e genes (*stx2A* and *stx2B*). We observed the ST4214 strains were recovered from different provinces (Shandong, Jiangsu and Liaoning) and years (2012, 2014, 2015, and 2016) in China, which indicated this clone strains have spread in swine and should be brought to our attention.

In addition, the clinical isolates of the *E. coli* CAU16175 and CAU16177 belonged to aEPEC, displaying different serotype and sequence type, O4:H11 and O103:H2, ST29 and ST20, respectively. For aEPEC, serotypes O45 and O123 are frequently occurring in diarrheagenic pigs (41, 42). The *E. coli* CAU16175 and CAU16177 also showed MDR to AMC, KAN, PB, TE, NAL, and CHL and carried 19/23 antimicrobial-resistant genes and 17/11 virulence genes. They both harbored beta-lactamase-resistant genes ($bla_{\mathrm{OXA-1}}$), aminoglycoside-resistant genes (*aac(3)-IVa*, *aac(6′)Ib-cr*, *aph(3′)-Ia* and *aph(4)-Ia*), phenicol-resistant genes (*cmlA1*, *catB3*, and *floR*), tetracycline-resistant genes [*tet*(A)], trimethoprim-resistant gene (*dfrA12*), sulfonamide-resistant genes (*sul1*, *sul2*, and *sul3*), rifampicin-resistant gene (*ARR-3*), and colistin-resistant genes (*mcr-1.1*). Interestingly, *E. coli* CAU16177 also harbored *mcr-3.1*. Four *mcr-1*– and *mcr-3*-positive *E. coli* have been reported, and three *E. coli* isolates were recovered from pigs (23). The *E. coli* CAU16175 and CAU16177 both harbored type III secretion system-associated virulence genes (*cif*, *espA*, *espB*, and *espJ*), non–LEE-encoded effector gene (*nleB*), glutamate decarboxylase gene (*gad*), increased serum survival gene (*iss*), a marker gene for EPEC, intimin gene (*eae*), and translocated intimin receptor gene (*tir*).

The *E. coli* CAU16175 carries IS*26*-enriched MDR region in pCAU16175_1 (IncHI2 type). The IncHI2-type plasmids have been discovered as genetic elements mediating the transmission of MDR genes (16). IS*26*-flanked Tns play an increasingly critical role in the mobilization and development of antimicrobial-resistant genes (43–45). So far, there are 51 Tns related with IS*26* from the Tn registry website. In an individual *E. coli* strain heterogeneous resistance-encoding plasmid, polymorphic MDR regions driven by IS*26*-flanked Tns have been detected (44). From the BLAST analysis, we found that there were abundant IS*26*-enriched MDR regions in *Salmonella* and *E. coli*, which were mostly isolated from humans and animals. The results showed that the plasmid carrying IS*26*-enriched MDR region has been widely distributed in humans and consumption animals (**Supplementary Table 1**). Combined with the antimicrobial susceptibility testing, we can deduce that the *E. coli* CAU16175 isolate has MDR and is difficult to control.

The *E. coli* CAU16175 isolate also carries the *mcr-1.1* gene in pCAU16175_3 (IncFII type). The emergence of the *mcr-1* gene can be traced back to the *E. coli* isolated in the 1980s, and the outbreak of chicken-derived *mcr-1*–containing *E. coli* started in 2009 (46).The *mcr-1* gene has been characterized in various genetic backgrounds and observed on a variety of plasmid type, including Incl2, IncX4, IncHI2, IncP, IncHI1, IncFII, IncFI, IncFIB, F18:A-:B+, IncY, IncK, and phage-like plasmid (29). Most of the recently reported the *mcr-1* genes were primarily mobilized by an IS*Apl1* composite Tns Tn*6330* and Tn*6390* (47, 48). The IS*Apl1* would be lost over time, leading to the stability of the *mcr-1* gene on plasmids (18, 49). Thus, only the truncated IS*Apl1* will lose the ability to transfer the *mcr-1* gene.

Furthermore, the *mcr-1.1* gene is located within the Tn*2* derivative in pCAU16175-3. From the Tn registry website (https://transposon.lstmed.ac.uk/), we noticed the *mcr-5* gene and the *mcr-3.6* gene could be mobilized by Tn*6452* and Tn*6518* (belonged to Tn*2* family). For the Tn*6452*, the *mcr-5* gene was embedded within a Tn*3*-family Tn with 38-bp inverted repeats and flanked by 5-bp DRs, which were usually generated during the insertion (50, 51). Although DRs appear at the flanking site of the Tn*2* derivative, the whole structure did not move by transposition because of truncated *tnpA* and deleted *tnpR*, which are essential to occur transposition for Tn*2*.

CONCLUSIONS

The current work shows the genetic characteristics of five DEC strains that exhibited MDR, including colistin resistance. Our data indicate that the ST4214 ETEC/STEC carried MDR and multivirulence genes and that the *E. coli* CAU16175 contains IS*26*-enriched MDR region and the *mcr-1.1* gene, which is located within a Tn*2* derivative. The coexistence of MDR and multivirulence in DEC may seriously compromise the effectiveness of clinical therapy, and heightened efforts are needed to control their dissemination.

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/, SRR10813965; https://www.ncbi.nlm.nih.gov/, SRR10828049; https://www.ncbi.nlm.nih.gov/, SRR10828048; https://www.ncbi.nlm.nih.gov/, SRR10828047; and https://www.ncbi.nlm.nih.gov/, SRR10828046.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of the China Agricultural University under the protocol CAU20140616-1.

AUTHOR CONTRIBUTIONS

LG and YZ conceived and designed the experiments. LG, JW, SW, JS, and XW performed the experiments. LG analyzed the sequencing data and wrote the manuscript. YZ revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key R&D Program of China (Project No. 2017YFD0502200) and the National Natural Science Foundation of China (Project Nos. 31873034 and 31672613).

ACKNOWLEDGMENTS

We would like to thank Prof. Wang Yang (China Agricultural University) for providing the *E. coli* J53 strain.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00503/full#supplementary-material

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 04 September 2020
doi: 10.3389/fvets.2020.00530

Phenotypic Characterization and Whole Genome Analysis of a Strong Biofilm-Forming *Staphylococcus aureus* Strain Associated With Subclinical Bovine Mastitis in Colombia

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 21 April 2020
Accepted: 08 July 2020
Published: 04 September 2020

Citation:
Torres G, Vargas K, Cuesta-Astroz Y, Reyes-Vélez J and Olivera-Angel M (2020) Phenotypic Characterization and Whole Genome Analysis of a Strong Biofilm-Forming Staphylococcus aureus Strain Associated With Subclinical Bovine Mastitis in Colombia. Front. Vet. Sci. 7:530. doi: 10.3389/fvets.2020.00530

Staphylococcus aureus represent a serious threat to public health due to food safety, antibiotic resistance, and the potential zoonotic transmission of strains between dairy cattle and humans. Biofilm formation by *S. aureus* results in chronicity of the infections which confers protection against the immune response and antibiotics. Likewise, biofilm allows the exchange of mobile genetic material among different strains through microbial interactions inside the matrix. In Colombia, where *S. aureus* continues to be one of the main pathogens isolated from bovine intramammary infections and where milking by hand is highly frequent, there are knowledge gaps on the zoonotic potential of the strains. Therefore, the aim of this work was to characterize genotypically and phenotypically the *S. aureus* Sa1FB strain with strong biofilm production and to perform genomic and phenotypic comparisons with other relevant *S. aureus* strains (native and references strains). These results show a highly productive strain of biofilm and a low ability of cell invasion compared to the other two native strains. In addition, high genomic similarity between *S. aureus* Sa1FB and the reference strains was observed, despite of the differences reported at the clinical level. However, Sa1FB exhibited special features in terms of mobile genetic elements, highlighting its ability to accept foreign genetic material. Indeed, this could increase mutation, pathogenesis, and adaptability to new hosts, representing a risk for people in contact with the milk obtained from animals infected with these strains. These results present the relevance of surveillance for early detection of emergent clones with zoonotic potential, which reduces the risk of occupational exposure and their spread in the community.

Keywords: biofilm, intramammary infections, mastitis, mobile genetic elements, *Staphylococcus aureus*, virulence factors, whole genome sequencing

INTRODUCTION

Staphylococcus aureus has been described as a commensal pathogen from humans, and different animal species such as dairy cattle and other livestock (1, 2). It is also the most frequent agent associated with bovine mastitis worldwide (3, 4). Moreover, this Gram-positive bacterium can cause in humans a wide variety of clinical conditions including skin diseases, bacteremia-toxic syndrome, and food diseases (2, 5). On the other hand, in dairy cattle, *S. aureus* mainly produces intramammary infections (IMI) (6).

S. aureus represent a serious threat to public health due to food safety, antibiotic resistance, and the potential zoonotic transmission of strains between dairy cattle and humans (7). Zoonotic transfer of this pathogen between both hosts can occur by direct contact or through the food chain (8).

In Colombian specialized dairy herds, hand milking has been reported on a range between 43.6 and 77.7% (9, 10), representing a high risk for milkers in acquiring the bacterium during the milk harvest. In addition, ∼41% of the raw milk obtained in the country is commercialized under informal conditions and without pasteurization, which increases the probability of spread of this pathogen among people without exposure to livestock (11).

As a versatile microorganism, *S. aureus* has become a more virulent and resistant pathogen to antimicrobials in both human and animal populations (5). This strong potential of multi-species colonization has been recognized as a product of the presence of multiple putative virulence factors (cell surface adhesins, extracellular enzymes, biofilm, cell invasion, and toxins). These virulence factors have a complex regulation network that allows the adaptation and survival in the host (7, 8). Within these virulence factors, biofilm formation is one of the main determinants, because this confers protection to both host's immune response and to antimicrobials (12). Biofilm formation results in chronicity of the infections and allows the development and transfer of antimicrobial resistance, due to microbial interactions inside the biofilm (12, 13). Another *S. aureus* factor associated with persistent infections, is the cell invasion, since this mechanism also allows it to evade the immune response and the antibiotics (14).

The whole genome sequence (WGS) analysis and the determination of the multilocus sequence typing (MLST) profile has been integrated to the genomic surveillance of potential zoonotic bacteria like *S. aureus* (15). Moreover, these methods had been used for the identification of the population structure and potential shifts in the genomic configuration (3, 8). Analyses of the MLST sequence types have revealed a highly clonal population (16) and clearly distinct clonal complexes (CCs) associated with specific hosts and environments (17–19).

The host diversity of *S. aureus* entails a direct zoonotic potential for humans, bringing the importance of its active surveillance for both public and animal hygiene (2). Therefore, a better knowledge of the adaption of *S. aureus* lineages is needed to assess the potential of host switching (15). The objective of this research was to characterize genotypically and phenotypically the *S. aureus* Sa1FB strain with strong biofilm production and to perform genomic comparisons with other relevant *S. aureus* genomes.

MATERIALS AND METHODS

Staphylococcus aureus Strains

The Sa1FB strain was characterized previously genotypically and phenotypically (20). The ability to form biofilm (phenotype) on microplates and the amplification of the biofilm-associated genes, *ica* and *bap*, (genotype) were carried out according to protocols earlier published (21, 22). The genotypes and phenotypes identified were: Sa1FB Strong biofilm-producing (*ica* $^{+}$–*bap* $^{+}$), Sa2FB Weak biofilm-producing (*ica* $^{+}$–*bap* $^{-}$), and Sa3NF Non biofilm-producing (*ica* $^{-}$–*bap* $^{-}$). The three strains were isolated from bovine subclinical mastitis in Antioquia (Colombia).

Cell Invasion Assay

The invasion assay was performed based on a previously described protocol (23). Clonal bovine mammary epithelial cells (MEC) (24) were cultured into a 24-well polystyrene culture plate (TrueLine, USA) using DMEM medium (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), 5 μg/mL insulin (Sigma-Aldrich, USA), and 1 μg/mL hydrocortisone (Sigma-Aldrich, USA). Once the cells were confluent (1.5 – 2.5 × 10^5 cells/well), these were independently co-cultured with each of the three native strains at a multiplicity of infection (MOI) of 10:1 and incubated for 2 h at 37°C in 5% CO_2.

After incubation, the wells were aspirated to remove non-internalized bacteria and non-attached to the cell surface. The supernatants were cultured in Tripticase soy agar (TSA) (Oxoid, United Kingdom). Then, the MEC were washed three times with sterile PBS (VWR, USA) and treated using DMEM supplemented with 100 μg/mL gentamicin (Sigma-Aldrich, USA) in order to kill the extracellular bacteria.

The plates were incubated again for 2 h at 37°C in 5% CO_2. Finally, the MEC were washed with sterile PBS and treated with 0.25% trypsin (AMRESCO, USA) and 0.1% EDTA (AMRESCO, USA) until cells detached, which were immediately lysed with 0.1% Triton X-100 (AMRESCO, USA). MEC lysates were diluted and plated on TSA. These plates were incubated at 37°C overnight. MEC without bacteria were used as negative control and the strains with DMEM as viability control. All the experiments were performed in triplicate and repeated two times.

Biofilm Formation Assay *in vitro*

The biofilm production of the strains was induced following the standard protocol previously reported with some modifications (20, 21). The strains were transferred from stock culture into TSA and incubated at 37°C overnight under aerobic conditions. These colonies were suspended in sterile distilled water until a turbidity comparable to 0.5 MacFarland scale (∼10^8 CFU/mL) was reached. This suspension was diluted 1:100 in TSB supplemented with 1% glucose (Merck, USA) to reach a bacterial concentration of ∼10^6 CFU/mL. Then, 2 mL from the diluted suspension was aliquoted into 12-well polystyrene tissue culture microtiter plate (TrueLine, USA). Each well at the bottom contained a glass

coverslip as a basis for biofilm formation. Experiments were performed in duplicate and repeated two times. The plates were incubated at 37°C for 24 h under static aerobic conditions. The next day, the wells were aspirated, and each well was washed three times with 2 mL sterile phosphate-buffered saline (PBS, pH 7.2). After washing, the glass coverslips were transferred to other plates and were washed again. The biofilm formation was verified using 2% crystal violet for 15 min. *S. aureus* strains V329 (*ica* and *bap* positive) and ATCC 6538 (*ica* positive and *bap* negative) were used as positive controls, whereas TSB with glucose was used as a negative control.

Visualization of Biofilms by Electronic Microscopy

The observation of biofilm formed on microplates by the three native strains evaluated was carried out using scanning electron microscopy (SEM). Briefly, samples were fixed with a glutaraldehyde solution at 2.5% overnight and subsequently dehydrated using a different concentration of ethanol (50, 75, 95, and 100%). Finally, the samples were critical point dried, coated with gold and visualized in a JEOL-JSM 6490LV microscope (JEOL, Japan).

DNA Extraction

The genomic DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen, Germany) according to protocol for Gram-positive bacteria. Concentration and quality of DNA were measured using NanoDrop (ThermoFisher Scientific, USA). Extracted DNA was stored at −80°C until use.

Genome Sequencing, Assembly, and Annotation

Staphylococcus aureus Sa1FB strain was sequenced (WGS) on the Illumina MiSeq platform. Pair reads of 300 bp in length were obtained after library preparation with the Illumina Nextera XT DNA Library preparation kit. *De novo* assembly was performed using the raw reads and the PATRIC (Pathosystems Resource Integration Center) (25) genome assembly service (revised service Dec 2019) using the SPAdes workflow (26), which integrates the trimming process of the reads using TrimGalore before assembly and Pilon (27) to correct assembly errors. A subsequent annotation was performed using Rapid Annotations using a Subsystems Technology tool kit (RASTtk) also found in PATRIC. The sequences were queried using the following tools available in PATRIC: VFDB and Victors (virulence factors) and CARD and NDARO (antibiotic resistance). To detect putative orthologs across genomes for comparing, we performed an OrthoMCL (28) cluster analysis using the default settings (*E-value* cutoff: 1e−5 and identity > 50%). The genome sequence of *S. aureus* Sa1FB was deposited in the public database PATRIC (https://www.patricbrc.org/view/Genome/1280.24396) with the genome ID: 1280.24396.

Whole Genome Alignment

The assembled and annotated *S. aureus* Sa1FB strain was aligned with the complete bovine genomes of *S. aureus* RF122 and *S. aureus* Newbould 305 with default parameters of progressiveMauve and the average nucleotide identity (ANI) was calculated by MUMmer using JSpeciesWS (29).

TABLE 1 | General genome characteristics of the *S. aureus* Sa1FB strain.

Feature	*S. aureus* Sa1FB strain
Genome ID (PATRIC)	1280.24396
Genome size (bp)	2,745,618
GC content (%)	32.8
Contigs	30
Total of CDS	2,632
Total of tRNA	57
Total of rRNA	9
Hypothetical proteins	507
Antibiotic resistance genes	60
Virulence factors	85
Pathogenicity island SaPIbov2	Presence
Sequence tipo (MLST)	ST126

RESULTS

General Genome Features

Table 1 shows the *S. aureus* Sa1FB strain genome features. The sequence of the *S. aureus* Sa1FB genome draft had an estimated length of 2,745,618 bp with GC content of 32.8% and 2,632 coding sequences (CDS) as shown also in **Figure 1** and **Supplementary Table 1**.

Using Subsystems Technology of the RAST server it was possible to know more about the annotated genes in different biological processes and metabolical pathways (**Figure 2**). The predicted genes included: 548 genes involved in metabolism, 120 genes involved in stress response, defense, and virulence, and 184 genes involved in energy among other biological processes (**Supplementary Table 2**).

Cluster of Orthologs Groups

OrthoMCL was used to arrange proteins into clusters and to identify groups of the most conserved proteins among proteomes of *S. aureus* Sa1FB, Newbould and RF122 strains. It was found that 1,745 (94%) out of the 1,857 orthologous groups were shared across three strains, showing that they probably carry similar functional capabilities (**Figure 3**). Twenty-six (1.4%) orthologous groups were exclusive in the *S. aureus* Sa1FB strain, which consisted of 12 protein-encoding prophage enzymes/proteins such as: helicase, terminase, endonuclease, tail tube protein, proteases, resolvase, polymerase, primase, and tail fiber proteins. Five proteins encoding for mobile element enzymes (transposases). Another finding was the identification of a hypothetical SAV0786 homolog in superantigen-encoding pathogenicity island SaPI (**Supplementary Table 3**).

Whole Genome Alignment

According progressiveMauve results, we found that most of the regions of the three genomes were highly conserved

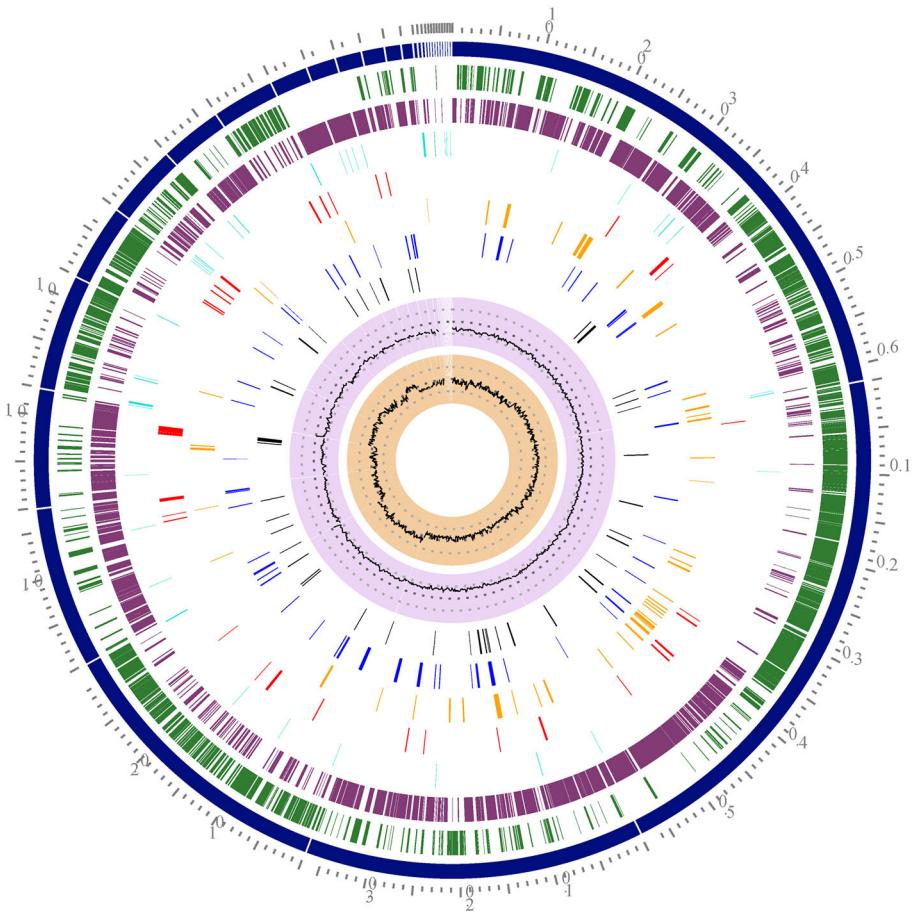

FIGURE 1 | Circular representation of the *S. aureus* Sa1FB draft genome. Tracks from the outermost are as follows: Contigs, Forward CDS, reverse CDS, RNA (tRNA and rRNA), AMR genes, virulence factors, transporters, and drug targets. The two inner tracks are G+C content and GC skew. The circular map was generated by using the circular viewer of PATRIC.

(**Figure 4**). Each locally collinear block (LCBs) in colors was a homologous region of sequences shared across the three genomes. Despite the rearrangements across the genomes, *S. aureus* Sa1FB and *S. aureus* Newbould exhibited the highest identity (98.96%) compared with *S. aureus* RF122 (97.92%) as shown by the ANI calculation prediction based on MUMmer by JSpeciesWS.

Biofilm Formation Genes

Subsystems Technology of the RAST identified the genes involved in the formation of biofilm in *S. aureus* Sa1FB and from these results it was possible to compare them with the biofilm formation genes predicted by the same tool available in PATRIC in the reference strains *S. aureus* Newbould and *S. aureus* RF122. The subsystems technology of RAST tool classified the biofilm formation genes in the following hierarchical classification: Superclass: Cellular processes; Class: Microbial communities; Subclass: Quorum sensing and biofilm formation; and Subsystem name: Biofilm formation in *Staphylococcus.*

For each strain (Sa1FB, Newbould, and RF122) it was possible to identify the same set of genes related with biofilm formation (**Supplementary Table 4**). The same 10 genes were identified across three strains and the sequence identity percentage was calculated as having *S. aureus* Sa1FB as a reference. Accordingly our annotation process was also able to identify another key gene for the biofilm formation, the gene *bap* (Biofilm associated protein) with the PATRIC identifier: fig|1280.24396.peg.2264. However, this gene was initially anotated as an hypothetical protein by the Rapid Annotations using Subsystems Technology tool kit (RASTtk) also found in PATRIC. In order to attribute function to this protein, we used a *bap* reference protein sequence deposited in the UniProt database (ID:Q79LN3) to align with our hypothetical protein, having a result of 99% of sequence identity.

Based on these results of the biofilm formation genes we could hypothesize that the *bap* gene is relevant for the biofilm formation in the Sa1FB strain, the presence of this gene being the main difference in the gene repertory related with biofilm formation across the three strains (Sa1FB, Newbould, and RF122).

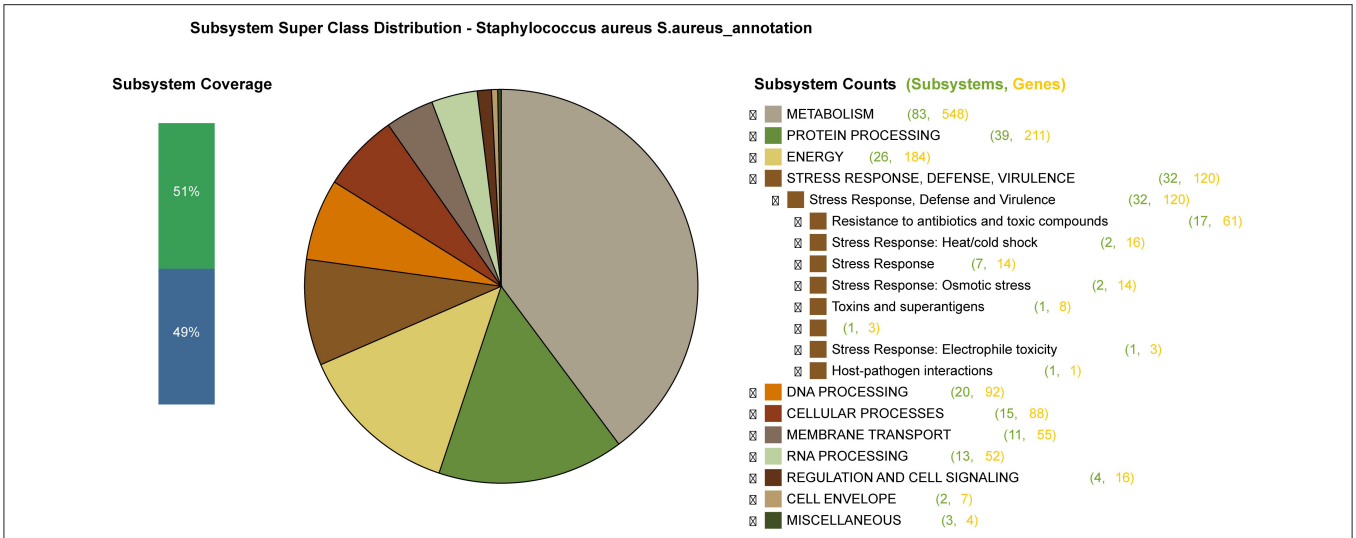

FIGURE 2 | An overview of the subsystem categories of the annotated draft whole-genome of *S. aureus* Sa1FB from the RAST server. The pie chart shows the counts of genes related to each subsystem. The bar graph (on the left) determines the subsystem coverage, this is the ratio of coding sequences annotated in the SEED subsystem (51%) and outside of the SEED subsystem (49%).

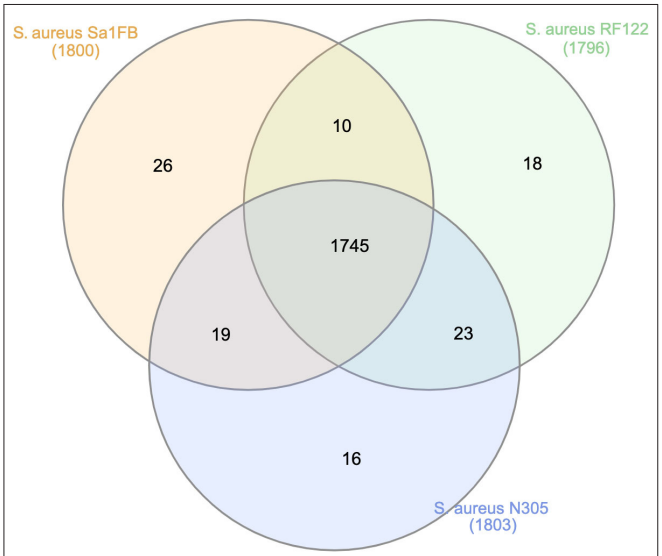

FIGURE 3 | Unique and shared orthologs among *S. aureus* Sa1FB, Newbould, and RF122 strains. N305: Newbould 305.

Antimicrobial Resistance

Antimicrobial resistance (AMR) phenotypes of *S. aureus* Sa1FB refer to the resistance or susceptibility to one or more antibiotics. According to the predictions on AMR phenotypes performed in PATRIC, *S. aureus* Sa1FB is susceptible to ciprofloxacin, clindamycin, erythromycin, gentamicin, methicillin, tetracycline, and trimethoprim sulfamethoxazole and is resistant to penicillin. These phenotypes were verified in the laboratory by Vitek®2 system (AST-GP79 card, bioMérieux, France), except for ciprofloxacin. In this case, two cephalosporins (cepaholotine and ceftiofur) were tested. AMR genes refer to genes implicated or associated with the resistance to one or more antibiotics. According to computational predictions based on CARD and NDARO databases *S. aureus* Sa1FB showed 60 genes likely related with resistance to different antibiotics (**Supplementary Table 5**).

Virulence Factors

Eighty-five genes were identified with potential virulence factors (**Supplementary Table 6**). Among the different features of the virulence factors it was possible to identify genes related with the following characteristics: immune evasion, toxins, secretion system type VII, adherence, iron and heme uptake, and proteases. *S. aureus* Sa1FB showed specific virulence factors compared with the other two bovine strains, these factors were: capsular polysaccharide synthesis enzyme Cap8E and fibronectin binding protein FnbB.

Cell Invasion

The percentage of cell invasion per each isolate ranged from 0.1 to 0.6%. **Table 2** shows the percentages of cell invasion per strain.

Biofilm Morphological Characteristics by Electronic Microscopy

Visual inspection of the three strains by SEM, allowed us to identify considerable morphological differences (phenotypes) among these strains (**Figure 5**). Interestingly, the Sa1FB strain formed a biofilm in which the bacterial cells were not embedded in an extracellular matrix, despite of being a carrier of the *ica* operon. In contrast, the bacterial aggregates were very compact and free of the matrix, which is a typical feature of strains that harbor the *bap* gene.

With respect to the Sa2FB strain, the bacterial cells appear to be embedded inside an extracellular matrix, a typical feature of this genotype (**Figure 5**). Regarding the cellular density, less

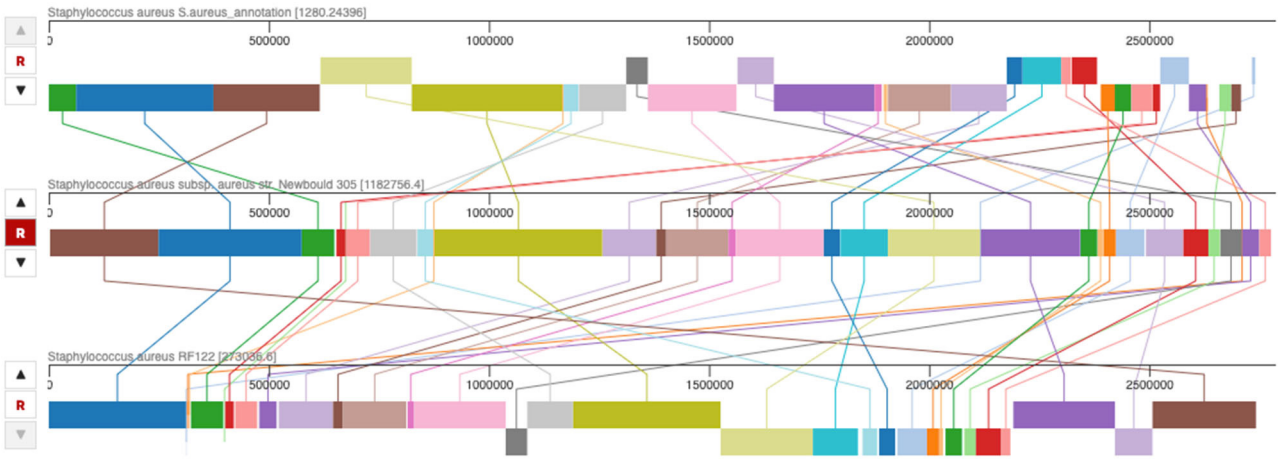

FIGURE 4 | Whole genome alignment among *S. aureus* Sa1FB, *S. aureus* RF122, and *S. aureus* Newbould 305 by progressiveMauve. The lines connecting between locally collinear blocks (LCBs) indicate the blocks were conserved among three genomes.

biomass was present in this isolate compared to Sa1FB, in agreement with the initial characterization of the isolates, which were weak and strong biofilm-forming, respectively.

The Sa3NF strain, corresponded to the genotype and phenotype described (*ica* $^{-}$–*bap* $^{-}$), which did not form biofilm (**Figure 5**).

TABLE 2 | Percentage of cell invasion by strain.

Strain	No. of CFU internalized	% of CFU internalized
Sa1FB	3,000	0.1
Sa2FB	6,000	0.2
Sa3NF	18,000	0.6

No, number; %, percentage; CFU, colony forming units.

DISCUSSION

The aim of this research was to explore both genotypic and phenotypic characteristics of the Sa1FB strain with strong biofilm production, and to make comparisons with other relevant *S. aureus* genomes available. These characterizations and comparisons would help to explore the zoonotic potential of this particular strain, given the virulence characteristics within the context of the milk production system in Colombia.

Alignment of the *S. aureus* Sa1FB genome with the Newbould 305 and RF122 bovine strains were recognized as causing clinical mastitis, sharing the majority of the locally collinear blocks (LCBs), indicating a substantial amount of conserved genetic information among these bovine strains. The predicted value of ANI among strains also showed a high similarity among these three strains at the genomic level. Likewise, no appreciable differences were observed among the virulence factors of the strains, agreeing with what was reported recently in a study carried out in Brazil, which compared four isolates, three of these belonging to ST126, with the same reference strains used in our study (30).

Sa1FB strain was classified as ST126, a genotype associated with infections in cows. This genotype has also been reported in the USA, Italy, and Brazil, where Brazil showed most of the reports (31–35). Furthermore, in Brazil, ST126 is one of the clones most frequently isolated from bovine mastitis and associated with persistent infections (32–34). According to PubMLST database, ST126 was assigned to CC97, since this matches at four loci with ST97 (central ST of CC97). Our results contrast with most of the reports, that place ST126 into CC126 (32–34, 36). These studies probably used other methodologies (e.g., eBURST—it is a single-linkage method) or database (www.mlst.net—currently not available) to assign the clonal complex. These discrepancies in the nomenclature could be a problem to epidemiologic surveillance of clones belonging to complexes with zoonotic potential, since CC97 is one of main *S. aureus* clonal complexes which cause bovine infections and an emerging cause of human infections. Although, the ST126 strain has only been associated with infections in cows to date, this does not guarantee that a spillover event (switch to humans) could not occur (8).

It is difficult to predict exactly when a strain will switch its genotype and adapt to a new host, because this event will depend on natural recombinations among different clones that could confer in them virulence, resistance, or immune evasion pathways (37, 38). The results obtained in this study do not predict the time it would take for the Sa1FB strain to evolve and adapt to humans, since the evidence has suggested that the recombinations can be higher *in vivo* than predicted *in vitro* or with bioinformatic tools (38). An *in vivo* study showed a high rate of transfer of mobile genetic elements (MGE) between strains isolated from animals and humans during the first 4 h of the co-colonization process (38). Also, exchange of extensive genomic regions (up to 20% of the genome) between distant

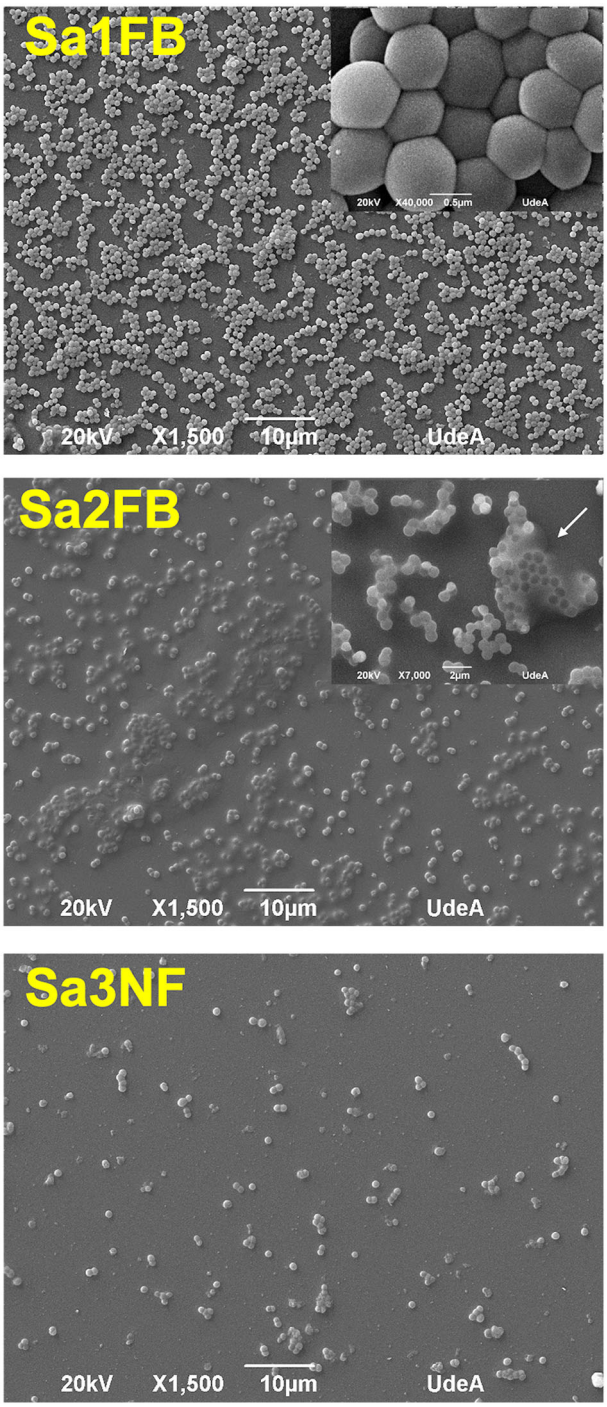

FIGURE 5 | Scanning electron microscopy images of the biofilms formed by Sa1FB and Sa2FB strains. The white arrow in the Sa2FB image shows the extracellular matrix. The three images showed the differences in the ability to form biofilm: Sa1FB was strong biofilm-forming, Sa2FB was weak biofilm-forming, and Sa3NF was non biofilm-forming.

lineages have been detected (37). Regarding clones of bovid origin, there are previous reports that highlight the emergence of clones that switched to humans (18, 31). One study showed the spread to humans of community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains of bovine origin belonged to CC97 (39, 40). These authors detected in the strains MGE that conferred antimicrobial resistance and the capacity to evade the immune response in humans, key factors to survive and transmit among humans (40). In Denmark, cases of infected humans with strains from CC97 increased 11-fold between 2007 and 2011 (40). Likewise, other studies have also highlighted the rapid increase of the number of people colonized with *S. aureus* of the ST398 (CC398), which is a genotype shared by pigs (primary host), cattle, chicken, horses, and humans (mainly in farmer working) (41, 42). The ST398 had been recognized as a livestock-associated MRSA (LA-MRSA) strain. This clone generally is transmitted from pigs to humans, but rarely from person to person (42). However, a recent report from Denmark described a severe case of infection with LA-MRSA CC398, which was presented with bacteremia and an epidural abscess (42). Since the patient was not exposed to livestock, the authors suggested that the transmission was from person to person (42). These findings demonstrate the high ability of adaptation in *S. aureus* and its pathogenic potential in different hosts. Also, it provides strong evidence that livestock could act as a potential source of new human-pathogenic *S. aureus* strains, like other clones belonging to CC97.

The genotypic and phenotypic analysis performed on our strain (Sa1FB) showed different virulence factors; among these, the high ability to form biofilm. This mechanism allows resistance to antibiotics and disinfectants, as well as the evasion of the immune response of the host. These characteristics allow this pathogen to persist for a long time in both biotic and abiotic environments (12). In infections caused by biofilm-forming *S. aureus* strains, the concentration of antibiotic required to kill them can be 10-fold higher compared to planktonic bacteria (43–45). In addition, exposure to antibiotic selection pressure in biofilms has also been linked to the development of antibiotic resistance in this pathogen (46). According to the phenotypic predictions performed in PATRIC and confirmed by Vitek®2 system, *S. aureus* Sa1FB is susceptible to different antibiotics and resistant to penicillin. Previous works performed in the region showed the same behavior (penicillin resistance) in the evaluated *S. aureus* strains (47, 48). Despite low antibiotic resistance found in Sa1FB, a study reported that *S. aureus* strains carrying the *bap* gene were less susceptible to antibiotic treatments when forming biofilm *in vitro* and more persistent in the bovine mammary gland (22).

We found that the Sa1FB strain was a carrier of *ica*, *fnbB*, and *bap* genes, which had been widely associated with the biofilm formation process (13, 22, 49, 50). Both *fnbB* and *bap* were only identified in Sa1FB but not in the reference strains included. This result is in agreement with what was identified in a study which compared eight *bap* positive strains with the reference strain V329. The authors also detected these genes in the isolates evaluated (35). The *bap* gene encodes the biofilm-associated protein (Bap), involved in intercellular adhesion and, subsequently, in bacterial accumulation. This gene generally confers a strong biofilm-forming phenotype and is located in a transposon inserted in the SaPIbov2 mobile pathogenicity island (22, 51). This gene was the main difference in the gene

repertory related with biofilm formation across three strains (Sa1FB, Newbould and RF122). In contrast to what is commonly reported, *bap* positive strains are usually isolated from bovine mastitis, a research carried out in Italy found this genotype in pigs, suggesting that they potentially have the ability of moving across different hosts (39). It has also been demonstrated in experimental infections that *bap* positive strains cause more persistent infections in bovines and present higher resistance to antibiotics in comparison to strains that did not harbor this locus (22). Recently, we highlighted the virulence potential of this genotype, since we reported that strains carried the *bap* gene were more capable of producing strong biofilms than *bap*-negatives (20).

Regarding *ica* operon, which encodes the polysaccharide intercellular adhesion (PIA) factor, which is the most common mechanism used by *S. aureus* for biofilm formation, since most of the clinical isolates involved in both human and animals infections carry this locus (13). Despite the Sa1FB strain also harboring the *ica* operon, the phenotype observed by electronic microcopy did not show the extracelullar matrix characteristic of PIA-dependent biofilm. In contrast, we observed a very compact bacterial aggregate where this matrix is not evident, suggesting that in the presence of both loci, *bap* gene drives the biofilm formation. These findings are consistent with other reports, who have described two biofilm phenotypes, one where cells are embedded by an extracellular matrix (PIA-dependent) and the other based on proteins such as fibronectin-binding proteins (FnBPs) and Bap (PIA-independent) (13, 49). A research also concluded that the *bap* gene was sufficient to generate biofilm, even in the absence of the *ica* operon (22). It is important to highlight that SEM does not determine the presence of PIA. For it is necessary to use other techniques such as antibodies PIA-specific, staining or quantification of sugar content in the matrix.

On the other hand, the *fnbB* gene encodes for fibronectin-binding protein B (FnBPB), a molecule that plays an important role in PIA-independent biofilms (13, 49, 52). This protein is involved in the primary attachment phase to fibronectin, elastin, and fibrinogen, as well as in the accumulation process in biofilm formation (13, 52). It has also been demonstrated that FnBPB favors cell invasion and protects against the antimicrobial activity of histones released during the neutrophils extracellular traps (NETs) (23, 53). Since we did not evaluate the presence of this marker in the other two native strains tested, we cannot discuss its influence in the invasion capacity. A study in which a mutant strain for *fnb* was generated showed significantly impaired colonization and biofilm formation in the animal model used (54). Some studies have concluded that genes that promote better colonization of *S. aureus* and better immune response evasion can help its spread from person to person (37, 55).

Biofilms have been recognized as niches where gene transfer among bacteria can occur, conferring them antimicrobial resistance and the capacity for adaptation to different hosts and environments (56–58). Several studies have revealed that horizontal gene transfer and biofilms are related processes and have high relevance in bacterial adaptation and evolution (56, 57). The acquisition of the great majority of MGE is performed through horizontal gene transfer, including the *S. aureus* pathogenicity islands (SaPIs), that can be disseminated in bacterial populations through phages (59). We exclusively found in Sa1FB material associated with MGE (prophages and transposases), confirming its ability to accept genetic material. MGE transfer is usually higher within biofilms than in planktonic cells, since biofilms provide optimal conditions for bacterial interactions, due to the close proximity of cells within this structure and because the matrix can accumulate different chemical compounds that facilitate the process (e.g., communication signals and extracellular DNA) (60). Hence, biofilms have a high impact on the dissemination of MGE in bacteria of clinical and industrial relevance, such as *S. aureus*. In a study performed in the United Kingdom demonstrated that biofilm formed by this specie significantly increased horizontal transfer of plasmid-borne antibiotic resistance (58). Other studies reported that staphylococci within biofilms increased mutability, which could also accelerate the spread of resistance and adaptability traits among strains that coexist in these biofilms matrices (61). Similarly, other authors detected some horizontal transfer and antibiotic resistance genes in the staphylococcal clinical strains collected from biofilms (57). According to published results in Chile, biofilms produced on surfaces from milking equipment can act as source of *S. aureus* contamination for bulk tank milk and cows. This same study found that several genotypes can inhabit inside the same biofilm simultaneously (62). Due to the high ability to form biofilm in our strain, it can generate this structure on surfaces in contact with milk during milking process or cooling, where it could interact and MGE exchange with zoonotic strains. Recently, we conducted a study in Antioquia (Colombia), in which 97 strains isolated from IMI were genotyped by spa typing. The results showed that 50% of bovines were infected with genotypes that also caused infections in humans, a fact that demonstrated close proximity between human and bovine strains in the region, as well as the high risk of a spillover event (63).

Cell invasion is another way in which *S. aureus* can avoid antibiotics and the immune response in hosts (14). In our study, the Sa1FB strain showed the lowest invasion percentage, supporting results previously informed by other authors, who evidenced that Bap promoted the adhesion and biofilm formation but prevented cellular internalization (64). We observed that as the ability to form biofilm in the strains tested decreased, the percentage of cell invasion increased, indicating that the adhesion and formation of bacterial accumulations on epithelial cells probably reduced the entry of *S. aureus* into them. Similar results were observed in a study conducted in Brazil, since they found that the strain with the largest production of biofilm presented the lowest invasion rate; whereas, the strain that showed poor biofilm formation had the highest invasion rate. However, they did not observe any relation between the invasion capacity and *bap* gene presence (65). Contrary to our finding, there are reports that did not find an association between capacity to produce biofilm and the invasiveness (23, 66).

In Colombia, where this pathogen has been reported as one of the main causes of IMI, the importance of cows as a source of zoonotic *S. aureus* strains is unclear. The risk of transmission of this pathogen to people working in close contact with livestock, especially for those who perform milking by hand, is probably high. These facts highlight the relevance of surveillance for early

detection of emergent clones and the application of biosecurity actions in the agricultural setting that reduces the risk of occupational exposure and their spread in the community (40).

CONCLUSIONS

This study shows the high genetic similarity between *S. aureus* Sa1FB and the reference strains, despite the differences reported at the clinical level. Nevertheless, the Sa1FB strain exhibited special features in terms of MGEs, highlighting its ability to accept foreign genetic material. Indeed, this could increase its mutability, pathogenesis, and adaptability to new hosts, which represents a risk for milkers and people in close contact with the milk obtained from animals infected with these strains. Furthermore, the high ability of Sa1FB to form biofilm would generate the proper environment where the exchange of genetic material among strains could occur.

These findings highlight the relevance of surveillance for the detection of emergent clones with zoonotic potential, which reduces the risk of occupational exposure and their spreading in the community.

DATA AVAILABILITY STATEMENT

The datasets generated in this study are deposited in PATRIC (patricbrc.org) under the genome ID: 1280.24396 (https://www.patricbrc.org/view/Genome/1280.24396).

AUTHOR CONTRIBUTIONS

GT and MO-A contributed to the conception, design of the study, and were responsible for funding acquisition. GT and KV carried out the experiments. GT, YC-A, JR-V, and MO-A carried out the analyses data. All authors wrote, contributed to manuscript revision, and approved the submitted version.

FUNDING

This study was supported by Colciencias (grant number 727, 2015), University of Antioquia (grant number 2017-15551), and Biogenesis Research Group (grant number ES84180138) from Colombia.

ACKNOWLEDGMENTS

We wish to thank Dr. Tatiana Ruiz (University of Antioquia) for providing the clonal bovine mammary epithelial cells.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00530/full#supplementary-material

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 29 September 2020
doi: 10.3389/fvets.2020.547891

Genomic Epidemiology of *Salmonella* Infantis in Ecuador: From Poultry Farms to Human Infections

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 01 April 2020
Accepted: 20 August 2020
Published: 29 September 2020

Citation:
Mejía L, Medina JL, Bayas R, Salazar CS, Villavicencio F, Zapata S, Matheu J, Wagenaar JA, González-Candelas F and Vinueza-Burgos C (2020) Genomic Epidemiology of Salmonella Infantis in Ecuador: From Poultry Farms to Human Infections. Front. Vet. Sci. 7:547891. doi: 10.3389/fvets.2020.547891

Salmonella enterica is one of the most important foodborne pathogens around the world. In the last years, *S. enterica* serovar Infantis has become an important emerging pathogen in many countries, often as multidrug resistant clones. To understand the importance of *S. enterica* in the broiler industry in Ecuador, we performed a study based on phenotypic and WGS data of isolates from poultry farms, chicken carcasses and humans. We showed a high prevalence of *S. enterica* in poultry farms (41.4%) and chicken carcasses (55.5%), but a low prevalence (1.98%) in human samples. *S.* Infantis was shown to be the most prevalent serovar with a 98.2, 97.8, and 50% in farms, foods, and humans, respectively, presenting multidrug resistant patterns. All sequenced *S.* Infantis isolates belonged to ST32. For the first time, a pESI-related megaplasmid was identified in Ecuadorian samples. This plasmid contains genes of antimicrobial resistance, virulence factors, and environmental stress tolerance. Genomic analysis showed a low divergence of *S.* Infantis strains in the three analyzed components. The results from this study provide important information about genetic elements that may help understand the molecular epidemiology of *S.* Infantis in Ecuador.

Keywords: *Salmonella* Infantis, ST32, broiler, WGS, Ecuador, megaplasmid, multidrug resistance (MDR)

INTRODUCTION

Foodborne infections caused by *Salmonella enterica* are of primary importance worldwide. The WHO estimates that *Salmonella* causes more than 153 million illnesses, 120,281 deaths, and 8.27 million disability-adjusted life years annually (1). As foodstuffs can be contaminated in several parts of the food chain, a "from farm to table" approach is necessary to understand the epidemiology of *Salmonella*. Although *Salmonella* can contaminate vegetables, food-producing animals, especially poultry, are considered important sources for human infections (2, 3). In Latin American countries, poultry is one of the main sources of protein of animal origin. This is the case of Ecuador, where poultry meat is the most consumed commodity with a yearly per capita consumption of 30.4 Kg (4).

Previous investigations in Latin America showed that *Salmonella enterica* serovar Infantis is an increasingly important serotype on poultry farms (5–9). Moreover, this serotype has also been reported to cause infections in local inhabitants and travelers that have visited Latin American countries (10–12). However, no genomic data considering isolates of *Salmonella* originated from animals, foodstuff, and humans have been released in Latin America so far.

Antimicrobials are commonly used in poultry production as both therapeutics and growth promoters. However, even when antimicrobials are used under technical criteria, they can select resistant strains of *Salmonella* that pose a public health problem when reaching consumers (13). This is of special concern in developing countries where the misuse of antimicrobials and lack of control is an issue to be addressed.

There is a wide diversity of virulence factors that are essential for pathogenicity of *Salmonella* in host cells. Among these factors, fimbriae, flagella, plasmids, pathogenicity islands, toxins, and secretion systems are the more frequently associated to pathogenic strains of *Salmonella* (14).

This research was aimed at describing by phenotyping methods and whole-genome sequencing, the antimicrobial resistance (AMR) characteristics and genetic profiles of *Salmonella* isolates obtained from broiler farms, broiler carcasses and humans in Quito—Ecuador.

MATERIALS AND METHODS

Study Design and Sampling

Samples for *Salmonella* isolation were collected weekly from November 2017 to November 2018 following the guidelines of the document: "Integrated Surveillance of Antimicrobial Resistance in Foodborne Bacteria" by the World Health Organization (15).

Poultry Farms: 133 flocks from 69 farms were sampled during the study period. For every sampled flock, 25 caeca from 25 chicken were randomly collected at the slaughterhouse level and transported to the laboratory in an ice-box within the next 2 h. Caecal samples are recommended by the WHO because they provide a higher recovery of isolates and better represent contamination of individual animals at the farm level (15). At the laboratory, a sample pool of 25 g was obtained for bacteriological isolation as previously described (8).

Chicken carcasses: 335 carcasses were collected in three kinds of markets as follows: 125 samples from supermarkets, 126 samples from small shops, and 84 samples from open markets. Sampling of chicken carcasses was performed alternately between the north and south of the city. Each carcass was collected in its original bag and transported to the laboratory in an ice-box within the next 2 h. At the laboratory, 25 g of breast skin of every carcass were aseptically collected for bacteriological analysis.

Human stool samples: 302 samples were evenly collected in two health care centers located in the urban periphery of Quito (Guamani health care center at the south and Calderon health care center at the north) from patients with two or more episodes of diarrhea or vomiting in the last 24 h. Human stool samples were transported to the laboratory in an ice-box within the next 2 h. Approximately 25 g of feces were collected for bacteriological analysis.

According to national legislation, ethics approval was not required for poultry farms and chicken carcasses sampling since no animals were sacrificed during this study. For the human component, the project was approved by the bioethics committee from the National Institute of Public Health "Leopoldo Izquieta Pérez" (Protocol ID:CEISH-INSPI-005). The participants were informed about the objective of the study and all volunteers provided a written consent. All personal information was anonymized.

Isolation and Identification of *Salmonella enterica*

Salmonella isolation was performed by a method based in the ISO 6579-1:2007 protocol. Briefly, 225 mL of Buffered Peptone Water (BPW; Difco, BD, Sparks, MD) was added to every sample, homogenized by hand for 1 min and incubated at 37°C for 20 h. Then, 100 μL of each enrichment was inoculated onto Modified Semi-solid Rappaport-Vassiliadis agar (MSRV; Oxoid, Basingstoke, UK) in three equidistant points and incubated at 42°C for 24 h. Afterwards, plates were examined for the presence of a white halo around of at least one inoculation point. A loopful taken from the edge of the white halo was streaked on a Xylose Lysine Deoxycholate agar (XLD, Difco) and incubated at 37°C for 24 h. After incubation, one suspect colony of *Salmonella* was biochemically confirmed by Triple Sugar Iron (TSI, Difco, BD), Iron Lysine (LIA, BBL, BD), Urea (BBL, BD), and Sulfur Indole Motility tests (SIM, BBL, BD). Isolated colonies were confirmed by PCR as previously described (16). The 95% confidence interval ($CI_{95\%}$) for the prevalence of *Salmonella* at each component was calculated.

Antimicrobial Susceptibility Testing

All *Salmonella* isolates were examined by the Kirby-Bauer disk diffusion method with the following antibiotics: sulfamethoxazole + trimethoprim (25 μg), gentamicin (10 μg), ciprofloxacin (5 μg), cefotaxime (30 μg), tetracycline (30 μg), streptomycin (10 μg), chloramphenicol (30 μg), cefoxitin (30 μg), amikacin (30 μg), nitrofurantoin (300 μg), azithromycin (15 μg), fosfomycin (200 μg), ertapenem (10 μg), amoxicillin + clavulanic acid (30 μg). *E. coli* ATCC 25922 strain was used as quality control. Results and methods were interpreted according to CLSI 2019 criteria considering all intermediate phenotypes as resistant for further analysis (17).

Detection of Extended Spectrum Beta-Lactamase (ESBL) Genes

Salmonella isolates that presented resistant phenotypes to beta-lactam antibiotics were further tested by PCR for the identification of ESBL genes. PCR conditions and primers were the ones described by Hasman et al. (18) for bla_{CTX-M}, Olesen et. al (19) for bla_{TEM}, Kruger et al. (20) for bla_{CMY} and Arlet et al. (21) for bla_{SHV}. Sub-families of bla_{CTX-M} genes were identified with PCR protocols described by Carattoli et al. (22) for $bla_{CTX-M-1}$, Jiang et al. (23) for $bla_{CTX-M-2}$, Hopkins et al. (24) for $bla_{CTX-M-8}$, Paauw et al. (25) for $bla_{CTX-M-9}$ and

Dierikx et al. (26) for $bla_{CTX-M-14}$. Amplification products were confirmed by gel electrophoresis using a 2% agarose gel. All PCR products were purified and sequenced at Macrogen Inc (Seul-South Korea). Obtained sequences were aligned against reference sequences with the online tool ResFinder v3.2 (27).

Whole Genome Sequencing

For whole genome sequencing (WGS), a selection of *Salmonella* isolates was made from the animal and food components. When selecting isolates from poultry farms the first positive sample of each farm was considered. For chicken carcasses, the first positive sample of every sampling week in each kind of market was selected. All non-*S.* Infantis isolates and all isolates from the human component were selected for WGS.

Genomic DNA was extracted using Invitrogen PureLink Genomic DNA Kit (Thermo Fisher Scientific, Walthman, MA, USA) following manufacturer's recommendations for Gram-negative bacterial cell lysates. DNA was quantified using Invitrogen Qubit 3.0 fluorometer (Thermo Fisher Scientific, Walthman, MA, USA), and sequenced with the Illumina NextSeq platform using Nextera XT Library Preparation Kit obtaining 150 × 2 bp paired-ends sequences (Illumina, San Diego, CA, USQ). Default parameters were used for all bioinformatic tools and programs unless otherwise specified. Reads were trimmed with Trimmomatic to remove ambiguous nucleotides and those with quality score values <20 (28). The programs Fastqc (29) and Multiqc (30) were used for quality assessment.

Serotype Identification

Salmonella serotypes were identified by PCR as described by Akiba et al. (16). Additionally, serotypes of isolates subjected to WGS were further confirmed by the analysis of their raw sequencing reads using the SeqSero pipeline (31).

MLST Analysis, Antimicrobial Resistance Genes, and Plasmid Detection

In order to identify MLST sequence types (ST), antimicrobial resistance genes and plasmid sequences, ARIBA (32) was used with PubMLST (33), ResFinder v3.2 (27), and PlasmidFinder 2.1 (34) databases, respectively. Phenotype resistance was compared with the presence of resistance genes found by WGS. Additionally, we performed a mapping against the megaplasmid p-F219 described by Vallejos-Sánchez et al. (35) using Burrows-Wheeler Aligner with BWA-MEM algorithm (36). BCFtools and vcfutils from SAMtools (37) were used to obtain the fastq files from SAM files, and the fasta sequences were transformed from fastq with Seqtk (38). The sequences were concatenated and a maximum likelihood phylogenetic tree was obtained with IQTREE2 software (39). As we obtained two well-defined clusters for *S.* Infantis, we annotated one representative of each plasmid cluster with Prokka (40) and performed an orthologous genes analysis (coverage of 90%, similarity on protein sequences of 80%) with Proteinortho5 (41). A manual comparison of all genes present in the plasmids was carried out. We performed the same analysis for non-Infantis isolates.

Megaplasmid Analysis

Two megaplasmids (pESI and p-F219) commonly associated to pathogenic and MDR strains of *S.* Infantis were analyzed in order to identify their relatedness. We used D-Genies (42) to obtain a dot plot of genome comparison, a genome alignment with progressiveMauve (43) in order to identify locally collinear blocks, and an ANI calculation (44) for computing average nucleotide identity in sequences shared by both plasmids.

Core Genome and Metadata Analysis

A Peruvian *S.* Infantis strain, FARPER-219 (35), and two Ecuadorian isolates (SRR4019589 and SRR4019602) analyzed by the US Centers for Disease Control and Prevention from two patients that developed salmonellosis after traveling to Ecuador (10) were added as references in the phylogenetic analysis of all *S.* Infantis isolates. From trimmed reads, Spades (45) was used to generate assemblies. Later, genome annotation was performed with Prokka (40). An orthologous genes analysis with the same conditions as for plasmid detection was performed with Proteinortho5 (41). The strict core genes, those present in all the isolates, were extracted with the Proteinortho tool: grab_proteins.pl. Mafft (46) and an in-house script were used for multiple alignment of every gene and subsequent concatenation in a single multiple alignment, respectively. The phylogenetic tree from the core genome alignment was obtained using IQTREE2 (39) with 1,000 bootstrap replicates. The metadata for sample origin, phenotypic antibiotic resistance patterns, and plasmid *in silico* detection was added to the final tree with iTol tools (47).

RESULTS

Salmonella Prevalence and Serotype Identification

Salmonella was present in 41.4% (55/133; $CI_{95\%}$:33–49.7), 55.5% (186/335; $CI_{95\%}$:50.2–60.8), and 1.98% (6/302; $CI_{95\%}$:0.4–3.6) in poultry farms, chicken carcasses and human stool samples, respectively. *S.* Infantis accounted for 98.2% ($n = 54$) of isolates from poultry farms, 97.8% ($n = 182$) of isolates from chicken carcasses, and one half ($n = 3$) of human sample isolates. Additionally, one isolate was typed as *S.* Enteritidis in broiler flocks; at the retail level one and three isolates were typed as *S.* Typhimurium and *S.* Enteritidis, respectively, while in the human stool samples two isolates were typed as *S.* Enteritidis and one isolate corresponded to monophasic *S.* Typhimurium 4,[5],12:i:- (**Supplementary Table 1**).

Antimicrobial Resistance

For *S.* Infantis isolates, antimicrobial resistance rates to nitrofurantoin, tetracycline, sulfamethoxazole + trimethoprim, streptomycin, gentamicin, cefotaxime, ciprofloxacin and chloramphenicol ranged from 64.8 to 100%. On the other hand, fosfomycin and azithromycin resistance rates were lower, ranging from 0 to 42.6%. Only one isolate from a stool sample presented phenotypic resistance to amikacin while none of the *Salmonella* isolates in this study was resistant to ertapenem (**Table 1**).

TABLE 1 | Number of *S.* Infantis isolates resistant to each tested antimicrobial.

Antimicrobial	Number (%) of resistant isolates		
	Poultry farms (farm)	Chicken carcasses (food)	Stool samples (human)
Nitrofurantoin	54 (100)	180 (99.4)	2 (66.7)
Tetracycline	54 (100)	176 (97.2)	3 (100)
Sulfamethoxazole + trimethoprim	44 (81.5)	158 (87.3)	1 (33.3)
Streptomycin	46 (85.2)	154 (85.1)	3 (100)
Gentamicin	45 (83.3)	155 (85.6)	2 (66.7)
Cefotaxime	51 (94.4)	150 (82.9)	1 (33.3)
Chloramphenicol	45 (83.3)	149 (82.3)	2 (66.7)
Ciprofloxacin	35 (64.8)	116 (64.1)	1 (33.3)
Fosfomycin	23 (42.6)	68 (37.6)	1 (33.3)
Azithromycin	10 (18.5)	31 (17.1)	0 (0)
Cefoxitin	7 (13)	11 (6.1)	0 (0)
Amoxicillin + clavulanic acid	7 (13)	7 (3.9)	0 (0)
Amikacin	0 (0)	0 (0)	1 (33.3)
Ertapenem	0 (0)	0 (0)	0 (0)

TABLE 2 | Antimicrobial resistance patterns of *S.* Enteritidis, *S.* Typhimurium, and monophasic *S.* Typhimurium 4,[5],12:i:-.

Resistant pattern	No. Antimicrobial clases	*S.* Enteritidis	*S.* Typhimurium	Monophasic *S.* Typhimurium 4,[5],12:i:-
SAQBTFN	7		1	
SAQTFNM	7	1		
SABTFNP	7	1		
SABTFN	6			1
QFM	3	1		
N	1	3*		

**1 isolate obtained from poultry farms and 2 isolates from human stool samples. Sulfonamide (S), aminoglycosides (A), quinolones (Q), Beta-lactams (B), tetracyclines (T), phenicol (F), nitrofuran (N), macrolides (M), Fosfomycin (P).*

Considering antimicrobial classes that were tested, *S.* Infantis isolates presented 43 antimicrobial resistance patterns. With the exception of one isolate from a stool sample, all isolates showed multidrug-resistant phenotypes. Importantly, 87 and 82% of isolates from poultry farms and chicken carcasses, respectively, presented resistance from 6 up to 9 classes of antimicrobials (**Supplementary Table 2**). One *S.* Infantis isolated from chicken carcasses could not be recuperated for this analysis.

Salmonella serotypes other than *S.* Infantis also presented multiresistant patterns, except for 3 *S.* Enteritidis isolates that were only resistant to one group of antimicrobials. For this set of isolates, every resistant pattern included isolates belonging to only one serotype (**Table 2**).

One isolate of *S.* Enteritidis, one of *S.* Typhimurium and one of Monophasic *S.* Typhimurium 4,[5],12:i:-; and 205 isolates of *S.* Infantis were identified as resistant to beta-lactam antibiotics.

Six *S.* Infantis isolates from chicken carcasses and one from poultry farms did not present any of the investigated ESBL genes. All other *S.* Infantis and one *S.* Enteritidis isolated from a carcass presented the $bla_{\mathrm{CTX-M-65}}$ gene.

Genomic Analysis

For WGS analysis, 144 isolates (40 from the animal component, 98 from the food component and six from the human component) were selected. Raw sequence data is available under bioproject PRJEB37560. The sequences from three samples were not enough to perform genomic analysis. The obtained average number of reads per strain was 1,356,678 (range 247,022–14,106,025) and after the quality control steps, the average number was 1,266,242 (range 228,263–13,094,594) (**Supplementary File 1**). Average Phred Score was Q34.

MLST typing showed that all *S.* Infantis isolates ($n = 137$) belonged to ST32. The five *S.* Enteritidis isolates belonged to ST11. Additionally, the single isolates of *S.* Typhimurium and monophasic *S.* Typhimurium 4,[5],12:i:- belonged to ST19 and ST2379, respectively (**Supplementary Table 1**).

The strict core genome of all *S.* Infantis included in the analysis corresponded to 3,552 genes and spanned 3,161,448 bp, 1,414 of which were variable (SNPs). The alignment of the concatenated genes present in this core was used to obtain a maximum-likelihood tree using FARPER 219 as outgroup (**Figure 1**). This strain was chosen because it was isolated in Peru, a neighbor country to Ecuador. Despite its inclusion in ST32, FARPER-219 presented genetic divergence with the Ecuadorian strains. The two *Salmonella* genomes from the USA (SRR4019589, SRR4019602) grouped indistinctive with some of the genomes of this study. Notably, the analyzed strains did not group according to their sampling origin or their phenotypic resistance patterns.

Genes of antimicrobial resistance were also confirmed with WGS data (**Supplementary File 2**). For most of the antimicrobial classes (folate pathway inhibition, aminoglycoside, beta-lactams, tetracyclines, fosfomycin, and phenicol) correspondence rates were higher than 80%. However, no genes responsible for the phenotypic resistance to quinolones, nitrofurans, and macrolides were found in sequenced isolates (**Table 3**).

Regarding virulence genes, *sifA, sseL, pipB, sopD2,* and *srlP* (part of SPI 2) were found in the core genome of all *S.* Infantis strains. The *lpf* operon that encodes the long polar fimbrae (LPF), the *fim* gene cluster that encodes type 1 fimbriae, and the *csg* operons that encodes the Tafi fimbriae (Thin aggregative fimbriae) were also present in these genomes.

The presence of plasmids was confirmed *in silico* by PlasmidFinder. Only one *S.* Infantis isolate (U2449s) presented one plasmid determinant (IncX1 and IncX1_1) despite the multidrug resistance patterns found in our isolates (**Figure 1** and **Supplementary File 2**). To further analyze the low incidence of plasmids, we mapped the raw reads from our samples against the megaplasmid p-F219 described by Vallejos-Sánchez et al. (35). We found two p-F219-like plasmids. The first one,

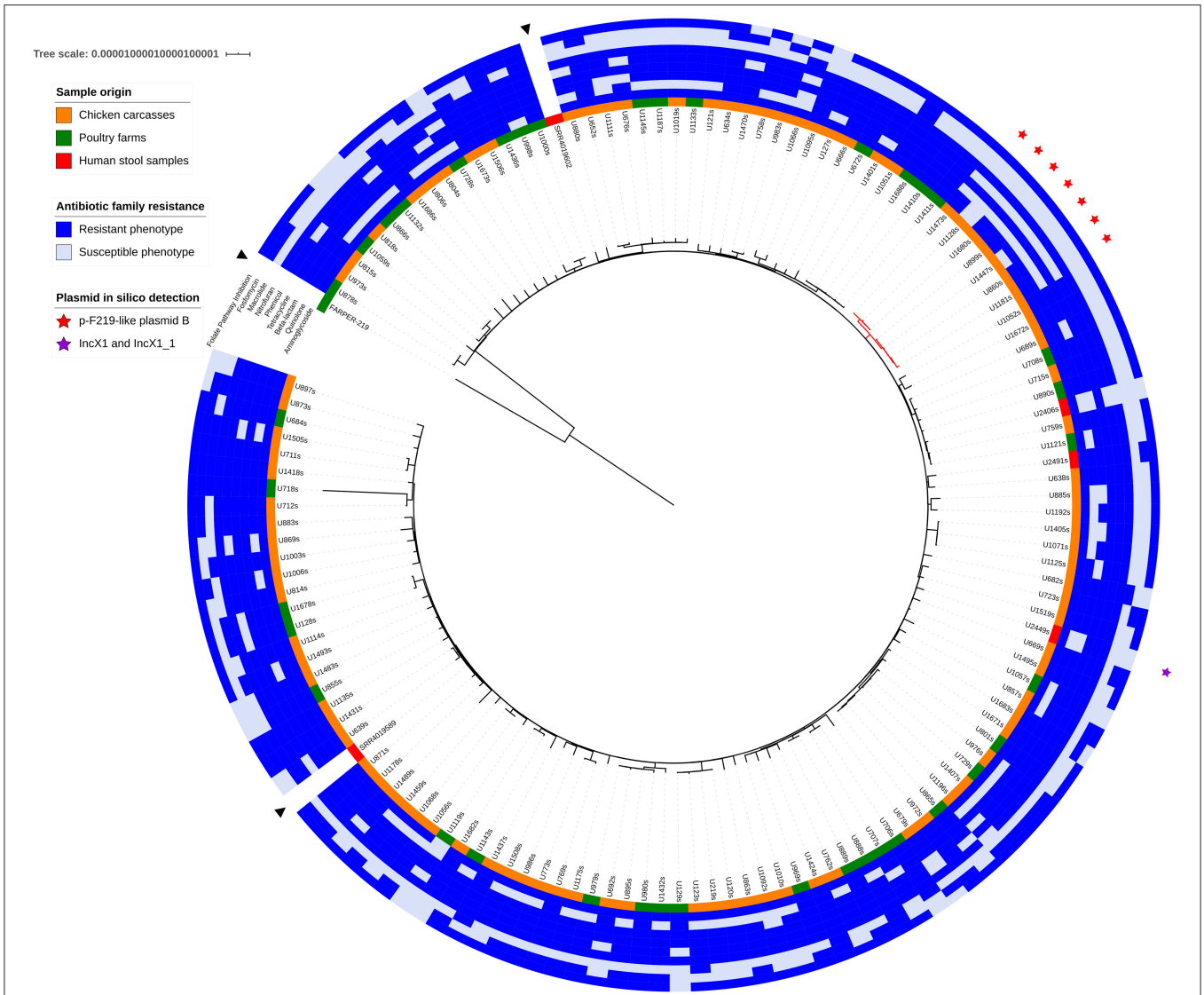

FIGURE 1 | Maximum likelihood phylogenetic tree of core genome alignment of all 137 *S.* Infantis isolates based on 3,552 genes. Two *S.* Infantis genomes from Ecuador detected in USA (SRR4019589, SRR4019602) and a Peruvian strain (FARPER-219) were included in the analysis and are indicated with a black triangle. The origin of each sample is colored in red for human stool samples, in green for poultry farms isolates and in orange for chicken carcasses strains. The phenotypic resistance for nine antibiotic families is marked with a blue box. Strains with p-F219-like plasmid B are marked with a red star. The rest of the samples harbor the p-F219-like plasmid A. The purple star indicates the presence of IncX1 and IncX1_1 plasmids in one of the strains. Digital version of the phylogenetic tree is available with iTOL login LMejia at https://itol.embl.de/shared_projects.cgi.

denoted as plasmid A, was present in most of the strains. This plasmid contained 338 genes shared with the p-F219 megaplasmid. The second one, denoted as plasmid B, was present in the remaining seven strains. Plasmid B lacked 72 of the genes present in plasmid A and presented six exclusive genes (**Supplementary File 3**). The strains that presented plasmid B belonged to a monophyletic clade (denoted with a red star in **Figure 1**). These strains also share susceptibility to fosfomycins, macrolides, phenicols, and beta-lactams. *Salmonella* strains harboring plasmid B were isolated from chicken carcasses sampled during different weeks of the year, different parts of the city and different types of retail stores (data not shown).

New hypothetical proteins (n = 147) were found in both plasmids; 43 exclusively found in plasmid A and five in plasmid B (**Supplementary File 3**).

The comparison of the p-F219 plasmid with another megaplasmid, commonly found in pathogenic *S.* Infantis strains, pESI plasmid, showed that they share more than 79% of their sequences (>75% of identity) (**Supplementary Figure 1**). Besides, we noticed a large genomic inversion in plasmid p-F219 when compared with the pESI plasmid that is also observable in progressiveMauve genome alignment (**Supplementary Figure 2**). From the ANI calculation, 99.41% of identity was found in the orthologous genes present in both samples.

TABLE 3 | Comparison of phenotypic AMR with AMR genes obtained from WGS data.

Antibiotic family	Phenotype (%)[a]	Phenotype + AMR gene[b] (%)	No phenotype + AMR gene[c] (%)
Folate pathway inhibition	84.56	92.17	7.83
Aminoglycoside	97.06	100	3.03
Quinolone	58.09	1.27	0
Beta-lactams	84.56	94.87	4.27
Tetracycline	97.06	99.24	0.76
Phenicol	80.88	80.91	6.36
Nitrofuran	98.53	0	0
Macrolide	17.65	0	0
Fosfomycin	37.5	90.2	41.18

[a] *Rate of isolates with phenotypic resistance.*
[b] *Rate of isolates with phenotypic resistance that presented a resistance gene by WGS analysis.*
[c] *Rate of isolates without phenotypic resistance that presented a resistance gene by WGS analysis.*

In silico plasmid detection in non-Infantis isolates showed the presence of two plasmids in all *S.* Enteritidis strains, while monophasic *S.* Typhimurium 4,[5],12:i:- and *S.* Typhimurium presented 1 and 5 plasmids, respectively (**Supplementary Table 1**).

DISCUSSION

In the last decades, there has been a clear rise in the prevalence of multidrug resistant *Salmonella enterica* worldwide, especially of serovar Infantis (11, 48–50).

To better explain the epidemiology of *Salmonella* in Ecuador, we studied *S. enterica* isolated from poultry farms, chicken carcasses and human stool samples in Quito. The prevalence of *Salmonella* was high in poultry farms and chicken carcasses, similarly to other studies in the region (6–8, 51). Although this research did not look for *Salmonella* in earlier stages of the broiler production chain, previous studies in Ecuador have reported the importance of compound feed, 1-day-old chicks and broiler pens in the *Salmonella* exposure of broilers (7, 52). These results highlight the necessity to improve broilers production systems in Ecuador toward a better control of *Salmonella* in the food chain.

In this study, it was also seen that samples from different kinds of markets delivered similar rates of *Salmonella* isolates, denoting that the geographical distribution of retailers does not influence the presence of *Salmonella* in carcasses (**Supplementary Table 3**). On the other hand, all human isolates originated in the southern health care center. However, it must to be considered that the low *Salmonella* prevalence in human samples could be influenced by the fact that other pathogens might be the main causes of diarrhea. This fact has been studied in Ecuador were other viruses, parasites and bacteria are the main cause of gastroenteritis cases (53–56). Additionally, the state of health carriers should be considered when accessing the real prevalence of *Salmonella* in humans (57). These circumstances represent limitations of this study and should be considered in future research.

The predominance of *S.* Infantis in this study is in accordance with other reports in the world that show that this serotype is becoming an emergent pathogen. For example, in Europe, *S.* Infantis has been reported to be one of the most common serovars in poultry and in humans (58). The same tendency has been reported in the neighboring country of Peru, where *S.* Infantis is the most prevalent serotype in broilers (6). However, a wider variety of serotypes has been reported in other Latin American countries (51, 59, 60). This could be explained by the fact that the poultry industry of Peru and Ecuador have close commercial interactions which could determine a common epidemiology of this pathogen. Nevertheless, a recent publication from Chile reported that 24% of broiler meat samples ($n = 361$) were positive to the isolation of *S.* Infantis (61). This data highlights the necessity of more research in the field to better understand the epidemiology of *Salmonella* in the region.

Several studies have shown that control programs of targeted *Salmonella* serotypes could have favored the occurrence of other serotypes (2, 62). This kind of shifts might explain to some extent the low presence of *S.* Enteritidis and Typhimurium among our samples but further research is needed to prove this hypothesis.

Most *Salmonella* isolates in this study presented multidrug resistance (MDR) phenotypes. This issue is especially evident in *S.* Infantis, as already been seen in Ecuador (7, 8) and other countries of the region (63–65). Although in a lower extent, this feature has also been reported in Europe where high rates of MDR (up to 85%) are reported in *S.* Infantis isolated from poultry (66). The high levels of resistance in *Salmonella* isolates in Latin America could be related to the intensive use of antimicrobials in poultry production as prophylactics, therapeutics, and growth promotors (67, 68).

S. Infantis isolates belonged to ST32, that is among those more frequently identified within this serovar (33, 69). All Ecuadorian *S.* Infantis isolates showed a high genomic similarity with an apparently common origin. However, in order to verify that all in fact share a common ancestor, a larger analysis including isolates from countries around the world is necessary for identifying the origin of this serovar in Ecuador. The close similarity found between isolates from farms, animals and humans show that this pathogen may be responsible for human infections through the food chain.

We did not observe any clear clustering of *S.* Infantis isolates and antibiotic phenotypic resistance patterns, what makes sense since the phylogenetic tree was obtained from the core genome and most of the antimicrobial resistance determinants are expected to be part of the accessory genome.

The resistance genes analysis carried out here could not successfully explain resistance to macrolides, nitrofurans, and quinolones in our isolates. In fact, phenotypic patterns and genetic detection correlation differences have been described previously (70–72), but point mutations not considered in this study may explain these phenotypes. Additional studies are still needed in order to identify the genes or mutations responsible for antimicrobial resistance in *S.* Infantis.

We found two p-F219-like plasmids (named A and B) present in all the analyzed isolates. Our samples share a significant number of genes with the p-F219 plasmid. The similarity between both p-F219-like plasmids requires a recent common ancestor from which they may have evolved. These plasmids may provide fitness and pathogenic advantages to the strains since they contain several genes for antimicrobial resistance, fimbriae, transposases, and environmental stress tolerance (**Supplementary File 3**). Other *S.* Infantis isolates around the world have been shown to also harbor pESI-related megaplasmids, that appear to be the difference between non-pathogenic and pathogenic isolates since it also provides antimicrobial resistance, oxidative stress tolerance, pathogenicity traits, and mercury environmental tolerance (69, 70, 73, 74). Besides, pESI has shown more pathogenicity and increased intestinal inflammation in experimental mice infections when compared to the plasmid-free isolates (73).

Both plasmids (p-F219 and pESI) share regions of very close similarity with an extensive genomic inversion. Genes present in both plasmids share more than 99% average nucleotide identity suggesting that p-F219 is actually a pESI-like plasmid and the variants found in our samples may also be cataloged as such. A genomic comparison between *S.* Infantis strains showed that large plasmids from multiple isolates actually share a pESI backbone with some genetic plasticity to add different mobile genetic elements due to insertion sequences (75). As pESI-like plasmids confer a MDR phenotype but also several virulence factors and tolerance to environmental stress, their acquisition may have been involved for making *S.* Infantis a successful emerging pathogen worldwide.

The negative results from the unmapped reads from *S.* Enteritidis and *S.* Typhimurium isolates against the p-F219 plasmid suggest that this plasmid may not be widespread among other *Salmonella* serovars, but more isolates need to be analyzed to confirm this hypothesis.

Other virulence genes in *Salmonella* are essential for pathogenicity and infection. We looked for some of the more relevant virulence and invasion determinants in the core genome of *S.* Infantis as determined by ProteinOrtho. Some genes that are usually identified as part of the *Salmonella* pathogenicity island 2 (SPI-2) were found: *sifA, sseL, pipB, sopD2, srlP* (75). The long polar fimbrae (LfP), encoded by the *lpf* operon, was found as putative proteins in all isolates; it is believed that this protein is involved in adhesion and growth on the small intestine mucosa (76), evasion of the host immune system (77). Moreover, variations in these genes may also impact the host range of the bacteria (78). We also found the *fim* gene cluster that encodes type 1 fimbriae and it is involved in the initiation of biofilm formation (79). Another determinant found in our strains was the *csg* operon that encodes the Tafi fimbriae (Thin aggregative fimbriae). Tafi is responsible of adhesive activities and biofilm formation (76). The presence of these genes in *S.* Infantis may suggest a pathogenic character of this serotype that, together with the multidrug resistant profiles, represent a potential public health concern.

To the best of our knowledge this is the first report based on *Salmonella enterica* in Ecuador that uses phenotypic and WGS information to analyze the relatedness of strains isolated from poultry, food and human samples. Isolates from this study show multidrug resistance patterns highlighting the importance of a reduced and better usage of antimicrobials in intensive poultry farms settings. Presence of related megaplasmids together with the core genome high similarity may suggest the dissemination of *S.* Infantis through the food chain to humans. The data presented here has shown the importance of *Salmonella enterica* serovar Infantis as a foodborne pathogen in Ecuador and provide critical information about its clonality and circulating strains.

DATA AVAILABILITY STATEMENT

Sequence data are available under bioproject PRJEB37560.

ETHICS STATEMENT

According to national legislation, ethics approval was not required for poultry farms and chicken carcasses sampling since no animals were sacrificed during this study. For the human component, the project was approved by the bioethics committee from the National Institute of Public Health Leopoldo Izquieta Pérez (Protocol ID was CEISH-INSPI-005). The participants were informed about the study's objective and all volunteers provided a written consent. All personal information was anonymized.

AUTHOR CONTRIBUTIONS

JM, JW, FV, and CV-B conception and design of the study. JLM, CS, FV, and RB performed laboratory analysis. JLM and CV-B performed the statistical analysis. JLM, LM, and CV-B organized the databases. LM and FG-C performed the bioinformatic and genomic analysis. CV-B and LM wrote the first draft of the manuscript. LM, SZ, FG-C, and CV-B contributed to the manuscript revision. All authors contributed to the article and approved the submitted version.

FUNDING

This project was funded by the WHO Advisory Group on Integrated Survillance on AMR (AGISAR) grant AGISAR-JM/lg and also funded by projects BFU2017-89594R from MICIN (Spanish Government) and PROMETEO2016-0122 (Generalitat Valenciana, Spain). WGS was co-financed by the European Union through the Operational Program of European Regional Development Fund (ERDF) of Valencia Region (Spain) 2014–2020. LM was recipient of a predoctoral fellowship from Fundación Carolina and Universidad San Francisco de Quito.

ACKNOWLEDGMENTS

Our acknowledgments to Dr. Gina Watson and Dr. Aida Soto from the OPS/OMS Ecuadorian office for the support in the logistical management of this project. We also thank Mariuxi Japon and Paola Damian for their support with the laboratory work and isolation of *Salmonella*. Whole genome

sequences were obtained at Servicio de Secuenciación y Bioinformática (FISABIO, Valencia). Action co-financed by the European Union through the Operational Program of European Regional Development Fund (ERDF) of Valencia Region (Spain) 2014–2020.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.547891/full#supplementary-material

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

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 06 November 2020
doi: 10.3389/fvets.2020.583930

Does Shiga Toxin-Producing *Escherichia coli* and *Listeria monocytogenes* Contribute Significantly to the Burden of Antimicrobial Resistance in Uruguay?

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OPEN ACCESS

Edited by:
Josefina Leon-Felix, Consejo Nacional de Ciencia y Tecnología (CONACYT), Mexico

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *15 July 2020*
Accepted: *09 October 2020*
Published: *06 November 2020*

Citation:
Mota MI, Vázquez S, Cornejo C, D'Alessandro B, Braga V, Caetano A, Betancor L and Varela G (2020) Does Shiga Toxin-Producing Escherichia coli and Listeria monocytogenes Contribute Significantly to the Burden of Antimicrobial Resistance in Uruguay? Front. Vet. Sci. 7:583930. doi: 10.3389/fvets.2020.583930

Shiga toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes* are worldwide recognized zoonotic pathogens. Recent reports have emerged about the circulation of antimicrobial-resistant STEC and *L. monocytogenes* isolates. To assess the frequency of antimicrobial resistance and related genes in these pathogens, we studied 45 STEC and 50 *L. monocytogenes* isolates locally recovered from different sources. Antimicrobial susceptibility testing was performed by disk-diffusion method, and the genomic sequences of three selected STEC and from all 50 *L. monocytogenes* isolates were analyzed for antibiotic resistance genes. Four STEC and three *L. monocytogenes* isolates were phenotypically resistant to at least one of the antibiotics tested. Resistance genes *aph*(3″)-Ib, *aph*(3′)-Ia, *aph*(6)-Id, bla_{TEM-1B}, *sul*2, *mef*(A), and *tet(*A) were found in a human STEC ampicillin-resistant isolate. All *L. monocytogenes* isolates harbored *fosX*, *lin*, *mdr*L, *lde fepA*, and *norB*. Overall resistance in *L. monocytogenes* and STEC was low or middle. However, the high load of resistance genes found, even in susceptible isolates, suggests that these pathogens could contribute to the burden of antimicrobial resistance.

Keywords: antimicrobial resistance, Shiga toxin-producing *Escherichia coli* (STEC), *Listeria monocytogenes*, zoonotic pathogens, resistance genes

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes* are well-recognized zoonotic pathogens circulating in Uruguay (1, 2). In humans, STEC can produce watery or bloody diarrhea (WD, BD) or even more severe conditions such as hemorrhagic colitis (HC) or hemolytic–uremic syndrome (HUS). HUS can be lethal in the early stages or leave long-term sequelae; ∼20% of children who suffer it require chronic dialysis or kidney transplant. STEC has also been rarely associated with urinary tract infections (3, 4). Cattle and other food production animals are the main known reservoir for STEC, and the transmission to humans occurs by direct contact with them or through the ingestion of foods or water contaminated with its feces (5). Although controversial, antibiotics such as gentamicin, azithromycin, fosfomycin, and meropenem are recommended to the treatment of human STEC infections to avoid the development of most severe diseases (6).

Listeria monocytogenes is the etiologic agent of invasive listeriosis, a severe food-borne disease that mainly affects elderly, immunocompromised people, pregnant women, and infants. *L. monocytogenes* is widely distributed in nature, including the bowel of cattle, so it has multiple opportunities to enter the food production and supply chain. Although human invasive listeriosis is rare, it has high rates of hospitalization and case fatality (7, 8).

Listeria monocytogenes is susceptible to most clinically relevant groups of antibiotics active against Gram-positive bacteria, except for intrinsic resistance to fosfomycin, older quinolones, sulfamethoxazole, oxacillin, and expanded-spectrum cephalosporins (8, 9). The first-line therapy for listeriosis is ampicillin or penicillin G, with or without the addition of gentamicin. For beta-lactam-allergic patients, the therapy of choice is trimethoprim-sulfamethoxazole or vancomycin (8–10).

Likewise, antibiotics are used in veterinary medicine for the treatment and prevention of infectious diseases, but also, they have been used for a long time for animal growth promotion and improved productivity. These situations contribute to the selection of resistant bacteria, including STEC and *L. monocytogenes*, which could then be transmitted to humans, also facilitating the spread of antibiotic resistance genes (6, 11).

Reports have emerged about the circulation of antimicrobial-resistant *L. monocytogenes* isolates worldwide (11). Similarly, antimicrobial-resistant STEC isolates were reported in Brazil and Mexico among other countries (6), highlighting the role as reservoir of resistance genes and recommending the surveillance of its susceptibility profiles.

The aim of this study was to assess the frequency of antibiotic resistance against different drugs used in human and veterinary medicine in a set of STEC and *L. monocytogenes* isolates and to analyze the presence of possible related genes.

MATERIALS AND METHODS

Bacterial Strains

We studied a collection of 45 STEC and 50 *L. monocytogenes* isolates. All of them were received at the Bacteriology and Virology Department (University of the Republic, School of Medicine) between 2010 and 2019 to confirm the identification and to determine pathotype and serotype. All STEC received until the end of 2017 were included in this study. STEC isolates were from different sources: human samples ($n = 7$), six isolates from feces of children $\leq$5 years old and one belonging to the serogroup O157 from urine of an adult woman; food samples ($n = 37$), all recovered from beef (*Bos taurus*); and animal sample ($n = 1$) isolated from feces of a healthy cow (see **Supplementary Table 1**).

Listeria monocytogenes isolates were selected as a convenience sample from a total of 498 isolates received, including different serotypes, sources, and year of isolation (see **Supplementary Table 2**). Human isolates ($n = 29$) were obtained from blood, placenta, amniotic fluid, and cerebrospinal fluid samples. The food isolates ($n = 21$) were recovered from frozen food, ready-to-eat food, deli meat, and cheese.

STEC isolates were serotyped and analyzed by PCR for the presence of *stx1/2*, *eae*, and *ehxA* virulence genes (1).

Listeria monocytogenes strains were serotyped using a combination of multiplex PCR and agglutination tests with commercially available *Listeria* antisera to one and four somatic antigens as we previously described (3).

Antimicrobial Susceptibility Testing

All STEC isolates were studied by disk-diffusion method according to the guidelines for *Enterobacteriaceae* of Clinical and Laboratory Standards Institute (CLSI) (12). We used Mueller–Hinton agar plates, and the antimicrobials tested were ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), fosfomycin-trometamol (FOT), cefepime (FEP), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), ceftriaxone (CRO), ciprofloxacin (CIP), gentamicin (CN), imipenem (IPM), meropenem (MEM), and trimethoprim-sulfamethoxazole (SXT) (Oxoid®). Plates were incubated at 35 ± 2°C in ambient air during 16–18 h, and the result interpretation was done according to Clinical and Laboratory Standards Institute (CLSI) breakpoints (Table 2A, *Enterobacteriaceae* M02 and M07) (12). *E. coli* ATCC 25922 was used as quality control.

Listeria monocytogenes antimicrobial susceptibility testing was performed by disk-diffusion method according to the recommendations for *L. monocytogenes* of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (13). Mueller–Hinton agar plates supplemented with 5% of mechanically defibrinated horse blood and 20 mg/L β-NAD (MH-F) were prepared "in-house." A panel of six antibiotics was tested: benzylpenicillin (1 μg), gentamicin (10 μg), trimethoprim-sulfamethoxazole (1.25 μg/23.75 μg), meropenem (10 μg), erythromycin (15 μg), and ciprofloxacin (5 μg) with Oxoid® disks. Cultures were incubated at 35°C with 5% CO_2 for 18–20 h.

Streptococcus pneumoniae ATCC 49619 and *Staphylococcus aureus* ATCC 25923 were used as quality control strains. *L. monocytogenes*-specific clinical breakpoints of EUCAST were used for penicillin, meropenem, erythromycin, and trimethoprim-sulfamethoxazole; for gentamicin and ciprofloxacin, interpretation was done according to the clinical breakpoint value for *Staphylococcus* spp.

Detection of Antimicrobial Resistance Genes

The genomic DNA from three selected human STEC isolates (corresponding to different serogroups, two fully susceptible, and one resistant only to ampicillin) and from all *L. monocytogenes* isolates was extracted with the DNA blood and tissue kit (Qiagen®) and subjected to whole-genome sequencing by Illumina MiSeq platform with Nextera XT library prep kits (USA) and TruSeq Nano library kit. The reads were *de novo* assembled with SPAdes version 3.13.1 (14). Genomic sequences of STEC and *L. monocytogenes* were analyzed for resistance genes using the software ABRicate with the databases ResFinder, CARD, NCBI AMRFinderPlus, and MEGARes (update April 19, 2020). For *L. monocytogenes*, we also searched for the antimicrobial resistance genes *fepA*, *lde*, and *pen*A using BLAST tool because these genes

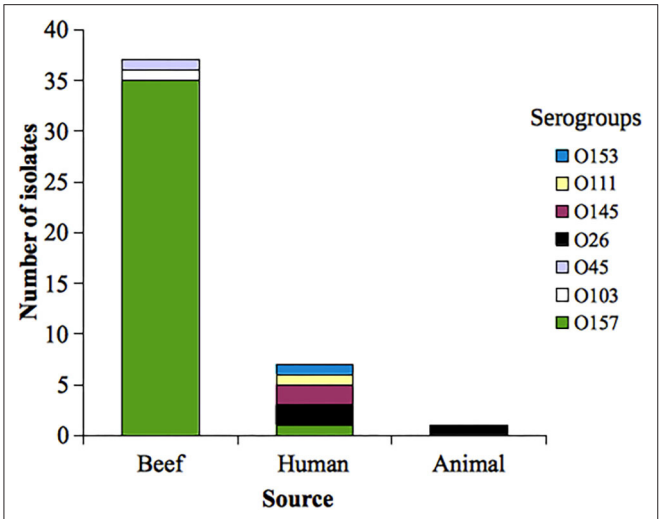

FIGURE 1 | Distribution of analyzed Shiga toxin-producing *Escherichia coli* (STEC) serogroups according to the source. Uruguay, 2010–2017.

TABLE 1 | Characteristics of resistant Shiga toxin-producing *Escherichia coli* (STEC) isolates analyzed.

Isolate identification	Source	Serotype	Virulence genes	Resistance profile
IH23	Beef	O157:H7	*stx*1/2, *eae*, *ehx*A	AMP, CN, SXT
IH12	Human, HUS	O26:H11	*stx*1/2, *eae*, *ehx*A	AMP, SXT
IH36	Healthy cow	O26:H11	*stx*1, *eae*, *ehx*A	AMP, SXT
IH7	Human, HUS	O111:HNM	*stx*1/2, *eae*, *ehx*A	AMP

Uruguay, 2010–2017.
AMP, ampicillin; CN, gentamicin; SXT, trimethoprim-sulfamethoxazole.

have been reported in these bacteria but were not included in the databases mentioned above.

RESULTS

Source, Serogroup Distribution, Antimicrobial Resistance, and Resistance Genes Found in STEC

STEC serogroup distribution was as follows: O157 (36 isolates), O26 (3), O145 (2), O45 (1), O103 (1), O111 (1), and O153 (1). Serogroup distribution according to the source is shown in **Figure 1**.

Four out of 45 STEC analyzed (8.8%) showed resistance to at least one of the antimicrobials tested (O26:H11, 2 isolates; O157:H7, 1 and O111:HNM, 1) (see **Table 1**).

Only one STEC O157 isolate (obtained from beef sample) was resistant (2.8%); on the other hand, three out of the nine non-O157 (32%) analyzed included were resistant. Resistance to ampicillin was observed in all ($n = 4$) the resistant STEC analyzed; additionally, three isolates were also resistant to trimethoprim-sulfamethoxazole and one to gentamicin (see **Table 1**).

TABLE 2 | Characteristics of resistant *Listeria monocytogenes* isolates analyzed.

Isolate identification	Source	Serotype	Resistance profile	Resistance genes
Ulm_70	Food	4b	CIP	*fos*X, *lin*, *nor*B, *lde*, *mdr*L, *fep*A
Ulm_74	Human	4b	E	*fos*X, *lin*, *nor*B, *lde*, *mdr*L, *fep*A
Ulm_77	Food	1/2b	CIP	*fosX, lin, norB, lde, mdrL, fepA*

Uruguay, 2010–2019.
CIP, ciprofloxacin; E, erythromycin.

Resistance genes found in the only sequenced resistant STEC isolate (serogroup O111, isolated from a child with HUS, see **Table 1**) were *aph*(3″)-Ib, *aph*(3′)-Ia, *aph*(6)-Id, bla_{TEM-1B}, *sul*2, and *tet*(A) (minimum identity and coverage of 88%). We did not find these genes in the other two susceptible STEC sequenced (see **Supplementary Table 1**). One STEC O145:H25 susceptible to all antibiotics tested bears the *fosA7* gene, associated with resistance to fosfomycin (see **Supplementary Table 1**). Also, the three STEC isolates sequenced carried among others the *mdfA*, *mphB*, and *mef*(A) genes.

Serotypes, Antimicrobial Resistance, and Resistance Genes Found in *L. monocytogenes*

Among *L. monocytogenes* isolates analyzed, 27 belonged to serotype 1/2b, 20 to 4b, and 3 to 1/2a. Forty-seven isolates of *L. monocytogenes* were susceptible to all antibiotics tested. Two isolates were resistant to ciprofloxacin (serotypes 4b and 1/2b, both from food origin), and one isolate was resistant to erythromycin (serotype 4b, human source) (see **Table 2**). Serotype distribution according to the source is shown in the **Figure 2**.

We identified the resistance genes *fos*X, *lin*, *nor*B, *lde*, *mdr*L, and *fep*A in all analyzed genomes, with a minimum identity and coverage of 90% (see **Supplementary Table 2**).

DISCUSSION

The overall resistance frequency found in STEC (8.8 %) suggests that its local contribution to the burden of antimicrobial resistance seems low and comparable to that previously reported in a similar study of Spain-País Vasco (15). Probably, the percentage of resistance could have been higher if we had included the tetracycline and chloramphenicol disks in the susceptibility assays (16).

Only 1 out of 36 STEC O157 isolates analyzed was resistant (2.8%); however, 3 of the 9 non-O157 (32%) were resistant. This figure coincides with results obtained by Sasaki et al. (17) and would be related to the fact that STEC non-O157 may acquire genes for antimicrobial resistance more easily than STEC O157 isolates do. Resistance to ampicillin was observed in all the resistant STEC isolates analyzed. Beta-lactamase TEM-1

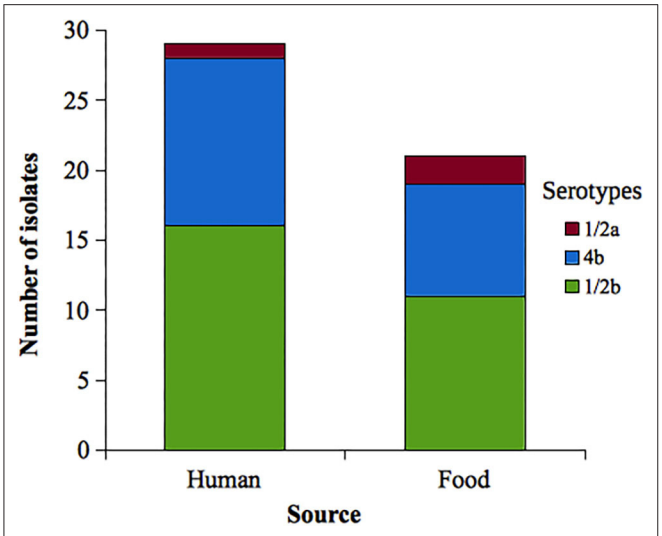

FIGURE 2 | Distribution of analyzed *Listeria monocytogenes* serotypes according to the source. Uruguay, 2010–2019.

is the most prevalent enzyme responsible for resistance to ampicillin in gram-negative bacteria, and the encoding genes are usually located in mobile genetic elements. In this sense, the resistance genes found *aph*(3″)-Ib, *aph*(3′)-Ia, *aph*(6)-Id, bla_{TEM-1B}, and *sul*2 are generally located in class 1 integrons (18) as was previously reported by Colello et al. in STEC isolates recovered from animals in neighboring Argentina (19). We also found *tet*(A), *fosA*7, and *mef*(A), *mphB* genes, responsible for tetracycline, fosfomycin, and macrolide resistance, respectively. Due to economic reasons, we could only analyze the genome of three STEC isolates. We hope to carry out the whole-genome sequencing (WGS) on the remaining STEC isolates to detect other resistance genes.

Taking together the STEC isolated from beef and animal source and assuming that all the beef isolates come from the bowel of the cattle, we noticed that only 2 of these 38 (5.2%) were resistant, whereas 2 of the 7 (28.5%) isolated from humans showed resistance. These figures are similar to those previously reported by Oporto et al. in Spain-País Vasco. The difference could be explained in part for which was said above about serogroup behavior and also by selection pressure due to the frequent use of aminopenicillins in humans, especially in children (15, 17, 20). However, we cannot rule out that STEC have been acquired by cross contamination during meat processing or handling.

Treatment with antibiotics in the HUS phase is controversial; some authors do not recommend them (21), and others suggest that the early use (e.g., BD stage) of azithromycin, fosfomycin, aminoglycosides, and meropenem can be a therapeutic option (22–24). In this set of STEC, one was resistant to gentamicin, and none showed resistance to meropenem nor fosfomycin by disk diffusion assay. However, one of these fosfomycin-susceptible isolates carried the *fosA*7 gene. According to this finding, that gene was also detected in a fosfomycin-susceptible *E. coli* obtained from a Japanese river. In this Japanese isolate, the *fosA* gene was truncated, thus explaining the observed phenotype. However, in our STEC isolate, the *fosA*7 gene was complete; therefore, the *in vitro* susceptibility to fosfomycin could be due to the fact that the gene is not fully expressed, or its level of expression is extremely low. Nevertheless, this finding highlights the role of STEC as a reservoir of transferable resistance genes (25).

The role of azithromycin in the prevention of HUS cases remains to be assessed knowing that *mef*(A), unlike *mph*(A) gene, has a poor role in resistance to this antibiotic (26).

The obtained results show that STEC deserves special attention considering the local circulation of antibiotic-resistant full-pathogenic strains, in both humans and animals, and knowing that some of them harbor transferable resistance genes. The spread of these strains and its resistance genes will surely continue and even increase if this situation is not addressed.

CLSI and EUCAST guidelines include minimal inhibitory concentration (MIC) breakpoints for three or four antibiotics, respectively, for *L. monocytogenes*, and some years ago, EUCAST incorporated the disk-diffusion method for the same antibiotics (13, 27). In both guidelines, the culture medium contains horse blood, which is not everywhere commercially available. These difficulties may have led researchers to use alternative culture media and/or to interpret their results based on criteria defined for other microorganisms.

The results of this study using EUCAST guidelines show that *L. monocytogenes* local isolates remain fully susceptible to penicillin, gentamicin, trimethoprim-sulfamethoxazole, and meropenem. We found a low frequency of ciprofloxacin (two isolates) and erythromycin resistance (one isolate). It is important to highlight that these antibiotics are not therapeutic options for treatment of invasive infections in humans. Resistance frequency found in our study was similar to those previously reported by other authors using microdilution methods according to CLSI or EUCAST recommendations for *L. monocytogenes*. In the USA, Davis et al. tested 90 *L. monocytogenes* isolates recovered from human, food, animal, and the environment and found only 2% of ciprofloxacin resistance but not resistance to penicillin G, ampicillin, erythromycin, gentamicin, and trimethoprim-sulfamethoxazole (28). In Poland, Kuch et al. analyzed 344 human isolates (recovered between 1997 and 2013) and did not find resistance to ampicillin, penicillin, meropenem, erythromycin, trimethoprim-sulfamethoxazole, levofloxacin, gentamicin, vancomycin, nor rifampicin (29). In Australia, Wilson et al. using gradient diffusion test, found resistance to ciprofloxacin (2%) and erythromycin (1%) among 100 *L. monocytogenes* isolates originating from food between 1988 and 2016; no resistance was observed to penicillin G or tetracycline (30).

On the other hand, in Argentina, Prieto et al. found higher frequency of resistance to erythromycin (30%) among 250 food and human disease-related *L. monocytogenes* isolates recovered between 1992 and 2012 but no resistance to penicillin G, ampicillin, trimethoprim-sulfamethoxazole, gentamicin, tetracycline, nor rifampin (31).

Our results and those of the aforementioned studies differ from others in which resistance to beta-lactams is reported with

high frequency, a cause for concern since this group of antibiotics is the first line of treatment for invasive listeriosis (32–35). However, these studies do not use standardized culture media for *L. monocytogenes* and interpret their results based on the criteria defined for *Staphylococcus* or *Enterococcus*; these factors may explain such discrepancies at least partially.

Genomic sequences analysis revealed the presence of the resistance genes *fosX*, *lin*, *norB*, *lde*, *mdrL*, and *fepA* in all *L. monocytogenes* strains studied.

Listeria monocytogenes is intrinsically resistant to fosfomycin due to the lack of expression of transport systems through the membrane. Also, the presence of *fos*X gene could explain another resistance mechanism in *L. monocytogenes*, since it was globally present in all strains analyzed here as well as in all the 100 studied by Hurley et al. (36). The FosX protein catalyzes the hydration of fosfomycin breaking the oxirane ring (37).

The *lin* gene was detected in all the analyzed strains and encoded for a lincomycin resistance ABC-F type ribosomal protection protein, a member of the ATP-binding cassette F (ABC-F) proteins (38). We did not find descriptions of this mechanism in *L. monocytogenes*, but we did find the *lin* gene in almost all genomes of this species in NCBI Pathogen Detection Isolates Browser (https://www.ncbi.nlm.nih.gov/pathogens/) suggesting that this mechanism could be involved in the natural resistance to lincomycin.

Macrolide resistance in *L. monocytogenes* has been linked to the methyl-transferase coding gene *erm*B and to efflux mechanisms mediated by multidrug efflux transporter of *Listeria* (MdrL) (39). We found the *mdrL* gene in all the analyzed genomes but not the *ermB* gene in any of them.

Fluoroquinolone resistance in *L. monocytogenes* seems to be primarily due to efflux pumps, principally through overexpression of the *lde* and *fepA* genes (31, 40, 41). NorB is a member of the major facilitator superfamily (MFS) of transporters that confers resistance to hydrophilic quinolones (norfloxacin and ciprofloxacin) and hydrophobic quinolones (sparfloxacin and moxifloxacin). The norB gene has been found by us and other authors in the analyzed genomic sequences of *L. monocytogenes* (30, 42).

The presence of the genes *mdrL*, *lde*, *fepA*, and *norB* coding for the respective efflux pumps seems to be universal in the *L. monocytogenes* isolates analyzed in this study; however, only two isolates were resistant to ciprofloxacin and one to erythromycin. Therefore, additional mechanisms or the level of expression of these genes could explain the differences in susceptibility to fluoroquinolones and macrolides. Likewise, 45 of the 50 strains analyzed had ciprofloxacin inhibition zones near the cut-off point (±5 mm) for *Staphylococcus* spp. (data not shown).

CONCLUSIONS

Antimicrobial-resistant *L. monocytogenes* and STEC isolates are present at a low-middle frequency. However, the high load of resistance genes found suggests that these pathogens could contribute to the local burden of antimicrobial resistance. A nationwide detailed study is necessary to determine the prevalence of resistant *L. monocytogenes* and STEC strains (including the resistance to antibiotics not tested in this work to STEC as tetracycline and chloramphenicol) and also to know the involved genes.

DATA AVAILABILITY STATEMENT

Genomic sequences of STEC and *Listeria monocytogenes* used in this study are available at https://www.ncbi.nlm.nih.gov/sra/ and the access numbers are in the **Supplementary Material** (see Table SRA with Accession numbers).

AUTHOR CONTRIBUTIONS

MM and SV performed the characterization of STEC and *Listeria monocytogenes*, DNA extraction, analysis of sequences, data processing, scientific discussion, and drafted the article. BD'A performed bioinformatic analysis of genomic sequences and drafted the article. CC, VB, and AC conducted the susceptibility studies to *L. monocytogenes* and STEC. LB performed scientific discussion and drafted the article. GV performed the characterization of STEC and *Listeria monocytogenes*, analysis of sequences, data processing, and scientific discussion and drafted the article. All the authors approved the manuscript to be published.

FUNDING

This work was partially supported by Comisión Sectorial de Investigación Científica (CSIC-UdelaR) program I+D grant N° 308 (responsable MM). SV received a Ph.D. grant from the National Agency for Research and Innovation (ANII).

ACKNOWLEDGMENTS

We thank Elton Burnett for allowing us the sequencing of 40 strains analyzed in this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.583930/full#supplementary-material

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

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 19 November 2020
doi: 10.3389/fvets.2020.588919

Transferable Resistance to Highest Priority Critically Important Antibiotics for Human Health in *Escherichia coli* Strains Obtained From Livestock Feces in Uruguay

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 29 July 2020
Accepted: 19 October 2020
Published: 19 November 2020

Citation:
Coppola N, Freire B, Umpiérrez A, Cordeiro NF, Ávila P, Trenchi G, Castro G, Casaux ML, Fraga M, Zunino P, Bado I and Vignoli R (2020) Transferable Resistance to Highest Priority Critically Important Antibiotics for Human Health in Escherichia coli Strains Obtained From Livestock Feces in Uruguay. Front. Vet. Sci. 7:588919. doi: 10.3389/fvets.2020.588919

The aim of this work was to detect *Escherichia coli* isolates displaying resistance to oxyimino-cephalosporins, quinolones, and colistin in feces from livestock in Uruguay. During 2016–2019, fecal samples from 132 broiler and layer chicken flocks, 100 calves, and 50 pigs, were studied in Uruguay. Samples were cultured on MacConkey Agar plates supplemented with ciprofloxacin, ceftriaxone, or colistin. *E. coli* isolates were identified by mass spectrometry and antibiotic susceptibility testing was performed by disk diffusion agar method and colistin agar test. Antibiotic resistance genes were detected by polymerase chain reaction and sequencing. The most frequently detected resistance gene was *qnrB19*, recovered from 87 animals. Regarding plasmid-mediated quinolone resistance genes, *qnrS1* was the second in prevalence (23 animals) followed by *qnrE1*, found in 6 chickens and two calves. Regarding resistance to oxyimino-cephalosporins, 8 different β-lactamase genes were detected: $bla_{CTX-M-8}$ and bla_{CMY-2} were found in 23 and 19 animals, respectively; next, $bla_{CTX-M-2}$ and bla_{SHV-12} in 7 animals each, followed by $bla_{CTX-M-14}$ in 5, $bla_{CTX-M-15}$ and bla_{SHV2a} in 2, and $bla_{CTX-M-55}$ in a single animal. Finally, the *mcr-1* gene was detected only in 8 pigs from a single farm, and in a chicken. Isolates carrying bla_{CMY-2} and bla_{SHV-12} were also found in these animals, including two isolates featuring the bla_{CMY-2}/*mcr-1* genotype. To the best of our knowledge, this is the first work in which the search for transferable resistance to highest priority critically important antibiotics for human health is carried out in chickens and pigs chains of production animals in Uruguay.

Keywords: swine, poultry, ESBL, *E. coli*, MCR-1, CTX-M-8, CMY-2, *qnrB19*

INTRODUCTION

The interaction between humans and animals is quite diverse and may lead to cases of zoonosis and/or anthropozoonosis (1). Over the past decades the aforementioned interaction has constantly increased worldwide partly due to animal husbandry practices, the growth of the companion animal market, climate change, and ecosystem disruption. In this context, bacterial transmission may occur through food products (e.g., meat or eggs) or through direct contact, in particular in farmers, veterinarians, or abattoir workers (2).

As the human-animal connection escalates, so does the threat of pathogen spread (2, 3). With current rapid transport systems, a pathogen emerging today in any given country can easily be carried unnoticed in people, animals, plants, or food products, to distant parts of the world in <24 h (4).

On the other hand, anthropogenic changes to the ecosystem increase the number of shared habitats between humans and animals, thus exposing both to new pathogens. In this regard, several authors have described the occurrence in humans and several animal species of the pandemic strain, *Escherichia coli* 025:H4 ST131, carrying the extended-spectrum beta-lactamase CTX-M-15. This particular event indicates an interspecies transmission from humans to pets and livestock and has been particularly described across Europe (5).

Recently, the WHO, FAO, and OIE organizations have coined the term "One Health" which regards the environment and human and animal health as a single entity. In this context, antibiotic resistance is a major concern (6).

The highest consumption of antibiotics occurs in animal husbandry, reaching in several countries 80% of the annual total antimicrobial consumption, with a higher consumption estimated by the year 2030. In this sense, the food industry may be accountable for the spread and increase of antibiotic resistance mechanisms (6, 7).

The impact on humans of antibiotic-resistant bacteria of animal origin is reflected in the increase of enteropathogens such as *Salmonella* spp. resistant to oxyimino-cephalosporins and fluoroquinolones, responsible among others for severe pediatric infections. Moreover, resistance to azithromycin and ciprofloxacin has been noted in species such as *Campylobacter jejuni*, and ESBL-producing *Escherichia coli* (8). Particularly in the United States, enterobacteria are responsible for 140,000 healthcare-associated infections annually, with 26,000 infections attributable to ESBL-producing enterobacteria, representing 19% of hospital-acquired infections.

In this respect, foodborne infections due to these resistant enteropathogens are a risk to humans due to possible therapeutic failure (8). Evidence indicates that a reduction in antibiotic consumption in animal husbandry would lead to a decrease in bacterial antibiotic resistance levels (9).

Antibiotics, in animal husbandry, are mainly used to treat infections, furthermore, they are also used as a prophylactic, through group treatment by incorporating them in drug premixes at relatively high concentrations, and more worryingly as growth promoters (although this practice has already been banned in some countries) (8).

Particularly noteworthy is the emergence of transferable resistance to critical antibiotics of highest priority for human medicine such as oxyimino-cephalosporins, fluoroquinolones and polymyxins, due to: extended spectrum β-lactamases (ESBLs) and plasmidic cephalosporinases (pAmpC); plasmid-mediated quinolone resistance (PMQR) genes [e.g., *qnr* and *aac(6′)-Ib-cr*]; and *mcr* alleles, respectively (10).

Infections caused by multidrug resistant microorganisms (MRM) lead to longer hospital stays (6.4–12.7 days), increased morbimortality (6.5%) and elevated economic costs (18,588–29,069 US$ per patient) (11, 12). Additionally, the occurrence of MRM in production animals may result in economic losses in trade and agriculture commerce; an example of the latter was observed in Norway, where the presence of resistant *E. coli* in retail chicken meat resulted in a 20% decrease in sales (13).

In Uruguay, measures have been taken to restrict the use of antibiotics, both in human health and in animal husbandry. The use of antibiotics as growth promoters in the latter is forbidden, and cattle under treatment are not destined for human consumption (https://www.proa.hc.edu.uy/), (Decree N° 98/011). In addition, in March 2019 Decree 141/019 was established, prohibiting the import, export, manufacture, sale, use, possession and marketing of veterinary products containing the substance "colistin" in its composition, either alone or associated with other chemicals (either as raw material or finished product), or incorporated into animal feed.

Finally, in 2017 Umpierrez et al. reported for the first time the occurrence of antibiotic- resistance genes in *E. coli* isolated from cattle in Uruguay. In that study, the authors detected multidrug-resistant *E. coli* isolated from calves carrying $bla_{CTX-M-14}$ and PMQR genes, among other resistance determinants (14).

The aim of this work was to detect *E. coli* isolates displaying resistance to oxyimino-cephalosporins, quinolones, and colistin in feces from livestock in Uruguay.

MATERIALS AND METHODS

Sampling and Transport

During 2016–2019, fecal samples from 282 animals were studied in Uruguay: 100 from calves, 132 from broiler and layer chicken flocks and 50 from pigs of different ages; samples were obtained from five, 13, and five establishments, respectively. Between 5 and 20 animals were sampled in each establishment (see **Table 1**).

Pig and bovine feces were collected wearing latex gloves directly from animals; conversely, chicken samples were taken directly from cloacae with sterile swabs. All samples were refrigerated at 4°C and sent, within 24 h, to Departamento de Bacteriología y Virología (Instituto de Higiene, Montevideo, Uruguay), or to Plataforma de Investigación en Salud Animal for processing (Colonia, Uruguay).

Samples were pre-enriched in Luria Bertani broth for 12 h at 37°C. Next, 10 µl of the broth were cultured on MacConkey Agar plates (Oxoid Ltd., Basingstoke, UK) supplemented with 0.125 mg/L ciprofloxacin (ION, Montevideo, Uruguay) or 1 mg/L ceftriaxone (Libra, Montevideo, Uruguay), or 3 mg/L colistin (Sigma-Aldrich St. Louis MO USA).

TABLE 1 | Main results from the studied establishments.

	Establishment	Studied animals	Animals w/cro R	Animals w/cip R	Animals w/col R	Selected *E.coli*	Resistance genes			Genotypes detected
							Bla	**PMQR**	***mcr***	
Calves	C1	20	0	8	0	11	–	$qnrB19^{(3)}$	–	$qnrB19^{(3)}$
	C2	20	0	5	0	6	–	$qnrB19^{(2)}$	–	$qnrB19^{(2)}$
	C3	20	0	16	0	20	–	$qnrB19^{(6)}$	–	$qnrB19^{(6)}$
	C4	20	0	7	0	7	–	–	–	–
	C5	20	1	19	0	29	$bla^{(1)}_{CTX-M-15}$	$qnrB19^{(8)}$, $qnrE1^{(2)}$, $qnrS1^{(1)}$	–	$bla_{CTX-M-15}/qnrB19^{(1)}$, $qnrB19^{(7)}$, $qnrE1^{(2)}$, $qnrS1^{(1)}$
	Total	100	1	55	0	73	1	22	0	
Poultry	P1	17	3	12	0	16	$bla^{(3)}_{CTX-M-2}$	$qnrB19^{(6)}$, $qnrE1^{(5)}$	–	$bla^{(2)}_{CTX-M-2}$, $bla_{CTX-M-2}/qnrE1^{(1)}$, $qnrE1/qnrB19^{(2)}$, $qnrB19^{(4)}$, $qnrE1^{(2)}$
	P2	10	3	9	0	12	$bla^{(3)}_{CTX-M-2}$	$qnrB19^{(4)}$	–	$bla^{(2)}_{CTX-M-2}$, $bla_{CTX-M-2}/qnrB19^{(1)}$, $qnrB19^{(3)}$
	P3	5	2	2	0	3	$bla^{(1)}_{CTX-M-8}$, $bla^{(1)}_{CTX-M-55}$	$qnrB19^{(2)}$	–	$bla^{(1)}_{CTX-M-8}$, $qnrB19^{(1)}$, $bla_{CTX-M-55}/qnrB19^{(1)}$
	P4	10	1	6	0	10	$bla^{(1)}_{CMY-2}$	$qnrB19^{(8)}$	–	$bla^{(1)}_{CMY-2}$, $qnrB19^{(8)}$
	P5	10	0	10	0	11	–	$qnrB19^{(8)}$	–	$qnrB19^{(8)}$
	P6	10	0	10	1	11	–	$qnrB19^{(1)}$	$mcr\text{-}1^{(1)}$	$qnrB19/mcr\text{-}1^{(1)}$
	P7	10	0	9	0	15	–	$qnrB19^{(9)}$	–	$qnrB19^{(9)}$
	P8	10	6	4	0	9	$bla^{(5)}_{CMY-2}$, $bla^{(1)}_{CTX-M-2}$	$qnrB19^{(1)}$	–	$bla^{(5)}_{CMY-2}$, $bla^{(1)}_{CTX-M-2}$, $qnrB19^{(1)}$
	P9	10	0	8	0	8	–	$qnrB19^{(4)}$	–	$qnrB19^{(4)}$
	P10	10	0	9	0	9	–	–	–	–
	P11	10	0	10	0	10	–	–	–	–
	P12	10	0	7	0	7		$qnrB19^{(3)}$	–	$qnrB19^{(3)}$
	P13	10	8	9	0	19	$bla^{(3)}_{CTX-M-8}$, $bla^{(3)}_{CMY-2}$, $bla^{(2)}_{SHV-2a}$	$qnrB19^{(9)}$, $qnrE1^{(2)}$	–	$bla^{(3)}_{CTX-M-8}$, $bla^{(2)}_{SHV-2a}$, $bla_{CMY-2}/qnrB19^{(1)}$, $bla^{(2)}_{CMY-2}$ $qnrB19^{(7)}$, $qnrB19/qnrE1^{(1)}$, $qnrE1^{(1)}$
	Total	132	23	105	1	140	23	62	1	
Swine	S1	10	6	8	0	17	$bla^{(6)}_{CTX-M-8}$	$qnrB19^{(4)}$, $qnrS1^{(1)}$	–	$bla^{(6)}_{CTX-M-8}$, $qnrB19^{(4)}$, $qnrB19/qnrS1^{(1)}$
	S2	10	7	8	0	17	$bla^{(1)}_{CTX-M-8}$, $bla^{(3)}_{CTX-M-14}$, $bla^{(1)}_{CTX-M-15}$, $bla^{(2)}_{CMY-2}$	$qnrB19^{(8)}$, $qnrS1^{(6)}$	–	$bla_{CTX-M-15}/qnrB19/qnrS1^{(1)}$, $bla^{(2)}_{CTX-M-14}$, $bla^{(2)}_{CMY-2}$, $bla_{CTX-M-14}/qnrB19^{(1)}$, $qnrB19/qnrS^{(4)}$, $bla^{(1)}_{CTX-M-8}$, $qnrB19^{(2)}$, $qnrS1^{(1)}$
	S3	10	10	10	7	26	$bla^{(8)}_{CMY-2}$, $bla^{(7)}_{SHV-12}$	$qnrB19^{(1)}$	$mcr\text{-}1^{(7)}$	$bla^{(7)}_{SHV-12}$, $mcr\text{-}1^{(5)}$, $mcr\text{-}1/bla^{(2)}_{CMY-2}$, $bla^{(6)}_{CMY-2}$, $qnrB19^{(1)}$
	S4	10	5	10	0	17	$bla^{(4)}_{CTX-M-8}$, $bla^{(1)}_{CTX-M-14}$	$qnrS1^{(8)}$		$bla^{(4)}_{CTX-M-8}$, $bla^{(1)}_{CTX-M-14}$, $qnrS1^{(8)}$
	S5	10	8	10	0	22	$bla^{(7)}_{CTX-M-8}$	$qnrB19^{(3)}$, $qnrS1^{(7)}$		$bla_{CTX-M-8}/qnrB19^{(1)}$, $qnrB19/qnrS1^{(1)}$, qnrS1$^{(6)}$ $bla_{CTX-M-8}$ $^{(7)}$
	Total	50	36	46	7	99	40	38	7	

The number in parentheses corresponds to the number of isolates containing that gene or genotype. cro, Ceftriaxone; R, Resistance; cip, Ciprofloxacin; col, Colistin; PMQR, plasmid-mediated quinolone resistance.

Identification and Antibiotic Susceptibility Testing

Putative *E. coli* colonies were identified by matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker, MA) in the facilities of Instituto de Higiene (Montevideo, Uruguay).

We then tested susceptibility to the following antibiotics: amoxicillin-clavulanic acid (AMC), ceftriaxone (CRO), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (CN), amikacin (AK), and trimethoprim-sulfamethoxazole (SXT). Results were interpreted according to the Clinical Standard Laboratory Institute 2020 guidelines (15); isolates displaying intermediate resistance levels were considered as resistant.

Isolates showing resistance to 3rd generation cephalosporins underwent phenotypic screening for ESBLs and/or AmpC β-lactamases with the synergy double-disc method, using ESBL and AmpC inhibitors (AMC and boronic acid, respectively) plus 3rd generation cephalosporins according to Cordeiro et al. (16).

Minimum inhibitory concentration to colistin was determined for *E. coli* isolates recovered from MacConkey supplemented with the aforementioned antibiotic, by colistin agar test according to CLSI (15); *E. coli* ATCC 25922 was used as a quality control.

Detection of ESBL, PMQR, and Colistin Resistance Genes

Resistance genes were sought by polymerase chain reaction (PCR) and confirmed by sequencing. We searched for the following resistance genes:

(a) oxyimino-cephalosporins
bla$_{\text{CTX-M-group-1}}$, *bla*$_{\text{CTX-M-group-2}}$, *bla*$_{\text{CTX-M-group-3}}$, *bla*$_{\text{CTX-M-group-4}}$, *bla*$_{\text{CTX-M-group-25}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{PER-2}}$, *bla*$_{\text{AmpC}}$ (17);

(b) quinolones
aac (15)Ib-cr, *qnrA*, *B, C, D, E, S, Vc*, *qepA* (17);

(c) colistin
Detection of plasmid-mediated colistin resistance genes (mcr-1, mcr-2, and mcr-3) was performed by Real-Time PCR according to Li et al. (18) and mcr 4 was sought by PCR according to Carattoli et al. (19).

The detailed list of primers used can be found in **Supplementary Table 1**.

RESULTS

General Results

During the present work, 687 *E. coli* isolates were recovered: 334 from calves, 200 from poultry and 153 from pigs.

The 687 isolates were subjected to antibiotic susceptibility determination and detection of antibiotic resistance genes, as described in the materials and methods section. However, to avoid duplicate results, for each animal studied, only those isolates that presented phenotypic or genotypic differences are presented. Accordingly, we selected 73 bovine isolates, 141 poultry isolates and 99 pig isolates (**Supplementary Table 2**).

The most frequently detected resistance was to fluoroquinolones, being present in all of the analyzed farms. In 214/282 (75.9%) of the studied animals, we detected ciprofloxacin-resistant *E. coli* isolates (observed in 55 calves, 110 chickens, and 49 pigs) (**Table 1**). The farms displaying the highest levels of resistance corresponded to those of pig farming, yielding resistant isolates in 98% of the studied animals (49/50), followed by poultry farming with 83.3% (110/132), and lastly calf stables with 55% of the studied animals.

Conversely, resistance to oxyimino-cephalosporins was detected in 60/282 animals (21.4%), albeit quite heterogeneously: 72% of the sampled swine yielded resistant isolates (36/50) distributed along all the analyzed farms, followed by poultry farms where 17.4% of the sampled animals harbored resistant isolates (23/132), in 6/13 studied farms. Finally, a single calf harboring resistant isolates was detected among the studied establishments.

Resistance to colistin was the lowest of the three tested antibiotics, only 8 animals carried resistant isolates (2.8%); seven corresponded to pigs and the remaining case to a chicken.

Detection of Resistance Genes

The most frequently detected resistance gene was *qnrB19*, which was present in *E. coli* isolates recovered from 87 animals belonging to the 3 production lines. Regarding PMQR genes, *qnrS1* was the second most frequent, being detected in 23 animals. Sparing a single case corresponding to a calf, the remaining 22 cases corresponded to isolates recovered from swine. The third gene in frequency was *qnrE1*, found in 8 isolates obtained from chickens and calves.

Regarding resistance to oxyimino-cephalosporins, 8 different β-lactamase genes were detected, the most frequent being *bla*$_{\text{CTX-M-8}}$ and *bla*$_{\text{CMY-2}}$ found in 22 and 19 animals, respectively; next, *bla*$_{\text{CTX-M-2}}$ and *bla*$_{\text{SHV-12}}$ were both detected in 7 animals, followed by *bla*$_{\text{CTX-M-14}}$ in 4, *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{SHV2a}}$ in 2, and *bla*$_{\text{CTX-M-55}}$ in a single animal (**Table 1**).

Finally, transferable colistin resistance genes were detected in 2 establishments, belonging to a pig and a poultry farm; in the former, *mcr-1* was found in 8 animals, whereas in the latter in a single animal. In 3 pig-derived isolates, *mcr-1* was found along with *bla*$_{\text{CMY-2}}$, while in the poultry isolate *mcr-1* was detected alongside *qnrB19*.

Assessment of Relative Frequencies of Transferable Resistance Genes

As previously mentioned, in the present work we selected 73 *E. coli* isolates obtained from 100 calves, 141 isolates obtained from 132 chickens, and 99 isolates obtained from 50 pigs, thus yielding genotype/studied animal ratios of 0.73 (73/100), 1.08 (140/132), and 1.77 (99/50) for calves, poultry, and swine, respectively. Additionally, 30% of the *E. coli* resistant genotypes recovered from calves were linked to transferable resistance genes (22/73); conversely, in poultry, and swine, the frequency of isolates harboring transferable resistance genes was 55% (78/140) and 76% (75/99), respectively (**Table 1**).

DISCUSSION

This is the first work tackling the presence of transferable resistance to antibiotics considered as highest priority critically important antibiotics for human health in three chains of production animals in Uruguay.

The most alarming situation was observed in swine husbandry, where the highest percentage of animals harboring resistant isolates, the highest number of bacterial genotypes per animal and the highest percentage of transferable resistance genes were observed. Taking into account this scenario it is possible to hypothesize that, from the three populations studied, the swine digestive tract is where the best condition for resistance genes horizontal transfer events occur.

Recently, Van Boeckel et al. have reported that the global consumption of antibiotics per kilogram of animal in cattle, poultry, and swine husbandry is 45, 148, and 172 mg, respectively (7). Our data show some degree of correlation with that study, in the sense that a greater use of antibiotics entails a higher detection of resistance mechanisms on account of the selective pressure imposed.

Nevertheless, resistance to colistin (at least in this study) is lower than values reported in other countries of our region and the rest of the world, such as Ecuador, Argentina, and Spain (20–22). During this work we detected the presence of 8/50 pigs (16%) carrying *mcr-1*-harboring *E. coli*, all of them corresponding to the same farm. In 5/7 animals, the genes *mcr-1* and $bla_{\text{CMY}-2}$ were found in separate *E. coli* isolates, yet in 3 animals those genes were found within the same isolate (**Supplementary Table 2**). Recently, we reported the detection of the first *E. coli* isolates of human origin carrying *mcr-1* in Uruguay. One of these isolates harbored $bla_{\text{CMY}-2}$ and *mcr-1,* albeit in different plasmids; in this sense, *mcr-1* was encoded in an IncI2-type plasmid (23). More studies are needed to determine if there is a relationship between these isolates (i.e., *mcr-1*–$bla_{\text{CMY}-2}$ bearing isolates of human and animal origin) or the genetic platforms that encode them. In addition, we also found an isolate obtained from poultry, carrying *mcr-1*. Presumably, the mandatory ban in Uruguay on veterinary usage of colistin, in any of its forms, will have beneficial effects by reducing the selection pressure on microorganisms carrying *mcr-1*. However, keeping in mind that *mcr* alleles are frequently associated with genes conferring resistance to other antibiotics widely used in veterinary medicine, such as quinolones and oxyimino cephalosporins, co-selection events are likely to occur. This has been demonstrated by the presence of the *mcr* gene along with ESBL, carbapenemases or plasmid-mediated quinolone resistance mechanisms such as *qnrB* or *qnrS* in different plasmids (IncI2, IncX4, y IncHI2) (24–27).

Regarding resistance to 3rd generation cephalosporins and quinolones in swine and poultry, the most frequently detected genes were $bla_{\text{CTX}-\text{M}-8}$ and $bla_{\text{CMY}-2}$, and *qnrB19*, respectively. A similar situation was found in human *Salmonella enterica* isolates in recent surveillance studies in our country (16). Since this microorganism is a primary pathogen and is associated with episodes of gastroenterocolitis, it could act as a doorway for resistance genes circulating in agricultural and veterinary environments. Notwithstanding, the intake of *E. coli* strains harboring the same or other resistance mechanisms could go unnoticed in the event of asymptomatic gut colonization.

In this concern, colonization with *E.coli* carrying resistance genes to antibiotics of critical use could be considered as a silent zoonosis, contributing to the resistance gene pool of microorganisms present in the gastrointestinal tract of animals and humans. It has been observed that gut colonization by *E. coli* strains with reduced susceptibility to fluoroquinolones can last from 2 weeks to 6 months, whereas the presence of β-lactamase-producing *E. coli* in outpatients can last up to 4 months in feces. Conversely, the persistence of ESBL-producing *E. coli* and *K. pneumoniae* in feces from recently discharged patients can last an average of 98 days (range 14–182 days) (28, 29).

Among the various ESBLs detected in poultry we also found $bla_{\text{CTX}-\text{M}-55}$. This β-lactamase had never been reported in our country; yet in our neighboring country Brazil, it has been reported both in poultry and humans, usually associated to the glutathione transferase gene *fosA3,* responsible of conferring resistance to fosfomycin (30, 31). In order to assess this probable association in our poultry-derived isolates, we also performed PCR detection of *fosA3*, obtaining positive results (data not shown).

Although the main mechanism of resistance to oxyimino-cephalosporins in enterobacteria of human origin is $bla_{\text{CTX}-\text{M}-15}$, this ESBL gene occasionally occurred in the isolates analyzed in the present work. In Uruguay $bla_{\text{CTX}-\text{M}-2}$, $bla_{\text{CTX}-\text{M}-8}$, $bla_{\text{SHV}-12}$, and $bla_{\text{SHV}-2}$ are among the most frequently detected β-lactamase genes, mainly in pediatric samples; more studies are needed to determine if there is any link with the microorganisms detected in this work or, perhaps, with the genetic structures that encode these genes (32–34).

In a previous report, we found *E. coli* isolates obtained from cattle showing some degree of resistance to fluoroquinolones, namely, 7.3% were carriers of PMQR (mainly *qnrB2* and *qnrS*) (14). In the present work, the number of isolates carrying PMQR genes rose to 31.5% (23/73); furthermore, we also detected a change in the circulating alleles. In this regard, gene *qnrB19* was the most frequently found, while the presence of *qnrE1* and *qnrS1* was detected in two and one isolate, respectively. The occurrence of *qnrE1* has been recently reported in isolates of *Salmonella enterica* from cattle in Brazil (35), however it has not been described outside the *Salmonella* genus in neither cattle nor poultry; thus, in this work we confirm the circulation of such gene in *E. coli* both in cattle and poultry.

The *qnrE1* gene was first reported in Argentina in 2017, in a human clinical isolate obtained in 2007 (36). Interestingly, in 2011 we reported the occurrence in Uruguay of a *qnrB* variant, termed *qnrBKp737* (defined by a partial sequence of 606 bp obtained from a PCR amplification product) (37). The *in silico* translation of the partial nucleotide sequence displayed 26 amino acid differences with QnrB1; further comparison showed that *qnrBKp737* was identical to *qnrE1* (unpublished data). Apparently, this resistance mechanism scarcely reported so far, is also a long-standing problem in the context of "One Health" in our region.

One limitation of our work is the fact that we did not conduct a study of risk factors to determine which variables influence the selection of resistant microorganisms.

In light of our results, it will be necessary to carry out new studies encompassing a greater number of establishments with a design that allows us to analyze these aspects.

Beyond these limitations, the wide distribution of fluoroquinolone-resistant isolates in the animals analyzed is alarming and may reflect the widespread use of antimicrobials such as enrofloxacin. Since this class of antibiotics is on the list of highest priority critically important antibiotics for human health, their use in veterinary medicine should be drastically limited.

In conclusion, we have detected transferable resistance genes to the three antibiotics considered critical to human health, present in feces from farm animals in Uruguay. Several of such genes have also been reported previously in microorganisms of human origin in our country. Tackling the problem of antimicrobial resistance requires comprehensive approaches, including prudent use of antibiotics and surveillance under the "One Health" concept.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

NC, AU, and PÁ: carrying out of antibiotic sensitivity studies and detection of resistance genes by PCR and preparation of the manuscript. BF: pig samples collection, antibiotic sensitivity studies, and detection of resistance genes by PCR. NFC: real-time PCR for mcr-1, 2, and 3, interpretation of results, and preparation of the manuscript. GT: poultry samples collection, interpretation of results, and preparation of the manuscript. GC: pig samples collection, interpretation of results, and preparation of the manuscript. MC and MF: calves sample collection, interpretation of results, and preparation of the manuscript. PZ: experimental design, general coordination, interpretation of results, and elaboration of the manuscript. IB and RV: experimental design, general coordination, sequence analysis, interpretation of results, and elaboration of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from Agencia Nacional de Investigación e Innovación (ANII) and Espacio Interdisciplinario Universidad de la República to RV. This work was funded by the project PL-15 from Instituto Nacional de Investigación Agropecuaria (INIA). This work was also funded by grant to NC from Comisión Sectorial de Investigación Científica (CSIC).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.588919/full#supplementary-material

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

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 25 November 2020
doi: 10.3389/fvets.2020.547843

Broiler Farms and Carcasses Are an Important Reservoir of Multi-Drug Resistant *Escherichia coli* in Ecuador

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 31 March 2020
Accepted: 29 October 2020
Published: 25 November 2020

Citation:
Ortega-Paredes D, de Janon S, Villavicencio F, Ruales KJ, De La Torre K, Villacís JE, Wagenaar JA, Matheu J, Bravo-Vallejo C, Fernández-Moreira E and Vinueza-Burgos C (2020) Broiler Farms and Carcasses Are an Important Reservoir of Multi-Drug Resistant Escherichia coli in Ecuador. Front. Vet. Sci. 7:547843. doi: 10.3389/fvets.2020.547843

Antimicrobial resistance (AMR) is a major health threat for public and animal health in the twenty-first century. In Ecuador, antibiotics have been used by the poultry industry for decades resulting in the presence of multi-drug resistant (MDR) bacteria in the poultry meat production chain, with the consequent risk for public health. This study evaluated the prevalence of ESBL/AmpC and *mcr* genes in third-generation cephalosporin-resistant *Escherichia coli* (3GC-R *E. coli*) isolated from broiler farms (animal component), broiler carcasses (food component), and human enteritis (human component) in Quito-Ecuador. Samples were collected weekly from November 2017 to November 2018. For the animal, food, and human components, 133, 335, and 302 samples were analyzed, respectively. Profiles of antimicrobial resistance were analyzed by an automated microdilution system. Resistance genes were studied by PCR and Sanger sequencing. From all samples, 122 (91.7%), 258 (77%), and 146 (48.3%) samples were positive for 3GC-R *E. coli* in the animal, food, and human components, respectively. Most of the isolates (472/526, 89.7%) presented MDR phenotypes. The ESBL $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{CTX-M-27}}$, and $bla_{\text{CTX-M-14}}$ were the most prevalent ESBL genes while $bla_{\text{CMY-2}}$ was the only AmpC detected gene. The *mcr*-1 gene was found in 20 (16.4%), 26 (10.1%), and 3 (2.1%) of isolates from animal, food, and human components, respectively. The implication of poultry products in the prevalence of ESBL/AmpC and *mcr* genes in 3GC-R must be considered in the surveillance of antimicrobial resistance.

Keywords: AmpC beta-lactamases, broiler farms, broiler carcasses, *E. coli*, extended-spectrum beta-lactamase (ESBL), human, mcr-1

INTRODUCTION

The World Health Organization recognizes antimicrobial resistance (AMR) as a major health threat in the 21st century (1). A global projection predicts that the increase of deaths linked to AMR will develop from 700,000 in 2016 to 10 million deaths per year in 2050 while 100 trillion USD could be lost by 2050 (2). In this scenario, the use of antibiotics in food animal production is one of the most important issues contributing to the AMR crisis. In fact, over 50% of antibiotic production is used by the meat industry and an increase of 50% in antibiotic usage for farming is estimated by 2030. Moreover, up to a 160% increase in antibiotic usage in food animals is expected in Latin American countries in absence of changes (3).

Poultry production is an important sector for the study of AMR because of the common usage of antibiotics in this industry. Additionally, it is expected that poultry will be the main animal production industry by 2025 (4). This issue is especially significant in developing countries where antimicrobials are not only used to treat infections but also prophylactically and as growth promoters (5). In Ecuador, poultry products are the most important source of animal protein with a per capita consumption of poultry meat of 30.4 kg/year (6).

In Ecuador, commonly used antibiotics in poultry production include quinolones, fosfomycin, and colistin, which are listed by WHO as critically important antimicrobials for human medicine, with quinolones and colistin even highest prioritized (7). This practice has promoted the dissemination of multi-drug resistant (MDR) bacteria, principally extended-spectrum β-lactamases and AmpC-producing (3GC-R) *Escherichia coli,* which is commonly studied as a sentinel organism to understand the epidemiology of AMR (8). In Ecuador, colistin was banned for use in food animals in 2019. However, *mcr* genes have been recently identified in *E. coli* isolated from animals, humans, and the environment (9–11).

A relationship between 3GC-R *E. coli* isolated from poultry products and humans has been suggested previously (12–16). In Ecuador, Vinueza-Burgos et al. described a high prevalence of 3GC-R *E. coli* and the presence of *mcr*-1 in poultry farms (17). However, there is no data about the prevalence of 3GC-R and colistin-resistant *E. coli* in broiler carcasses and humans in this location. Therefore, this study aimed to evaluate the state of 3GC-R and *mcr* genes in *E. coli* in broiler farms, chicken carcasses at retail level and human stool samples in Ecuador.

MATERIALS AND METHODS

Study Design and Sampling

Samples for *E. coli* isolation were collected weekly from November 2017 to November 2018. Under local legislation, ethical approval was not required for collecting chicken caeca and carcasses during sampling. The participants for the human component were informed about the objective of the study; the participation was voluntary (all volunteers provided a written consent) and all personal information was anonymized. This project was approved by the committee of bioethics from the National Institute of Public Health "Leopoldo Izquieta Pérez" (Protocol ID: CEISH-INSPI-005). The sample distribution for each component considered local characteristics (location of farms in the zone of Quito, distribution of retail shops in the city, and location of healthcare centers).

The sample size for the animal component was calculated considering the number of industrial farms close to Quito, the number of batches that these farms produce in 1 year, and the prevalence of 3GC-R *E. coli* in poultry farms previously reported in this zone (17). For the food and human components, the minimum sample size was calculated considering an infinite population and at a 0.9 of confidence.

For the animal component,133 flocks from 69 farms close to Quito were sampled during the study period. For every sampled flock, 25 caeca from 25 chicken were randomly collected at the slaughterhouse and transported to the laboratory in an icebox within 2 h. At the laboratory, a sample pool of 25 g. was obtained and homogenized by hand as previously described (17).

For the food component, 335 carcasses were collected in three kinds of markets as follows: 125 samples from supermarkets, 126 samples from small shops, and 84 samples from open markets. Sampling places were distributed in both northern and southern areas of Quito. A sampling of chicken carcasses was performed alternately between the north and south of the city. Each sample consisted of one carcass collected in its original bag and transported to the laboratory in an icebox within 2 h. At the laboratory, 25 g of breast skin of every carcass were aseptically collected for bacteriological analysis.

For the human component, stool samples were collected in two health care centers located in the urban periphery of Quito. The inclusion criteria of the patients from whom samples were taken were: individuals with two or more episodes of diarrhea or vomiting in the last 24 h. Stool samples were transported to the laboratory in an icebox within 2 h and 25 g of feces were collected for bacteriological analysis.

Isolation and Identification of Cefotaxime-Resistant *E. coli*

All samples were homogenized with 225 ml of buffered peptone water (BPW; Difco, BD, Sparks, MD) and incubated at 37°C for 18–24 h. A loopful of each sample was streaked onto chromogenic Tryptone Bile X-Glucuronide (TBX) agar (BioRad, Hercules, California, USA) supplemented with cefotaxime (3 mg/l). Positive plates were considered when at least one typical colony could be selected (when available, two colonies were selected for further analysis) and confirmed to be *E. coli* using Triple Sugar Iron (TSI) agar (Difco, BD, Detroit, USA) and by PCR as described elsewhere (18). From the TSI medium, one loopful was suspended in 300 μl of sterile water and used to extract DNA by the boiling method. Another loopful was used to subculture the isolate in trypticase soy broth (TSB) (Difco, BD, Detroit, USA) and stored with glycerol (60%) at −80°C.

Antimicrobial Susceptibility Testing and Resistance Genes Screening

Resistance profiles to antibiotics commonly tested for *Enterobacteraceae* were obtained for all isolates using the Vitek®

2 system with AST-N271 cards (BioMérieux, Marcy-l'Étoile, France). The following antibiotics were tested: ampicillin, ampicillin + sulbactam, cephalothin, cefuroxime, ceftriaxone, cefotaxime, ceftazidime, cefepime, ertapenem, meropenem, amikacin, gentamicin, ciprofloxacin, norfloxacin, fosfomycin, nitrofurantoin, and trimethoprim + sulfamethoxazole. The MIC for colistin was tested in isolates positive for *mcr* genes by PCR using BD Phoenix™ M50 with NMIC/ID 94 panel (Becton Dickinson, Nueva Jersey, USA). Antimicrobial resistance phenotypes were obtained following the manufacturer's instructions. *E. coli* ATCC 25922 was used as a quality control strain. Results were evaluated using the clinical breakpoints recommended by CLSI (19). Isolates resistant to at least 3 antibiotic classes were considered as MDR. The *mcr* 1 to 5, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{CMY} genes were tested by PCR in all isolates as previously described (17, 20). Obtained amplicons were sequenced at Macrogen (Seoul-South Korea). Sequences were analyzed using Genious Prime software with the ResFinder database (21).

Statistical Analysis

Significate differences of the prevalence of 3GC-R *E. coli* within and among each component were calculated using a χ^2 test. This statistical test was also used to assess differences in antibiotics resistance rates and the presence of resistance genes variants as well as determining differences among farms, types of markets, and location of health care centers ($p < 0.05$). The 95% confidence intervals ($CI_{95\%}$) for the prevalence of 3GC-R *E. coli* was obtained by Binomial "exact" calculation. All tests were carried out in RStudio V.1.2.

RESULTS

Isolation of *Escherichia coli* Resistant to Cefotaxime

A total of 526 samples were positive for 3GC-R *E. coli*. The highest prevalence was identified in the animal component (91.7%; $CI_{95\%}$: 90.8–92.7), followed by the food component (77%; $CI_{95\%}$: 76.3–77.8) and the human component (48.3%; $CI_{95\%}$: 47.9–48.8). χ^2 test identified significant differences among components ($p < 0.05$). However, no significant differences were observed within farms, types of shops, and locations of health centers ($p > 0.05$).

Antimicrobial Susceptibility Testing

All the isolates were resistant to ampicillin, cephalothin, cefuroxime, ceftriaxone, cefotaxime, and trimethoprim + sulfamethoxazole. High resistance rates (more than 80%), were registered for cefepime and ceftazidime in the three components. Resistance to ciprofloxacin and norfloxacin ranged from 70 to 80% in the animal and food components and were significantly higher than rates in the human component, where half of the isolates were resistant. AMR to the combination β-lactam + β-lactamase inhibitor (Ampicillin + Sulbactam) was around 55% in the three components.

Resistance to fosfomycin, nitrofurantoin, and gentamicin showed significant differences between the three components ranging from 30 to 50% in animal and food components and from 24 to 30% in the human component, while for cefepime the resistance rate was higher in humans ($p < 0.05$). Five isolates (two from the animal, one from food, and two from human components) were resistance to carbapenems (ertapenem and/or meropenem). None of the isolates were resistant to Amikacin (**Table 1**). Additionally, 89.7% of the isolates (472/526), presented MDR patterns with three up to seven groups of antibiotics. The most frequent pattern included resistance to β-lactams, fluoroquinolones, and folate pathway inhibitors (**Table 2**, **Supplementary File 1**). Distribution of MIC values for every tested antibiotic in each component shown in **Supplementary File 4**.

Resistance Genes

Group 1 of $bla_{\text{CTX-M}}$ genes was the most prevalent family of ESBL genes, followed by group 9, group 2, and group 8. The allele $bla_{\text{CTX-M-55}}$, belonging to group 1, was identified as the most prevalent variant in the three components, followed by $bla_{\text{CTX-M-3}}$ in animal and food components and $bla_{\text{CTX-M-15}}$ in the human component. Among $bla_{\text{CTX-M}}$ group 9, the most frequent allele in the three components was $bla_{\text{CTX-M-65}}$ followed by $bla_{\text{CTX-M-27}}$ (animal and food components) and $bla_{\text{CTX-M-14}}$ (human component). The $bla_{\text{CTX-M-2}}$ gene was present in the animal and food components, while the $bla_{\text{CTX-M-8}}$ gene was detected in animal and human components. Moreover, ESBL variants of bla_{SHV} and broad-spectrum β-lactamases bla_{TEM} were frequently identified in the three components. Additionally, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-17}}$, and $bla_{\text{CTX-M-2}}$ were registered only in poultry isolates, $bla_{\text{CTX-M-8}}$ was identified in food and human components, and $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CTX-M-27}}$ were identified only in the human component. Finally, only the AmpC gene $bla_{\text{CMY-2}}$ was detected in the samples from the three components (**Table 3**, **Supplementary File 2**).

The *mcr*-1 gene was found in 16.4% (20/122), 10.1% (26/258), and 2.1% (3/146) of isolates from the animal, food, and human components, respectively. Colistin resistant isolates showed MIC values from 2 to 4 μg/ml. In these isolates, the allele $bla_{\text{CTX-M-55}}$ was dominant in animal and food components, followed by $bla_{\text{CTX-M-65}}$, $bla_{\text{CTX-M-2}}$, and $bla_{\text{CTX-M-3}}$. Additionally, $bla_{\text{CMY-2}}$ and $bla_{\text{SHV-5}}$ genes were detected in these components while the alleles $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$ were the only ones detected *mcr*-1 positive isolates from humans (**Supplementary File 3**).

DISCUSSION

Studies of 3GC-R *E. coli* in poultry production remain scarce in the Andean region of South America (15, 20). Additionally, only a few of these studies have evaluated the prevalence of *mcr* genes in 3GC-R *E. coli* from poultry (17). To the best of our knowledge, this issue has not been evaluated with a multi-component approach in this region.

The high prevalence of 3GC-R *E. coli* in poultry showed in this study is in concordance with a previous report (17). These issues could be related to the rapid dissemination of 3GC-R mediated by horizontal transfer in broiler farms (22). On the other hand, the

TABLE 1 | Antibiotic resistance rates in each component.

Antibiotic	Animal component *n* = 122	Food component *n* = 258	Human component *n* = 146	Total *n* = 526
Ceftazidime	111 (91%)	244 (94,6%)	142 (97,3%)	497 (94,5%)
Cefepime	100 (82%)	225 (87,2%)	133 (91,1%)	458 (87,1%)
*Ciprofloxacin	*92 (75,4%)	*209 (81%)	86 (58,9%)	387 (73,6%)
*Norfloxacin	*86 (70,5%)	*187 (72,5%)	76 (52,1%)	349 (66,3%)
Ampicillin + Sulbactam	65 (53,3%)	147 (57%)	83 (56,8%)	295 (56,1%)
*Fosfomycin	*62 (50,8%)	*127 (49,2%)	43 (29,5%)	232 (44,1%)
*Nitrofurantoin	44 (36,1%)	*105 (40,7%)	40 (27,4%)	189 (35,9%)
*Gentamicin	36 (29,5%)	*96 (37,2%)	35 (24%)	167 (31,7%)
Ertapenem	2 (1,6%)	1 (0,4%)	2 (1,4%)	5 (1%)
Meropenem	1 (0,8%)	0	0	1 (0,2%)

**Antibiotics in which significant differences ($p < 0.05$) were identified.*
Antibiotics with resistance rates or 100% and 0% were not included in this table.

TABLE 2 | Antimicrobial resistance patterns by antibiotic family.

Nº antibiotic families	Pattern	Animal component	Food component	Human component	Total
7	BEAFPNS		1		1
6	BAFPNS	10	17	8	35
5	BAFNS	9	14	6	29
5	BAFPS	8	25	5	38
5	BFPNS	10	29	3	42
5	BEAFS			1	1
5	BEFPS	1			1
5	BEPNS	1			1
5	BAPNS		1		1
4	BFNS	13	39	8	60
4	BFPS	14	31	13	58
4	BAFS	7	25	12	44
4	BAPS		7	1	8
4	BPNS	1		1	2
4	BEFS			1	1
3	BPS	17	16	12	45
3	BAS	2	6	2	10
3	BFS	20	28	29	77
3	BNS		4	14	18
2	BS	9	15	30	54
Total		122	258	146	526

B, β-lactams from first line to third generation cephalosporin; E, carbapenems; A, Aminoglycosides; F, fluoroquinolones; P, Phosphonates; N, Nitrofurans; S, Folate pathway inhibitor.

prevalence in the food component was significantly lower than in the animal component. This fact has been observed in Brazil, where 3GC-R *E. coli* was isolated from 54.2 and 29.2% of samples coming from animals and chicken carcasses, respectively (23). Besides, around 50% of the human stool samples were positive for 3GC-R *E. coli*. Prevalence of 3GC-R *E. coli* in healthy carriers has been reported in the Netherlands, Japan, India, Libya, and Sweden ranging from >5 to 19% (24). Moreover, *E. coli* ESBL has been registered in 18.8% of pediatric patients with diarrhea (25). Our results suggest that the poultry environment in Ecuador is a reservoir of 3GC-R *E. coli*.

Carbapenem resistance mediated by $bla_{\text{KPC-2}}$, $bla_{\text{KPC-3}}$, $bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{VIM-2}}$ has been reported in *Enterobacteriaceae* in South American countries in human isolates (26). In Ecuador, $bla_{\text{KPC-2}}$ is the most prevalent carbapenemase gene detected in *E. coli* in hospitals (27, 28), and recently it was detected in an urban river in *Citrobacter freundii* (29). However, there are no reports of carbapenemases from poultry in our region (carbapenems are not used for poultry production). In our study, the isolates resistant to imipenem/meropenem from the three components were negative for the bla_{KPC} gene by PCR testing (data not shown). Therefore, this resistance could be related to not tested carbapenemase genes or to a combination of ESBL enzymes and porin loss (30).

In this study, resistance to fluoroquinolones was significantly higher in isolates originated in poultry (up to 80%) than in isolates originated in the human component (55%). These data are concordant with the common use of quinolones in the poultry industry in Ecuador (31). Additionally, ciprofloxacin is commonly used as an empiric treatment of community-acquired *E. coli* infections in humans. These practices could explain the high resistance rates observed for fluoroquinolones which has also been reported in other countries of the region (32). Even though resistance to fluoroquinolones could be explained by chromosomal point mutations (33), quinolone resistance mediated by mobile resistance genes (*qnr*) has been frequently associated with ESBL production in Enterobacteriaceae (34) suggesting that the co-transference of these genes is a common event.

Additionally, all the isolates in this study were resistant to trimethoprim + sulfamethoxazole. This finding could be explained by the presence of class 1 integrons. These site-specific recombination systems typically have a *sul* gene in their 3′CS end, and frequently present aminoglycoside, quinolone, and β-lactam

TABLE 3 | Prevalence of ESBL/AmpC genes in the cefotaxime-resistant *Escherichia coli* isolates.

Gen family	Allele	Animal component	Food component	Human component	Total
bla_{CTX} group 1	*nd*		3		3
	$bla_{CTX\text{-}M\text{-}1}$		1		1
	$bla_{CTX\text{-}M\text{-}123}$	1	3		4
	$bla_{CTX\text{-}M\text{-}15}$		2	26	28
	$bla_{CTX\text{-}M\text{-}27}$			1	1
	$bla_{CTX\text{-}M\text{-}3}$	6	17	13	36
	$bla_{CTX\text{-}M\text{-}55}$	50	109	35	194
bla_{CTX} group 9	$bla_{CTX\text{-}M\text{-}14}$			6	6
	$bla_{CTX\text{-}M\text{-}17}$	1			1
	$bla_{CTX\text{-}M\text{-}27}$			19	19
	$bla_{CTX\text{-}M\text{-}65}$	27	56	21	104
bla_{CTX} group 2	$bla_{CTX\text{-}M\text{-}2}$	9	11		20
bla_{CTX} group 8	$bla_{CTX\text{-}M\text{-}8}$		1	2	3
bla_{CTX} group nd	–	1		2	3
CMY	*nd*		4		4
	$bla_{CMY\text{-}2}$	19	38	21	78
*TEM**	*nd*		2	3	5
	$bla_{TEM\text{-}1}$	19	74	42	135
	$bla_{TEM\text{-}166}$			1	1
	$bla_{TEM\text{-}176}$		2		2
	$bla_{TEM\text{-}1b}$	1	4	11	16
SHV	*nd*		1	2	3
	$bla_{SHV\text{-}12}$	3	4	11	18
	$bla_{SHV\text{-}2a}$	2		1	3
	$bla_{SHV\text{-}5}$	3	1	6	10
nd	–	8	15	7	30
Total		122	258	146	526

**The* bla_{TEM} *alleles identified are not ESBL.*
nd, not determined.

resistance genes in the variable region. These features promote the selection and evolution of these genetic platforms in mixed antibiotic pressure environments (35), which could explain our findings. However, a genetic analysis to test those elements is necessary to confirm this hypothesis.

The resistance rates to nitrofurantoin reported in this study are higher than the ones reported previously in Ecuador and Colombia (36). However, this antibiotic is not used in the poultry industry. The mechanism of resistance and the reason for the increase of resistance rates reported in this study remain unknown. Our results also showed that half of the isolates from poultry and about 30% of human isolates were resistant to fosfomycin. In South American countries resistance to fosfomycin in *E. coli* isolated from human infections has been reported ranging from 2 to 3% (37, 38). On the other hand, a study carried out in Brazil reported a lower prevalence of this resistance in poultry. There are no other reports of fosfomycin resistance in poultry in neighboring countries, but a close relation of Enterobacteria isolated from poultry between Peru and Ecuador has been described before, proposing the hypothesis of a common epidemiology of these bacteria in the Andes region (30, 31). Nitrofurantoin and fosfomycin are antibiotics prescribed for the treatment of infections caused by MDR and extremely resistant enterobacteria (39, 40). Our findings highlight the urgency of a better regulation of the usage of these antibiotics in Ecuador.

Finally, the aminoglycoside amikacin is not used in poultry production and is restricted to complicated infections in humans (41), so the susceptibility of all isolates to amikacin is not surprising.

In this study, $bla_{\text{CTX-M-55}}$ was the most prevalent allele of the $bla_{\text{CTX-M}}$ group, followed by $bla_{\text{CTX-M-65}}$ and $bla_{\text{CTX-M-2}}$ in the poultry components. These results show a change in the prevalence of $bla_{\text{CTX-M}}$ genes compared with the report a previous report where $bla_{\text{CTX-M-65}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-3}}$ were the most prevalent alleles in poultry isolates (17). These outcomes differ with reports in other Latino-American countries as Colombia, where the most dominant allele was $bla_{\text{CMY-2}}$ (42), and Brazil where $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-2}}$ are the most important alleles in poultry (37, 38, 43, 44). Additionally, in the human component $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-65}}$ were the most prevalent alleles. Based on clinical evidence, the change of the dominance of $bla_{\text{CTX-M-15}}$ to $bla_{\text{CTX-M-55}}$ in humans in Ecuador was already hypostatized in 2016 by Zurita et al. (45).

The presence of component-specific alleles of $bla_{\text{CTX-M}}$ genes also suggests the existence of specific reservoirs. Moreover, it has been hypothesized that the ecological characteristics were animal husbandry is carried out in the region (e.g., altitude, lack of seasons, etc.) could be related to specificities in the epidemiology of these genetic determinants (15, 29). Additionally, it should be noted that the methods used to screen the presence of 3GC-R *E. coli* could give biased information. For example, the use of selective media containing ceftazidime could contribute to recover isolates carrying ceftazidimases with low affinity to cefotaxime; principally alleles of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{VEB}, bla_{TLA}, and $bla_{\text{GES/IBC}}$ (46). On the other hand, media supplemented with cefoxitin could be used for the proper isolation of AmpC β-lactamase-producing *E. coli*.

In Ecuador the *mcr* genes have been reported before in *E. coli* isolated from humans and animals (9–11, 15); but, to the best of our knowledge, this is the first time that genetic determinants for colistin resistance are studied in a multiple-component frame in our region. From all *mcr* genes tested in this study, only the *mcr*-1 gene was detected. This gene was reported in a previous study with a lower prevalence (17) which could indicate that the prevalence of *mcr*-1 in the poultry production has increased. On the other hand, the prevalence of *mcr*-1 in the human component

remains low. This relation has been emphasized before, pointing out the animal production origin of this gene (47). Although the use of colistin as a growth promotor has been recently banned in Ecuador, the former intensive use of this antibiotic in the poultry production could be seen as the main trigger for the prevalence observed in the animal component. Our results show that poultry production is an important reservoir of 3GC-R *E. coli* that harbors the *mcr*-1 gene.

It is worth to mention that, as some of the tested antibiotics in this study are not used in poultry production (e.g., cephalosporins and nitrofurantoin), the resistance of *E. coli* to these drugs could be mediated by co-selection events (selection of mixed AR under the pressure of single agent) (48). Genomic elements as conjugating plasmids, insertion sequences, and integrons could play a main role in the dissemination and accumulation of AMR determinants in the poultry production environment. Therefore, co-selection and co-resistance processes should be considered when implementing strategies for AMR control. Besides, environmental factors as contamination of upstream rivers with antibiotics and resistant bacteria should be considered in this analysis (49).

Poultry production has been recognized as an important environment for the evolution of AMR worldwide (50, 51). Indeed, new configurations of resistance genes have been described from poultry production. Important examples are the colocation of *mcr*-1 and *mcr*-3 in plasmids (52), and the emergence of *E. coli* strains co-carrying ESBL and *fos*A3 genes (38). These findings suggest that the antibiotic pressure in poultry production promotes the active recombination and selection of MDR genotypes. Therefore, the presence of strains with new combinations of genetic determinants is a real possibility that should be further studied. In fact, the MDR patterns found in this study suggest the presence of multiple resistance mechanisms that deserve a deeper analysis at the genomic level.

Concluding Remarks

The high prevalence of 3GC-R *E. coli* reported in this study is worrisome in terms of public health and highlights the need for health policies to prevent the increase of AR in the country.

Additionally, the high prevalence of 3GC- R *E. coli* registered in our study poses a risk of transmission to humans via the food chain. However,

the implication of poultry products in the epidemiology of 3GC-R *E. coli* needs further research since other sources for the human acquisition of these bacteria should be considered.

To the best of our knowledge, this study shows for the first time, data on 3GC-R *E. coli* with a multi-component approach in Latin America. We stress the importance of MDR phenotypes and genetic determinants that are spreading rapidly worldwide.

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

DO-P: sampling, interpreted the results, and wrote the manuscript. SJ and KDLT: sampling, interpreted the results, and database management. FV and JV: designed the study, sampling, and performed the experiments in human isolates. JW and JM: designed the study. EF-M: designed the study, provided the critical input, and interpreted the results. CB-V and KR: performed the colistin phenotyping experiments. CV-B: designed the study, provided the critical input, interpreted the results, and wrote the manuscript. KR and KDLT: performed critical laboratory analysis and data analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This project was funded by the WHO Advisory Group on Integrated Surveillance on AMR (AGISAR) grant AGISAR-JM/lg.

ACKNOWLEDGMENTS

The authors want to thank José Luis Medina, Janina Mantilla, Vladimir Naranjo, and Andrea Márquez for their kind help in sample processing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.547843/full#supplementary-material

Supplementary File 1 | Antimicrobial resistance patterns by Antibiotic.

Supplementary File 2 | Genotyping of the isolates.

Supplementary File 3 | Phenotyping and genotyping of *mcr*-1 positive strains.

Supplementary File 4 | Distribution of MIC values.

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

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 26 November 2020
doi: 10.3389/fvets.2020.575848

First Case Report on Quantification of Antimicrobial Use in Corporate Dairy Farms in Pakistan

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Intensive livestock farming has become indispensable to meet the rapidly increasing demand for animal-based nutrition in low- and middle-income countries (LMICs) where antimicrobials are frequently used for treatment and prophylactic or metaphylactic purposes. However, very little is known about the trends of antimicrobial use (AMU) in dairy animals in LMICs. The objective of this study was to quantify AMU in two large commercial dairy farms in Pakistan. A retrospective study was conducted at two large corporate commercial dairy farms located in Punjab province for the year 2018. AMU was calculated using three metrics: active ingredient (AI; kg) and milligrams per population unit (mg/PU; mg/kg), which quantifies the amount of AI used, and antimicrobial treatment incidence (ATI; DDDA/1,000 cow-days), which estimates the per-day number of treatments to 1,000 cows. Total on-farm AMU was found to be 138.34 kg, 65.88 mg/kg, and 47.71 DDDA/1,000 cow-days. Measured in ATI, aminoglycosides (11.05 DDDA/1,000 cow-days), penicillins (8.29 DDDA/1,000 cow-days), and tetracyclines (8.1 DDDA/1,000 cow-days) were the most frequently used antimicrobial classes. A total of 42.46% of all the antimicrobials used belonged to the critically important antimicrobials for human medicine as defined by the World Health Organization. Considerably high AMU was found compared to other farm-level studies across the world. This was the first study to quantify AMU in the dairy industry in Pakistan. Our results showed that corporate commercial dairy management practices are associated with increased antimicrobial consumption and highlight the need for antimicrobial stewardship programs to encourage prudent use of antimicrobials in commercial dairy.

Keywords: antimicrobial use, quantification, LMICs, corporate dairy, Pakistan

OPEN ACCESS

Edited by:
Gabriel Arriagada, Universidad de O'Higgins, Chile

Reviewed by:
Laurel Redding, University of Pennsylvania, United States
Lothar Kreienbrock, University of Veterinary Medicine Hannover, Germany

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *24 June 2020*
Accepted: *26 October 2020*
Published: *26 November 2020*

Citation:
Umair M, Abdullah RM, Aslam B, Nawaz MH, Ali Q, Fatima F, Ali J, Zahoor MA and Mohsin M (2020) First Case Report on Quantification of Antimicrobial Use in Corporate Dairy Farms in Pakistan. Front. Vet. Sci. 7:575848. doi: 10.3389/fvets.2020.575848

INTRODUCTION

Antimicrobial resistance (AMR) has been considered a global health problem, and the situation is worse in low- and middle-income countries (LMICs) due to lack of responsible antimicrobial use (AMU) and inadequate antimicrobial stewardship (1). Owing to the increasing demand of animal protein, antimicrobials are extensively used in food animals for treatment and prophylactic reasons

(2, 3). Global data highlighted that the overall consumption of antimicrobials in food animals far exceeds consumption in human medicine because of larger biomass and non-therapeutic AMU in food animals (4–6). It has been suggested that the overall burden of AMR has increased due to the contribution of AMU from food animals (7). In addition, AMU in animals has become a worldwide concern as in most of the countries, more than 50% of the medically important antimicrobials are being used in livestock (5). In efforts to meet the ever-increasing animal protein demand in LMICs, a shift towards intensive livestock farming has resulted in irrational AMU (3, 8). However, data on AMU in LMICs are often not available due to weak regulatory infrastructure, over-the-counter sale of antimicrobials, and inappropriate prescription practices (9). Surveillance of AMU in animal production systems is one of the key objectives of the Global Action Plan on AMR (GAP-AMR) proposed by the World Health Organization (WHO) in the 68th World Health Assembly in 2015 (10). As a WHO member state, Pakistan has drafted its National Action Plan on AMR (NAP-AMR) in 2017 (11).

The dairy sector in Pakistan plays a significant role in its agriculture-based economy. Pakistan is one of the world's top milk producers, with an estimate of 45.8 million tons of milk produced in the year 2018 (12). The majority of dairy milk is produced from small dairy holders (one to four animals) throughout the country; however, due to the increasing demand of milk intensive and semi-intensive dairy farming is becoming increasingly popular (13, 14).

According to FAOSTAT 2018, Pakistan stands at 3rd (13.6 million cattle heads) and 11th (16.8 million tons) in terms of the number of cattle and per-year milk production, respectively, but 128th (3.4 L/day) in terms of yield per animal (12). Livestock in Pakistan contributes to 60.54% in the agriculture sector and 11.22% in the country's GDP with a growth of 4% during the fiscal year 2018–2019. The livestock wing under the Ministry of National Food Security and Research has taken several measures for the growth of the dairy sector in terms of improving per-unit productivity by allowing the import of high-yielding exotic dairy breeds (Holstein-Friesian and Jersey), their genetic material (embryos and semen), feedstuff, and farm equipment at low import duties. Dairy production in Pakistan can be classified into five major systems, i.e., small holder subsistence or market-oriented production system, rural or peri-urban commercial production system, and corporate sector production system. The corporate sector represents <1% of the country's dairy and maintains high-producing exotic cattle breeds (Holstein-Friesian and Jersey) with an average herd size of 2,000–5,000 animals. Currently, only 15 such large corporate farms are operating in the country (14, 15).

Although NAP-AMR urged the monitoring and reduction in the level of AMU in animals, nationwide surveillance to monitor AMU in livestock has not been established yet (16). Therefore, this study is designed to quantify AMU on a convenience sample of two large corporate commercial dairy farms for 1 year to provide the first baseline study on AMU at the farm-level in Pakistan.

TABLE 1 | Adjusted animal number (ANadj) as per the weights of dairy cattle defined by Jensen et al. (17).

Category	Count[a]	Weight/Head (kg)	ANadj	Biomass (kg)
Farm 1				
Cows	624	600	624	374,400
Heifers	576	300	288	172,800
Calves	528	100	88	52,800
Farm 2				
Cows	1,856	600	1,856	1,113,600
Heifers	930	300	465	279,000
Calves	1,074	100	179	107,400
Total	5,588		3,500	2,100,000[b]

[a] *Animal count taken as year average.*
[b] *Population unit (PU); composite weight (Wc) of all the animals under study.*

MATERIALS AND METHODS

Study Design

To evaluate quantitative AMU, a retrospective study was conducted in two large corporate commercial dairy farms located in Punjab province for the year 2018. Both farms were automated semi-controlled and had maintained exotic cattle (Holstein-Friesian) with a total animal count of 5,588, consisting of 2,480 milking cows, 1,506 heifers, and 1,602 calves, taken as average for the year 2018 (**Table 1**). As a standard commercial operation, inventory records were maintained at both farms.

Drug inventory output records (drugs issued from inventory intended to be used at animals kept on the farm) from January 1st to December 31st for the year 2018 were accessed after signing an agreement permitting drug inventory data access and ensuring farm anonymity and secrecy in any form of publication. For the detailed product composition and dosage information, market, and respective product websites were visited. Data were maintained in columns as product name, active ingredients (AIs), concentration, and labeled dosage. Calculations were made for each AI, categorized regarding its class and the labeled treatment route using Microsoft Office Excel 2016. AMU was calculated in three different metrics: 1) AI, 2) antimicrobial treatment incidence (ATI), and 3) milligrams per population unit (mg/PU). Metrics 1 and 3 quantify the amount of AI used, whereas metric 2 estimates the number of treatments per day over 1,000 cows.

Active Ingredient

The total amount of AI in milligrams (mg) for each product is determined using the labeled concentration and quantity used. For products comprising more than one AI, mg were calculated separately for each ingredient. For the prodrug compositions and the concentrations given in international units (IU), AI mg were calculated using the methodology defined by World Organization for Animal Health (OIE) and European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) [European Medicine Agency (EMA)] (18, 19). For each

antimicrobial, AI values were then added up and expressed in kilograms (kg).

Antimicrobial Treatment Incidence

Total amount of AI used in terms of the number of treatments as per the defined daily dose animal (DDDA) over 1,000 cows per day is evaluated as ATI (**Equations 1 and 2**). One DDDA (mg/cow-day) is defined as the average labeled daily dose, recommended to be administered per day, in mg per kg of the animal body weight multiplied by the approximate body weight of a dairy cow taken as 600 kg (**Equation 3**) (17). For long-acting compositions, DDDA was calculated as per-day average according to the labeled duration of action (20). For products containing more than one AI, DDDA for each AI were calculated according to the labeled daily dose mentioned for the respective product. For intramammary compositions, one intramammary tube was considered as one DDDA (21, 22). As the drug inventory records at both farms were maintained for the entire on-farm herd population, the total animal biomass is adjusted against the weight of one dairy cow (23), and the total number of animals was calculated to be 3,500 termed as adjusted animal number (ANadj) (**Table 1**) (**Equation 4**). ANadj is used for the calculation of ATI for each AI against every product in the drug inventory (ATI for all AIs in a single product will be the same) (**Equations 1–4**). Individual ATIs were then added up for each antimicrobial and drug class with respect to the labeled route of treatment.

$$ATI_{DDDA/1{,}000 cow-days} = TF \times 1{,}000_{cow-days} \quad (1)$$

$$TF = \frac{Total\ amount\ of\ individual\ AI\ for\ each\ brand\ used_{mg}}{DDDA_{mg/cow-day} \times ANadj_{\ cows} \times Days\ of\ study_{days}} \quad (2)$$

$$DDDA_{mg/cow-day} = Labeled\ daily\ dose_{mg/kg} \times 600_{kg} \quad (3)$$

$$ANadj = \frac{Total\ biomass\ (Wc_{kg})}{600_{kg}} \quad (4)$$

Treatment fraction (TF) is a decimal ratio between the actual numbers of treatments and the maximum possible number of treatments within the days of study, i.e., *ANadj* × *Days of study*, also termed as animal-days at risk. TF when multiplied by 1,000 cow-days gives the number of treatments using DDDA per thousand cows in 1 day, i.e., DDA/1,000 cow-days.

Milligrams per Population Unit

Population unit (PU) is defined as the composite weight (Wc) in kg of all the animals in the study. mg/PU is the amount of AI in mg used per kg of PU. PU was considered constant throughout the study period.

$$mg/PU_{mg/kg} = \frac{AI_{mg}}{Wc_{kg}} \quad (5)$$

RESULTS

A total of 42 antimicrobial products (parenteral, intramammary, and intrauterine) were used, containing 28 different AIs (belonging to 13 antimicrobial classes) during the year 2018 (**Tables 2**, **3**, **Supplementary Table 1**). Of the 42 antimicrobial products used, 15 contained a single antimicrobial agent (five each, belonging to aminoglycosides, cephalosporins, and tetracyclines), whereas 27 were combination products (**Table 3**). In terms of ATI metric, aminoglycosides (11.05 DDDA/1,000 cow-days), penicillins (8.29 DDDA/1,000 cow-days), and tetracyclines (8.1 DDDA/1,000 cow-days) were the most frequently used antimicrobial classes, whereas sulfonamides (34.16 kg, 16.27 mg/kg), aminoglycosides (33.17 kg, 15.79 mg/kg), and tetracyclines (31.42 kg, 14.96 mg/kg) were the most highly used antimicrobial classes when measured in quantities as AI and mg/PU. Parenterally administered antimicrobials gave the highest total ATI, i.e., 23.49 DDDA/1,000 cow-days followed by intramammary, oral, and intrauterine routes with a total ATI of 21.07, 3.05, and 0.1 DDDA/1,000 cow-days, respectively (**Table 2**).

AI analysis showed that in terms of ATI, oxytetracycline (7.02 DDDA/1,000 cow-days), penicillin G (6.24 DDDA/1,000 cow-days), and cefalonium (4.27 DDDA/1,000 cow-days) were the most frequently used antimicrobial AIs. Oxytetracycline (7.02 DDDA/1,000 cow-days), gentamicin (3.34 DDDA/1,000 cow-days), and enrofloxacin (3.11 DDDA/1,000 cow-days) were the most frequent antimicrobials used via parenteral administration. ATI values for the antimicrobials cefalonium (4.27 DDDA/1,000 cow-days), penicillin G (3.21 DDDA/1,000 cow-days), and neomycin (2.56 DDDA/1,000 cow-days) used in intramammary compositions were higher than those for the parenteral compositions, i.e. 0, 3, and 0 DDDA/1,000 cow-days, respectively. In terms of the AI quantities, oxytetracycline (31.15 kg, 14.83 mg/kg), streptomycin (21.35 kg, 10.17 mg/kg), and sulfadimidine (19.99 kg, 9.52 mg/kg) were commonly used antimicrobials (**Supplementary Table 1**). A total of 138.34 kg of antimicrobials was used at the studied farms during the year 2018 with ATI and mg/PU of 47.71 DDDA/1,000 cow-days and 65.88 mg/kg, respectively (**Table 2**, **Supplementary Table 1**).

A total of 21% (6/28) of the antimicrobials used at studied farms belonged to the critically important with highest priority (CIA-HtP) category of antimicrobials for human medicine, while 32% (9/28) belonged to the high priority (CIA-HhP) category according to WHO (**Table 4**) (24). ATI quantities for CIA-HtP and CIA-HhP were 7.03 and 19.91 DDDA/1,000 cow-days, whereas quantities used were 12.24 and 46.49 kg, respectively (**Table 4**).

DISCUSSION

Globally, several initiatives have called for the prudent use of antimicrobials in food animals to prevent AMR crisis (25, 26). The Indian subcontinent (India and Pakistan) is one of the largest dairy milk-producing regions in the world (12). However, to the best of our knowledge, there is no study on quantification of AMU at the farm level from the entire region of the Indian subcontinent.

A shift toward intensive livestock farming due to an increase in animal-based protein demand in LMICs has been positively

TABLE 2 | Active ingredient (AI), treatment fraction (TF), antimicrobial treatment incidence (ATI), and milligrams of active ingredient used per kilogram of total population weight (mg/PU) of different antimicrobial classes in the study.

Antimicrobial class	Parenteral			Intramammary			Intrauterine			Oral			Total			
	AI[a]	TF[b]	ATI[c]	AI	TF	ATI	AI	TF	ATI	AI	TF	ATI	AI	TF	ATI	mg/PU[d]
Aminocoumarins	–	–	–	0.17	0.0013	1.32	–	–	–	–	–	–	0.17	0.0013	1.32	0.08
Aminoglycosides	31.55	0.0044	4.43	0.91	0.0056	5.57	0.01	0	0.03	0.7	0.001	1.02	33.17	0.0111	11.05	15.79
Aminopenicillins	4.72	0.0007	0.73	0.18	0.0019	1.88	–	–	–	–	–	–	4.9	0.0026	2.61	2.33
Aminopenicillins+β-lactam inhibitors	0.0088	–	0.0013	–	–	–	–	–	–	–	–	–	0.0088	–	0.0013	0.0042
Antifungals	0.0009	–	0.03	–	–	–	–	–	–	–	–	–	0.0009	–	0.03	0.0004
Cephalosporins	0.73	0.001	0.96	1.52	0.0049	4.9	–	–	–	–	–	–	2.25	0.0059	5.85	1.07
Fluoroquinolones	6.9	0.0037	3.72	–	–	–	–	–	–	–	–	–	6.9	0.0037	3.72	3.29
Macrolides	3.96	0.0015	1.47	–	–	–	–	–	–	–	–	–	3.96	0.0015	1.47	1.89
Penicillins	8.11	0.003	3	0.84	0.0053	5.25	0.01	–	0.03	–	–	–	8.96	0.0083	8.29	4.27
Phenicols	11.75	0.0012	1.18	–	–	–	–	–	–	–	–	–	11.75	0.0012	1.18	5.6
Polymyxins	0.65	0.0009	0.86	–	–	–	–	–	–	–	–	–	0.65	0.0009	0.86	0.31
Polypeptides	–	–	–	0.04	0.0011	1.08	–	–	–	–	–	–	0.04	0.0011	1.08	0.02
Sulfonamides	8.92	0.0001	0.08	–	–	–	0.35	–	0.03	24.89	0.002	2.03	34.16	0.0022	2.15	16.27
Tetracyclines	31.15	0.007	7.02	0.28	0.0011	1.08	–	–	–	–	–	–	31.42	0.0081	8.1	14.96
Total	108.45	0.0235	23.49	3.93	0.0211	21.07	0.37	0.0001	0.1	25.59	0.0031	3.05	138.34	0.0477	47.71	65.88

[a] *AI: the amount of active ingredient used in kilogram.*

[b] *TF: a ratio between the actual numbers of treatments and the maximum possible number of treatments and has no unit. TF values are rounded to four digits after decimal; complete values are given in* ***Supplementary Table 1****.*

[c] *ATI: the number of antimicrobial treatments per 1,000 cow–days in DDDA/1,000 cow-days.*

[d] *mg/PU: milligrams of active ingredient used per kilogram of total animal biomass in milligrams per kilogram.*

TABLE 3 | Formulations of 42 products used at the studied farms.

	AMG	AMG	AMG	AMG	AMG	AMP	CEP	FLQ	MAC	MAC	PEN	PEN	PHN	SUL	SUL	TET	
	5					2	5	3			1		3	1		5	
AMM		1[a]															**PEN**
AMG						1	1		1	1	1	1					
AMP											1						
MAC													1				
PEN			2	1											1		
POP																	
SUL															2		
TET					1												
β-L						1											
		AMG	**PEN**	**ANF**	**POP**					**POM**		**PEN**			**AMG**		

[a] *Table key: In one product containing four active ingredients, classes are mentioned in order of decreasing concentration (mg/ml), i.e., AMG, AMM, AMG, and PEN. Numbers at the intersection of more than one antimicrobial class represent products with more than one antimicrobial active ingredient.*

AMG, Aminoglycosides; AMP, Aminopenicillins; CEP, Cephalosporins; FLQ, Fluoroquinolones; MAC, Macrolides; PEN, Penicillins; PHN, Phenicols; SUL, Sulfonamides; TET, Tetracyclines; AMM, Aminocoumarins; POP, Polypeptides; β-L, β-Lactams; ANF, Antifungals; POM, Polymyxins.

linked with the excessive use of antimicrobials in food animals. In this study, we found an excessive amount of antimicrobial consumption in two of the corporate dairy farms, a growing industry in Pakistan. The total AMU of 66 mg/kg identified in this report is substantially higher than the global average of 45 mg/kg in cattle (4).

The number of on-farm treatments, quantified by the number of DDDA/1,000 cow-days, can be indicative of herd health status and the rationality of AMU. Indeed, a herd with poor health will receive more treatments and have higher ATI values than a herd with good health, and a herd with a good health status (i.e., low disease incidence rate) but relatively high ATI values suggests inappropriate metaphylactic or prophylactic AMU. ATI overestimation was checked by adjusting the weight of young stock to that of adult dairy cattle and by calculating per-day average DDDA for long-term preparations as reported previously

TABLE 4 | Total active ingredient (AI) kilogram (kg) and percentage (%), antimicrobial treatment incidence (ATI), and milligrams of active ingredient used per kilogram of total population weight (mg/PU) by WHO Critically Important Antimicrobial (CIA) for Human Medicine, sixth revision (24).

WHO CIA Category	Antimicrobials used[a]	AI (kg)	AI (%)	ATI (DDDA/1,000 cow-days)	mg/PU (mg/kg)
Critically Important Antimicrobials with Highest Priority (CIA-HtP)	Ceftiofur, cefquinome, tylosin, colistin, enrofloxacin, marbofloxacin	12.24	8.85	7.03	5.83
Critically Important Antimicrobials with High Priority (CIA-HhP)	Dihydrostreptomycin, framycetin, gentamicin, neomycin, streptomycin, amoxicillin, ampicillin, clavulanic acid, penicillin G	46.49	33.61	19.91	22.14
Highly Important Antimicrobials (HIA)	Cefalonium, cephalexin, cloxacillin, oxytetracycline, tetracycline, sulphadiazine, sulfadimidine, sulfathiazole	67.65	48.9	17.16	32.21
Important Antimicrobials (IA)	Bacitracin, florfenicol, thiamphenicol	11.79	8.52	2.26	5.61
Others	Novobiocin, methyl hydroxybenzoate	0.17	0.12	1.35	0.08

[a] ***Supplementary Table 1***.

(20, 23). However, antimicrobial overdosing or underdosing could not be accessed by ATI as no treatment records were maintained at farms and calculations were made using the labeled daily dose. In several cases, there was a discrepancy between the ATI and the AI. For example, unlike for parenteral treatments, because of low DDDA values, the ATIs of intramammary treatments were higher than the AIs (**Table 2**). Similarly, the observed number of ATIs for each AI in combination products was higher than that observed for single-ingredient products. Thereby, ATI is a measure of the number of treatments with reference to the number of cow-days, regardless of the amount of AI or DDDA used. A decimal ratio between the amount of AI used (AI) and the number of treatments per 1,000 cow-days (ATI), i.e., AI/ATI, reflects the relative value of the DDDA. A low AI/ATI ratio will indicate a low value for DDDA; in contrast, a high ratio will be suggestive of a high value for DDDA.

Total on-farm AMU (47.71 DDDA/1,000 cow-days and 65.88 mg/kg) was considerably higher than that reported from other countries, i.e., 4.2 DDDA/1,000 animal-days and 5.43 DDDA/cow/year or 14.88 DDDA/1,000 cow-days reported from Pennsylvania and Wisconsin, United States, respectively (15, 27); 14.35 DDDA/1,000 cow-days from Canada (28); 5.86 DDDA/cow/year or 16.05 DDDA/1,000 cow-days from Netherlands (23); 5.21 DDDA/LC/year or 14.27 DDDA/1,000 cow-days from Argentina (29); 20.78 DDDA/1,000 cow-days from Belgium (20); 1.27 $PrDD_{LU}/LU_{pop-risk}$/year, which would be equivalent to 3.48 DDDA/1,000 cow-days from Austria (30); 3.24 DDDvet/cow/year or 8.87 DDDA/1,000 cow-days from the United Kingdom (31); and 8.65 mg/kg from New Zealand (32).

We identified aminoglycoside and penicillin as the most commonly used antimicrobials in our study. In contrast, similar studies from European regions have shown penicillins and cephalosporins to be the most common antimicrobials used in the dairy sector (20, 23, 33–35). However, our results are in correspondence with studies from African LMICs where tetracyclines, aminoglycosides, and penicillins are the most highly used antimicrobials (36). The majority of antimicrobials were used for parenteral treatments followed by intramammary treatments, in line with the observations made in a Canadian study (28). Low ATI for intrauterine treatments is probably an underestimation as some of the parenteral antimicrobials might be used off-label for intrauterine therapies.

In this study, we observed a high intensity of AMU in the two corporate commercial dairy farms, with 91.4% of treatments consisting of critically and highly important antimicrobials for human medicine (**Table 4**). This finding highlights the fact that monitoring of AMU in commercial farming is crucial to the national efforts aiming to promote prudent use of antimicrobials and related stewardship programs (16).

The lack of internationally accepted standard methodology and of an abbreviation system for reporting AMU at the farm level hinders the quantification and comparison of data among different locations (17, 20, 29, 30, 32, 33, 37). However, the EMA has approved the number of DDDA/1,000 cow-days as a standard measure to report AMU in Europe (20). A system of high-resolution units quantifying AMU at the animal/herd-level where treatment data are available or contrariwise, along with their abbreviations, must be defined by OIE to streamline AMU data from different sources and regions of the world.

One of the main limitations of our study is that it was based on a convenient sample of farms that are not representative of the country's commercial dairy sector. Our results are therefore not generalizable to the rest of the Pakistani dairy sector. Another limitation is that, as the AMU calculations were based on inventory record data and the animal number was adjusted for a single dairy cow, AMU for each age group (calves, heifers, and cows) is not available, which may have led to the underestimation or overestimation of AMU in cows.

CONCLUSION

This is the first attempt to calculate AMU in dairy animals in Pakistan. The total AMU was considerably higher when compared to that in international studies, with a large percentage of animal use of critically important antimicrobials for human

medicine. Our baseline data will help policymakers to devise suitable antimicrobial stewardship programs for the emerging corporate commercial dairy sector in Pakistan.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MM: conceived and supervised the study. MM and MU: methodology. MN, RA, QA, JA, and MU: data collection and validation. MU and FF: data analysis and results interpretation. MM, MU, BA, MZ, and QA: manuscript writing and review. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors are grateful to the dairy producers for openly sharing their drug inventory data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.575848/full#supplementary-material

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 15 January 2021
doi: 10.3389/fvets.2020.595149

Antimicrobial Usage Factors and Resistance Profiles of Shiga Toxin-Producing *Escherichia coli* in Backyard Production Systems From Central Chile

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 15 August 2020
Accepted: 17 December 2020
Published: 15 January 2021

Citation:
Pavez-Muñoz E, González C, Fernández-Sanhueza B, Sánchez F, Escobar B, Ramos R, Fuenzalida V, Galarce N, Arriagada G, Neira V, Muñoz-Aguayo J, Flores-Figueroa C, Johnson TJ and Alegría-Morán R (2021) Antimicrobial Usage Factors and Resistance Profiles of Shiga Toxin-Producing Escherichia coli in Backyard Production Systems From Central Chile. Front. Vet. Sci. 7:595149. doi: 10.3389/fvets.2020.595149

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic pathogen and important cause of foodborne disease worldwide. Many animal species in backyard production systems (BPS) harbor STEC, systems characterized by low biosecurity and technification. No information is reported on STEC circulation, antimicrobial resistance (AMR) and potential drivers of antimicrobial usage in Chilean BPS, increasing the risk of maintenance and transmission of zoonotic pathogens and AMR generation. Thus, the aim of this study was to characterize phenotypic and genotypic AMR and to study the epidemiology of STEC isolated in BPS from Metropolitana region, Chile. A total of 85 BPS were sampled. Minimal inhibitory concentration and whole genome sequencing was assessed in 10 STEC strain isolated from BPS. All strains were cephalexin-resistant (100%, $n = 10$), and five strains were resistant to chloramphenicol (50%). The most frequent serotype was O113:H21 (40%), followed by O76:H19 (40%), O91:H14 (10%), and O130:H11 (10%). The *stx*1 type was detected in all isolated strains, while *stx*2 was only detected in two strains. The Stx subtype most frequently detected was *stx*1c (80%), followed by *stx*1a (20%), *stx*2b (10%), and *stx*2d (10%). All strains harbored chromosomal bla_{AmpC}. Principal component analysis shows that BPS size, number of cattle, pet and horse, and elevation act as driver of antimicrobial usage. Logistic multivariable regression shows that recognition of diseases in animals ($p = 0.038$; OR = 9.382; 95% CI: 1.138–77.345), neighboring poultry and/or swine BPS ($p = 0.006$; OR = 10.564; 95% CI: 1.996–55.894), visit of Veterinary Officials ($p = 0.010$; OR = 76.178; 95% CI: 2.860–2029.315) and close contact between animal species in the BPS ($p = 0.021$; OR = 9.030; 95% CI: 1.385–58.888) increase significantly the risk of antimicrobial use in BPS. This is the first evidence of STEC strains circulating in

BPS in Chile, exhibiting phenotypic AMR, representing a threat for animal and public health. Additionally, we identified factors acting as drivers for antimicrobial usage in BPS, highlighting the importance of integration of these populations into surveillance and education programs to tackle the potential development of antimicrobial resistance and therefore the risk for ecosystemic health.

Keywords: antimicrobial resistance, Shiga toxin-producing *Escherichia coli*, backyard production systems, zoonoses, one health, antimicrobial use

INTRODUCTION

Shiga toxin-producing *Escherichia coli* is considered one of the most common causes of foodborne disease worldwide, causing diarrhea with or without blood and potentially hemolytic uremic syndrome (HUS) in people (1). In the last decade, STEC has become much more prevalent in developing countries, with variations in the age distribution, geographic region and socioeconomic factors (2), which has led to its consideration as an emerging pathogen (3). Estimates indicate over 2.8 million annual acute illnesses worldwide, and up to 4,000 annual cases of HUS associated to STEC infection (4).

STEC strains are usually detected in ground beef and ready-to-eat food or drink (5), derived from domestic animals specially raised in intensive production farms (6, 7). Also, there is evidence about STEC in backyard production systems (BPS), but the information is scarce (8). BPS are considered as one of the most common forms of animal production worldwide, with particular importance in developing countries (9). These animal husbandry systems, which involve carrying out agricultural and livestock activities in a common space, constitute a part of the family farming system corresponding to a fragment of the family income source. As such, it implies that the activities and times allocated to animals breeding are conditioned by other household activities (10).

BPS are defined as small-scale production systems, not exceeding 100 animals, which are mainly poultry and pigs (11), among other species maintained (12). Their main features are the low levels of biosecurity, technological development and veterinary assistance, resulting in a close contact between humans and these animals, leading to pathogen transmission and dissemination. This could potentially increase the risk of failures in early detection of zoonotic and non-zoonotic outbreaks (11, 13–15). Ill animals from BPS are usually sold, slaughtered, and consumed, without considering the risk of zoonotic infections, increasing the risk of human infection (16). In this context, some of the most important diarrheagenic bacteria have been described in BPS throughout the world, including *Campylobacter* spp., *Salmonella enterica*, and STEC, all of which have also been associated with outbreaks in people (11, 17–21). Therefore, BPS could be an important source of pathogens to people. In this context, reports from BPS estimate STEC prevalence between 0.2 and 74% in dairy cattle (22), over 70% in sheep and goats (23), and even a 4% in captive wild birds (24), with several other reports in different animal species with close in-contact with humans (25, 26). Information about positivity to STEC in Latin America is scarce, but a report described STEC isolation in alpacas, raised under small farmer condition in Peru (25).

The identification and characterization of STEC is based in the detection of the Shiga toxins (Stx), with two types (Stx1 and Stx2), further classified into four subtypes for Stx1 (Stx1a, Stx1c, Stx1d, and Stx1e) and 12 for Stx2 (Stx2a-l) (27, 28). Nevertheless, little information is available for STEC characterization in the BPS context in Latin America, while available information worldwide reports a variable carriage of Stx virulence genes in isolates from backyard animals (8).

The extended use of antimicrobial drugs in the food animals' industries, including fish, cattle, swine and chicken, has led to an increase of the antimicrobial resistance (AMR) in zoonotic bacteria. These phenotypes may be transferred, as well as their resistance encoding genes, to humans directly by contact or throughout the food chain (29, 30). AMR in bacteria from the *Enterobacteriaceae* family is a sign of the emergence of resistant bacterial strains in the environment (31, 32), including *E. coli, Klebsiella* spp., *Proteus* spp., and *Salmonella* spp. (19, 33, 34). Additionally, significant losses in terms of morbidity and mortality have been reported due to multi-drug resistance (MDR) in bacterial infections (35, 36). Moreover, in the last 5–10 years a growing demand for "organic foods" has been reported, including animals sourced from backyard production systems (37, 38). Literature supports the importance of "organic" animal foods, particularly poultry, both in terms of food safety and its economic impact in low income populations when compared to conventional foods (10, 39). This may be due to the fact that BPS do not use antibiotics or synthetic growth promoters systematically and routinely, and its animals are fed in an open pasture system (40, 41). Additionally, the growing access to, and the use of, antimicrobials (either through prescription or non-prescription), in both people and animals, leads to an increase in multidrug resistance among several pathogens (42). Several genes have been linked to AMR in bacteria isolated from backyard animals, humans and even seafood, against tetracycline (*tet*A, *tet*B, *tet*C, and *tet*G) (43), amoxicillin, amoxicillin+clavulanic acid, ampicillin, and ceftiofur ($bla_{\mathrm{PSE-1}}$, bla_{TEM}, and bla_{CMY}) (44), among other resistance genes related to antimicrobials widely used (45). Thus, MDR STEC strains have been described as a major public health threat worldwide and in Chile (46, 47). In this context, Adesiji et al. (43) described an increase in the incidence of AMR in developing countries, related to inappropriate or uncontrolled use of these drugs in farming practices.

The aim of this study was to asses epidemiology of STEC strains isolated from animals raised in BPS from central Chile and AMR, in order to improve understanding and knowledge about these neglected animal population and their impact under a One Health approach.

MATERIALS AND METHODS

Sample Collection

A total of 85 BPS were included in this study, located in Metropolitana de Santiago region during 2019. A proportional stratified random sampling approach was used, based on the six provinces included in the study area (**Table 1**), using a random allocation of sampling points, as previously described (11). BPS farm that breed poultry and/or pigs up to a maximum of 100 birds or 50 pigs were considered in this study.

Poultry cloacal samples were collected using sterile swabs with Cary-Blair transport medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For pigs and any other animal, non-poultry, present at the BPS, rectal samples were collected under the same conditions. In a selection of BPS, based on viability, for environmental samples were collected including fresh feces, nesting material, floors of the poultry or pig, and other animal pens, using sterile swabs with Cary-Blair transport medium. All samples were labeled with the identification of the BPS and animal species, stored at 4°C and transported to the laboratory and kept refrigerated until processing.

Sample Processing

STEC Isolation and Identification

Samples were processed according to protocols previously described (7). Briefly, swabs were suspended into 9 mL tryptone soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), homogenized and incubated overnight at 42°C for enrichment. Subsequently, 25 μL of each culture were plated onto MacConkey agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates then incubated at 37°C for 18–24 h. An aliquot from the confluent area of bacterial growth was then suspended in 500 μL of sterile nuclease-free water and boiled for 15 min at 100°C. Tubes were then centrifuged at 26,480 g for 5 min at room temperature. Concentration and quality (260/280 absorbance ratio) of the obtained extracted DNA was measured in a nanodrop (NANO-400 micro-spectrophotometer, Hangzhou Allsheng Instruments Co., Hangzhou, China). Samples with an absorbance ratio closest to the optimal range (1.8–2.0) were kept at −20°C for further analyses (48). Presence of *stx*1 and/or *stx*2 genes was assessed by PCR with primer sets and reaction conditions following protocols previously described (49). As positive control, a previously characterized STEC strain was used (STEC 97) (50), and *E. coli* ATCC 25922 as negative control. PCR products (5 μL) were separated by electrophoresis on a 2% (wt/vol) agarose gel and visualized under LED light (GelDock, Maestrogen Inc., Hsinchu City, Taiwan) by SYBR® Safe DNA Gel Stain 10,000X (Thermo-Fisher Scientific, Waltham, MA, USA). Product size was determined using Accuruler 100 bp Plus DNA ladder (Maestrogen Inc., Hsinchu City, Taiwan). For each PCR positive, a maximum of 30 colonies (*E. coli* phenotype) were individually plated onto MacConkey agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates and subjected to the multiplex PCR in order to identify the colony harboring *stx* genes. If this was not possible, isolation was repeated from the confluent growing zone.

Once the colonies possessing the *stx*1 and/or *stx*2 genes were detected, they were identified as *E. coli* using the VITEK®2 system (bioMérieux) and the GN VITEK®2 card, according to the manufacturer's instructions.

TABLE 1 | Demographic distribution of BPS and sample size by province, Metropolitana region, Chile.

Region	Province	N° of BPS breeding birds	N° of BPS breeding pigs	Sample size
Metropolitana	Melipilla	1,910	202	34
	Chacabuco	426	78	13
	Santiago	244	61	10
	Cordillera	237	29	5
	Talagante	387	36	7
	Maipo	632	92	16
Total		3,836	498	85

Phenotypic Antimicrobial Resistance Characterization

Minimal inhibitory concentration (MIC) analysis were performed to characterize phenotypic antimicrobial resistance using the VITEK2 system (bioMérieux, Marcy-l'Étoile, France) and the AST-GN98 card, according to the manufacturer's instructions. Clinical cut-off values were applied according to the Clinical and Laboratory Standards Institute guidelines (51). The antimicrobials (AM) used for the analyses included aminoglycosides (amikacin and gentamicin), β-lactams (amoxicillin-clavulanic acid, ampicillin, cephalexin, cefovecin, cefpodoxime, ceftazidime, ceftiofur and imipenem), folate synthesis inhibitors (trimethoprim-sulfamethoxazole), nitrofurans (nitrofurantoin), phenicols (chloramphenicol), quinolones (ciprofloxacin, enrofloxacin, and marbofloxacin), tetracyclines (doxycycline), and also cefepime, cefotaxime, ceftazidime alone, and in combination with clavulanic acid for the detection of extended-spectrum β-lactamase (ESBL). MDR was determined if an isolated strain presented resistance to three or more antibiotics of different classes (52). Intermediate strains were classified as resistant.

Whole Genome Sequencing (WGS) and Assembly

From all isolated STEC strains, genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega), following manufacturer's instructions. Genomic DNA libraries were created using the QIAseq FX DNA library kit (QIAGEN) and MiSeq Reagent kit v3 600 cycles (Illumina), and sequencing was performed using 2 × 300-bp dual-index runs on an Illumina

MiSeq at the University of Minnesota Mid-Central Research and Outreach Center. All raw FASTQ files were trimmed and quality filtered using Trimmomatic (v0.33) (53), specifying removal of Illumina Nextera adapters, a sliding window of 4 with an average Phred quality score of 20, and 36 as the minimum read length. Trimmed reads were *de novo* assembled using the Shovill pipeline (v1.0.4), which utilizes the SPAdes assembler (54), with default parameters (https://github.com/tseemann/shovill).

In silico STEC Typing and Genotypic Antimicrobial Resistance

VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/) (55) was used to identify *stx* type and subtype genes. Molecular serotype was inferred with the SerotypeFinder 2.0 (https://cge.cbs.dtu.dk/services/SerotypeFinder/), based on the sequences of the O-antigen processing and the flagellin genes (56). Resistance genes were identified using ResFinder3.2 (https://cge.cbs.dtu.dk/services/ResFinder/) (57) and ABRicate (v.0.8.13) (https://github.com/tseemann/abricate/). These analyses were performed with a default setting of a 90% of identity threshold and 60% minimum gene length overlap, and the presence of these genes was confirmed when a coverage and identity >90% was identified.

Epidemiological Data Collection

A survey was conducted on each BPS by a semi-structured interview with BPS owners, after they consent voluntarily to be part of this study. Data was collected in relation to infrastructure, biosecurity, animal production practices, and public health.

Statistical Analysis

Descriptive statistics analysis was conducted to summarize data about antimicrobial use and about infrastructure, biosecurity and trade practices of BPS. BPSs were then classified as positive or negative for the presence of STEC.

TABLE 3 | Serotype and *Stx* type and subtype genes detected in STEC strains isolated from animals raised in backyard production systems from Metropolitana region.

Strain code	*Stx* type	*Stx* subtype	Serotype
RA-2	*stx*1	*stx*1c	O113:H21
RA-3	*stx*1	*stx*1c	O113:H21
RA-4	*stx*1	*stx*1c	O76:H19
RA-5	*stx*1	*stx*1c	O76:H19
RA-6	*stx*1	*stx*1c	O76:H19
RA-7	*stx*1/*stx2*	*stx*1a/*stx2*b	O91:H14
RA-8	*stx*1	*stx*1c	O76:H19
RA-10	*stx*1	*stx*1c	O113:H21
RA-12	*stx*1	*stx*1c	O113:H21
RA-13	*stx*1/*stx2*	*stx*1a/*stx2d*	O130:H11

TABLE 2 | MICs of selected antimicrobials against STEC strains isolated from animals raised in BPS from Metropolitana region, Chile.

Strain	RA-2	RA-3	RA-4	RA-5	RA-6	RA-7	RA-8	RA-10	RA-12	RA-13
Origin	**Sheep**	**Sheep**	**Sheep**	**Sheep**	**Sheep**	**Sheep**	**Sheep**	**Sheep**	**Sheep**	**Cow**
Antimicrobial*										
ESBL	–	–	–	–	–	–	–	–	–	–
AN	≤2	≤2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
AMC	≤2	≤2	≤2	≤2	4	≤2	≤2	≤2	≤2	≤2
AM	8	8	4	4	8	≤2	4	8	8	4
CN	8^+	8^+	16^+	16^+	16^+	8^+	16^+	8^+	8^+	8^+
CFO	≤0.5	1	≤0.5	≤0.5	1	≤0.5	≤0.5	1	1	≤0.5
CPD	≤0.25	≤0.25	0.5	0.5	0.5	≤0.25	0.5	≤0.25	≤0.25	≤0.25
CAZ	≤0.12	≤0.12	≤0.12	≤0.12	0.25	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
CFT	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
C	8^+	16^+	8^+	4	16^+	≤2	4	4	8^+	4
CIP	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
DO	1	1	1	≤0.5	1	≤0.5	1	1	1	1
ENR	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
GM	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
IPM	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
MRB	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
FT	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16
SXT	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20	≤20

**AN, Amikacin; AMC, amoxicillin-clavulanic acid; AM, ampicillin; CN, cefalexin; CFO, cefovecin; CPD, cefpodoxime; CAZ, ceftazidime; CFT, ceftiofur; C, chloramphenicol; CIP, ciprofloxacin; DO, doxycycline; ENR, enrofloxacin; GM, gentamicin; IPM, imipenem; MRB, marbofloxacin; FT, nitrofurantoin; SXT, trimethoprim-sulfamethoxazole.*
$^+$Antimicrobial resistance.

To establish the influence on AM usage of animal maintenance-related variables in BPS, elevation (meters above sea level), and surface (acres), a principal component analysis (PCA) was carried out using "deftools," "factoextra," and "ggbiplot" packages of R statistical software (58). PCA was also performed in order to determine the existence of grouping among the same variables on BPS that report different AM management intervention (AM, medicinal herbs, mixed, no intervention). Given the nature of the dependent variable (use AM or not), PCA was used as an indicator of continuous variables to include in the multivariable logistic model.

Due to the nature of the information (binary or dichotomous outcome) a logistic regression model analysis was fitted to investigate factors that determine AM use in BPS, where the response can have only two values, representing the use ($Y = 1$) or not ($Y = 0$) of antimicrobials (AM). The construction of the model was performed following previously reported methods (11), briefly, as a first step a simple logistic regression was performed in order to select the variables to be included in further analyses, including the results from PCA. Variables with a $p \leq 0.15$ in this preliminary analysis were selected for the multivariable logistic regression model. The model with the lowest log Likelihood Ratio Test (LRT) was selected for the final model (59), using a stepwise backward elimination procedure removing those variables whose regression coefficients were not significant ($p > 0.05$). The convergence of the models was set at *epsilon* (ε) $= e^{-16}$, in order to present stricter conditions for determining statistically significant factors. Non-significant variables whose elimination induced a change of 20% in the regression coefficients of the significant variables when removed, were retained in the model to adjust for confounding factors. Biologically and epidemiologically coherent interactions were evaluated (60). Goodness-of-fit was assessed using the Hosmer and Lemeshow Test (61, 62). This considering the role of the misuse or misinformed use of AM in the potential generation of AMR among the circulating pathogens in these neglected animal population.

RESULTS

Seven hundred and twelve (712) samples were collected from animals raised in BPS (63) located in the Metropolitana region, Chile. Of these, 531 (74.6%) corresponds to hens samples, followed by 55 (7.7%) duck samples, 25 (3.5%) swine samples, 20 (2.8%) goose samples, and 81 (11.4%) samples belonging to small ruminants, horses, and other domestic animals that represent <2% of the total samples each one. A total of 20 samples (2.81%) belonging to 10 BPS (11.76%) were detected positive to STEC by PCR. Positivity to STEC was detected in 9 sheep (45%), 3 dairy cattle (15%), 3 ducks (15%), 2 goats (10%), 2 hens (10%), and 1 swine (5%). No environmental samples were detected as STEC positive. From the PCR positive samples, only 13 colonies (1.83%), each from different samples, were successfully isolated and of them 10 (1.40%) proceed to MIC and WGS analysis. Samples that have a *stx* positive PCR, but no isolation was possible, the diagnosis was considered as presumptive, because other bacterial species can also carry *stx* genes.

TABLE 4 | Summary table of principal component analysis (PCA), indicating the importance of each quantitative variable of animal maintenance in BPS, elevation (meters above sea level) and surface (acres) on the use of antimicrobials, standard deviation (SD), and the percentage of explanation of variation linked to each principal component.

Quantitative variables[a]	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11
Principal component eigenvectors											
Elevation	0.17	−0.71	0.00	−0.08	0.24	−0.59	0.03	−0.18	−0.17	−0.04	0.02
Surface	−0.73	0.18	−0.43	0.22	0.03	−0.25	−0.10	0.25	−0.01	−0.11	−0.25
N° of birds	−0.45	0.16	−0.10	0.39	0.71	0.16	0.19	−0.20	0.02	0.05	0.03
N° of swine	−0.37	−0.60	0.36	−0.04	−0.04	0.02	0.50	0.33	0.12	−0.00	0.00
N° of horse	−0.52	0.10	−0.26	−0.71	−0.02	0.03	0.17	−0.30	0.11	0.07	−0.11
N° of sheep	−0.54	0.03	−0.24	0.45	−0.56	−0.14	0.17	−0.26	0.01	0.11	0.10
N° of goat	−0.62	−0.46	0.29	0.01	0.08	−0.06	−0.42	−0.01	0.35	0.07	0.07
N° of cow	−0.82	0.12	−0.21	−0.29	0.04	0.05	−0.08	0.19	−0.27	−0.07	0.24
N° of rabbit	−0.39	−0.66	0.34	0.10	−0.12	0.36	−0.13	−0.16	−0.29	−0.01	−0.15
N° of dog	0.29	−0.50	−0.70	−0.02	0.05	0.11	−0.06	0.19	−0.04	0.36	−0.01
N° of cat	0.23	−0.57	−0.65	0.05	−0.03	0.22	0.02	−0.09	0.15	−0.33	0.06
Principal component eigenvalues											
SD	1.67	1.47	1.27	1.00	0.95	0.81	0.75	0.71	0.60	0.53	0.42
% of Variance	25.35	19.60	14.56	9.17	8.25	5.91	5.12	4.59	3.25	2.58	1.60
Cumulative %	25.35	44.95	59.52	68.69	76.94	82.86	87.98	92.57	95.82	98.40	100.00

[a]*PC, Principal component; SD, Standard deviation.*

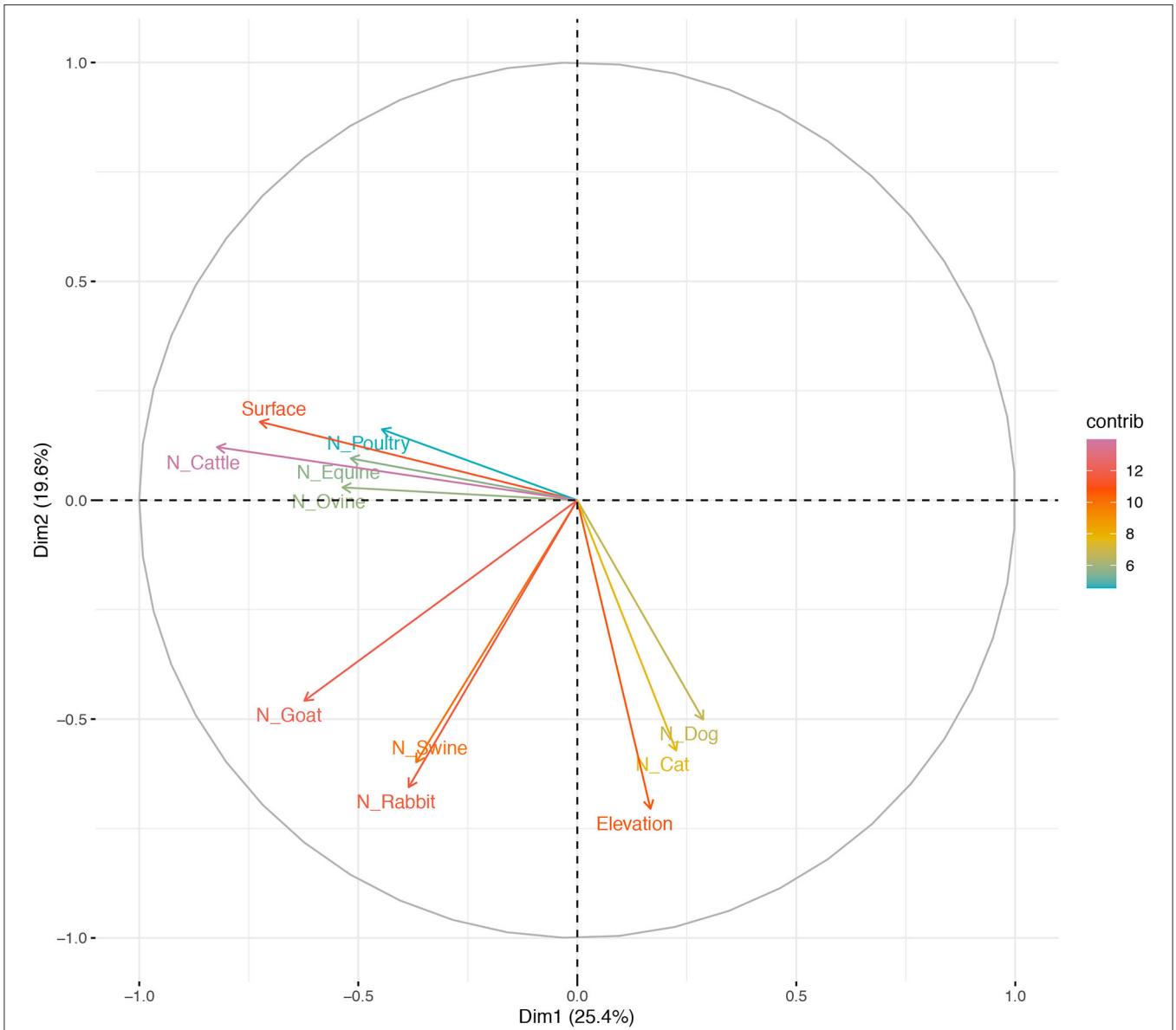

FIGURE 1 | Distribution of antimicrobial use in BPS from Metropolitana region, on the first two principal components (PC) extracted from survey response on Elevation (meters above sea level), Surface (acres), N° of Poultry, N° of Cattle, N° of Equine, N° of Ovine, N° of Goat, N° of Swine, N° of Rabbit, N° of Dog and N° of Cat maintained at BPS. Space distribution of quantitative animal and BPS-related variables according to dimensions 1 and 2 (Dim 1 and 2), plotted as their eigenvectors.

From the original positive samples, a total of 10 STEC positive samples were analyzed by MIC. Detail of AMR is summarized in **Table 2**. The STEC strains were susceptible to most of the AM included in the analysis. However, all of them were resistant to cephalexin (100%, $n = 10$) and five strains were resistant to chloramphenicol (50%).

From the 13 STEC strains, 10 were successfully sequenced and upload to Enterobase repository (https://enterobase.warwick.ac.uk/species/index/ecoli) (**Supplementary Material 1**). Whole Genome sequences has also been deposited at GenBank under the accession JAEDXK000000000 to JAEDXT000000000, BioProject PRJNA682583. Molecular serotyping detected by WGS showed the presence of non-O157 strains, predominantly O113:H21 (40%, $n = 4$), O76:H19 (40%, $n = 4$), O91:H14 (10%, $n = 1$), and O130:H11 (10%, $n = 1$) serotypes. Additionally, *stx*1 was detected in all isolated strains, *stx*2 was only detected in two strains (**Table 3**). The Stx subtype most frequently detected was *stx*1c (80%, $n = 8$), followed by *stx*1a (20%, $n = 2$), *stx*2b (10%, $n = 1$), and *stx*2d (10%, $n = 1$) (**Table 3**). Using the ABRicate tools, all strains harbored the chromosomal bla_{AmpC} (100%, $n = 10$). No other AMR encoding genes were detected.

Variance (measure by eigenvalues) for the first four components (PC1–PC4), also named dimensions (dim) were >1 and they explained around 68% of the variability found in the use of AM in BPS from Metropolitana region. These components allowed us to summarize our data into multivariate linear

FIGURE 2 | Distribution of antimicrobial use in BPS from Metropolitana region, on the first two principal components (PC) extracted from survey response on Elevation (meters above sea level), Surface (acres), N° of Poultry, N° of Cattle, N°. of Equine, N° of Ovine, N° of Goat, N° of Swine, N° of Rabbit, N° of Dog, and N° of Cat maintained at BPS. Space distribution of quantitative variables according to dimensions 1 and 2 (Dim 1 and 2), plotted as their eigenvectors and differentiated by type of intervention (AM, medicinal herbs, mixed, no intervention).

regression analyses, without losing information or minimizing such loss. In particular, the values for these components (expressed as percentages) were 25.35, 19.60, 14.56, and 9.17%, respectively. Eigenvectors (**Table 4**) from these four components confirm that some of this variable are related to antimicrobial use by the BPS owner, specifically: PC1 is dominated by the N° of cattle and surface (acres), indicating that smaller BPS and the ones with lower number of cattle tend to have more chances of using AM; PC2 is dominated by the elevation of the BPS (**Figure 1**), this means that BPS located closer to 0 meters above sea level have more chances of using AM; PC3 is dominated the number of domestic animals (dogs and cats), indicating that the presence of pets, decrease the probability of AM usage at BPS; and PC4 is dominated by the number of horse, indicating that lower number of horses increase the risk of AM usage. No evidence of significative grouping in terms of different AM management intervention in BPS was detected (**Figure 2**). Furthermore, PCA results were used to determine the inclusion of quantitative variable into the multivariable model.

Variables retained in the final multivariable logistic regression model for antimicrobial use in BPS located in Metropolitana region can be observed in **Table 5**. A total of 97 variables were collected throughout the survey. Only eight variables were retained in the final model. Among them, the recognition of diseases in animals ($p = 0.038$; OR = 9.382; 95% CI: 1.138–77.345), the maintenance of poultry and/or swine in neighboring BPS ($p = 0.006$; OR = 10.564; 95% CI: 1.996–55.894), the visit of Official Veterinary Officials ($p = 0.010$; OR = 76.178; 95% CI: 2.860–2,029.315) and the close contact between different animal species in the BPS ($p = 0.021$; OR = 9.030; 95% CI: 1.385–58.888) increase significantly the risk of antimicrobial use by BPS owners. Several non-significant variables were retained in the final model, in order to account for potential confounding factors. Interaction terms were evaluated but none of them was significant at the LRT.

DISCUSSION

Previous evidence reported the social and economic impact that BPS play in rural households, representing a risk for animal and human health by becoming a hot-spot of human-wildlife-domestic species contact (9, 10). One of the main features of BPS is low biosecurity measures or standards, and the maintenance of several animal species. Of these, small-scale poultry production is the most important, together with swine, cattle, and other small ruminants (11). These production systems have been linked to the occurrence of several zoonotic and non-zoonotic outbreaks worldwide (11, 14, 15, 64). BPS maintain a wide spectrum of species that harbor STEC, including cattle (22), sheep and goats (23, 65), and poultry and captive wild birds (11, 24). Positivity reported by this study (11.76%) is similar to what has been reported in productive animal species (cattle and swine) under industrialized conditions in Chile (7). As far as we know, this is the first report of STEC positivity in animals raised under BPS condition in Chile, detecting positivity in sheep (35%), cattle (25%), duck (15%), goat (10%), hens (10%), and swine (5%), highlighting the importance of BPS in terms of animal and public health. Serotypes reported by this study are commonly detected in animals or animal products (66, 67), and linked to animal and human diarrhea (68) and HUS, under particular conditions (69, 70). It is important to highlight that no O157 STEC strains were detected in this study, high morbidity, and mortality serotype linked to animal transmission (71). The *stx* subtype genes profile detected in this study, is consistent with that reported previously for most STEC isolates, both in animals from intensive farming systems and people (7, 72). Even so, it is important to highlight that O113:H21 have been linked to severe human diseases and HUS (73).

TABLE 5 | Results of the multivariable model for antimicrobial usage in BPS from Metropolitana region, Chile.

Variable	Classification	OR	95% CI		*p*-value
			Lower	Upper	
(Intercept)		0.006	0	0.125	0.001
Last poultry keeping	Current		Reference		
	0–1 year	0.609	0.081	4.579	0.63
	2–5 years	5.146	0.547	48.455	0.152
	> 5 years	14.345	1.279	237.054	0.063
N° of sheep		0.968	0.655	1.43	0.869
Responsible for poultry management	Family		Reference		
	Man	1.101	0.156	7.755	0.923
	Woman	0.194	0.037	0.939	0.054
Recognize diseases in animals	No		Reference		
	Yes	9.382	1.138	77.345	0.038
Veterinary/ zootechnician visit	No		Reference		
	1 per year	0.113	0.004	2.971	0.191
	more than one time per year	2.673	0.112	63.824	0.544
Neighbors keep poultry and/or swine	No		Reference		
	Yes	10.564	1.996	55.894	0.006
Visit of the Official Veterinary Service	No		Reference		
	Yes	76.178	2.86	2029.315	0.01
Animal contact in BPS	No		Reference		
	Yes	9.03	1.385	58.888	0.021

Little information is known regarding AMR of STEC strains and other enteropathogens isolated from animals raised in BPS in Latin America. Regarding phenotypic AMR in the STEC isolated strains analyzed, our results show phenotypical resistance against cephalexin in all the STEC strains isolated from animals raised in BPS, similar results to reports for cattle and swine samples under industrialized production systems in the same region of Chile (47). Even though cephalexin resistance is reported as a common feature in STEC isolates and is an antimicrobial of non-frequent use in animals or humans, non all STEC strains show this feature (74), suggesting that this resistance pattern is a threat to global health (75, 76). This could be due to the chance of exchanging resistance elements with other bacteria that share hosts with STEC or throughout other mechanisms (77). Similar resistance patterns, including β-lactamases and particularly to cephalexin, has been described for piglets, humans, free-range birds, water sources, and even STEC strains isolated from flies (78–80). Resistance to chloramphenicol was reported in five STEC strains, being different from that reported for industrialized species in Chile, where AMR was detected for a wider variety of drugs at phenotypical analysis (47). Resistance to the phenicols is mainly due to the presence of *cat* genes, encoding for chloramphenicol acetyltransferases, specific to chloramphenicol, or to the presence of *cml* genes, encoding for efflux pumps, among other mechanism, such as *nfs*B nitroreductase expression (81).

A gap in the knowledge is recognized in terms of AM usage in BPS (82, 83), leading to a potential misuse of AM in these settings. AM usage as disease preventers under BPS or similar low technification productive systems is well described (84, 85), based on the socio-economic impact of this animal housekeeping production (10). Other use reported is as growth promoters, reported in small-scale poultry production systems improving feed conversion ratios and overall productivity (63, 86), even when it has been banned in several countries, including Chile (87, 88). A lack of understanding of the public health outcomes related to BPS antimicrobial usage in this neglected population, including both animal and humans (89, 90), creates a perfect scenario for antibiotic misuse resulting in AMR generation on high impact pathogens (91). Recent reports highlights the use of AM in animal production under low and middle-income countries, a proxy to the BPS conditions, reporting that AM use was greatest in chickens, followed by swine, and dairy cattle, however, per kg of meat produced, AMU was highest in swine, followed by chickens and cattle (92), situation that could be similar under Chilean BPS conditions, if this neglected animal

population was involved actively in surveillance programs of AMR or animal health (93, 94).

The PCA analysis suggest that some of the continuous variable measures during sampling, can have implications on the decision of AM usage, among them, the number of cattle raised at the BPS shows importance on the determination of AM use, as reported widely, mainly linked to the eagerness of livestock producers to meet high demand by using AM as promoters of animal growth and disease prevention, arising AMR (95), as it has been reported for *E. coli* in calves from India, observing presence of several resistance genes for carbapenems, drugs not used in food animal treatment, hence carbapenem-resistant strains in calves could possibly by originated from the natural environment or human contact (96). It has also been described to BPS pig farmers, that presents low training on animal raising, with low knowledge on AM, engaging in several irrational AM use practices (97). Surface of the BPS, measure on acres, can be a proxy of flock size or total number of animals raised in the production systems, as previously reported (98), unit size as been reported as an element of inclusion for surveillance of AM usage in animal production from low and middle-income countries (99).

Even when no significant result was detected for the maintenance of a wide diversity of animal species in a BPS and its association with AM usage, PCA and evidence suggest an important role in the maintenance and transmission of several pathogens, particularly STEC, as all these species have been reported has of STEC and *Salmonella* spp. reservoirs in Chile and worldwide (7, 11, 100, 101). In this sense logistic multivariable model highlights the role of within-BPS animal contact increasing the risk of AM usage 9.03 times, perhaps due to an increase in the probability of becoming infected with a pathogen, potentially leading to clinical signs or a decrease in the productive indicators (102), also it can be related to the presence of several potential host and therefore reservoir for a wide number of pathogens (103, 104).

Logistic multivariable regression model, also detected significant association between recognizing diseases in animals, increasing the probability of AM usage in over 9.38 times, this could be explained in a two-way direction, either BPS owner are aware of disease and also on how to treat infected animals (105, 106) or these treatments are due to a lack of knowledge on how to deal with diseases and are linked with misuse of AM, potentially leading to the development of AMR (107, 108). Linked to this risk factor, our model detected statistically significant association of AM usage with the visit of a Veterinary Officer to BPS, establishing an increase in the probability of AM usage over 70 times, it is important to highlight that Veterinary Officers only visit BPS in the presence of an outbreak of some high impact pathogens (e.g., highly pathogenic avian influenza, PRRS) (14, 15) and only return to BPS if sample results are positive to these pathogens, under this conditions, AM usage can be increased or explained due to BPS sanitary status, but should be following the guidelines and assistance of the Veterinary Officers. Model also detected significance to a 10.56 increase in the probability of AM usage when neighbors of a BPS also maintain hens or swine, the existence of animals in the vicinity plus low biosecurity measures increase the chance of pathogen transmission (11, 109) due to free animal movements, leading to the potential use of AM (110, 111), other potential explanation to this phenomena, could be related to BPS location within family groups, working under the existence of cooperation groups or by social/cultural influence of neighbors (112, 113).

This study corresponds to the first AMR report (phenotypic and genotypic) in circulating STEC strains under backyard production systems in Chile and the first epidemiological approach to understand AM usage under this animal production conditions.

DATA AVAILABILITY STATEMENT

The data generated in this study has been deposited into BioProject (accession: PRJNA682583, JAEDXK000000000, JAEDXT000000000).

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institucional de Cuidado y Uso de Animales of the Universidad de Chile (permit code 18205-VET-UCH) for obtaining rectal samples from backyard production systems animals. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

RA-M, NG, FS, and EP-M contributed to conception and design of the study. RA-M, NG, GA, VN, and TJ contributed with resources to the study. RA-M, EP-M, CG, BF-S, FS, BE, VF, RR, NG, JM-A, CF-F, and TJ performed the laboratory analyses. RA-M performed the statistical analysis. GA and VN revised sections of the manuscript. RA-M wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) grant number 11180476.

ACKNOWLEDGMENTS

The authors acknowledge all staff from the Zoonotic Agents Epidemiology Group for their support in field activities and sample processing. The authors also want to thank Dr. Shona Jane Lee for proof-reading the manuscript and English language edition.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.595149/full#supplementary-material

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