



# **"ONE HEALTH" APPROACH FOR REVEALING RESERVOIRS AND TRANSMISSION OF ANTIMICROBIAL RESISTANCE**

EDITED BY: Ziad Daoud and Jean-Marc Rolain  
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# "ONE HEALTH" APPROACH FOR REVEALING RESERVOIRS AND TRANSMISSION OF ANTIMICROBIAL RESISTANCE

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# Table of Contents

- 06 Editorial: “One Health” Approach for Revealing Reservoirs and Transmission of Antimicrobial Resistance**  
Ziad Daoud and Jean-Marc Rolain
- 09 Seasonal Occurrence and Carbapenem Susceptibility of Bovine *Acinetobacter baumannii* in Germany**  
Peter Klotz, Paul G. Higgins, Andreas R. Schaubmar, Klaus Failing, Ursula Leidner, Harald Seifert, Sandra Scheufen, Torsten Semmler and Christa Ewers
- 20 Discovery of Novel Antibiotic Resistance Determinants in Forest and Grassland Soil Metagenomes**  
Inka Marie Willms, Aysha Kamran, Nils Frederik Aßmann, Denis Krone, Simon Henning Bolz, Fabian Fiedler and Heiko Nacke
- 31 Whole Genome Sequence Analysis Reveals Lower Diversity and Frequency of Acquired Antimicrobial Resistance (AMR) Genes in *E. coli* From Dairy Herds Compared With Human Isolates From the Same Region of Central Zambia**  
Geoffrey Mainda, Nadejda Lupolova, Linda Sikakwa, Emily Richardson, Paul R. Bessell, Sydney K. Malama, Geoffrey Kwenda, Mark P. Stevens, Barend M. deC. Bronsvort, John B. Muma and David L. Gally
- 41 The Emergence of Chromosomally Located *bla*<sub>CTX-M-55</sub> in *Salmonella* From Foodborne Animals in China**  
Chuan-Zhen Zhang, Xiao-Min Ding, Xiao-Ling Lin, Ruan-Yang Sun, Yue-Wei Lu, Run-Mao Cai, Mark A. Webber, Huan-Zhong Ding and Hong-Xia Jiang
- 49 Clonal Spread of Extended-Spectrum Cephalosporin-Resistant Enterobacteriaceae Between Companion Animals and Humans in South Korea**  
Jun Sung Hong, Wonkeun Song, Hee-Myung Park, Jae-Young Oh, Jong-Chan Chae, Saeam Shin and Seok Hoon Jeong
- 57 Effect of Single Dose of Antimicrobial Administration at Birth on Fecal Microbiota Development and Prevalence of Antimicrobial Resistance Genes in Piglets**  
Mohamed Zeineldin, Ameer Megahed, Brandi Burton, Benjamin Blair, Brian Aldridge and James F. Lowe
- 72 Dissemination of Multidrug-Resistant Commensal *Escherichia coli* in Feedlot Lambs in Southeastern Brazil**  
Katia Suemi Gozi, Juliana Rodrigues Froes, Luana Perpetua Tobias Deus Ajude, Caroline Rodrigues da Silva, Rafaela Speranza Baptista, Juliana Regina Peiró, Marcia Marinho, Luiz Claudio Nogueira Mendes, Mara Corrêa Lelles Nogueira and Tiago Casella
- 83 Whole Genome Sequencing of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Escherichia coli* Isolated From a Wastewater Treatment Plant in China**  
Xiawei Jiang, Xinjie Cui, Hao Xu, Wenhong Liu, Fangfang Tao, Tiejuan Shao, Xiaoping Pan and Beiwen Zheng



- 92 ***Bacterial Heavy-Metal and Antibiotic Resistance Genes in a Copper Tailing Dam Area in Northern China***  
Jianwen Chen, Junjian Li, Hong Zhang, Wei Shi and Yong Liu
- 104 ***Characterization of a NDM-1- Encoding Plasmid pHFK418-NDM From a Clinical Proteus mirabilis Isolate Harboring Two Novel Transposons, Tn6624 and Tn6625***  
Dandan Dong, Manli Li, Zhenzhen Liu, Jiantao Feng, Nan Jia, Hui Zhao, Baohua Zhao, Tingting Zhou, Xianglilan Zhang, Yigang Tong and Yuanqi Zhu
- 114 ***A State-of-Art Review on Multi-Drug Resistant Pathogens in Foods of Animal Origin: Risk Factors and Mitigation Strategies***  
Fernando Pérez-Rodríguez and Birce Mercanoglu Taban
- 121 ***Emergence and Characterization of a Novel IncP-6 Plasmid Harboring bla<sub>KPC-2</sub> and qnrS2 Genes in Aeromonas taiwanensis Isolates***  
Xinjun Hu, Xiao Yu, Yibing Shang, Hao Xu, Lihua Guo, Yile Liang, Yixin Kang, Li Song, Jifeng Sun, Feng Yue, Yimin Mao and Beiwen Zheng
- 128 ***Genetic Features of mcr-1 Mediated Colistin Resistance in CMY-2-Producing Escherichia coli From Romanian Poultry***  
Iuliana E. Maciucă, Max L. Cummins, Andreea P. Cozma, Cristina M. Rimbu, Eleonora Guguianu, Carmen Panzaru, Monica Licker, Edit Szekely, Mirela Flonta, Steven P. Djordjevic and Dorina Timofte
- 143 ***Emergence of Genetic Diversity and Multi-Drug Resistant Campylobacter jejuni From Wild Birds in Beijing, China***  
Juan Du, Jing Luo, Jingjing Huang, Chengmin Wang, Meng Li, Bojun Wang, Bo Wang, Han Chang, Jianwei Ji, Keya Sen and Hongxuan He
- 157 ***Petting Zoo Animals as an Emerging Reservoir of Extended-Spectrum  $\beta$ -Lactamase and AmpC-Producing Enterobacteriaceae***  
Anat Shnaiderman-Torban, Amir Steinman, Gal Meidan, Yossi Paitan, Wiessam Abu Ahmad and Shiri Navon-Venezia
- 166 ***Resistance Reservoirs and Multi-Drug Resistance of Commensal Escherichia coli From Excreta and Manure Isolated in Broiler Houses With Different Flooring Designs***  
Bussarakam Chuppava, Birgit Keller, Amr Abd El-Wahab, Christian Sürle and Christian Visscher
- 177 ***Multidrug-Resistant and Clinically Relevant Gram-Negative Bacteria are Present in German Surface Waters***  
Linda Falgenhauer, Oliver Schwengers, Judith Schmiedel, Christian Baars, Oda Lambrecht, Stefanie Heß, Thomas U. Berendonk, Jane Falgenhauer, Trinad Chakraborty and Can Imirzalioglu
- 188 ***Gene Transmission in the One Health Microbiosphere and the Channels of Antimicrobial Resistance***  
Fernando Baquero, Teresa M. Coque, José-Luis Martínez, Sonia Aracil-Gisbert and Val F. Lanza
- 202 ***Characterization and Bio-Typing of Multidrug Resistance Plasmids From Uropathogenic Escherichia coli Isolated From Clinical Setting***  
Sandip Kumar Mukherjee and Mandira Mukherjee

- 211 One Health Approach Reveals the Absence of Methicillin-Resistant *Staphylococcus aureus* in Autochthonous Cattle and Their Environments**  
Susana Correia, Vanessa Silva, Juan García-Díez, Paula Teixeira, Kevin Pimenta, José E. Pereira, Soraia Oliveira, Jaqueline Rocha, Célia M. Manaia, Gilberto Igrejas and Patrícia Poeta
- 216 Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in Patients Admitted to Kuwait Hospitals in 2016–2017**  
Samar S. Boswihi, Edet E. Udo, Bindu Mathew, Bobby Noronha, Tina Verghese and Sajida B. Tappa
- 226 Bacteria From the Multi-Contaminated Tinto River Estuary (SW, Spain) Show High Multi-Resistance to Antibiotics and Point to *Paenibacillus* spp. as Antibiotic-Resistance-Dissemination Players**  
Benedito Eduardo-Correia, Héctor Morales-Fillooy and José P. Abad
- 244 Heavy Metal Toxicity in Armed Conflicts Potentiates AMR in *A. baumannii* by Selecting for Antibiotic and Heavy Metal Co-resistance Mechanisms**  
Wael Bazzi, Antoine G. Abou Fayad, Aya Nasser, Louis-Patrick Haraoui, Omar Dewachi, Ghassan Abou-Sitta, Vinh-Kim Nguyen, Aula Abara, Nabil Karah, Hannah Landecker, Charles Knapp, Megan M. McEvoy, Muhammad H. Zaman, Paul G. Higgins and Ghassan M. Matar
- 256 Genome-Based Analysis of Extended-Spectrum  $\beta$ -Lactamase-Producing *Escherichia coli* in the Aquatic Environment and Nile Perch (*Lates niloticus*) of Lake Victoria, Tanzania**  
Zebedayo Baniga, Yaovi M. Gildas Hounmanou, Egle Kudirkiene, Lughano J. M. Kusiluka, Robinson H. Mdegela and Anders Dalsgaard
- 267 Point Deletion or Insertion in *CmeR*-Box, A2075G Substitution in 23S rRNA, and Presence of *erm(B)* Are Key Factors of Erythromycin Resistance in *Campylobacter jejuni* and *Campylobacter coli* Isolated From Central China**  
Yiluo Cheng, Wenting Zhang, Qin Lu, Guoyuan Wen, Zhongzheng Zhao, Qingping Luo, Huabin Shao and Tengfei Zhang
- 276 Characterizing Antimicrobial Resistant *Escherichia coli* and Associated Risk Factors in a Cross-Sectional Study of Pig Farms in Great Britain**  
Manal AbuOun, Heather M. O'Connor, Emma J. Stubberfield, Javier Nunez-Garcia, Ellie Sayers, Derick W. Crook, Richard P. Smith and Muna F. Anjum





# Editorial: “One Health” Approach for Revealing Reservoirs and Transmission of Antimicrobial Resistance

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## Editorial on the Research Topic

### “One Health” Approach for Revealing Reservoirs and Transmission of Antimicrobial Resistance

The goals of medicine have mostly aimed at relieving pain and suffering, promoting health and preventing diseases, as well as curing diseases when possible and providing care when cure is not achievable (Cassell, 1998). Medicine has always been focused on human beings, and this has not changed. However, what has changed, or evolved, is our understanding of the interactions existing between humans and their environments, including nature, animals, societies, and even inanimate objects. The field of Microbiology and Immunology has been pioneering in investigating and understanding how humans react and interact with their environments. Since Edward Jenner (1749–1823) discovered “the cowpox protection against smallpox” (Riedel, 2005) and Louis Pasteur (1822–1895) confirmed the “Germ theory of disease” (Williamson, 1955), scientists have made huge contributions to the field.

Over the last 40 years, Multi-Drug Resistant Gram-Negative Bacilli including Extended-Spectrum Beta-Lactamase (ESBLs), carbapenemase producers, Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococcus (VREs), as well as many other resistant organisms have been extensively reported in patients and humans (Fournier et al., 2012; Stokle et al., 2013; Creighton and Howard, 2017). In this context, data from patients is tremendous and has reached an advanced level of understanding of the molecular mechanisms behind resistance in these bacteria. Resistance has been shown to be disseminated in humans, but also animals, and the environment (Daoud et al., 2018). In many cases, microorganisms isolated from humans and animals share the same mechanisms of resistance (Baquero et al.). Many studies investigating resistance in the environment have also reported resistant bacteria in wastewaters and natural environments. More recently, resistance to colistin has been detected and reported in several hospital and community environments such as animal farms. In 2010, The Food and Agriculture Organization, World Organization for Animal Health, and World Health Organization initiated programs of collaboration for “Sharing responsibilities and coordinating global activities to address health risks at the animal-human-ecosystems interfaces<sup>1</sup>.”

The present Research Topic ““One Health” Approach for Revealing Reservoirs and Transmission of Antimicrobial Resistance” shows that understanding the epidemiology, characteristics, mechanisms, and elements of resistance in different microenvironments is imperative for the

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<sup>1</sup> [https://www.who.int/influenza/resources/documents/tripartite\\_concept\\_note\\_hanoi/en/](https://www.who.int/influenza/resources/documents/tripartite_concept_note_hanoi/en/)

successful control of the global spread of resistant bacteria and resistance genes between the three components of One Health: Humans, Environment, and Animals.

At the Environment level, the study "*Bacteria From the Multi-Contaminated Tinto River Estuary (SW, Spain) Show High Multi-Resistance to Antibiotics and Point to *Paenibacillus* spp. as Antibiotic-Resistance-Dissemination Players*" of Eduardo-Correia et al. found that *Paenibacillus* isolates share many resistance mechanisms with several other genera and suggests a role for this bacterium in the inter-genus dissemination of antibiotic resistance. For the first time, a possible hotspot of resistance interchange in a particular environment was detected. This leads to the possibility that one bacterial member of the community could be responsible for the promotion of antibiotic resistance in this environment. Chen et al. show co-occurrence of heavy metals and genes of resistance in a specific environment. The study *Bacterial Heavy-Metal and Antibiotic Resistance Genes in a Copper Tailing Dam Area in Northern China* suggests that heavy Metal Resistance genes (MRGs) and antimicrobial resistance genes (ARGs) can be co-selected in soil contaminated by heavy metals. These findings shed light on the relationship between heavy metals and antimicrobial resistance, a Research Topic where final and clear conclusions have not been yet fully described.

The spread of resistance genes in the environment, specifically in forest and grassland soil, as well as in rivers is addressed in this Research Topic in two studies. The first on the "*Discovery of Novel Antibiotic Resistance Determinants in Forest and Grassland Soil Metagenomes*" of Willms et al. reports for the first time on the presence of non-mobile dihydropteroate synthase (DHPS) genes conferring resistance to sulfonamides in forest soil with no history of exposure to these synthetic drugs. The second "*Emergence and Characterization of a Novel IncP-6 Plasmid Harboring blaKPC-2 and qnrS2 Genes in *Aeromonas taiwanensis* Isolates*" of Hu et al. detected blaKPC-2 and qnrS2 genes on a non-conjugative plasmid in three carbapenem resistant isolates of *Aeromonas taiwanensis* from river sediments samples. These studies conclude that the spread of genes of resistance and resistant organisms in natural environments form a close monitoring system to control the occurrence and spread of such MDR mechanisms.

In parallel, the results of another study assessing the occurrence of ARGs in Waste Water Treatment Plants (WWTPs) "*Whole Genome Sequencing of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Escherichia coli* Isolated From a Wastewater Treatment Plant in China*" of Jiang et al. are in full agreement with the previous two studies. The Whole Genome Sequencing results suggest that pharmaceutical WWTP can play a significant role in the emergence of ARGs and suggest the need to strengthen active surveillance of ARB and ARGs from the pharmaceutical industry.

Studies exploring resistance in hospitals and humans are countless, fewer are those exploring animals and nature. More importantly, in view of the geographic and cultural changes imposed by war migrations, an in-depth assessment of the interlinkages existing between humans, animals, and environments is needed. The large use of animal products in

human agriculture, the carriage of resistant bacteria, and the spread of antimicrobial resistance in the environment is posing a potential risk for transmitting resistance from poultry and poultry products to the human population. Descriptive studies of prevalence and surveillance of resistant bacteria, identification of new/undescribed reservoirs and mechanisms of resistance, channels of transmission, and subsequent alterations of the flora, are also instrumental in our battle against bacterial resistance.

In addition to the environment, animals constitute the second determinant of One Health. In this context, the use of antibiotics in animal farms is a major issue that needs more attention. A comparative molecular characterization of Enterobacteriaceae carrying Extended-spectrum  $\beta$ -lactamase (ESBL) and AmpC, isolated from human stool samples, rectal swabs from companion animals, and swabs from the environment of veterinarian hospitals in South Korea was performed by Hong et al. The results showed that while in animals blaCMY-2-like, blaCTX-M-55, and blaCTX-M-14 (16.1%) were the most commonly found genes of resistance, blaCTX-M-15 was predominant in human samples. The pulsotypes of *E. coli* isolates from dogs and humans showed more than 85% similarity, suggesting a direct transmission and dissemination of extended spectrum cephalosporin-resistant Enterobacteriaceae between humans and companion animals. Similarly, a study performed on patients hospitalized in Kuwait "*Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in Patients Admitted to Kuwait Hospitals in 2016–2017*" of Boswihi et al. identified four Livestock Associated MRSA clones with CC97-MRSA-V [fusC+] as the dominant one, and suggested an independent acquisition from different sources. Another research work from Beijing-China reports the detection of Multi-Drug Resistant *Campylobacter jejuni* from the wild birds "silver pheasant," which might lead to potential public health threats as vectors of this organism.

Many studies in this Research Topic addressed resistance in animal farms. According to AbuOun et al., animals carrying resistance at the start of the fattening period can be a reservoir and the starting point for the transmission of bacterial resistance to the other animals in the same flock. On the other hand, the study "*Characterizing Antimicrobial Resistant *Escherichia coli* and Associated Risk Factors in a Cross-Sectional Study of Pig Farms in Great Britain*" reports that *E. coli* isolates from pig farms were "phylogenetically diverse harboring a variety of AMR profiles with widespread resistance to old antibiotics with no concurrent resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides."

Interestingly, Schnaiderman-Torban et al. found a significant correlation between petting zoos and the spread of antibiotics-resistant ESBL/AmpC-producing bacteria, including highly virulent, disease-associated MDR *E. coli* strains. This highlights the importance of having infection control guidelines for such environments where individuals, children, and families interact directly and indirectly with the animals.

Gozi et al. conclude that feedlot lambs act as reservoirs of commensal multidrug-resistant *E. coli*, and antibiotic resistance can reach humans through the food chain. Their study "*Dissemination of Multidrug-Resistant Commensal *Escherichia coli* in Feedlot Lambs in Southeastern Brazil*" constitutes the first



report of a so broad characterization of antimicrobial resistant *E. coli* isolated from sheep.

The Research Topic of antimicrobial use in preventive/curative treatment in farms can have several side effects and can lead to collateral damage. The study by Zeineldin et al. evaluated the impacts of early-life antimicrobial intervention on fecal microbiota development, and the prevalence of selected ARGs in neonatal piglets. Their results demonstrate that the shifts in fecal microbiota structure caused by perinatal antimicrobial intervention are modest and limited to groups of microbial taxa. Maciuca et al. come up with concurring results showing that high prevalence of mcr-1 plasmid mediated colistin resistance in commensal AmpC producing *Escherichia coli* from poultry suggests the selection of these isolates by prophylactic and/or therapeutic farm use of colistin and/or

cephalosporins. In this same context, our correspondence to The Lancet Infectious Diseases (Olaitan et al., 2021) concluded that colistin use in farms is leading to a spread of colistin resistance, and suggested that a ban of colistin use as a feed additive for growth promotion would slow or "possibly stop" the spread of plasmid-mediated colistin resistance.

Studies exploring the discovery of new antibacterial molecules are highly needed for treating human and animal infections. In addition, studies involving new means of breaking the cycle of transmission of resistance, are highly encouraged.

## 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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# Seasonal Occurrence and Carbapenem Susceptibility of Bovine *Acinetobacter baumannii* in Germany

Peter Klotz<sup>1\*</sup>, Paul G. Higgins<sup>2,3</sup>, Andreas R. Schaubmar<sup>4</sup>, Klaus Failing<sup>4</sup>, Ursula Leidner<sup>1</sup>, Harald Seifert<sup>2,3</sup>, Sandra Scheufen<sup>1,5</sup>, Torsten Semmler<sup>6</sup> and Christa Ewers<sup>1</sup>

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*Acinetobacter baumannii* is one of the leading causes of nosocomial infections in humans. To investigate its prevalence, distribution of sequence types (STs), and antimicrobial resistance in cattle, we sampled 422 cattle, including 280 dairy cows, 59 beef cattle, and 83 calves over a 14-month period. Metadata, such as the previous use of antimicrobial agents and feeding, were collected to identify putative determining factors. Bacterial isolates were identified via MALDI-TOF/MS and PCR, antimicrobial susceptibility was evaluated via VITEK2 and antibiotic gradient tests, resistance genes were identified by PCR. Overall, 15.6% of the cattle harbored *A. baumannii*, predominantly in the nose (60.3% of the *A. baumannii* isolates). It was more frequent in dairy cows (21.1%) than in beef cattle (6.8%) and calves (2.4%). A seasonal occurrence was shown with a peak between May and August. The rate of occurrence of *A. baumannii* was correlated with a history of use of 3rd generation cephalosporins in the last 6 months prior to sampling. Multilocus sequence typing (Pasteur scheme) revealed 83 STs among 126 unique isolates. Nine of the bovine STs have previously been implicated in human infections. Besides known intrinsic resistance of the species, the isolates did not show additional resistance to the antimicrobial substances tested, including carbapenems. Our data suggest that cattle are not a reservoir for nosocomial *A. baumannii* but carry a highly diverse population of this species. Nevertheless, some STs seem to be able to colonize both cattle and humans.

**Keywords:** ESKAPE, *Acinetobacter baumannii*, antimicrobial susceptibility, MLST, cattle, epidemiology

## INTRODUCTION

*Acinetobacter* is widespread in the microbiota of animals, plants and the environment (Doughari et al., 2011). Several species are able to cause opportunistic, mainly hospital-acquired infections. In contrast to many other *Acinetobacter* species, *Acinetobacter baumannii* is mainly associated with clinical environments and hospital outbreaks (Towner, 2009). Nevertheless, the bacterium

**Abbreviations:** FS, pen-floor fecal sample; NS, nasal swab; RS, rectal swab.



is sporadically found in samples of cattle (Hamouda et al., 2008, 2011; Nam et al., 2009, 2010; Al Bayssari et al., 2015; Rafei et al., 2015; Fernando et al., 2016) and even carbapenem-resistant strains have been reported (Al Bayssari et al., 2015; Pailhoriès et al., 2016; Webb et al., 2016). The increase of carbapenem-resistant *A. baumannii* isolates implicated in human infections causes a serious limitation of treatment options and has been associated with high mortality rates (Falagas and Rafailidis, 2007; Perez et al., 2007). In *Acinetobacter* spp., carbapenem-resistance is mostly mediated by acquired class D beta-lactamases, so called oxacillinases (mainly OXA-23, OXA-40, and OXA-58). A further mechanism is the insertion of a genetic element (e.g., IS*Aba1*) upstream of intrinsic resistance genes, such as the  $\beta$ -lactamase gene *bla*<sub>OXA-51-like</sub> which results in its overexpression (Turton et al., 2006). *A. baumannii* strains involved in nosocomial infections frequently belong to certain clonal groups. Eight different so called international clones (ICs) have been identified worldwide, among which the most important groups are IC1 and IC2 corresponding to the multilocus sequence types ST1 and ST2 of the Pasteur scheme (Higgins et al., 2010a). In addition to these major clones important further groups are responsible for mostly regionally distributed outbreaks, e.g., IC7 (corresponding to ST25). Some clonal complexes have not yet been linked to certain ICs although they are also responsible for nosocomial infections and outbreaks, e.g., CC32 which does not belong to IC1-8 (Da Silva et al., 2014; Sahl et al., 2015). *A. baumannii* has also been associated with infections in hospitalized cats, dogs and horses (Endimiani et al., 2011; Zordan, 2011; Smet et al., 2012; Belmonte et al., 2014; Pomba et al., 2014; Ewers et al., 2017) and recent studies have even described the emergence of carbapenem-resistant *A. baumannii* isolates belonging to ST1 and ST2 in companion animals (Pomba et al., 2014; Ewers et al., 2016, 2017). According to the few recent reports, mostly novel STs have been identified among bovine *A. baumannii* isolates, while strains of the dominant clonal lineages were only rarely identified (Lupo et al., 2014; Al Bayssari et al., 2015; Rafei et al., 2015). In order to elucidate the occurrence of *A. baumannii* in livestock, we performed an explorative representative study in the federal state of Hesse, Germany, including the collection of metadata concerning the animals and farms. By that we were able to determine the prevalence of *A. baumannii* in cattle in Hesse and could provide an insight into the phylogenetic diversity of the isolates and the antimicrobial resistance features of bovine *A. baumannii* in German cattle. The analysis of metadata allowed us to identify factors influencing the prevalence of *A. baumannii* and provides important insights for future investigations concerning the prevalence and origin of *A. baumannii*.

## MATERIALS AND METHODS

### Study Design

We designed our study population based on the total number of 467,787 animals obligatory registered in the national database for

cattle<sup>1</sup> in Hesse in October 2014. The cattle population in Hesse consists of various breeds, whereof a minority is usually held extensively (Salers, White Park, Scottish Highland, Welsh-Black, Galloway, Belted Galloway, Luing, Small Zebu, White Galloway, Longhorn, Brahman, Heck cattle, Beefalo, Water Buffalo, Bison, European Bison, other crossbreds, and other taurine cattle). Due to a high level of time and effort for sampling these animals, we excluded them from our study. To determine a representative sample size for the Hessian cattle population, the 383,870 animals of usually non-extensively held breeds were then defined as the sampling population. We did not define further exclusion criteria. Taking into account retrospective data from the microbiological diagnostics unit of our institute (P. Klotz, E. Prenger-Berninghoff, S. Scheufen, and C. Ewers, unpublished data), we estimated a response distribution for the occurrence of *A. baumannii* of 1%. Accordingly, a sample size of 380 animals was calculated (confidence level: 95%, margin of error: 1%). Stratification of the sample was done by using the categories “dairy” (female individuals of dairy breeds >7 months), “beef” (male individuals of beef cattle breeds >7 months) and “calf” (male and female individuals <8 months). Furthermore, stratification of the random sample was done according to the number of registered animals in the respective 22 districts (**Supplementary Table S1**). All animals of the study population were listed by their stratification criteria, and random numbers were assigned to each individual. By sorting the animals according to their random numbers, the animals and their corresponding farms were selected, beginning with the smallest random individual number. Due to matters of time and availability, the individual animals were then conveniently selected by the sampler at the farm. The number of animals on each farm was dependent on the original random list.

### Bacterial Strains, Species Identification

From January 2015 to February 2016 NS and RS from cattle ( $n = 422$ ) as well as FS from the corresponding stables ( $n = 353$ ) were collected in Hesse, Germany (**Supplementary Table S4**). The FSs were collected at five locations in the stable to increase the chance of finding isolates. The number of 422 animals includes 42 additional samples (28 dairy cows, 9 beef cattle, and 10 calves) to the determined sample size ( $n = 380$ ) due to variable sampling conditions on different farms. The samples were cultured on blood agar (blood agar base by Merck Chemicals, GmbH, Darmstadt, supplemented with 5% sheep blood), Water-blue Metachrome-yellow Lactose Agar (Oxoid, Wesel, Germany), and MacConkey Agar (Oxoid, Wesel, Germany) containing 1 mg/L cefotaxime (Sigma-Aldrich/Merck, Darmstadt, Germany). Colonies morphologically similar to *A. baumannii* were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS; Bruker Daltonics, Bremen, Germany, Database V4.0.0.1). Score values >2.000 were accepted for species identification. The MALDI-TOF/MS results were verified by multiplex PCR targeting different portions of the *gyrB* gene (Higgins et al., 2010b).

<sup>1</sup>www.hi-tier.de

## Metadata and Statistical Methods

Metadata concerning the animals and farms were collected and assessed via a questionnaire (Supplementary Table S2). Among other things, questions addressed animal age, sex and breed, farm size, animal feeding, use of sewage sludge as fertilizer, and use of antimicrobials previous to the sampling of animals. For statistical analyses, putative determining factors were identified (Supplementary Table S3). To assess the association of these factors and the prevalence of *A. baumannii* (animals positive either in nasal or RS), a logistic regression model analysis using the generalized linear model (glm) family was performed in two steps. First, due to the high number of variables, the putative determining factors were included separately in the model (single-factor analysis) to evaluate raw associations with the prevalence of *A. baumannii*. Secondly, variables with more than 380 observations (90% of 422 possible observations) and statistical relevant values in the single-factor model ( $p \leq 0.05$ ) were included in a multiple logistic regression model together. Factors closely associated to the variable category were excluded from the multiple model. As the prevalence of *A. baumannii* was significantly higher in dairy cows, we also performed single-factor and multiple logistic regression analysis exclusively on this group. Criteria for inclusion of variables into the multiple model were a minimum of 252 observations (90% of 280 possible observations) and statistical relevant values ( $p \leq 0.05$ ) in the single-factor regression analysis. In the same manner we analyzed the occurrence of *A. baumannii* in FSs. Here, variables with more than 318 observations (90% of 353 possible observations) were included to the multiple model. Comparison of the prevalence of *A. baumannii* in different sample locations was conducted via frequency tables and Pearson's chi-squared test or the Fisher's exact test for count data. The statistical analyses were done by means of the statistical program package R (Free Software Foundation's GNU project, official homepage<sup>2</sup>).

## Screening for Carbapenem Non-susceptible Strains, Determination of Minimum Inhibitory Concentrations (MICs)

Screening for carbapenem non-susceptible *A. baumannii* was done by streaking the isolates on Mueller-Hinton agar plates (Oxoid, Wesel, Germany) containing meropenem (Sigma-Aldrich/Merck, Darmstadt, Germany) at different concentrations (2 mg/L and 4 mg/L). Minimum inhibitory concentrations (MICs) were determined by using the VITEK2 system and antimicrobial susceptibility testing card AST-GN38 (bioMérieux, Nürtingen, Germany). Imipenem MICs for *A. baumannii* isolates that showed growth on Mueller-Hinton agar containing meropenem were additionally evaluated by using antibiotic gradient agar diffusion method (Liofilchem, Roseto degli Abruzzi, Italy). MICs were interpreted according to CLSI breakpoints defined for human *Acinetobacter* spp. (CLSI, 2014) with exception of nitrofurantoin (breakpoints for Enterobacteriaceae, CLSI), cefpodoxime and ceftiofur

(breakpoints for ceftazidime, CLSI), ceftiofur (breakpoints for ceftiofur, CLSI), enrofloxacin and marbofloxacin (breakpoints for ciprofloxacin, CLSI). Intrinsic resistance was assumed according to the definitions described in the EUCAST and CLSI guidelines (CLSI, 2014; EUCAST, 2018).

## Analysis of Antimicrobial Resistance Genes

All isolates were screened for carbapenemase genes *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-40</sub>, *bla*<sub>OXA-58</sub> via PCR (Gröbner et al., 2009). PCRs targeting the *ISAbal* region upstream of intrinsic *bla*<sub>OXA-51</sub>-like genes (Turton et al., 2006) and *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub> (Gröbner et al., 2009), *bla*<sub>OXA-48</sub> (Poirel et al., 2004), *bla*<sub>NDM</sub> (Pfeifer et al., 2011), *bla*<sub>IMP</sub>, *bla*<sub>GIM</sub>, *bla*<sub>SIM</sub>, *bla*<sub>SPM</sub> (Mendes et al., 2007), and *bla*<sub>GES</sub>

**TABLE 1A** | Difference and dependency of the prevalence of *Acinetobacter baumannii* in nose swabs vs. rectal swabs.

	Negative NS	Positive NS	Sum
Negative RS	357 (84.6%)	60 (14.2%)	417 (98.8%)
Positive RS	1 (0.2%)	4 (0.9%)	5 (1.2%)
Sum	358 (84.8%)	64 (15.2%)	422 (100%)

$\chi^2$ -squared = 11.8252, df = 1, p-value = 0.00058.

Fisher's Exact Test for Count Data: p-value = 0.00216.

McNemar's chi-squared = 55.1475, df = 1, p-value = 0.0001.

NS, nasal swabs (n = 422); RS, rectal swabs (n = 422); FS, Pen-floor fecal sample (n = 353).

Only one FS was taken per farm so in some cases several NS and RS confer to the same FS.

**TABLE 1B** | Difference and dependency of the prevalence of *A. baumannii* in nose swabs vs. pen-floor fecal samples.

	Negative NS	Positive NS	Sum
Negative FS	341 (80.8%)	42 (10%)	383 (90.8%)
Positive FS	17 (4%)	22 (5.2%)	39 (9.2%)
Sum	358 (84.8%)	64 (15.2%)	422 (100%)

$\chi^2$ -squared = 53.3386, df = 1, p-value = 0.0001.

McNemar's chi-squared = 9.7627, df = 1, p-value = 0.00178.

NS, nasal swabs (n = 422); RS, rectal swabs (n = 422); FS, pen-floor fecal sample (n = 353).

Only one FS was taken per farm so in some cases several NS and RS confer to the same FS.

**TABLE 1C** | Difference and dependency of the prevalence of *A. baumannii* in rectal swabs vs. pen-floor fecal samples.

	Negative RS	Positive RS	Sum
Negative FS	381 (90.3%)	2 (0.5%)	383 (90.8%)
Positive FS	36 (8.5%)	3 (0.7%)	39 (9.2%)
Sum	417 (98.8%)	5 (1.2%)	422 (100%)

$\chi^2$ -squared = 10.0217, df = 1, p-value = 0.00155.

Fisher's Exact Test for Count Data: p-value = 0.00643.

McNemar's chi-squared = 28.6579, df = 1, p-value = 0.0001.

NS, nasal swabs (n = 422); RS, rectal swabs (n = 422); FS, pen-floor fecal sample (n = 353).

Only one FS was taken per farm so in some cases several NS and RS confer to the same FS.

<sup>2</sup><http://www.r-project.org>

(Rieber et al., 2017) were performed on isolates which showed growth on Mueller-Hinton agar containing meropenem, which was used to screen for putative carbapenem-resistant strains.

## Clonal Analysis, Assignment of International Clones and Multilocus Sequence Typing

To identify copy strains among isolates originating from the same farm, the same animal or the same sample location, pulsed-field gel electrophoresis (PFGE) of *Apal*-restricted whole genomic DNA was performed (Seifert et al., 2005). International clones I-III were identified via PCR (Turton et al., 2007). Multilocus sequence typing (MLST) was performed according to the Pasteur scheme (Diancourt et al., 2010). A minimum spanning tree based on MLST allele profiles was created with Bionumerics version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). eBurst v3 was used to assess clonal groups based on allele sequence profiles of 1161 currently available STs<sup>3</sup> (date of accession: 12-05-2018).

## RESULTS

### Prevalence of *A. baumannii* in 422 Cattle

From 1197 samples (844 animal samples and 353 FSs), we obtained 144 *A. baumannii* isolates. As PFGE analysis identified 18 of these isolates as copy strains (identical PFGE pattern – a maximum of three strains per animal), further analysis was performed with 126 “unique” strains. The overall prevalence of *A. baumannii* in 422 cattle was 15.6% (CI: 95%, 12.3–19.5%). The rate of false positive *A. baumannii* identification with MALDI-TOF was 2.3% (two *A. nosocomialis*, one *A. calcoaceticus*). We identified considerable differences in the prevalence of *A. baumannii* with respect to the sample location (*p*-values of <0.0001 to 0.0010, **Table 1**). The highest prevalence was determined for NS (15.2%), followed by FSs (FS: 9.2%) and RSs (RS 1.2%). The occurrence in each sample location was dependent on the occurrence in all other sample locations (*p*-values of <0.0001 to 0.0064, **Table 1**). The frequency of *A. baumannii* was highest in the category “dairy cows” followed by “beef cattle” and “calves” (21.1, 6.8, and 2.4%, *p*-value: <0.0001). The three animal categories also differed in further aspects, e.g., the systemic use of 3rd generation cephalosporins in a 6 months period prior to sampling on the farm (23% in dairy cattle, 4% in beef cattle, and 21% in calves) and naturally, the age of animals (means of 58 months in dairy cattle, 19 months in beef cattle and 2 months in calves). The prevalence also differed depending on the season of sampling but not for the different geographical regions. The prevalence was highest in the 2nd trimester of the year (May–August, 44.3%), followed by the 3rd trimester (September–December, 15.3%) and the 1st trimester (January–April, 5.1%, **Figure 1**). At the farm level, 45 of 353 farms were positive for *A. baumannii* in the FS (12.8%), again with the highest prevalence in the 2nd trimester

(26.2%) followed by the 3rd and the 1st trimester (12.3 and 2.5%, respectively).

### Analysis of Putative Determining Factors Prevalence in Cattle (All Categories Included)

The single-factor logistic regression analysis revealed nine factors that may have an influence on the occurrence of *A. baumannii* in the animals (**Table 2**). However, for only three of these factors significant values remained in the multiple logistic regression model (*n* = 387), namely systemic use of 3rd generation cephalosporins in a 6 months period prior to sampling on farm level (*p*-value: 0.0069, OR = 2.6), sampling trimester (May–August: *p*-value: <0.0001, OR = 18; September–December: *p*-value: 0.0168, OR = 2.9), and the category (calf: *p*-value: 0.0012, OR = 0.08).

### Occurrence in Dairy Cows

In dairy cows, the single-factor logistic regression analysis revealed five putative determining factors (**Table 2**). Out of these factors the season of sampling (May–August: *p*-value < 0.0001, OR = 22; September–December: *p*-value: 0.0130, OR = 3.7) and the use of 3rd generation cephalosporins in a 6 months period prior to sampling (*p*-value: 0.0071, OR = 2.8) were consistently significant in the multiple model (*n* = 245).

### Occurrence in Pen-Floor Fecal Sample

The single-factor logistic regression analysis revealed four factors that may have an influence on the occurrence of *A. baumannii* in the FSs (**Table 2**). In the multiple model (*n* = 306) only the trimester of sampling was significant (2nd Trimester May–August; *p*-value: 0.0013; OR = 7.2).

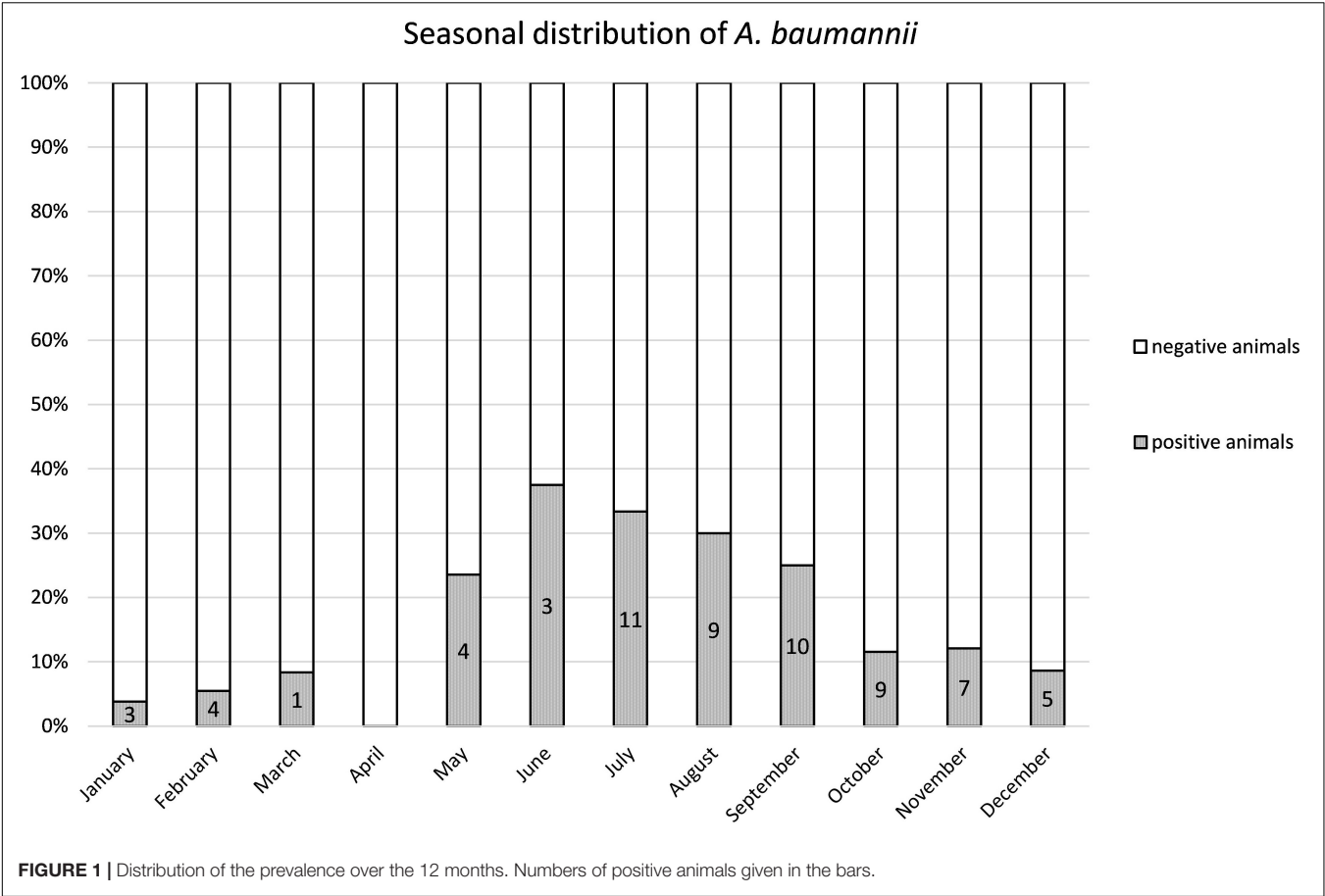
### Antimicrobial Susceptibility and Resistance Genes

All 126 *A. baumannii* isolates showed resistance against ampicillin, amoxicillin-clavulanic acid, cefalexin, ceftiofur, nitrofurantoin and chloramphenicol. Intermediate resistance was determined for piperacillin (6%) and rifampicin (25%). All isolates were susceptible to aminoglycosides, fluoroquinolones, polymyxin B and carbapenems (**Supplementary Table S5**). According to PCR analysis, neither an acquired carbapenemase gene nor insertion sequences upstream of *bla*<sub>OXA-51</sub> were present among the isolates.

### Phylogenetic Analysis

According to PCR analyses, 19 *A. baumannii* isolates (15%) belonged to IC2 and 16 isolates (13%) to IC3. The remaining 91 isolates were non-IC1-3 strains. The isolates did not cluster in any clonal complex (CC) that has previously been associated with IC1 to IC8 according to MLST analysis. We identified 83 different STs whereof 67 were newly described, like the most frequently identified ST1027 which forms a new CC together with its single locus variants (SLVs) ST1026, ST1033, and ST1070 (all newly described). Nine STs, including ST155, ST80, ST504, and ST690 have been previously described for human clinical *A. baumannii* strains and these STs are indicated in red in **Figure 2**. According to eBurst analysis, the majority

<sup>3</sup><http://pubmlst.org>



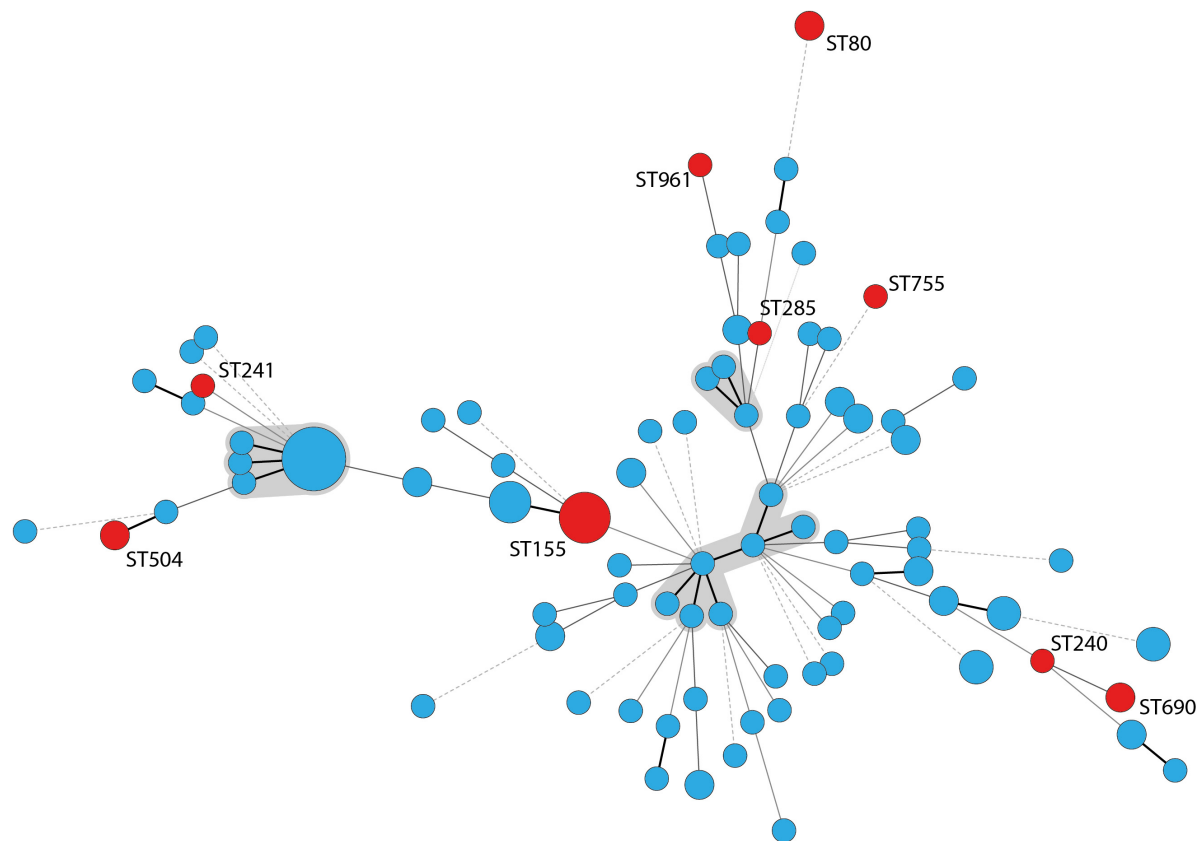
of the bovine *A. baumannii* strains did not form CCs, but mostly appeared as singletons (**Supplementary Figure S1**). However, some bovine STs grouped together with STs recently determined for strains isolated from human specimen. For example, IHIT31974 and IHIT32879 (ST690) that were isolated from the nose of two cattle on the same farm are SLVs of ST402, which belongs to IC7 and CC25, a clonal group that has recently been linked with hospital-associated infections in humans (Sahl et al., 2015). The disperse grouping distant from the important clinical STs 1, 2, and 25 is also demonstrated by an alignment of the concated sequences of the MLST alleles (**Supplementary Figure S2**).

**TABLE 2 |** Results of the single-factor logistic regression for the occurrence of *A. baumannii*.

Putative determining factor	All categories		Dairy cows		Pen-floor fecal samples	
	p-value	nObs	p-value	nObs	p-value	nObs
Age of the animal	0.0002	422	n.s.			
Category (dairy/beef/calf)	0.0003	422				
Feeding of corn silage	0.00705	367	0.0270	266		
Feeding of straw	0.0215	105	n.s.			
Local use of 1st CEPH on farm level	n.s.		n.s.		0.0458	330
Sex of the animal	0.0028	422	n.s.			
Systemic use of 3rd CEPH on farm level	0.0010	387	0.0040	255	0.0372	319
Systemic use of florfenicol on farm level	n.s.		0.0500	255	n.s.	
Systemic use of penicillin on farm level	0.0369	387	n.s.		n.s.	
Trimester	<0.0001	422	<0.0001	280	0.0004	353
Use of sewage sludge	<0.0001	263	0.0013	169	0.0173	210

nObs, number of observations; 3rd CEPH, 3rd generation cephalosporins; 1st CEPH, 1st generation cephalosporins; n.s., not significant.





**FIGURE 2 |** Minimum spanning tree of 126 *A. baumannii* cattle field isolates. STs that have been previously found in human samples are labeled with the ST number and circles indicating the STs are marked in red; clonal complexes are shaded gray; thick lines: single locus variant, thin lines: double locus variant, dashed line: multiple locus variants.

## DISCUSSION

### Prevalence of *A. baumannii* in Hessian Cattle

The aim of the present study was to assess the prevalence of *A. baumannii* in the German state of Hesse. Only a few studies investigated the prevalence of *A. baumannii* or other *Acinetobacter* spp. in cattle before. These are only to a limited extend comparable to our study. Either sample material was restricted to fecal samples and selective media was used for initial screening, resulting in a very low recovery rate compared to our study of 0.6% (Webb et al., 2016), or information needed to determine the prevalence of *A. baumannii* in the respective species and sample locations is not provided (Hamouda et al., 2011). One study reports a very high isolation rate of 83% in mouth swabs from cattle from the Reunion Islands (Pailhoriès et al., 2016), but it only included six animals on a single farm. Probably this is not representative for a larger population of cattle in this region. A frequency similar to our results (isolation rates up to 44.3% in the second trimester) is reported by Wilharm et al. (2017) in stork samples collected in Poland with an isolation rate of 25% and local rates up to 48%.

### Prevalence in Different Categories and Different Sample Locations

With dairy cows, beef cattle, and calves, we included three different categories of cattle which differ in relevant aspects, such as feeding, lifetime, and antimicrobial treatment, probably influencing the frequency of *A. baumannii*. The highest prevalence of *A. baumannii* was observed in dairy cows (21.1%). The longer life span of dairy cows compared to beef cattle and calves, combined with a selection pressure due to use of antimicrobials (e.g., 3rd generation cephalosporins) could lead to a higher chance for dairy cows to acquire *A. baumannii* and to establish a stable colonization in the nose or gut. We isolated *A. baumannii* mainly from the nose, which could suggest that the bacterium has a tropism to the nasal mucosa. Consistent with this, *A. baumannii* has been isolated from human nasal samples with rates from 54 to 92% in long term facilities (Liou et al., 2017). However, it has been shown in another study that the colonization of the human digestive tract was also high (41%) in a nosocomial context (Corbella et al., 1996). Few studies investigated the occurrence of the bacterium in skin, nose, and fecal swabs from healthy humans and reported recovery rates of 0–10.4% (Berlau et al., 1999; Patil and Chopade, 2001; Dijkshoorn et al., 2005; Griffith et al., 2006). The high

prevalence of *A. baumannii* in the cattle nose may be due to repeated acquisition of the bacterium probably by uptake or inhaling of feed particles or surrounding air contaminated with *A. baumannii*. Indeed, airborne transmission has been shown for *A. baumannii* in hospitals (Shamsizadeh et al., 2017; Solomon et al., 2017), and contamination of air samples from animal stables with *Acinetobacter* spp. has been reported (Andersson et al., 1999; Zucker et al., 2000). Moreover, *Acinetobacter* spp. have been identified in plants, including the rhizosphere of maize and others (Gulati et al., 2010; Dematheis et al., 2012), maize silage (Li and Nishino, 2011) and soybean diet in fish (Catalán et al., 2017), all of which are used as feed in cattle production.

## Identification of Putative Determining Factors

The aim of the multiple logistic regression analysis was to identify putative determining factors that could influence the occurrence of *A. baumannii* in our study population.

### Seasonality

The analysis suggested that the season is a significant factor for the occurrence of *A. baumannii*, probably due to prolonged survival and increased growth of the bacterium associated with higher temperature and humidity. This could also explain the high prevalence in the study from the tropical Reunion Islands mentioned above (Pailhoriès et al., 2016). The seasonality of *Acinetobacter* has furthermore been reported in publications from human medicine (Retailliau et al., 1979; Poutanen et al., 1997; Perencevich et al., 2008). According to Perencevich et al. (2008) monthly infection rates for *A. baumannii* can rise by 17% for each 5.6°C increase in temperature. Interestingly, it was demonstrated that non-MDR *A. baumannii* were identified more frequently in warm months, while MDR strains showed less seasonal variation (Fukuta et al., 2012).

## Use of 3rd Generation Cephalosporins

The chance of isolating *A. baumannii* in our sample was also higher when 3rd generation cephalosporins were used on the farm during the 6 months prior to sampling. Due to the resistance of the bovine *A. baumannii* against many cephalosporins, including the 3rd gen. cephalosporin ceftiofur, which is widely used in dairy production (24.8% of 387 animals of the sample population), selective antibiotic pressure could be a relevant factor in the maintenance of *A. baumannii* on cattle farms.

## Use of Sewage Sludge as Fertilizer

Another interesting determining factor was the use of sewage sludge as a fertilizer on the farm. Due to the reduced number of observations (<90% of the possible observations) it was excluded from the multiple models. Though, this variable showed significant *p*-values in all single-factor analyzes and also if included to the multiple models (data not published). Distribution of *A. baumannii* via sewage sludge on feeding plants via fertilization seems possible as it has been demonstrated that it can be emitted from hospitals into the environment via wastewaters and urban sewage (Seruga Music et al., 2017) and is able to persist through the wastewater treatment process (Hrenovic et al., 2016). Our further analysis of the bovine *A. baumannii* showed that the isolates differ from human clinical isolates concerning antimicrobial resistance and phylogeny. Therefore, further research is needed to clarify (a) the role of the use of sewage sludge fertilization as a putative determining factor for *A. baumannii* in cattle and (b) the phylogeny of human non-clinical isolates.

## Antimicrobial Susceptibility

Although 50 isolates showed growth on Mueller–Hinton agar containing 2 mg/L meropenem and 10 isolates showed growth on Mueller–Hinton agar containing 4 mg/L meropenem, we could not detect phenotypic resistance to carbapenems

**TABLE 3 |** Sequence types of bovine *A. baumannii* isolates with known occurrence in human isolates.

STPast	Number of cattle isolates	Human sample location	Acquired $\beta$ -lactamase	Year of isolation	Country	Reference
ST80	2	Unknown	OXA-40	1999–2010	Spain	Villalón et al., 2011; Mosqueda et al., 2014
ST155	8	Wound	PER-1	2002, 2009, 2012	United States, China, Italy	http://pubmlst.org/abaumannii/
ST240	1	Unknown	Unknown	2010, 2013	Japan	Higuchi et al., 2014
ST241	1	Blood, sputum, stool	Unknown	Unknown	United States, Brazil	http://pubmlst.org/abaumannii/
ST285	1	Urine, sputum	OXA-40	2010	United States, Lebanon	Rafei et al., 2014
ST504	2	Perirectal	Unknown	Unknown	United States	Gregorio et al., 2015
ST690	2	Wound	Unknown	2002, 2014	Spain, Lebanon	http://pubmlst.org/abaumannii/; Al Atrouni et al., 2016a
ST755	1	n.n.	OXA-40	2009–2012	Vietnam	Schultz et al., 2016
ST961	1	Wound	Unknown	2006	United States	http://pubmlst.org/abaumannii/

in our isolates, as determined by a reference method for susceptibility testing. This is in concordance with other studies investigating the occurrence of *A. baumannii* in cattle without using selective media (Hamouda et al., 2011; Rafei et al., 2015). This situation seems to distinguish the cattle isolates from highly resistant strains found in companion animals and humans in a nosocomial background (Francey, 2000; Ewers et al., 2017). Regarding their antimicrobial resistance and phylogenetic diversity, *A. baumannii* strains from cattle rather resemble environmental strains and isolates from wildlife animals (Pailhoriès et al., 2015; Bardbari et al., 2017; Wilharm et al., 2017).

## Phylogenetic Analyzes

Multilocus sequence typing analysis underlined the huge diversity of *A. baumannii* strains in the investigated cattle population. Similar results were obtained in studies from Lebanon and France (Al Atrouni et al., 2016b; Pailhoriès et al., 2016). Notably, some of our bovine strains showed phylogenetic proximity to successful clinical STs. For example, IHIT32879 (ST690: 3-3-2-1-7-2-14) is a SLV of the imipenem-susceptible human clinical isolate LUH7841 (ST402: 3-3-2-1-7-2-4), which belongs to the globally distributed clinically relevant CC25 (Sahl et al., 2015). This CC corresponds to IC7 and is frequently found in human isolates (Chagas et al., 2014; Sahl et al., 2015). Several other STs have recently been isolated from humans, including carbapenem resistant isolates. ST155 has been isolated in China, the United States, and Italy from human samples and was in one occasion associated with a PER-1 gene<sup>4</sup>. ST80 has been identified for the first time in 2014 in OXA-40 producing carbapenem-resistant isolates from human patients in Spain (Mosqueda et al., 2014). A study from Spain identified ST80 as an important group in a Spanish hospital besides ST2, ST3, and ST15 (Villalón et al., 2015). The association to OXA-40 has also been shown for ST285 and ST755, isolated from the United States, Lebanon, and Vietnam (Table 3 and Figure 2). IHIT31924 (ST1074) is a SLV of ST135 which is part of the CC499. The other members of CC499 have already been found in human specimens and OXA-23 has been identified in one of its members (ST192). Cattle might thus host carbapenem-susceptible progenitors of clinically relevant human *A. baumannii* strains and putative transmission routes should be part of future investigations. On the other hand, the lack of the dominant human lineages such as ST2 among our bovine field isolates suggests a selection of these strains inside the hospitals rather than a transition from the cattle population. This represents a notable difference to the *A. baumannii* population in hospitalized companion animals. These animals often show the same STs (or at least ICs) and resistance mechanisms as human nosocomial isolates, indicating interspecies transmission (Endimiani et al., 2011; Zordan, 2011; Pomba et al., 2014; Ewers et al., 2016, 2017).

## CONCLUSION

Our data show that *A. baumannii* is a frequent *Acinetobacter* species in cattle. In contrast to human and small veterinary medicine where primarily carbapenem-resistant isolates belonging to IC1, IC2, and IC7 cause epidemic and endemic outbreaks, the population of bovine *A. baumannii* is highly diverse and still susceptible to many antibiotics which is similar to those found in avian sources (Wilharm et al., 2017). Nevertheless, a minority of strains is phylogenetically connected to clinical isolates, but still lack acquired carbapenemase genes. These strains are a potential threat for public health especially if they enter the clinical environment and acquire resistance genes. Our data strongly suggest that the seasonal occurrence of *A. baumannii* should be taken into account in future study designs. A more detailed, genome-based characterization of bovine isolates in the context of *A. baumannii* strains isolated from humans would be of utmost importance for public health issues. It will help to understand the evolution of *A. baumannii* and might contribute to the identification of factors responsible for the assumed shift from environmental strains toward nosocomial lineages. It remains unclear where the bovine strains originate from and if they colonize the animals transiently or for a longer period. The high prevalence of *A. baumannii* in cattle nose samples, the diversity of strains isolated from individual animals, and their seasonal isolation peaks, point toward a temporarily colonization and a frequent exchange with the environment. The analysis of the meta-data hint toward uptake via the feed possibly enhanced through the fertilization with sewage sludge. This possibly forms a continuous circle of reinfection inside the cattle population.

## Limitations

Our study design comprises limitations concerning technical and statistical aspects: for productivity reasons only one colony among similar morphologies was chosen for further analysis and selective media for carbapenem-resistant strains were not included. Thus, we accepted possible underestimation of diversity and phenotypic detection of carbapenem resistant strains. Metadata were collected via questionnaires which are inevitable reliant on subjective assessments of the farmers. This resulted in missing values and mandatory exclusion of some variables from the single-factor logistic regression to the multiple models. As we did not distinguish between calves from dairy cows and beef cattle the impact of different management variables in these categories cannot be tested in our model but are met to some extend in our metadata analysis without a significant impact on the prevalence of *A. baumannii*.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the directive 2010/63/EU of the European parliament and of the council and the German animal welfare law. According to the assessment of the animal welfare

<sup>4</sup><https://pubmlst.org/abaumannii/>

officer of the Faculty of Veterinary Medicine of Giessen in 2014 the animals did not experience pain, suffering, distress, or lasting harm due to the sampling. An ethical committee statement was therefore not necessary.

## AUTHOR CONTRIBUTIONS

PK, KF, SS, TS, and CE contributed conception and design of the study. PK collected the samples, organized the database, and wrote the first draft of the manuscript. KF wrote sections of the manuscript. PK, KF, and AS performed the statistical analyzes. PK, PH, and HS performed the MLST analyzes. PK and UL performed the PFGE and PCR experiments. All authors contributed to manuscript revision, read and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00272/full#supplementary-material>

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# Discovery of Novel Antibiotic Resistance Determinants in Forest and Grassland Soil Metagenomes

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Soil represents a significant reservoir of antibiotic resistance genes (ARGs), which can potentially spread across distinct ecosystems and be acquired by pathogens threatening human as well as animal health. Currently, information on the identity and diversity of these genes, enabling anticipation of possible future resistance development in clinical environments and the livestock sector, is lacking. In this study, we applied functional metagenomics to discover novel sulfonamide as well as tetracycline resistance genes in soils derived from forest and grassland. Screening of soil metagenomic libraries revealed a total of eight so far unknown ARGs. The recovered genes originate from phylogenetically diverse soil bacteria (e.g., Actinobacteria, Chloroflexi, or Proteobacteria) and encode proteins with a minimum identity of 46% to other antibiotic resistance determinants. In particular forest soil ecosystems have so far been neglected in studies focusing on antibiotic resistance. Here, we detected for the first time non-mobile dihydropteroate synthase (DHPS) genes conferring resistance to sulfonamides in forest soil with no history of exposure to these synthetic drugs. In total, three sulfonamide resistant DHPs, differing in taxonomic origin, were discovered in beech or pine forest soil. This indicates that sulfonamide resistance naturally occurs in forest-resident soil bacterial communities. Besides forest soil-derived sulfonamide resistance proteins, we also identified a DHPS affiliated to Chloroflexi in grassland soil. This enzyme and the other recovered DHPs confer reduced susceptibility toward sulfamethazine, which is widely used in food animal production. With respect to tetracycline resistance, four efflux proteins affiliated to the major facilitator superfamily (MFS) were identified. Noteworthy, one of these proteins also conferred reduced susceptibility toward lincomycin.

**Keywords:** soil metagenome, functional metagenomics, antibiotic resistance, dihydropteroate synthase, tetracycline resistance, sulfonamide resistance

## INTRODUCTION

Pathogenic bacteria resistant to multiple classes of antibiotics pose risks to public health and are considered as one of the major global challenges within the 21st century. Some of the antibiotic resistance genes (ARGs) carried by these bacteria have been traced to soil origins (Forsberg et al., 2012) and can potentially spread via e.g., groundwater or wildlife (Davies and Davies, 2010).

Nevertheless, in contrast to clinical pathogens, bacterial communities inhabiting complex environments such as soil have been rarely considered within studies focusing on antibiotic resistance (Walsh, 2013b). To assess risks of environmental resistomes and develop strategies to tackle antibiotic resistance, an improved knowledge on the ecology of resistance determinants including their origins, diversity and underlying resistance mechanisms is urgently required (Allen et al., 2009; Wang et al., 2017).

Among Earth's microbial habitats, soil harbors the highest diversity of prokaryotes including numerous multi-resistant bacteria (Delmont et al., 2011; Walsh and Duffy, 2013; Nesme and Simonet, 2015). The synthesis of antibiotics likely evolved in this habitat and promoted the development of different antimicrobial compound-specific resistance mechanisms (D'Costa et al., 2007; Walsh, 2013a). Previously unknown soil-derived ARGs were recovered from both, pristine and intensively managed sites, by function-based screening of metagenomic libraries (Allen et al., 2009; Perron et al., 2015; Lau et al., 2017). In contrast to sequence-based metagenomic library screening, this culture-independent approach is not based on conserved DNA regions and therefore allows the identification of entirely novel target genes (Nacke and Daniel, 2014; Cheng et al., 2017). For instance, a so far unknown peptide-associated macrolide resistance mechanism was uncovered by coupling function-based metagenomic library screening and high-resolution proteomics analysis (Lau et al., 2017). Besides dependence on conserved DNA regions, the fact that various resistance genes show high levels of similarity to genes encoding other cellular functions (Martínez, 2008; Perron et al., 2015) represents another limitation of sequence-based resistome analysis. An illustrative example are efflux pumps of the resistance-nodulation-division (RND) superfamily, which can confer antibiotic resistance, but can also transport proteins involved in cell division and nodulation, or both (Piddock, 2006; Perron et al., 2015).

In this study, we used function-based metagenomic library screening to identify so far unknown tetracycline and sulfonamide resistance genes in forest and grassland soil. Due to an excellent therapeutic index, few side effects, oral administration and low cost, tetracyclines belong to the most widely used classes of broad spectrum antibiotics in clinic (Thaker et al., 2010; Wang et al., 2017). After more than 60 years of excessive tetracycline usage, tetracycline resistance became one of the most abundant antibiotic resistances among clinical and commensal microbes (Wang et al., 2017). Another class of antibiotics, sulfonamides, is also commonly prescribed to people suffering from infections (Landers et al., 2012).

ARGs present in forests and grasslands, belonging to the most abundant terrestrial ecosystems worldwide, might become clinically relevant as they can potentially spread via lateral gene transfer. Here, we report the identification of four novel tetracycline and four previously unknown sulfonamide resistance genes derived from these ecosystems. Most of the proteins encoded by the novel ARGs showed low identity to already known antibiotic resistance determinants.

## MATERIALS AND METHODS

### Site Description, Soil Sampling, and Metagenomic Library Construction

Soil samples were derived from forest and grassland sites of the German Biodiversity Exploratories Schorfheide-Chorin and Schwäbische Alb (Fischer et al., 2010). The land use intensity index (LUI) (Blüthgen et al., 2012) was calculated for all grassland sites. To account for interannual variation in management practices, the LUI was calculated from 2006 to 2008 (sampling year) (Table 1). LUI allows separate analysis of the intensity of grazing (calculated by considering numbers of grazing cattle, horses, or sheep, and duration of grazing with respect to each site), the mowing frequency, and the intensity of fertilization. Forest plots were dominated by European beech (*Fagus sylvatica*) or Scots pine (*Pinus sylvestris*) (Table 1).

The collection of the samples was performed previously as described by Nacke et al. (2011a). Descriptions of the soil characteristics are provided in Table 2. Total microbial community DNA was isolated from collected soil by employing the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, United States) and metagenomic libraries, named AEG2, AEG3, and SEG8 were generated as described by Nacke et al. (2011b). The metagenomic libraries AEW9, SEG6, SEW2, and SEW5 were previously constructed (Nacke et al., 2011b). Names of constructed metagenomic libraries refer to the designation of the samples from which the libraries were derived.

### Antibiotic Resistance Screening and Sequence Analysis

The function-based screening was based on the ability of metagenomic library-bearing *Escherichia coli* clones to form colonies on LB agar medium containing 50 mg/L kanamycin, which selects for the screening vector pCR-XL-TOPO (Thermo Fisher Scientific, Braunschweig, Germany), and 5 mg/L tetracycline or 250 mg/L sulfamethoxazole. Colonies formed after incubation for 1–3 days at 37°C under aerobic conditions were picked for further study.

The recombinant plasmids derived from positive clones were sequenced by Microsynth Seqlab (Göttingen, Germany) using Sanger sequencing technology. All plasmid inserts were taxonomically classified using the software KAIJU (Menzel et al., 2016). An initial prediction of ORFs located on the inserts was performed by employing the ORF finder tool provided by the National Center for Biotechnology Information (NCBI) and the Artemis program (Rutherford et al., 2000; Wheeler et al., 2003). The results were verified and improved manually by e.g., GC frame plot and ribosome-binding site analysis. Subsequently, blast (Altschul et al., 1990) search against the NCBI non-redundant protein sequence database was performed. In addition, Resfams (Gibson et al., 2015), a recently generated database of protein families and associated profile hidden Markov models, representing all major ARG classes, was used for sequence comparisons. Blast searches against the ACLAME database (Leplae et al., 2010) version 0.4 and the Gypsy database (Llorens et al., 2011) release 2.0 were performed to identify



TABLE 1 | Characteristics of the study sites.

Site	Land use	Management	Treatment	Tree species	LUI (grazing, mowing, fertilization)
AEG2	Grassland	Meadow	Fertilized	NA	0.00, 2.07, 1.27
AEG3	Grassland	Meadow	Fertilized	NA	0.00, 2.76, 2.06
AEW9	Forest	Unmanaged forest	NA	Beech	NA
SEG6	Grassland	Mown pasture	Non-fertilized	NA	0.29, 1.38, 0.00
SEG8	Grassland	Pasture	Non-fertilized	NA	0.14, 0.69, 0.00
SEW2	Forest	Age class forest	NA	Pine	NA
SEW5	Forest	Age class forest	NA	Beech	NA

The table lists the sites, land use, management type, treatment, dominant tree species, and LUI, land use index (calculated for 2006–2008) for grassland samples. AEG/AEW: sites located in the Biodiversity Exploratory Schwäbische Alb; SEG/SEW: sites located in the Biodiversity Exploratory Schorfheide-Chorin.

TABLE 2 | Basic properties of soil samples.

Sample	Soil type	pH	OC (g kg <sup>-1</sup> )	Total N (g kg <sup>-1</sup> )	C:N ratio
AEG2	Leptosol	6.9	72.3	7.2	10.1
AEG3	Leptosol	6.3	53.7	5.2	10.4
AEW9	Leptosol	6.4	60.0	4.5	13.4
SEG6	Histosol	5.2	284.1	23.9	11.9
SEG8	Gleysol	7.4	73.2	7.1	10.4
SEW2	Cambisol	3.5	17.0	1.0	16.7
SEW5	Cambisol	3.1	29.6	1.6	18.3

AEG/AEW: soil samples derived from the Biodiversity Exploratory Schwäbische Alb; SEG/SEW: soil samples derived from the Biodiversity Exploratory Schorfheide-Chorin.

mobile genetic elements. Moreover, the IS finder (database from 2018-09-11) (Siguier et al., 2006) was employed for identification of bacterial insertion sequences.

A neighbor-joining phylogenetic tree was constructed in MEGA (version 7.0) (Kumar et al., 2016) based on a ClustalW (Thompson et al., 1994) alignment of dihydropteroate synthase (DHPS) sequences. A total number of 1,000 bootstrap samplings were carried out to test the tree topology. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the number of differences method.

### Subcloning of ORFs Potentially Encoding Antibiotic Resistance

To verify if candidate ORFs encode antibiotic resistance, they were subcloned into vector pCR4-TOPO (Thermo Fisher Scientific) and subsequently introduced into *E. coli* TOP10. Two insert sequences (corresponding plasmids, pLAEG3\_tet01 and pLSEG6\_tet01) encoded proteins with similarity to members of the TetR family of regulators. In this case, the gene encoding the regulator as well as the potential ARG were subcloned together. In a first step, PCR was performed for amplification of candidate ORFs (including sequences potentially comprising promoters) from plasmid DNA. PCR primers are listed in Table 3. The PCR reaction mixture (50 µl) contained 10 µl 5-fold Phusion GC buffer, 200 µM of each of the four deoxynucleoside triphosphates, 5% DMSO, 0.2 µM of each primer, 1 U of Phusion HF DNA polymerase (Thermo Fisher Scientific), and approximately 20 ng of plasmid DNA. The following thermal cycling scheme was used: initial denaturation at 98°C for 1 min, 20 cycles of denaturation at 98°C for 1 min, annealing for

45 s (annealing temperatures, see Table 3), and extension at 72°C for 30 s per kb, followed by a final extension period at 72°C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Subsequently, a deoxyadenosine was added to the 3' termini of the DNA as described by Nacke et al. (2011b) to facilitate cloning by the TA method. The DNA was then purified using the QIAquick PCR purification kit (Qiagen) and inserted into vector pCR4-TOPO (Thermo Fisher Scientific) as described by the manufacturer. Transformation of resulting vectors into *E. coli* TOP10 chemically competent cells was performed according to the protocol of the manufacturer.

### Antibiotic Susceptibility Analysis

Antibiotic susceptibility assays were conducted by using the 2-fold serial microtiter broth dilution method by considering the Clinical and Laboratory Standards Institute (CLSI) guidelines document M100-S24 (2014) and the MICs were recorded after 20 h of incubation at 37°C. The antibiotics cefotaxime, chloramphenicol, erythromycin, gentamicin, lincomycin, rifampicin, sulfadiazine, sulfamethoxazole, sulfamethazine, sulfisoxazole, tetracycline, and tylosin were considered. All assays were performed in duplicate. In addition, the susceptibility to different sulfonamides was further analyzed by spotting serial dilutions of cultures with starting OD<sub>600</sub> of 0.5 onto Iso-Sensitest agar (Thermo Fisher Scientific) supplemented with sulfamethoxazole, sulfamethazine, sulfisoxazole or sulfadiazine. *E. coli* TOP10 carrying vector pCR4-TOPO (Thermo Fisher Scientific) was used as control.

**TABLE 3 |** Primer sets designed in this study and corresponding templates.

Template	Oligonucleotide	Sequence (5' to 3')	Annealing temperature (°C)
pLAEG2_dhps01	AEG2_dhps01_for_150	GATACCCTAACGTAACCGC	55
	AEG2_dhps01_rev	TCAGCGCGGATTGCTTC	55
pLAEW9_dhps01	AEW9_dhps01_for_150	CCTGATCGGTCAGGTCCTTA	55
	AEW9_dhps01_rev	TTACGCCGTTTGCCCC	55
pLSEW2_dhps01	SEW2_dhps01_for_150	CCGCCCGCCGTGTG	60
	SEW2_dhps01_rev	TTATGAAGCGGCGATAGCAGTAATAAC	60
pLSEW5_dhps01	SEW5_dhps01_for_104	GGTCATCGCGACAAAGGGTG	60
	SEW5_dhps01_rev	CTATACAGGCCGTCAGCTGC	60
pLAEG3_tet01	AEG3_tet01b_for	CTATTGCTTGACGCGATCG	55
	AEG3_tet01a_rev	CTATTCGCGCGGCTCAG	55
pLSEG6_tet01	SEG6_tet01b_for	TTATCCTCGACGCGCCTTG	60
	SEG6_tet01a_rev	TCAGCCCGGAGCCAAGG	60
pLSEG8_tet01	SEG8_tet01_for_150	GGATTGGAACAGACATATAGTG	55
	SEG8_tet01_rev	TTACCGGTTCCCCACTG	55
pLSEG8_tet02	SEG8_tet02_for_150	TTTAAGAGAATTTTCAGGATAAAG	50
	SEG8_tet02_rev	TTAACCATGCTTTGTGACG	50

## Accession Numbers

The insert sequences of the plasmids carried by metagenomic library clones showing decreased susceptibility to sulfamethoxazole or tetracycline have been submitted to GenBank under accession numbers MK159018 to MK159025.

## RESULTS AND DISCUSSION

In order to discover so far unknown ARGs in environmental resistomes, soil metagenomic libraries were subjected to function-based screening. As sequence information is not required before screening, this is the only strategy that bears the potential to discover entirely novel ARGs (Simon and Daniel, 2009). In addition, it is selective for full-length genes and functional gene products. The soil used for construction of metagenomic libraries was derived from forest and grassland varying in land use history. Fertilized and non-fertilized grassland sites as well as pristine and age class forest sites, harboring different dominant tree species, were considered (Table 1). This enabled the identification of ARGs in soils from hardly as well as intensively managed ecosystems.

Metagenomic libraries contained approximately 39,800–559,000 clones (Table 4). The quality of the libraries was controlled by determining the average insert sizes and the percentage of insert-bearing *E. coli* clones. The average insert sizes of metagenomic DNA-containing plasmids ranged from 2.6 to 6.0 kb and the frequency of clones carrying plasmid inserts was at least 73% (Table 4).

### Novel ARGs Derived From Phylogenetically Divergent Soil Bacteria

The soil-derived metagenomic libraries were screened for resistance against tetracycline and sulfamethoxazole using selective agar medium. We recovered eight positive *E. coli* clones, harboring plasmids listed in Table 4, from functional

screens. The entire inserts of these plasmids were sequenced and taxonomically classified, which revealed in all cases a bacterial origin (Supplementary Table S1). Some of the insert sequences are affiliated to Gram-negative bacterial phyla including Bacteroidetes and Proteobacteria whereas others belong to Actinobacteria (Supplementary Table S1). Noteworthy, one of the insert sequences was affiliated to the poorly characterized candidate phylum Zixibacteria.

Forsberg et al. (2014) reported that bacterial phyla, which were abundant in soil samples as determined by 16S rRNA gene sequencing, were also well-represented among taxa inferred from antibiotic resistance-conferring metagenomic library inserts derived from the same samples. Previously, we detected Proteobacteria, Actinobacteria, Bacteroidetes, and Chloroflexi among the dominant phyla in soils of our study sites via pyrosequencing of 16S rRNA genes (Kaiser et al., 2016). These phyla were also covered by the antibiotic resistance-conferring inserts described in this study (see Supplementary Table S1). Despite their high-GC content and predicted transcriptional incompatibilities with *E. coli*, also Actinobacteria were represented with respect to inserts of positive clones reported here and by Forsberg et al. (2014). The taxonomic origins of our resistance-conferring inserts show that the metagenomic library host *E. coli* allows identification of ARGs carried by phylogenetically divergent soil bacteria.

### Forest Soil Not Exposed to Synthetic Drugs Harbors Sulfonamide-Resistant DHPs

Sulfonamides are synthetic antimicrobial compounds targeting the folic acid pathway enzyme DHPs. Although all forest sites analyzed in this study exhibit no history of exposure to these synthetic compounds, three genes, *AEW9\_dhps01*, *SEW2\_dhps01*, and *SEW5\_dhps01*, conferring sulfonamide resistance, were recovered from beech or pine forest soil (Tables 1, 5 and Figure 1). Furthermore, with respect to both

**TABLE 4 |** Characterization of soil metagenomic libraries and designation of plasmids harbored by positive clones.

Library	Number of clones	Average insert size (kb)	Insert frequency (%)	Estimated library size (Gb)	Plasmids of positive clones
AEG2	115965	3.6	73	0.30	pLAEG2_dhps01
AEG3	40095	5.8	85	0.20	pLAEG3_tet01
AEW9*	100950	2.6	89	0.23	pLAEW9_dhps01
SEG6*	39825	6.0	91	0.22	pLSEG6_tet01
SEG8	559000	4.8	86	2.30	pLSEG8_tet01-02
SEW2*	135240	5.7	95	0.73	pLSEW2_dhps01
SEW5*	166040	4.0	95	0.63	pLSEW5_dhps01

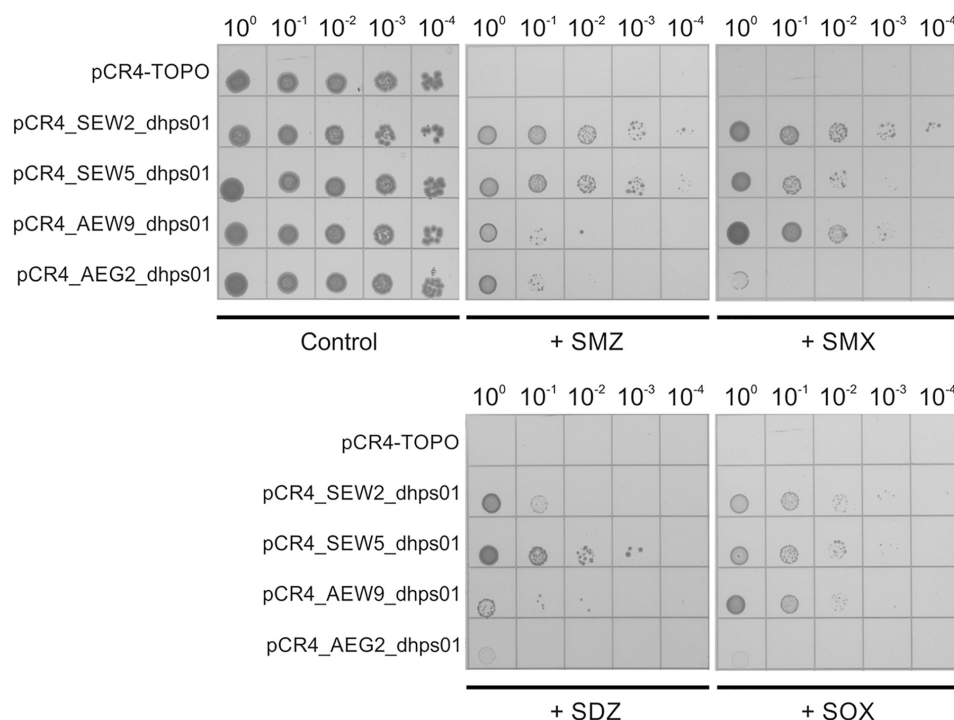
AEG/AEW: metagenomic libraries derived from the Biodiversity Exploratory Schwäbische Alb; SEG/SEW: metagenomic libraries derived from the Biodiversity Exploratory Schorfheide-Chorin. \*Previously generated libraries. Names of constructed metagenomic libraries refer to the designation of the samples from which the libraries were derived (see **Table 2**).

forest sites (SEW2 and SEW5) located in the Schorfheide-Chorin exploratory (Northeastern Germany), as well as the forest site (AEW9) located in the Schwäbische Alb exploratory (Southwestern Germany), to our knowledge soils were not exposed to chemicals that resemble sulfonamides in their molecular structure. Especially, in case of the site AEW9 it is

unlikely that such chemicals were spread, as this site belongs to an unmanaged beech forest. Resistance to sulfonamides is commonly mediated by the mobile DHPS-encoding genes *sul1*, *sul2*, or *sul3* (Sköld, 2000; Perreten and Boerlin, 2003), which have been detected in various environments such as shrimp ponds, swine farm wastewater and manured soil

**TABLE 5 |** Proteins encoded by genes associated with antibiotic resistance and their observed sequence identities.

Plasmid	Gene	No. of encoded amino acids	Closest similar non-hypothetical protein, accession no. (no. of encoded amino acids), organism	E-value	Percent identity to the closest similar protein (Blast)	Percent identity to the closest similar protein (ClustalW alignment)
pLAEG2_dhps01	<i>AEG2_dhps01</i>	286	Dihydropteroate synthase, WP_116719066 (292), Anaerolineaeles bacterium	3e-151	213/282 (76%)	74%
pLAEW9_dhps01	<i>AEW9_dhps01</i>	273	Sulfonamide-resistant dihydropteroate synthase Sul3, WP_106052391 (263), Victivallales	1e-73	123/264 (47%)	46%
pLSEW2_dhps01	<i>SEW2_dhps01</i>	269	Dihydropteroate synthase, OGQ04760 (263), Deltaproteobacteria bacterium	3e-77	140/270 (52%)	52%
pLSEW5_dhps01	<i>SEW5_dhps01</i>	271	Dihydropteroate synthase, QJU07522 (270), Alphaproteobacteria bacterium 64-11	3e-99	159/259 (61%)	58%
pLAEG3_tet01	<i>AEG3_tet01a</i>	403	MFS transporter, AIA16595 (403), uncultured bacterium	0.0	398/403 (99%)	98%
	<i>AEG3_tet01b</i>	230	Bacterial regulatory protein of tetR family, AIA16695 (190), uncultured bacterium	2e-127	179/190 (94%)	94%
pLSEG6_tet01	<i>SEG6_tet01a</i>	408	MFS transporter, WP_078811785 (418), <i>Prostheobacter debontii</i>	2e-128	200/383 (52%)	49%
	<i>SEG6_tet01b</i>	197	TetR family transcriptional regulator, PZN78209 (205), Proteobacteria bacterium	4e-63	109/194 (56%)	54%
pLSEG8_tet01	<i>SEG8_tet01</i>	432	MFS transporter, WP075350247 (408), <i>Algoriphagus marinus</i>	3e-174	250/402 (62%)	61%
pLSEG8_tet02	<i>SEG8_tet02</i>	405	Tetracycline resistance MFS efflux pump, AIA16766 (418), uncultured bacterium	0.0	272/402 (68%)	67%



**FIGURE 1 |** Resistance against sulfonamide antibiotics mediated by *SEW2\_dhps01*, *SEW5\_dhps01*, *AEW9\_dhps01*, and *AEG2\_dhps01*. Five microliters of serially diluted *E. coli* TOP10 cultures with starting OD<sub>600</sub> of 0.5 were spotted onto Iso-Sensitest agar plates supplemented with 1000 mg/L sulfamethazine (+ SMZ), 250 mg/L sulfamethoxazole (+ SMX), 250 mg/L sulfadiazine (+ SDZ) or 500 mg/L sulfisoxazole (+ SOX). Iso-Sensitest agar plates with no sulfonamide added (control) were also included. *E. coli* TOP10 cultures carrying the cloning vector pCR4-TOPO, pCR4\_SEW2\_dhps01, pCR4\_SEW5\_dhps01, pCR4\_AEW9\_dhps01 or pCR4\_AEG2\_dhps01 were considered.

(Phuong Hoa et al., 2008; Wang et al., 2014), but also in clinical isolates (Grape et al., 2003). To our knowledge, we report here for the first time the presence of functional non-mobile sulfonamide-resistant DHPSs in forest soil ecosystems. The deduced gene products of *AEW9\_dhps01*, *SEW2\_dhps01*, and *SEW5\_dhps01* showed only 46 to 58% amino acid sequence identities to the closest known DHPSs over the full length proteins (Table 5). Furthermore, *AEW9\_dhps01* harbors the alternative start codon GTG (all other detected *dhps* genes harbored the start codon ATG).

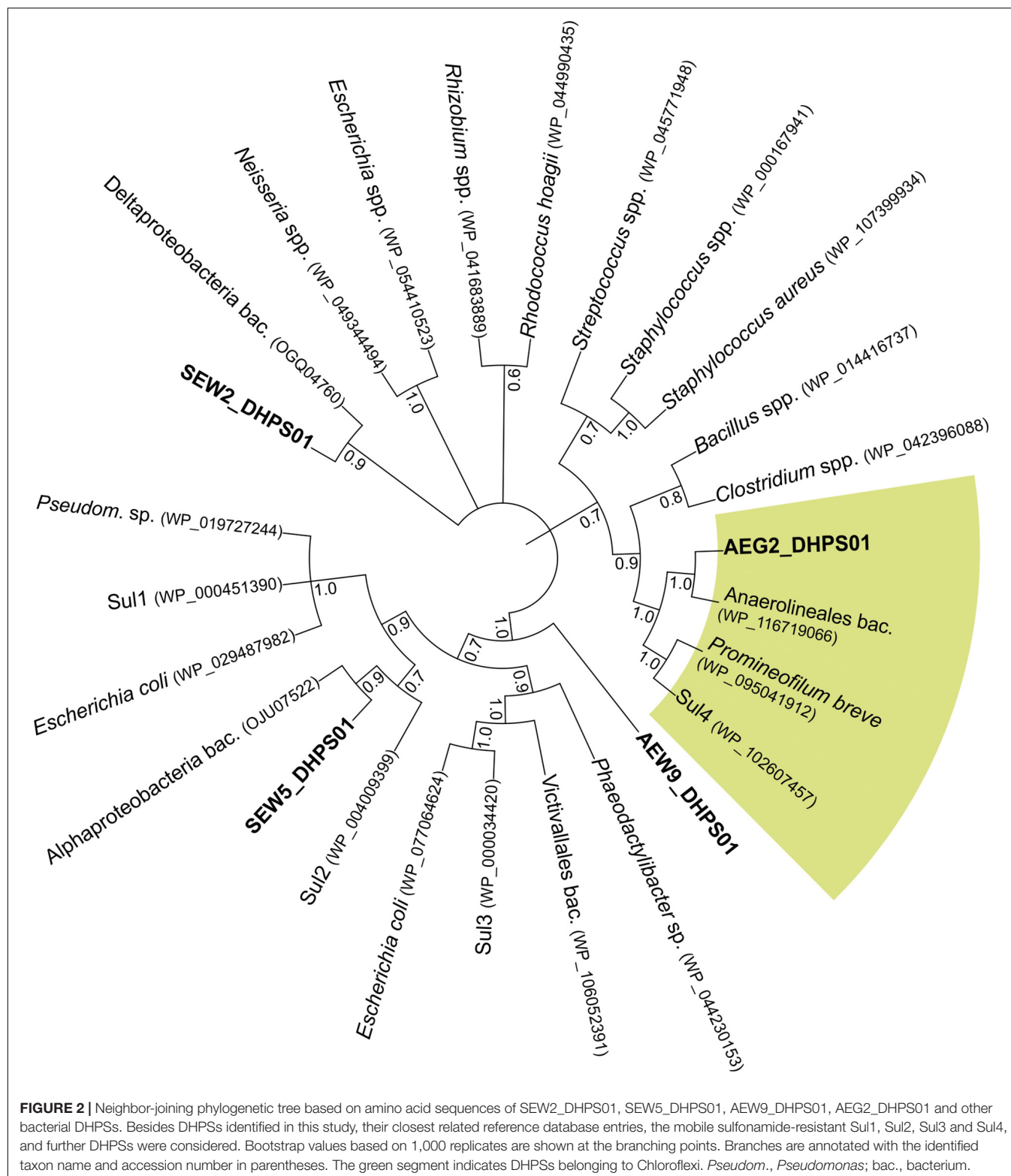
Phylogenetic analysis revealed that *SEW2\_DHPS01* exhibits homology with a putative DHPS affiliated to Deltaproteobacteria (Figure 2). Nevertheless, it has so far not been analyzed if this putative enzyme represents a functional DHPS, which can confer resistance to sulfonamides. In contrast to *SEW2\_DHPS01*, *AEW9\_DHPS01* showed low identity (46%) to a DHPS with confirmed sulfonamide resistance, but branched separately from this enzyme affiliated to the Lentisphaerae (family Victivallales), in a phylogenetic tree (Figure 2). The remaining sulfonamide resistance-conferring enzyme identified in forest soil, *SEW5\_DHPS01*, was most similar (58% identity) to a DHPS from Alphaproteobacteria. Strikingly, no mobile genetic elements were predicted with respect to the inserts comprising *AEW9\_dhps01*, *SEW2\_dhps01*, and *SEW5\_dhps01*. This indicates that different bacterial phyla colonizing forest soil ecosystems harbor DHPSs, which are naturally insensitive to the inhibitory

effects of sulfonamides. Furthermore, our results show that forest soil-derived DHPSs can provide high-level resistance in *E. coli* TOP10 (Figures 1, 3) and therefore potentially also in clinically relevant Enterobacteriaceae. As sulfonamides are used to treat gastrointestinal or urinary infections in human and belong to the most commonly sold and administered veterinary antibiotics (De Briyne et al., 2014; Santman-Berends et al., 2014), mobilization and spread of so far unknown genes conferring resistance to these synthetic compounds would have severe consequences, especially for the animal sector. In particular, *SEW2\_DHPS01* and *SEW5\_DHPS01* exhibited high-level resistance toward sulfamethazine (Figure 1), which is widely used in food animal production (Lau et al., 2017).

## Discovery of a Grassland Soil-Derived DHPS Affiliated to Chloroflexi

Recently, a fourth mobile sulfonamide resistance gene (*sul4*), encoding a DHPS phylogenetically related to representatives of the phylum Chloroflexi, has been discovered in polluted Indian river sediment (Razavi et al., 2017). This gene is flanked by an ISCR element, which is known to be involved in horizontal gene transfer (Razavi et al., 2017). In this study, we identified an enzyme (*AEG2\_DHPS01*) with reduced susceptibility toward sulfonamides (Figure 1 and Table 6), showing similarity to DHPSs from Chloroflexi, in a fertilized grassland soil.





AEG2\_DHPS01 shares 76% sequence identity with a DHPS from a member of the Anaerolineae (Table 5) and clusters with different Chloroflexi DHPSs including Sul4 in a phylogenetic tree (Figure 2).

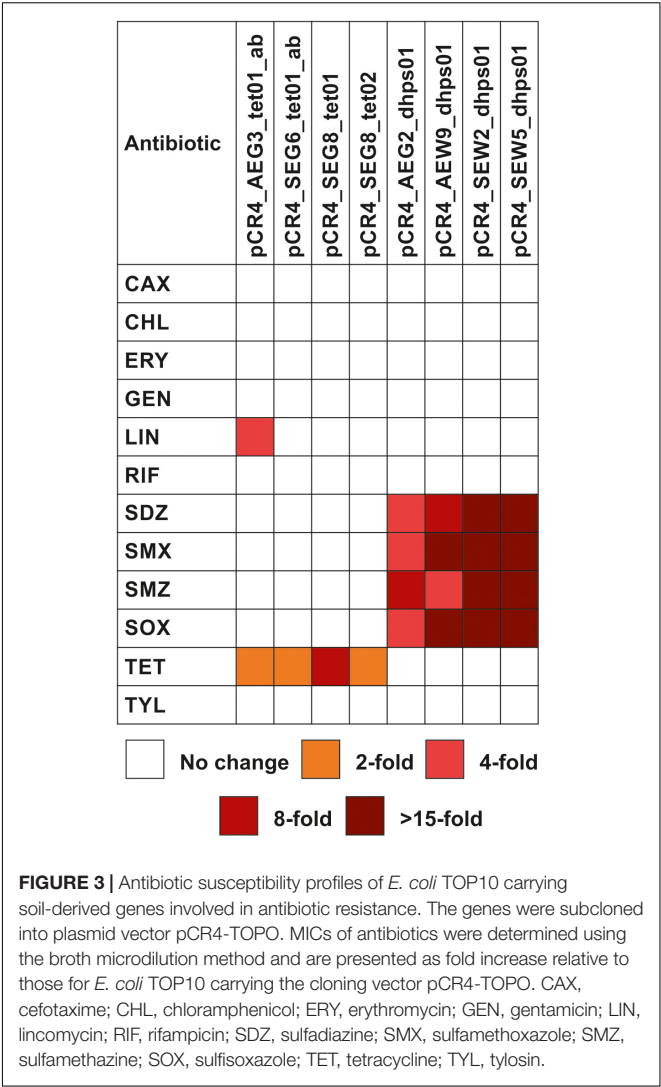
As *sul4* is flanked by a partial *folK* ORE, it might have been decontextualized from a set of chromosomal genes involved in folate synthesis (Razavi et al., 2017). Nevertheless, Razavi et al. (2017) pointed out that further investigations on Chloroflexi

could provide additional hints about the original host of *sul4* and how it has been decontextualized. With respect to the insert carrying *AEG2\_dhps01*, no genes potentially involved in folate synthesis were identified. Instead, *AEG2\_dhps01* is flanked by an ORF encoding a putative gene product with low similarity (23% identity) to a primosomal protein N' (replication factor Y) – superfamily 2 helicase from an *Anaerolineae* bacterium (Supplementary Table S2). It is possible that this gene product can contribute to horizontal gene transfer between Chloroflexi and other bacterial taxa as helicases play a major role in replication, recombination, and repair of nucleic acid substrates (Flechsig et al., 2011; Byrd and Raney, 2012). Besides the potential helicase gene, *AEG2\_dhps01* is flanked by an ORF encoding a gene product with similarity to a hypothetical protein of an *Anaerolineales* bacterium.

Taxonomic analysis of the complete insert carrying *AEG2\_dhps01* confirmed that its original host belongs to the Chloroflexi (Supplementary Table S1). Thus, besides *Sul4*, *AEG2\_DHPS01* represents the so far only identified DHPS showing reduced susceptibility toward sulfonamides (Table 6), which is affiliated to the Chloroflexi. In order to analyze if sulfonamide resistance is a common characteristic of Chloroflexi, isolates belonging to this phylum should be analyzed with respect to susceptibility toward synthetic drugs in future surveys. Apart from sulfonamides, no decreased susceptibility toward other tested antibiotics was detected with respect to *E. coli* TOP10 carrying the subcloned *dhps* genes (Figure 3 and Table 6).

### An Efflux Protein Conferring Reduced Tetracycline and Lincomycin Susceptibility

We identified four plasmids, pLAEG3\_tet01, pLSEG6\_tet01, pLSEG8\_tet01, and pLSEG8\_tet02, conferring efflux-mediated tetracycline resistance. All of these plasmids encode gene products with similarity to major facilitator superfamily (MFS) efflux proteins (Table 5). MFS efflux systems are widely distributed in both Gram-positive and Gram-negative bacteria (Sun et al., 2014). Accordingly, Wang et al. (2017) reported that 21 out of 24 tetracycline resistance genes, identified by functional



**FIGURE 3 |** Antibiotic susceptibility profiles of *E. coli* TOP10 carrying soil-derived genes involved in antibiotic resistance. The genes were subcloned into plasmid vector pCR4-TOPO. MICs of antibiotics were determined using the broth microdilution method and are presented as fold increase relative to those for *E. coli* TOP10 carrying the cloning vector pCR4-TOPO. CAX, cefotaxime; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; LIN, lincomycin; RIF, rifampicin; SDZ, sulfadiazine; SMX, sulfamethoxazole; SMZ, sulfamethazine; SOX, sulfisoxazole; TET, tetracycline; TYL, tylosin.

metagenomics in Chinese soils, were affiliated to the MFS. The proteins encoded by these 21 genes showed identities  $\geq 78\%$  to

**TABLE 6 |** Antibiotic susceptibility of plasmid-carrying *E. coli* clones.

Plasmid	Minimal inhibitory concentration (μg/ml)											
	CAX	CHL	ERY	GEN	LIN	RIF	SDZ	SMX	SMZ	SOX	TET	TYL
Cloning vector	<0.125	2	1024	4	512	8	15.625	7.8125	62.5	15.625	1	512
pCR4_AEG2_dhps01	<0.125	2	1024	4	512	8	<b>62.5</b>	<b>31.25</b>	<b>500</b>	<b>62.5</b>	1	512
pCR4_AEW9_dhps01	<0.125	2	1024	4	512	8	<b>125</b>	<b>500</b>	<b>250</b>	<b>1000</b>	1	512
pCR4_SEW2_dhps01	<0.125	2	1024	4	512	8	<b>500</b>	<b>500</b>	<b>&gt;1000</b>	<b>1000</b>	1	512
pCR4_SEW5_dhps01	<0.125	2	1024	4	512	8	<b>&gt;1000</b>	<b>500</b>	<b>&gt;1000</b>	<b>1000</b>	1	512
pCR4_AEG3_tet01ab	<0.125	2	1024	4	<b>2048</b>	8	15.625	7.8125	62.5	15.625	<b>2</b>	512
pCR4_SEG6_tet01ab	<0.125	2	1024	4	512	8	15.625	7.8125	62.5	15.625	<b>2</b>	512
pCR4_SEG8_tet01	<0.125	2	1024	4	512	8	15.625	7.8125	62.5	15.625	<b>8</b>	512
pCR4_SEG8_tet02	<0.125	2	1024	4	512	8	15.625	7.8125	62.5	15.625	<b>2</b>	512

CAX, cefotaxime; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; LIN, lincomycin; RIF, rifampicin; SDZ, sulfadiazine; SMX, sulfamethoxazole; SMZ, sulfamethazine; SOX, sulfisoxazole; TET, tetracycline; TYL, tylosin. Bold values indicate an increase in minimal inhibitory concentration compared to the control strain carrying the cloning vector pCR4-TOPO.

the closest related reference database entries (Wang et al., 2017). In contrast, three out of four MFS representatives identified in this study shared  $\leq 67\%$  identity with their closest related proteins (Table 5). Besides an MFS representative, two insert sequences (corresponding plasmids, pLAEG3\_tet01 and pLSEG6\_tet01) encoded proteins with similarity to members of the TetR family of regulators (Table 5). These regulators are associated with antibiotic resistance and are known to control expression of MFS members (Cuthbertson and Nodwell, 2013). Noteworthy, the insert of plasmid pLtetSEG8\_02 encodes a protein with similarity to an endonuclease (Supplementary Table S2), which might contribute to horizontal gene transfer.

McGarvey et al. (2012) identified a tetracycline-resistant metagenomic library clone, harboring a MFS representative, with reduced susceptibility toward rifampicin. Here, no resistance toward rifampicin was detected with respect to recombinant MFS producing *E. coli* clones (Figure 3). Nevertheless, the tetracycline-resistant clone carrying plasmid pCR4\_AEG3\_tet01ab showed reduced susceptibility toward lincomycin (Figure 3 and Table 6). The gene product AEG3\_Tet01a encoded by this plasmid shows 98% identity to a soil-derived MFS from an uncultured bacterium (Table 5), which confers resistance to chloramphenicol. So far, it has not been analyzed if this chloramphenicol resistance mediating MFS identified by Forsberg et al. (2014) also encodes lincomycin resistance.

## CONCLUSION

Our findings highlight the vast potential of functional metagenomics for the discovery of so far unknown antibiotic resistance determinants in environmental resistomes. We recovered several soil-derived target genes and proteins with low similarity to reference database entries from hardly as well as intensively managed forest and grassland, indicating that the resistance reservoir of the uncultured microbial majority is far from being extensively explored. As we detected here for the first time non-mobile DHPs conferring resistance to sulfonamides in forest soil with no history of exposure to these synthetic drugs, it is possible that this characteristic naturally occurs in complex bacterial communities. Most of the detected antibiotic resistance determinants were not flanked by potential mobile genetic elements. Nevertheless, the recent finding of a fourth mobile sulfonamide resistance gene indicates ongoing forces that introduce, mobilize and maintain antibiotic resistance determinants in bacterial communities (Razavi et al., 2017). Considering, that several ARGs reported here conferred high-level resistance to non-pathogenic *E. coli*, it can be assumed that this could also be the case with respect to clinically

relevant Enterobacteriaceae. In order to predict the emergence of antibiotic resistance, an extensive knowledge on environmental resistomes will be required, which might also direct the design of novel antibiotics that are less susceptible to resistance.

## DATA AVAILABILITY

The datasets generated for this study can be found in GenBank, MK159018 to MK159025.

## AUTHOR CONTRIBUTIONS

HN designed the study. IW, AK, NA, DK, SB, FF, and HN carried out field and laboratory work. IW and HN prepared and analyzed the data. All authors interpreted the results and wrote the paper.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00460/full#supplementary-material>

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# Whole Genome Sequence Analysis Reveals Lower Diversity and Frequency of Acquired Antimicrobial Resistance (AMR) Genes in *E. coli* From Dairy Herds Compared With Human Isolates From the Same Region of Central Zambia

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Antibiotic treatment of sick dairy cattle is critical for the sustainability of this production system which is vital for food security and societal prosperity in many low and middle-income countries. Given the increasingly high levels of antibiotic resistance worldwide and the challenge this presents for the treatment of bacterial infections, the rational use of antibiotics in humans and animals has been emphatically recommended in the spirit of a “One Health” approach. The aim of this study was to characterize antimicrobial resistance (AMR) genes and their frequencies from whole genome sequences of *Escherichia coli* isolated from both dairy cattle and human patients in central Zambia. Whole genome sequences of *E. coli* isolates from dairy cattle ( $n = 224$ ) and from patients at a local hospital ( $n = 73$ ) were compared for the presence of acquired AMR genes. In addition we analyzed the publicly available genomes of 317 human *E. coli* isolates from over the wider African continent. Both acquired antibiotic resistance genes and phylogroups were identified from *de novo* assemblies and SNP based phylogenetic analyses were used to visualize the distribution of resistance genes in *E. coli* isolates from the two hosts. Greater acquired AMR gene diversity was detected in human compared to bovine *E. coli* isolates across multiple classes of antibiotics with particular resistance genes for extended-spectrum beta lactamases (ESBL), quinolones, macrolides and fosfomycin only detected in *E. coli* genomes of human origin. The striking difference was that the Zambian or wider African human isolates were significantly more likely to possess multiple acquired AMR genes compared to the Zambian dairy cattle isolates. The median number of resistance genes in the Zambian cattle cohort was 0 (0–1 interquartile range), while in the Zambian human and wider African cohorts the medians and interquartile ranges were 6 (4–9) and 6

(0–8), respectively. The lower frequency and reduced diversity of acquired AMR genes in the dairy cattle isolates is concordant with relatively limited antibiotic use that we have documented in this region, especially among smallholder farmers. The relatively distinct resistant profiles in the two host populations also indicates limited sharing of strains or genes.

**Keywords:** AMR, *E. coli*, antibiotic, cattle, dairy

## INTRODUCTION

Antimicrobial resistance (AMR), in particular to antibiotics, has placed a huge burden on public health delivery systems. AMR contributes to treatment failure or prolonged hospitalization of human patients and the global extent of the issue has been captured by WHO reports including a definition of the priority resistant pathogens (Tacconelli et al., 2013; World Health Organization [WHO], 2017a,b). These include multi-drug resistant Enterobacteriaceae, including *Escherichia coli*, which combined with its relative ease of isolation and capacity for gene exchange, makes it an important sentinel organism to understand the epidemiology of AMR in different environments and animal hosts. AMR is an increasing threat to human and animal life and this is exacerbated in regions of the globe by inadequate health facility infrastructures and lower sanitation and hygiene.

Antibiotic accessibility is variable across the planet, for both economic and legislative reasons. The emergence and spread of AMR in animals, humans and the environment is extremely complex and it is very difficult to demonstrate where reduced use would be most effective in terms of restricting AMR (Woolhouse et al., 2015). As a consequence, a precautionary principle is being applied in livestock (O'Neill, 2014), including legislation, rotational practices, and promotion of the use of disease preventive measures such as vaccines to reduce the use of antibiotics, especially those of last resort in the treatment of human infections. While this must be supported, antibiotics are critical for the effective treatment of livestock diseases and help preserve the economic viability of small-scale livestock practices in many low and middle income countries. As such there is an argument that “the genie is out of the bottle” and the sporadic use of 1st and 2nd generation antimicrobials for production animals to treat infections is critical and is unlikely to impact on human infection treatments (van Bunnik and Woolhouse, 2017). Toward this, more information is required about resistance genes in livestock species and humans in different settings across the globe.

In Zambia, farming at different scales is central to the country's economy and the livelihoods of many smallholders and their families and communities. There is limited information from Zambia on AMR levels in farmed livestock. In one recent study (Chishimba et al., 2016) just over 20% of *E. coli* isolates from chickens surveyed were shown to contain extended-spectrum  $\beta$ -lactamases (ESBLs) indicating they are a reservoir of important AMR genes, although the risk of strain and/or gene transfer to humans is unknown. Our recent work in central Zambia established that antibiotic use in the dairy sector was relatively well controlled and phenotypic AMR in bovine *E. coli* was

statistically associated with prescribing trends, use of introduced exotic (higher production) breeds, and treatment of lumpy skin disease but not bacterial mastitis (Mainda et al., 2015). Recent developments in sequencing have revolutionized the diagnosis of infectious and non-infectious diseases in public health and, when applied in the context of AMR, makes it possible to identify different resistance genes and also to generate refined dissemination genetic maps and study the phylogenetics of the resistomes of the microorganisms involved (Crofts et al., 2017; van Bunnik and Woolhouse, 2017; Baker et al., 2018). Here, the sequencing of *E. coli* isolates from dairy cattle from over 100 smallholdings/farms within a radius of 120 km around Lusaka provided an opportunity to analyze the AMR genotypes of the isolates. During this work, we were informed of a small-scale study collecting *E. coli* from human patients presenting with diarrhea at a referral hospital in Lusaka (University Teaching Hospital). This allowed a comparison of the frequency and diversity of acquired AMR genes associated with *E. coli* in the two hosts in this region. This analysis was then extended to include *E. coli* genomes from humans available from the Enterobase (Alikhan et al., 2018) database across from multiple African countries.

## MATERIALS AND METHODS

### Sample Selection and Whole Genome Sequencing

The cattle isolates were from a previously described study, with the main sampling from 376 dairy cattle covering 104 farms in Lusaka and surrounding areas over a 4-month period in early 2014 (Mainda et al., 2015). A total of 371 *E. coli* isolates were tested phenotypically for resistance to a panel of six different antibiotics and 61/371 (16.4%) were positive for resistance to at least one of the antibiotic classes. The resistance prevalence estimates were as published previously (Mainda et al., 2015) and the proportions of isolates resistant or susceptible to the tested antibiotics are shown as **Supplementary Data Sheet S1**. All 61 isolates exhibiting phenotypic resistance were sequenced to capture their resistance gene diversity. For comparison, a further 125 randomly sampled Zambian cattle *E. coli* isolates with no phenotypic resistance were also sequenced from the remaining 310 isolates. A previously published study (Mainda et al., 2016) examining the zoonotic threat of Shiga toxin positive *E. coli* from these Zambian cattle samples involved the sequencing of 41 of these isolates (without phenotypic resistance) and so these genomes were also included in our analysis. Overall there were 227/371 cattle isolates that were

genome sequenced and 224 were of sufficient quality for analysis of acquired AMR genes. This breakdown of *E. coli* strains for sequencing is shown as **Supplementary Data Sheet S2**. We appreciate that the *E. coli* isolates selected for sequencing from cattle overestimates the actual levels of phenotypic resistance (32.2% compared to 16.4%) but ensured that the bovine *E. coli* AMR gene diversity was captured and there was phylogenetic context for both resistant and sensitive bovine *E. coli* isolates.

*Escherichia coli* were also isolated from patients with diarrhea at the University Teaching Hospital in Lusaka using the methodologies applied to our cattle work (Maininda et al., 2015). Lusaka is the central urban environment to the cattle sampling area so we considered this population of isolates valid for examining relationships between resistance genes in cattle and human *E. coli* isolates in the study area. The human *E. coli* isolates ( $n = 79$ ), were collected between December 2014 and January 2015 and therefore are concurrent with the sampling frame for the cattle *E. coli* isolates (Maininda et al., 2015). Information on their phenotypes to tested antibiotics is provided as **Supplementary Data Sheet S1**. Informed consent was obtained from all subjects. All these isolates were submitted for genome sequencing, six did not provide DNA of sufficient quality for AMR gene analysis.

DNA preparation and sequencing methodologies are as published (Maininda et al., 2016). DNA was extracted from the *E. coli* isolates using the Qiagen DNA extraction kit as per manufacturer's instructions. The DNA was then quantified by Spectrophotometer® Nanodrop and sequenced at Edinburgh Genomics<sup>1</sup>. The Miseq Illumina platform was used for whole genome sequencing which will capture both plasmid and chromosome based sequences with 224 genomes of *E. coli* isolates from cattle and 73 genomes of *E. coli* from humans available for analysis. Quality control of sequence reads was performed using the software FASTQC (Andrews and Fast, 2010) and when necessary the trimming was done with cutadapt (Martin, 2011). The raw reads sequences were *de novo* assembled using Spades software (Bankevich et al., 2012).

In addition, for comparative purposes, genome sequences from 317 *E. coli* indicated to have been isolated from humans on the African continent were downloaded from Enterobase (Alikhan et al., 2018) and analyzed as for the Zambian cattle and human isolate sequences in this study. The downloaded sequences were from 15 countries, including Somalia, Egypt, Nigeria, Madagascar, Tanzania, Morocco, Burkina Faso, Senegal, Kenya, Algeria, Mali, Democratic Republic of the Congo, South Africa, Zambia and Gabon. The majority originate from Tanzania relating to studies on *E. coli* across livestock and human samples ( $n = 258$ ) (Moremi et al., 2016; Mshana et al., 2016). The isolates relating to these genomes were collected over a much wider timeframe, from 1973 to 2017. The project numbers for these downloaded genomes are provided as **Supplementary Data Sheet S3**.

The three sets of genomes in this study were then analyzed for the presence and absence of resistance genes. The resistance

genes were initially detected using the Short Read Sequence Type 2 (SRST2) software in a Linux environment (Inouye et al., 2014) but all analyses published here were carried out with outputs obtained for AMR genes within the ResFinder 3.0 database (Zankari et al., 2012). This version of the database included horizontally acquired resistance genes and not resistance conferred by mutations, for example in housekeeping genes. In the current study, acquired AMR genes were analyzed in the context of different *E. coli* phylogroups (Clermont et al., 2015) as *E. coli* has been traditionally clustered by phylogroup and these groupings have a good association with isolates being a commensal or pathogen. The phylotyping method described by Clermont et al. (2013) was performed *in silico*. In short, based on the presence or absence of 4 genes: *chuA*, *yjaA*, *tspE4.C2*, *arpA* isolates initially can be separated into 4 phylogroups (A, B1, B2, D). To further distinguish between groups and assign strains to an additional 4 phylogroups (C, E, F, and cryptic clades) it is necessary to check for the presence of a fifth gene *trpA* and/or distinguish the specific alleles for the above genes. The workflow is as published (Clermont et al., 2013). The identification of the genes and genetic fragments in the isolate genomes was carried out with blastn 2.2.28+ with sequence similarity and length coverage defined as 98 and 99%, respectively. After performing all the steps each *E. coli* sequence was assigned to one of the eight possible phylogroups.

## Statistics

Statistical analyses and visualization were performed in R 3.4.4<sup>2</sup>. *p*-values for resistant genes distribution in different population and in different phylogroups were obtained using “prop.test.” Co-occurrence analysis was carried out using R package “cooccur.” The relationships between different isolates were analyzed using RAxML 8.0.0 with 500 bootstrap samples and the results are presented as phylogenetic trees.

## RESULTS

### Diversity of AMR Genotypes in the Studied *E. coli* Genomes Cattle Isolates

As described in the Section “Materials and Methods,” a total of 224 *E. coli* whole genome sequences from cattle were analyzed, which included 61 isolates with established phenotypic resistances to capture resistance gene diversity from our initial pool of 371 cattle isolates (**Supplementary Data Sheet S1**; Maininda et al., 2015). One phenotypically resistant isolate produced poor quality reads and so was not included. Resistance genes were detected in 66/224 (29.5%) of the genomes, with 51/60 of these from the phenotypically resistant subset. There were 9/60 isolates that were resistant to the antibiotics tested but for which no resistance gene was detected. The six most common resistance genes detected were: *strB1* (19%), *sul2* (17%), *tetA* (16%), *strA4* (13%) *bla*<sub>TEM-1</sub> (13%), and *tetB* (10%). A detailed list of the

<sup>1</sup><http://genomics.ed.ac.uk/>

<sup>2</sup>[www.r-project.org/](http://www.r-project.org/)



different resistance genes and their frequencies are presented as **Supplementary Data Sheet S4**. The numbers and percentages of isolates with 0, 1–5, 6–10, and > 10 resistance genes, respectively, are shown **Figure 1** (Zambian Bovine–ZB).

### Human Isolates

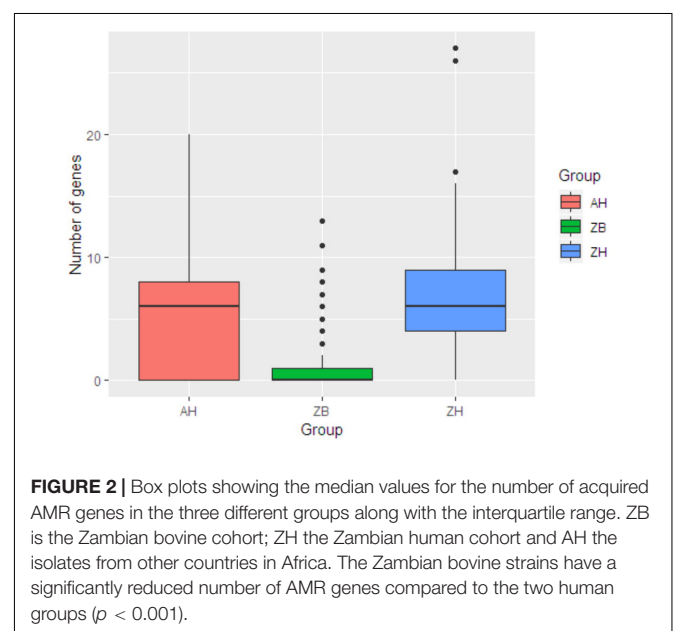
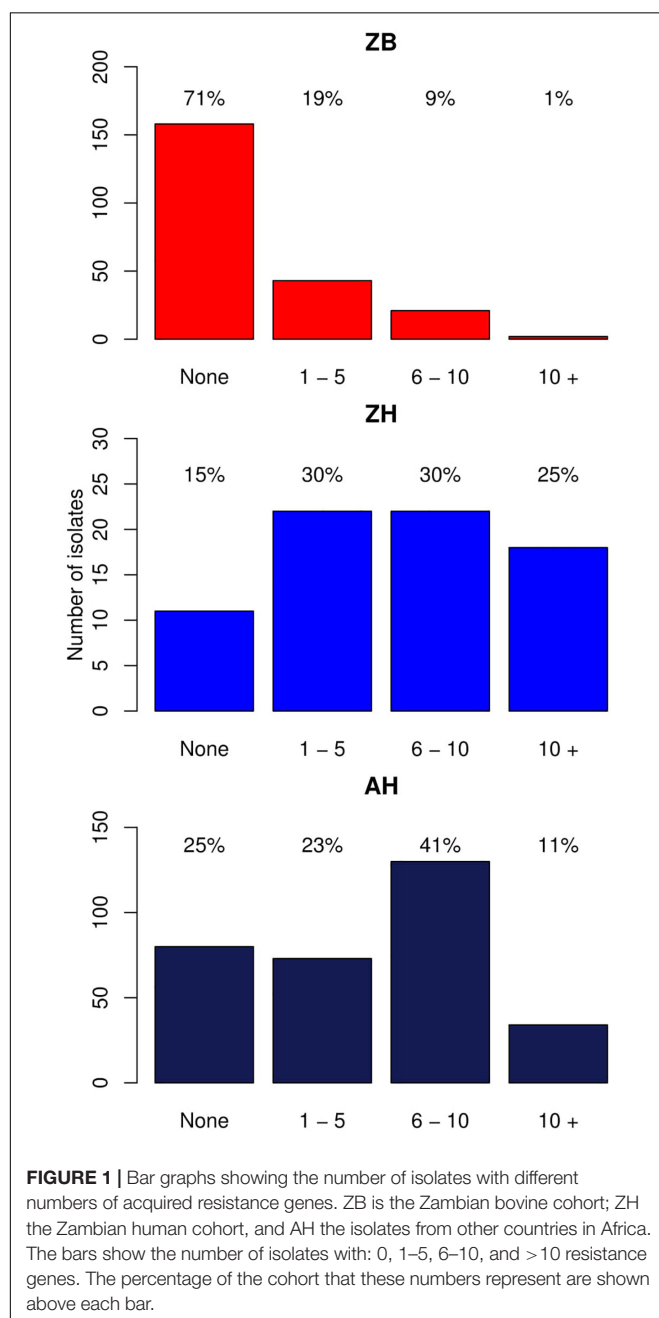
Whole genome sequences were analyzed for 73 human isolates from the Lusaka hospital. The phenotypic resistances and comparison where possible with the cattle isolates is presented as **Supplementary Data Sheet S1**. Where the same antibiotics were tested in both host groups (ampicillin, tetracycline, gentamicin, ciprofloxacin, and cefpodoxime) the human *E. coli* isolates

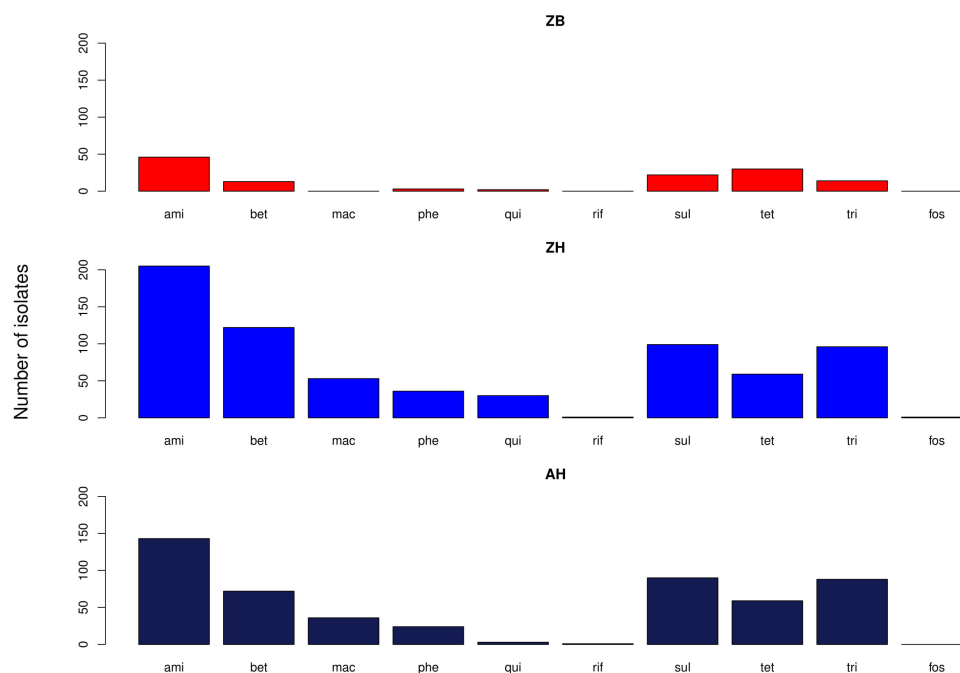
had significantly higher proportions of resistance than the cattle isolates ( $p < 0.0001$ ) for each one (**Supplementary Data Sheet S5**). Resistance genes were identified in 62/73 (84.9%) of the *E. coli* genomes from humans, although all human isolates had some level of phenotypic resistance to one or more of the antibiotics tested. The frequency and combinations of resistance genes were then analyzed as for the cattle isolates. The six most common resistance genes were: *sul2* (66%), *strB1* (64%), *strA4* (57%), *bla<sub>TEM-1</sub>* (56%), *aadA* (38%), and *tetA* (32%); gene patterns and frequencies are shown in **Supplementary Data Sheet S5**.

This was then compared with resistance genes present in 317 human isolates submitted to Enterobase that were from the African continent. From these 237/317 (74.8%) had at least one resistance gene and the patterns plus frequencies are shown in **Supplementary Data Sheet S6**. The numbers and percentages of isolates with 0, 1–5, 6–10, and > 10 resistance genes are plotted in **Figure 1** (Zambian Human–ZH and African Human–AH). Despite the sub-sampling bias for WGS toward phenotypically resistant cattle *E. coli* isolates, the carriage of multiple AMR genes was significantly higher in *E. coli* populations from humans (Zambian or African) than in isolates of dairy cattle origin ( $p < 0.001$ ) (**Figure 2**). The human isolates were therefore much more likely to encode multiple resistance genes.

### Comparison of Acquired AMR Genes From Analyzed Genomes of Cattle and Human *E. coli* Isolates

The proportion of isolates in the different host groups encoding each of the main antibiotic classes was then analyzed (**Figure 3**). While the general pattern is similar, the overall proportions in the cattle population were lower. Determinants of aminoglycoside resistance were the most frequent gene type identified in either cattle or human *E. coli* populations. Macrolide resistance was





**FIGURE 3 |** Bar graphs showing the number of isolates in each of the three groups with genes relating to resistance of the specified antibiotic groups. ami – aminoglycosides; bet – Beta-lactams; mac – macrolides; phe – phenolics; qui – quinolones; rif – rifampicin; sul – sulphonamides; tet – tetracyclines; tri – trimethoprim. ZB is the Zambian bovine cohort; ZH the Zambian human cohort and AH the isolates from other countries in Africa.

relatively absent in the cattle population while, by contrast, tetracycline resistance was over-represented in the cattle isolates (Figure 3). The presence of multiple acquired resistance genes in the same strains and the possibility of particular combinations is important for clinical and molecular epidemiology and to guide antibiotic treatment in a region. In the genomes of *E. coli* isolates from the sampled Zambian dairy cattle, there were 42 different resistance genotype patterns (Supplementary Data Sheet S4) compared with 52 in the Lusaka human isolates (Supplementary Data Sheet S5) and 138 in the wider African human *E. coli* isolates (Supplementary Data Sheet S6). Most of these patterns were not repeated showing the diversity of AMR genotypes captured in this analysis. The most common positive or negative co-associations for resistance genes are shown for the Zambian cattle and Zambian human groups (Figures 4A,B). There were 58 paired co-associations in the Zambian human isolates compared with 14 in the bovine isolates with only 3 being shared between the two groups, these were: *blaTEM-1B* with *strB*; *blaTEM-1B* with *dfrA8*; *strA* with *strB*. This analysis demonstrates the distinct resistance profiles in the *E. coli* strains from the two different hosts and that co-associations are much more likely in human strains that encode multiple resistance genes.

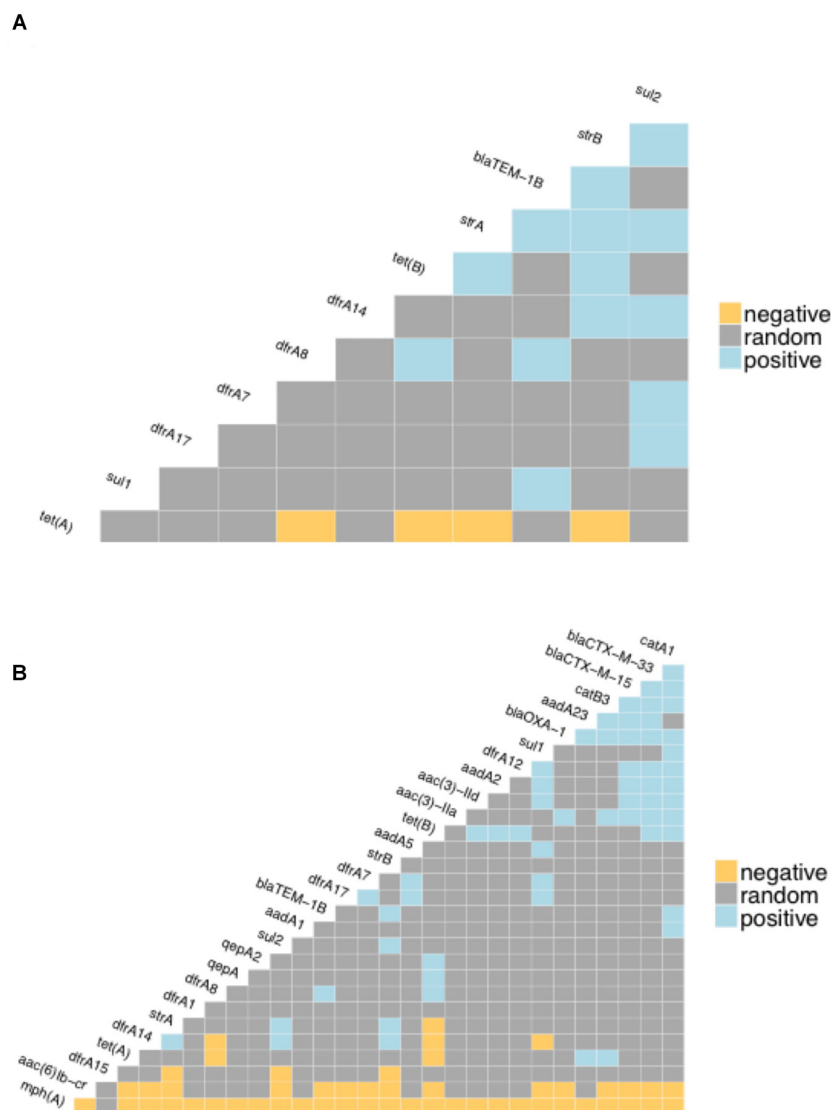
## Phylogenomic Assessment of the Resistome of *E. coli* Isolated From Cattle and Humans

The genomes from the two Zambian groups were clustered according to single nucleotide polymorphisms in core genes

(Figure 5). The genomes from the Zambian human *E. coli* isolates tended to cluster together often away from those of cattle origin and this in part reflects differences in phylogroups between the two Zambian sample sets (Figure 6). The distribution and number of acquired resistance genes were then plotted on to the relationship tree and this provides a clear visual representation of the multiple resistance genes in the Zambian human compared to the bovine *E. coli* isolates (Figure 5). The majority of cattle *E. coli* with resistance genes were assigned to phylogroup B1 indicating that these isolates were largely commensals (Figure 6). However, the isolates with multiple AMR resistance genes from the Zambian human isolates were distributed across the major phylogroups including those associated with more pathogenic strains (Phylogroups B2 and D). The human strains originating from across Africa followed a similar trend to the group of Zambian human isolates although no phylogroup D strains were detected. It is striking that the bovine *E. coli* isolates in phylogroups associated with pathogenesis, B2 and D, had negligible AMR levels in contrast to the human isolates in these groups (Figure 6). Conversely, the proportion of isolates with AMR genes in the “commensal” B1 cluster was significantly higher for the two sets of sequenced human isolates compared to the sequenced bovine isolates.

## DISCUSSION

Our previous study examined antibiotic use in cattle on different sized dairy units, including smallholdings in a region

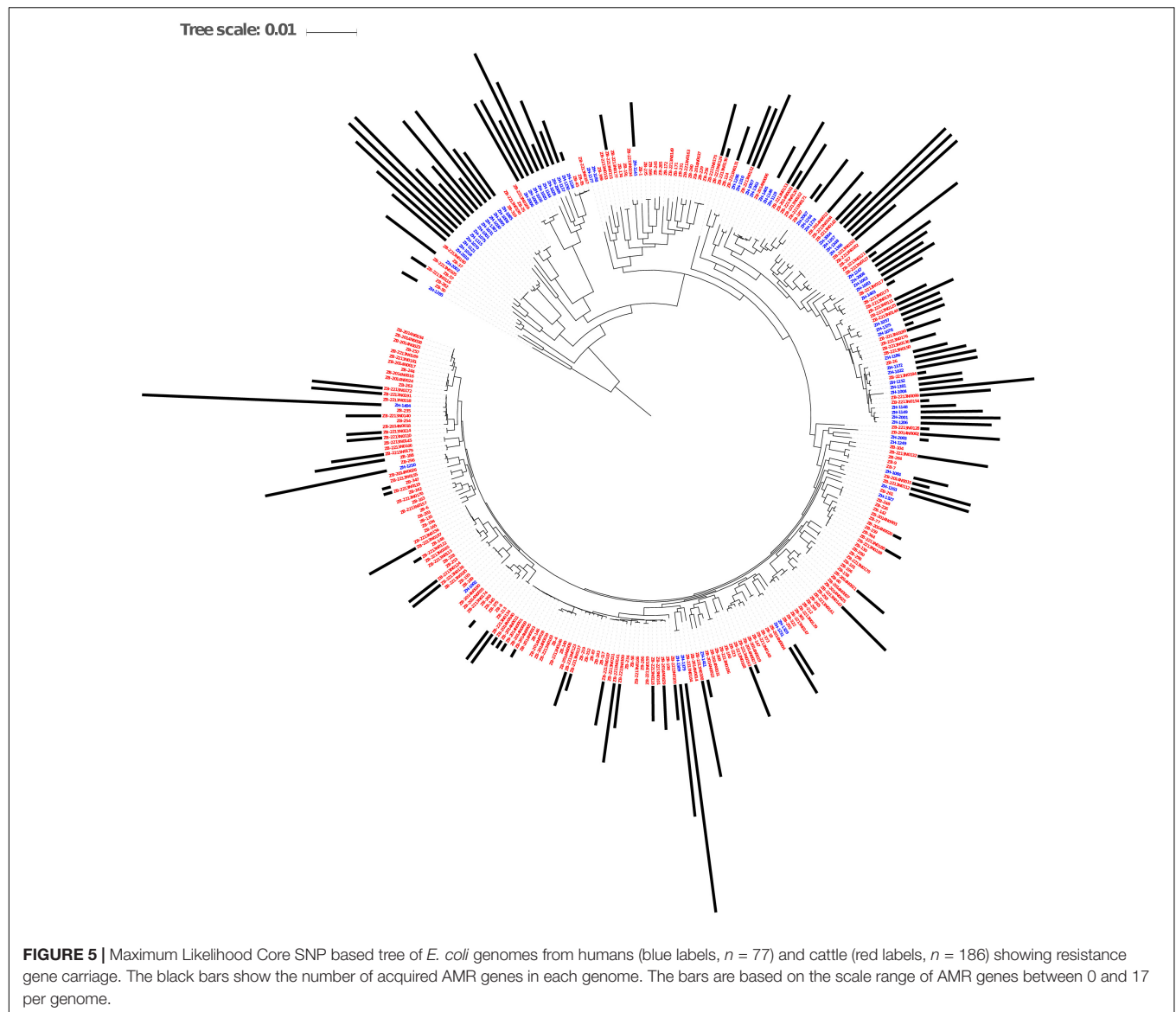


**FIGURE 4 |** Co-occurrence matrices for acquired antibiotic resistance genes. The matrices show the likelihood of negative or positive co-occurrence of the specified resistance genes among: **(A)** the Zambian bovine *E. coli* genomes; **(B)** the Zambian human genomes. The analysis was carried with the  $p < 0.05$  as the threshold for negative or positive.

surrounding Lusaka in Zambia. That study demonstrated phenotypic resistance commensurate with tetracycline and penicillins as the main antibiotics used in cattle in this region, with the majority of isolates exhibiting no resistance to the antibiotics tested (Mainda et al., 2015). All resistant isolates and randomly selected non-resistant isolates from our previous study were sequenced here to obtain genomic information in relation to the acquired resistance genes present. During the study we were also able to obtain a group of human *E. coli* isolates selected from people presenting with diarrhea at a University Teaching Hospital in Lusaka over the same timeframe (2014). Phenotypic testing for resistance demonstrated significantly higher levels of resistance amongst the human isolates when the same antibiotics were tested (**Supplementary Data Sheet S1**). Sequencing of

these isolates then provided an opportunity to compare the type and distribution of acquired resistance genes in *E. coli* isolates originating from the two hosts. While we appreciate the caveats of such an analysis, especially the relatively small number of human isolates and their *post hoc* addition, we consider the comparative analysis has real value to address questions around resistance gene epidemiology in *E. coli* as a sentinel species in the two hosts from this area of Africa. To bolster the analysis, we have also included comparison with 317 *E. coli* genomes isolated from humans in different African countries. In general, these have provided similar results to the Zambian human cohort which we consider as a validation of this study.

While we would not anticipate *E. coli* to be the aetiological agent responsible for diarrhea in the majority of patients, it would



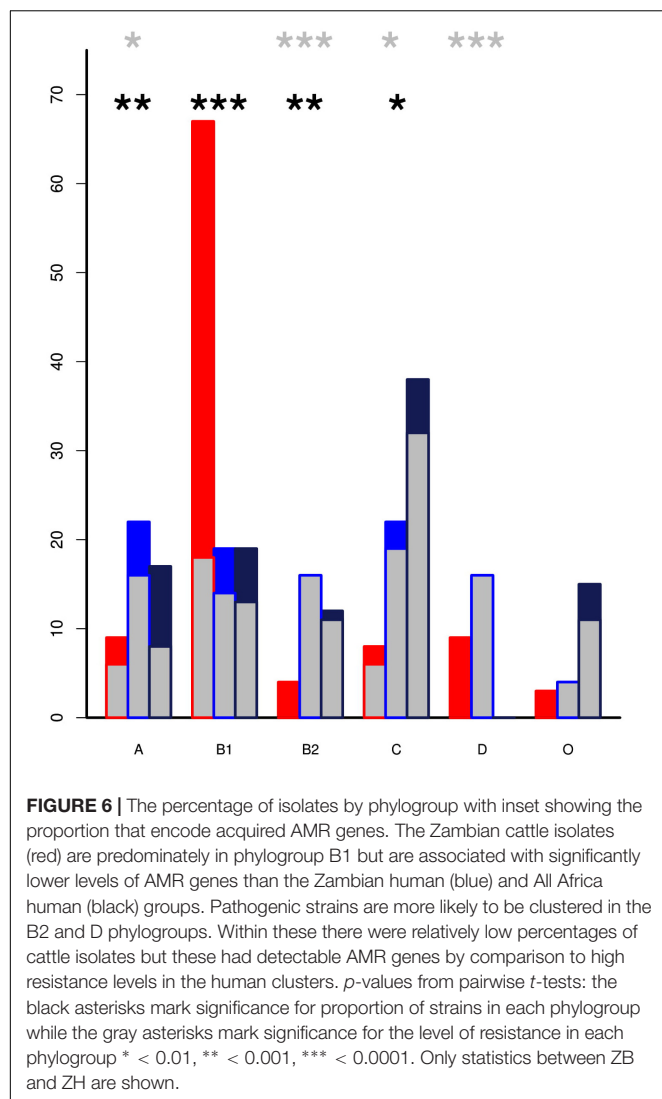
be reasonable to expect that the human cohort may contain a higher proportion of potentially pathogenic *E. coli* compared to the cattle set. This was apparent from an analysis of the phylogroups of the isolates (**Figure 6**) with, in fact, both human cohorts containing higher proportions of phylogroups associated with pathogenic strains (B2 and/or D) when compared with the cattle isolates which were predominately phylogroup B1, more associated with a commensal existence. While we do not have the antimicrobial treatment data for the Zambian human isolates we would anticipate that we are still capturing many isolates in these groups that are not associated with disease.

Despite the pre-selection of all phenotypically resistant isolates for sequencing in the cattle set it was evident that resistance genes were significantly more frequent in the *E. coli* isolates from the Zambian human population compared to *E. coli* isolates from local dairy cattle. The majority of human isolates encoded resistance genes in stark contrast to the cattle isolates

and the human isolates carried a significantly higher mean number of resistance genes than the cattle isolates (**Figure 1**). As a consequence, *E. coli* isolates encoding multiple drug resistance were much more common in the human cohort. The human isolates also had a significantly higher frequency of acquired AMR genes compared to the cattle isolates even when considering those assigned only to phylogroup B1, generally associated more with commensal strains.

There has been considerable debate over the emergence of AMR in humans that may be linked to antibiotic use in production animals (Goodyear, 2002; Gillings, 2013; O'Neill, 2014; Woolhouse et al., 2015; van Bunnik and Woolhouse, 2017). Whilst this study covers only one area of one country, it is evident from our findings that the resistance genotypes present in the human isolates were much more diverse than those found in cattle with resistance genes identified in the human isolates not found in cattle isolates. In general there was no clear evidence





to indicate exchange of combined AMR genes between the two hosts or sharing of specific resistant strains. However, many AMR genes are present on highly mobilizable genetic elements including plasmids (Woolhouse et al., 2015) so ready exchange of these to generate different AMR combinations in different strains can occur and the original sources of such genes is virtually impossible to define. Beyond this and a study in poultry in Zambia (Chishimba et al., 2016) the significance of other reservoirs of AMR in the region have not been investigated. In addition, while we have collected data on the types of antibiotics used in the cattle population (Mainda et al., 2015), we do not have equivalent data for other production systems and the human population in Lusaka and so the selection pressures in these communities on AMR by antibiotic use are not defined (Mshana et al., 2013).

Despite this lack of information our study indicates that the *E. coli* of human origin and those of cattle origins are under different and largely independent AMR selection pressures. This is evidenced by the presence of rare but important

clinical AMR genes encoding beta lactamases such as *bla*<sub>NDM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub> and fluoroquinolones resistance encoding genes *qnr*, *aac* in *E. coli* of human origin that were absent in the *E. coli* of cattle origin. However, the resistance genes encoding older and commonly applied antibiotics such as *tetA* and *tetB* for tetracycline, *strA* and *strB* for streptomycin, *sul1* and *sul2* for sulphonamides and *dfrA* for trimethoprim were detected in both populations, which is an indication that such resistances are common. However, such selection could still be driven by independent pressures in each population rather than cross-over between them. It is also useful evidence that certain clinically important antibiotics such as cephalosporins and fluoroquinolones are still being mostly used in the treatment of human infection and rarely used in dairy cattle of the study area. This could be attributed to the high cost of such antibiotics when compared to the first generation antibiotics (tetracycline, penicillin, and sulphonamides) which are less expensive and the ones commonly used in cattle (Mainda et al., 2015). Further, the human hosts were likely to have been treated with different types of antibiotics than the cattle host as shown by the wide diversity of resistance genes.

A recent study that examined antibiotic resistance in atypical enteropathogenic *Escherichia coli* from both sub-Saharan Africa and Asia (Ingle et al., 2018) indicated high levels of AMR in these human isolates concordant with our study. In particular they noted certain co-associations, particularly of *strA* and *strB* with *sul2*, with *bla*<sub>TEM</sub> and *dfrA*<sub>14</sub> being linked in a further subset. These associations match those shown to be prevalent in our study in both cattle and human isolates and probably indicate the longer term evolution of combined resistances to antibiotics that have been applied over longer timeframes.

More studies need to be carried out to analyze the distribution of strains and resistance genes in species that are in physical co-association as even in the context of *E. coli* we are still trying to determine if AMR transfer is primarily one of strain acquisition and strain maintenance in that host or, perhaps more likely, temporary transmission to a new host and then dissemination of the AMR gene(s) to a more host-adapted strain. This dynamic will vary depending on the bacterial species being investigated, but species that we know can be promiscuous and zoonotic, such as *E. coli*, are a logical point of study until metagenome sequencing costs allow a wider picture of AMR dynamics in complex populations to be gathered. Even then, better methods need to be developed to associate AMR genes with the carrying organism.

The limited sharing of strains and resistance genes between the dairy cattle and human populations in the study area and sampling interval serves as an important reminder of the challenge of establishing transmission routes, and of attributing the AMR problem in humans to antibiotics use in farmed animals. As genome sequence data accrue it should be feasible to understand the direction, nature and frequency of gene flow between animal and human populations in greater detail. At the same time, metadata related to antibiotic use will be key to

understand the selective pressures and impact of management strategies. Our main message from this study is that some agricultural sectors are behaving responsibly through both choice and necessity which helps maintain the incredible value of many antimicrobials for treatment of livestock diseases which reduce disease, improve animal welfare and help maintain the livelihoods of the many smallholders and farmers which are dependent on their productivity.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the, Tropical Diseases Research Centre (TDRC) Ethics Review Committee guidelines at [https://healthresearchweb.org/en/zambia/ethics\\_1007](https://healthresearchweb.org/en/zambia/ethics_1007), with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The study involving human isolates was approved by the “Tropical Diseases Research Centre Ethical Committee” Clearance #: STC/2015/12. The animal sampling study was carried out in accordance with the recommendations of the University of Edinburgh’s “Animal Welfare and Ethics Committee” (AWERB) at <https://www.ed.ac.uk/research/animal-research/animal-welfare-ethics> and the protocol was approved by the UoE AWERB. The project work also aligned to permissions under a licence to Professor Mark Stevens: Mark Stevens PPL 60/4420.

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## AUTHOR CONTRIBUTIONS

GM, LS, PB, MS, BB, JM, and DG conceived and planned the experiments. GM, LS, DG, SM, and GK carried out the field work and microbiology. NL, GM, ER, and PB carried out the main sequence and data analyses. GM, NL, and DG contributed to the interpretation of the results. GM and DG took the lead in writing the manuscript with most figures produced by NL. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01114/full#supplementary-material>

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# The Emergence of Chromosomally Located *bla*<sub>CTX-M-55</sub> in *Salmonella* From Foodborne Animals in China

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The emergence and increase in prevalence of resistance to cephalosporins amongst isolates of *Salmonella* from food animals imposes a public health threat. The aim of the present study was to investigate the prevalence and characteristics of CTX-M-producing *Salmonella* isolates from raw meat and food animals. 27 of 152 (17.76%) *Salmonella* isolates were ESBL-positive including 21/70 (30%) from food animals and 6/82 (7.32%) from raw meat. CTX-M-55 was the most prevalent ESBL type observed (12/27, 44.44%). 7 of 12 CTX-M-55-positive *Salmonella* isolates were *Salmonella* Indiana, 2 were *Salmonella* Typhimurium, 2 were *Salmonella* Chester, and the remaining isolate was not typeable. Eight CTX-M-55-positive *Salmonella* isolates were highly resistant to fluoroquinolones (MIC<sub>CIP</sub> = 64 µg/mL) and co-harbored *aac*(6')-Ib-cr and *oqxAB*. Most of the CTX-M-55 positive isolates (11/12) carried *bla*<sub>CTX-M-55</sub> genes on the chromosome, with the remaining isolate carrying this gene on a transferable 280 kb IncHI2 plasmid. A chromosomal *bla*<sub>CTX-M-55</sub> gene from one isolate transferred onto a 250 kb IncHI2 plasmid which was subsequently conjugated into recipient strain J53. PFGE and MLST profiles showed a wide range of strain types were carrying *bla*<sub>CTX-M-55</sub>. Our study demonstrates the emergence and prevalence of foodborne *Salmonella* harboring a chromosomally located *bla*<sub>CTX-M-55</sub> in China. The co-existence of PMQR genes with *bla*<sub>CTX-M-55</sub> in *Salmonella* isolates suggests co-selection and dissemination of resistance to both fluoroquinolones and cephalosporins in *Salmonella* via the food chain in China represents a public health concern.

**Keywords:** chromosomal, *bla*<sub>CTX-M-55</sub>, *Salmonella*, Indiana, transfer

## INTRODUCTION

*Salmonella* species are the second most common bacterial cause of foodborne gastroenteritis worldwide and almost 80.3 million foodborne illness per year are caused by non-typhoid *Salmonella* infections (Majowicz et al., 2010). Extended-spectrum cephalosporins (ESCs) are effective drugs of choice in children for treatment of non-typhoid salmonellosis, due to the contraindication for use in children of fluoroquinolones (FQs), the classical first-line antibiotics. The emergence of *Salmonella* isolates resistant to ESC is a worldwide public health concern (Arlet et al., 2006). Resistance to these



drugs is mainly mediated by the bacterial production of extended-spectrum  $\beta$ -lactamases (ESBLs) with CTX-M-type enzymes being the most common.

CTX-M genes have successfully disseminated globally and are common in clinical settings, communities, livestock and companion animals. There are many CTX-M variants of which, CTX-M-15 and CTX-M-14 are the most prevalent (Zhao and Hu, 2013; de Jong et al., 2014). However, the epidemiology of CTX-M-type ESBLs is evolving rapidly. A number of minor allelic variants have been described and classified as belonging to one of six groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and KLUC, named after the archetypal enzymes of each group) that differ from each other by  $\geq 10\%$  amino acid residues (D'Andrea et al., 2013).

CTX-M-55 is a CTX-M-15 variant that contains a substitution of A80V within the  $\beta$ -lactamase possessing enhanced cephalosporin-hydrolyzing activity (He et al., 2015) and has been detected as increasing rapidly in prevalence, especially in *Escherichia coli* from animals (Zheng et al., 2012; Cunha et al., 2017; Norizuki et al., 2018). Whilst the *bla*<sub>CTX-M-55</sub> gene is less commonly detected in *Salmonella* from animals or humans, the first report was from human isolates in the United States and China in 2011 (Sjolund-Karlsson et al., 2011; Yu et al., 2011). Since then CTX-M-55 producing *Salmonella* have been identified from a number of different serotypes from Switzerland (Gallati et al., 2013), Japan (Imoto et al., 2014), China (Wong et al., 2015), Korea (Kim et al., 2017), Denmark (Torpdahl et al., 2017), and Thailand (Luk-In et al., 2018). CTX-M-55 carrying isolates from animals have been isolated from fish, pork and chicken (Nguyen et al., 2016; Nadimpalli et al., 2018). Since CTX-M-55 *Salmonella* isolates are increasingly detected and show high-level resistance to ESCs and are often cross-resistant to FQs, these *Salmonella* strains represent a potentially severe clinical and food safety issues and this warrants investigation of the prevalence of *bla*<sub>CTX-M-55</sub>-harboring *Salmonella*.

Carriage of CTX-M genes is mostly associated with a diverse set of transmissible plasmids (Canton et al., 2012). However, a small number of chromosomal CTX-M genes have been identified in several studies in *E. coli* where transfer into the chromosome was mediated by transposons or insertion sequences (Fabre et al., 2009; Hamamoto and Hirai, 2018).

In the present study, we investigated the prevalence of CTX-M-type ESBL-producing *Salmonella* isolates from food animals and raw meat in Guangdong province during 2015 and 2017, analyzed the characteristics of these CTX-M-55-positive strains including phenotypes, genotypes, genetic relatedness, and plasmid profiles.

## MATERIALS AND METHODS

### Sample Collection and *Salmonella* Isolation, Identification

A total of 891 specimens were collected from the Guangdong province of China between 2015 and 2017. Of these samples, 453 fecal swabs from free-range food animals (84 from chickens, 249 from ducks, 107 from pigs, and 13 from geese) were obtained

from veterinary clinics. 438 raw meat samples (156 from chicken meat, 35 from duck meat, and 247 from pork) were collected from different supermarkets. Fecal swabs and meat samples (cut into pieces) were placed into sterile selenite cysteine broth and incubated for 24 h at 37°C. Aliquots were then streaked on chromogenic medium selective for *Salmonella* (CHROMagar Microbiology, France) and incubated for another 24 h at 37°C. One purple colony was selected from each plate and then confirmed using the API20E system (bioMérieux, Marcy L'Étoile, France) and identified by MALDI-TOF MS (Axima-Assurance-Shimadzu). All isolates identified as *Salmonella* were stored at  $-80^{\circ}\text{C}$  in Luria-Bertani (LB) broth containing 30% glycerol.

### Antimicrobial Susceptibility Testing, Detection of ESBL Genes and ESBL Production Verified by Phenotype

The minimum inhibitory concentrations (MICs) of cefotaxime (CTX) and ciprofloxacin (CIP) were determined in triplicate for each bacterial strain using the agar dilution method on Mueller-Hinton agar plates according to the CLSI reference method (CLSI-M100-S26). *E. coli* ATCC 25922 was used as the quality control strain. *Salmonella* isolates showing resistance to cefotaxime (with MIC  $\geq 4$   $\mu\text{g/mL}$ ) were screened for the presence of the ESBL-genes *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA</sub>, *bla*<sub>CTX-M</sub> and *bla*<sub>CMY-2</sub> by PCR using the primers and conditions described previously (Jiang et al., 2012). Amplified PCR products were submitted to BGI Life Tech Co., Ltd. (Beijing, China) for DNA sequencing and the identity of specific  $\beta$ -lactamase genes were determined using the protein BLAST algorithm<sup>1</sup>. Double disk synergy tests were performed to further verify ESBL production by using a central amoxicillin/clavulanic acid (AMC) disk, 15 and 20 mm (center to center) separately away from cefotaxime disks. Synergy was interpreted by a clear-cut enlargement of the inhibition zone of CTX disk near the inhibition zone of AMC disk (Jarlier et al., 1988).

### Detection of PMQR Genes, Mutations Within Quinolone Resistance-Determining Region (QRDR) of Target Genes and Serotyping of CTX-M-55-Positive Isolates

The presence of PMQR genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, *qepA*, and *oqxAB* from *bla*<sub>CTX-M-55</sub>-positive isolates was also investigated by PCR using primers and conditions as previously described (Jiang et al., 2012). Mutations in QRDRs of the target genes *gyrA*, *gyrB*, *parC*, *parE* were confirmed by PCR and sequencing and their DNA sequences were compared with the *Salmonella* Typhimurium LT2 genome as a reference.

CTX-M-55-producing *Salmonella* isolates were serotyped using *Salmonella* specific O and H antigens (Statens Serum Institute, Denmark) by the slide agglutination test according to the Kauffmann-White scheme.

<sup>1</sup><https://blast.ncbi.nlm.nih.gov>

## Pulsed-Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST)

Genetic relatedness of all *bla*<sub>CTX-M-55</sub>-harboring isolates were analyzed by pulsed-field gel electrophoresis (PFGE) of XbaI-digested genomic DNA using a CHEF-MAPPER System (BioRad Laboratories, Hercules, CA, United States) as previously described (Jiang et al., 2014). PFGE patterns were compared using the Dice similarity coefficient with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

MLST was carried out by PCR and DNA sequence analysis of 7 housekeeping genes *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA* to determine the allelic profiles using software available at <http://mlst.warwick.ac.uk/mlst/dbs/Senterica>.

## Conjugation Experiments and Plasmid Analysis

Conjugation experiments of *bla*<sub>CTX-M-55</sub> gene positive *Salmonella* isolates were conducted by liquid mating in LB broth using sodium azide-resistant *E. coli* J53 as the recipient strain. Transconjugants were selected on MacConkey agar containing cefotaxime (2 µg/mL) and sodium azide (300 µg/mL). The presence of *bla*<sub>CTX-M-55</sub> in transconjugants was verified by PCR and sequencing as described. PFGE analysis was conducted using S1 nuclease (Takara Biotechnology, Dalian, China) digested genomic DNA as previously described (Barton et al., 1995) to identify the genetic location of *bla*<sub>CTX-M</sub> genes. Primers used for *bla*<sub>CTX-M</sub>-probes were the same as those used to amplify CTX-M encoding genes. The resulting gels were analyzed by Southern blotting after transfer to Hybond-N+ membranes (GE Healthcare, Little Chalfont, United Kingdom) and probing with a DIG-labeled *bla*<sub>CTX-M</sub> gene fragment according to the manufacturer's instructions (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Applied Science, Mannheim, Germany). Restriction fragments of agarose-embedded DNA of strain H9812 digested with XbaI (Takara) at 37°C for 4 h was used as DNA size marker during electrophoresis.

## Whole Genome Sequencing

To characterize the genetic context of *bla*<sub>CTX-M</sub> genes DNA from selected isolates was used to sequence whole genomic content. This was done by MajorBio Co., Shanghai, China. The resulting reads were trimmed and genomes assembled using "SPAdes v 3.11.0" (Nurk et al., 2013), annotated using "Prokka v 1.13" (Seemann, 2014) and mapped reads against reference genomes using "Bowtie2 v 2.3.4.3" (Langmead and Salzberg, 2012). Annotations and alignments were visualized in "Artemis."

## RESULTS

### *Salmonella* Isolation and Antimicrobial Susceptibility Phenotypes

We collected 891 samples for this study and 152 *Salmonella* were identified by MALDI-TOF MS of which, 46.05% (*n* = 70) were isolated from animals (13 from chickens, 43 from ducks, 11

from pigs, and 3 from geese). The remaining 82 samples came from raw meat (including 37 chicken meat, 5 duck meat, and 40 pork supermarket samples). The isolation rate of *Salmonella* strains from raw meat (17.90%) was similar to that from the farm animals (15.45%).

We also examined susceptibility of the 152 *Salmonella* isolates to cefotaxime and ciprofloxacin. Resistance rates to cefotaxime and ciprofloxacin were 26.97% (*n* = 41) and 30.26% (*n* = 46), respectively. In the 70 *Salmonella* isolates from animals, 30 displayed cefotaxime resistance (42.86%) and 34 ciprofloxacin resistant (48.57%). The 82 *Salmonella* isolates from raw meat, contained 11 isolates resistant to cefotaxime (13.43%) and 12 resistant to ciprofloxacin (14.63%). The rate of cross-resistant to both antibiotics were significantly higher for the animal isolates (32.86%) than for raw meat (4.88%).

### ESBL Characterization and Production, Serotyping of CTX-M-55-Producing Isolates

A total of 27 CTX-M ESBLs producing isolates were confirmed among the *Salmonella* isolates. Of these, 21/70 (30%) were from animals and 6/82 (7.32%) were from meat. We found that 12/27 (44.44%) were CTX-M-1 group members and all were confirmed as being *bla*<sub>CTX-M-55</sub>. There were also 12/27 that belonged to the CTX-M-9 group and were assigned as *bla*<sub>CTX-M-27</sub> (*n* = 8), *bla*<sub>CTX-M-14</sub> (*n* = 2) and *bla*<sub>CTX-M-65</sub> (*n* = 2). The remaining 3 CTX-M-encoding genes were all identified as the hybrid allele *bla*<sub>CTX-M-64</sub>. We also found that 14/41 cefotaxime-resistant isolates were CTX-M negative.

Of the *bla*<sub>CTX-M-55</sub> positive isolates, 10/12 were from animals and the remaining 2 were from meat samples. A clear-cut extension of the edge of the inhibition zone of CTX disk toward the AMC disk was seen from each *bla*<sub>CTX-M-55</sub> carrying *Salmonella* strain, consistent with ESBL production. These isolates were also serotyped and 7 were *S. Indiana*, 2 *S. Typhimurium*, 2 *S. Chester* and 1 was untypeable (Table 1).

### Detection of Ciprofloxacin Resistance Mechanisms in CTX-M-55-Producing Isolates

Multiple QRDR mutations in *gyrA* and *parC* were detected in the high-level quinolone resistant isolates that co-harbored *aac*(6')-Ib-cr and *oqxAB*. However, strains with an MIC<sub>CIP</sub> in the 0.5 to 4 µg/mL range did not contain mutations in the QRDRs of target genes, but all contained *qnrS* (Table 1).

The 12 CTX-M-55 producers were all ciprofloxacin resistant and 8 exhibited high-level resistance (MIC<sub>CIP</sub> = 64 µg/mL), 4 isolates demonstrated lower resistance (MIC<sub>CIP</sub> = 0.5–4 µg/mL). For PMQR determinants, 11 of 12 *bla*<sub>CTX-M-55</sub>-positive isolates were found to harbor at least one PMQR gene. The combination of *aac*(6')-Ib-cr + *oqxAB* (*n* = 7) dominated followed by *qnrS* (*n* = 3) and *oqxAB* + *qnrS* (*n* = 1). One isolate completely lacked any PMQR genes (Table 1).

TABLE 1 | Characteristics of *bla*<sub>CTX-M-55</sub> gene-harboring *Salmonella* isolates.

Strains	Serotype	Year	Sources	Cephalosporins resistance characterization			Quinolone resistance characterization				
				MIC <sub>CTX</sub>	β-lactamase genes	<i>bla</i> <sub>CTX-M-55</sub> location	MIC <sub>CIP</sub>	PMQR genes	Mutations in GyrA/GyrB	Mutations in ParC/ParE	MLST
HZP3	Typhimurium	2016	pork	128	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>TEM-1</sub>	1	<i>oxyAB</i> + <i>qnrS</i>	–/–	–/–	ND
LWP4	Typhimurium	2016	pork	128	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>TEM-1</sub>	0.5	<i>qnrS</i>	–/–	–/–	ST34
OJM1	Indiana	2017	Chicken meat	256	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>OXA-1</sub>	64	<i>aac</i> (6')- <i>lb-cr</i> + <i>oxyAB</i>	S83F D87N/–	T57S S80R/–	ST17
PJM1	ND	2017	Chicken meat	128	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>TEM-1</sub>	4	<i>qnrS</i>	–/–	T57S/–	ST321
OYM4	Indiana	2017	duck	256	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>OXA-1</sub>	64	<i>aac</i> (6')- <i>lb-cr</i> + <i>oxyAB</i>	S83F D87N/–	T57S S80R/–	ST17
OYM6	Indiana	2017	duck	256	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>OXA-1</sub>	64	<i>aac</i> (6')- <i>lb-cr</i> + <i>oxyAB</i>	S83F D87N/–	T57S S80R/–	ST17
OYM8	Indiana	2017	duck	256	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>OXA-1</sub>	64	<i>aac</i> (6')- <i>lb-cr</i> + <i>oxyAB</i>	S83F D87N/–	T57S S80R/–	ST17
OYM9	Indiana	2017	duck	256	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>OXA-1</sub>	64	<i>aac</i> (6')- <i>lb-cr</i> + <i>oxyAB</i>	S83F D87N/–	T57S S80R/–	ST17
OYM10	Chester	2017	duck	256	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>OXA-1</sub>	64	–	D87N/–	T57S S80R/–	ST343
OYM13	Indiana	2017	duck	256	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>OXA-1</sub>	64	<i>aac</i> (6')- <i>lb-cr</i> + <i>oxyAB</i>	S83F D87N/–	T57S S80R/–	ST17
OYZ3	Indiana	2017	duck	256	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>OXA-1</sub>	64	<i>aac</i> (6')- <i>lb-cr</i> + <i>oxyAB</i>	S83F D87N/–	T57S S80R/–	ST17
OYZ4	Chester	2017	duck	128	<i>bla</i> <sub>CTX-M-55</sub>	<i>bla</i> <sub>TEM-1</sub>	4	<i>qnrS</i>	–/–	–/–	ST27

ND, not determined.

## Genetic Relatedness and Molecular Typing Analysis of CTX-M-55-Producing Isolates

The 12 CTX-M-55 isolates produced 11 different profiles that were divided into 9 different PFGE clusters designated 1–9 with 85% genetic similarity. Five MLST profiles were determined including ST17, ST27, ST34, ST321, and ST343. ST17 was most prevalent ( $n = 7$ , 58.3%), ST27, ST34, ST321, and ST343 were represented by one isolate each and no ST could be determined for one isolate (Figure 1).

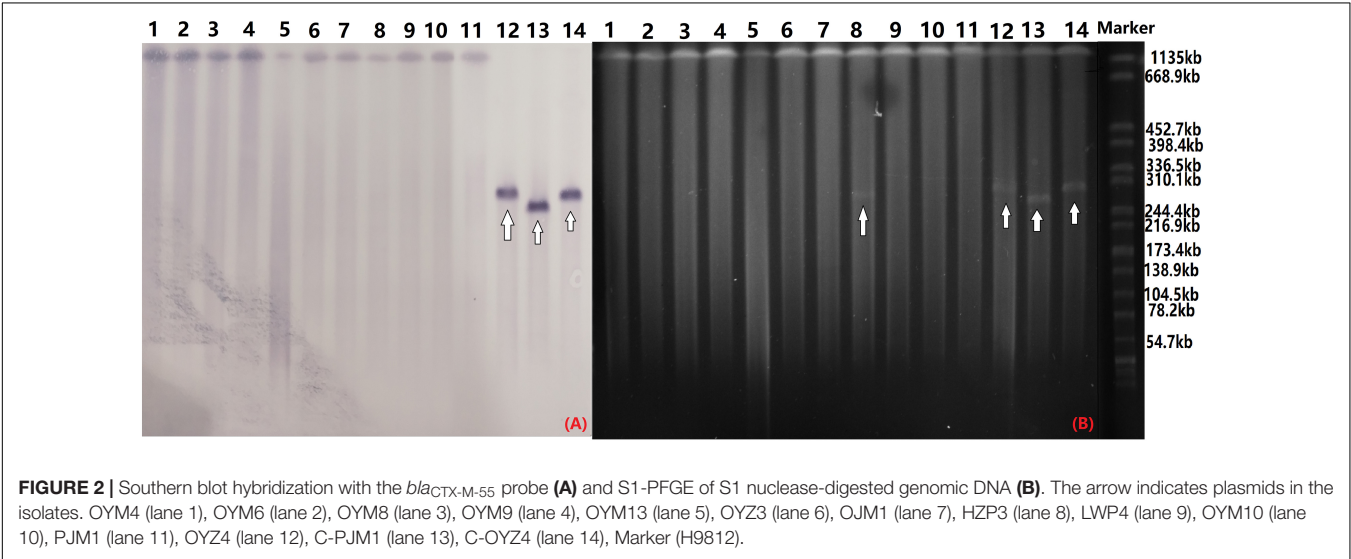
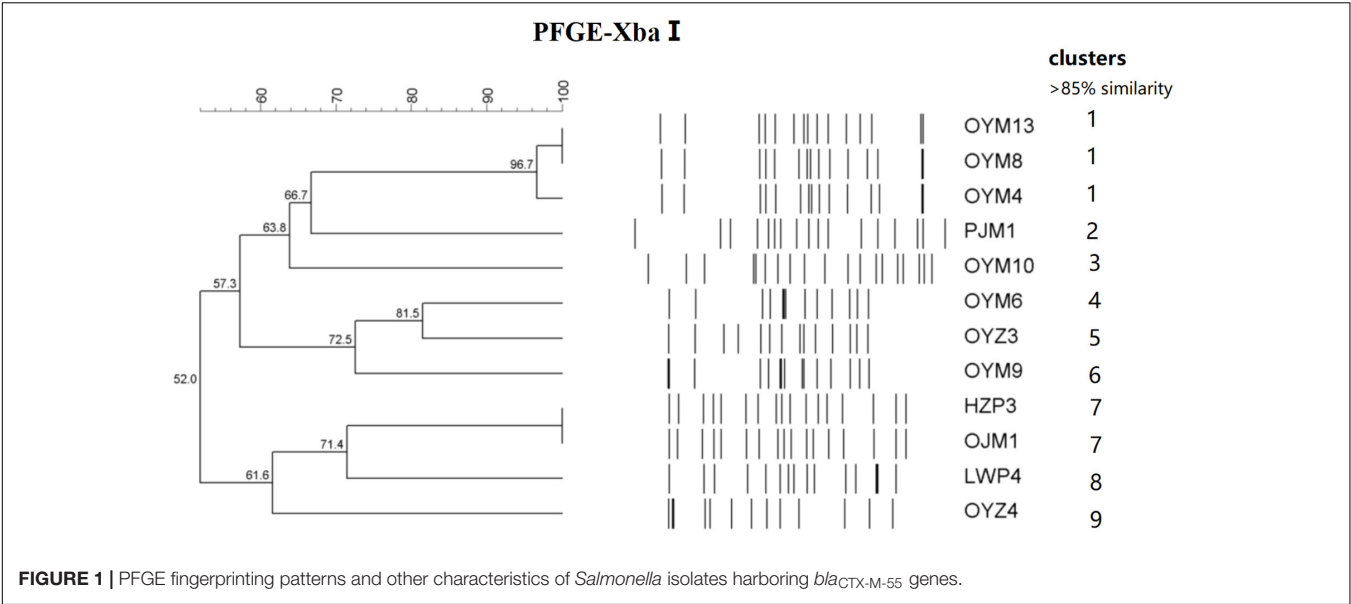
## *bla*<sub>CTX-M-55</sub> Hybridization and Plasmid Analysis

S1-PFGE and southern hybridization analysis of all 12 *bla*<sub>CTX-M-55</sub>-positive *Salmonella* isolates and 2 transconjugants found that *bla*<sub>CTX-M-55</sub> was chromosomally located in 11 of the isolates and in one isolate was present on a 280 kb plasmid (Figure 2). Interestingly, we successfully obtained one transconjugant from PJM1 whose *bla*<sub>CTX-M-55</sub> gene was chromosomal. This transconjugant carried the *bla*<sub>CTX-M-55</sub> gene on a 250 kb IncHI2 plasmid suggesting a mechanism where transfer from the chromosome onto a plasmid was followed by conjugation of this plasmid, now carrying the resistance gene (Figure 2). Two narrow-spectrum β-lactamase gene, *bla*<sub>TEM-1</sub> and *bla*<sub>OXA-1</sub> and PMQR gene *qnrS* also co-transferred with *bla*<sub>CTX-M-55</sub> in the transconjugants (Table 2). Analysis of the whole genome assembly of PJM1 identified the *bla*<sub>CTX-M-55</sub> present within an 11 kb contig with 100% identify to plasmid pCSFA1096, previously identified from *Salmonella* in China. However, mapping of all reads from the total genome sequencing of PJM1 against the pCSFA1096 genome identified alignments only over the elements carrying the resistance genes and the rest of the plasmid backbone was not present. This suggests the *bla*<sub>CTX-M-55</sub> gene originally transferred into the chromosome of PJM1 within a larger mobile element which retains the capacity to be mobilized. This supports our proposed model of transfer of this chromosomal element from PJM1 onto a plasmid and subsequent onward transmission.

## DISCUSSION

In the present study we found that 27 (65.85%) cefotaxime-resistant *Salmonella* strains produced CTX-M-type ESBLs. In this group, 21 were from food animals and 6 were from meat. The CTX-M-producing strains included 17 that were ciprofloxacin-resistant and 9 with decreased susceptibility to ciprofloxacin. In addition, at least one PMQR gene was detected in each of the 21 CTX-M isolates from animals and these results were similar to our previous study (Zhang et al., 2016). Together these studies suggest that the co-existence or co-transfer of PMQR genes in CTX-M-producing *Salmonella* strains increase their probability of survival in the presence of quinolones and/or cephalosporins (Liu et al., 2013).

The cefotaxime-resistant *Salmonella* strains included 5 CTX-M subtypes and CTX-M-55 was the most prevalent ( $n = 12$ ).



This was inconsistent with our previous studies that CTX-M-27 was the most prevalent ESBL in *Salmonella* strains isolated in 2009, 2010, and 2014 (Jiang et al., 2014; Zhang et al., 2016). In the current study, all the 12 CTX-M-55-producing strains were simultaneously non-susceptible to ciprofloxacin and almost all harbored PMQR genes. PMQR gene *qnrS* was only detected in low level ciprofloxacin resistant strains which contained no resistance-associated mutations in the QRDR. Though PMQR determinants only confer low level fluoroquinolone resistance, their existence (especially *qnr*) provide strains with a selective advantage under fluoroquinolones exposure and can accelerate the development of chromosome-mediated quinolone resistance (Robicsek et al., 2006; Strahilevitz et al., 2009).

Plasmids are key vectors in the global dissemination of antibiotic resistance genes in Gram-negative bacteria. Plasmid families including IncF, IncI1, IncI2, IncX, IncA/C, and IncHI2 play important roles in ESBL gene spread (Wang et al., 2018b). The *bla*<sub>CTX-M-55</sub> genes are the second most abundant ESBL

**TABLE 2 |** Characteristics of *bla*<sub>CTX-M-55</sub> gene-positive transconjugants.

Transconjugants	Origin	Inc type	ESBL genes	PMQR genes	Plasmid size	Transfer rate
C-PJM1	chicken	IncHI2	<i>bla</i> <sub>CTX-M-55</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>TEM-1</sub>	<i>qnrS</i>	250 kb	58.9%
C-OYZ4	duck	IncHI2	<i>bla</i> <sub>CTX-M-55</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>TEM-1</sub>	<i>qnrS</i>	280 kb	74.4%



subtype in the Enterobacteriaceae (Zhang et al., 2014; Lupo et al., 2018). This is especially true of *E. coli* from both humans and animals in Asia and these are usually found on IncF and IncI1 plasmids (Zhang et al., 2014; Lupo et al., 2018; Wang et al., 2018a). Additional data from our laboratory presented evidence that the prevalence of *bla*<sub>CTX-M-55</sub> in *E. coli* from both livestock and human origin is increasing. The F33:A-B- and IncI1 plasmids have driven the spread of these genes in China. F33:A-B- plasmids impart a significant biological advantage to their host and thus contribute to the increasing distribution of *bla*<sub>CTX-M-55</sub> (Wang et al., 2018b).

In the present study, we found chromosomal copies of *bla*<sub>CTX-M-55</sub> in 11/12 CTX-M-55-producing *Salmonella* strains. We speculate that the cross-species dissemination of *bla*<sub>CTX-M-55</sub> from plasmids in *E. coli* to *Salmonella* chromosomes contributes to the spread and stable persistence of this gene in *Salmonella* (Wong et al., 2015). Following the first isolation from a food animal in 2010 in China (Yan et al., 2010), the detection rate of *S. Indiana* increase d rapidly, especially from veterinary clinics and food-producing animals. The highly fluoroquinolone and  $\beta$ -lactam-resistant *S. Indiana* ST17 is the most prevalent sequence type of this serovar in China. This may suggest an increasing disseminating trend of ST17 CTX-M-55-encoding *S. Indiana*. The highly drug-resistant *S. Indiana* ST17 is one of the most prevalent antimicrobial-resistant foodborne pathogens in China so that its isolation from animals is a public health concern (Wang et al., 2017; Zhao et al., 2017; Cao et al., 2018). Additionally, the monophasic variant of *S. Typhimurium* ST34 has already emerged in Europe and Asia (Arnott et al., 2018). A comparison of the genomes of a pork meat and a human isolate revealed only 10 single nucleotide polymorphisms (SNP). This indicated that human bacterium was acquired from pork meat (Arnott et al., 2018).

A major mechanism underpinning the global dissemination of  $\beta$ -lactam resistant bacteria is their possession of resistant plasmids with low fitness cost or stable carriage of ESBL genes in the chromosome although the later are currently uncommon. Before 2013, strains harboring chromosome-located ESBL encoding genes were sporadically detected in *Escherichia coli* (Garcia et al., 2005; Coque et al., 2008), *Salmonella* Concord (Fabre et al., 2009), *Klebsiella pneumoniae* (Coelho et al., 2010), *Proteus mirabilis* and *Morganella morganii* (Harada et al., 2012; Mahrouki et al., 2012). Since then, highly prevalent *E. coli* strains possessing chromosomal CTX-M-14 and CTX-M-15  $\beta$ -lactamases were identified in 2013 (Hirai et al., 2013), 2016 (Hamamoto et al., 2016) and 2018 (Hamamoto and Hirai, 2018) separately. There may be a chromosomal *bla*<sub>CTX-M</sub> transpositional unit responsible for the global dissemination of CTX-M-14 in *E. coli*. However, the nature and significance of the spread of chromosomally located *bla*<sub>CTX-M</sub> genes remains unclear. We found most isolates carrying CTX-M-55 had the gene in a chromosomal context and these were from a diverse set of strain types. Transfer was however, possible from a strain (PJM1) with a chromosomal gene and the transconjugants carried the gene on a 280 kb IncHI2 plasmid. Together these data suggest that CTX-M-55 readily incorporates into the chromosome of *Salmonella* and that this is associated with PMQR

carriage but that the element carrying the CTX-M-55 gene can move onward onto plasmids for further dissemination.

A recent study in Cambodia demonstrated that spread of CTX-M-55-type *S. enterica* from pork and fish samples was mediated by MDR IncA/C2 and IncHI2 plasmids (Nadimpalli et al., 2018). IncHI2 plasmids are the fifth most widely disseminated plasmid type that mediate transmission of antibiotic resistance genes. These are primarily found in *Salmonella*, *E. coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae* of human and avian sources (Garcia Fernandez et al., 2007; Li et al., 2013; Haenni et al., 2016; Zhang et al., 2016).

## CONCLUSION

In conclusion, we identified foodborne *Salmonella* harboring chromosomally located *bla*<sub>CTX-M-55</sub> from China. These strains are simultaneously non-susceptible to fluoroquinolones. The co-existence of PMQR genes and CTX-M ESBL genes indicated co-selection for these determinants which may accelerate the dissemination of multi-drug resistance. Importantly, these strains may promote the development of isolates resistant to both cefotaxime and ciprofloxacin. The determination of the mechanisms and dissemination routes of ESBL-producing *Salmonella* is critical for animal and human health and understanding the interplay between movement of resistance genes between plasmids and chromosomal locations is important to understand the dynamics and evolutionary consequences of spread of antimicrobial resistance (AMR). This would also provide useful information to effectively control the development of antibiotic resistance to cephalosporins and fluoroquinolones.

## ETHICS STATEMENT

This study protocol was approved by the South China Agriculture University Animal Ethics Committee. The strains of free-range food animal origin were isolated from fecal swabs of healthy chickens, pigs, ducks, and geese and the owners of the animals gave permission for their animals to be used in this study.

## AUTHOR CONTRIBUTIONS

C-ZZ, H-ZD, and H-XJ conceived and designed the experiments. C-ZZ, X-MD, X-LL, R-YS, Y-WL, and R-MC performed the experiments. C-ZZ, MW, H-ZD, and H-XJ analyzed the data. C-ZZ, X-MD, X-LL, Y-WL, and R-MC contributed reagents, materials, and analysis tools. C-ZZ, MW, and H-XJ wrote the manuscript.

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**Conflict of Interest Statement:** 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.

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# Clonal Spread of Extended-Spectrum Cephalosporin-Resistant *Enterobacteriaceae* Between Companion Animals and Humans in South Korea

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Extended-spectrum cephalosporin (ESC)-resistant *Enterobacteriaceae* is an increasingly important problem in both human and veterinary medicine. The aims of this study were to describe a comparative molecular characterization of *Enterobacteriaceae* carrying ESC resistance genes, encoding extended-spectrum  $\beta$ -lactamase (ESBL) and AmpC, isolated from human stool samples, rectal swabs from companion animals, and swabs from the environment of veterinarian hospitals in South Korea, and to examine their possible dissemination and transmission. The ESC resistance genes were identified by PCR and sequencing. Isolates with the predominant ESC resistance genes were assessed for their genetic relatedness by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing. A total of 195 *Escherichia coli* and 41 *Klebsiella pneumoniae* isolates that exhibited ESC resistance were recovered on CHROMagar ESBL from human, companion animal, and the veterinary hospital environmental samples. In companion animals, most of the ESC resistance genes were *bla*<sub>CMY-2-like</sub> (26.4%), followed by *bla*<sub>CTX-M-55</sub> (17.2%) and *bla*<sub>CTX-M-14</sub> (16.1%), whereas *bla*<sub>CTX-M-15</sub> (28.6%) was predominant in human samples. The epidemiological relatedness of isolates carrying ESC resistance genes, including 124 *E. coli* and 23 *K. pneumoniae* isolates carrying CMY-2-like, DHA-1-like, or/and CTX-M-type, were analyzed by PFGE. The pulsotypes of five *E. coli* isolates (three from dogs and two from humans) carrying *bla*<sub>CMY-2-like</sub>, which were attributed to sequence type 405, from different veterinary clinics showed >85% similarity. Our results indicate direct transmission and dissemination of ESC-resistant *Enterobacteriaceae* between humans and companion animals.

**Keywords:** *Enterobacteriaceae*, ESBL, AmpC, companion animal, human, environment



## INTRODUCTION

The concept of “One Health,” which is the integration of human, animal, environmental, and ecosystem health, has recently emerged (Takashima and Day, 2014). One issue that should be addressed through a One Health approach is antimicrobial resistance (AMR). Therefore, we need to take steps to address the dissemination of AMR through the adoption of a One Health approach, promoting the integration of human and animal health, food safety, and environmental surveillance (Roca et al., 2015; Sikkema and Koopmans, 2016).

*Enterobacteriaceae* are among the most commonly reported causes of bacterial infections in humans and animals (Schmiedel et al., 2014). Notably, *Enterobacteriaceae* carrying extended-spectrum  $\beta$ -lactamases (ESBLs) and AmpC  $\beta$ -lactamases (AmpCs) are broadly distributed among extended-spectrum cephalosporin (ESC)-resistant bacteria (Kameyama et al., 2013). In both humans and animals, CTX-M-type enzymes are the most common ESBLs, whereas CMY-, and DHA-type enzymes are the most prevalent plasmid-mediated AmpCs (Liebana et al., 2004; Jacoby, 2009; Matsumura et al., 2012). These enzymes are able to inactivate ESCs and are normally encoded on mobile genetic elements, thus they can be transmitted to the same or different bacteria in humans, animals, foods, or the environment through either directly (transmission of AMR bacteria), or indirectly (transfer of AMR genes) (Haenni et al., 2014; Hong et al., 2016). Companion animals could play a role as a reservoir of AMR bacteria, as they are in close association with humans, living in their homes, and near their food. In addition, the widespread use and misuse of antibiotics in both human and veterinary medicine is increasing the spread of AMR bacteria (Dorado-García et al., 2018; Melo et al., 2018; Pulss et al., 2018). However, the impact of companion animals on human health in terms of attributing to exchange and share AMR determinant is not yet clear. Therefore, systematic control and prevention, through implementation of a national AMR surveillance program, are greatly needed and should be applied in the fields of human, and veterinary clinical medicine.

In this study, we represented a molecular characterization of ESC-resistant *Enterobacteriaceae* isolates collected from companion animals, humans, and veterinary hospital environments as part of a national surveillance program at 36 veterinary hospitals in South Korea and examined their epidemiological relatedness.

## MATERIALS AND METHODS

### Bacterial Profiles

We collected *Escherichia coli* and *Klebsiella pneumoniae* isolates from the rectal swabs of companion animals, including dogs ( $n = 315$ ) and cats ( $n = 74$ ); stool samples of humans, including pet owners ( $n = 48$ ) and medical staff ( $n = 33$ ); and 352 swabs of veterinary hospital environmental surfaced, including examination tables, cages, water bowls, scales, microscopes, keyboards, and switches, at 36 veterinarian hospitals of various regions in South Korea during July to November, 2017. All

samples were cultured on CHROMagar ESBL (CHROMagar, Paris, France) for use in the selection of *E. coli* (dark pink to reddish colony) and *Klebsiella/Enterobacter/Citrobacter* species (metallic blue colony). After pure sub-culture for a single colony on each blood agar plate (SPL Life Sciences, Gyeonggi-do, South Korea) per a given sample, *E. coli* and *K. pneumoniae* isolates were selected and identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) with a Vitek-MS (bioMérieux, Marcy-l'Etoile, France).

### Antimicrobial Susceptibility Testing

The isolates were tested for antimicrobial susceptibility to the following antimicrobial agents: ampicillin, piperacillin, ampicillin-sulbactam, cefazolin, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole, by the agar disk diffusion method on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, United States) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018b). The minimal inhibitory concentration of colistin was determined by broth microdilution using the criteria of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2018).

### Detection of Antimicrobial Resistance Genes

Template DNA was prepared by the boiling method. PCR and DNA sequencing of various AMR genes, including *bla*<sub>CTX-M-1</sub> group, *bla*<sub>CTX-M-2</sub> group, *bla*<sub>CTX-M-9</sub> group, *bla*<sub>CTX-M-25</sub> group, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>DHA</sub>, *bla*<sub>CMY-1</sub>, *bla*<sub>CMY-2</sub>, *bla*<sub>ACC</sub>, *bla*<sub>ACT</sub>, *bla*<sub>FOX</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM</sub>, *bla*<sub>KPC</sub>, *bla*<sub>GES</sub>, and *bla*<sub>OXA-48-like</sub>, were performed as described previously (Lee et al., 2018a). The sequences were compared to published DNA sequences using BLAST<sup>1</sup>.

### ESBL/AmpC Production Phenotypic Testing

ESC-resistant isolates with ESBL/AmpC PCR-negative were performed phenotypic disk diffusion test to confirm for ESBL/AmpC production (Song et al., 2007a,b).

### Nucleotide Sequence-Based Bacterial Typing

The epidemiological relationships among the CTX-M-type or/and CMY-2-like-producing isolates, which being collected from humans, companion animals, and the environment, were analyzed by pulsed-field gel electrophoresis (PFGE) using *Xba*I restriction enzyme. Then after, multilocus sequence typing (MLST) was performed for *E. coli* and *K. pneumoniae* strains with representative PFGE profiles as described previously (Nemoy et al., 2005; Jeong et al., 2016). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 were included as quality controls.

<sup>1</sup><https://blast.ncbi.nlm.nih.gov/Blast.cgi>

## Statistical Analysis

Statistical analysis was performed using SPSS Statistics version 24.0.0 (IBM Corp., Armonk, NY, United States). For comparison of the ESC-resistance rates between groups, we used the chi-square test. All *p* values were two-sided, and values less than 0.05 were considered statistically significant.

## RESULTS

### ESC Resistance and Antimicrobial Susceptibility

Among 389 samples of companion animals (315 dogs and 74 cats), 81 of humans (48 owners and 33 staffs), and 352 of veterinary hospital environmental surface, a total of 236 non-duplicated ESC-resistant isolates, including 195 *E. coli*, and 41 *K. pneumoniae* isolates were recovered. There were some cases that both ESC-resistant *E. coli* and *K. pneumoniae* isolates were simultaneously selected from 17 dogs, one cat, and one the environmental sample (Supplementary Table S1). Of the 195 ESC-resistant *E. coli* isolates, 174 (44.7%) were recovered from companion animals (389 total samples), 14 (17.3%) were recovered from human stool samples (81 total samples), and 7 (2%) were recovered from the environment (352 total samples). Among the *E. coli* isolates from companion animals, the ESC-resistance rate for canines (49.2%) was significantly higher than the rate for feline isolates (25.7%) (*p* value = <0.001). Of the 41 ESC-resistant *K. pneumoniae* isolates, 29 (7.5%) were recovered from companion animals, and 12 (3.4%) were recovered from the environment. Among the *K. pneumoniae* isolates from companion animals, the ESC-resistance rate for canines (8.3%) was not statistically different from the rate of felines (4.1%) (*p* value = 0.216). None were recovered from human-derived samples (Table 1). The AMR profiles of the ESC-resistant *E. coli* and *K. pneumoniae* isolates are listed in Supplementary Tables S2, S3, respectively.

### Genotypes of ESC Resistance

We performed genotype characterization of ESC resistance for all ESC-non-susceptible 195 *E. coli* and 41 *K. pneumoniae* isolates, regardless of isolation of both *E. coli* and *K. pneumoniae* isolates in same sample. Among the ESC-non-susceptible 195 *E. coli*, 171 (87.7%) isolates harbored known ESC resistance genes (Table 1), including nine different ESBL types and four AmpC types. A few isolates harbored both ESBL and AmpC (seven isolates carried CTX-M-type and CMY-2-like genes, and one isolate carried CTX-M-type and DHA-1-like genes). Of the 174 ESC-resistant *E. coli* isolates categorized from companion animals, *bla*<sub>CMY-2-like</sub> gene (*n* = 46, 26.4%) was most common ESC resistance determinant, followed by CTX-M-55 (*n* = 30, 17.2%), CTX-M-14 (*n* = 28, 16.1%), and CTX-M-15 (*n* = 20, 11.5%), which were the dominant ESBL genes. Interestingly, for the ESC-resistant *E. coli* isolates in companion animals, NDM-5 was detected in four isolates along with *bla*<sub>CTX-M-15</sub> and *bla*<sub>CMY-2-like</sub>. Of the 14 ESC-resistant *E. coli* isolates from humans, CTX-M-15 was predominant,

which accounted for 28.6% of the total (*n* = 4). One isolate was positive for SHV-190, and remaining 24 isolates were negative for both known ESBLs and AmpCs by the primers used in this study.

Among the ESC-non-susceptible 41 *K. pneumoniae* isolates, six different ESBL types and three AmpC types were detected in all 41 (100%) isolates (Table 1). In samples from companion animals and the environment, CTX-M-15 (*n* = 25) and DHA-1-like (*n* = 21) were dominant genotypes, followed by SHV-types (*n* = 5), and CTX-M-14 (*n* = 5). The presence of both ESBL- and AmpC-type genes was detected in 21 *K. pneumoniae* isolates (51.2%), and in 8 *E. coli* isolates (4.1%). No ESC-resistant *K. pneumoniae* isolates were recovered from human stool samples (Table 1).

### Macro-Restriction of ESC-Resistant *Enterobacteriaceae*

To determine the epidemiological relatedness among ESC-resistant isolates from companion animals, humans, and various hospital areas in veterinarian clinics, 124 *E. coli* and 23 *K. pneumoniae* isolates carrying the predominant ESC resistance genes (*E. coli* carrying CTX-M-15, CTX-M-55, CTX-M-14, or/and CMY-2-like genes, and *K. pneumoniae* carrying CTX-M-15 or/and DHA-1-like genes) were subjected to PFGE analysis. Among the 124 *E. coli* isolates, similar PFGE patterns were observed only between isolates from companion animals and humans (Supplementary Figure S1). Five CMY-2-like harboring *E. coli* isolates without other resistance genes belonged to one pulsotype, with >85% similarity, which was attributed to sequence type 405 (ST405) by MLST analysis. Three of the five isolates were derived from samples obtained at the same hospital, two were from humans (E246 was from a veterinarian and E249 was from a nurse) and the third was from a rectal swab from a dog (E245) admitted to the above hospital. The two remaining isolates (E92 and E118) were collected from two dogs at different veterinary clinics (Figure 1). For the 23 *K. pneumoniae* isolates that mostly produced CTX-M-15 or/and DHA-1-like enzymes in companion animals and the environment, 7 pulsotypes were identified which corresponded to ST307 (d, e, and f PFGE-types), ST392 (a, b, and c PFGE-types), and ST950 (g PFGE-type) (Figure 2).

## DISCUSSION

Antimicrobial resistance surveillance systems of clinical isolates in South Korea have been well-documented in both the Korea antimicrobial resistance monitoring system (KARMS) and Korea global antimicrobial surveillance system (Kim et al., 2017; Lee et al., 2018b). However, this is the first report on the prevalence and molecular characterization of ESC-resistant *Enterobacteriaceae* isolates collected from companion animals, humans, and the veterinarian hospital environment for national surveillance in South Korea. Previously, the KARMS study for human clinical isolates reported that the rate of AMR to cefotaxime was 35.0% in *E. coli* and 41.0% in *K. pneumoniae* (Kim et al., 2017). Among the *E. coli* isolates collected from rectal swabs

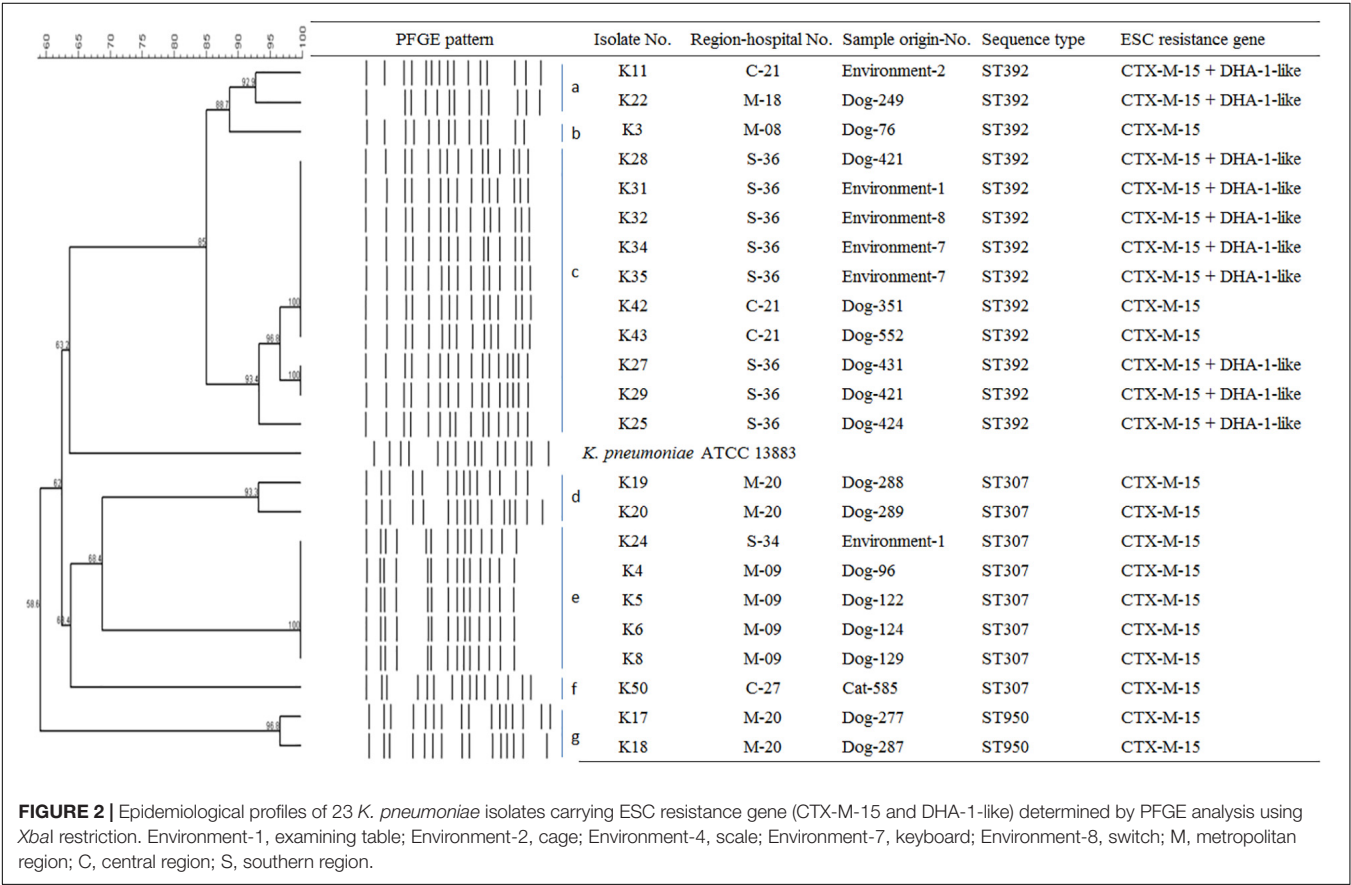
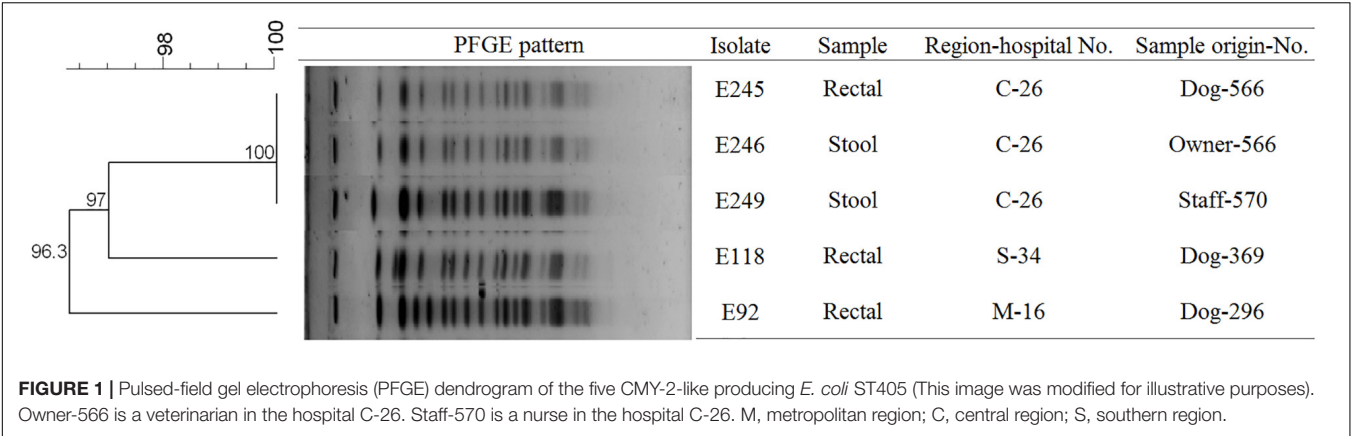
of dogs and cats obtained in this study, the rates of ESBL- or/and AmpC-producing *E. coli* isolates were 43.5% (137/315) for dogs and 24.3% (18/74) for cats, respectively. In previous study showed that the rate of ESBL or/and AmpC-producing *E. coli* isolates in dogs had a 38.1% (So et al., 2012), which represented currently increasing incidence in South Korea.

CTX-M type ESBL genes in the fields of human medicine, and CMY type AmpC genes in both human and veterinary medicine, are dominant in *E. coli* worldwide including South Korea (Kameyama et al., 2013; Lee et al., 2018a). Especially, CTX-M-15- and CMY-2-producing *E. coli* isolates are the most frequently detected genotypes associated with ESC resistance in companion

**TABLE 1 |** Genotypes of  $\beta$ -lactamases in extended-spectrum cephalosporin-resistant *E. coli* and *K. pneumoniae* isolates from companion animals, humans, and the hospital environment.

Organism	ESC resistance gene <sup>a</sup>			No. (%) isolates					
	ESBL <sup>b</sup>	AmpC	Carbapenemase	Companion animal		Human (n = 81)	Environment (n = 352)	Total (n = 822)	
				Dog(n = 315)	Cat (n = 74)				
<i>E. coli</i>	CTX-M-1			3	1	0	0	4	
	CTX-M-3			4	0	0	1	5	
	CTX-M-15			11	1	4	0	16	
	CTX-M-15	CMY-2-like	NDM-5	4	0	0	0	4	
	CTX-M-55			26	1	0	0	27	
	CTX-M-55 + CTX-M-14			0	1	0	0	1	
	CTX-M-55	CMY-2-like		2	0	0	0	2	
	CTX-M-14			25	2	1	1	29	
	CTX-M-14	CMY-2-like		1	0	0	0	1	
	CTX-M-14	DHA-1-like		1	0	0	0	1	
	CTX-M-24			3	2	0	0	5	
	CTX-M-27			5	1	2	0	8	
	CTX-M-65			6	1	0	0	7	
	SHV-190			1	0	0	0	1	
		CMY-2-like		40	6	2	3	51	
		CMY-2-like+ DHA-1-like		0	2	0	0	2	
		CMY-4		1	0	0	0	1	
		DHA-1-like		3	0	2	0	5	
		DHA-9		1	0	0	0	1	
		Unidentified	Unidentified	Unidentified	18	1	3	2	24
	Total			155 (49.2)	19 (25.7)	14 (17.3)	7 (2.0)	195 (23.7)	
<i>K. pneumoniae</i>	CTX-M-15			10	1	0	1	12	
	CTX-M-15	DHA-1-like		6	0	0	6	12	
	CTX-M-15 + CTX-M-14	DHA-1-like		1	0	0	0	1	
	CTX-M-55	DHA-1-like		0	0	0	1	1	
	CTX-M-55	DHA-9		0	0	0	1	1	
	CTX-M-14			2	0	0	1	3	
	SHV-12	DHA-1-like		2	0	0	2	4	
	SHV-26	DHA-1-like		2	0	0	0	2	
	SHV-28			1	0	0	0	1	
		CMY-2-like		1	1	0	0	2	
		DHA-1-like		0	1	0	0	1	
		CMY-2-like + DHA-1-like		1	0	0	0	1	
		Total			26 (8.3)	3 (4.1)	0 (0)	12 (3.4)	41 (5.0)

<sup>a</sup>ESC, extended-spectrum cephalosporin. <sup>b</sup>ESBL, extended-spectrum  $\beta$ -lactamase.



animals and humans, respectively (Matsumura et al., 2012; Woerther et al., 2013; Haenni et al., 2014; Dorado-García et al., 2018). Because companion animals are in close contact with humans, the genotypes of companion animals in this study were initially expected to be similar to the genotypes of humans disseminated in South Korea. The total number of CMY-2-like-producing *E. coli* isolates was greater than the number of CTX-M-type (CTX-M-55-, CTX-M-14, and CTX-M-15)-producing isolates from the rectal swabs of companion animals, which was consistent with the numbers described in previous reports of AmpC in animals, suggesting that it is an important mechanism

of resistance to ESC (So et al., 2012; Dorado-García et al., 2018). Instead, CTX-M-15 was more detected than other ESC-resistant determinants in humans, but CTX-M-55 and CTX-M-14 were more prevalent than CTX-M-15 in companion animals in this study. The CTX-M-55 and CTX-M-14 were previously detected in food-producing animals and turkey meat, respectively (Kiratisin et al., 2008; Randall et al., 2011; Matsumura et al., 2012; Liao et al., 2015). These findings indicate that the ESC resistance gene variants are not limited to certain hosts, emphasizing the need for coordinated control in the concept of One Health. In addition, in previous studies, CTX-M-14 were the most



common ESC resistance genotypes, whereas CTX-M-55 was rarely detected in companion animals in South Korea (So et al., 2012; Tamang et al., 2012). Therefore, the observed increase in CTX-M-55 in companion animals in South Korea may also affect AMR transmission between humans and companion animals.

All 41 ESC-resistant *K. pneumoniae* isolates harbored ESC-resistance genes. In contrast, of the 195 ESC-resistant *E. coli* isolates, 24 (12.3%) isolates did not identify for ESBL, and/or AmpC type in this study. These isolates were susceptible to cefoxitin and were positive for ESBL production (disk diffusion test), suggesting the presence of an ESBL variant not detected by the primers used in this study. We performed PFGE to understand the relatedness between ESC-resistant *E. coli* and *K. pneumoniae* isolates from humans, companion animals, and the environment in veterinary hospitals during 5 months. Most of the ESC-resistant isolates had no evidence of clonal spread between humans and companion animals. Instead, the five CMY-2-producing *E. coli* ST405 isolates from two humans and three dogs showed high identity, which suggested the possibility of direct transmission between humans, and companion animals. The spread of *E. coli* ST405 carrying CTX-M-15 has been frequently described in humans as an epidemic lineage, along with *E. coli* ST131 (Coque et al., 2008). However, we detected *E. coli* ST405 carrying CMY-2-like genes in this study.

In this study, CTX-M-15 (61.0%,  $n = 25/41$ ), either alone or in combination with DHA-1-like, was mostly detected in ESC-resistant *K. pneumoniae* isolates from companion animals and the environmental samples. Recently, CTX-M-15- and DHA-1-coproducing *K. pneumoniae* ST11 have emerged in human patients and are being disseminated (Cha et al., 2018; Lee et al., 2018a), while there was no report that described in *K. pneumoniae* isolate carrying CTX-M-15 from dogs and cats in South Korea. CTX-M-15 was essentially associated with ST11, ST15, ST307, and ST392 clones in *K. pneumoniae*, which have been frequently detected in other part of the world (Ewers et al., 2014; Wyres et al., 2019). ST307 is also frequent among carbapenemase producers in South Korea, suggesting a wider dissemination in different setting in our country (Yoon et al., 2018a,b). There was no transmission between humans and companion animals, however, clonal spread was observed among companion animals and between companion animals and the environment in this study. Based on the observation of environmental colonization of AMR *K. pneumoniae* in veterinary hospitals, infection control for the environment should be carefully considered in the veterinary field.

Our results have limitations: (i) the number of stool specimens from humans was less than that of companion animals and the isolation of ESBL/AmpC producing *K. pneumoniae* was unusually more frequent than *E. coli* with ESBL/AmpC in the environmental samples. Unfortunately, we do not know the reason why for this, (ii) plasmid distribution was not included in this study, which is well known to contribute to ESBL distribution in *Enterobacteriaceae*. Nevertheless, our findings illustrate the importance of infection control strategies for usage of antibiotics and demonstrate the need for further cooperation among the

fields of human and veterinary medicine and environmental science in the One Health perspective.

## DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendation of ethical guidelines of Konkuk University College of Veterinary Medicine, South Korea. Individual written informed consent for the use of samples was obtained from all the animal owners and veterinarian.

## AUTHOR CONTRIBUTIONS

WS was responsible for the study design, data analysis, and proofreading of the manuscript. JH performed examination of molecular work (resistance gene, PFGE, and MLST), analyzed the experimental data, and wrote the manuscript. H-MP, J-CC, and SJ designed the sample collection and experiments. J-YO designed and performed the experiments. SS conducted the statistical analysis and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01371/full#supplementary-material>

**FIGURE S1** | Epidemiological profiles of 124 ESBL/AmpC (CTX-M-15, CTX-M-55, CTX-M-14, or/and CMY-2-like) producing *E. coli* isolates from humans, companion animals (green box), and the environment (blue box) determined by PFGE analysis using XbaI restriction. Red box represented five CMY-2-like producing *E. coli* isolates related closely. *E. coli* ATCC strain used in this study was *E. coli* ATCC 25922.

**TABLE S1** | Samples that grew both *E. coli* and *K. pneumoniae* isolates from dog, cat, and the environment.

**TABLE S2** | Antimicrobial susceptibilities of extended-spectrum cephalosporin-resistant *E. coli* isolates from humans, companion animals, and the hospital environment.

**TABLE S3** | Antimicrobial susceptibilities of extended-spectrum cephalosporin-resistant *K. pneumoniae* isolates from companion animals and the hospital environment.

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# Effect of Single Dose of Antimicrobial Administration at Birth on Fecal Microbiota Development and Prevalence of Antimicrobial Resistance Genes in Piglets

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Optimization of antimicrobial use in swine management systems requires full understanding of antimicrobial-induced changes on the developmental dynamics of gut microbiota and the prevalence of antimicrobial resistance genes (ARGs). The purpose of this study was to evaluate the impacts of early life antimicrobial intervention on fecal microbiota development, and prevalence of selected ARGs (*ermB*, *tetO*, *tetW*, *tetC*, *sull*, *sulll*, and *blaC<sub>TX-M</sub>*) in neonatal piglets. A total of 48 litters were randomly allocated into one of six treatment groups soon after birth. Treatments were as follows: control (CONT), ceftiofur crystalline free acid (CCFA), ceftiofur hydrochloride (CHC), oxytetracycline (OTC), procaine penicillin G (PPG), and tulathromycin (TUL). Fecal swabs were collected from piglets at days 0 (prior to treatment), 5, 10, 15, and 20 post treatment. Sequencing analysis of the V3-V4 hypervariable region of the 16S rRNA gene and selected ARGs were performed using the Illumina Miseq platform. Our results showed that, while early life antimicrobial prophylaxis had no effect on individual weight gain, or mortality, it was associated with minor shifts in the composition of fecal microbiota and noticeable changes in the abundance of selected ARGs. Unifrac distance metrics revealed that the microbial communities of the piglets that received different treatments (CCFA, CHC, OTC, PPG, and TUL) did not cluster distinctly from CONT piglets. Compared to CONT group, PPG-treated piglets exhibited a significant increase in the relative abundance of *ermB* and *tetW* at day 20 of life. Tulathromycin treatment also resulted in a significant increase in the abundance of *tetW* at days 10 and 20, and *ermB* at day 20. Collectively, these results demonstrate that the shifts in fecal microbiota structure caused by perinatal antimicrobial intervention are modest and limited to particular groups of microbial taxa. However, early life PPG and TUL intervention could promote the selection of ARGs in herds. While additional investigations are required to explore the consistency of these findings across larger populations, these results could open the door to new perspectives on the utility of early life antimicrobial administration to healthy neonates in swine management systems.

**Keywords:** antimicrobial, microbiota, neonatal piglets, resistance genes, fecal



## INTRODUCTION

The widespread use of injectable antimicrobials in the treatment and prevention of human and animal diseases continues to rise globally (MacKie et al., 2006). Numerous concerns related to human and animal health have been raised regarding the long-term sequelae of this trend, including emergence of antibiotic-resistant bacteria, dissemination of ARGs into the environment, perturbations of the gut microbiota-ecosystem and increased risk of diseases (Chee-Sanford et al., 2001; Hoelzer et al., 2017). Antimicrobial resistance develops when the administered antimicrobial eliminates susceptible microorganisms but leaves behind resistant strains that continue to grow and multiply (Wegener, 2003). These resistant bacteria transmit their genetic resistance characteristics to their progeny through vertical evolution, or to other bacterial species through horizontal evolution (Holmes et al., 2016). Recently, several lines of evidence indicate that extensive use and misuse of existing antimicrobials increases the numbers of ARG copies and risk of their spread among commensal bacterial population (Roca et al., 2015; Czaplewski et al., 2016; Zeineldin et al., 2019a).

Traditionally, the majority of studies evaluating the effect of antimicrobial administration on emergence of antibiotic resistant bacteria and ARGs have focused on pathogenic organisms using culture-based methods (Thanner et al., 2016). While this approach has enhanced our understanding of the nature of antimicrobial resistance in a single class of organisms, it is limited in its ecosystem-level application. Advancements in culture independent techniques such as next generation sequencing have allowed for the determination of microbial diversity in several animal biogeographic niches and have helped in the assessment of antimicrobial resistance determinants at the microbial ecosystem-level (Zhao et al., 2017; Zeineldin et al., 2019b).

Immediately after birth, the swine gastrointestinal tract is colonized by a complex microbial ecosystem, that plays a crucial role in the intestinal configuration, immune system maturation, and host gene expression (Zhang et al., 2016; Zeineldin et al., 2019c). During this stage, the microbial ecosystem is unstable and highly susceptible to various environmental factors, including antimicrobial administration, dietary intervention and stress exposure (Schokker et al., 2014). Given the instability of microbiota at this phase, the microbial population has the potential to disseminate and transfer ARGs, which could have significant effects on the development of metabolic and immune disorders (Gibson et al., 2015; Su et al., 2017). In intensive swine management systems, newborn piglets are frequently administered antimicrobials to prevent outbreaks of infectious diseases; however, the effects of early life antimicrobial prophylaxis on the emergence of ARGs and its connection with the gut microbial community in piglets are poorly understood. Recently, a study of early life antimicrobial intervention showed long-lasting impacts on the gastrointestinal microbial diversity and composition in newborn piglets (Schokker et al., 2014). In our previous study, we explored the change in the fecal microbiota of 8-weeks-old piglets in response to parenteral antimicrobial administration and we found that the fecal microbiota showed antimicrobial-specific variation in both

duration and extent (Zeineldin et al., 2018a). To gain further insight into the swine gut ecosystem and to find alternatives to antimicrobials, it is crucial to understand the developmental dynamics of the gut microbiota and prevalence of ARGs in response to perinatal antimicrobial administration in piglets. Consequently, the aim of this study was to investigate the short-term impact of commonly used antimicrobials during early life on the developmental dynamics of the fecal microbiota, and relative abundance of selected ARGs (*ermB*, *sullI*, *sullII*, *tetC*, *tetO*, *tetW*, and *blaCTX-M*) in suckling piglets using high-throughput sequencing analysis.

## MATERIALS AND METHODS

### Ethics Statement

This study was conducted in compliance with the recommendations of the guidelines for the care and use of animals of University of Illinois at Urbana-Champaign. The protocol was approved by the Ethical Committee for Institutional Animal Use and Care of the University of Illinois at Urbana-Champaign.

### Experimental Design and Samples Collection

The experiment was conducted in a commercial swine farm in the Midwestern US with consent from the facility owner. A total of 48 l were used in this study based on a randomized complete block design with farrowing day and dam parity group as blocks. Approximately five days before farrowing, the pregnant sows were transferred to a farrowing pen and kept there until the end of the experiment. Sows were fed a standard lactation diet, provided *ad libitum* access via an automatic dry feeding system, and were given *ad libitum* access to water from a nipple drinker. Directly after birth, litters were randomly assigned into one of six groups ( $n = 8$  per group); control (CONT), ceftiofur crystalline free acid (CCFA), ceftiofur hydrochloride (CHC), oxytetracycline (OTC), procaine penicillin G (PGP), and tulathromycin (TUL). Littermates were used to minimize differences arising from maternal microbiota. After farrowing (day 0), all piglets were ear tagged and treatments were applied. All piglets in a litter were assigned to a single treatment group. The dosage schedule for each treatment group was as follow; CONT (saline 1cc IM), CCFA (5.0 mg /kg of body weight IM), CHC (5 mg/kg of body weight IM), OTC (22 mg/kg of body weight IM), PPG (33,000 units/kg of body weight) and TUL (2.5 mg/kg of body weight IM). CCFA and CHC are third-generation cephalosporins with a broad-spectrum activity against both Gram-positive and Gram-negative bacteria (Chander et al., 2011). OTC is a tetracycline antibiotic that also directly targets both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). PGP is one of the beta-lactam antibiotics that targets Gram-positive and Gram-negative bacteria (Ranheim et al., 2002). TUL is one of macrolide antibiotics that inhibit bacterial essential protein biosynthesis of both Gram-positive and Gram-negative bacteria

(Schokker et al., 2014). The antimicrobial classes in this study are considered the most popular approved antibiotics used in the swine industry for the control and treatment of swine diseases (Schwarz et al., 2001).

The treated piglets were housed in a conventional farrowing pen that was approximately 1.9 m × 2.6 m where the sow was confined so that she could not turn around, and the sidewall penning for the piglets was solid to prevent contact between litters. All piglets were allowed to suckle colostrum and piglets were not added to the birth litter (some were removed prior to treatment if there were more pigs than available mammary glands). The antimicrobial dosages and routes of administration were based on the manufacturer label instructions. The piglet's tails were not docked, and teeth were not clipped. All piglets were weighed individually at days 0 and 20 of life, and dead piglets were recorded throughout the study. Deep fecal swabs (Pur-Wraps®, Puritan Medical Products, Guilford, ME, United States) were collected immediately prior to treatment (day 0), and again on days 5, 10, 15, and 20 after dosing. The fecal swabs were snap-frozen in sterile containers and transported to the laboratory on the same day. Samples were kept at −80°C pending further processing.

## Extraction of Genomic DNA and Illumina Sequencing

Four clinically healthy piglets from each group (CONT, CCFA, CHC, OTC, PPG, and TUL) at the different sampling days (0, 5, 10, 15, and 20) were selected for the microbiota analysis. Negative control samples were also obtained from cotton swabs and extraction kit reagents. In a decontaminated sterile environment, microbial DNA was extracted from all selected samples using commercially available kits (MO BIO Laboratories, Inc., Carlsbad, CA, United States) (Zeineldin et al., 2017b, 2018b). Briefly, the swabs were mixed with 750 µl of Bead Solution (MO BIO Laboratories, Inc.), and bead beating was carried out in Bullet Blender 24 Gold tube holder machine (Next Advance, Inc., Averill Park, NY, United States) for 10 min. Then the extraction process was completed according to the manufacturer's manual. The concentration and integrity of DNA were assessed using a Nanodrop™ spectrophotometer (NanoDrop Technologies, Rockland, DE, United States), and agarose gel electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, United States). Additionally, the extracted DNA concentration was assessed on a Qubit (Life Technologies, Grand Island, NY, United States) using the High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, United States). The extracted DNA was then subjected to Fluidigm Access Array Amplification (Fluidigm Corporation, South San Francisco, CA, United States). The primer sequences F357 -for (CCTACGGGNGGCWGCAG) and R805-rev (GACTACHVGGGTATCTAATCC) were designed with an attached eight base barcode sequence that was unique to each sample to amplify the V3-V4 hypervariable region of the 16S rRNA gene. Additionally, a total of seven primer sets targeting seven different ARGs conferring resistance to the most popular antimicrobial classes used in the swine industry, were used (**Supplementary Table S1**). Additionally, the primer

sequences F357 -for (CCTACGGGNGGCWGCAG) and R805-rev (GACTACHVGGGTATCTAATCC) were designed with an attached eight base barcode sequence that was unique to each sample to amplify the V3-V4 hypervariable region of the 16S rRNA gene. The mastermix for PCR amplification was prepared using the Roche High Fidelity Fast Start Kit and 20x Access Array loading reagent according to Fluidigm protocols. PCR reactions consisted of DNA sample, 20X Access Array Loading Reagent, forward and reverse primer, Fluidigm Illumina linkers with unique barcode, and water to a final volume of 100 µl. PCR reactions were performed on a Fluidigm Biomark HD™ PCR machine (Fluidigm Corporation, South San Francisco, CA, United States; **Supplementary Table S2**). Amplicons were purified on a Fragment Analyzer (Advanced Analytics, Ames, IA, United States) to confirm amplicon size. The final fluidigm pools were quantitated by qPCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, United States). Samples were then pooled in equimolar ratio, spiked with 15% non-indexed PhiX control library, and loaded onto the MiSeq V3 flowcell at a concentration of 8 pM for cluster formation and sequencing. The final genomic libraries were then sequenced from both ends following manufacturer's guidelines (Illumina, Inc., San Diego, CA, United States) at the DNA Services lab at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign, Urbana, IL, United States).

## Sequence Data Processing and Microbial Community Analysis

The raw sequence data were preprocessed from Illumina base call (bcl) files into compressed paired-end read fastq files (2 × 300) using bcl2fastq 1.8.4 (Illumina, San Diego, CA, United States) without demultiplexing, and then sorted by initial PCR-specific primer using a custom in-house pipeline. The generated bcl files were converted into demultiplexed compressed fastq files using bcl2fastq 1.8.4 (Illumina, San Diego, CA, United States). A secondary pipeline decompressed the fastq files, generated plots with quality scores using FastX Tool Kit<sup>1</sup>. Trimmomatic (v. 0.38) was used to trim the low-quality base at the overlapping end of the raw sequence reads (Bolger et al., 2014). Barcode and sequencing primers were also trimmed from the raw sequence reads. After preprocessing, the 16S rRNA gene sequences were analyzed using Quantitative Insights into Microbial Ecology (QIIME v.1.9.1) software<sup>2</sup> (Caporaso et al., 2010). Raw sequence reads were quality filtered using the following quality criteria; minimum sequence length equal 200, maximum sequence length equal 1000, a Phred score of less than 25, maximum number of ambiguous bases equal 6 and homopolymer runs of >6 bp (Bokulich et al., 2012). The open-reference operational taxonomic unit (OTU) clustering was conducted in QIIME at 97% similarity using UCLUST clustering (v1.2.22q) (Edgar, 2010), and taxonomy was assigned using the Silva reference database (v.132) (Quast et al., 2013). Chimeric sequences were detected and removed using UCHIME

<sup>1</sup>[http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)

<sup>2</sup><http://qiime.org/>

(v. 6.1) prior to downstream analysis (Edgar et al., 2011). One sample from the TUL-treated piglets was not included in the analysis due to unsuccessful sequencing. Two OTUs detected as a contaminant in negative controls (classified as *Stenotrophomonas* and *Xanthomonas*) were removed prior to analyses. The alpha diversity (within community) were calculated within QIIME using the number of OTUs per sample and the Shannon diversity index. To standardize our analysis due to uneven sequencing depth, all samples were randomly subsampled to 1358 sequences per sample. To compare overall microbiota composition among groups, a beta diversity analysis was performed considering the abundance of each detected OTUs in each sample using weighted UniFrac distances and was displayed using principal coordinate analysis (PCoA). Finally, a Venn diagram was generated for graphical descriptions of the number of unique and shared OTUs between treatment groups.

Statistical analysis and graphing were performed using PAST version 3.13, JMP 13 (SAS Institute Inc.) and RStudio (version 1.1.383, R Studio, Inc., Boston, MA, United States). Data were logarithmically transformed or ranked when necessary to achieve normality and homogeneity of variance prior to statistical analyses. Significance difference was stated at  $P < 0.05$ . Statistical comparisons of weighted UniFrac distances between treatment groups at different sampling days were determined using analysis of similarity (ANOSIM) with 9999 permutations and Bonferroni corrected  $P$ -values in PAST version 3.13. Due to the fact that the same piglets were sampled repeatedly over the course of the study, repeated measures ANOVA with *post hoc* Tukey's honestly significant difference (HSD) pairwise comparisons were performed to compare the difference in microbial relative abundance and alpha diversity indices between the treatment groups. To further identify taxa that were significantly different between the different time points in the same groups and between the groups at the same time point, the OTUs abundance were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) pipeline in Galaxy<sup>3</sup> (Segata et al., 2011). We then compared the overall microbial communities between the treatment groups using stepwise discriminant analysis in JMP 13 (SAS Institute Inc.). For this analysis, the relative abundances of different bacterial genera in each group were used as a covariate, and treatment groups were used as the categorical variable. The discriminant analysis was used to determine how equivalent samples, from animals in different groups, were differentiated from one another, and was illustrated using canonical loading plots.

## Prediction of the Metagenome Functions Profiles

The metagenomic prediction of functional profiles based on 16S rRNA gene composition was done with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt v1.0.06) (Langille et al., 2013). Closed reference OTUs were taxonomically assigned against the Greengenes (v13.5) database, normalized by copy number, and gene features were predicted at level 2 and level 3 Kyoto Encyclopedia of Genes

and Genomes (KEGG) orthology groups (Kotera et al., 2012). The unclassified functional categories were eliminated from the analysis. The difference in overall predictive function gene profiles among groups were compared with Statistical Analysis of Metagenomic Profiles software (STAMP v2.1.3) (Parks et al., 2014). Two-sided Welch's  $t$ -test and Benjamini-Hochberg FDR correction were used in two-group analysis and ANOVA with the Tukey-Kramer test and Benjamini-Hochberg correction were chosen for multiple-group analysis. Differences were considered significant at  $P < 0.05$ . Principal component analysis (PCA) and heatmap diagram were also performed using STAMP and MicrobiomeAnalyst respectively (Dhariwal et al., 2017).

## Selected Antimicrobial Resistance Genes Quantification

For ARGs sequence classification, we have developed a customized version of the Antibiotic Resistance Gene Database (ARG-ANNOT) that incorporated all sequences of the seven ARGs that used in this study. The customized ARG-ANNOT database was used to align the seven ARGs raw sequences reads obtained from the Illumina sequencing according to the used primers. The ARG sequence depth and coverage for each ARG were also counted. To avoid bias, normalization of the ARGs reference sequence length by the 16S rRNA gene sequence length was conducted. The abundance of ARGs was expressed as ARG copy number per 16S rRNA gene copy. The relative abundance of ARGs was calculated using the following equation (Li B. et al., 2015):

$$Abundance = \sum_1^n \frac{N_{ARG-like\ sequence} \times L_{reads}/L_{ARG\ reference\ sequence}}{N_{16S\ sequence} \times L_{reads}/L_{16S\ sequence}}$$

The difference in ARGs abundance, between treatment groups at different sampling days were analyzed using repeated measures ANOVA with pairwise *post hoc* Tukey's HSD comparisons in PAST version 3.13. Dunnett's multiple comparisons procedure was also used to compare the mean ARGs abundance in different treatment groups (CCFA, CHC, OTC, PPG, and TUL) at each sampling day (0, 5, 10, 15, and 20), against the CONT group at the same time point. The difference in overall ARGs abundance among treatment groups were compared using PCA fitted in STAMP software (Parks et al., 2014). Differences with a value of  $P < 0.05$  were considered significant.

## Accession Numbers

Raw paired-end Fastq sequence data obtained in this study were submitted to the Sequence Read Archive of the NCBI under bio-project accession number PRJNA407634.

## RESULTS

### Impact of Antimicrobial Treatment on Body Weight Gain and Overall Mortality Ratio

There were no significant differences in the average daily weight gain between the treatment groups (CCFA, CHC, OTC, PPG,

<sup>3</sup><https://huttenhower.sph.harvard.edu/galaxy/>



and TUL) and CONT over the first 20 days of life ( $P > 0.05$ , **Supplementary Figure S1A**). Compared to CONT, the treated piglets showed non-significant changes in the overall mortality ratios ( $P > 0.05$ , **Supplementary Figure S1B**). However, TUL-treated piglets showed an increase in the mortality during the time period from 15 to 20 days of life (**Supplementary Figure S1B**). Our results showed that the early life antimicrobial intervention failed to affect mortality or the average daily weight gain in the neonatal piglets.

## Summary of Sequence Data Analysis

After quality filtering and removal of low-quality sequences, a total of 2,508,268 sequences were obtained from all samples. The number of sequences per sample ranged from 5307 to 48524 (mean  $\pm$  SD,  $15201.624 \pm 7324.965$ ). Using 97% similarity, 1296 OTUs were identified among all samples. Collectively, most OTUs were shared among the treatment groups with only 8, 5, 18, 11, 4 and 7 OTUs uniquely identified in piglets from the CONT, TUL, CCFA, CHC, PPG, and OTC group, respectively (**Supplementary Figure S2**).

## Microbial Taxa Affected by Early Life Antimicrobial Intervention

At the phylum level, the microbial composition in all treatment groups varied according to ages (**Figure 1**). At day 0, Proteobacteria was the most predominant phylum, representing 79, 76, 82, 85, 85, and 91 % of all bacterial populations in CONT, CCFA, CHC, OTC, PPG, and TUL respectively. While at day 20, Firmicutes was the most relatively abundant phylum, representing 61, 43, 47, 40, 32, and 41% of all bacterial populations in CONT, CCFA, CHC, OTC, PPG, and TUL, respectively. Compared to the CONT group, TUL-treated piglets exhibited a lower relative abundance of Actinobacteria at day 5 ( $P = 0.029$ ). Furthermore, CONT piglets had a higher relative abundance of Firmicutes compared to those in PPG group at days 15 and 20 ( $P = 0.031$  and  $0.016$ ), respectively.

At the genus level, a total of 189 genera were identified from all samples. The core fecal microbial community (defined as the genera found at a relative abundance of  $> 1\%$  in all treatment groups) at the baseline (day 0) was comprised of common fecal microbial genera including *Escherichia-Shigella* (41.24%), *Clostridium* (17.33%), *Fusobacterium* (4.58%), *Bacteroides* (3.39%), *Actinobacillus* (3.04%), *Streptococcus* (3.01%), and *Lactobacillus* (2.44%). A hierarchically clustered heatmap of the most predominant microbial communities at the genus level is shown in (**Figure 2**). Compared to CONT, the TUL-treated piglets showed a decline in the relative abundance of *Ruminococcus* at day 15 and *Actinomyces* at days 10 and 20 of life. In contrast, the TUL-treated piglets had an increased proportion of *Escherichia-Shigella* at day 5 and *Bacteroides* at day 14. In CCFA group, the treated piglets had an increased proportion of *Campylobacter* at day 5, *Rikenellaceae RC9 gut group* at day 15 and a reduction in the proportion of *Lactobacillus* at day 5, *Streptococcus* at day 5, *Prevotella* at day 15. In CHC group, the piglets had a lower relative abundance of *Streptococcus* at day 5 and an increased proportion of *Campylobacter* at day 10.

The OTC-treated piglets exhibited an increase in the relative abundance of *Escherichia-Shigella* at day 5, *Bacteroides* at day 15, and a reduction in the relative abundance of *Lactobacillus* at day 5. In PPG group, the piglets showed a reduction in the proportion of *Fusobacterium* at day 10 and *Clostridium* at day 20. The PPG-treated piglets had an increased proportion of *Olsenella* at day 15 and day 20, *Escherichia-Shigella* at day 15, and *Bacteroides* at day 15 and day 20.

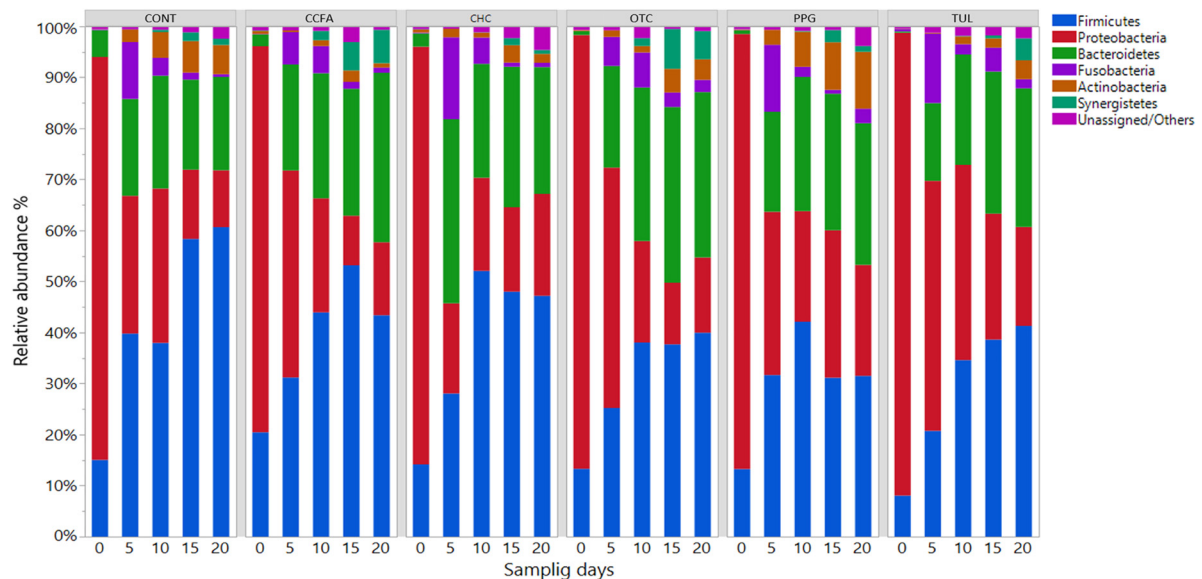
Alpha-diversity was computed using the number of OTUs per sample and the Shannon diversity indices (**Figure 3**). Collectively, the microbial diversity indices increased with age ( $P < 0.001$ ). Alpha diversity metrics showed non-significant changes between the CONT and treatment groups (**Figure 3**). Beta diversity analysis showed that the overall fecal microbiota structure at baseline (day 0) did not differ by treatment group (ANOSIM,  $P = 0.17$ ; **Figure 4**). The early life antimicrobial-induced changes in the microbial community composition were not sufficient to cluster samples by treatment at the different time points as shown by PCoA (ANOSIM,  $P > 0.05$ ; **Figure 4**). However, there was a significant effect of sampling time on the overall microbial community composition ( $P < 0.0001$ ,  $R^2 = 0.36$ ) (**Supplementary Figure S3**).

To further evaluate the potential changes in fecal microbiota associated with early life antimicrobial administration and to determine indicator taxa in each group, differences in the relative abundance of taxa between CONT and treated piglets were compared using LEfSe. Compared to CONT group, 15, 6, 14, 8, and 9 OTUs were identified as indicator taxa in CHC, OTC, TUL, PPG, and CCFA treated piglets respectively (**Supplementary Figure S4**). Additionally, a number of potential indicator taxa that were differentially represented in the treatment groups at the same age with their LDA scores are depicted in (**Supplementary Figure S5**). Collectively, the changes in the fecal microbiota structure caused by perinatal antimicrobials intervention are limited to a particular group of microbial taxa.

## Relationships Among the Overall Microbiota Composition of the Six Treatment Groups

A multiple group similarities tree was constructed using the Unifrac distance metrics to identify the similarities and differences among the antimicrobial treatment (**Figure 5A**). Collectively, comparison of the microbiota composition of different treatments group (CCFA, CHC, OTC, PPG, and TUL) showed no significant changes when compared to CONT group (ANOSIM,  $P > 0.05$ , **Supplementary Table S3**). However, the taxonomic composition of TUL-treated piglets was separated from the compositions of the CHC and CCFA treated piglets (ANOSIM,  $P = 0.024$  and  $0.015$ ) respectively (**Figures 5A,B**). The microbial community structure of the PPG-treated piglets was closest to OTC-treated piglets, indicating a closely community structure between these two treatments (**Figures 5A,B**). Similarly, samples from the CCFA and CHC piglets were also clustered together indicating that these two treatments resulted in similar community structures (**Figures 5A,B**).





**FIGURE 1 |** Taxonomic classification of 16S rRNA gene sequences at the phylum level for control (CONT,  $n = 4$ ), ceftiofur crystalline free acid (CCFA,  $n = 4$ ), ceftiofur hydrochloride (CHC,  $n = 4$ ), oxytetracycline (OTC,  $n = 4$ ), procaine penicillin G (PPG,  $n = 4$ ) and tulathromycin (TUL,  $n = 4$ ) treated piglets at each sampling time points. Only those bacterial phyla that averaged more than 1% of the relative abundance across all samples are displayed.

## Effect of Early Life Antimicrobial on Predicted Microbial Functional Profiles

Predicted functional profiles of the fecal microbial communities in the six groups (CONT, CCFA, CHC, OTC, PPG, and TUL) at the level 2 KEGG pathway were investigated using PICRUSt (Figure 6). Altogether, the PCA plot revealed that the predicted functional genes in each sample varied significantly with age (ANOSIM,  $P < 0.0001$ , Supplementary Figure S6A). Only, the TUL and CONT groups showed significant differences in the overall predicted KEGG pathways (level 2), particularly in carbohydrate metabolism, glycan biosynthesis, and metabolism and nucleotide metabolism (Supplementary Table S4). Furthermore, PCA of the predicted KEGG pathways (level 2) revealed that samples from CONT and TUL groups were clustered into two distinct groups (ANOSIM;  $P = 0.017$ ; Supplementary Figure S6B). The overall predicted KEGG pathways (level 2) of CCFA, CHC, OTC, and PPG treated piglets showed no significance differences when compared to CONT (ANOSIM;  $P > 0.05$ , Supplementary Figure S7). Detailed PICRUSt results of the functional gene profiles at KEGG level 3 are depicted in (Supplementary Table S5).

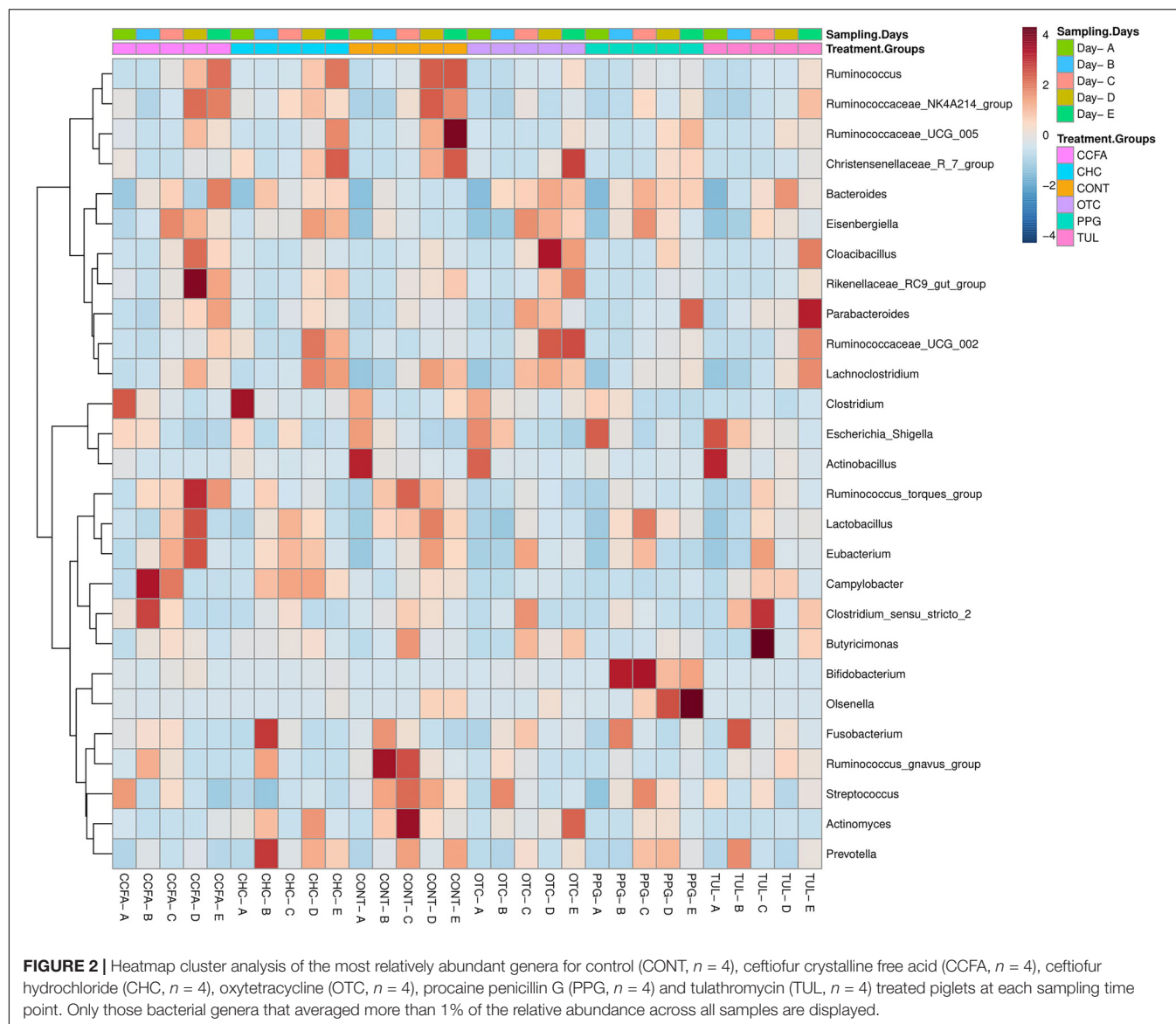
## Effect of Early Life Antimicrobial Administration on Selected ARGs

In this study, we quantified seven ARGs in relation to the bacterial 16S rRNA gene. All ARGs were detected with the exception of the *bla*<sub>CTX-M</sub>, which was below the limit of quantification in all samples. Across all samples, the most relatively abundant ARGs were *ermB* (33.85%), *tetW* (11.65%), and *SulII* (9.06%) (Figure 7). Compared to CONT, the early

life TUL intervention resulted in a significant increase in the abundance of *tetW* at days 10 and 20 ( $P < 0.05$ ), and *ermB* at day 20 ( $P < 0.05$ ) (Figures 8A,B). PPG-treated piglets exhibited a significant increase in the relative abundance of *ermB* and *tetW* at day 20 of life ( $P < 0.05$ ) (Figures 8C,D). In CCFA, CHC, and OTC groups, comparisons of ARGs abundance showed no significant differences after antimicrobial administration compared to CONT group at the same time point ( $P > 0.05$ ). PCA of the overall ARGs relative abundance (*ermB*, *tetO*, *tetW*, *tetC*, *sulI*, and *sulII*) revealed that samples from CONT and TUL groups clustered into two distinct groups (ANOSIM;  $P = 0.015$ , Supplementary Figure S8). The overall relative abundance of ARGs in the CCFA, CHC, OTC, and PPG treated piglets showed no significance difference when compared to CONT group (ANOSIM;  $P > 0.05$ , Supplementary Figure S8).

## DISCUSSION

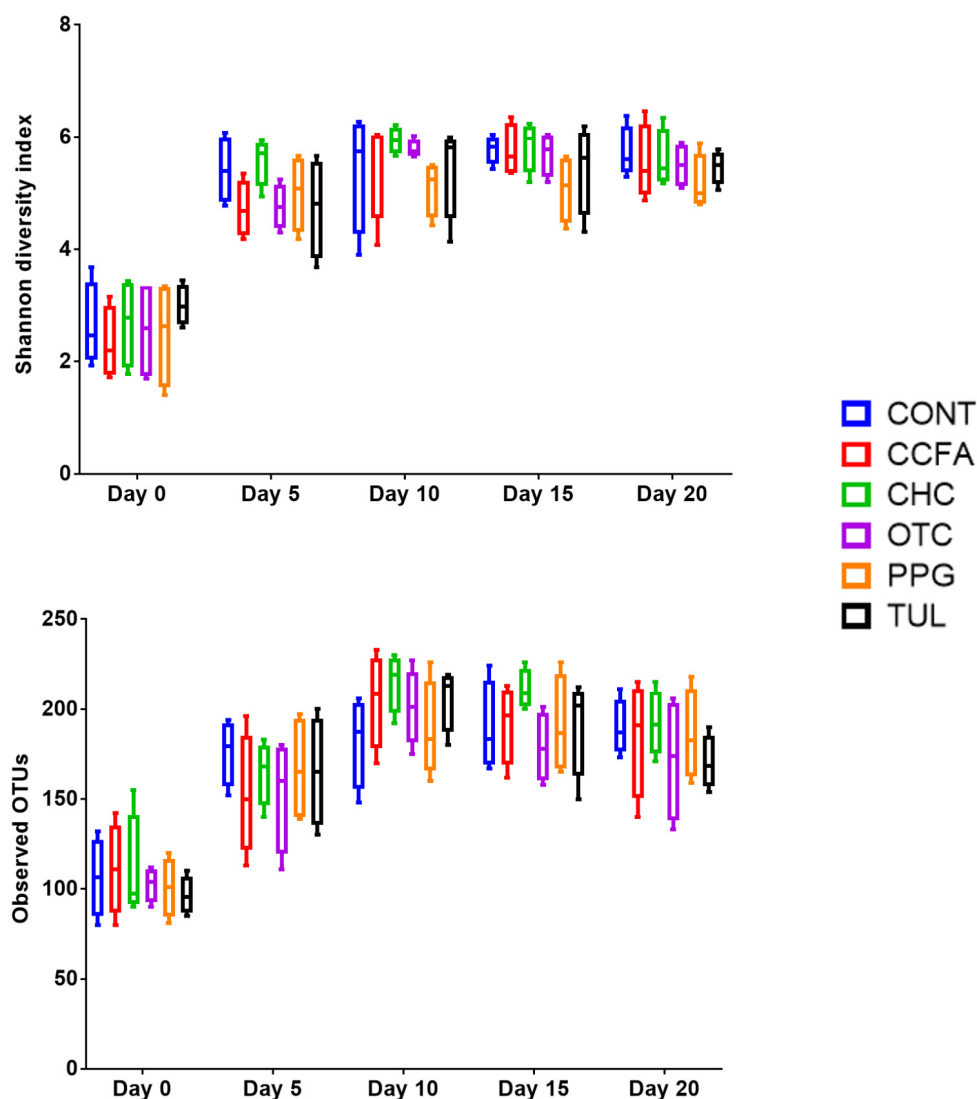
The extensive use of antimicrobials has led to emergence of antimicrobial-resistant bacteria and ARGs in the environment, which is thought to pose an imminent threat to animal and human health (Berendonk et al., 2015). Several research studies have also revealed microbial shifts in the swine gastrointestinal microbiota after antimicrobial administration (Kim et al., 2012; Zeineldin et al., 2018a; Zhao et al., 2018). In order to overcome these problems, production systems must adapt to reduce the use of antimicrobials. A key step in reducing antimicrobial use is understanding the mechanism and magnitude by which antimicrobial administration affects the microbial ecosystem, emergence of ARGs, and overall



host health. Multiple lines of evidence indicate that the gastrointestinal tract of swine has a complex and diverse microbial ecosystem, where extensive communication between host, mucosal communities, and surrounding environment, occur (Li K. et al., 2017; Zeineldin et al., 2017a). It is therefore crucial to understand how common management practices, including early life antimicrobial administration, may influence this complex ecosystem in animals raised in intensive production systems.

Antimicrobials are used parentally in swine production to control and prevent infectious disease (Pyörälä et al., 2014). Sound scientific evidence shows that antimicrobial intervention can have both detrimental and beneficial effects on host health (Phillips et al., 2004), but this has not been widely studied in neonates. This study used 16S rRNA gene sequencing to quantify the impacts of a single dose of early life antimicrobial on the fecal microbiota structure,

and relative abundance of selected ARGs (*ermB*, *tetW*, *tetO*, *tetC*, *sull*, *sullI*, and *bla<sub>CTX-M</sub>*) in neonatal piglets. In line with other studies, our results demonstrated that the fecal microbial communities in all treatment groups were dominated by Firmicutes, Proteobacteria, and Bacteroidetes at the phylum level (Maradiaga et al., 2018), and by *Escherichia-Shigella*, *Bacteroides*, *Lactobacillus*, *Clostridium*, and *Streptococcus* at the genus level (Maradiaga et al., 2018). In terms of temporal changes, time-dependent dynamics of the piglet's fecal microbiota were observed. The neonatal piglets at day 0 had a significantly greater proportion of *Escherichia-Shigella*, *Fusobacterium*, *Clostridium*, and *Actinobacillus*. The piglets fecal microbiota composition observed in this study at day 0 after birth was similar to that published by Kubasova et al. (2017). Although *Escherichia* and *Clostridium* are often the first genera to colonize the gastrointestinal tract in different animal species



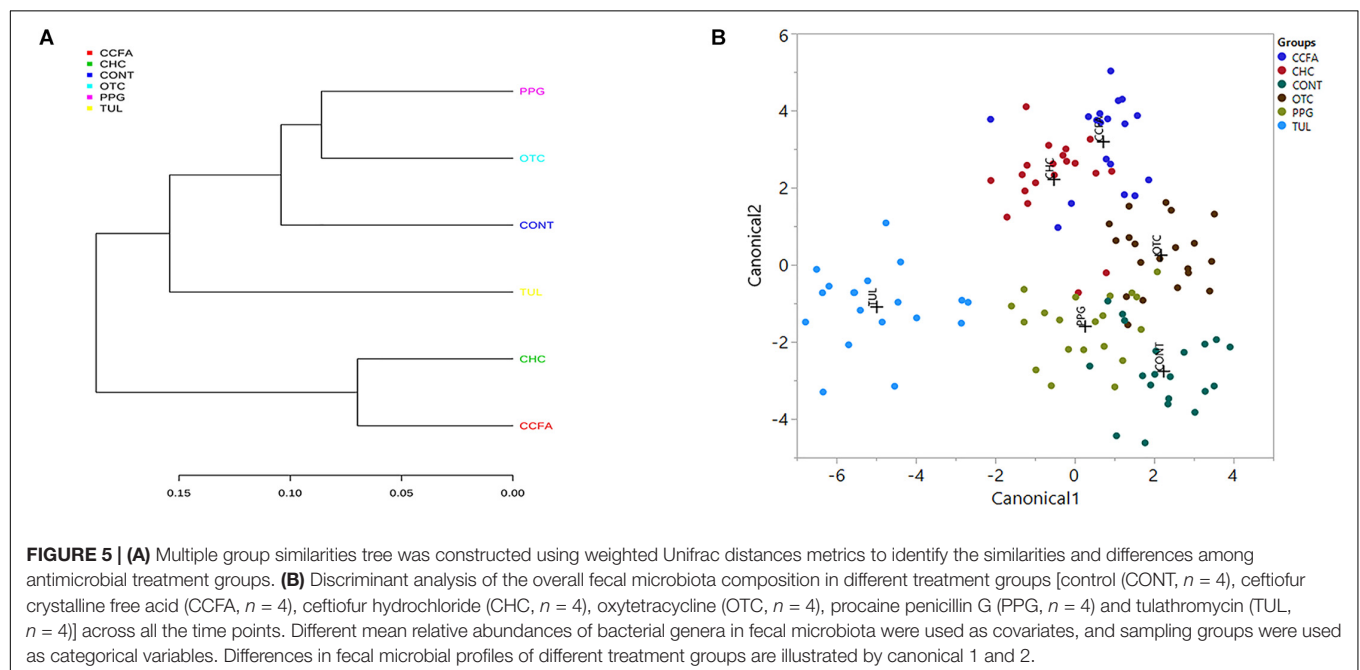
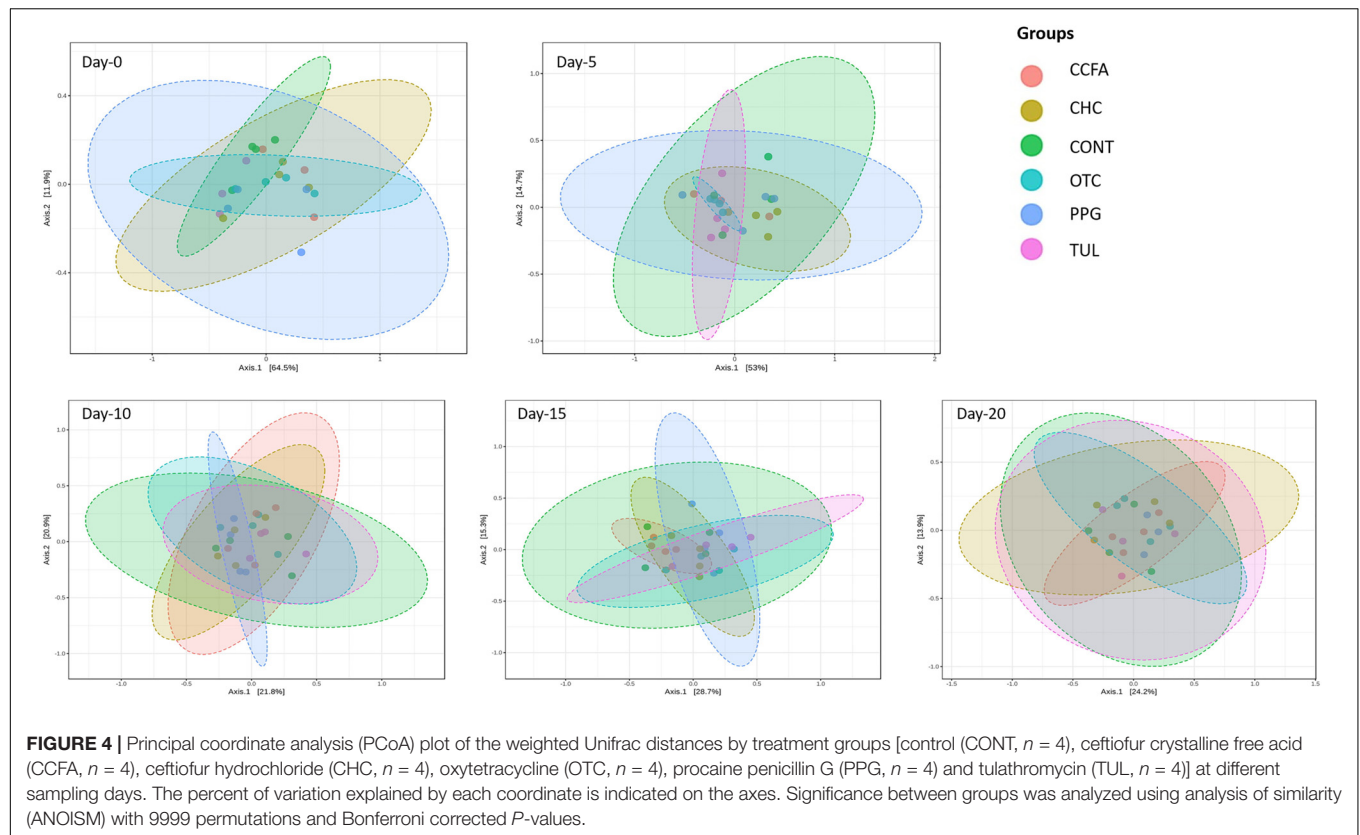
**FIGURE 3 |** Bacterial diversity indices by treatment groups control (CONT,  $n = 4$ ), ceftiofur crystalline free acid (CCFA,  $n = 4$ ), ceftiofur hydrochloride (CHC,  $n = 4$ ), oxytetracycline (OTC,  $n = 4$ ), procaine penicillin G (PPG,  $n = 4$ ), and tulathromycin (TUL,  $n = 4$ ) at different time points (days 0, 5, 10, 15, and 20).

(Rodríguez et al., 2015; Slifierz et al., 2015), the presence of *Fusobacterium* in the gut microbiota of 0 day old piglets is of concern since some *Fusobacterium* spp. have been linked to swine dysentery (Durmic et al., 1998). In 20-day-old piglets, *Lactobacillus*, *Bacteroides*, *Ruminococcus*, and *Ruminococcaceae* UCG-005 were the most relatively abundant genera, which is similar to previous reports (Slifierz et al., 2015; Kubasova et al., 2017).

Contrary to the disruption of the swine gut microbiota that can result from in feed antimicrobial exposure (Kim et al., 2012, 2016; Looft et al., 2012; Zhao et al., 2018), The observed changes in the developmental dynamics of the fecal microbiota showed antimicrobial-specific variations in both duration and extent. Our findings are generally in line with a previous study that evaluated the impact of antimicrobial treatment on the microbiota composition and resistance

gene reservoir (Choo et al., 2018). Using 16S rRNA gene sequencing, Choo et al. (2018) demonstrated that the disruption in the oropharyngeal microbiota composition of humans was restricted to a relatively small group of *Actinomyces* species (Choo et al., 2018). In the present study, the reduction in the relative abundance of *Actinomyces* population in response to TUL treatment are in agreement with other *in vitro* studies (Smith et al., 2005). *Actinomyces* spp. are Gram-positive facultative anaerobes that consume lactate and frequently reside in the female genital tract, and gastrointestinal tract of healthy individuals (Takahashi and Yamada, 1999; Smith et al., 2005).

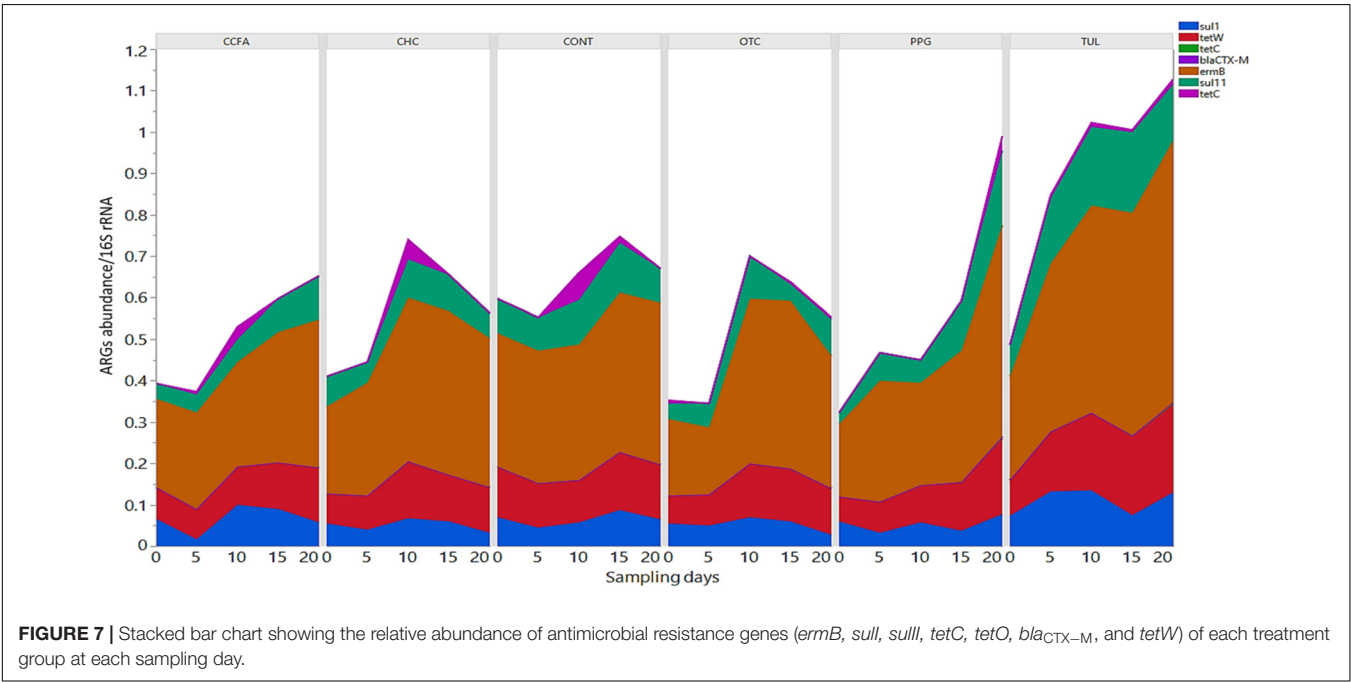
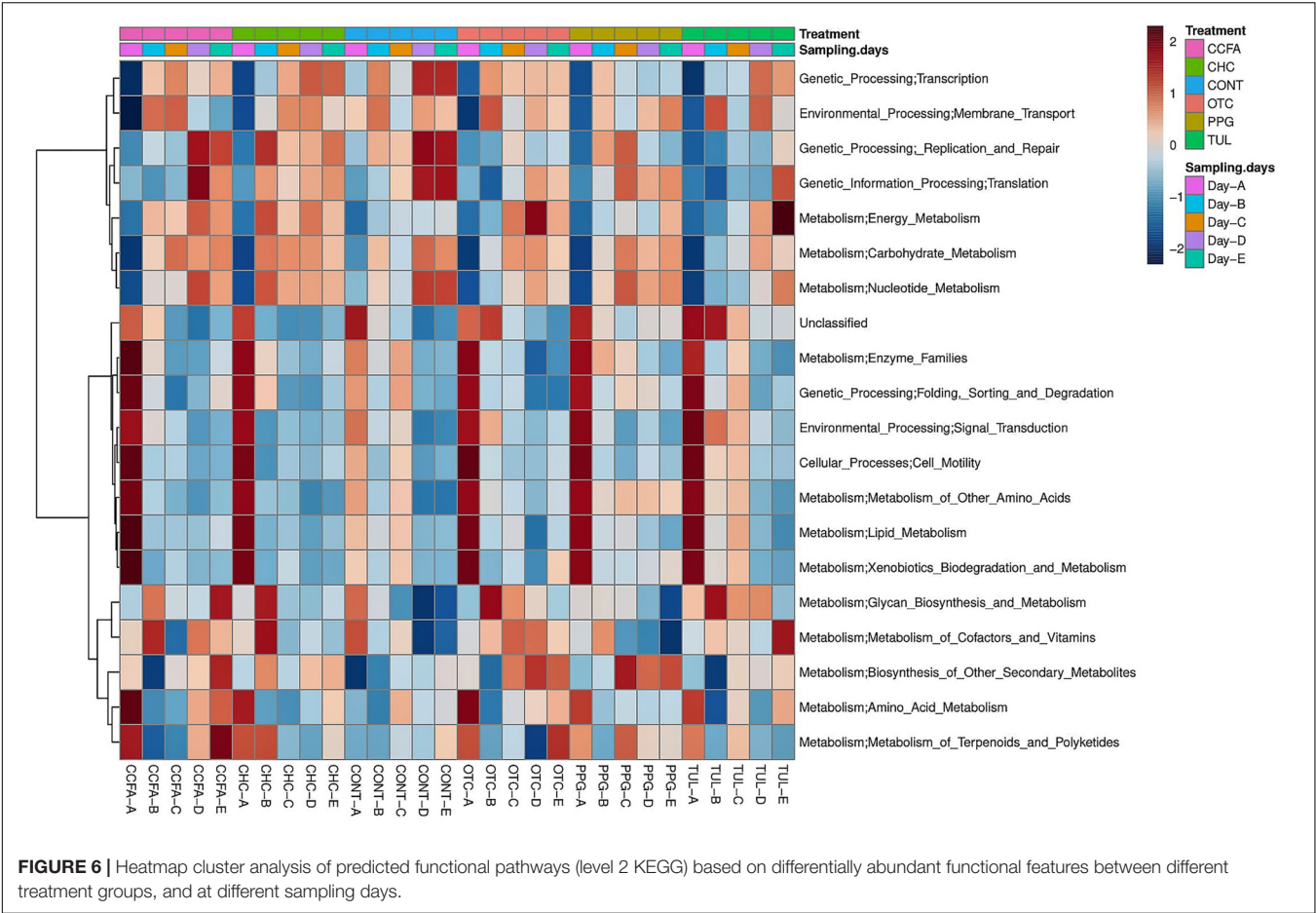
Of particular interest in our results is the decrease in the proportion of *Ruminococcus* in the CCFA, CHC and TUL-treated piglets compared with CONT group. Members of *Ruminococcus* genera are commonly associated with gut

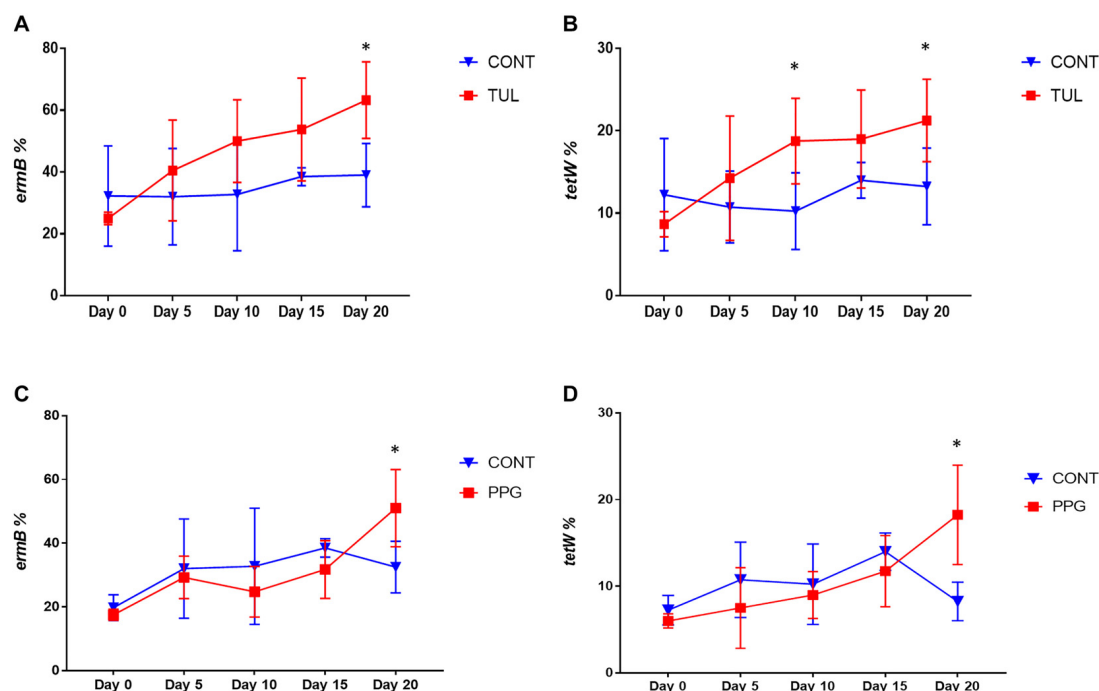


health, through generation of short-chain fatty acid that play an important role in reduction of colonization of many opportunistic pathogens (Yu et al., 2018). Additionally, TUL, PPG and OTC treated piglets showed an increased abundance of *Escherichia-Shigella* and *Bacteroides*. *Escherichia* spp. are

commonly found in farm environment and are considered indigenous to the piglets gut microbiota (Yang et al., 2004). *Escherichia* spp. can be pathogenic, and include many species associated with neonatal and post weaning diarrhea in swine (Bischoff et al., 2002; Chapman et al., 2006). Similarly, an







**FIGURE 8 | (A,B)** Line graphs illustrating the difference in relative abundance of *ermB* and *tetW* between the control (CONT,  $n = 4$ ) group, and tulathromycin (TUL,  $n = 4$ ) group at different sampling days. **(C,D)** Line graphs illustrating the difference in abundance of antimicrobial resistance genes (*ermB* and *tetW*) between the control (CONT,  $n = 4$ ) group, and procaine penicillin G (PPG,  $n = 4$ ) treated piglets at each sampling day. \* $P < 0.05$ .

increase in the abundance of *Bacteriodes* spp. during early life is considered disease predisposing condition (Korpela et al., 2016; Tran et al., 2018). Further studies evaluating the role of *Bacteriodes* and *Escherichia*, either as markers of gastrointestinal dysbiosis after antimicrobial treatment, are warranted. The PPG-treated piglets also exhibited an increase in the proportion of *Olsenella* at day 15 and day 20. The *Olsenella* genus was first proposed by (Dewhirst et al., 2001), and has recently been reclassified to the *Atopobiaceae* family within the *Coriobacteriales* order and *Coriobacteriia* class (Gupta et al., 2013). Members of the *Olsenella* genus are Gram positive rods that produce skatole, a compound responsible for boar taint and off-flavor taint, which released upon heating meat from male pigs (Li X. et al., 2015). In the CCFA and CHC groups, piglets had an increased proportion of *Campylobacter* at day 5 and day 10 respectively. *Campylobacter* spp., are considered one of the common causes of human enteritis (Dicksveld et al., 2014) and swine dysentery (Yang et al., 2017). Taken together, our result suggests that early life antimicrobial intervention may make the gastrointestinal tract more susceptible to potential pathogenic bacteria. While it is difficult to understand whether the short-term moderate changes in the developmental dynamics of gastrointestinal microbiota observed in this study have any significant long-term impacts on the health and production of the growing piglets, the significance of antimicrobial-induced microbial shift have been well documented by other researchers (Schokker et al., 2015).

Bacterial diversity is often used as a crucial measure of functional resilience and homeostasis of gastrointestinal microbial ecosystem (Lozupone et al., 2012). Bacterial diversity indices suggest that the piglet fecal microbiota was rich and diverse and underwent intricate development during the first 20 days of life. Similarly, the gastrointestinal tract of piglets during early life, showed an age-dependent manner of microbial population evolution and acquisition (Bian et al., 2016). In line with other studies, our result showed that there was no significant changes in the overall microbial community composition between treatment groups at each time point as measured by beta diversity analysis (Zhang et al., 2016; Li P. et al., 2017). In contrast, Looft et al. (2014) observed a significant changes in diversity indices after early life carbadox administration of in 6-weeks-old piglets. The discrepancies between the present study and previous research might have resulted from the use of different type of antimicrobial, dosage, route of administration, and different environmental conditions (Li P. et al., 2017).

To identify indicator taxa that are significantly discriminated between CONT and other treatment groups, we used a well-established approach, LefSe, to identify bacterial taxa of interest for further analysis (Segata et al., 2011). In this study, LefSe revealed 15, 6, 14, 8, and 9 OTUs as indicator taxa in CHC, OTC, TUL, PPG, and CCFA treated piglets respectively. These results further support the concept that the shifts in the fecal microbiota structure caused by perinatal

antimicrobial intervention are modest and are limited to a particular group of microbial taxa. We also used PICRUST to predict the fecal metagenome and identified potential functional pathways that were significantly different between treatment groups. Similar to highly diverse and developed fecal microbiota, predicted functional pathways differed by time point. While these are only presumptions, based on the predicted functional features of the taxonomically assigned microbial population in our study, similar shift have been noted after different antimicrobial therapy in humans (Pérez-Cobas et al., 2013). The significant enrichment in some functional pathways after different antimicrobial administration implied that these functional features might play a crucial role under stress conditions (Wang and Quinn, 2010). Similarly, Perez-Cobas et al. (2012) demonstrated an increase in functional genes belonging to carbohydrate metabolism and energy metabolism/sugars category during antimicrobial treatment (Perez-Cobas et al., 2012). Further investigations into the functional profiles associated with microbial community changes (i.e., which community members have the same functional features and could alternate for one another), either by shotgun metagenomics, direct metabolites measurement or by transcriptome analysis, will be an essential next step to better understand the effect of early life antimicrobial interventions on microbiota function in piglets.

In this study, we assessed carriage of seven different ARGs genes (*tetW*, *tetO*, *tetC*, *sull*, *ermB*, *sullI*, and *bla<sub>CTX-M</sub>*) in relation to the bacterial 16S rRNA gene, based on their identification in previous research (Supplementary Table S1). The tested ARGs belongs to the most abundant type of these ARGs confer resistance to macrolides, beta lactams, sulfonamide and tetracycline, and can be carried by common members of the gut microbiota (Li et al., 2016). Our results demonstrate that the ARGs were present in the piglet's gut microbiota from the first day of life. Compared to CONT group, the TUL and PPG treated piglets exhibited a significant increase in the relative abundance of the *ermB* gene. This finding is in line with the increased carriage of *ermB* after long-term administration of erythromycin in healthy individuals (Choo et al., 2018). The *ermB* gene can be horizontally transferred between the commensal microbiota via transformation or conjugation, permitting commensal microbiota to serve as a resistance reservoirs (Roberts et al., 2011). Additionally, the TUL and PPG treated piglets exhibited a significant increase in the relative abundance of *tetW*, which encodes for a ribosomal protection protein. Interestingly, the change in the proportion of *ermB* and *tetW* had a similar temporal pattern. This might indicate that these genes are linked together on the same mobile genetic element (Rubio-Cosials et al., 2018). These findings suggest that single dosages of TUL and PPG can increase the relative abundance of ARGs conferring resistance to antimicrobials that are not administrated. Moreover, increases in the levels of transmissible ARGs within the developing fecal microbiota highlight the potential of the gut to act as a resistance reservoir (Looft et al., 2012).

Our study had a number of experimental limitations that should be considered. The sequencing analysis was conducted on a relatively small number of piglets, though similar to other published sequencing studies (Yu et al., 2018). Furthermore, our analysis focused on short-term impacts of antimicrobial administration on the fecal microbiota. It would have been interesting to continue to sample the piglets for a longer period after weaning to define how these changes impact future health and productivity of growing piglets. Finally, our study focused on identification of selected ARGs, and we did not evaluate change in the resistome using non-targeted sequencing. Despite these experimental limitations, our study results provide preliminary insight into an area of investigation that could be of great relevance to the swine gut health. Understanding the factors that influence the developmental dynamics of gut microbiota is important for establishing which management approaches could be used to promote and maintain a stable microbial ecosystem during this important phase of production.

## CONCLUSION

This study demonstrated that antimicrobial intervention had relatively minor effects on the gut microbiota development during early life in comparison to control piglets but alterations were noticeable in particular taxa. However, early life TUL and PPG intervention could promote selection of ARGs in herds. This knowledge may help us to understand the impacts of early antimicrobial exposure on gut microbial composition and development of ARGs in swine management system. Understanding when and how and the gut microbiota changes in response to antimicrobial administration will aid in the development of new antimicrobial alternatives.

## AUTHOR CONTRIBUTIONS

JL and BA designed the experiment. BrB, AM, BeB, MZ, and JL conducted the experiment. MZ, AM, BrB, and BeB carried out the laboratory analyses. MZ and AM conducted the data analysis. MZ wrote the manuscript. All authors edited and approved the manuscript for submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01414/full#supplementary-material>

**FIGURE S1 | (A)** Bar graph illustrating body weight (kg) at day 0 and day 20, and average weight gain from day 0 to day 20 in different treatment groups. **(B)** Bar graph illustrating the mortality percent of piglets from day 0 to day 5 (day 0–5), from day 5 to day 10 (day 5–10), from day 10 to day 15 (day 10–15), and from day 15 to day 20 (day 15–20) in different treatment groups. There was no significant change in the average daily weight gain and overall mortality ratio ( $P > 0.05$ ).

**FIGURE S2 |** Venn diagram depicting the common and unique OTUs among the different treatment groups (CONT, CCFA, CHC, OTC, PPG, and TUL). A total of 842 OTUs were represented core microbiota and shared between all treatment groups.

**FIGURE S3 |** Principal coordinate analysis (PCoA) for different sampling days (0, 5, 10, 15, and 20). The percent variation explained by each coordinate is indicated on the axes. The individual data points from which represent total fecal microbiota compositions of each piglet are also depicted. Significance between groups was analyzed using analysis of similarity (ANOSIM) with 9999 permutations and Bonferroni corrected  $P$ -values.

**FIGURE S4 |** LDA Effect Size (LEfSe) analysis of fecal microbiota depicting the top OTUs with the highest linear discriminant analysis LDA score  $\log_{10} \geq 2.0$  that discriminate between the CONT group and CCFA, CHC, OTC, PPG, and TUL treated piglets. Each color refers to each group and its corresponding indicator taxa.

**FIGURE S5 |** Identification of indicator bacterial taxa associated with statistically significant differential abundance between the different treatment groups (CONT,

CCFA, CHC, OTC, PPG, and TUL) at different sampling days. The top OTUs with the highest LDA score  $\log_{10} \geq 2.0$  that discriminate between the CONT group and CCFA, CHC, OTC, PPG, and TUL treated piglets at each time point are depicted. Each color refers to each group and its corresponding indicator taxa.

**FIGURE S6 | (A)** Principal component analysis for overall functional gene profiles at different sampling days (days 0, 5, 10, 15, and 20). **(B)** PCA for overall functional gene profiles between the CONT and TUL-treated piglets. The percent variation explained by each principal component is indicated on the axes.

**FIGURE S7 |** Principal component analysis for the overall predicted functional gene pathways between CONT and different treatment groups (CCFA, CHC, OTC, and PPG) across all time points.

**FIGURE S8 |** Principal component analysis for overall selected antimicrobial resistance genes (*ermB*, *sull*, *sulll*, *tetC*, *tetO*, and *tetW*) between CONT group and CCFA, CHC, OTC, PPG, and TUL treated piglets.

**TABLE S1 |** Primers targeting selected antibiotic resistance genes used in this study.

**TABLE S2 |** Access Array cycling program without imaging (Fluidigm Biomark HD PCR machine) for amplifying the primer/sample combinations.

**TABLE S3 |** The result of nonparametric ANOSIM test (analysis of similarities) with 9999 Monte Carlo permutations to evaluate the UniFrac distances significance between different treatment groups.

**TABLE S4 |** The difference in mean relative abundance of function gene profiles in fecal microbiota at level 2 KEGG pathway between the CONT and TUL groups.

**TABLE S5 |** Summarized PICRUST results of mean relative abundance of function gene profiles in fecal microbiota at level 3 KEGG pathway in all treatment groups (CONT, CCFA, CHC, OTC, PPG, and TUL).

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# Dissemination of Multidrug-Resistant Commensal *Escherichia coli* in Feedlot Lambs in Southeastern Brazil

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Antimicrobial resistance (AR) is a public health issue since it limits the choices to treat infections by *Escherichia coli* in humans and animals. In Brazil, the ovine meat market has grown in recent years, but studies about AR in sheep are still scarce. Thus, this study aims to investigate the presence of AR in *E. coli* isolated from lambs during feedlot. To this end, feces from 112 lambs with 2 months of age, after weaning, were collected on the first day of the animals in the feedlot (day 0), and on the last day before slaughtering (day 42). Isolates were selected in MacConkey agar supplemented with 4 mg/L of ceftiofur and identified by biochemical methods. Isolates were submitted to an antimicrobial susceptibility test by disc-diffusion and PCR to investigate genes for phylogenetic group, virulence determinants and resistance to the several antimicrobial classes tested. The genetic localization of the *bla* genes detected was elucidated by S1-PFGE followed by Southern blot-hybridizations. The isolates were typed by *Xba*I-PFGE and MLST methods. Seventy-eight *E. coli* were isolated from 8/112 (7.1%) animals on day 0, and from 55/112 (49.1%) animals on day 42. Since only *fimH* was present in almost all *E. coli* (97.4%) as a virulence gene, and also 88.5% belonged to phylogroups B1 or A, we consider that isolates represent intestinal commensal bacteria. The dendrogram separated the 78 non-virulent isolates in seven clusters, two of which comprised 50 *E. coli* belonging to ST/CC 1727/446 or ST 3994 recovered on day 42 commonly harboring the genotype *bla*<sub>CMY-2</sub>-*aac*(3)-*Ila* -*tetA*-*sul1*-*sul2*-*floR*-*cmlA*. Special attention should be given to the presence of *bla*<sub>CTX-M-15</sub>, a worldwide gene spread, and *bla*<sub>CTX-M-14</sub>, a hitherto undetected gene in *Enterobacteriaceae* from food-producing animals in Brazil. Importantly, *E. coli* lineages and plasmids carrying *bla* genes detected here have already been reported as sources of infection in humans either from animals, food, or the environment, which raises public health concerns. Hence, two types of commensal *E. coli* carrying important AR genes clearly prevailed during feedlot, but lambs are also reservoirs of bacteria carrying important AR genes such as *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub>, mostly related to antimicrobial treatment failure.

**Keywords:** *Escherichia coli*, sheep, multidrug resistance, cephalosporin, aminoglycoside, tetracycline, trimethoprim/sulfamethoxazole, phenicol

## INTRODUCTION

The use of antimicrobial agents in humans and animals can cause the emergence and dissemination of antimicrobial resistance (AR) in pathogens, which may compromise the effective treatment of infections in humans (Kaesbohrer et al., 2012). International public health agencies have reported the potential link and risks between the overuse or misuse of antimicrobials in veterinary practices and the emergence of human resistant pathogens, which encourage surveillance of AR and antimicrobial use worldwide (EFSA, 2011; WHO, 2017). Human exposure to AR bacteria through direct contact with animals, consumption and handling of contaminated food, and bacteria released into the environment may contribute to the spread of AR determinants (Kaesbohrer et al., 2012).

Infections caused by AR *E. coli* and their isolation from food-producing animals are increasing worldwide (EFSA, 2008, 2011; Kaesbohrer et al., 2012). This scenario is regarded as a consequence of the selective pressure exerted on the gastrointestinal tract (GIT) of the animals by the overuse of antimicrobials (Graham et al., 2017). During slaughtering, the carcass may be contaminated and AR commensal or pathogenic bacteria might reach humans through the food chain (Cyoia et al., 2019; Projahn et al., 2019). The relationship between AR strains isolated from humans and the food chain has been already reported (Belmar Campos et al., 2014). Therefore, the monitoring of commensal bacteria is important since it constitutes a reservoir of AR genes, which allows the tracking of emerging resistance in livestock and possible spread to animal-derived food and other zoonotic pathogens (EFSA, 2008; Kaesbohrer et al., 2012; Madec and Haenni, 2018).

The majority of studies about antimicrobial use and resistance in food-producing animals are carried out on cattle, chickens, and pigs, but in regard to other food-producing flocks, such as sheep, information is scarce. Little is known about AR in sheep in Brazil, despite the increased consumption of lamb meat (FAO, 2018). Therefore, this study aims to determine the distribution of AR *E. coli* in the fecal microbiota of feedlot lambs in Brazil.

## MATERIALS AND METHODS

### Study Population

A special feedlot comprising 140 lambs with 2 months of age, after weaning, coming from 35 different farms in the State of São Paulo, Southeastern Brazil was chosen for this study. Stool samples were collected weekly from the rectum of the animals for parasitological screening between September 14, 2016 and October 27, 2016, under the Ethics Committee approval number FOA00845-2017. Trimethoprim/sulfamethoxazole was used to prevent and to treat clinical manifestations of respiratory disease, and florfenicol was used to treat infectious keratoconjunctivitis. Stool samples from 112 lambs were collected immediately after the arrival of the sheep at the feedlot (day 0) and then on the day before the slaughtering of the animals (day 42) to further investigate the presence of AR *E. coli*.

### Bacterial Culture, Identification and Antimicrobial Susceptibility

About one gram of feces was diluted in 5 mL of sterile NaCl 0.9% and directly inoculated onto MacConkey agar (Oxoid) supplemented with 4 mg/L of ceftiofur (Lapisa). Following incubation at 37°C for 18–24 h, one of each of the different presumptive *E. coli* colonies (i.e., pinkish round colony due to lactose fermenting, dry to little mucous aspect, and characteristic odor) were selected for identification by biochemical essays using a commercial kit (NewProv) and further characterization described below.

Antimicrobial susceptibility testing was performed following the Clinical and Laboratory Standards Institute (CLSI, 2017) guidelines using the disc diffusion method. Bacterial susceptibility to 13 beta-lactam and non-beta-lactam antibiotics (Oxoid) of veterinary and human interest was tested: amoxicillin/clavulanate, ceftazidime, cefotaxime, ceftiofur, cefoxitin, ertapenem, amikacin, gentamicin, enrofloxacin, nalidixic acid, tetracycline, trimethoprim/sulfamethoxazole, florfenicol, and chloramphenicol. Parallel to the antimicrobial susceptibility test, the phenotypic test for production of extended-spectrum beta-lactamase was performed by the Modified Double Disc Synergy Test (Kaur et al., 2013). *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control strains.

### AR and Virulence Genes, and Phylogenetic Grouping

Investigation of the main plasmidial genes associated with cephalosporins resistance (*bla*<sub>CTX-M</sub> and *bla*<sub>CMY</sub>), aminoglycosides resistance [*aac*(3)-Ia, *aac*(3)-IIa, *acc*(6')-Ih, *ant*(2'')-Ia, *aph*(3')-VI, *aph*(3')-Ia and *aac*(6')-Ib], quinolones resistance (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepAB*, and *oqxAB*), tetracycline resistance (*tetA*, *tetB*, and *tetC*), trimethoprim resistance (*dfr* Ia, *dfr* VII, and *dfr* XII), sulphas resistance (*sul*1 and *sul*2), and phenicols resistance (*floR*, *cat* and *cmlA*) was performed in each respective resistant strain by PCR according to previous protocols (Supplementary Table 1). Products of *bla* genes were sequenced using the corresponding primers in order to identify the variant detected and analyzed using BLAST<sup>1</sup>.

The following 20 virulence genes, which have been associated with Extraintestinal Pathogenic *E. coli* strains, were investigated by PCR as previously described (Supplementary Table 1): *fimH*, *papEF*, *papG* I, *papG* II, *papG* III, *sfa/focDE*, *sfaS*, *focG*, *afa/draBC*, *nfaE*, *kpsMT* K1, *kpsMT* K5, *hlyA*, *cnf1*, *cdtB*, *sat*, *vat*, *fyuA*, *iutA*, and *iroN*. Since sheep are known as an important source of Shiga-toxin producing-*E. coli* (STEC) (Vettorato et al., 2009), the *stx1* and *stx2* genes, as well as the *aggR* and the *eae* genes, associated with Enterotoxigenic *E. coli* (EAEC) and Enteropathogenic *E. coli* (EPEC), respectively, were additionally searched by PCR according to previous protocols (Supplementary Table 1). *E. coli* isolates were also submitted to phylogenetic grouping for predicting of commensal or pathogenic isolates as previously described (Clermont et al., 2000; Doumith et al., 2012).

<sup>1</sup><http://blast.ncbi.nlm.nih.gov/>



## Plasmids Typing

Replicon of the plasmids of the isolates was detected by PCR-based Replicon Typing scheme (Carattoli et al., 2005; Villa et al., 2010) using the PBRT 2.0 kit (DIATHEVA). S1 enzyme (Promega) was used for 45 min to linearize plasmids and results were visualized in *Pulsed-Field Gel Electrophoresis* (S1-PFGE) for 20 h with initial switch time = 1 s and final switch time = 30 s on an electric field of 6 V/cm. Southern blot-hybridization analysis on S1-PFGE gels was performed using adequate probes and the kits Amersham™ AlkPhos Direct Labeling Reagents and Amersham™ CDP-Star™ Detection Reagent (GE Healthcare).

## Isolates Typing

Bacterial DNA was typed by restriction with *Xba*I (Thermo Scientific) followed by a PFGE (*Xba*I-PFGE) for 22 h with initial switch time = 2.2 s and final switch time = 54.2 s, and 6 V/cm. The software BioNumerics™ version 7.6.3 (Applied Maths) was used for dendrogram construction and clustering based on the band-based Dice's similarity coefficient and the unweighted pair group method using arithmetic averages. Isolates were considered to belong to the same cluster when the similarity coefficient was  $\geq 90\%$ .

*Escherichia coli* isolates were additionally submitted to *Multilocus Sequence Typing* according to the Achtman's scheme<sup>2</sup>.

## Nucleotide Sequence Accession Number

The *bla* genes sequences reported in this study have been deposited to GenBank under accession numbers MK896925 to MK896944 and MK917695 to MK917713.

## RESULTS

Eight CTX-M-producing *E. coli* were isolated from eight animals on day 0, and 70 CTX-M- or CMY-2-producing *E. coli* were isolated from 55 lambs on day 42 (Figure 1 and Table 1). All 78 isolates presented resistance to at least one of the third-generation cephalosporins – 3GC tested (ceftazidime, cefotaxime, ceftiofur). The 53 CMY-2-producing *E. coli* presenting resistance to amoxicillin/clavulanic acid also presented resistance to the cephamycin cefoxitin (Figure 1). More than 80% of the isolates presented additional resistance to at least one of the phenicols tested (68, 87.2%), to tetracycline (66 isolates, 84.6%), to trimethoprim/sulfamethoxazole (65, 83.3%), and at least one of the aminoglycosides tested (64, 82.0%). Only seven isolates (9.0%) presented resistance to nalidixic acid and/or enrofloxacin, and all *E. coli* were susceptible to ertapenem (Table 1).

In total, 18 genes responsible for antimicrobial resistance were detected in this study, and all of the 78 isolates presented *bla*<sub>CMY-2</sub> or *bla*<sub>CTX-M</sub> genes (Figure 1 and Table 2). The genes *bla*<sub>CTX-M-8</sub>, *bla*<sub>CTX-M-14</sub>, and *bla*<sub>CTX-M-15</sub> were identified in the isolates from day 0 harbored by plasmids IncI1 of ~97 kb or IncHI1 ~194 kb for *bla*<sub>CTX-M-8</sub>, and plasmid FII of about 97 kb for *bla*<sub>CTX-M-15</sub>. We could not detect plasmids harboring *bla*<sub>CTX-M-14</sub>. The *bla*<sub>CTX-M-2</sub>, *bla*<sub>CTX-M-8</sub>, *bla*<sub>CTX-M-15</sub>, and

*bla*<sub>CMY-2</sub> were identified in isolates recovered on day 42 inserted into the chromosome in the case of *bla*<sub>CTX-M-2</sub>, and harbored by plasmids IncI1 of about 95 kb or 97 kb for *bla*<sub>CTX-M-8</sub>, plasmid IncHI2 of ~335 kb for *bla*<sub>CTX-M-15</sub>, and plasmid IncA/C of ~170 kb for all *bla*<sub>CMY-2</sub> (Table 3). Regarding resistance to aminoglycosides, especially gentamicin, the *aac*(3)-*Ila* gene was detected in 60 isolates (76.9%) on days 0 and 42 of feedlot while the *ant*(2'')-*Ia* gene was detected only in five isolates (6.4%) on day 42. The *qnrB* gene was the only one detected as responsible for quinolone non-susceptibility, present in six isolates (7.7%) obtained on day 42. The *tetA* and *tetB* genes, responsible for tetracycline resistance, were detected in 54 (69.2%) and 10 (12.8%) isolates, respectively, on days 0 and 42 of feedlot. Concerning resistance to trimethoprim, the *dfr* VII gene was detected only on day 0 of feedlot in four isolates (5.1%), and *dfr* Ia and *dfr* XII were detected only on day 42 in 16 (20.5%) and 21 (26.9%) isolates, respectively. Resistance to sulphas was detected at both the first and last days of feedlot, with 61 isolates (78.2%) carrying *sul*I, and 65 (83.3%) carrying the *sul*2 gene. Lastly, in regard to phenicols resistance, the *floR* and the *cmlA* genes were detected in 65 (83.3%) and 67 (85.9%) isolates, respectively, while the *cat* gene was detected in only eight isolates (10.3%); all recovered on both days 0 and 42 of feedlot (Figure 1 and Table 2).

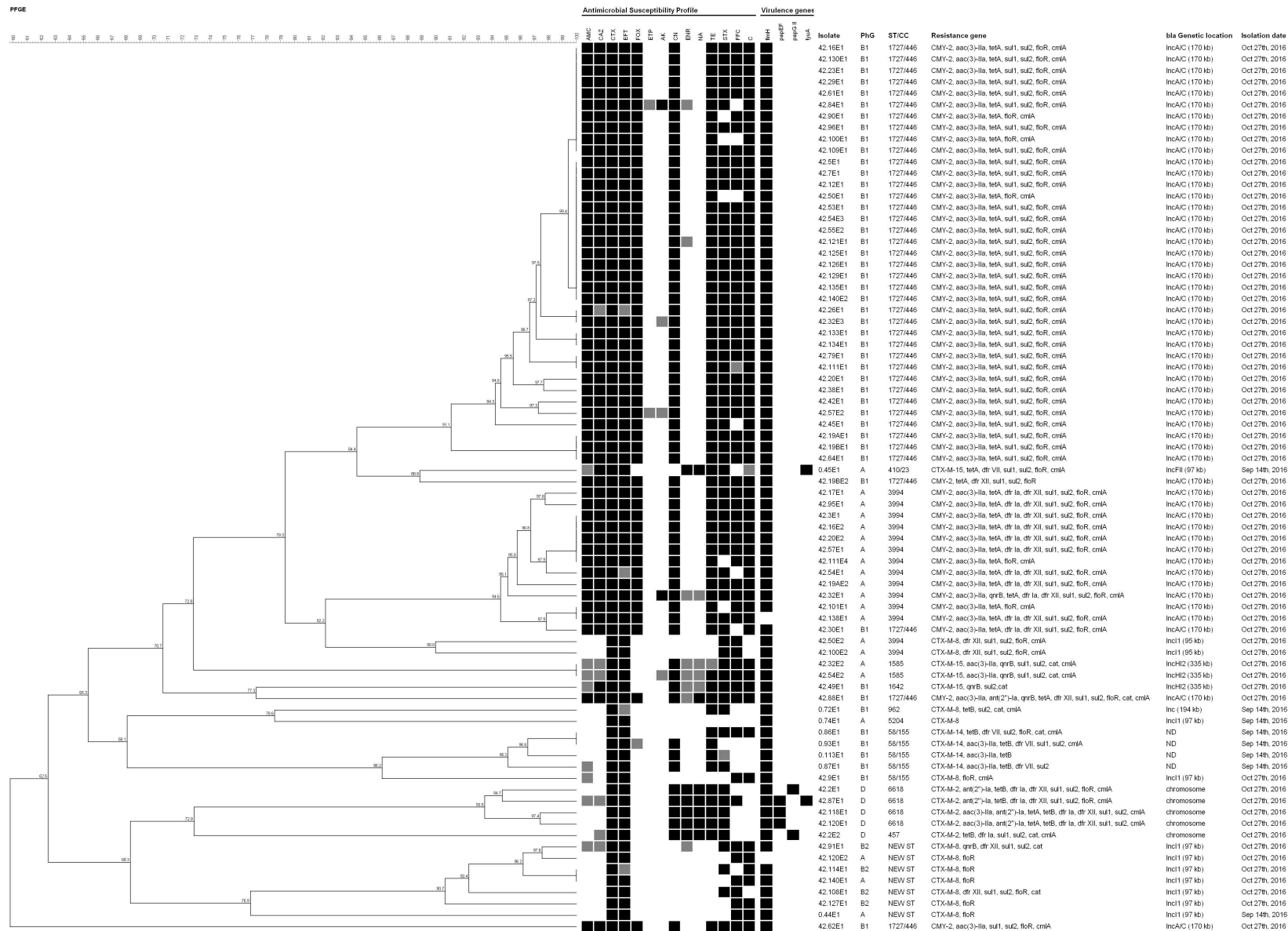
Four virulence genes were detected, but only *fimH* was present in the majority (76 isolates, 97.4%). The *papEF* was detected in 3 isolates (3.8%), and *papG* II and *fyuA* in 2 (2.6%), as presented in Figure 1. Furthermore, five genotypes concerning virulence were detected, including the absence of any gene, the presence of only *fimH* or a combination of it and the other genes detected (Table 4). No genes predictive of STEC, EAEC or EPEC were detected. Forty-eight (61.5%) *E. coli* belonged to phylogroup B1, 21 (27.0%) to phylogroup A, 5 (6.4%) to phylogroup D, and 4 (5.1%) to phylogroup B2. Phylogroup D was related only to the *bla*<sub>CTX-M-2</sub> gene (Table 4).

The *Xba*I-PFGE typing distinguished the 78 isolates in seven major clusters. Two lambs (animals #45 and #87) presented AR *E. coli* (isolates 0.45E1 and 42.45E1, and 0.87E1 and 42.87E1, respectively) on both days 0 and 42, but the strains are not similar by *Xba*I-PFGE and neither carry the same AR genes (Figure 1). Typing with the MLST scheme revealed 10 Sequence Types (ST) of *E. coli* in the studied feedlot, and one new allele profile in seven *bla*<sub>CTX-M-8</sub>-carrying isolates (0.44E1, 42.91E1, 42.108E1, 42.114E1, 42.120E2, 42.127E1, and 42.140E1) recovered on the first and last days of feedlot. The ST 1727 Clonal Complex (CC) 446 was predominant and present only on day 42 in 52.6% of the total isolates regarding just *bla*<sub>CMY-2</sub>-carrying *E. coli* from phylogenetic group B1, followed by the ST 3994, the new combination of MLST alleles, ST/CC 58/155, ST 6618, ST 1585, and the ST/CC 410/23, ST 457, ST 962, ST 1642, ST 5204 (Table 5).

## DISCUSSION

Seventy-four (94.9%) *E. coli* isolates presented a multidrug-resistant antibiotic type (MRAb) according to the antimicrobial

<sup>2</sup>[https://pubmlst.org/bigdb?db=pubmlst\\_mlst\\_seqdef](https://pubmlst.org/bigdb?db=pubmlst_mlst_seqdef)



**FIGURE 1 |** Dendrogram obtained from *Xba*I-PFGE typing of the 78 *E. coli* isolated. Dendrogram was constructed using Optimization 0% and Tolerance 1.5%. AMC, amoxicillin/clavulanate; CAZ, ceftazidime; CTX, cefotaxime; EFT, ceftiofur; FOX, cefoxitin; ETP, ertapenem; AK, amikacin; CN, gentamicin; ENR, enrofloxacin; NA, nalidixic acid; TE, tetracycline; STX, trimethoprim/sulfamethoxazole; FFC, florfenicol; C, chloramphenicol. Antimicrobial Susceptibility Profile squares: black, resistance; gray, intermediate resistance; white, susceptibility. Virulence genes squares: black, presence; white, absence. PhG, phylogenetic group. ST/CC, Sequence Type/Clonal Complex. ND, localization not detected. Isolation dates “Sep 14th, 2016” and “Oct 27th, 2016” refers to “day 0” and “day 42,” respectively.

**TABLE 1** | Isolates presenting resistance to each antimicrobial class among the 78 *E. coli* from stools of sheep in Southeastern Brazil.

Antimicrobial class	N isolates (%)	<i>bla</i> gene associated
Penicillin + beta-lactamase inhibitors	53 (68.0)	CMY-2
Third-generation cephalosporins	78 (100.0)	CTX-M-2, -8, -14, -15, CMY-2
Cepharmycin	53 (68.0)	CMY-2
Aminoglycosides	64 (82.0)	CTX-M-2, -14, -15, CMY-2
Quinolones	7 (9.0)	CTX-M-2, -15, CMY-2
Tetracycline	66 (84.6)	CTX-M-2, -8, -14, -15, CMY-2
Folate pathway inhibitors	65 (83.3)	CTX-M-2, -8, -14, -15, CMY-2
Phenicol	68 (87.2)	CTX-M-2, -8, -14, -15, CMY-2

The *bla* genes associated with each antimicrobial class resistance are also presented.

susceptibility test, with the exception of the isolate 0.74E1, recovered on day 0 from animal #74, and the isolates 42.120E2, 42.127E1, 42.140E1, obtained on day 42 from animals #120, #127 and #140. Interestingly, all the non-MRab *E. coli* were associated with *bla*<sub>CTX-M-8</sub> gene (Figure 1). The high percentage of MRab isolates illustrates the potential for spread of AR bacteria through a flock. Studies have already reported that the resistance rate to some antimicrobials rises during cattle or pig feedlot because of antimicrobial usage (Benedict et al., 2015; Gibbons et al., 2016; Weinroth et al., 2018). However, a Canadian study surveyed sheep flocks over a 1-year period and found no significant difference between the initial and the final visits (Scott et al., 2012), which is in disagreement with this study.

No isolate presented resistance to ertapenem (Table 1), which could be related to the fact that carbapenems are not approved for use in animals (OIE, 2018). The detection of AR *E. coli* in 55 animals after feedlot, in comparison to eight animals on day 0, indicates a selection pressure acting on the flock. Beta-lactams, florfenicol, macrolides, quinolones, tetracycline,

**TABLE 2** | Antimicrobial resistance genes distribution among the 78 *E. coli* from stools of sheep in Southeastern Brazil, according to the animals (ID) and the day of feedlot they were detected.

Antimicrobial class	Resistance gene	Distribution (%)	Animal ID (n)	Day
Third-generation cephalosporins	<i>bla</i> <sub>CTX-M-2</sub>	5 (6.4)	2, 87, 118, 120 (4)	42
	<i>bla</i> <sub>CTX-M-8</sub>	12 (15.4)	9, 44, 50, 72, 74, 91, 100, 108, 114, 120, 127, 140 (12)	0, 42
	<i>bla</i> <sub>CTX-M-14</sub>	4 (5.1)	86, 87, 93, 113 (4)	0
	<i>bla</i> <sub>CTX-M-15</sub>	4 (5.1)	32, 45, 49, 54 (4)	0, 42
	<i>bla</i> <sub>CMY-2</sub>	53 (68.0)	3, 5, 7, 12, 16, 17, 19A, 19B, 20, 23, 26, 29, 30, 32, 38, 42, 45, 50, 53, 54, 55, 57, 61, 62, 64, 79, 84, 88, 90, 95, 96, 100, 101, 109, 111, 121, 125, 126, 129, 130, 133, 134, 135, 138, 140 (45)	42
Aminoglycoside	<i>aac(3)-IIa</i>	60 (76.9)	2, 3, 5, 7, 12, 16, 17, 19A, 19B, 20, 23, 26, 29, 30, 32, 38, 42, 45, 49, 50, 53, 54, 55, 57, 61, 62, 64, 79, 84, 87, 88, 90, 93, 95, 96, 100, 101, 109, 111, 113, 118, 120, 121, 125, 126, 129, 130, 133, 134, 135, 138, 140 (52)	0, 42
	<i>ant(2'')-Ia</i>	5 (6.4)	2, 87, 88, 118, 120 (5)	42
Quinolone	<i>qnrB</i>	6 (7.7)	32, 49, 54, 88, 91 (5)	42
Tetracycline	<i>tetA</i>	54 (69.2)	3, 5, 7, 12, 16, 17, 19A, 19B, 20, 23, 26, 29, 30, 32, 38, 42, 45, 50, 53, 54, 55, 57, 61, 64, 79, 84, 88, 90, 95, 96, 100, 101, 109, 111, 120, 121, 125, 126, 129, 130, 133, 134, 135, 138, 140 (45)	0, 42
	<i>tetB</i>	10 (12.8)	2, 72, 86, 87, 93, 113, 118, 120 (8)	0, 42
Trimethoprim	<i>dfr Ia</i>	16 (20.5)	2, 3, 16, 17, 19A, 20, 30, 32, 54, 57, 87, 95, 118, 120, 138 (15)	42
	<i>dfr VII</i>	4 (5.1)	45, 86, 87, 93 (4)	0
	<i>dfr XII</i>	21 (26.9)	2, 3, 16, 17, 19A, 19B, 20, 30, 32, 50, 54, 57, 87, 88, 91, 95, 100, 108, 118, 120, 138 (21)	42
Sulphas	<i>sul1</i>	61 (78.2)	2, 3, 5, 7, 12, 16, 17, 19A, 19B, 20, 23, 26, 29, 30, 32, 38, 42, 45, 50, 53, 54, 55, 57, 61, 62, 64, 79, 84, 87, 88, 91, 93, 95, 96, 100, 108, 109, 111, 118, 120, 121, 125, 126, 129, 130, 133, 134, 135, 138, 140 (50)	0, 42
	<i>sul2</i>	65 (83.3)	2, 3, 5, 7, 12, 16, 17, 19A, 19B, 20, 23, 26, 29, 30, 32, 38, 42, 45, 49, 50, 53, 54, 55, 57, 61, 62, 64, 72, 79, 84, 86, 87, 88, 91, 93, 95, 96, 100, 108, 109, 111, 118, 120, 121, 125, 126, 129, 130, 133, 134, 135, 138, 140 (53)	0, 42
Phenicol	<i>floR</i>	65 (83.3)	3, 5, 7, 9, 12, 16, 17, 19A, 19B, 20, 23, 26, 29, 30, 32, 38, 42, 44, 45, 50, 53, 54, 55, 57, 61, 62, 64, 79, 84, 86, 87, 88, 90, 95, 96, 100, 101, 108, 109, 111, 114, 120, 121, 125, 126, 127, 129, 130, 133, 134, 135, 138, 140 (53)	0, 42
	<i>cat</i>	8 (10.3)	2, 32, 49, 54, 86, 88, 91, 108 (8)	0, 42
	<i>cmIA</i>	67 (85.9)	2, 3, 5, 7, 9, 12, 16, 17, 19A, 19B, 20, 23, 26, 29, 30, 32, 38, 42, 44, 45, 50, 53, 54, 55, 57, 61, 62, 64, 72, 79, 84, 86, 87, 88, 90, 93, 95, 96, 100, 101, 109, 111, 118, 120, 121, 125, 126, 129, 130, 133, 134, 135, 138, 140 (54)	0, 42

**TABLE 3 |** Genetic localization of *bla* genes detected in the 78 *E. coli* isolates.

<i>bla</i> gene	Localization	N isolates	Day
CTX-M-2	chromosome	5	42
CTX-M-8	IncI1 (95 kb)	2	42
	IncI1 (97 kb)	9	0, 42
	IncHI1 (194 kb)	1	0
CTX-M-14	ND*	4	0
CTX-M-15	IncFII (97 kb)	1	0
	IncHI2 (335 kb)	3	42
CMY-2	IncA/C (170 kb)	53	42

The size of plasmids is described between parentheses and represents an approximation according to S1-PFGE gels and the molecular reference.  
\*Not detected.

and trimethoprim/sulfamethoxazole are administered in sheep (OIE, 2018). In fact, some animals included in this study received florfenicol or trimethoprim/sulfamethoxazole, and this could explain the presence of the AR *E. coli* because of direct or co-selection of resistance determinants in the GIT of the animals (Collignon et al., 2016; Makita et al., 2016; Knudsen et al., 2018).

The set of genes codifying beta-lactamase enzymes carried by the *E. coli* isolated on the 2 days of analysis was diverse. On day 0, 7.1% (8/112 animals) of the sampled lambs presented *E. coli* harboring some *bla*<sub>CTX-M</sub>-variant. However, after 42 days of feedlot the majority of *E. coli* isolated (53/78, 68.0% of the total) harbored the *bla*<sub>CMY-2</sub> gene, comprising essentially the two great clusters of the dendrogram and the ST/CC 1727/446 and ST 3994 (Figure 1). Besides, *bla*<sub>CMY-2</sub>, the *bla*<sub>CTX-M-2</sub> gene was detected only on day 42, while *bla*<sub>CTX-M-14</sub> was detected in isolates recovered only on day 0 (Table 2). It seems that the first two genes entered into the flock during feedlot by some external factor such as surrounding animals, insects, or the environment (Blaak et al., 2015; Huijbers et al., 2015; Solà-Ginés et al., 2015), and the latter disappeared during feedlot perhaps because of competition between the *bla*<sub>CTX-M-14</sub>-carrying *E. coli* and other more successful strains, possibly the *bla*<sub>CMY-2</sub>-carrying *E. coli*. On the other hand, *bla*<sub>CTX-M-8</sub> and *bla*<sub>CTX-M-15</sub> were present on the first day of feedlot and persisted until the end (Table 2), which is clearly not linked to the maintenance of isolates into the feedlot, since the CTX-M-8- and the CTX-M-15-producing *E. coli* isolated on days 0 and 42 are not clonally related by PFGE or MLST (Figure 1). However, the majority of *bla*<sub>CTX-M-8</sub> detected in isolates from day 42 are harbored by IncI1 plasmids of ~97 kb, the same as two detected on day 0, which illustrates the

**TABLE 5 |** Sequence Types and Clonal Complexes detected for the *E. coli* isolated from sheep in Southeastern Brazil.

ST/CC <sup>a</sup>	N isolates (%)	<i>bla</i> gene associated	Phylogroup	Day
58/155	5 (6.4)	CTX-M-8, CTX-M-14	B1	0
410/23	1 (1.3)	CTX-M-15	A	0
457	1 (1.3)	CTX-M-2	D	42
962	1 (1.3)	CTX-M-8	B1	0
1585	2 (2.6)	CTX-M-15	A	42
1642	1 (1.3)	CTX-M-15	B1	42
1727/446	41 (52.6)	CMY-2	B1	42
3994	14 (17.9)	CTX-M-8, CMY-2	A	42
5204	1 (1.3)	CTX-M-8	A	0
6618	4 (5.1)	CTX-M-2	D	42
NEW <sup>b</sup>	7 (8.9)	CTX-M-8	A, B2	0, 42

STs are linked to the number of isolates belonging to each lineage as well as to the *bla* genes associated, the phylogenetic group and the day of feedlot each ST was identified. <sup>a</sup>ST/CC, Sequence Type/Clonal Complex. <sup>b</sup>New allelic combination: *adk*(295), *fumC*(54), *gyrB*(535), *icd*(767), *mdh*(260), *recA*(40), *purA*(83).

maintenance and spreading of that plasmid through the feedlot. On the other hand, the *bla*<sub>CTX-M-15</sub> gene identified in three *E. coli* recovered on day 42 probably entered the feedlot at some point since they are harbored by plasmid IncHI2 of ~335 kb, differently from the *bla*<sub>CTX-M-15</sub> harbored by an IncFII of ~97 kb on day 0 (Table 3). Remarkably, some animals (2, 16, 19A, 19B, 20, 32, 50, 54, 57, 100, 111, 120, 140) carried more than one CMY-2 or CTX-M-producing *E. coli* on day 42, which are also present in other animals (Figure 1 and Table 2), which demonstrates the exchanging of commensal GIT bacteria among animals in the feedlot.

The use of a 3GC to enrich medium for recovery of *E. coli* from the feces of broilers induced a positivity of 99% of the samples containing *bla*<sub>CMY-2</sub>- and/or *bla*<sub>CTX-M</sub>-isolates (Verrette et al., 2019), which could be the explanation for the high percentage of such *E. coli* in our study. The *bla*<sub>CMY-2</sub> gene has been reported as frequent in *E. coli* isolates causing urinary tract infections in Brazil (Rocha D. A. C. et al., 2016), and CMY-2- and CTX-M-producing *E. coli* were already isolated from poultry and buffalo in the country (Aizawa et al., 2014; Casella et al., 2018; Hoepers et al., 2018) but never in sheep. Apart from the prevalence of isolates presenting the *bla*<sub>CMY-2</sub> gene, the occurrence of *bla*<sub>CTX-M-14</sub>- and *bla*<sub>CTX-M-15</sub>-carrying *E. coli* in this study is remarkable. Those genes are the dominant *bla*<sub>CTX-M</sub>

**TABLE 4 |** Genotypes detected concerning virulence genes in the 78 *E. coli* isolates from stools of sheep in Southeastern Brazil.

Virulence genotypes	N isolates	Phylogroup	<i>bla</i> gene	Animal	Day
–	2	A	CTX-M-8, CMY-2	#120, #138	42
<i>fimH</i>	70	A, B1, B2	CTX-M-8, –14, –15, CMY-2	all others	0, 42
<i>fimH</i> <i>papEF</i>	2	D	CTX-M-2	#118, #120	42
<i>fimH</i> <i>papG II</i>	2	D	CTX-M-2	#2	42
<i>fimH</i> <i>fyuA</i>	1	A	CTX-M-15	#45	0
<i>fimH</i> <i>papEF</i> <i>fyuA</i>	1	D	CTX-M-2	#87	42

Genotypes are described according to the number of isolates, the phylogenetic groups and the *bla* genes related to each one, and also according to ID of the animals (#) and the day in which the *E. coli* with each combination of virulence genes were isolated.



variants in most regions worldwide, concerning isolates from human infections and food-producing or companion animals (Zhao and Hu, 2013; Bevan et al., 2017; Chong et al., 2018; Dandachi et al., 2018). This means that the studied lambs represent a potential source of hard-to-treat infections caused by *E. coli* or at least a reservoir of important AR genes that could reach human pathogens. The *bla*<sub>CTX-M-8</sub> gene was the second most detected in the studied population after *bla*<sub>CMY-2</sub>, present on both first and last days of feedlot (Table 2). CTX-M-8 was firstly identified in Brazil (Bonnet et al., 2000) and is still frequent in isolates from food-producing animals and meat in the country (Fernandes et al., 2016; Ferreira et al., 2016). However, it is thought to have a relatively low prevalence in other territories and is supposed to be transmitted by travelers or contaminated food (Dhanji et al., 2010; Egervärn et al., 2014; Eller et al., 2014).

Both genes *aac*(3)-IIa and *ant*(2'')-Ia codify resistance to gentamicin, and are present in plasmids (Ramirez and Tolmasky, 2010; Norris and Serpersu, 2013; Cox et al., 2015). In this study, *aac*(3)-IIa clearly predominated in relation to *ant*(2'')-Ia (Table 2). Notably, both genes reported here are clearly related to *E. coli* associated with infections (Miró et al., 2013; Fernández-Martínez et al., 2015). Resistance to phenicol was detected on the first and last days of feedlot, with *floR* and *cmlA* present in higher frequencies than the *cat* gene (Figure 1 and Table 2). A Portuguese study found only *cmlA* in *E. coli* isolated from sheep (Ramos et al., 2013), and a Brazilian study carried out with *Salmonella* Typhimurium isolated from humans and food revealed *floR* associated with food isolates and the *cat* gene associated with human *Salmonella* (Almeida et al., 2018). Furthermore, *cmlA* has already been reported in *E. coli* from chicken meat in the country (Casella et al., 2017a). Sixty-five isolates (83.3%) presented resistance to trimethoprim/sulfamethoxazole, but more than 60% of the *E. coli* presented at least one of the *sul* genes screened while 33.3% presented some *dfr* gene. Both *sul1* and *sul2* have been detected in *E. coli* isolated from sheep in Portugal (Ramos et al., 2013), and those genes have already been reported in *E. coli* isolated from clinical specimens (Oliveira-Pinto et al., 2017), chicken meat (Casella et al., 2015) and even surface water (Canal et al., 2016) in Brazil, but once again we know nothing about the subject in sheep. Resistance to tetracycline was detected during the entire feedlot stay of the lambs, with *tetA* and *tetB* detected on days 0 and 42, with considerable predominance of the first (Figure 1 and Table 2). Interestingly, the isolate 42.120E1 carried *tetA* and *tetB*, which is unexpected since both express the same tetracycline efflux mechanism (Thaker et al., 2010). *tetA* and *tetB* have already been detected in high frequencies in *E. coli* isolated from sheep (Ramos et al., 2013). The rising in the content of genes codifying resistance to tetracycline has been observed during bovine feedlot (Weinroth et al., 2018), but to our knowledge, there is no report of such an event concerning resistance to other antimicrobial classes in general, as observed in this study. In fact, the use of tetracyclines and trimethoprim/sulphonamides in sheep has already been reported as presenting a significant association with tetracycline resistance (Scott et al., 2012), and the *tetA* gene was positively associated with *bla*<sub>CMY-2</sub> after ceftiofur followed chlortetracycline treatment in cattle (Kanwar et al., 2013), which

is in agreement with our study. The *qnrB* gene was detected in six isolates recovered only on day 42, with all but one presenting intermediate resistance to the quinolones (Figure 1 and Table 2). A Chinese study reported *qnrB* as low-frequency among the genes detected in *E. coli* recovered from swine (Liu et al., 2018), and a recent study conducted in Brazil showed *E. coli* isolates carrying *qnrB* associated with the genes *bla*<sub>CTX-M-2</sub> and *bla*<sub>CMY-2</sub> in poultry (Ferreira et al., 2019). In our study, resistance to quinolones had little importance as a disseminated mechanism through the feedlot. Therefore, the presence of such genes codifying resistance to different antimicrobial classes in commensal isolates of food-producing animals as lambs raises public health concerns. The occurrence of MRAB *E. coli* in the studied lambs may be caused by the presence of animals and insects carrying these bacteria in the surroundings of the feedlot or even the environment (Blaak et al., 2015; Huijbers et al., 2015; Chong et al., 2018). Since we have collected feces from 112/140 flock animals, another possibility is a lamb not sampled as the source of that *E. coli*. Indeed, *bla*<sub>CMY-2</sub>-*floR*-*tetA*-*sul2*-harboring plasmids have already been identified in food-producing animals (Fernández-Alarcón et al., 2011) and could represent a similarity found in this study.

Regarding virulence genes, most isolates presented only *fimH* (Table 4), which is related to adhesion and is necessary for GIT colonization (Waksman and Hultgren, 2009). The absence of other virulence genes is not surprising, considering that the *E. coli* were isolated from feces of healthy animals and represent the GIT microbiota of the lambs. Instead of a known source of STEC strains in Brazil (Vettorato et al., 2009), sheep studied here did not present any evidence of carrying diarrheagenic *E. coli* (DEC). Nevertheless, all isolates were primarily selected from stools with the 3GC ceftiofur, which could represent a bias in the absence of STEC, EAEC or EPEC strains since such DEC could be present but do not carry genes for 3GC-resistance. The majority of the isolates (61.5%) belong to the phylogenetic group B1, 27.0% were classified as A, and 11.5% belong to phylogroups B2 or D (Figure 1 and Table 4). These results are in agreement with another study (Ramos et al., 2013), in which 61.1% of *E. coli* isolated from sheep were classified as phylogroup B1, 31.5% were phylogroup A, and 7.4% as phylogroups B2 or D. Traditionally, phylogenetic groups A and B1 are associated with commensal *E. coli*, while B2 and D with pathogenic isolates (Clermont et al., 2000), which is also in concordance with the few virulence genes detected.

Although the CMY-2-producers were distributed in different clusters according to *Xba*I-PFGE and belong to two different lineages according to MLST, the IncA/C plasmid of about 170 kb was confirmed as responsible for *bla*<sub>CMY-2</sub> mobilization. This fact also illustrates the dissemination of that plasmid through the feedlot, which was indeed related to *bla*<sub>CMY-2</sub> mobilization in food-producing animals and meat before, suggesting spread of the plasmid worldwide and in Brazil (Guo et al., 2014; Casella et al., 2017b; Dame-Korevaar et al., 2017). *bla*<sub>CTX-M-8</sub> was carried by an IncI1 plasmid of ~97 kb in isolates recovered on days 0 and 42, which seems to be responsible for the maintenance of that gene in the feedlot during the period analyzed. *bla*<sub>CTX-M-8</sub>-IncI1 plasmids have already been reported in *E. coli* isolated from

humans, wastewater, food-producing animals and meat, and appear to be more responsible for the mobilization of that gene in several countries, including Brazil (Ferreira et al., 2014b; Dropa et al., 2016; Norizuki et al., 2017; Casella et al., 2018; Dantas Palmeira et al., 2018). The *bla*<sub>CTX-M-15</sub> gene was carried by very different plasmids on the first and last days of feedlot (Table 3), which means that the *bla*<sub>CTX-M-15</sub>-IncFII present on day 0 probably disappeared and the *bla*<sub>CTX-M-15</sub>-IncHI2 entered the feedlot at any time point during the period. Since both plasmids are carried by extremely different *E. coli*, according to *Xba*I-PFGE and MLST methodologies (Figure 1), it seems that the change on plasmids responsible for *bla*<sub>CTX-M-15</sub> mobilization was due to the disappearance and entry of respective strains into the feedlot, contrary to what happened to the *bla*<sub>CTX-M-8</sub>-IncI1 plasmids of about 97 kb mentioned above. IncHI2 plasmids have also been reported as responsible for mobilization of *bla*<sub>CTX-M-15</sub> in several *Enterobacteriaceae* species isolated from humans or animals (Kariuki et al., 2015; Haenni et al., 2016) and have been detected in 3/4 of the CTX-M-15-producing *E. coli* in this study. The CTX-M-2-producers identified in this study seem to carry the *bla*<sub>CTX-M-2</sub> inserted into the chromosome. This is not a rare event nowadays and is plausible since it has already been reported in *E. coli* isolated from chickens and chicken meat in Brazil (Ferreira et al., 2014a; Casella et al., 2018). In addition to that, *bla*<sub>CTX-M-2</sub>-carrying *E. coli* were isolated just on day 42 and were clonally related by *Xba*I-PFGE and MLST, with the exception of isolate 42.2E2 (Figure 1). Finally, we could not detect the plasmid linked to *bla*<sub>CTX-M-14</sub>, and this gene has already been described inserted into the chromosome (Hamamoto et al., 2016; Hamamoto and Hirai, 2019), which could be the explanation for the present isolates. Further studies are required to elucidate this subject.

*Xba*I-PFGE typing grouped most of the *bla*<sub>CMY-2</sub>-carrying *E. coli* in the two major clusters, composed of 37 and 13 *E. coli* that carry essentially *bla*<sub>CMY-2</sub>-*aac*(3)-*Ila*-*tetA*-*sul1*-*sul2*-*floR*-*cmlA*, with exceptions, belonging to phylogroups B1-ST/CC 1727/446 or A-ST 3994, respectively (Figure 1). Strains belonging to the later cluster additionally carry the *dfr* *Ia* and *dfr* *XII* genes. This finding indicates that two strains have spread among animals throughout the feedlot, but all harboring the same *bla*<sub>CMY-2</sub>-carrying plasmid as mentioned above. Interestingly, two lineages were detected carrying different *bla* genes, such as ST/CC 58/155 presenting *bla*<sub>CTX-M-8</sub> or *bla*<sub>CTX-M-14</sub> and ST 3994 presenting *bla*<sub>CTX-M-8</sub> or *bla*<sub>CMY-2</sub>, and both groups have a considerable relationship within isolates (Figure 1). *E. coli* ST/CC 58/155 has already been reported harboring *bla*<sub>CTX-M-14</sub> and others from clinical specimens and healthy people in several countries (Gerhold et al., 2016; Kawamura et al., 2017). In Brazil, this lineage has already been reported carrying *bla*<sub>CTX-M-8</sub> or *bla*<sub>CMY-2</sub> in dogs, and the *bla*<sub>CTX-M-8</sub> gene was also harbored by an IncI1 plasmid (Melo et al., 2018), as in this study. Furthermore, the same Brazilian study showed an *E. coli* phylogroup D-ST 457 isolated from a diseased dog carrying the *bla*<sub>CTX-M-2</sub> inserted in the chromosome, the same as the only CTX-M-2-producing isolate ST 457 in this study, which demonstrates the presence of that clone in different

animals in the country. Contrary to the clonality described above regarding ST/CC 58/155, the *E. coli* ST/CC 1727/446 isolated in this study carry only *bla*<sub>CMY-2</sub>, but isolates were not clonally related according to *Xba*I-PFGE typing (Figure 1). This could represent micro-evolution occurring in the *E. coli* strains in the feedlot during the period of 42 days. The new combination of alleles (new ST) found in seven related *bla*<sub>CTX-M-8</sub>-carrying isolates was the unique lineage recovered on days 0 and 42, which means that the clone remained in the studied feedlot lambs carrying the same *bla*<sub>CTX-M-8</sub>-IncI1 plasmid (Figure 1 and Table 5).

## CONCLUSION

In conclusion, feedlot lambs act as reservoirs of commensal multidrug-resistant *E. coli*, and those AR genes or bacteria can reach humans through the food chain. The presence of *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub> deserves special attention since they are the genes most related to human infections worldwide. To the best of our knowledge, this is the first report of *bla*<sub>CTX-M-14</sub> in *Enterobacteriaceae* isolated from food-producing animals in Brazil. Additionally, *E. coli* ST lineages and plasmids harboring the *bla* genes detected have already been identified in humans, animals, meat and the environment, which demonstrates the concern for their dissemination and for public health. Further studies are needed in order to determine the reasons for the success of the *bla*<sub>CMY-2</sub>-*aac*(3)-*Ila*-*tetA*-*sul1*-*sul2*-*floR*-*cmlA*-carrying *E. coli* in the studied feedlot. To the best of our knowledge, this is the first study reporting such a broad characterization of antimicrobial resistant *E. coli* isolated from sheep.

## DATA AVAILABILITY

The datasets generated for this study can be found in GenBank, MK896925 to MK896944, and MK917695 to MK917713.

## ETHICS STATEMENT

Ethics Committee approval number FOA00845-2017. Universidade Estadual Paulista (UNESP) "Júlio de Mesquita Filho," campus de Araçatuba, Faculdade de Medicina Veterinária.

## AUTHOR CONTRIBUTIONS

TC, MN, and LM designed the study. KG, JF, LDA, and CS performed all the laboratorial experiments. RB handled with the animals and their stools. TC, MN, LM, JP, and MM wrote and revised the whole manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01394/full#supplementary-material>

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# Whole Genome Sequencing of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Escherichia coli* Isolated From a Wastewater Treatment Plant in China

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**Background and Objectives:** Wastewater treatment plants (WWTPs) are one of the major reservoirs for antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARGs) in the environment. Thus, the investigation on ARB and ARGs from WWTPs has attracted increasing attention in recent years. In order to uncover the resistome in a WWTP treating effluents from a pharmaceutical industry in China, the extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* strains were isolated and their whole genome sequences were obtained and analyzed. Moreover, metagenomic sequencing was applied to give a comprehensive view of antibiotic resistance in this WWTP.

**Methods:** 18 ESBL-producing *E. coli* strains were isolated from a WWTP located in Taizhou, China on April, 2017. All strains were sequenced using Illumina HiSeq 2000 sequencer. The whole genome sequences were assembled using SPAdes software and annotated with RAST server. Sequence types (STs), plasmids, ARGs and virulence genes were predicted from the genomes using MLST, Plasmid Finder, ResFinder and Virulence Finder, respectively. Metagenomic DNA of the same sample was extracted and sequenced using Illumina HiSeq X Ten platform. Metagenomic sequences were assembled using SOAPdenovo software.

**Results:** All 18 ESBL-producing *E. coli* strains were resistant to ampicillin, cefazolin, and ceftriaxone. Analysis of their genomes revealed that all strains carried beta-lactamase encoding genes and the most prevalent type was *bla*<sub>CTX-M</sub>. Various virulence genes and ARGs confronting resistance to other types of antimicrobial agents were also predicted. Further investigation on the metagenomics data indicated 11 ARGs with high amino acid identities to the known ARGs. Five of these ARGs, *aadA1*, *aac(6')-Ib-cr*, *flo(R)*, *sul2* and *sul1*, were also present in the genomes of the ESBL-producing *E. coli* isolated from the same sample.

**Conclusion:** Our study revealed the resistome of a pharmaceutical WWTP by both culture-dependent and metagenomic methods. The existence of ESBL-producing *E. coli* strains, indicating that pharmaceutical WWTP can play a significant role in the emergence of ARB. The occurrence of ARGs annotated from the metagenomic data suggests that pharmaceutical WWTP can play a significant role in the emergence of ARGs. Our findings highlight the need for strengthening the active surveillance of ARB and ARGs from pharmaceutical industry.

**Keywords:** waste water treatment plants, ESBL-producing *Escherichia coli*, antimicrobial resistance genes, metagenomics, *bla<sub>CTX-M</sub>*

## INTRODUCTION

The rapid emergence and global spread of antimicrobial resistant bacteria (ARB) worldwide has long been recognized as a threat to human health, and one of the most studied bacterial groups is extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae (Bush and Fisher, 2011). An epidemiological study has reported that ESBL-producing Enterobacteriaceae, especially *Escherichia coli*, is an important cause of community-acquired infection (Stadler et al., 2018). ESBL enzymes produced by resistant strains are capable of inactivating beta-lactam drugs (e.g., third-generation cephalosporins) by hydrolyzing their beta-lactam rings (Bush and Bradford, 2016). Some of these ESBL-producing *E. coli* may also carry virulence genes. The products of these virulence genes enhance their pathogenicity. For instance, virulence genes *eilA*, *air*, and *lphA* encode for proteins that are related to adherence of bacteria (Doughty et al., 2002; Sheikh et al., 2006). Virulence genes *senB* and *astA* encode for enterotoxins (Dubreuil et al., 2016). Some virulence genes, such as *gad*, are involved in the survival of bacteria in harsh conditions such as the extremely acidic environments in stomach (Capitani et al., 2003). Thus, the infections caused by ESBL-producing *E. coli* strains which also harbor virulence genes are always associated with increased mortality and cost burden (Schwaber et al., 2006). In addition, ESBL genes are often encoded on plasmids that can be readily exchanged between species (Carattoli, 2013). Moreover, ESBL genes have been found not only in *E. coli* strains from clinical origin, but also from environmental origin (Runcharoen et al., 2017; Zheng et al., 2017a).

Wastewater treatment plants (WWTPs) have been regarded as vast reservoirs and environmental suppliers of ARB and antimicrobial resistance genes (ARGs) (Guo et al., 2017; Jałowiecki et al., 2018). Majority of the research has primarily focused on municipal WWTP receiving hospital and household wastewater (Yu et al., 2016; Szekeres et al., 2017; Galler et al., 2018). In recent years, the issue of pharmaceuticals in the environment has been gaining more attention worldwide and there has been growing interest in the WWTPs treating effluents from pharmaceutical industries with antimicrobial agents production. For example, several studies revealed the environmental impacts and the resistome of antimicrobial agents contaminated effluents from pharmaceutical industries in India and Croatia (Fick et al., 2009; Rutgerström et al., 2014;

Bielen et al., 2017; Razavi et al., 2017; González-Plaza et al., 2018). A recent study in Nigeria has applied a culture-dependent method to isolate ARB in wastewater from pharmaceutical facilities (Obayiuwana et al., 2018). In China, studies have revealed the existence of ARGs in WWTPs that treated wastewater from antimicrobial agents producing factories (Li et al., 2010; Zhai et al., 2016; Tang et al., 2017). It is suggested that environmental pollution from pharmaceutical manufacturing can promote the spread of antimicrobial resistance, the induction of direct biostatic or bio-toxic effects to diverse organisms, and the alteration of species distribution (Bielen et al., 2017). Thus, there is a clear need for more research in this area.

The aim of the present study was to investigate the resistome in a WWTP of pharmaceutical industry in China. We used whole genome sequencing (WGS) to provide insight into genomic determinants of the ESBL-producing *E. coli* strains isolated from this WWTP and reveal their sequence types (STs), ARGs, virulence genes and plasmids. In addition, we applied a metagenomic approach to determine the microbial diversity and the occurrence of ARGs in this WWTP. The importance and originality of this study are derived from the identification of ARB and ARG in pharmaceutical WWTP using both WGS and metagenomic sequencing. Ultimately, it is hoped that this research will contribute to a deeper understanding of the ARB and ARGs released from pharmaceutical WWTP.

## MATERIALS AND METHODS

### Sample Collection

Sludge samples were collected from a WWTP located in Taizhou, China. This WWTP employed a conventional activated sludge process to treat wastewater from a beta-lactam antibiotics manufacturing company. A number of antimicrobial agents were produced by this company, including cefaclor, ceftizoxime, ceftibuten, cefuroxime sodium, cefprozil, cefdinir, and cefixime. There was an ongoing production at the time of sampling. Three independent samples (500 g for each sample) of aerobic active sludge were collected via the inspection opening of WWTP aeration tank in April 2017. All samples stored in sterilized tubes were transported to the laboratory on ice within 24 h.

## Isolation and Antimicrobial Susceptibility Testing of ESBL-Producing *E. coli*

For pre-enrichment step, the samples were cultured overnight in Mueller-Hinton broth without antimicrobial agents. Subsequently, 500 µl enriched sample were spread on chromID® ESBL (bioMérieux, France) plates (the selective chromogenic medium for ESBL-producing enterobacteria screening). The plates were cultivated at 37°C under aerobic conditions. *E. coli* colonies were picked up from each plate according to the manufacturer's instructions (pink/burgundy) after 48 h. All these strains were re-cultivated on chromID® ESBL plates for confirmation. The identification of isolated bacteria was carried out using a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) as described previously (Zheng et al., 2017b). Antimicrobial susceptibility testing was determined on all strains by VITEK 2 system employing panel AST-GN-16 (bioMérieux, France). The results were then interpreted according to the standards of the Clinical and Laboratory Standards Institute (Clsi., 2017). *E. coli* ATCC 25922 was used as control.

## Whole Genome Sequencing and Bioinformatic Analysis

Genomic DNA of the strains were extracted using QIAamp DNA Mini Kit (Qiagen, Germany) and were sent to Novogene Bioinformatics Technology, Co., Ltd. (Beijing, China) immediately on dry ice for WGS. Sequencing was carried out using the Illumina Hiseq 2000 sequencer (Illumina, United States) with a high-throughput 2 × 100 bp pair end sequencing strategy. Reads were filtered as described previously (Zheng et al., 2017b) and the resulting clean reads were assembled using SPAdes software (Bankevich et al., 2012). The draft genomes were annotated by RAST server (Aziz et al., 2008), while the 16S rRNA of strains were identified using EzBioCloud (Yoon et al., 2017). STs of the strains were predicted using MLST 2.0 (Larsen et al., 2012). Plasmids, ARGs and virulence genes were predicted from the genomes using PlasmidFinder 2.0 (Carattoli et al., 2014), ResFinder 3.1 (Zankari et al., 2012) and Virulence Finder 2.0 (Joensen et al., 2014) with nucleotide identity threshold of 90% and minimum length of 60%, respectively. Average nucleotide identity (ANI) between strains was calculated using their genomic sequences by Orthologous Average Nucleotide Identity Tool (OAT) with OrthoANI algorithm (Lee et al., 2016).

## Metagenomic DNA Extraction

Metagenomic DNA was extracted from the samples upon arrival at the laboratory. DNA extraction was performed using FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, United States) according to the manufacturer's instructions. The quality and integrity of DNA samples were checked by 1% agarose gel electrophoresis. Meanwhile, DNA yield and purity were measured using NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, United States). After extraction,

three DNA samples were mixed into one sample prior to metagenomic sequencing.

## Metagenomic Sequencing and Bioinformatic Analysis

Metagenomic DNA was transported to Zhejiang Tianke High-Tech Development, Co., Ltd. (Hangzhou, China) on dry ice immediately after exaction. High-throughput sequencing was conducted using Illumina Hiseq Xten platform with PE150 (pair-end sequencing, 150 bp reads) sequencing mode. Raw reads generated from the sequencing were filtered by removing the reads with more than 40 bp low quality nucleotides, quality value lower than 38, more than 10 ambiguous nucleotides, and more than 15 bp overlapped with adapter. After filtering, the remaining clean reads were assembled using SOAPdenovo2 software (Luo et al., 2012). Different K-mer sizes (49, 55, and 59) were chosen for the assembly with the parameters of -d 1, -M 3, -u and -F, while the highest scaffold N50 value was selected for further analysis. The obtained scaffolds were then broken down at position N, resulting in the formation of continuous sequences within scaffolds called Scaffigs. Then, all the clean reads were aligned to Scaffigs using SOAPaligner software (Gu et al., 2013). Only Scaffigs longer than 500 bp were retained for further analysis. Open reading frames (ORFs) were predicted from the assembled Scaffigs using MetaGeneMark with default parameters (Zhu et al., 2010). CD-HIT was used to obtain a non-redundant gene catalog (Fu et al., 2012). The retained clean reads were aligned to the gene catalog by using SOAPaligner, in order to calculate the number of matching reads (Gu et al., 2013). Unigenes were then obtained by removing the gene catalogs with less than three mapped reads. These unigenes were aligned against NCBI-NR database by DIAMOND software with the parameters of blastp, e-value ≤ 1e-5 (Buchfink et al., 2015) and further annotated with LCA algorithm using MEGAN (Huson et al., 2016). Further, ARGs were predicted by ResFinder 3.1 with 90% identity cutoff and 60% minimum length (Zankari et al., 2012).

## RESULTS

### Antimicrobial Resistance Profiles of *E. coli* Strains

A total of 18 pink colonies were picked up after spreading the samples on chromID® ESBL agar plates. By MALDI-TOF MS and 16S rRNA sequence analysis, all strains were identified as *E. coli*. As shown in Table 1, all strains were resistant to ampicillin, cefazolin and ceftriaxone, but not tigecycline.

### Genomic Features of *E. coli* Strains

Draft genome sequences of 18 *E. coli* strains were obtained through WGS and assembling. The sequences had been deposited in DDBJ/ENA/GenBank under bioproject (PRJNA433283) with an individual accession number. Genome sizes ranged from 4.67 to 5.33 Mb, with an estimated GC content between 50.3 and 50.9%. A total of 4,576 to 5,240 coding sequences, 92 to 118 RNAs were annotated via RAST server. Sequence typing was



**TABLE 1** | MIC values of all 18 *E. coli* strains.

Antibiotics ( $\mu\text{g/ml}$ )	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R16	R18	R19	R20
Ampicillin	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$	$\geq 32$
Amoxicillin	8	4	$\leq 2$	16	16	4	16	16	4	4	$\leq 2$	8	16	8	4	4	8	16
Piperacillin	$\leq 4$	$\leq 4$	$\leq 4$	64	64	$\leq 4$	64	64	$\leq 4$	$\leq 4$	$\leq 4$	$\leq 4$	64	$\leq 4$	$\leq 4$	$\leq 4$	$\leq 4$	64
Cefazolin	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$
Cefoxitin	$\leq 4$	$\leq 4$	$\leq 4$	$\geq 64$	$\geq 64$	16	$\geq 64$	$\geq 64$	$\leq 4$	8	$\leq 4$	$\leq 4$	$\geq 64$	$\leq 4$	16	16	$\leq 4$	$\geq 64$
Ceftriaxone	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$	$\geq 64$
Cefepime	$\leq 1$	2	$\leq 1$	$\geq 64$	$\geq 64$	2	$\geq 64$	$\geq 64$	4	2	$\leq 1$	4	$\geq 64$	$\leq 1$	2	$\geq 64$	2	$\geq 64$
Aztreonam	$\leq 1$	2	2	$\geq 64$	$\geq 64$	2	$\geq 64$	$\geq 64$	4	16	$\leq 1$	4	$\geq 64$	16	16	16	4	$\geq 64$
Ertapenem	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$
Imipenem	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$
Amikacin	8	$\leq 2$	$\leq 4$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$
Gentamicin	$\geq 16$	$\leq 1$	$\leq 1$	$\geq 16$	$\leq 1$	$\geq 16$	$\geq 16$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\geq 16$	$\leq 1$	$\geq 16$	$\leq 1$	$\leq 1$
Tobramycin	$\geq 16$	$\leq 1$	$\leq 1$	8	$\leq 1$	8	8	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\leq 1$	$\geq 16$	$\leq 1$	$\geq 16$	$\leq 1$	$\leq 1$
Ciprofloxacin	$\geq 4$	$\leq 0.25$	$\geq 4$	$\geq 4$	$\geq 4$	0.5	$\geq 4$	$\geq 4$	$\leq 0.25$	$\leq 0.25$	$\geq 4$	$\leq 0.25$	$\geq 4$	1	$\leq 0.25$	$\geq 4$	$\leq 0.25$	$\geq 4$
Levofloxacin	$\geq 8$	$\leq 0.25$	4	4	$\geq 8$	1	$\geq 8$	$\geq 8$	$\leq 0.25$	$\leq 0.25$	$\geq 8$	$\leq 0.25$	$\geq 8$	1	$\leq 0.25$	$\geq 8$	$\leq 0.25$	$\geq 8$
Tigecycline	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$
Nitrofurantoin	$\leq 16$	$\leq 16$	64	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	$\leq 16$	128	32	$\leq 16$
Trimethoprim	$\leq 20$	$\leq 20$	$\geq 320$	$\geq 320$	$\leq 20$	$\leq 20$	$\geq 320$	$\leq 20$	$\leq 20$	$\leq 20$	$\leq 20$	$\leq 20$	$\leq 20$	$\geq 320$	$\leq 20$	80	$\leq 20$	$\leq 20$

The MIC values labeled with gray background indicate the resistance to the antimicrobial agents.

conducted with the whole genome sequences of the strains, generating eight different STs (Table 2). The strains that classified to ST405 and ST155 were accounted for 55.56% ( $n = 10$ ). The STs represented by two strains were ST1193 ( $n = 2$ ) and ST167 ( $n = 2$ ). The remaining strains ( $n = 4$ ) were singletons and represented their own ST.

## ARGs in *E. coli* Strains

In order to detect the genes corresponding to the ESBL phenotype, we screened the genome sequences of the 18 *E. coli* strains for the presence of ARGs. The results indicated that all *E. coli* strains harbored *bla*<sub>CTX-M</sub> gene (Table 2). Further analysis of the genetic environment of *bla*<sub>CTX-M</sub> gene revealed that strains with same STs carried the same *bla*<sub>CTX-M</sub> subtypes. For example, Strains R2, R9, R12 and R19, designated as ST155, harbored *bla*<sub>CTX-M-14</sub> gene, while strains R5, R10, R13 and R20, which were classified as ST405, carried two *bla*<sub>CTX-M-15</sub> genes and shared the same genetic environments (Figure 1). With such high similarities in gene arrangements among some strains, we wondered whether these strains were copy isolates identified in the samples. Therefore, we calculated the ANI values between the 18 isolates. The result showed that some strains shared 100% ANI, which might infer that they were actually copy isolates (Supplementary Figure S1). The genes encoding for mobile elements, transposases and plasmid conjugative transfer proteins were located near *bla*<sub>CTX-M</sub> gene in majority of *E. coli* strains (Figure 1 and Supplementary Table S1). Besides beta-lactamase genes, most strains also carried genes conferring resistance to many other classes of antimicrobial agents. For instance, 12 strains harbored aminoglycoside resistance genes with *aph*(3'')-Ib and *aph*(6)-Id as the most abundant types; sulfonamide resistance genes (e.g., *sul1*, *sul2*, or *sul3*) were found in 12 strains. Eleven strains carried genes encoding for

tetracycline resistance, including *tet*(A), *tet*(B), *tet*(D), and *tet*(M). Moreover, resistance genes conferring resistance to phenicols, trimethoprim, macrolides, lincosamides and streptogramin B (MLS), fluoroquinolones, and fosfomycins were detected in some of the strains.

## Virulence Genes in *E. coli* Strains

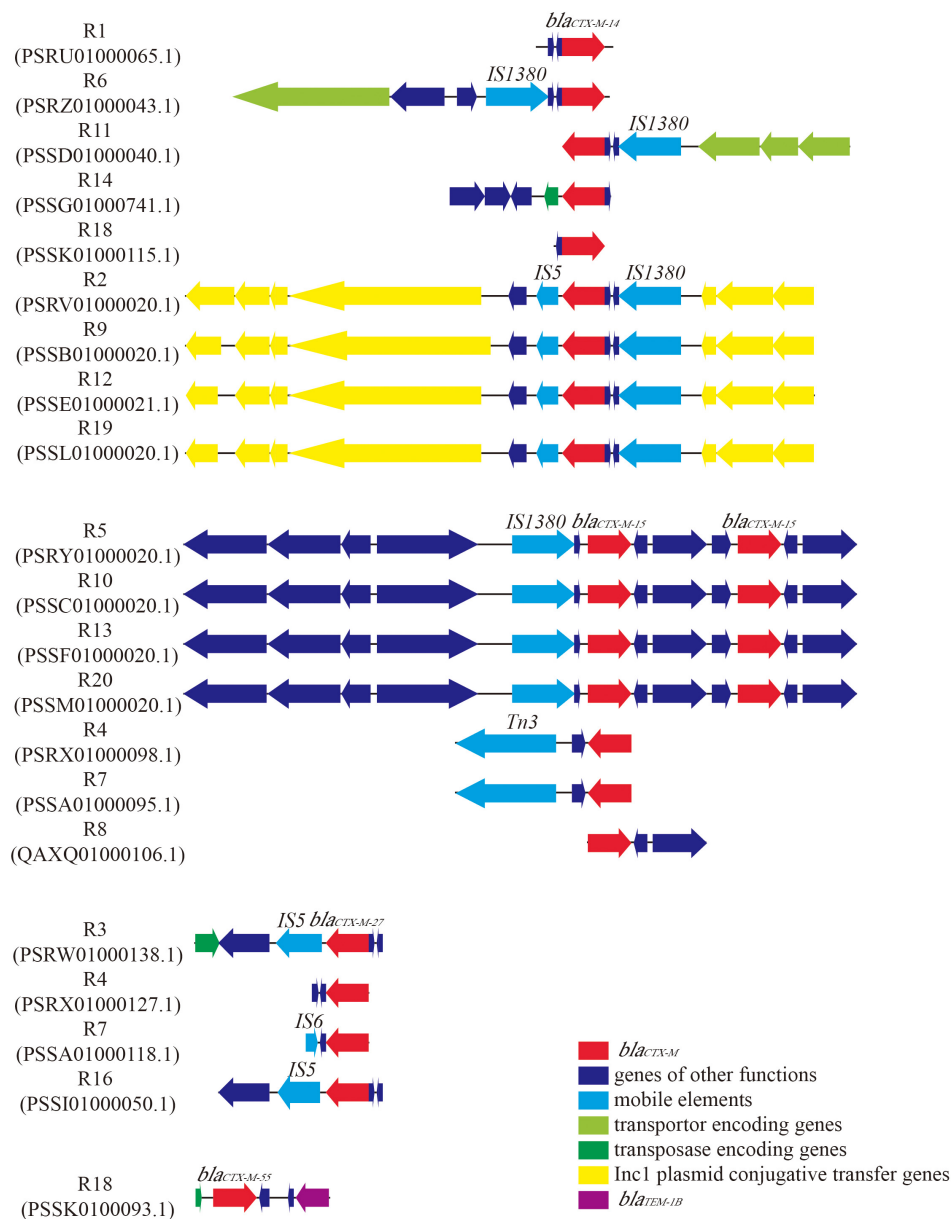
Virulence genes were detected in all *E. coli* strains and the numbers of virulence genes were different among the strains with different STs. One ST88 strain, R14, that encoded seven virulence genes (*gad*, *iroN*, *mchF*, *ireA*, *iss*, *celB*, and *tsh*), harbored more virulence genes than other STs strains. While the two ST167 strains, R4 and R7, only harbored *gad* gene. Of all the virulence genes, the glutamate decarboxylase encoding gene *gad* was the most common virulence gene presented in the 16 out of 18 strains (88.89%) (Table 2). The second predominant type was *eilA* (encoding for Salmonella HilA homolog), followed by *air* (encoding for Enterotoxigenic immunoglobulin repeat protein) and *lpfA* (encoding for long polar fimbriae). Two enterotoxin genes, namely, *astA* and *senB*, were found in some *E. coli* strains.

## Plasmids in *E. coli* Strains

17 out of 18 strains were predicted to harbor plasmids by PlasmidFinder (Supplementary Table S2). The Col156 plasmid replicon type was observed to be the most prevalent type among these strains ( $n = 13$ ). The five *E. coli* ST405 strains (R5, R8, R10, R13, and R20) carry one Col(MP18) plasmid replicon type and one IncY plasmid incompatibility groups, with strain R5 and R13 additionally harboring one Col(156) replicon type. The four *E. coli* ST155 strains (R2, R9, R12, and R19) were identified to carry the same four plasmids: Col(MP18), Col(156), IncB/O/K/Z and IncY. However, another ST155 strain, R18, was identified

**TABLE 2 |** ARGs and virulence genes identified in the genomes of *E. coli* strains.

Strain	STs	Virulence genes	Acquired antimicrobial resistance genes of different antimicrobial classes								
			Beta-lactam	Aminoglycoside	Fluoroquinolone	Fosfomycin	Macrolide, lincosamide and streptogramin B (MLS)	Phenicol	Sulfonamide	Tetracycline	Trimethoprim
R1	ST1193	<i>gad senB vat iha sat</i>	<i>bla</i> <sub>CTX-M-14</sub> <i>bla</i> <sub>TEM-1B</sub>	<i>aph</i> (3'')-Ib <i>aac</i> (3)-IId <i>aph</i> (6)-Id <i>aacA4</i>	<i>aac</i> (6')-Ib-cr			<i>cmlA1</i>	<i>sul2</i>	<i>tet</i> (A)	
R2	ST155	<i>gad lpfA</i>	<i>bla</i> <sub>CTX-M-14</sub>								
R3	ST450	<i>gad iha senB</i>	<i>bla</i> <sub>CTX-M-27</sub>	<i>aph</i> (3'')-Ib <i>aadA5</i> <i>aph</i> (6)-Id			<i>mph</i> (A)		<i>sul1 sul2</i>	<i>tet</i> (A)	<i>dfrA17</i>
R4	ST167	<i>gad</i>	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>CTX-M-27</sub>	<i>aadA5 aac</i> (3)-IIa			<i>mph</i> (A) <i>erm</i> (B)		<i>sul1</i>	<i>tet</i> (B)	<i>dfrA17</i>
R5	ST405	<i>gad eilA air</i>	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>CTX-M-15</sub>	<i>aph</i> (3'')-Ib <i>aph</i> (6)-Id				<i>floR</i>	<i>sul2</i>	<i>tet</i> (A)	
R6	ST38	<i>gad astA iss eilA</i>	<i>bla</i> <sub>CTX-M-14</sub>	<i>aadA5 aac</i> (3)-IId			<i>mph</i> (A)		<i>sul1</i>		<i>dfrA17</i>
R7	ST167	<i>gad</i>	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>CTX-M-27</sub>	<i>aadA5 aac</i> (3)-IIa			<i>mph</i> (A) <i>erm</i> (B)		<i>sul1</i>	<i>tet</i> (B)	<i>dfrA17</i>
R8	ST405	<i>eilA air</i>	<i>bla</i> <sub>CTX-M-15</sub>	<i>aph</i> (3'')-Ib <i>aph</i> (6)-Id				<i>floR</i>	<i>sul2</i>	<i>tet</i> (A)	
R9	ST155	<i>gad lpfA</i>	<i>bla</i> <sub>CTX-M-14</sub>								
R10	ST405	<i>gad eilA air</i>	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>CTX-M-15</sub>	<i>aph</i> (3'')-Ib <i>aph</i> (6)-Id				<i>floR</i>	<i>sul2</i>	<i>tet</i> (A)	
R11	ST1193	<i>gad vat sat iha</i>	<i>bla</i> <sub>CTX-M-14</sub>								
R12	ST155	<i>gad lpfA</i>	<i>bla</i> <sub>CTX-M-14</sub>								
R13	ST405	<i>gad eilA air</i>	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>CTX-M-15</sub>	<i>aph</i> (3'')-Ib <i>aph</i> (6)-Id				<i>floR</i>	<i>sul2</i>	<i>tet</i> (A)	
R14	ST88	<i>gad iroN mchF ireA iss celb tsh</i>	<i>bla</i> <sub>CTX-M-14</sub>	<i>aph</i> (4)-Ia <i>aac</i> (3)-IVa <i>aph</i> (3')-Ia <i>aadA2 aadA1</i>		<i>fosA3</i>		<i>floR cmlA1</i>	<i>sul2 sul3</i>	<i>tet</i> (B)	<i>dfrA12</i>
R16	ST1722	<i>eilA air astA lpfA iss</i>	<i>bla</i> <sub>CTX-M-27</sub>								
R18	ST155	<i>gad lpfA astA</i>	<i>bla</i> <sub>CTX-M-14</sub> <i>bla</i> <sub>CTX-M-55</sub> <i>bla</i> <sub>TEM-1B</sub>	<i>aph</i> (3'')-Ib <i>aph</i> (6)-Id <i>aph</i> (3')-IIa <i>aph</i> (3')-Ia <i>aadA1 aph</i> (4)-Ia <i>aac</i> (3)-IVa, <i>aadA2</i>	<i>oqxA oxqB</i>	<i>fosA3</i>	<i>mph</i> (A) <i>erm</i> (42)	<i>floR cmlA1</i>	<i>sul1 sul2 sul3</i>	<i>tet</i> (A) <i>tet</i> (M)	<i>dfrA17</i>
R19	ST155	<i>gad lpfA</i>	<i>bla</i> <sub>CTX-M-14</sub>								
R20	ST405	<i>gad eilA air</i>	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>CTX-M-15</sub>	<i>aph</i> (3'')-Ib <i>aph</i> (6)-Id				<i>floR</i>	<i>sul2</i>	<i>tet</i> (A)	



**FIGURE 1 |** Genetic environment of *bla*<sub>CTX-M</sub> in 18 *Escherichia coli* strains. GenBank accession numbers of the contigs which contain *bla*<sub>CTX-M</sub> were given below the strain names. ORFs were represented by arrows. The color of the arrow indicated the function of the gene. Subtypes of *bla*<sub>CTX-M</sub> and insertion sequence (IS) were labeled above the arrows.

to carry eight plasmids [Col(MG828), IncFII(pHN78A), IncHI2, IncHI2A, IncI1, IncQ1, IncX1, and P0111].

## ARGs in WWTP

By metagenomics sequencing, a total of 109,523,076 clean reads were generated and were then deposited in NCBI Sequence Read Archive with accession number of SRP132643. 11 ARGs with high identities were annotated using the assembled sequence generated by metagenomic sequencing. These ARGs are involved in the resistance to various types of antimicrobial agents including aminoglycosides, fluoroquinolones, macrolides,

phenicols, and sulfonamides (**Supplementary Table S3**). Five ARGs identified in metagenomic data were also carried by the *E. coli* strains isolated from the same WWTP (**Table 2**).

## DISCUSSION

In recent years, a growing number of studies have shown that WWTPs may function as major reservoirs of ARB and ARGs (Conte et al., 2017; Guo et al., 2017; Szekeres et al., 2017). Although most studies have reported ARB and ARGs

from municipal and hospital WWTP, some researchers focused on the pharmaceutical industry WWTPs for the wastewater discharged from pharmaceutical industry may contain high concentrations of hazardous substances, including antimicrobial agents. High abundance of resistant strains has been observed in pharmaceutical WWTPs. In particular,  $63.6 \pm 6.4\%$  of the bacterial strains isolated from penicillin production wastewater (Li et al., 2009) and 86% of the bacterial strains from a WWTP of multiple bulk drug manufacturers (Marathe et al., 2013) were resistant to many antimicrobial agents. Notwithstanding this, most of the bacterial strains isolated from these WWTPs were non-pathogenic. For instance, only two strains were identified as *E. coli* among the 189 strains isolated from a WWTP treating oxytetracycline, avermectin, and ivermectin production wastewater (Li et al., 2010). Therefore, in our study, we used selective plates for the isolation of ESBL-producing *E. coli* from the pharmaceutical WWTP. In total, we recovered 18 ESBL-producing *E. coli* strains. The results of antimicrobial susceptibility testing demonstrated that all strains displayed resistance to beta-lactams such as ampicillin, cefazolin, and ceftriaxone (Table 1).

In order to investigate the ARGs carried by these strains, WGS technology was applied to retrieve the draft genome sequences of all the 18 strains. Not surprisingly, all of the strains were found to carry at least one beta-lactamase gene, and the most prevalent ARGs type was *bla*<sub>CTX-M</sub> (Table 2). Previous studies have demonstrated that *bla*<sub>CTX-M</sub> is the most common ARGs type in ESBL-producing *E. coli* strains of human (Campos et al., 2018) and animal origin (Wu et al., 2018). The molecular homology analysis of *bla*<sub>CTX-M</sub> positive *E. coli* strains further suggested a transmission of pathogens across water, food animals and human (Hu et al., 2013). Our finding in accordance with other studies and revealed that *bla*<sub>CTX-M</sub> was the most dominant ARGs type in WWTPs of industrial origin. The results of sequence alignments indicated *bla*<sub>CTX-M</sub>-containing contigs share a high degree of similarity among the strains with the same STs (Figure 1 and Table 2). Further investigation of genetic environment revealed that the genes encoding for mobile elements, transporters, transposase and Inc11 plasmid conjugative transfer proteins located near the *bla*<sub>CTX-M</sub> in most of the strains, suggesting the transfer potential of *bla*<sub>CTX-M</sub> (Figure 1). In addition, plasmid prediction revealed that *bla*<sub>CTX-M-14</sub> genes and IncB/O/K/Z plasmids were located in the same contigs of strains R2, R9, R12, and R19. Likewise, *bla*<sub>TEM-1B</sub> gene and plasmid IncFIA were predicted in the same contig of strain R1. Further indicating the transfer potential of these ARGs via plasmids.

Numerous virulence genes were detected in the genomes of the 18 ESBL-producing *E. coli* strains. For instance, all ST405 strains (R5, R8, R10, R13, and R20) harbored *eilA* and *air* genes which encoded for Salmonella HilA homolog and Enteraggregative immunoglobulin repeat protein, respectively. The long polar fimbriae encoding gene *lpfA* was detected in all ST155 strains (R2, R9, R12, R18, and R19). These genes were demonstrated to play a role in the adherence of the bacteria (Doughty et al., 2002; Sheikh et al., 2006). Strain R14 which belonged to ST88, harbored the highest number of virulence genes among all the strains. The possession of a high

number of virulence traits in ST88 strains was also reported in previous studies (Oteo et al., 2014). Among these virulence genes, *gad* was the most prevalent type (Table 2). The *gad* gene enables the bacteria to survive in harsh acidic conditions in gastric environment by helping them to maintain a near-neutral intracellular pH (Capitani et al., 2003). Genes encoded for enterotoxins were also discovered. Strain R1 and R3 carried *senB* gene which encoded for plasmid-encoded enterotoxin. While strain R6, R16, and R18 harbored the heat-stable enterotoxin 1 encoding gene *astA*. These enterotoxins are responsible for fluid secretion and may associated with diarrhea (Dubreuil et al., 2016). With all these virulence genes, the *E. coli* strains isolated from the pharmaceutical WWTP may pose a potential threat to human health.

In addition to metagenomic sequencing, numerous studies have employed quantitative polymerase chain reaction (qPCR) for the detection of ARGs and their relative abundance (Szekeres et al., 2017; Zheng et al., 2017c). However, due to the limited availability of qPCR primers, metagenomic sequencing may be more appropriate for the screening of both known and novel ARGs (Schmieder and Edwards, 2012; Berglund et al., 2017; Marathe et al., 2018). Nevertheless, a combination of WGS and metagenomic sequencing can be an effective strategy to unravel the resistome in WWTP. To further reveal ARGs in this WWTP, a high-throughput metagenomic sequencing technology was implemented. The occurrence of 11 ARGs was analyzed from the metagenomic sequence with high amino acid identities (Supplementary Table S3). Five of these ARGs, *aadA1*, *aac(6')-lb-cr*, *flo(R)*, *sul2* and *sul1*, were also present in the genomes of the *E. coli* isolated from the same WWTP. However, we failed to find any beta-lactam resistance genes in the metagenomic data, while these beta-lactam resistance genes were carried by all the 18 *E. coli* isolated from the same sample. That may due to the low abundance of these beta-lactam resistance genes in the metagenomics data. By taxonomy annotation, only three out of 105,892 unigenes were annotated as *E. coli*, indicating that *E. coli* was of very low abundance in the WWTP. This low abundance of *E. coli* in WWTP was also observed in previous study (Li et al., 2010). Only those *E. coli* that exhibit antimicrobial resistance can survive in this WWTP which treats the antimicrobial agents producing pharmaceutical wastewater. The present work reported the isolation and analysis of ESBL-producing *E. coli* in a pharmaceutical WWTP and presented their antimicrobial phenotypes as well as genotypes. However, the background of *E. coli* in this geographic area is still unknown. In order to draw a whole picture of the prevalence of ESBL-producing *E. coli* and to evaluate the potential risk of their transmission in this area, further study needs to be done to elucidate the diversity of *E. coli* in this area.

## CONCLUSION

In present study, we applied both WGS and metagenomics sequencing technologies to uncover the antimicrobial resistance profiles of ESBL-producing *E. coli* strains and the antimicrobial resistome in a pharmaceutical WWTP. Our findings indicate



that pharmaceutical WWTP can play a significant role in the emergence of ARB and ARGs. In order to prevent the spread of ARB and ARGs, the sewage sludge produced by this WWTP are treated through dewatering and incineration. Thus, further research is required to investigate the ARB and ARGs in the sludge dewatering streams.

## AUTHOR CONTRIBUTIONS

XJ and BZ designed the study. XC and HX performed the experiments. WL, FT, TS, and XP contributed reagents, materials, and analysis tools. XJ and BZ analyzed the data and wrote the manuscript. All authors revised the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01797/full#supplementary-material>

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# Bacterial Heavy-Metal and Antibiotic Resistance Genes in a Copper Tailing Dam Area in Northern China

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Heavy metal resistance genes (MRGs) and antibiotic resistance genes (ARGs) in bacteria can respond to the inducement of heavy metals. However, the co-occurrence of MRGs and ARGs in the long-term heavy metal contaminated area is still poorly understood. Here, we investigated the relationship between the abundance of soil bacteria MRGs, ARGs and heavy metal pollution in a copper tailing dam area of northern China. We found that *arsC* and *ereA* genes coding for resistance mechanisms to arsenic and to macrolides, respectively, are the most abundant MRG and ARG in the study area. The abundance of MRGs is positively correlated with cadmium (Cd) concentration, and this indicates the importance of Cd in the selection of MRGs. The network analysis results show that *suIII* and MRGs co-occur and *copB* occur with ARGs, which suggests that MRGs and ARGs can be co-selected in the soil contaminated by heavy metal. The network analysis also reveals the co-occurrence of Cd and MRGs, and thus heavy metal with a high 'toxic-response' factor can be used as the indicator of MRGs. This study improves the understanding of the relationship between bacterial resistance and multi-metal contamination, and underlies the exploration of the adaptive mechanism of microbes in the multi-metal contaminated environment.

**Keywords:** metal resistance genes, antibiotic resistance genes, mobile genetic element, co-occurrence, metal pollution, risk index, copper tailing

## INTRODUCTION

The rapid industrialization has led to a series of ecological and environmental problems (Sawut et al., 2018). One of the most urgent issues is heavy metal contamination that is harmful to both ecosystem functions and human health. Heavy metals are widely distributed in almost all types of soils (Zhao et al., 2012), sediment (Gati et al., 2016), and water bodies (Tang et al., 2014). Some heavy metals are essential micronutrients for several cellular functions and components of biological macromolecules [for example, zinc (Zn) is an important component of DNA-polymerases] (Seiler and Berendonk, 2012), but can be toxic when accumulated to a certain concentration (for example, the excessive uptake of Zn leads to zinc-induced copper deficiency) (Fosmire, 1990; Zhao et al., 2012). In recent years, industrial (e.g., mining) and agricultural (e.g., land application of metal contained fertilizers) activities contribute to heavy metal accumulation (Charlesworth et al., 2011; Chen et al., 2015; Li L.G. et al., 2017).

Environmental compartments are subject to anthropogenic pressures (Berendonk et al., 2015). As a vital component of terrestrial ecosystems, soil microbes play a significant role in the material

and energy cycle. However, microbial communities are highly sensitive to environmental changes (Kandeler et al., 2000; Blakely et al., 2002; Kelly et al., 2003; Epelde et al., 2015). The excessive heavy metals found in the soil may impose selection pressures on soil microbes (Berendonk et al., 2015) and even change the diversity of soil microbial communities (Kandeler et al., 1996, 2000; Kelly et al., 2003; Epelde et al., 2015). To cope with these situations, an effective strategy for microorganisms is to evolve a system based on biochemical and genetically encoded mechanisms (Aka and Babalola, 2017). This has been found in many bacteria strains isolated from different heavy metal polluted scenarios (Zafar et al., 2007; Sabry et al., 2010; Rehman and Anjum, 2011; Muñoz et al., 2012). Bacteria can thus be used for the remediation of heavy metal polluted areas (Aka and Babalola, 2017; Chen et al., 2018). Nevertheless, present efforts of heavy metal resistance are mainly centered on single isolated strains (Zafar et al., 2007; Sabry et al., 2010; Rehman and Anjum, 2011; Muñoz et al., 2012). A better understanding of the distribution of resistance in heavy metal contaminated area, especially the long-term polluted sites, is critical to optimize such remediation schemes.

Antibiotic resistant genes (ARGs) have been reported as a new pollutant by the World Health Organization because of their emerging prevalence and wide distribution (World Health Organization [WHO], 2014). This is a threat to public health (Rodriguez-Mozaz et al., 2015). The increasing ARGs have been recognized as a consequence of the massive use of antibiotics in therapeutics and agriculture (Levy and Marshall, 2004; Huerta et al., 2013). However, more evidence shows that the dissemination of ARGs can also be influenced by heavy metal contamination (Pruden et al., 2006; Knapp et al., 2017; Tan et al., 2018). As early as in the 1970s, it was found that heavy metal resistance and antibiotic resistance can be selected simultaneously in the heavy metal contaminant ecosystem (Timoney et al., 1978). These phenomena can be interpreted on the molecular level as co-selection (two or more genetically linked resistance genes) or cross-selection (single genetic element provides tolerance to more than one antimicrobial agents) (Baker-Austin et al., 2006; Seiler and Berendonk, 2012). The co-selection of heavy metal resistance genes (MRGs) and ARGs have been reported in agriculture (Hu et al., 2016), animal husbandry (Zhu et al., 2013), wastewater treatment system (Cesare et al., 2016), and sediment (Wright et al., 2010). However, bacterial communities are shaped by a complex array of evolutionary, ecological, and environmental factors (Berendonk et al., 2015). Patterns of MRGs and ARGs in the area of long-term heavy metal contamination are still poorly studied.

Zhongtiaoshan Copper Mine is the largest non-coal underground mine in northern China (Liu et al., 2018). A huge tailing dam was built as a result of the long-term mining processes. The body of the dam was formed by large amounts of waste residue containing multiple heavy metals. As a result, the surrounding area of the dam is contaminated by heavy metals through wind or seep water.

The purpose of this study is to assess the heavy metal and antibiotic resistance in the downstream region of the tailing dam from the following aspects: (1) what the heavy metal

contamination level is in the region, (2) what the main MRGs types are and how they are distributed in this region, and (3) what the co-occurrence patterns of MRGs and ARGs are in this region. The objective of this study is to reveal the occurrence of MRGs and ARGs and the relationships between heavy metal and MRGs/ARGs in this copper tailing dam area.

## MATERIALS AND METHODS

### Soil Sampling

The study area (35°15'N, 111°39'E) is located in a copper tailing dam area in the southern part of Shanxi Province, China. The climate is monsoonal climate, with average annual temperature 14°C, mean annual rainfall 780 mm and annual frost-free period more than 200 days (Liu et al., 2018). The tailing dam has been used since 1972 (Figure 1). At the bottom of the tailing dam, a stream forms due to the seeping water. Four sampling sites TD0, TD1, TD2, and TD3 were selected along the stream in this study. TD0 site is on the top of the tailing dam and is covered by herbs. TD1 site is at the bottom of the dam and has been associated with poplar plantation of about 15 years. TD2 site, adjacent to TD1 site, is farmland. TD3 site is the most far away from the dam and has been associated with poplar plantation of about 10 years.

Sampling was carried out in June 2016, just after the wheat harvest. The upper 20 cm of soil was collected from three random locations at the TD0 site and nine random locations at each of the other three sites. All of the soil samples were sieved, thoroughly homogenized, and divided into two portions. One portion was stored at −80°C for molecular analysis, and the other was air-dried for chemical analysis. Soil pH in ddH<sub>2</sub>O was measured at a soil:solution ratio of 1:2.5 (w:v). Soil total carbon (TC), total nitrogen (TN), and total sulfur (TS) were determined by vario macro cube elemental analyzer (vario macro cube, elemental, Germany) (Table 1).

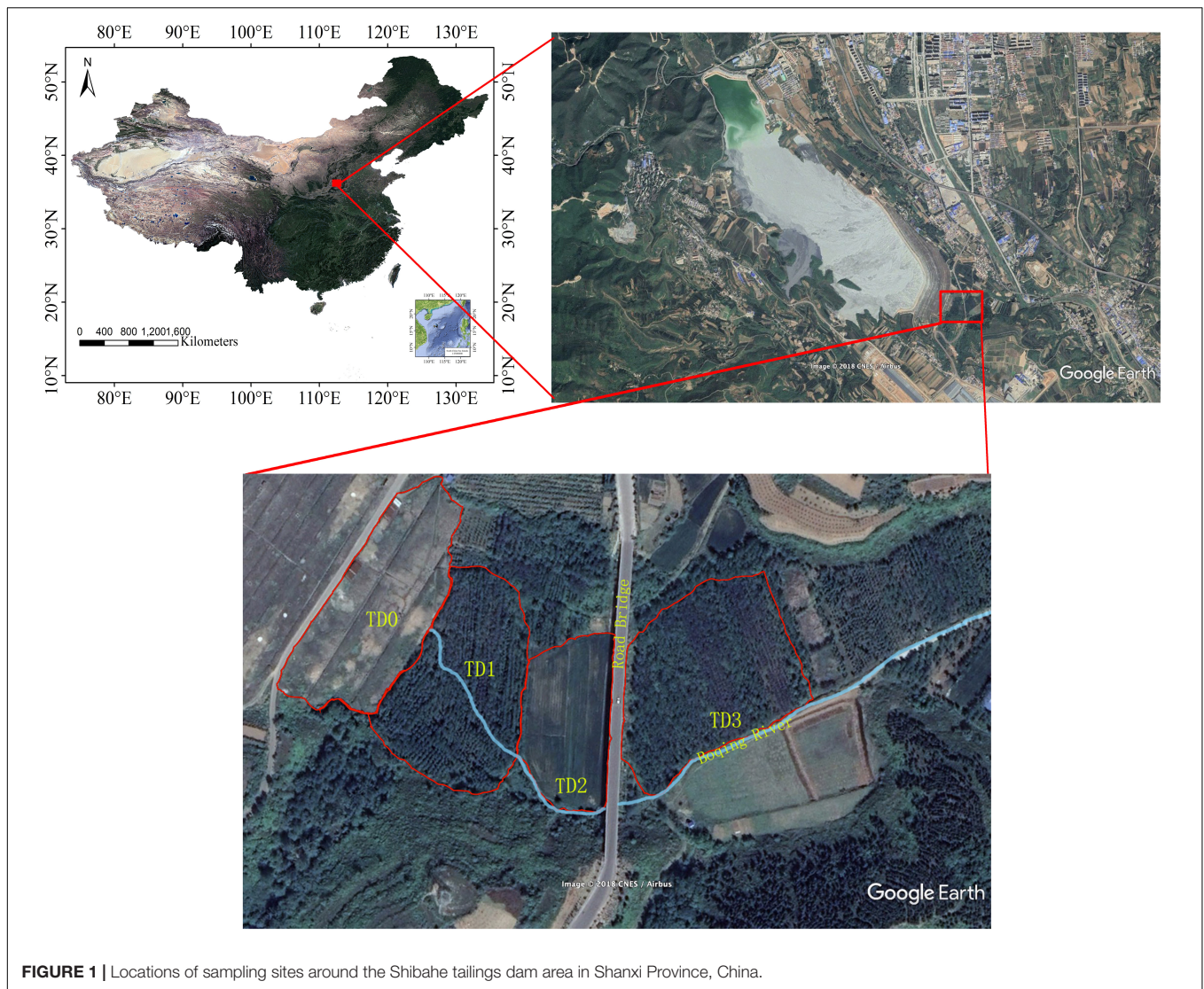
### Heavy Metal Content Analysis

Soil samples were digested using the HNO<sub>3</sub>-HF-H<sub>2</sub>O<sub>2</sub> method (Raven and Loeppert, 1997) in a microwave digesting apparatus (CEM MARs XPRSS, United States). The digested samples were diluted with deionized water to 50 mL. The concentrations of heavy metals (As, Cr, Cd, Cu, Ni, Pb, and Zn) in the solutions were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, iCAP 6000, Thermo Fisher, United Kingdom). A mixed standard solution including all the heavy metals and reagent blanks were carried out through digestion and analyzed as part of the quality control protocol. Results were adopted when the measured concentrations in the reference materials were within one standard deviation of the certified values (Li et al., 2012).

### Ecology Risk Analysis

The potential ecological risk index (*RI*) is used to characterize the toxicity level of heavy metals. *RI* is the potential ecological risk caused by overall contamination (Hakanson, 1980), which covers a variety of research domains (e.g., biological toxicology, environmental chemistry, and ecology) (Zhai et al., 2014). *RI* can





**FIGURE 1 |** Locations of sampling sites around the Shibahe tailings dam area in Shanxi Province, China.

be used to evaluate the comprehensive ecological risks caused by a single pollutant and the overall risk from various pollutants. The assessment methods of *RI* are shown as follows:

$$C_f^i = \frac{C_D^i}{C_R^i}$$

$$RI = \sum_i^n E_r^i = \sum_i^n (T_r^i \times C_f^i)$$

Where  $C_f^i$  is the single heavy metal pollution index,  $C_D^i$  is the concentration of individual heavy metal in samples,  $C_R^i$  is the reference value of heavy metal and usually uses the soil background value,  $E_r^i$  is the monomial potential ecological risk factor of individual heavy metal, and  $T_r^i$  is the heavy metal toxic response factor. The response values for each heavy metal are in the order of  $Zn = 1 < Cr = 2 < Cu = Pb = Ni = 5 < As = 10 < Cd = 30$  (Hakanson, 1980).

## DNA Extraction and Quantitative Real Time PCR

Total genomic DNA was extracted from 0.25 g fresh soils using the Qiagen DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA, United States) according to the manufacturer's instructions. DNA concentration and purity were assessed by Infinite 200 PRO (TECAN, Sweden) and the DNA concentration of each sample was adjusted to yield a concentration of  $10 \text{ ng } \mu\text{L}^{-1}$ . DNA extractions were stored at  $-20^\circ\text{C}$  for further analysis.

The heavy MRGs were selected according to the findings of Li L.G. et al. (2017) that 9 MRGs types have distinct preference for ARG types as their closest genetic neighbor, and we also selected to test the antibiotic resistance genes that are widely detected in many long-term contaminated fields in China (Hu et al., 2016, 2017). Here, we quantified the absolute abundances of 13 MRGs, 28 ARGs, and 2 mobile genes elements (MGEs) through quantitative real-time PCR (qPCR). The MRGs include 5 copper resistance genes (*copA*,

**TABLE 1** | Soil physicochemical characteristics and heavy metal content (mg kg<sup>-1</sup> dry soil) at each sampling site.

Sampling site	TD0	TD1	TD2	TD3	Background value
pH	8.00 ± 0.02 <sup>ab</sup>	7.76 ± 0.20 <sup>c</sup>	8.04 ± 0.21 <sup>a</sup>	7.80 ± 0.11 <sup>bc</sup>	–
TN (g/kg)	0.58 ± 0.03 <sup>c</sup>	0.89 ± 0.16 <sup>b</sup>	1.12 ± 0.06 <sup>a</sup>	0.89 ± 0.07 <sup>b</sup>	–
TC (g/kg)	12.28 ± 0.26 <sup>c</sup>	16.20 ± 1.27 <sup>a</sup>	13.95 ± 0.46 <sup>b</sup>	15.86 ± 1.52 <sup>a</sup>	–
TS (g/kg)	1.51 ± 0.71 <sup>a</sup>	0.73 ± 0.10 <sup>b</sup>	0.59 ± 0.02 <sup>b</sup>	0.68 ± 0.11 <sup>b</sup>	–
As	6.94 ± 2.42 <sup>b</sup>	12.28 ± 4.32 <sup>a</sup>	7.15 ± 3.44 <sup>b</sup>	10.52 ± 3.96 <sup>ab</sup>	9.1
Cd	0.17 ± 0.08 <sup>c</sup>	1.25 ± 0.66 <sup>a</sup>	0.54 ± 0.34 <sup>bc</sup>	0.76 ± 0.25 <sup>ab</sup>	0.1
Cr	47.24 ± 20.04 <sup>b</sup>	381.91 ± 176.75 <sup>a</sup>	371.90 ± 169.15 <sup>a</sup>	339.05 ± 174.71 <sup>a</sup>	55.3
Cu	338.00 ± 184.21 <sup>a</sup>	323.28 ± 157.59 <sup>a</sup>	122.10 ± 36.15 <sup>b</sup>	157.91 ± 36.37 <sup>b</sup>	22.9
Ni	34.17 ± 7.63 <sup>a</sup>	79.51 ± 55.13 <sup>a</sup>	77.89 ± 44.53 <sup>a</sup>	101.79 ± 67.16 <sup>a</sup>	29.9
Pb	756.76 ± 221.91 <sup>a</sup>	1227.24 ± 360.65 <sup>a</sup>	983.42 ± 292.03 <sup>a</sup>	1098.53 ± 349.17 <sup>a</sup>	14.7
Zn	33.43 ± 18.91 <sup>b</sup>	77.17 ± 36.37 <sup>a</sup>	89.23 ± 23.84 <sup>a</sup>	64.67 ± 16.73 <sup>ab</sup>	63.5

Data are means ± standard deviation. The different letters indicate that the means are significantly different among soils ( $P < 0.05$ ) with Duncan test. TN, TC, and TS represent soil total nitrogen, soil total carbon and soil total sulfur, respectively.

*copB*, *pcoA*, *pcoC*, and *pcoD*), 3 multiple heavy MRGs (*czcA*, *czcC*, and *czcD*), 2 arsenic resistance genes (*arsB* and *arsC*) and 3 resistance genes (*nccA*, *pbrT*, and *chrB* to nickel, lead, chromium, respectively). The ARGs include 15 tetracycline resistance genes (*tetA*, *tetC*, *tetE*, *tetK*, *tetL*, *tetA/P*, *tetG*, *tetM*, *tetO*, *tetQ*, *tetS*, *tetT*, *tetW*, *tetB/P*, and *tetX*), 4 β-lactam resistance genes (*bla<sub>CTX-M</sub>*, *bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, and *bla<sub>ampC</sub>*), 3 sulfonamide resistance genes (*sulI*, *sulII*, and *sulIII*), 3 quinolone resistance genes (*qnrA*, *qnrB*, and *qnrS*), and 3 macrolides resistance genes (*ereA*, *ereB*, and *mphA*). The MGEs include an integrase gene of class 1 integrons (*intI1*) and a transposon-transposase gene (*tnpA*).

The qPCR analysis was conducted using the iCycler iQ5 thermocycler (Bio-Rad, Hercules, CA, United States). Each sample was amplified in triplicate with 10 μL reaction mixture that consisted of 5 μL of SYBR Premix Ex Taq<sup>TM</sup> (Takara Bio, Inc., Shiga, Japan), 0.5 μL of each primer (10 mM), 3 μL of diluted template DNA, and microbial DNA-free water. The primers and thermal cycling conditions of MRGs, ARGs and MGEs are described in **Supplementary Table S1**. A negative control was included in each run. Melting curve analysis was performed at the end of each qPCR run to check the specificity of the amplicons.

## Data Analysis

The data were square root- or log-transformed to improve normality and reduce heteroscedasticity. MRGs and ARGs data were converted to relative abundance (normalized per 16S rRNA genes) because the bacterial population size has not been taken into account for the non-normalized data. The 16S abundance rRNA genes were used to assess the size of the bacterial population. Normalizing MRGs or ARGs count to 16S rRNA genes presents an approximate proportion of bacteria that carry the corresponding genes.

One-way analysis of variance (ANOVA) was used to assess the differences of heavy metals, *RI*, 16S rRNA genes, MRGs,

ARGs and MGEs among the study sites. Spearman linear correlation analysis was conducted to determine whether there were significant correlations between heavy metals, *RI*, MRGs, ARGs, and MGEs. Network analysis was used to explore the co-occurrence patterns of *RI*, MRGs, and ARGs.

ANOVA and Spearman linear correlation analysis were conducted in SPSS version 13.0 and R software version 3.4.4. Network analysis was performed and visualized with Gephi version 0.9.2.

## RESULTS

### Physicochemical Characteristics and Risk Assessment

Soil total carbon (TC) and total nitrogen (TN) at the TD0 site are significantly lower than those at the other three sites, while the TS shows the opposite. Soil pH is significantly higher at the TD2 site than at the TD1 and TD3 sites (**Table 1**). There is no difference in the concentrations of Ni and Pb among the four sites; however, the concentrations of other metals (As, Cd, Cr, Cu, and Zn) are significantly different among the four sites ( $P < 0.05$ , **Table 1**). The concentrations of all the metals measured are highest at the TD1 site except the concentrations of Cu, Ni, and Zn that are highest in TD0, TD3, and TD2, respectively. The analysis of the overall risk shows that *RI* is significantly higher at the TD1 site than at the other three sites ( $P < 0.05$ , **Table 2**), while the TD0 site represents the lowest potential ecological risk of all the sampling sites.

### Absolute Abundance of 16S rRNA Genes, MRGs, ARGs, and MGEs

Quantitative PCR was performed to analyze the abundances of total bacteria (by targeting the bacterial 16S rRNA genes), MRGs, ARGs, and MGEs. The abundance of 16S rRNA genes at the TD0 site is significantly lower than at the other sites ( $P < 0.01$ ,

**TABLE 2** | Potential ecology risk index (*RI*) at each sampling site.

Sampling site	TD0	TD1	TD2	TD3	Contribute to <i>RI</i>
<i>RI</i> -As	7.63 ± 2.66 <sup>b</sup>	13.5 ± 4.74 <sup>a</sup>	7.86 ± 3.79 <sup>b</sup>	11.56 ± 4.35 <sup>ab</sup>	1.63%
<i>RI</i> -Cd	49.00 ± 23.73 <sup>c</sup>	367.91 ± 192.79 <sup>a</sup>	159.13 ± 100.72 <sup>bc</sup>	223.98 ± 73.14 <sup>ab</sup>	32.08%
<i>RI</i> -Cr	1.71 ± 0.72 <sup>b</sup>	13.81 ± 6.39 <sup>a</sup>	13.45 ± 6.12 <sup>a</sup>	12.26 ± 6.32 <sup>a</sup>	1.65%
<i>RI</i> -Cu	73.80 ± 40.22 <sup>a</sup>	70.59 ± 34.41 <sup>a</sup>	26.66 ± 7.89 <sup>b</sup>	34.48 ± 7.94 <sup>b</sup>	8.24%
<i>RI</i> -Ni	2.29 ± 0.51 <sup>a</sup>	5.32 ± 3.69 <sup>a</sup>	5.21 ± 2.98 <sup>a</sup>	6.81 ± 4.49 <sup>a</sup>	0.79%
<i>RI</i> -Pb	257.40 ± 75.48 <sup>a</sup>	417.43 ± 122.67 <sup>a</sup>	334.50 ± 99.33 <sup>a</sup>	373.65 ± 118.77 <sup>a</sup>	55.45%
<i>RI</i> -Zn	0.53 ± 0.30 <sup>b</sup>	1.22 ± 0.57 <sup>a</sup>	1.41 ± 0.38 <sup>a</sup>	1.02 ± 0.26 <sup>ab</sup>	0.17%
<i>RI</i>	392.35 ± 37.34 <sup>c</sup>	889.78 ± 278.7 <sup>a</sup>	548.21 ± 119.19 <sup>bc</sup>	663.76 ± 148.39 <sup>b</sup>	–

Data are means ± standard deviation. The different letters indicate that the means are significantly different among soils ( $P < 0.05$ ) with Duncan test. Contribute to *RI* means the percentage of the single metal ecological risk to the overall risk.

**Figure 2A**). A total of 10 MRGs, 17 ARGs, and 2 MGEs (out of 13 MRGs, 28 ARGs, and 2 MGEs targeted) are detected in this study. The lowest abundances of MRGs and ARGs are found at the TD0 site (**Figures 2B,C**). The highest abundance of MRGs is found at the TD1 site, while the highest abundance of ARGs is found at the TD3 site. There is a significant difference for MGEs among all the sampling sites ( $P < 0.05$ ). The MGEs shows the lowest level at the TD0 site, and it has not been detected for the *intI1* gene at the TD0 site (**Figures 2D,E**).

## The Distribution Patterns of MRGs

Of all the tested MRGs genes, *arsB*, *arsC*, *pbrT*, *czcA*, and *copA* genes are major components at all the sampling sites. In particular, the *arsC* gene (coding for resistance mechanism to arsenic) accounts for nearly 46.13, 65.65, 68.48, and 65.52% of the total MRGs at the TD0, TD1, TD2, and TD3 site, respectively (**Figure 3A**). *copB* genes have not been detected at the TD0 and TD1 sites, and *czcC* genes have only been detected at the TD1 site. In accordance with the sampling along the seeping stream, an increasing trend of all the copper resistance genes (*pcoA*, *copA*, and *copB*) is observed, while all the other resistance genes is found at the highest abundance at the TD1 site and there are significant differences in these genes among all the sampling sites ( $P < 0.01$ ) (**Figure 3B** and **Supplementary Table S2**).

## The Distribution Patterns of ARGs

The main genes of ARGs detected in this study are five tetracyclines resistance genes (*tetA*, *tetC*, *tetG*, *tetS*, and *tetX*), one sulfonamides resistance gene (*suII*), and one macrolides resistances gene (*ereA*). They represent 98.08, 95.21, 94.07, and 95.66% of the total ARGs at the TD0, TD1, TD2, and TD3 sites, respectively (**Figure 3C**). The *ereA* gene has the largest distribution at each site, and it contributes to 48.04, 29.83, 29.22, and 28.00% of the total ARGs at each site, respectively (**Figure 3C**). When all ARGs are concerned, there are significant differences in *tetC*, *tetG*, *tetA/P*, *tetX*, *tetM*, *tetO*, *tetQ*, *tetW*, *tetB/P*, *mphA*, and *suII* genes among the sampling sites ( $P < 0.05$ , **Supplementary Table S3**).

The ARGs patterns are more complex than MRGs. ARGs patterns can be classified as the following aspects: (1) there is no difference in the relative abundance of *tetA*, *tetK*, *tetS*, *ereA*, *bla<sub>CTX-M</sub>*, and *bla<sub>SHV</sub>* genes among the sampling sites, and the

highest abundance of these genes is found at the TD0 site apart from *tetK* and *bla<sub>CTX-M</sub>* genes. (2) Similar to the majority of MRGs, the *suII* gene is significantly more abundant at the TD1 site than at the other three sites. (3) The *tetO*, *tetW*, and *tetB/P* genes, which are not detected at the TD0 site, are significantly more abundant at the TD2 site than at the TD1 and TD3 sites. (4) There are significant differences in the relative abundance of the *tetC*, *tetG*, *tetA/P*, *tetX*, *tetM*, *tetQ*, and *mphA* genes among the sampling sites, with the highest abundance of those genes at the TD3 site (**Figure 3D**).

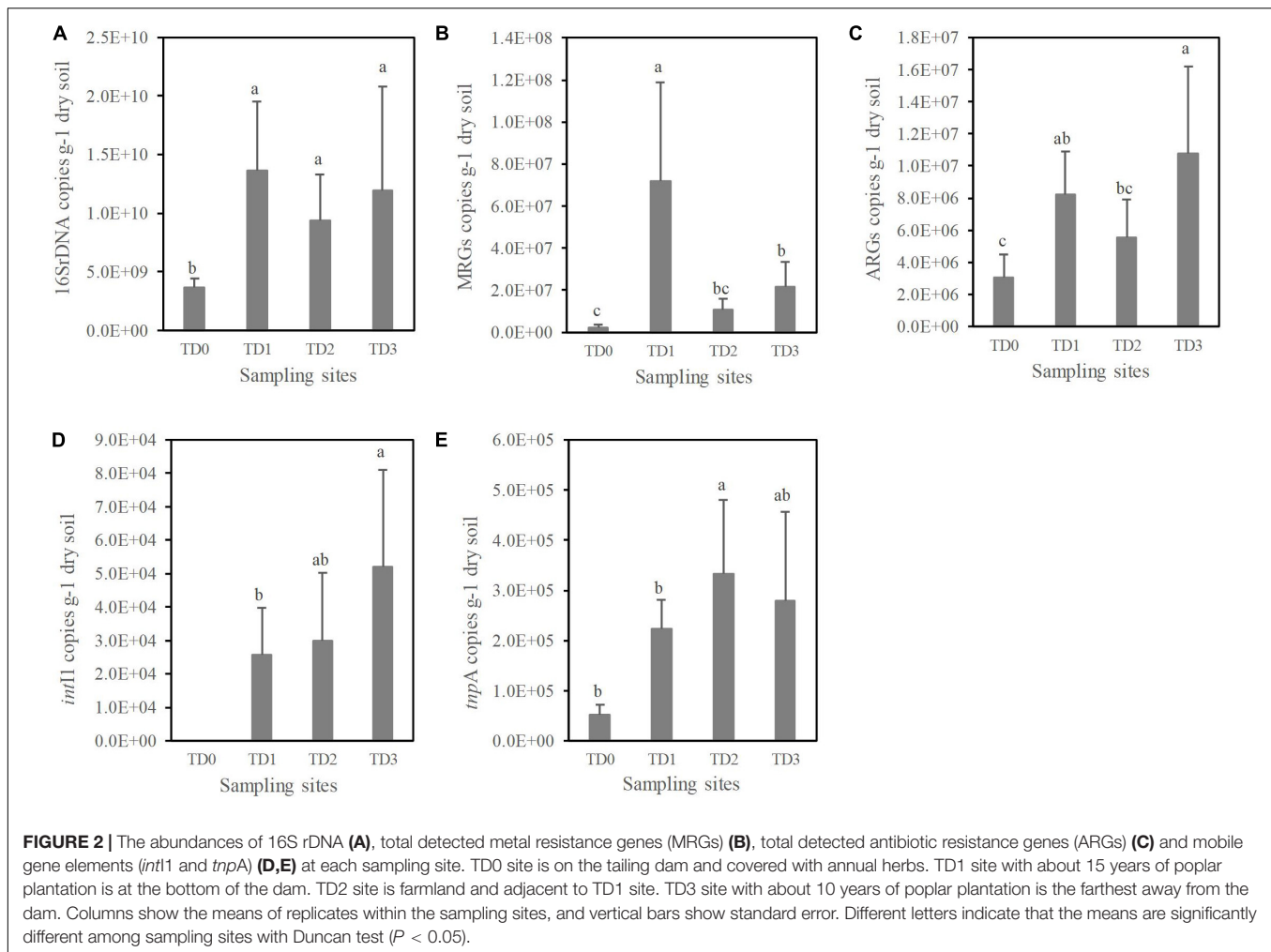
## Correlations Between the *RI*, MRGs, ARGs, and MGEs

The potential *RI* is positively correlated with soil TC, the abundance of MRGs, and 16S rRNA genes ( $P < 0.01$ ), and is negatively correlated with soil pH ( $P < 0.01$ , **Figure 4**). The abundance of MRGs is strongly positively correlated with TC, the abundance of ARGs and 16S rRNA genes ( $P < 0.01$ ), and is negatively correlated with soil pH ( $P < 0.01$ ). The abundance of ARGs is correlated with soil TN, TC, and the abundance of 16S rRNA genes. The abundance of *intI1* gene is positively correlated with the abundance of the *tnpA* gene ( $P < 0.01$ ) and TN ( $P < 0.05$ ), and negatively correlated with soil TS. The abundance of the *tnpA* gene is positively correlated with soil pH ( $P < 0.05$ ) and negatively correlated with TS ( $P < 0.01$ ). The abundance of MGEs has no significant correlation with 16S rRNA genes ( $P > 0.05$ ).

## Co-occurrence Patterns of MRGs and ARGs

Network analysis is conducted to explore the co-occurrence patterns among the relative abundance of MRGs, ARGs, and MGEs based on significant correlation analysis by Spearman correlation ( $P < 0.05$ ). The resultant network consists of 37 nodes (8 *RI*s, 10 MRGs, 17 ARGs, and 2 MGEs) and 144 edges, and can be clearly separated into five modules (**Figure 5**). Genes in the same module may co-occur under the same environmental pressure. The majority of MRGs and ARGs coexist within their internal genes, while the *copA* and *pcoA* may co-exist with many ARGs, and *suII* may co-exist with many MRGs. Genes co-existence with the MGEs indicate the mobility of these genes in the same module. The most densely connected node in each





module is defined as ‘hubs,’ for example, in module III, *bla<sub>ctx-M</sub>* may be the hub. The hubs can act as indicators to represent the quantity of other co-occurring genes in the same module.

## DISCUSSION

### Heavy Metal Contamination and Bacterial Abundance

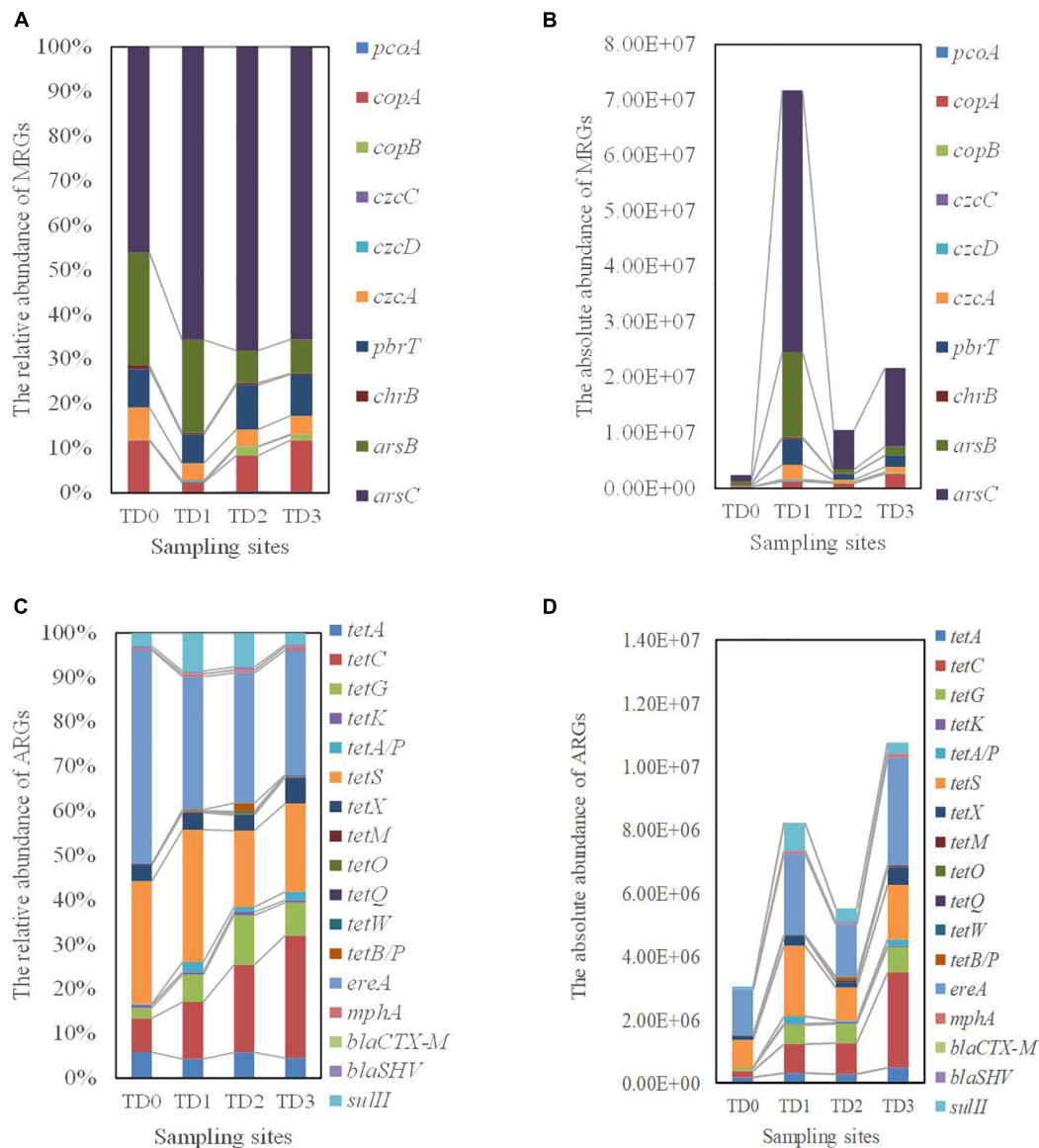
The *RI* results of heavy metals in the study area show that the TD1 site is suffering more ecological risk than the other three sites, and the TD0 site is at the lowest ecological risk level (Table 2). This distribution pattern results mainly from the accumulations of As, Cd, Cr, and Pb at the TD1 site (Table 1). This is probably related with the unique terrain conditions of this sampling site and the migration of heavy metals. There are two main sources of heavy metals for the TD1 site, of which one is the dissolved load with the leachate from the tailing dam (TD0) and the other is the sediment transported with the wash load (Merrington and Alloway, 1994). A similar accumulation pattern was observed in a similar area (Merrington and Alloway, 1994). The migration of metals in a dissoluble state in such an area can be achieved

through the oxidation of metal sulfides, which aggravates the process of leaching when the solubility of heavy metal is increased in the form of metal sulfates (Simón et al., 2001).

The TS concentration is significantly higher at the TD0 site than at the other three sites (Table 1), and the soil at the TD0 site is alkalescence with the pH  $8.00 \pm 0.02$  (Table 1). The high concentration of sulfur and the alkaline conditions indicate that the heavy metals exist in the form of metal sulfides at the TD0 site, which limits the migration of heavy metals to some extent. This interpretation is also supported by the significantly negative correlation between pH and *RI* (Figure 4). Thus, the transport of heavy metals from the TD0 site to the TD1 site is mainly the result of wash load. Evidence also shows that afforestation could effectively inhibit the oxidation of metal sulfides in the mining wasteland and reduce the production of acid wastewater and the release of heavy metals (Néel et al., 2003; Changul et al., 2010; Yang et al., 2010). After the accumulation of wash load, heavy metals can be fixed in the soil of the TD1 site under the influence of vegetation.

Although the highest heavy metal content is observed at the TD1 site, the TD1 site has the highest abundance of bacteria indicated by the abundance of the 16S rRNA genes





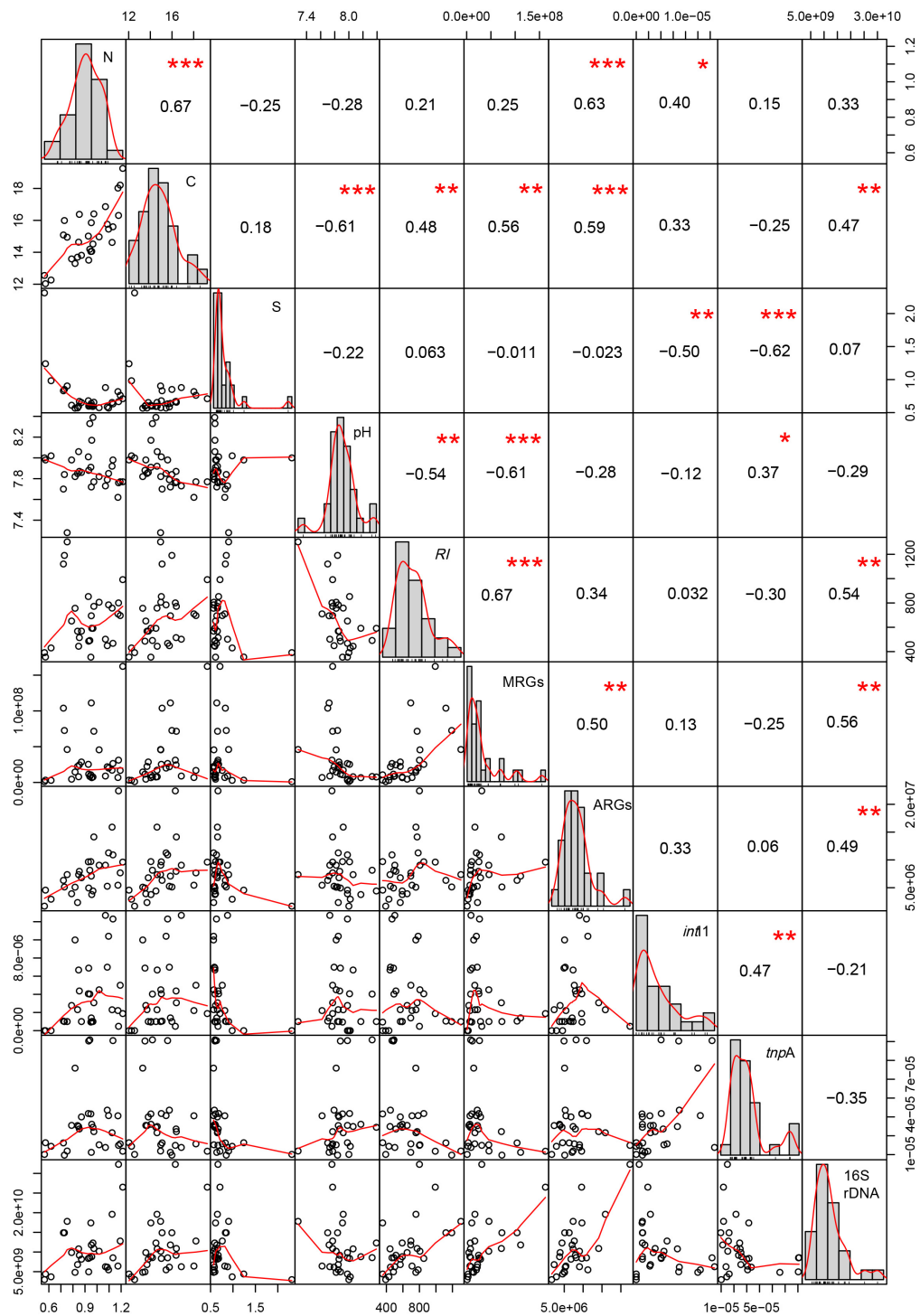
**FIGURE 3 |** The distribution of Metal resistance genes (MRGs) and antibiotic resistance genes (ARGs) detected at each sampling site. TD0 site is on the tailing dam and covered with annual herbs. TD1 site with about 15 years of poplar plantation is at the bottom of the dam. TD2 site is farmland and adjacent to TD1 site. TD3 site with about 10 years of poplar plantation is the farthest away from the dam. **(A)** The distribution of relative abundance of MRGs. **(B)** The distribution of absolute abundance of MRGs. **(C)** The distribution of relative abundance of ARGs. **(D)** The distribution of absolute abundance of ARGs.

(Figure 2A). Heavy metal contamination modifies the microbial diversity and biomass, because bacteria community is sensitive to heavy metals (Giller et al., 1998; Azarbad et al., 2015). Most studies have shown that high contamination of heavy metals can significantly decrease the soil bacterial biomass (Fließbach et al., 1994; Chander et al., 1995; Stefanowicz et al., 2010), but these studies focused on the short-term impacts of toxic pollutants. However, under a long-term polluted circumstance, Bourceret et al. (2015) found high richness and diversity of the bacterial community, and this is because edaphic parameters (nutrients like soil organic matter, soil available phosphorous, etc.) are increased simultaneously over the time of remediation. In this

study, we also found a significant correlation between the soil bacterial abundance and TC (Figure 4).

## Heavy Metal and Metal Resistance

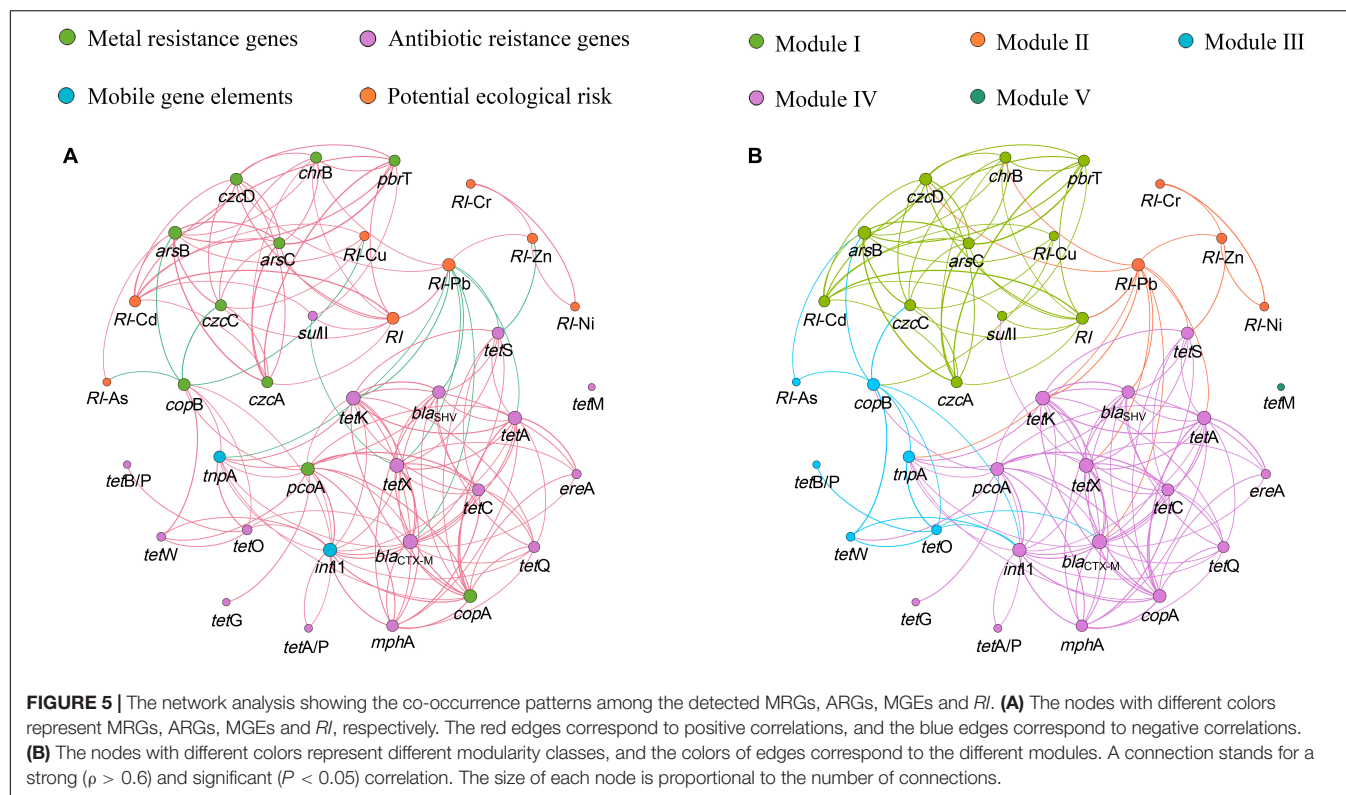
Heavy metals can form complex compounds, of which some are important for micronutrient physiological activities in all living microorganisms (Nies, 1999). However, most complex compounds are toxic at high concentrations (Nies, 1999). It is critical for microbial communities to develop the resistant systems (specific or unspecific) under long-term heavy metal stresses (Nies, 1999; Hemme et al., 2010; Chen et al., 2018). Such systems with the genetic basis of MRGs are inducible



**FIGURE 4 |** Correlations between soil physicochemical characteristics, *RI*, MRGs, ARGs, MGes and 16S rDNA. \*\*\* Means that correlation is significant at the 0.001 level (two-tailed). \*\* Means that correlation is significant at the 0.01 level (two-tailed). \* Means that correlation is significant at the 0.05 level (two-tailed).

under adverse circumstances (Nies and Silver, 1995). The MRGs at the TD1 site are more abundant than at the other three sites (Figure 2B); this difference is also revealed by *RI* at the four sampling sites. This suggests that high

*RI* would induce more bacterial resistance in the long-term contaminated area, which is also supported by the significant correlation between the abundance of MRGs and *RI* ( $P < 0.001$ ) (Figure 4).



Soil copper in the form of divalent copper is dominant in our study site; both *copA* and *pcoA* function by the way of oxidizing  $\text{Cu}^+$  to  $\text{Cu}^{2+}$  for the purpose of detoxifying (Rensing and Grass, 2003). Therefore, both *pcoA* and *copA* genes are not significantly correlated with soil Cu contents (Supplementary Table S4). This relationship was also observed in sediment (Roosa et al., 2014). The *copB* gene in TD2 and TD3 is associated with low Cu concentration (Table 1 and Supplementary Table S2), indicating that *copB* system may be functioned in low Cu circumstance.

Among these MRGs at all the sampling sites, *arsB* and *arsC* genes are the main components and they have the similar distribution pattern with arsenic and *RI* (Tables 1, 2, Figure 3A, and Supplementary Table S2). Arsenic is positively correlated with the abundances of *arsB* and *arsC* genes (Supplementary Table S4). The significantly positive correlation between arsenic and *ars* gene was also found in the batch growth experiments (Poirel et al., 2013). Arsenic can induce the expression of *ars* genes (Tauriainen et al., 1997; Lópezmaury et al., 2003; Ordóñez et al., 2005), and *ars* genes may be suitable for exploring the microbial response to arsenic stress.

There are significant correlations between Cd and its target resistant genes (*czcA*, *czcC*, *czcD*). Meanwhile, Cd is also significantly correlated with *pbrT*, *chrB*, *arsB*, and *arsC*, respectively ( $P < 0.05$ ) (Supplementary Table S4). This can be explained by the fact that different resistance genes may be located on the same unit of selection such as the same plasmid, transposon, or genome (Monchy et al., 2007). Van Houdt et al. (2009) reported that both Pb and Zn resistance increased because the transposon named *Tn6048* proliferated

under the Pb contaminated environment. Cd with a 'toxic-response' factor equal to 30 and Pb with a 'toxic-response' factor equal to 5 (Hakanson, 1980) contribute 32.08 and 55.45% to *RI*, respectively (Table 2). However, the abundance of MRGs is strongly correlated with Cd rather than Pb (Supplementary Table S4), and the network analysis reveals that MRGs and *RI*-Cd, not *RI*-Pb, were found in Module I (Figure 5). This indicates that heavy metal with high 'toxic-response' factor may be an indicator of the MRGs in the heavy metal contaminated area.

## Heavy Metal and the Co-occurrence of MRGs and ARGs

There are 17 ARGs detected in the copper tailing dam area (Supplementary Table S3), and ARGs exist in many metal contaminated circumstances (Hu et al., 2016, 2017). Correlations exist between metals and ARGs, but the trends are diversified (Knapp et al., 2017). In this study, significant correlations are observed between specific ARGs and heavy metals (Figure 5 and Supplementary Table S5). For example, Zn is significantly correlated with *sulIII*. Ji et al. (2012) reported that Zn was positively correlated with *sulI* and *sulIII*, rather than *sulII* in agriculture soil and manure samples. However, *sulI* and *sulIII* are not detected in this study. The different samples may account for the distinct results. Previous studies have reported that Zn directly triggers the selection of tetracycline resistance genes including *tetA*, *tetC*, and *tetG*, genes functioning as an efflux pump system (Yamaguchi et al., 1990; Palm et al., 2008). We find that Zn is significantly correlated with *tetW* and *tetB/P*, genes

encoding ribosomal protection proteins, which are detected in metal polluted areas (Berg et al., 2010; Knapp et al., 2011, 2017). This indicates that Zn may induce both the efflux pump system and ribosomal protection proteins.

The network analysis reveals strong correlation between *sulII* and MRGs in Module I. *pcoA* and most of ARGs are detected in Module III (Figure 5), suggesting the possibility of the co-occurrence of MRGs and ARGs in soil microbe. The co-occurrence of MRGs and ARGs is verified by an IncA/C plasmid carrying *mer* operon (coding for resistance mechanism to mercury) and ARGs including *sulII* harbored in the *Aeromonas salmonicida* sub sp. *salmonicida* strains isolated from aquaculture facilities (McIntosh et al., 2008). *IntI1* and *tnpA* are in the different modules, and show different correlations with each specific MRGs and ARGs (Figure 5). The similar correlations between MGEs and ARGs were observed in greenhouse soils with long-term dairy cattle and chicken manure (Li J. et al., 2017). Previous studies reported that the same ARGs or MRGs could be located in the same or different conjugative MGEs (Gillings et al., 2008; Herrick et al., 2014). Future studies are necessary to carry out detailed investigations of the horizontal transfer mechanism of MRGs and ARGs.

## CONCLUSION

This study selected a long-term heavy metal contaminated area, the Shibahe copper tailing dam area in Mountain Zhongtiaoshan of northern China, analyzed the levels of the contamination of multiple metals, and explored the relationship between soil microbial resistance and multi-metal contamination. Based on *in situ* monitoring, *RI* is adopted to evaluate the level of contamination. The abundance of soil bacteria is high at the high-*RI* site, and this is probably attributed to high soil nutrient and strong bacterial resistance. Both MRGs and ARGs exist in the multi-metal polluted soil. Of the MRGs, *arsC* coding for resistance mechanism to arsenic is at the highest abundance; *ereA*, which belongs to a deactivate system against macrolides, is the most abundant among the ARGs. The abundance of MRGs is positively correlated with Cd concentration, indicating that Cd plays a key role in the selection of MRGs. For ARGs, the abundances of *tetW* and *tetB/P* are significantly correlated with Zn concentration, which indicates that Zn may induce the antibiotic resistance of ribosome protection in such a long-term copper tailing dam area. The network analysis results show that *sulII* and MRGs co-occur and *copB* occur with ARGs, and this suggests that MRGs and ARGs can be co-selected in

the heavy metal contaminated soil. The network analysis also reveals the co-occurrence of Cd and MRGs, and accordingly heavy metal with a high 'toxic-response' factor can be used to indicate the occurrence of MRGs. In all, this study highlights the necessity to consider the contamination of multi heavy metals when assessing microbial resistance, and further improves the understanding of the relationship between bacterial resistance and multi-metal contamination. Meanwhile, our findings are alarming for the future evaluation of public health risk associated with heavy metal-induced selection of ARGs in multi-metal polluted environments.

## AUTHOR CONTRIBUTIONS

JC wrote the main manuscript and prepared the figures. JL helped with the sampling design and prepared the data analytical methods. WS helped in the field work, sampling, and soil parameter achieving. HZ prepared the assessment method. YL was responsible for project administration and funding acquisition. HZ and YL provided many suggestions for the experimental design and implementation. All authors reviewed the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01916/full#supplementary-material>

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# Characterization of a NDM-1-Encoding Plasmid pHFK418-NDM From a Clinical *Proteus mirabilis* Isolate Harboring Two Novel Transposons, Tn6624 and Tn6625

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Acquisition of the *bla*<sub>NDM-1</sub> gene by *Proteus mirabilis* is a concern because it already has intrinsic resistance to polymyxin E and tigecycline antibiotics. Here, we describe a *P. mirabilis* isolate that carries a pPrY2001-like plasmid (pHFK418-NDM) containing a *bla*<sub>NDM-1</sub> gene. The pPrY2001-like plasmid, pHFK418-NDM, was first reported in China. The pHFK418-NDM plasmid was sequenced using a hybrid approach based on Illumina and MinION platforms. The sequence of pHFK418-NDM was compared with those of the six other pPrY2001-like plasmids deposited in GenBank. We found that the multidrug-resistance encoding region of pHFK418-NDM contains  $\Delta$ Tn10 and a novel transposon Tn6625. Tn6625 consists of  $\Delta$ Tn1696, Tn6260, In251,  $\Delta$ Tn125 (carrying *bla*<sub>NDM-1</sub>),  $\Delta$ Tn2670, and a novel *mph*(E)-harboring transposon Tn6624. In251 was first identified in a clinical isolate, suggesting that it has been transferred efficiently from environmental organisms to clinical isolates. Genomic comparisons of all these pPrY2001-like plasmids showed that their relatively conserved backbones could integrate the numerous and various accessory modules carrying multifarious antibiotic resistance genes. Our results provide a greater depth of insight into the horizontal transfer of resistance genes and add interpretive value to the genomic diversity and evolution of pPrY2001-like plasmids.

**Keywords:** *Proteus mirabilis*, *bla*<sub>NDM-1</sub>, transposons, plasmids, multidrug-resistant

## INTRODUCTION

Urinary tract infections (UTIs) are the most common bacterial infections (Gastmeier et al., 1998). Cases of UTIs can be classified as uncomplicated or complicated (Beahm et al., 2017). Clinically, *Proteus mirabilis* is most frequently a pathogen of UTIs, particularly in patients suffering from complicated cUTIs (Schaffer and Pearson, 2015). Although *Escherichia coli* is the primary urinary tract pathogen, *P. mirabilis* ranks third as the cause of UTIs and accounts for 4.1% of urinary tract infection isolates in CANWARD surveillance study, 4.6% in southern China, respectively (Karlowsky et al., 2011; Li et al., 2017). Because this pathogen is intrinsically resistant

to nitrofurantoin, polymyxin, and tigecycline antibiotics (Ramos et al., 2018), acquiring additional carbapenemase antibiotic resistance is worrisome (Reffert and Smith, 2014). Currently, fosfomycin, which is previously used mainly as oral treatment for UTIs, has gained clinicians' attention worldwide because of its activity against multidrug-resistant bacteria (Reffert and Smith, 2014; Giske, 2015). In addition, fosfomycin resistance rates are generally low but substantially higher when carbapenemase producers are considered (Giske, 2015). One such resistance gene is *bla*<sub>NDM-1</sub> (New Delhi metallo- $\beta$ -lactamase), which was initially identified in a *Klebsiella pneumoniae* strain (Yong et al., 2009). Isolates of this species that harbor the *bla*<sub>NDM-1</sub> gene can hydrolyze nearly all  $\beta$ -lactam antibiotics except aztreonam. Therefore, the acquisition of *bla*<sub>NDM-1</sub> by *P. mirabilis* would be problematic, as it would greatly reduce the therapeutic options for treating infections caused by it.

The *bla*<sub>NDM-1</sub> gene is mainly and widely spread by an IS*Aba125*-bounded composite transposon Tn125 (Poirel et al., 2012; Ranjan et al., 2016), and *bla*<sub>NDM-1</sub>-carrying plasmids are commonly found in IncA (Solgi et al., 2017), IncC (Harmer and Hall, 2017), IncT (Mataseje et al., 2016), IncR (Gamal et al., 2016), IncFII (Lin et al., 2016), IncX (Wang et al., 2018), and IncN (Wang et al., 2018) incompatible groups. However, *bla*<sub>NDM-1</sub>-carrying plasmids have gradually appeared in some unknown incompatibility groups. The *bla*<sub>NDM-1</sub>-harboring pHFK418-NDM plasmid and six other plasmids have been assigned into the same unknown incompatibility group based on their replicons. The six plasmids are pPrY2001 (Accession no. KF295828) (Mataseje et al., 2014), p06-1619-1 (Accession no. KX832929) (Marquez-Ortiz et al., 2017), p16Pre36-NDM (Accession no. KX832927) (Marquez-Ortiz et al., 2017), pPp47 (Accession no. MG516912) (Dolejska et al., 2018), pPm60 (Accession no. MG516911) (Dolejska et al., 2018), and pC131 (Accession no. KX774387). The earliest reported plasmid, pPrY2001, is considered to be the reference plasmid, so the above-named plasmids are called pPrY2001-like plasmids (Marquez-Ortiz et al., 2017; Dolejska et al., 2018). Up to now, no studies in the published scientific literature have thoroughly analyzed and compared in detail the structures and genomes of this unknown incompatibility group.

Here, we studied the *bla*<sub>NDM-1</sub>-harboring plasmid, pHFK418-NDM, a known pPrY2001-like plasmid according to its replicon, which was first isolated from a clinical *P. mirabilis* HFK418 strain in China. We elucidated the complete sequence of pHFK418-NDM (which carries two novel transposons, Tn6624 and Tn6625) and compared it with six other pPrY2001-like plasmids to obtain insight into the horizontal transfer of resistance genes and the diversity and evolution of pPrY2001-like plasmids.

## MATERIALS AND METHODS

### Species Identification and Antimicrobial Susceptibility Testing

The study was approved by the Medical Ethics Committee at the Affiliated Hospital of Qingdao University, China, and written informed consent was received from the patient. The *P. mirabilis*

HFK418 strain was isolated from the urine specimen of a patient with epidemic encephalitis at the Affiliated Hospital of Qingdao University, China, in 2017. Referring to the method described in Ranjan et al. (2016), this strain was multiple tested for purity by routine laboratory methods, then the pure strain was cryopreserved at  $-80^{\circ}\text{C}$  in 50% glycerol. The pure isolate was revived in Luria-Bertani (LB) broth (BD Biosciences, United States) with 4  $\mu\text{g/ml}$  meropenem to experiments. The *P. mirabilis* HFK418 isolate was identified and subjected to antimicrobial susceptibility testing using the VITEK compact-2 automated system (bioMérieux, France). In addition, fosfomycin MICs were further determined by fosfomycin *E*-tests (bioMérieux). CLSI (Clinical and Laboratory Standards Institute) 2018 breakpoints were used (M100-S28) (CLSI, 2018).

### Antimicrobial Resistance Gene Screening and Plasmid Conjugal Transfer

The major acquired extended-spectrum  $\beta$ -lactamase (Dallenne et al., 2010; Hussain et al., 2014; Ranjan et al., 2017), fosfomycin (Dantas Palmeira et al., 2018), chloramphenicol (White et al., 1999), lincosamide (Garcia-Martin et al., 2018), and carbapenemase genes (Chen et al., 2015; Ranjan et al., 2016, 2017) were detected by PCR, after which all the PCR amplicons were sequenced on the ABI 3730 platform (Applied Biosystems, United States). The sodium azide-resistant *E. coli* J53Azi<sup>R</sup> strain was used as the recipient and the *P. mirabilis* HFK418 isolate as the donor for the conjugative transfer of the plasmids. The conjugal transfer tests were performed as described previously (Srijan et al., 2018), and the conjugation frequency was calculated as transconjugants divided by number of donors.

### Carbapenemase Activity Assay

To determine whether the *bla*<sub>NDM-1</sub> gene was expressed in both *P. mirabilis* HFK418 and the *E. coli* J53Azi<sup>R</sup> transconjugant HFK418-NDM-J53 strain, we performed an imipenem-EDTA *E*-test (AB-BioMérieux, Sweden) to assess the class B carbapenemase activity.

### Sequencing and Sequence Assembly

Bacterial genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, United States), followed by the MiSeq (Illumina, United States) and MinION (Oxford Nanopore, United Kingdom) sequencing. The short Illumina reads were trimmed to remove the poor quality sequences, and the resultant contigs were assembled using Newbler3.0 (Nederbragt, 2014). The longest single read obtained by the MinION sequencer was 98 kb, thereby crossing the repetitive shuffle regions in the plasmid (Laver et al., 2015). The long reads from the MinION combined with the short Illumina reads were hybrid assembled using SPAdesv3.11.1 (Bankevich et al., 2012). Hybrid assembly produced several scaffolds and BLASTN analysis confirmed that the scaffold in our study has the highest similarity to the plasmid p16Pre36-NDM (Accession no. KX832927) with coverage of 69% and identity of 96%. As most of the published plasmids are in a circle form, further bioinformatics analysis confirmed that



this scaffold can be successfully cyclized using our in-house script. The correctness was then proved by mapping the high-throughput sequencing reads to the cyclized scaffold using CLC Genomics Workbench 9.0, with a mean reads mapping coverage of 111x. The consensus sequence acquired from CLC Genomics Workbench 9.0 was finally treated as the complete sequence of our plasmid pHFK418-NDM.

## Sequence Annotation and Genome Comparisons

Open reading frames (ORFs) and pseudogenes that were predicted by RAST2.0 (Brettin et al., 2015) were further annotated using BLASTP/BLASTN (Boratyn et al., 2013) against the RefSeq databases (O'Leary et al., 2016) and UniProtKB/Swiss-Prot (Boutet et al., 2016). Mobile elements, resistance genes, and other features were annotated by INTEGRALL (Moura et al., 2009), ISfinder (Siguiet et al., 2006), ResFinder (Kleinheinz et al., 2014), PlasmidFinder (Carattoli et al., 2014), and the Tn Number Registry (Roberts et al., 2008) online databases. Comparisons of the multiple and paired sequences were conducted using MUSCLE 3.8.31 and BLASTN, respectively. Gene organization diagrams were drawn in Inkscape0.48.1<sup>1</sup>.

## Nucleotide Sequence Accession Number

The complete nucleotide sequence of plasmid pHFK418-NDM has been deposited in the National Center for Biotechnology Information nucleotide database<sup>2</sup> under accession number MH491967.

## RESULTS AND DISCUSSION

### Characterization of *P. mirabilis* HFK418

Plasmid pHFK418-NDM from *P. mirabilis* HFK418 was transferable to *E. coli* J53Azi<sup>R</sup> in the conjugation experiments, thereby generating the *bla*<sub>NDM-1</sub>-positive *E. coli* J53Azi<sup>R</sup> transconjugant HFK418-NDM-J53 strain. The conjugation frequency was  $1.5 \times 10^{-2}$ .

Imipenem-EDTA E-tests were positive in both *P. mirabilis* HFK418 and HFK418-NDM-J53. These two strains were highly resistant to ampicillin, cefazolin, cefuroxime, ceftazidime, ceftriaxone, imipenem, and meropenem, but not to aztreonam, revealing that pHFK418-NDM is a conjugative NDM-encoding plasmid with carbapenemase activity (Table 1 and Supplementary Figure S1).

### Overview of Plasmid pHFK418-NDM

PCR screening for antimicrobial resistance genes showed that *P. mirabilis* HFK418 carries *bla*<sub>NDM-1</sub>, *bla*<sub>OXA-1</sub>, *bla*<sub>CTX-M-65</sub>, *fosA3*, *catB5*, *lnu*(G), and *bla*<sub>OXA-10</sub> genes. The complete sequence of pHFK418-NDM is 145,619 bp with a mean G + C content of 42.8%, and 157 ORFs (Table 2 and Supplementary Figure S2). Based on the replicon, pHFK418-NDM was

**TABLE 1 |** Antimicrobial susceptibility profiles.

Antibiotics	MIC (mg/L)/antimicrobial susceptibility*		
	HFK418	HFK418-NDM-J53	J53
Ampicillin	≥32/R	≥32/R	=8/S
Cefazolin	≥64/R	=32/R	≥4/S
Cefuroxime	≥64/R	≥64/R	=4/S
Ceftazidime	≥64/R	≥64/R	≤1/S
Ceftriaxone	≥64/R	≥64/R	≤1/S
Imipenem	≥16/R	≥16/R	≤1/S
Meropenem	≥16/R	≥16/R	≤0.25/S
Aztreonam	≤1/S	≤1/S	≤1/S
Gentamicin	≥16/R	=2/S	≤1/S
Ciprofloxacin	≥4/R	≤0.25/S	≤0.25/S
Levofloxacin	≥8/R	≤0.25/S	≤0.25/S
Fosfomycin	≥1024/R	=4/S	=2/S
Nitrofurantoin	≥512/R	=64/I	≤16/S
Trimethoprim/sulfamethoxazole	≥320/R	=40/S	≤20/S

\*The interpretation is derived from the Clinical and Laboratory Standards Institute Guidelines (CLSI, 2018) (S, sensitive; R, resistant; I, intermediately resistant).

assigned into the unknown incompatibility group of pPrY2001-like plasmids. The linear genomic comparison conducted between pHFK418-NDM and six other pPrY2001-like plasmids [pPrY2001 (Mataseje et al., 2014), p06-1619-1 (Marquez-Ortiz et al., 2017), pC131, pPp47 (Dolejska et al., 2018), pPm60 (Dolejska et al., 2018), and p16Pre36-NDM (Marquez-Ortiz et al., 2017)] showed that the highest sequence homology belonged to pHFK418-NDM with >69% query coverage and >99% identity (Supplementary Figure S3 and Supplementary Data Sheet S2).

The genomic structures of the pPrY2001-like plasmids comprised two major regions: the backbone and accessory module. The backbone could be further divided into three parts: the replication genes (*repA* and its iterons), the conjugal transfer genes (*tiv*, *rlx*, and *cpl*), and the plasmid maintenance genes (*parFG*, *MazFE*, *stbB*, *ssb*, and *flhC*). Each plasmid's backbone was able to integrate two or more accessory modules by transposition or recombination events. pHFK418-NDM contains two accessory modules, the Tn6901 related region and the multidrug-resistant (MDR) region, while the MDR region contains Tn6625 and ΔTn10 (Supplementary Figures S2, S3).

### Backbone Regions in the pPrY2001-Like Plasmids

Our pairwise comparison analysis of the pPrY2001-like plasmids backbones showed that they shared >96% nucleotide identity across >42%, indicating that their backbones were relatively conserved. However, there were three major differences among all their backbones. (I) the *parC* gene (centromere, binding sites for *parG*) did not exist in pPrY2001, and the copy numbers of the 8-bp tandem repeat (TGTGTata) within the *parC* gene varied among the other plasmids (4 for p06-1619-1, pC131, and pPm60; 5 for pPp47, pHFK418-NDM, and p16Pre36-NDM). (II) Compared with the conjugal transfer region in the other plasmids, the *rlx* gene from pPrY2001 is disrupted into Δ*rlx*-3'

<sup>1</sup><https://inkscape.org/en/>

<sup>2</sup><https://www.ncbi.nlm.nih.gov/WebSub/?form=history&session=new&tool=genbank>

TABLE 2 | Major features of pPrY2001-like plasmids in this work.

Category	pPrY2001-like plasmids						
	pPrY2001	p06-1619-1	pC131	pHFK418-NDM	pPp47	pPm60	p16Pre36-NDM
Accession number	KF295828	KX83299	KX77437	This study	MG516912	MG516911	KX83297
Strain	<i>P. rettgeri</i>	<i>P. rettgeri</i>	<i>P. rettgeri</i>	<i>P. mirabilis</i>	<i>P. mirabilis</i>	<i>P. mirabilis</i>	<i>P. rettgeri</i>
Source	Clinical	Clinical	Clinical	Clinical	Wildlife	Wildlife	Clinical
Country	Canada	American	Brazil	China	Australia	Australia	American
Total length(bp)	113, 295	90, 666	118, 501	145, 619	142, 085	113, 297	244, 116
Total number of ORFs	123	97	125	157	161	127	270
Mean G + C content,%	41.3	37.5	40.8	42.8	42.7	40.9	47.9
Length of the backbone (bp)	74, 670	72, 067	77, 414	69, 823	69, 543	68, 879	150, 505

and  $\Delta rlx-5'$  by insertion of ISPrre5 (named in this study). (III) The hybrid backbone of plasmid p16Pre36-NDM was acquired from a pPrY2001-like plasmid and the IncC2 plasmid (the *orf1847* and *rhs2* marked genes) (Supplementary Figure S3).

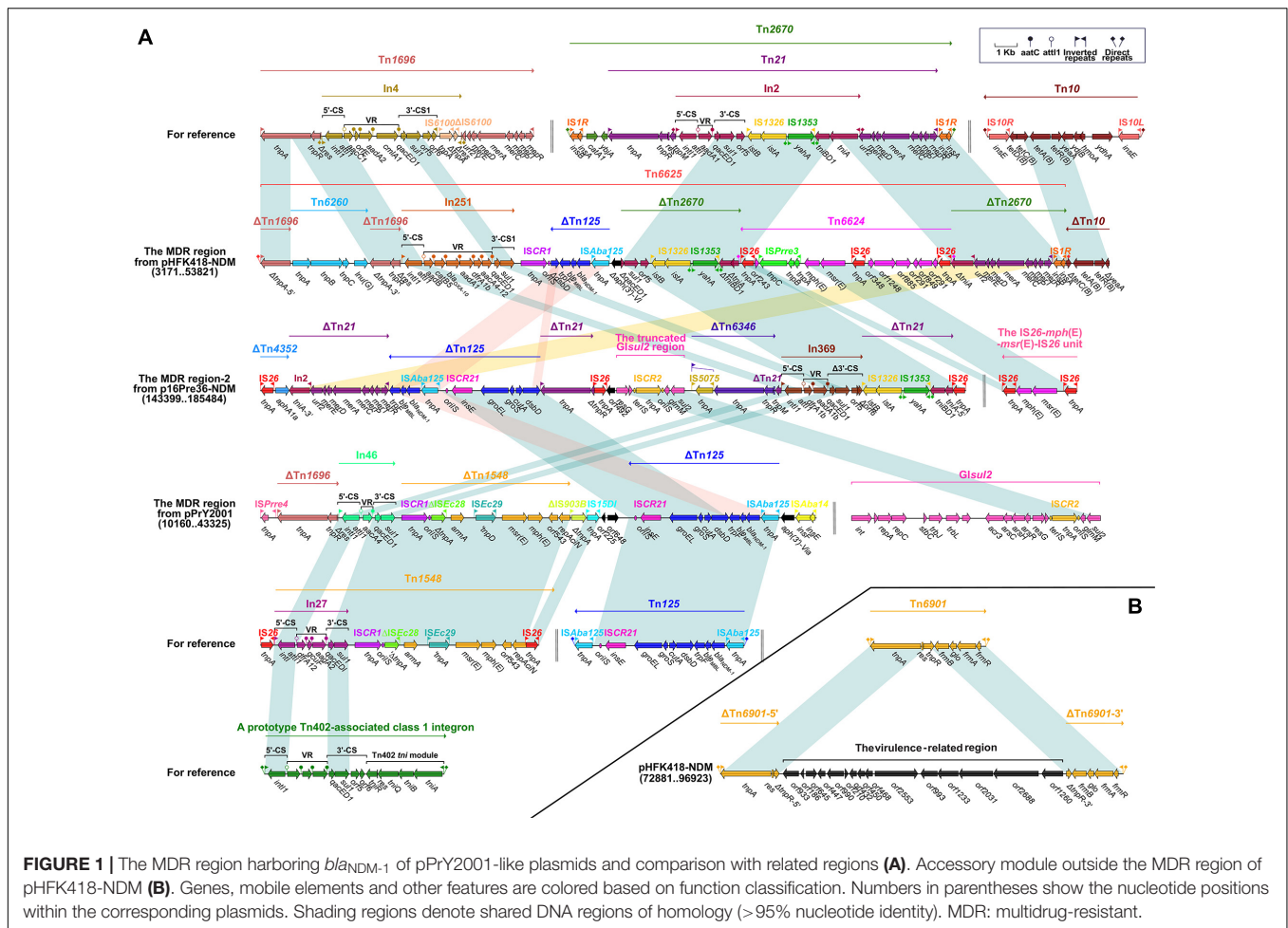
### The MDR Region Harbors the *bla*<sub>NDM-1</sub> Gene From pPrY2001-Like Plasmids

We found that the *bla*<sub>NDM-1</sub>-carrying  $\Delta$ Tn125 transposon is present in the MDR region of pHFK418-NDM, p16Pre36-NDM (the MDR region-2), and pPrY2001. Tn125, an IS*Aba*125-bounded composite transposon in plasmid pNDM-BJ01, was acquired from *Acinetobacter lwoffii* (Poirel et al., 2012). It is made up of IS*Aba*125, *bla*<sub>NDM-1</sub>, *ble*<sub>MBL</sub> (bleomycin resistance), *trpF*, *dsbD*, *cutA*, *groES*, *groEL* and ISCR21, and is bordered by 3-bp direct repeats (DRs: target site duplication signals for transposition). In the MDR region of these three plasmids,  $\Delta$ Tn125 has undergone the deletion of IS*Aba*125 downstream of ISCR27. In addition,  $\Delta$ Tn125 from pHFK418-NDM and p16Pre36-NDM contain the following differences:  $\Delta$ Tn125 in pHFK418-NDM has a  $\Delta$ *dsbD-trpF-ble*<sub>MBL</sub>-*bla*<sub>NDM-1</sub>-IS*Aba*125 structure, while the ISCR21-*groEL-groES-cutA-dsbD* fragment, which occurs upstream of *bla*<sub>NDM-1</sub> in p16Pre36-NDM, was generated by complex recombination events (Figure 1A).

Integron In251, which is located upstream of  $\Delta$ Tn125 in pHFK418-NDM, belongs to the prototypic Tn402-associated class 1 integron. This class 1 integron can be divided sequentially into an IRI (inverted repeat at the integrase end), a 5'-conserved segment (5'-CS: *intI1-attI1*), a variable region (VR: containing one or more resistant genes), a 3'-conserved segment (3'-CS: *qacED1-sul1-orf5-orf6*), the Tn402 *tni* module (*tniA-tniB-tniQ-res-tniR*) and IRT (inverted repeat at the *tni* end), and is surrounded by 5-bp DRs. Furthermore, In369 (in MDR region-2 from p16Pre36-NDM), In46 (in the MDR region from pPrY2001), In809 (in the MDR region-1 from pPm60), and In1129 (in the MDR region-1 from p16Pre36-NDM) are also different derivatives from the prototypical Tn402-associated class 1 integron. The structures of In251, In369, and In46 are arranged as IRI, 5'-CS, VR (*aadB-catB5-bla*<sub>OXA-10</sub>-*aadA1-dfrA1-aacA4-12* in In251, *dfrA1b-aadA1b* in In369, and *aacA4* in In46), and  $\Delta$ 3'-CS (*qacED1-sul1* in In251 and In46, *qacED1-sul1-orf5- $\Delta$ orf6* in In369), without the Tn402 *tni* module and IRT. The Tn402 *tni* module and IRT have been replaced downstream by

other mobile elements. In809 and In1129 each have the following common structure: IRI, 5'-CS, VR, 3'-CS, and IRT, and their Tn402 *tni* module has been lost during the evolutionary process. A difference between In809 and In1129 is apparent in the variable region (*dfrA1-aadA27c* in In1129, *bla*<sub>IMP-4</sub>-*qacG2-aacA4-catB3* in In809).  $\Delta$ Tn1696 is embedded upstream of the class 1 integrons In251, In46, In809, and In1460 (in the MDR region-1 from pPp47). The Tn1696 prototype comprises an IRL (inverted repeat left)-*tnpA* (transposase)-*tnpR* (resolvase)-*res* (resolution site)-*mer* (mercury resistance)-IRR (inverted repeat right) structure, and a *res* site is interrupted by insertion of In4 into 75-bp  $\Delta$ *res-5'* and 45-bp  $\Delta$ *res-3'*. Compared with the structure of Tn1696,  $\Delta$ Tn1696 has the same IRL-*tnpA-tnpR- $\Delta$ res-5'* module in the MDR region of pHFK418-NDM, pPrY2001, pPm60, and pPp47. The  $\Delta$ Tn1696 *tnpA* from pHFK418-NDM and pPm60 is segmented into two fragments,  $\Delta$ *tnpA-5'* and  $\Delta$ *tnpA-3'*, by insertion of Tn6260. Belonging to the Tn554 family, Tn6260 consists of *tnpA*, *tnpB*, *tnpC*, and *lnu(G)* (lincosamide resistance), as identified in *Enterococcus thailandicus* a523 (Ybazeta et al., 2017), *Virgibacillus halodenitrificans* PDB-F2 (Tao et al., 2016), and *E. faecalis* E531 (Zhu et al., 2017). Up until now, Tn6260 only appeared in pPrY2001-like plasmids when pHFK418-NDM and pPm60 were present. Moreover, IS*Pmi3* split *tnpB* of Tn6260 from pPm60 into two parts,  $\Delta$ *tnpB-5'* and  $\Delta$ *tnpB-3'*, which are surrounded by 8-bp DRs (Figures 1A, 2).

$\Delta$ Tn2670 from pHFK418-NDM is integrated downstream of  $\Delta$ Tn125. Flanked by 9-bp DRs, Tn2670 is organized as IS*I*R, *catA1* (chloramphenicol resistance), *ybjA* (acetyl transferase), Tn21, and IS*I*R, and was initially discovered in plasmid R100 from *Shigella flexneri* (Partridge and Hall, 2004). Tn21, a Tn3-family transposon unit, contains an IRL-*tnpA-tnpR-res-tnpM* (modulator protein)-In2-*urf2*-the *mer operon*-IRR module, and a presumed ancestral *urf2M* gene is interrupted by insertion of In2 to generate *tnpM* and *urf2* (Liebert et al., 1999). In2 comprises IRI, 5'-CS, VR (*aadA1*), 3'-CS, IS1326, IS1353, the *tni* module, and IRT, and is delimited by 5-bp DRs. In terms of the structure of Tn2670,  $\Delta$ Tn21 can be divided into four segments in the MDR region from p16Pre36-NDM; namely, (I) IRL, *tnpA*, and  $\Delta$ *tnpR*, (II) *tnpM*, (III), In2 (IS1326, IS1353, the disrupted *tni* module), and (IV), In2 (the disrupted *tni* module and IRT), *urf2*, the *mer operon*, and IRR. These four segments fall within different positions by virtue of transposition or recombination events. In pHFK418-NDM,  $\Delta$ Tn2670 reserves a fragment from



the 3'-CS of In2 to IS1R, but its *tniA* gene is segmented into two fragments ( $\Delta tniA_{In2-5'}$  and  $\Delta tniA_{In2-3'}$ ) by insertion of Tn6624 (Figure 1A).

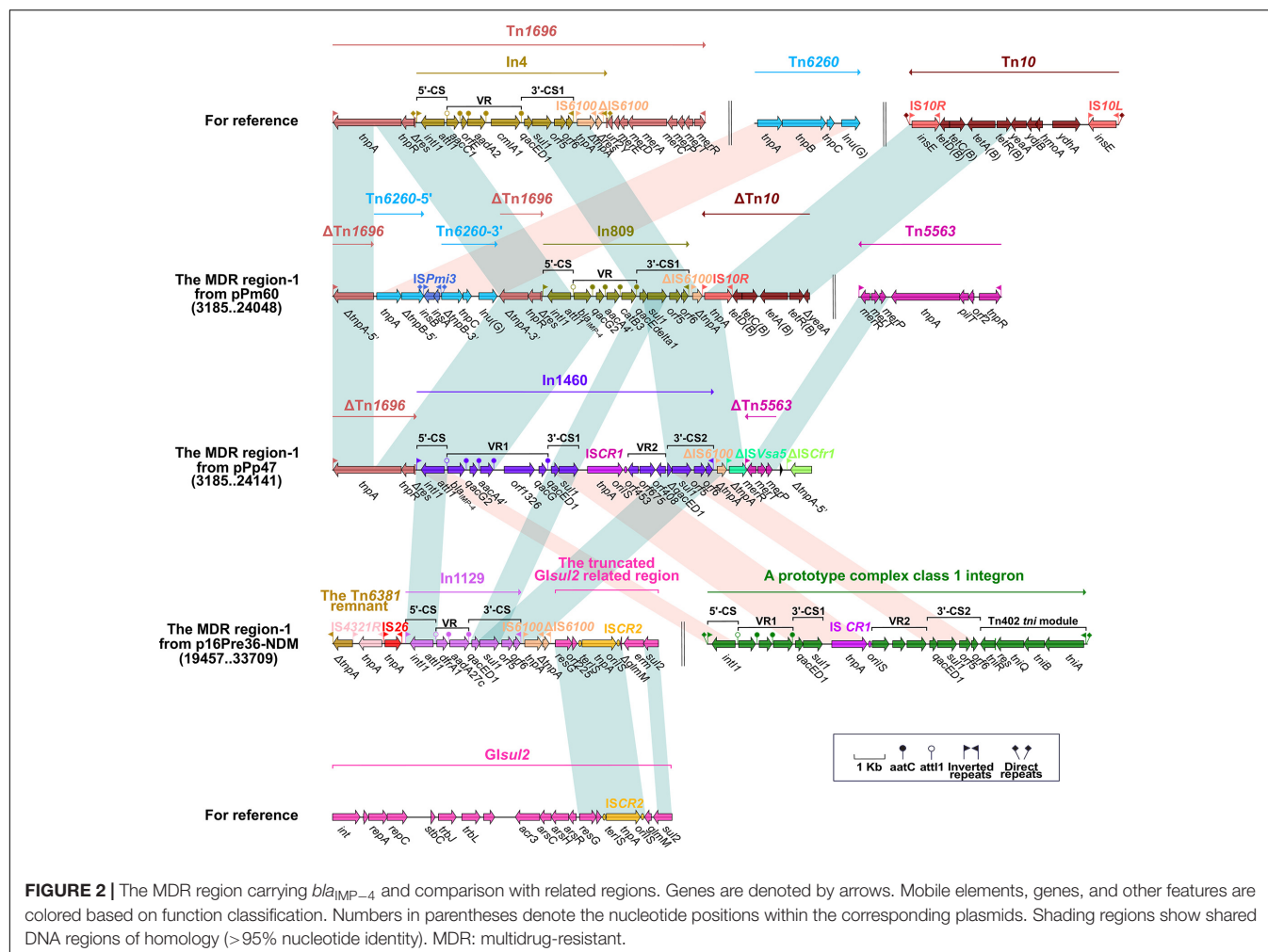
Tn6624, a novel IS26-based transposon unit, has been inserted into the pHFK418-NDM plasmid from *P. mirabilis* HFK418. Delimited by 8-bp DRs (CATCGGCG), it has the following mosaic structure: IS26, a novel IS66-family ISPrre3, *mph*(E) (macrolide resistance), *msr*(E) (macrolide efflux protein), IS26, a fragment with an unknown function, and IS26. The *mph*(E)-*msr*(E)-IS26 fragment originated from the IS26-*mph*(E)-*msr*(E)-IS26 transposon unit and was initially identified in the chromosomal integrative conjugative element from *Pasteurella multocida* (Michael et al., 2012). Three copies of IS26 are present in Tn6624, which promotes the formation and transposition of Tn6624. Another novel 48,068 bp multidrug resistance transposon, Tn6625, was found in the pHFK418-NDM plasmid from *P. mirabilis* HFK418. The  $\Delta$ Tn1696, Tn6260, In251,  $\Delta$ Tn125, Tn6624, and  $\Delta$ Tn2670 mobile elements have been described in detail above, and all of them are included in the large composite Tn6625 transposon. Tn6625 carries twelve resistance genes, bounded by 3-bp DRs (TTG). Tn6625 contains integron In25, which has so far only been found in wastewater-isolated *Providencia* VIGAT3

(Guo et al., 2011). Thus, In251 was first isolated from clinical *P. mirabilis* HFK418, suggesting that it has been efficiently transferred from environmental micro-organisms to clinical isolates (Figure 1A).

The MDR region of pHFK418-NDM includes Tn6625 and  $\Delta$ Tn10. Delimited by 9-bp DRs, Tn10 is arranged sequentially as IS10L, *ydH*A, *hmo*A, *ydj*B, *yea*A, *tet*R, *tet*A (tetracycline resistance), *tet*C, *tet*D, and IS10R, as identified in the conjugative R27 plasmid from *Salmonella typhi* (Lawley et al., 2000).  $\Delta$ Tn10 was found in the MDR region of pHFK418-NDM, pPp47, and pPm60, and comprises a common fragment (*tet*D-*tet*C-*tet*A-*tet*R- $\Delta$ *yea*A). But IS10R is absent in pHFK418-NDM, truncated in pPp47, and intact in pPm60. Tn10 is also integrated between *orf*153 and *orf*489 in the backbone of p16Pre36-NDM, bracketed by 9-bp DRs. Tn10 is an integral transposon in p16Pre36-NDM, but its IS10R has two segments ( $\Delta$ IS10R-5' and  $\Delta$ IS10R-3') and is disrupted by insertion of ISKpn26 with 4-bp DRs (Figures 1A, 2, 3).

There are other transposon units also ( $\Delta$ Tn6346, the truncated *Glsul*2 region, and  $\Delta$ Tn1548) in the MDR region of p16Pre36-NDM and pPrY2001, except as described above.  $\Delta$ Tn6346 and the truncated *Glsul*2 region are embedded in the MDR region of p16Pre36-NDM. Tn6346,





a Tn3-family transposon, was discovered in heavy metal-tolerant *Achromobacter* AO22 (Ng et al., 2009). In the MDR region-2 of p16Pre36-NDM,  $\Delta$ Tn6346 is arranged in turn as the IS5075 interrupted-IRL, *tnpA*, *tnpR*, and 121-bp  $\Delta$ res, and the lost *mer* operon and IRR were replaced by *tnpM* from  $\Delta$ Tn21. *Glsul2* is arranged sequentially as *int* (integrase), several conjugation transfer genes, *resG* (resolvase), *ISCR2*, *glmM* (phosphoglucosamine mutase) and *sul2*, which are found in various bacterial species (Nigro and Hall, 2011). In the MDR region-2 from p16Pre36-NDM,  $\Delta$ Glsul2 comprises *resG*, *orf225*, *ISCR2*, *glmM*, and *sul2*. In the MDR region-1 of p16Pre36-NDM, the truncated Glsul2 related region has a *resG*-*orf225*-*ISCR2*- $\Delta$ *glmM*-*erm* (rRNA adenine N-6-methyltransferase)-*sul2* structure. The *erm* resistance gene is present 100-bp downstream of *ISCR2*. The truncation of *glmM* and the appearance of *erm* are correlated with *ISCR2*-mediated transposition.  $\Delta$ Tn1548 is present in the MDR region of pPrY2001, and Tn1548 was initially discovered in plasmid pCTX-M3 from *Citrobacter freundii* (Dolejska et al., 2013). Compared with the structure of Tn1548,  $\Delta$ Tn1548 comprises *ISCR1*,  $\Delta$ ISEc28, *armA* (aminoglycoside resistance), *ISEc29*, *msr*(E), *mph*(E), *orf543*, and  $\Delta$ repAci (Figure 1A).

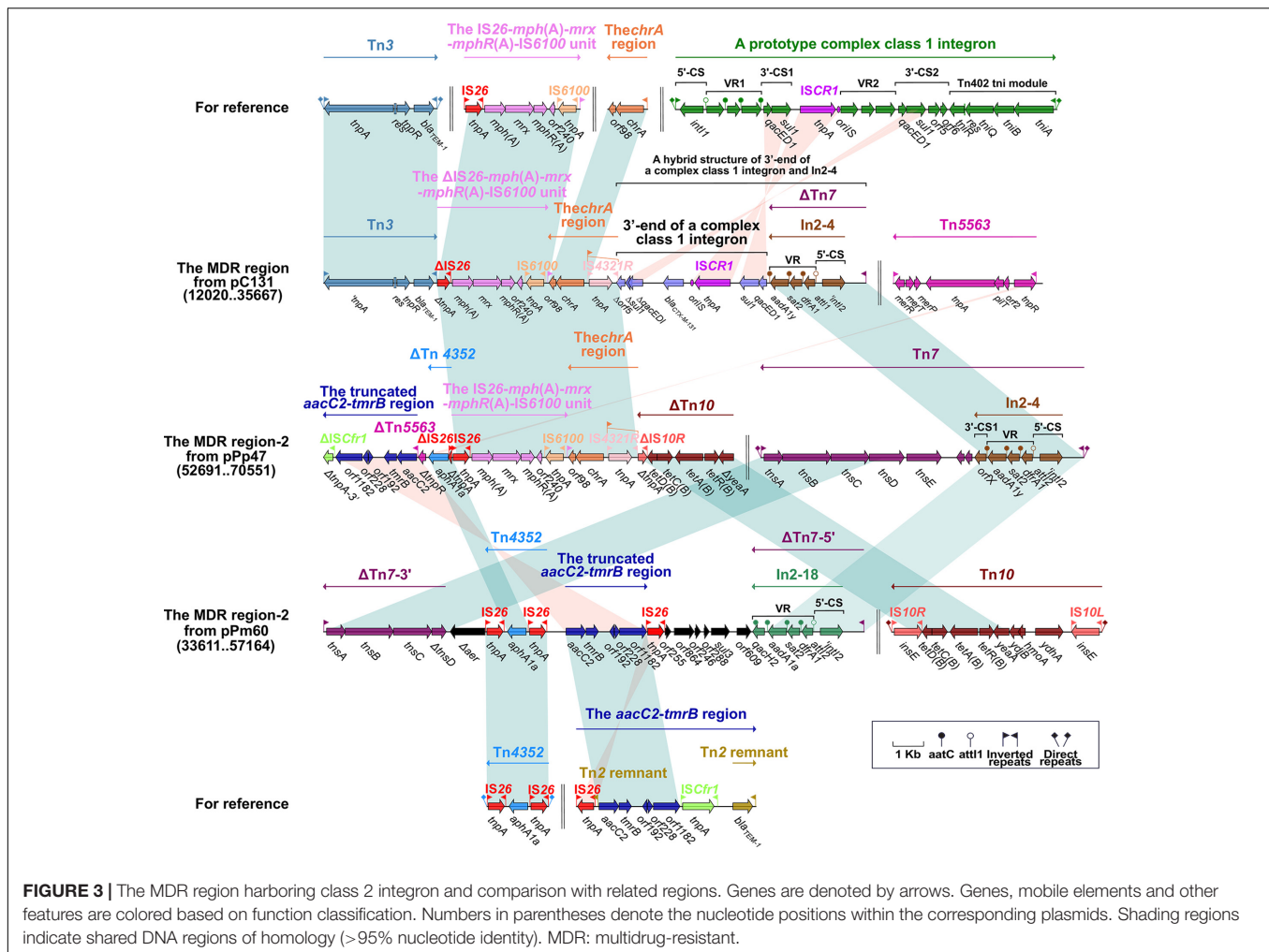
## The MDR Region Harbors the *bla*<sub>IMP-4</sub> Gene From pPrY2001-Like Plasmids

The *bla*<sub>IMP-4</sub> gene is integrated into the integron (In809 and In1460) of the MDR region-1 from pPm60 and pPp47. In809 is a prototype Tn402-associated class 1 integron, and its VR region includes *bla*<sub>IMP-4</sub>, *qacG2*, *aacA4'*, and *catB3*. In1460 is a complex class 1 integron made up of IRI, 5'-CS, VR1 (variable region 1), 3'-CS1 (*qacED1-sul1*), *ISCR1* (common region), VR2 (variable region 2), 3'-CS2 (*qacED1-sul1-orf5-orf6*), the Tn402 *tni* module and IRT, bounded by 5-bp DRs. In1460 comprises IRI, 5'-CS, VR1 (*bla*<sub>IMP-4</sub>-*qacG2*-*aacA4'*-*orf1326-qacG*), 3'-CS1 (*qacED1-sul1*), *ISCR1*, VR2 ( $\Delta$ *orf453-orf675-orf408*), 3'-CS2 ( $\Delta$ *qacED1-sul1-orf5-orf6*), and IRT. The insertion of VR2 between *ISCR1* and 3'-CS2 truncates *qacED1* at the 5' terminal of the 3'-CS2 (Figure 2).

## The MDR Region Harbors a Class 2 Integron From pPrY2001-Like Plasmids

In2-4 (in the MDR region from pC131) and In2-18 (in the MDR region-2 from pPm60) can be classified as class 2 integrons, embedded in  $\Delta$ Tn7. The class 2 integron is found





in transposon Tn7 and its derivatives (Hansson et al., 2002). Bracketed by 5-bp DRs, Tn7 contains IRL–In2-4–the *tns* module (*tnsE*–*tnsD*–*tnsC*–*tnsB*–*tnsA*)–IRR (Peters, 2014). Integron In2-4 contains 5′-CS (*intI2*–*attI2*), VR (*dfrA* [dihydrofolate reductase]–*sat2* [streptothricin acetyltransferase]–*aadA1y* [aminoglycoside adenylyltransferase]), and 3′-CS (*ybeA*, also known as *orfX*) (Hansson et al., 2002). ΔTn7 from pC131 has a core module (from IRL to the VR of In2-4), and its missing portion has been replaced by the 3′-end of a complex class 1 integron. The 3′ end of the complex class 1 integron includes 3′-CS1, ISCR1, VR2 (*bla*<sub>CTX-M-131</sub>), and Δ3′-CS2 (Δ*qacED1*–Δ*sul1*–Δ*orf5*); the truncated 3′-CS2 results from its positioning between VR2 and the *chrA* region. It is apparent that ΔTn7 from pPm60 is arranged as an IRL, In2-18 (5′-CS and VR [*dfrA1*–*sat2*–*aadA1a*–*qacH2*]), a truncated *tns* module (Δ*tnsD*–*tnsC*–*tnsB*–*tnsA*), and an IRR. Insertion of the accessory region (from Δ*aer* to *orf609*) means that ΔTn7 is split into two separate portions; namely, ΔTn7-5′ and ΔTn7-3′ (Figure 3).

The transposon unit IS26–*mph(A)*–*mrx*–*mphR(A)*–IS6100 and the *chrA* region were found to be inserted into the upstream region of the 3′-end of a complex class 1 integron in pC131. The macrolide resistance unit IS26–*mph(A)*–*mrx*–*mphR(A)*–IS6100

is considered to be a mobile element, and the *mph(A)*–*mrx*–*mphR(A)* operon encodes a phosphotransferase, a positive regulator factor, and a negative transcription factor (Partridge, 2011). This transposon unit is truncated in the MDR region of pC131, but it is present as an intact structure in the MDR region-2 of pPp47, while in pC131, the transposon unit (ΔIS26–*mph(A)*–*mrx*–*mphR(A)*–IS6100) is situated between Tn3 and the *chrA* region. Tn3 carries the class A beta-lactamase-encoding *bla*<sub>TEM-1</sub> gene, which was initially observed as an R1 plasmid in *E. coli* (Bailey et al., 2011). Here, Tn3 is an unabridged transposon in pC131, but its *tnpA* is a pseudogene. The *chrA* region (IRL<sub>chrA</sub>–*chrA* [chromate resistance]–*orf98*) is derived from a Tn21-like transposon in plasmid pCNB1 from *Comamonas*, and is often closely linked to IRT–IS6100 (Partridge, 2011). The *chrA* region, which is connected with the IS26–*mph(A)*–*mrx*–*mphR(A)*–IS6100 unit in pC131 and pPp47, has arisen through IS6100-mediated recombination. The *chrA* region includes the IS4321R interrupted-IRL<sub>chrA</sub>, *chrA* and *orf98* in pC131 and pPp47. Insertion of IS4321R, IRL<sub>chrA</sub> is disrupted and forms two parts, ΔIRL<sub>chrA</sub>-5′ and ΔIRL<sub>chrA</sub>-3′ (Figure 3).

We found that Tn4352 and the truncated *aacC2*–*tnrB* region are integrated between ΔTn7-3′ and ΔTn7-5′ in pPm60.

Flanked by 8-bp DRs at both ends, Tn4352 is an IS26-bounded structure (IS26-*aphA1a*-IS26), and the *aphA1a* resistance gene confers resistance to kanamycin and neomycin (Wrighton and Strike, 1987). Although Tn4352 is complete in the MDR region-2 from pPm60, it is truncated in the MDR region-2 from p16Pre36-NDM and pPp47. Furthermore, the structure of  $\Delta$ Tn4352 is IS26-*aphA1a* in p16Pre36-NDM and  $\Delta$ IS26-*aphA1a* in pPp47. The orientation of Tn4352 in p16Pre36-NDM is direct, but reversed in pPp47 and pPm60. The *aacC2*-*tmrB* region is present in plasmids pCTX-M3 and pU302L, is derived from transposon Tn2 from the Tn3-family, and contains a IS26 mobile element at its right-hand end (Partridge, 2011). The *aacC2* and *tmrB* genes account for aminoglycoside and tunicamycin resistance, respectively. The truncated *aacC2*-*tmrB* region in pPm60 is composed of an *aacC2*-*tmrB*-*orf192*-*orf228*-*orf1182* segment. The direction of the truncated *aacC2*-*tmrB* region is direct in pPm60, but reversed in pPp47. Owing to the insertion of a 28,064 bp exogenous region (with an unknown function), the truncated *aacC2*-*tmrB* region from pPp47 is segmented into two parts:  $\Delta$ ISCfr1-3' exists in the MDR region-1, while a fragment from  $\Delta$ ISCfr1-5' to the *aacC2* gene is embedded in the MDR region-2. Similarly,  $\Delta$ Tn5563 is also located in the two MDR regions of pPp47. Tn5563 was originally discovered in plasmid pRA2 from *Pseudomonas aeruginosa* (Yeo et al., 1998), and two segments of  $\Delta$ Tn5563 in pPp47 are arranged as follows: the reverse segment (the *mer* operon and IRR) is present in MDR region-1 and the direct fragment (IRL and  $\Delta$ *tnpR*) is present in MDR region-2 (Figures 1A, 2, 3).

### Other Accessory Modules Outside the MDR Region of pPrY2001-Like Plasmids

We found that Tn6901 has a complete structure in pHFK418-NDM, but it is interrupted by insertion of the virulence-related region to generate two segments,  $\Delta$ Tn6901-5' and  $\Delta$ Tn6901-3'. Tn6901 is made up of an IRL-*tnpA*-*res*-*tnpR*-*frmB* (S-formylglutathione hydrolase)-*glo* (glyoxalase resistance)-*frmA* (S-glutathione dehydrogenase)-*frmR* (negative transcriptional regulator)-IRR structure in plasmid Rts1 from *Proteus vulgaris*, flanked by 5-bp DRs (Murata et al., 2002). Tn6901 is inserted between *orf1528* and *orf942* in the backbone of pHFK418-NDM, bracketed by 5-bp DRs. pHFK418-NDM, a pPrY2001-like plasmid, is the only virulence gene-carrying plasmid, indicating that this plasmid can not only carry a large number of drug resistance genes, but also integrate virulence genes within it (Figure 1B).

It is known that *xerC* and *xerD* genes are site-specific recombinases in the lambda integrase family, where it was found that *xer*-mediated recombination events resulted in the transmission of resistance gene between plasmids and chromosomal locations (Merino et al., 2010). The *dfrA6*-*ereA* region is located downstream of the conjugal transfer region in p16Pre36-NDM, and has undergone *xer*-mediated recombination. The *dfrA6*-*ereA* region consists of *xerC*, *recD*, *xerD*, *dfrA6* (trimethoprim resistance), *ereA* (erythromycin resistance), and *dinB* (Supplementary Figure S2).

## CONCLUSION

The *bla*<sub>NDM-1</sub>-harboring pHFK418-NDM plasmid, a pPrY2001-like plasmid group member, was first recovered from a clinical multidrug resistant *P. mirabilis* HFK418 isolate in China. Our data have revealed that the pHFK418-NDM plasmid contains two novel transpositions, Tn6624 and Tn6625. Tn6625, a large composite transposon, has integrated a variety of mobile elements, such as the *bla*<sub>NDM-1</sub>-carrying  $\Delta$ Tn125, *mph*(E)-harboring Tn6624, and In251. In251 was first identified from the above-mentioned clinical isolate, suggesting that it had been efficiently transferred from environmental organisms to clinical isolates. The pHFK418-NDM plasmid was found to have the ability for conjugal transfer, and to harbor a large numbers of resistance and virulence genes.

The pPrY2001-like plasmids described above harbor a wide variety of antimicrobial resistance genes, with the exception of p06-1619-1. Their relatively conserved backbones have integrated a great variety of accessory modules in the form of resistance genes, gene clusters, insertion sequences, transposons, and integrons, all of which enhance the diversification and evolution of the pPrY2001-like plasmids. Our findings augment our current understanding on the horizontal transfer of resistance genes and the genetic diversity and evolution of pPrY2001-like plasmids.

## AUTHOR CONTRIBUTIONS

YZ, YT, and XZ conceived the study and designed the experimental procedures. DD, ZL, JF, NJ, and HZ performed the experiments. DD and ML analyzed the data. YZ, YT, XZ, BZ, and TZ contributed to reagents and materials. YZ, YT, and DD wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02030/full#supplementary-material>

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# A State-of-Art Review on Multi-Drug Resistant Pathogens in Foods of Animal Origin: Risk Factors and Mitigation Strategies

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Most of the foodborne microbial diseases are linked to foods of animal origin such as milk, meat, and poultry. Nowadays, the presence of multi-drug resistant (MDR) pathogens in foods is becoming an increasingly public health concern worldwide due to the overuse of antimicrobial drugs in animal feed. MDR pathogens can enter the food chain by posing a significant risk to both animals and consumers. MDR pathogens causing infections are untreatable due to their resistance to various antibiotics, primarily cephalosporin and carbapenems and to their extended-spectrum beta-lactamase (ESBL)-producing capability. In addition, foods of animal origin and food-related environments can be likely vehicles for spreading of multi-drug resistance genes, which accelerates the thriving of global antibiotic resistance. This paper reviews the role of foods of animal origin as a vehicle for MDR pathogens, stressing the contribution of food processes, environments, and storage conditions in dissemination and reduction of antimicrobial resistances (AMRs). Controlling the growth of MDR microorganisms and limiting the transmission/expression of AMR genes in food ecosystems could be an effective mitigation strategy, putting the focus on food processes as a part of the solution for AMR in foods. Bioprotective cultures are also a promising and environmentally friendly technology to reduce the incidence of MDR pathogens, though caution is taken as microbial starters and probiotics can also carry AMR. Finally, applying Whole Genome Sequencing (WGS) and predictive microbiology, within a Risk Assessment framework, is key to get insight into those mechanisms and conditions along the food chain favoring or reducing AMR.

**Keywords:** antimicrobial resistance, bacteriocin, biocontrol, lactic acid bacteria, food safety, food-related environment, microbial interaction, risk assessment

## INTRODUCTION

The emergence of multi-drug resistant (MDR) pathogens has been one of the most critical public health problems in the last decades. In addition to occurrence of nosocomial infections with high mortality rates due to the dissemination of ESKAPE pathogens [*Enterococcus* (E.) *faecium*, *Staphylococcus* (S.) *aureus*, *Klebsiella* (K.) *pneumoniae*, *Acinetobacter* (A.) *baumannii*, *Pseudomonas* (P.) *aeruginosa*, and *Enterobacter* species which can “escape” from the biocidal action of multiple types or classes of antibiotics] both in humans and animals (Rice, 2008;

Pendleton et al., 2013; Santajit and Indrawattana, 2016), more recent challenges such as mobile colistin-resistant (mcr) strains and New Delhi metallo- $\beta$ -lactamase-1 (NDM-1)-producing strains in food-producing animals (FPA) are emerging as reservoirs of colistin resistance and  $\beta$ -lactam antibiotics including the last-resort antibiotic carbapenem resistance respectively in the public nowadays (Al-Tawfiq et al., 2017; Khan et al., 2017; Ghafur et al., 2019). The incidence of these pathogens varies based on the bacterial species, antimicrobial groups, and the most of all the geographical location in the world. According to the Centers for Disease Control and Prevention (CDC) report in 2013, at least 23,000 people died from more than 2 million people infected with antibiotic-resistant bacteria each year in the USA and the etiological agents in those rapidly increasing rates of infections were mostly found as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *E. faecium* (VRE), and fluoroquinolone-resistant *P. aeruginosa* (Centers for Disease Control, 2004, 2013). Moreover, data of European Antimicrobial Resistance Surveillance Network (EARS-Net) showed that high levels of antibiotic resistance had still remained in the European Union (EU) for several bacterial species, and their percentages were generally higher in southern and south-eastern Europe than in northern Europe between the years of 2014 and 2017. As an example, more than one-third of the *K. pneumoniae* were reported as resistant to at least one of the antibiotic groups of aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems. Besides, combined resistance of *Escherichia (E.) coli* and *K. pneumoniae* to several antimicrobial groups by production of extended-spectrum beta-lactamase (ESBL) was also commonly determined. While carbapenem resistance percentage of *K. pneumoniae* was reported as almost 10%, it was at higher percentages for *A. baumannii* and *P. aeruginosa*, including a significant increase from 10.4% in 2014 to 14.9% in 2017 for vancomycin-resistant *E. faecium*. Likewise, prevalence of plasmid-borne *mcr-1* and *bla<sub>NDM</sub>* genes particularly in *E. coli* strains has undermined the antimicrobial effectiveness of colistin and carbapenems. This has resulted in treatment failures due to a lack of effective therapeutic alternatives for microbial diseases. On the contrary, although the resistance situation of MRSA isolates appeared to continue, a decrease from 19.6% in 2014 to 16.9% in 2017 was reported (EARS-Net, 2018).

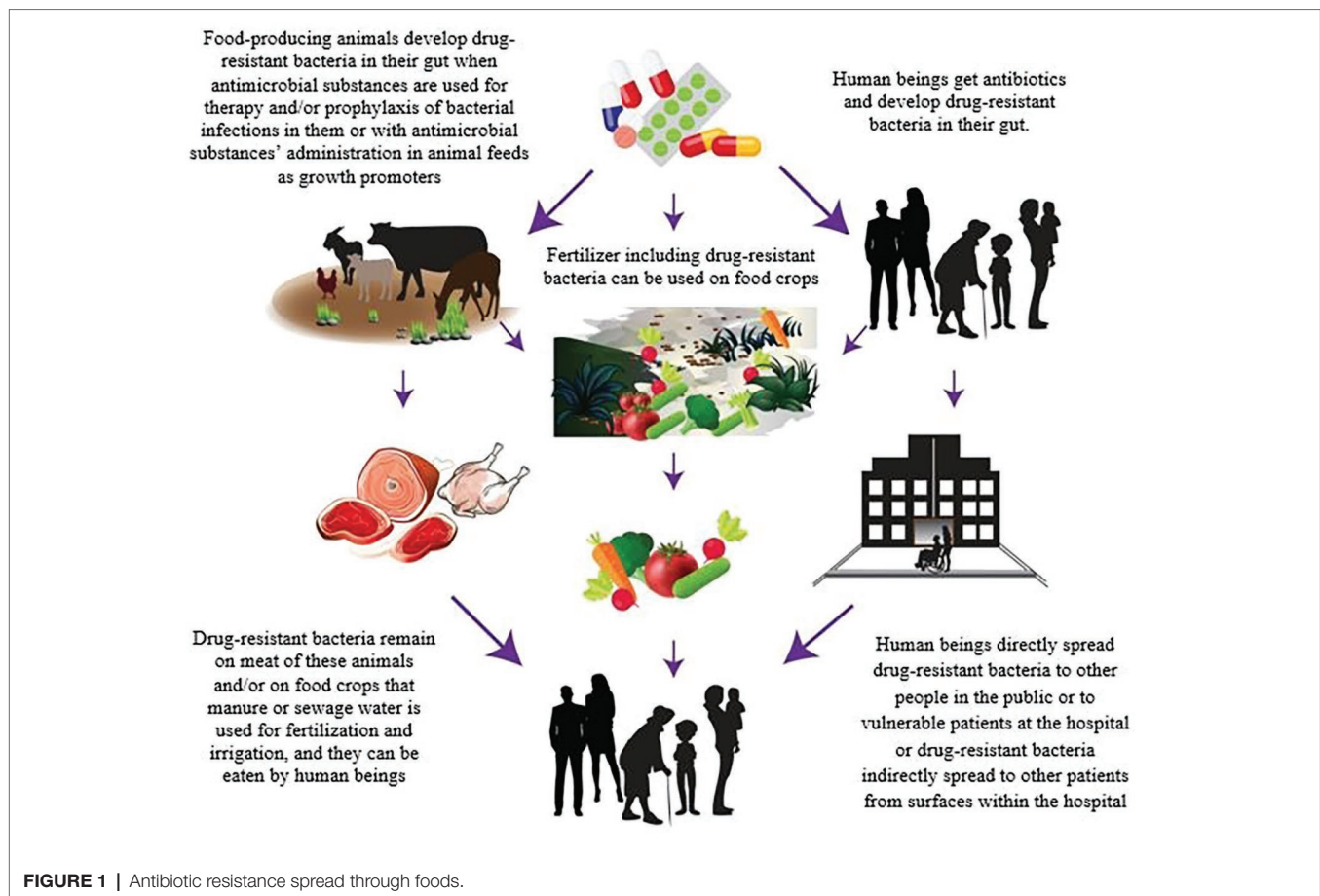
The presence of the MDR pathogens in foods of animal origin such as milk, meat, and poultry has dramatically increased over the last few years. More important is their ability in evolving to gain novel characteristics, particularly multi-drug resistance. These recently emerging MDR pathogens were previously unknown to the animal food industry since only few reports on their presence in foods of animal origin were available, but the epidemiological circumstances have changed with the advent and spread of them in foods of animal origin due to the overuse of antimicrobial drugs in FPA; thus, MDR zoonotic bacteria are able to reach the intestinal tract of humans (Barber et al., 2003; Muloi et al., 2018). Therefore, although there is an acceptance that the transmission of MDR pathogens primarily occurs from infected person to other persons, highly resistant bacterial infections are no longer limited to hospital-acquired infections since foods of animal origin are frequently

contaminated with MDR pathogens and, hence, has started to become the possible source for the exposure of not only high-risk groups like vulnerable patients, but also whole public (Lee, 2003; Centers for Disease Control, 2013).

The awareness of the potential risks of antimicrobial resistance (AMR) due to the severity of the diseases that MDR zoonotic pathogens may cause is a growing concern for food industry, which could lead to a loss of consumer confidence and accordingly a fall in demand of foods of animal origin. From this perspective, the increasing number of MDR pathogens in foods of animal origin not only imposes a significant burden on the global food industry, but also causes significant but avoidable economic losses. Therefore, there is an urgent need for a better understanding of the risk factors along the food chain. This review has been designed to shed light on the role of foods of animal origin as a vehicle for bacteria-specific antibiotic resistance, including the possible contamination routes of MDR pathogens and proposing the Risk Assessment (RA) framework as basis for the study. The review is also focused on the mitigation strategies such as the possibility of use of bioprotective cultures to prevent and/or control the incidence of MDR pathogens in foods and hence to combat this growing threat through reducing pathogen growth and limiting dissemination of antibacterial resistance genes in the food environment.

## THE ROLE OF FOODS OF ANIMAL ORIGIN ON MULTI-DRUG RESISTANCE DISEASES AND RISK FACTORS

In order to control the dissemination of MDR diseases through the foods of animal origin, the sources of contamination must be identified. There are several complex routes of transmission of these resistant bacteria and/or AMR genes along the food chain. However, the relative contribution of foods to the global burden of infections caused by MDR pathogens has not been estimated yet (Likotrafiti et al., 2018). Animal production and aquaculture are known as the primary sectors where the large majority of the antibiotics are used (Verraes et al., 2013). The overuse or misapplication of antimicrobial drugs for therapy and prophylaxis of bacterial infections in FPA or with their use in animal feeds as growth promoters in food animal production are leading to the development of MDR in pathogens during livestock production (Figure 1; Barber et al., 2003; Aarestrup, 2005; Normanno et al., 2007; Verraes et al., 2013; Schrijver et al., 2018). van Boeckel et al. (2015) presented the first global map of antibiotic consumption in livestock and provided a baseline estimate of its global importance in the future. It is also now generally accepted that more antibiotics are used in FPA than the amount administered in humans (Centers for Disease Control, 2013). This has unavoidably led to the emergence of resistant pathogenic bacteria in animals' gut, and because of their resistance to commonly used therapeutic antibiotics, these bacteria may cause infections for which there are limited therapeutic options. In other words, every time that the antibiotic, which is not needed, is administered to FPA, it creates a risk of development of a resistant infection in humans in the near future



**FIGURE 1** | Antibiotic resistance spread through foods.

(Smith et al., 2002; Silbergeld et al., 2008; Callens et al., 2018). Besides that, MDR pathogens can also reach crops and plants through contaminated manure or sewage water that is used for fertilization and irrigation (Figure 1; Nilsson, 2012; Centers for Disease Control, 2013; Pruden et al., 2013; Durso and Cook, 2014). In this respect, a recent report published by the European Food Safety Authority (EFSA) and European Center for Disease Prevention and Control (ECDC) presented scientific evidence of the existence of a linkage between the use of antibiotics in livestock production and AMR in foodborne pathogens (European Food Safety Authority/European Centre for Disease Prevention, 2018). It was also demonstrated that the use of third- and fourth-generation cephalosporins to treat *E. coli* infections in livestock was related to resistances of *E. coli* found in humans. In addition, ciprofloxacin-resistant *Salmonella*, and macrolides and fluoroquinolones-resistant *Campylobacter* strains are on the rise in FPA (Kumar et al., 2012; Mukherjee et al., 2013).

## The Impact of the Food Chain on the Antimicrobial Resistance Transmissions

The food chain is one of the main driving forces in the transmission of MDR due to the fact that foods are not sterile and usually present microbiological contamination or can become contaminated at specific stages along the food chain (i.e., cross contamination, recontamination) (Cahill et al., 2017).

Indeed, the food chain can act as a booster for MDR dissemination, allowing for survival or even increase of MDR pathogens. MDR transmission is not exclusive for food of animal origin or fish, and plant foods may also harbor MDR microorganisms as vegetables can be contaminated during primary production, through water contaminated with fecal material from effluent of surrounding farms. In general, pathogens are not frequent in foods, and the major risk arises from the high prevalence of non-pathogenic microorganisms that can transfer AMR genes to other microbial species, including foodborne pathogens. It is well documented that genes encoding resistance are transmissible between different bacteria in FPA and also from them to bacteria in foods and in humans by horizontal gene transfer (i.e., transformation, conjugation, and transduction) (Aarestrup, 2005; Appelbaum, 2006; Hammerum et al., 2010; Verraes et al., 2013). In a study, identical gentamicin resistance gene was found in *Enterococcus* spp. isolated from FPA, retail foods, and humans from geographically different areas (Donabedian et al., 2003) which can also be accepted as a supporting result of the dissemination of gentamicin-resistant *Enterococcus* spp. from FPA to humans via the food chain. Liu et al. (2019) also showed that mobile elements harboring *mcr-1* and *bla<sub>NDM</sub>* acquired by FPA strains are the ways of their transmission to our foods and then from foods to humans, after finding structurally similar *mcr-1* and *bla<sub>NDM</sub>* bearing plasmids both in foods and in clinical isolates. In addition, identical *mcr-1* genes were reported in



21% healthy swine at slaughter, 15% marketed pork and chicken meat, and 1% patients in China in 2016 (Yi-Yun et al., 2016). On the other hand, AMR gene transfer is not only specific to live cells; conversely, stressed or partially inactivated cells are able to confer resistance to other microorganisms, including pathogens, or microbiota in general and, after ingestion, mobilized to intestinal microbiota in humans through the gene transfer mentioned above (Verraes et al., 2013).

Notwithstanding the foregoing, we should not narrow the origin of drug resistances to primary production as the food chain can generate resistances by itself. The pressure exerted by the wide use of biocides for food production such as disinfectants, preservatives, and other chemicals or even environmental and process conditions applied through the food distribution chain has been proved to trigger the adaption of microbial populations by developing transient resistances (Capita and Alonso-Calleja, 2013; Händel et al., 2013).

## Multi-Drug Resistant Foodborne Pathogens From Farm to Fork

The transmission routes of MDR foodborne pathogens along the food chain and how they can reach consumers are not clear at present. The relevance of the mechanisms mentioned above with respect to the possibility that resistant pathogens can be originated at primary production and spread through the food chain is also unknown. However, it is reasonable to consider that MDR pathogens from a very early food stage (i.e., primary production) can enter and remain in the food systems and (re)contaminate, survive, and/or grow in food or food environments resulting in their presence of both raw food and ready-to-eat products at the consumption stage (Figure 1; Lee, 2003; Verraes et al., 2013). Although the use of preservation technologies and/or food-processing technologies (i.e., hurdle technology concept) can play a similar role to that found for non-resistant pathogens by inactivating populations, and reducing risk (Mathur and Singh, 2005; Lester et al., 2006; Verraes et al., 2013), it has been also suggested that the sublethal conditions induced by preservation methods can stimulate the horizontal transmission of plasmids containing AMR genes (McMahon et al., 2007). Even the complete elimination of bacteria by lethal treatments does not assure that AMR is not transmitted, since DNA released from lysed cells can still be transferred, by the process of transformation to living microorganisms (pathogenic or commensal) on foods or in human digestive system. In addition, environmental stress produced by these technologies can drive pathogens to adapt to the stressful environment by evoking the expression of the resistance genes, and, as a consequence, enhanced resistance capacity and changes in virulence and infectivity of the populations (Horn and Bhunia, 2018). In spite of the few studies carried out hitherto, it has been proved that sublethal stress produced by thermal, acidic, and saline conditions can affect the phenotypic resistance (Verraes et al., 2013). For example, there are data suggesting that sublethal high temperatures can reduce the presence of phenotypic resistance while increase in salt concentrations or reduction of pH is rather related to its increase (McMahon et al., 2007).

## Lactic Acid Bacteria as Reservoirs of Antimicrobial Resistance

The fermentative processes carried out in foods such as technological processes or those occurring naturally are also a key aspect in the transmission of resistances. Lactic acid bacteria (LAB), one of the most common microbiota in foods, and widely used as starters for specific food products (yoghurt, cheese, dry-cured meat, etc.) can also act as reservoirs of AMR genes similar to those found in clinical pathogens and hence cause a spread of resistance genes to foodborne pathogenic bacteria (van den Bogaard and Stobberingh, 2000; Smith et al., 2002). As an example, identical tetracycline-, erythromycin-, and vancomycin-resistant genes that were found in clinical bacterial species were also detected in *Lactococcus lactis* and *Lactobacillus* species isolated from fermented meat and milk products (Mathur and Singh, 2005). Besides, some human commensal bacteria revealed the presence of AMR determinants within their genomes and therefore they show intrinsic AMR (Cox and Wright, 2013). Among the LAB isolated from fermented foods, transferable resistance genes were rare (European Food Safety Authority, 2008) and mostly *Enterococcus* spp. was found to have antibiotic resistance, especially to vancomycin although resistance to chloramphenicol and erythromycin was also observed (Teuber et al., 1999). Therefore, the presence of AMR genes in starter, adjunct, and/or probiotic cultures that are intentionally added during animal food processing can also pose a substantial risk for increasing foodborne diseases that cannot be treated by current antibiotics (Verraes et al., 2013).

## MOVING TOWARD A QUANTITATIVE RISK ASSESSMENT APPROACH TO DEVELOP A BETTER CONTROL OF MULTI-DRUG RESISTANT ALONG THE FOOD CHAIN

Risk Assessment is a structured and science-based method intended to estimate human risk associated with exposure to foodborne hazards (Codex Alimentarius Commission, 2014). Microbiological Risk Assessment (MRA) has been widely used to estimate risk and risk factors linked to microbiological hazards along the food chain, being a pillar in the development of food policy and control measures for the most relevant foodborne pathogens (World Health Organization/Food and Agriculture Organization, 2002, 2016; Pérez-Rodríguez et al., 2017). The application of MRA in the context of MDR foodborne pathogens is needed more than before in order to shed light on the main transmission routes of MDR and identify the relevant factors along the food chain (Claycamp and Hooberman, 2004; Geenen et al., 2010). By using a Risk Analysis framework, efficient mitigation strategies for antimicrobial resistant microorganisms could be developed and implemented to fight against resistant foodborne pathogens. One of the most critical elements in MRA is the generation of data that allow to accomplish all MRA steps properly. For that, the development of surveillance programs that can incorporate reliable data on the presence of resistance microorganisms and genes throughout



the food chain is a primary requirement. Whole Genome Sequencing (WGS) analysis is proposed as a key tool to accomplish this task, providing data on complex and non-culturable communities in addition to providing an information basis to determine the linkage between MDR microorganisms in foods and observed foodborne disease cases. On the other hand, the interpretation of genomic data into the current MRA paradigm is complex, but MRA is estimated to move to the study of microbial behavior (i.e., expression of genes) rather than the taxonomic and genotypic identification (Cocolin et al., 2018). Therefore, incorporation of studies of predictive microbiology (Pérez-Rodríguez, 2013), based on the mathematical characterization of microbial response in ecofood systems, becomes an innovative discipline that may be applied to better elucidate the main transmission routes and food process parameters contributing to MDR dissemination. In this sense, it is also reported that antimicrobial resistant mutants can show a different phenotype than wild microorganisms due to the metabolic cost produced by the mutations (Schulz zur Wiesch et al., 2010; Händel et al., 2013). This fact leads us to formulate the hypothesis that if there are some food process parameters and conditions, or the combination of them, more affecting the viability and spread of MDR microorganisms, maybe, this information could be exploited to develop more effective control measures to minimize their transmission along the food chain.

## MITIGATION STRATEGIES TO COMBAT GROWING THREAT OF MULTI-DRUG RESISTANT PATHOGENS IN FOODS

The presence of MDR pathogens in foods of animal origin could safe food production in future. Taking an *One Health* perspective of the problem is proposed to address the AMR problem, considering the food chain as a paramount factor for reducing and controlling resistance transmissions (European Commission, 2017; World Health Organization, 2018). Therefore, changes are required primarily in animal and plant production, by applying Good Agricultural Practices (GAP), and later in the animal food industry in the way of production, storage, processing, and distribution of foods of animal origin. FPA-human transmission of AMR increases the pressure of a reduced antimicrobial use in those animals. Therefore, prudent antimicrobial use in animal husbandries and control procedures targeting all foods of animal origin throughout the processing are the main effective intervention strategies to prevent the transmission of resistant bacteria from foods to humans and vice versa (Muaz et al., 2018). Furthermore, efforts to prevent such a challenge should also be built on application of effective food safety management, including Good Manufacturing Practices (GMP) and Good Hygienic Practices (GHP) during production and between food industry workers carrying the resistant strains so that person-to-person spread of these pathogens in animal food sector can be reduced (Lee, 2003; Normanno et al., 2007).

The use of bioprotective cultures is also proposed as a sustainable alternative to antibiotics in livestock production (Castellano et al., 2017). The antagonistic effect of LAB, as biopreservatives, is mainly due to a decrease of pH values in foods as well as the antibacterial activity of organic acids or peptides (bacteriocins) and bacteriocin-like inhibitory substances (BLISs), which can be exploited against foodborne pathogens (Gálvez et al., 2010). However, special caution should be taken to minimize the potential role of LAB as carriers or vectors of transferable AMR genes in ecofood systems. Therefore, the selection of the bacterial species that are intentionally added to foods should be based on Generally Recognized as Safe (GRAS) principle and supported by a thorough study of their biochemical and genetic characteristics in order to determine the presence of AMR genes or potential to act as carriers.

the strategies designed to combat this growing threat of MDR pathogens, face a particular challenge as there is a rapid dissemination of resistance genes between bacteria. Preventing the dissemination of antibiotic resistance genes can only be achieved with the continuous awareness of people working in agriculture and food sectors, and sustainable hygiene and sanitary practices (Centers for Disease Control, 2013).

## CONCLUDING REMARKS

Antibiotics are powerful drugs that are usually safe in treating microbial diseases in FPA for improving the health and welfare of animals; however, the indiscriminate use of antibiotics can lead to develop antibiotic resistance in animal microbiota. Due to the linkage between antibiotic use in FPA and the occurrence of antibiotic resistance diseases in humans *via* food chain, a judicious strategy regarding the use of antibiotics under only veterinary supervision in FPA should be promoted and reinforced. Rather than an “AMR amplifier,” we suggest that the food chain could act as a “resistances’ modulator” to reduce the incidence of resistant microorganisms by properly controlling food process parameters. Nevertheless, further research is needed to determine the further research is needed to determine both the mechanisms involved in the transmission of resistance and the food processing and storage conditions that are significant in their mitigation. Then, on this basis, robust control programs might be designed and put in place for the prevention of dissemination of the AMR genes between the bacteria along the food chain. The scientific basis to develop such mitigation strategies should be underpinned by a MRA scheme, whereby risk factors and transmission routes can be identified based on the combination of genomic data and predictive microbiology outcomes.

## 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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# Emergence and Characterization of a Novel IncP-6 Plasmid Harboring *bla*<sub>KPC-2</sub> and *qnrS2* Genes in *Aeromonas taiwanensis* Isolates

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The dissemination of *Klebsiella pneumoniae* carbapenemases (KPCs) among Gram-negative bacteria is an important threat to global health. However, KPC-producing bacteria from environmental samples are rarely reported. This study aimed to elucidate the underlying resistance mechanisms of three carbapenem-resistant *Aeromonas taiwanensis* isolates recovered from river sediment samples. Pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS) analysis indicated a close evolutionary relationship among *A. taiwanensis* isolates. S1-PFGE, Southern blot and conjugation assays confirmed the presence of *bla*<sub>KPC-2</sub> and *qnrS2* genes on a non-conjugative plasmid in these isolates. Plasmid analysis further showed that pKPC-1713 is an IncP-6 plasmid with a length of 53,205 bp, which can be transformed into DH5 $\alpha$  strain and mediated carbapenems and quinolones resistance. The plasmid backbone of p1713-KPC demonstrated 99% sequence identity to that of IncP-6-type plasmid pKPC-cd17 from *Aeromonas* spp. and IncP-6-type plasmid: 1 from *Citrobacter freundii* at 74% coverage. A 14,808 bp insertion sequence was observed between *merT* gene and hypothetical protein in p1713-KPC, which include the quinolone resistance *qnrS2* gene. Emergence of plasmid-borne *bla*<sub>KPC</sub> and *qnrS2* genes from *A. taiwanensis* isolates highlights their possible dissemination into the environment. Therefore, potential detection of such plasmids from clinical isolates should be closely monitored.

**Keywords:** *Aeromonas taiwanensis*, *bla*<sub>KPC-2</sub>, *qnrS2*, whole-genome sequencing, plasmid analysis

## INTRODUCTION

The global spread of *Klebsiella pneumoniae* carbapenemases (KPCs) among Gram-negative bacteria, has become a major public health concern in recent decades (Grundmann et al., 2017). Infections caused by KPC-producing bacteria have been associated with a high mortality rate of 53%, which presents tremendous challenges for clinicians and healthcare providers (Munoz-Price et al., 2013). Although most KPCs are commonly found in *Enterobacteriaceae*, the production



of KPCs appeared to be less dominance in *Aeromonadaceae* spp. (Picao et al., 2013; Montezzi et al., 2015; Lamba and Ahammad, 2017; Paschoal et al., 2017; Tuo et al., 2018; Xu et al., 2018; Zheng et al., 2018b). The emergence of *bla*<sub>KPC</sub> gene on broad-host-range plasmids has facilitated its rapid dissemination to *Enterobacteriaceae* and other Gram-negative families (van Duin and Doi, 2017). More importantly, KPC-type carbapenemases are often associated with quinolone resistance determinants, which can be modulated by *qnrS* gene (Montezzi et al., 2015). The quinolone resistance gene, *qnrS2*, shares 92% identity with the *qnrS* gene, was first detected on a highly mobile plasmid that is isolated from wastewater treatment plant bacterial population (Bonemann et al., 2006). Thus far, the co-existence of *bla*<sub>KPC</sub> and *qnrS2* in the same plasmid has not been reported.

The objective of the present study was to identify plasmid-borne *bla*<sub>KPC-2</sub> genes in *Aeromonas taiwanensis* from river sediments in China and to analyze the plasmids carrying them. Three strains were isolated and found to harbor IncP-6 plasmids carrying both *bla*<sub>KPC-2</sub> and *qnrS2*. We characterized the phenotypic and molecular features of these strains in order to assess their genomic epidemiology profiles. Additionally, the complete nucleotide sequence of p1713-KPC was determined by plasmid sequencing. To the best of our knowledge, this is the first study to indicate the co-occurrence of *bla*<sub>KPC-2</sub> and *qnrS2* genes in the same plasmid and this plasmid harboring these two gene were founded in *A. taiwanensis* isolates for the first time.

## MATERIALS AND METHODS

### Bacterial Isolation and Pulsed-Field Gel Electrophoresis (PFGE)

The KPC-2-producing *A. taiwanensis* isolates were previously identified from river sediment samples from China (Xu et al., 2018). A total of three *A. taiwanensis* isolates were further characterized to uncover the underlying resistance mechanisms. The genomic diversity of *A. taiwanensis* isolates was assessed by *Xba*I-pulsed-field gel electrophoresis (PFGE) as described previously (Zheng et al., 2016). A dendrogram of PFGE profiles was constructed with BioNumerics v7.6 by using UPGMA (unweighted pair group method with averages) clustering. Isolates with a similarity cut-off of  $\geq 80\%$  were considered as pulsotypes.

### Whole-Genome Sequencing (WGS) and Assembly

To characterize the genomic features and resistome of *A. taiwanensis* isolates, WGS was conducted on all the three isolates. Genomic DNA was extracted and sequenced with HiSeq X Ten sequencing platform (Illumina, San Diego, CA, United States). Subsequently, *de novo* genome assembly and bioinformatics analysis were carried out as previously described (Xu et al., 2018; Zheng et al., 2018a). After obtaining the raw reads, the genome sequence of pKPC-1713 was assembled using plasmidSPAdes (Antipov et al., 2016).

## Phylogenetic Reconstruction and Analysis

The pan-genome analysis was performed with Roary: the pangenome pipeline (version 3.6.0) using the Prokka annotation (Seemann, 2014; Page et al., 2015). The complete genome sequences of all 20 *Aeromonas* spp. strains were downloaded from NCBI genome database (current as of March 30, 2019). Roary software was used to cluster the above genomes. The software clustered the genomes based on the genes carried by each strain. According to the distribution of each gene among the strains, the genes were divided into core genes and accessory genes. Core genes were defined as genes carried by 95% or more of the strains. Accessory genes were those carried by less than 95% of the strains. Then the core genome of these strains is obtained. A whole-genome phylogenetic tree was built from the core-genome SNPs of *Aeromonas* spp. and the three studied isolates. Phylogenetic reconstruction and analysis was performed using the R package phangorn (Schliep, 2011).

## Plasmid Characterization

The size of plasmid was determined by S1-nuclease PFGE analysis, as previously described (Zheng et al., 2015). Southern blot hybridization of plasmid DNA was performed on *Aeromonas hydrophila* isolates by using DIG-labeled *bla*<sub>KPC</sub>- and *qnrS*-specific probes, according to manufacturer's instructions (Roche Diagnostics, Germany) (Zheng et al., 2016). Plasmid conjugation experiments were carried out by filter mating using *Escherichia coli* J53 and EC600 as recipient strains, at a ratio of 1:1 in broth culture (Zheng et al., 2015). The pKPC-1713 plasmid was transferred into chemically competent *E. coli* DH5 $\alpha$  cells via transformation process. The transformants were selected on Luria-Bertani agar plates supplemented with 2 mg/L meropenem. The presence of *bla*<sub>KPC</sub> and *qnrS2* genes was then screened by PCR and sequencing. Plasmid DNA was extracted from conjugants with Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA). To obtain the complete sequence of the plasmid co-expressing *bla*<sub>KPC</sub> and *qnrS2*, the combined application of PCR walking method with targeted primers (Supplementary Table S1) and Illumina sequencing technique were performed to specifically investigate the representative plasmid pKPC-1713. The RAST annotation pipeline was chosen to perform rapid annotation of the plasmids (Overbeek et al., 2014). Plasmid replicons and antibiotic resistance genes were identified using CGE server<sup>1</sup>. The sequence of pKPC-1713 was BLAST for homology against the NCBI plasmids database.

## Susceptibility Testing

The isolated *A. taiwanensis* 1713, *E. coli* DH5 $\alpha$  and DH5 $\alpha$ : pKPC-1713 were cultured overnight, while *E. coli* ATCC 25922 was used as a quality control. Minimum inhibitory concentrations (MICs) of amikacin, ampicillin, ampicillin-sulbactam, aztreonam, cefazolin, ceftazidime, cefotetan, ceftriaxone, ciprofloxacin, ertapenem, gentamicin, imipenem, levofloxacin, nitrofurantoin, piperacillin-tazobactam, tobramycin, and trimethoprim

<sup>1</sup><https://cge.cbs.dtu.dk/services/>

antibiotics were determined by VITEK 2 system with AST-GN16 panel. The results of antimicrobial susceptibility testing were interpreted according to 2017's CLSI guidelines.

## Plasmid Mobilization

Plasmid conjugation was performed as previously described with some modification (Yi et al., 2012). *A. taiwanensis* isolates were electrotransformed with a plasmid pEC1002-MCR(CP021205) which contains a *tra* module. Isolates containing the resident and pEC1002-MCR plasmids were used as donor strains. Plasmid conjugative transfer was performed by using donor and recipient sodium azide resistant *E. coli* J53 cells mixed in a 1:1 ratio as described previously. Transconjugants were selected on MacConkey agar containing 2 mg/L imipenem and 100 mg/L sodium azide. Transconjugants were confirmed by susceptibility testing and PCR.

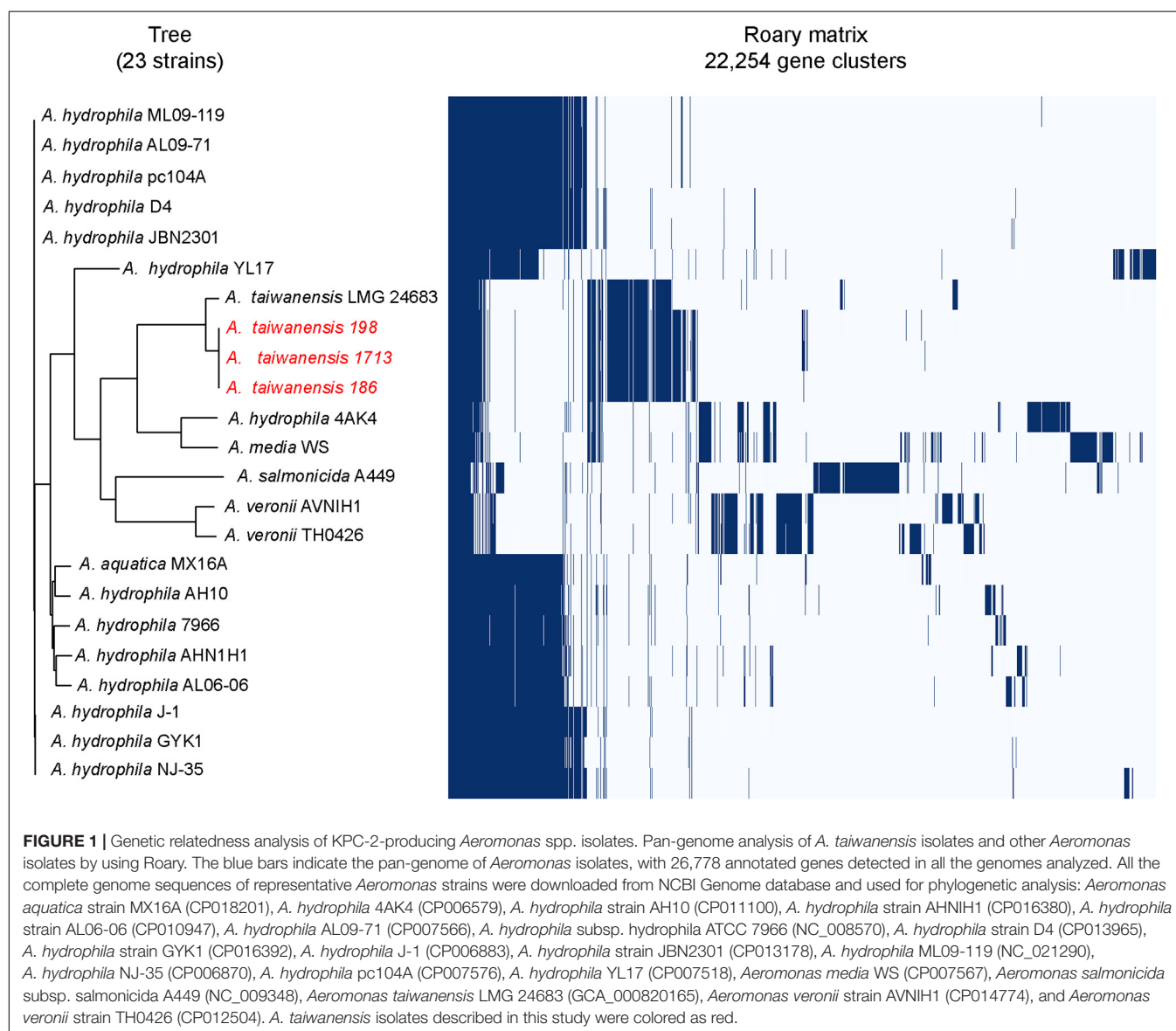
## Accession Numbers

The whole genome shotgun project of the *A. taiwanensis* isolates has been deposited into DDBJ/EMBL/GenBank under the Bioproject accession number PRJNA478520. The nucleotide sequence of plasmid p1713-KPC has been deposited into the GenBank database under GenBank accession number MH624132.

## RESULTS AND DISCUSSION

### Comparative Genomic Analysis of Carbapenem-Resistant *A. taiwanensis* Isolates

Pulsed-field gel electrophoresis analysis revealed that two pulsotypes from *A. taiwanensis*, namely 198 and 186, exhibited



**FIGURE 1 |** Genetic relatedness analysis of KPC-2-producing *Aeromonas* spp. isolates. Pan-genome analysis of *A. taiwanensis* isolates and other *Aeromonas* isolates by using Roary. The blue bars indicate the pan-genome of *Aeromonas* isolates, with 26,778 annotated genes detected in all the genomes analyzed. All the complete genome sequences of representative *Aeromonas* strains were downloaded from NCBI Genome database and used for phylogenetic analysis: *Aeromonas aquatica* strain MX16A (CP018201), *A. hydrophila* 4AK4 (CP006579), *A. hydrophila* strain AH10 (CP011100), *A. hydrophila* strain AHN1H1 (CP016380), *A. hydrophila* strain AL06-06 (CP010947), *A. hydrophila* AL09-71 (CP007566), *A. hydrophila* subsp. *hydrophila* ATCC 7966 (NC\_008570), *A. hydrophila* strain D4 (CP013965), *A. hydrophila* strain GYK1 (CP016392), *A. hydrophila* J-1 (CP006883), *A. hydrophila* strain JBN2301 (CP013178), *A. hydrophila* ML09-119 (NC\_021290), *A. hydrophila* NJ-35 (CP006870), *A. hydrophila* pc104A (CP007576), *A. hydrophila* YL17 (CP007518), *Aeromonas media* WS (CP007567), *Aeromonas salmonicida* subsp. *salmonicida* A449 (NC\_009348), *Aeromonas taiwanensis* LMG 24683 (GCA\_000820165), *Aeromonas veronii* strain AVNIH1 (CP014774), and *Aeromonas veronii* strain TH0426 (CP012504). *A. taiwanensis* isolates described in this study were colored as red.

relatively identical PFGE patterns (Supplementary Figure S1). Meanwhile, Roary matrix-based gene sequence analysis generated a large pan-genome matrix of 26,778 gene clusters across 23 genomes (Figure 1). Moreover, the 186, 198, and 1713 isolates were found to be genetically closely related by Roary matrix-based gene sequence analysis (Figure 1). These findings are consistent with the results of PFGE profiles, suggesting a possible clonal spread of KPC-producing *A. taiwanensis*. So far, only few reports described the detection of plasmid-mediated *bla*<sub>KPC</sub> determinants in *Aeromonadaceae* (Picao et al., 2013; Montezzi et al., 2015). Notably, the presence of *bla*<sub>KPC</sub> in *Aeromonadaceae* considered predominantly environmental is remarkable, and the spread of KPC-producing *A. taiwanensis* in aquatic environments deserves attention.

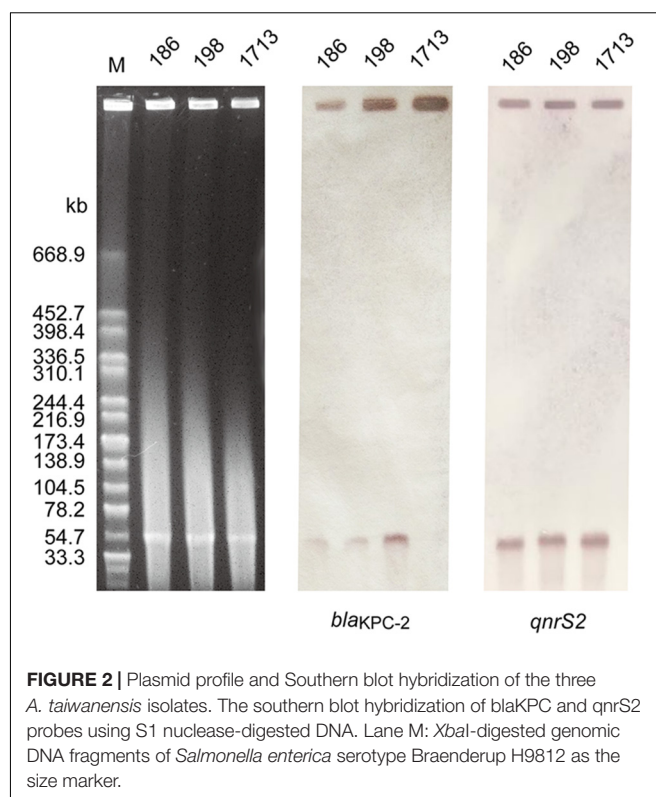
## Identification of the Plasmid Harboring Both *bla*<sub>KPC-2</sub> and *qnrS2* Genes

S1-PFGE and Southern blot analysis demonstrated that all *A. taiwanensis* isolates contained a ~54 kb plasmid, harboring both *bla*<sub>KPC-2</sub> and *qnrS2* genes (Figure 2). Moreover, nearly identical plasmid sequences of the three isolates were assembled from WGS data by SPAdes (data not shown), suggesting that these isolates shared the same plasmid profile. Notably, none of the plasmids could be transferred to recipient strains via conjugation, despite repeated attempts, which suggests that *bla*<sub>KPC-2</sub> and *qnrS2* are located on a non-conjugative plasmid. Occasionally, *qnrS2* has been identified in the plasmids of *Aeromonas* spp. (Arias et al., 2010; Marti and Balcasar, 2012; Marti et al., 2014; Yang et al., 2017); however, the co-occurrence of *bla*<sub>KPC</sub> and *qnrS2* genes in the same plasmid has never been described before. To our knowledge, this is the first study to indicate the co-occurrence of *bla*<sub>KPC</sub> and *qnrS2* genes in the same plasmid. As a consequence, we selected a representative sample of 1713 isolate for further plasmid characterization.

Plasmid transformation and MICs determination revealed that carbapenem and quinolone resistant genes were successfully transferred from a donor strain to *E. coli* DH5 $\alpha$ . Antibiotic susceptibility testing showed that the MIC values of ertapenem, imipenem, ciprofloxacin, and levofloxacin were increased from 0.125 to 8 mg/L, 0.125 to 16 mg/L, 0.25 to 8 mg/L, and 1 to 4 mg/L, respectively (Table 1). These results confirmed that carbapenem and quinolone resistance were successfully transferred to recipient cells.

## Complete Sequence of pKPC-1713 Plasmid

Sequence analysis of representative pKPC-1713 plasmid revealed the total size of 53,205 bp with an average G + C content of 58% and 66 open reading frames. The plasmid pKPC-1713 harbored five functional regions of genes, including *bla*<sub>KPC-2</sub> encoding region, IS5045 elements, an insertion region, genes involved in plasmid maintenance, and plasmid replication (Figure 3). The search against nr/nt database revealed a 99% identity with *Aeromonas* sp. ASNIH3 IncP-6-type plasmid pKPC-cd17 at 74% coverage, and a 99% identity with *Citrobacter freundii* IncP-6-type plasmid: 1 at 74% coverage (Figure 3).



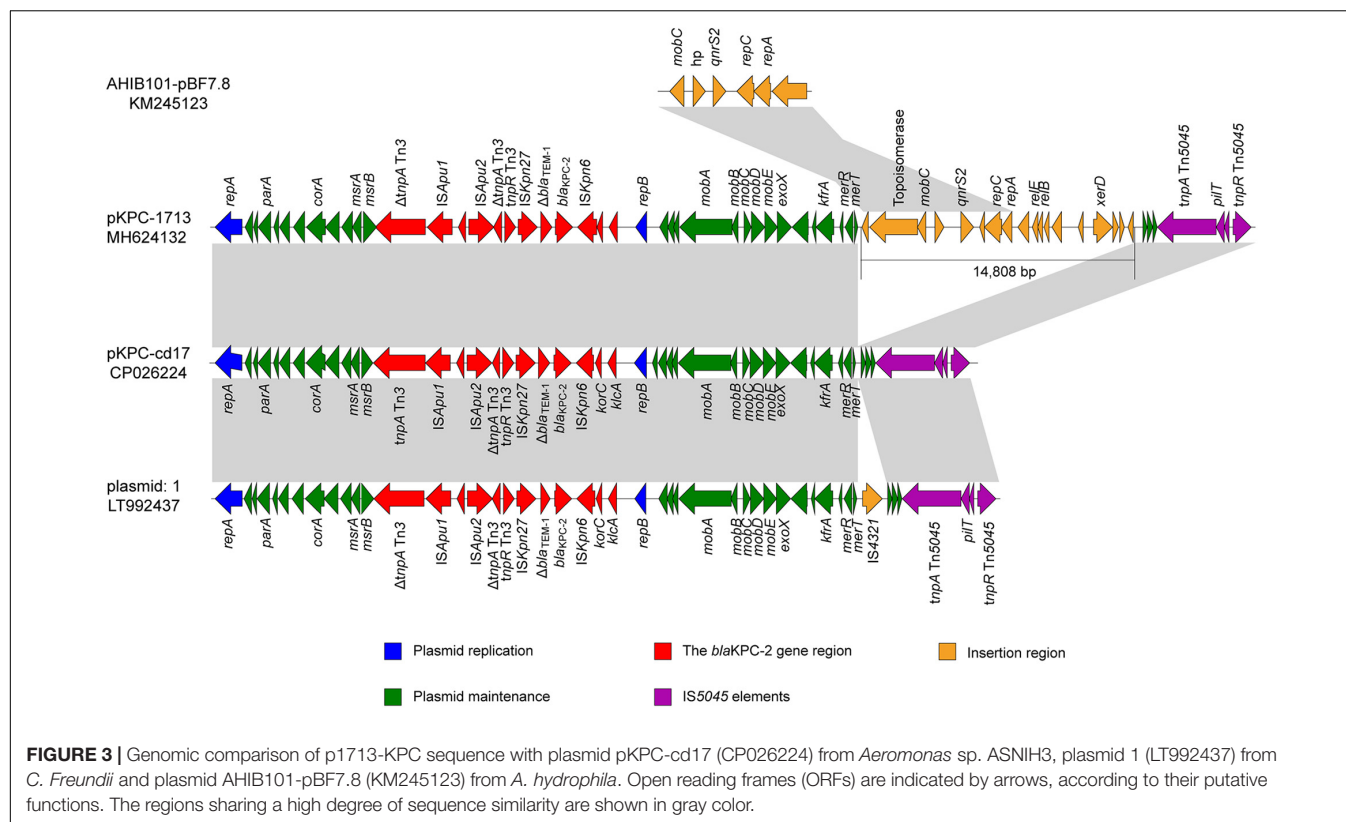
**FIGURE 2 |** Plasmid profile and Southern blot hybridization of the three *A. taiwanensis* isolates. The southern blot hybridization of *bla*<sub>KPC</sub> and *qnrS2* probes using S1 nuclease-digested DNA. Lane M: *Xba*I-digested genomic DNA fragments of *Salmonella enterica* serotype Braenderup H9812 as the size marker.

**TABLE 1 |** Characteristics of *A. taiwanensis* 1713 and its transformant strain<sup>a</sup>.

Antibiotics	<i>A. taiwanensis</i> 1713	DH5 $\alpha$ : pKPC-1713	<i>E. coli</i> E DH5 $\alpha$
Ampicillin	≥128/R	≥128/R	1/S
Amoxicillin/clavulanate	≥128/R	64/R	1/S
Piperacillin/tazobactam	≥128/R	≥128/R	1/S
Cefazolin	≥128/R	≥128/R	1/S
Ceftriaxone	≥128/R	≥128/R	0.125/S
Ceftazidime	16/R	16/R	0.25/S
Ceftaxitin	≥128/R	≥128/R	0.25/S
Aztreonam	≥128/R	≥128/R	0.25/S
Ertapenem	8/R	8/R	0.125/S
Imipenem	16/R	16/R	0.125/S
Ciprofloxacin	8/R	8/R	0.25/S
Levofloxacin	4/I	4/I	1/S
Amikacin	1/S	0.25/S	0.25/S
Gentamicin	0.5/S	0.25/S	0.25/S
Tobramycin	8/I	0.25/S	0.25/S
Tigecycline	1/S	0.25/S	0.125/S
Nitrofurantoin	16/S	16/S	16/S
Trimethoprim/ sulfamethoxazole	≥320/R	20/S	20/S

<sup>a</sup>MIC for antimicrobial drugs tested, mg/L. The MICs were interpreted according to the CLSI guidelines.

In contrast to pKPC-cd17, an insertion of 14,808 bp DNA fragment and IS4321 gene were observed between *merT* gene and hypothetical protein in p1713-KPC and plasmid: 1, respectively. The insertion sequence of p1713-KPC harbored 18 different



genes, which include the quinolone resistance *qnrS2* gene. Interestingly, BLAST search of this insertion sequence revealed a 99% identity with *A. hydrophila* IB101 plasmid AHIB101-pBF7.8 at 100% coverage (Figure 3). We speculate that p1713-KPC has been formed by an IncP-6 plasmid fusing with the *qnrS* plasmid after one of them has acquired extra DNA with *xerD* etc. Furthermore, *bla*<sub>KPC-2</sub> gene was found to be located on a 14.5 kb Tn3 transposon element in p1713-KPC with the linear structure:  $\Delta tnpA$  Tn3-IS<sub>Apu1</sub>-IS<sub>Apu2</sub>- $\Delta tnpA$ -Tn3-*tnpR* Tn3-IS<sub>Kpn27</sub>- $\Delta bla_{TEM-1}$ -*bla*<sub>KPC-2</sub>- $\Delta ISKpn6$  (Figure 3). Previous studies of *bla*<sub>KPC-2</sub> have indicated its association with Tn3-based transposon and responsible for its widespread among *Enterobacteriaceae* in different geographical locations of China (Shen et al., 2009; Yang et al., 2013).

## Plasmid Mobilization

IncP-6 plasmids are found in various species from both clinical and environmental sources, suggesting that they have a broad host range and potential for long-term persistence in the environment (Naas et al., 2013; Dai et al., 2016; Wang et al., 2017; Yao et al., 2017). Interestingly, most of these *bla*<sub>KPC-2</sub> bearing IncP-6 plasmids were detected in China. Our findings further revealed that *bla*<sub>KPC-2</sub> carrying IncP-6 plasmid has moved into *Aeromonas* spp. Of note, p1713-KPC is lacking of a *tra* module encoding primary pilus, which explains the failure of plasmid conjugation. Studies had shown that plasmids could be able to transfer by conjugation if the right self-transmissible plasmids are present (Smillie et al., 2010). However, due to unknown reasons,

mobilization transfer from donor *A. taiwanensis*, which harbored resident plasmid and electrotransformed plasmid containing a *tra* module, to the recipient cells was not successful. Nevertheless, acquisition of free DNA is a general feature of *Aeromonas* isolates (Huddleston et al., 2013). And the IncP plasmid, which is a broad-host-range incompatibility plasmid, has also been proved with the potential to mediate the dissemination of antibiotic resistant genes from the *Enterobacteriaceae* to other Gram-negative bacteria, such as *Pseudomonas aeruginosa* (Zhao et al., 2017). Since the complex mechanism of plasmid transfer is still not fully understood and these 3 IncP plasmids do encode mobilization functions, potential detection of such incP plasmids from clinical isolates should be closely monitored. Therefore, it is suggested that IncP-6 plasmids may act as an important vector responsible for the genetic transmission of *bla*<sub>KPC</sub> and *qnrS2* among the aquatic *Aeromonas* spp.

## CONCLUSION

The analysis of plasmids carrying different resistance genes is pivotal for characterization of bacterial isolates, since they play a significant role in transmission of antibiotic resistance (Galata et al., 2018). In this study, we reported the complete sequence of p1713-KPC, a novel IncP-6 plasmid identified from environmental *A. taiwanensis* isolates. To our knowledge, this is the first report describing the co-existence of *bla*<sub>KPC-2</sub> and *qnrS2* on the same plasmid in *A. taiwanensis* isolates. The emergence and dissemination of such plasmids in environmental



isolates deserve special attention. Given that *Aeromonas* spp. are ubiquitous organisms isolated from a wide range of environmental niches, they might act as important vectors for the dissemination of plasmid-mediated carbapenem- and quinolone-resistance genes. Therefore, potential detection of such plasmids from clinical isolates should be closely monitored.

## DATA AVAILABILITY

The whole genome shotgun project of the *A. taiwanensis* isolates has been deposited into DDBJ/EMBL/GenBank under the Bioproject accession number PRJNA478520. The complete nucleotide sequence of plasmid p1713-KPC has been deposited into the GenBank database under GenBank accession number MH624132.

## AUTHOR CONTRIBUTIONS

XH, YM, and BZ conceived and designed the experiments. XH, XY, YS, HX, YL, YK and LS performed the experiments. LG, JS, and FY analyzed the data. BZ and XH wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02132/full#supplementary-material>

**FIGURE S1 |** PFGE profiles of *A. taiwanensis* strains. A dendrogram of PFGE profiles was constructed with BioNumerics v7.6 by using UPGMA (unweighted pair group method with averages) clustering. Isolates with a similarity cut-off of  $\geq 80\%$  were considered as pulsotypes. The scale bar indicates the percentage of genetic relatedness.

**TABLE S1 |** Primers used in the plasmid sequencing of novel IncP-6 plasmid pKPC-1713, harbouring *bla*<sub>KPC-2</sub> and *qnrS2* genes.

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# Genetic Features of *mcr-1* Mediated Colistin Resistance in CMY-2-Producing *Escherichia coli* From Romanian Poultry

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Colistin is a last resort antibiotic used for the treatment of human infections associated with carbapenemase-producing Enterobacteriales. Here, we evaluated the occurrence of *mcr-1* and -2 plasmid-mediated colistin resistance in colistin and/or carbapenem resistant human clinical Enterobacteriales and other gram-negative bacteria ( $n = 543$ ) as well as third generation cephalosporin-resistant (3GCR) *Escherichia coli* isolates from poultry abattoir workers ( $n = 15$ ) and poultry fecal samples ( $n = 92$ ) collected from two geographically separate abattoirs in Romania. which revealed that *mcr-1* was present within four sequence types (STs): ST744 ( $n = 7$ ), ST57 ( $n = 7$ ), ST156 ( $n = 2$ ), and ST10 ( $n = 1$ ). Within STs, serotypes were conserved and, notably, all except one of the *mcr-1*-positive isolates were found to exhibit fluoroquinolone-resistance (FQR) associated SNPs in both *gyrA* and *parC*. While there were variations in genotypes, all isolates belonging to ST744, ST57, and ST156 were rich in resistance determinants, carrying aminoglycoside-modifying enzymes genes, sulfonamide resistance gene *bla*<sub>TEM-1</sub> as well as *bla*<sub>CMY-2</sub> AmpC  $\beta$ -lactamase resistance genes. They also exhibited high similarity in carriage of virulence genes; ST10, however, only carried the *mcr-1* gene. Whole genome sequencing (WGS) analysis also revealed that although the *mcr-1* gene was identified in a diverse population of *E. coli*, two STs (ST57 and ST744) predominated and interestingly, were found in isolates across both abattoirs providing evidence for clonal transmission. Also, two main genomic contexts of *mcr-1* isolates were revealed with all ST57 isolates harboring the *mcr-1* gene between two copies of IS*Ap1* (or the Tn6330 transposon) whilst a common *mcr-1* containing scaffold, highly similar to IncX type *mcr-1*-bearing plasmids (pWI2-*mcr*, Accession number: LT838201), was present among *mcr-1* isolates of varying phylogenetic backgrounds (ST10, ST744 and ST156). The high prevalence of the *mcr-1* gene in poultry *E. coli* isolates with

co-resistance to cephalosporins and quinolones, in a country where antimicrobial use in food production species is poorly regulated, is concerning and the findings from this study should lead to better surveillance of antimicrobial resistance (AMR) in food-production animals in Romania.

**Keywords:** colistin-resistance, plasmid-mediated, *mcr-1* gene, poultry, humans, Romania

## INTRODUCTION

The emergence and spread of carbapenem-resistance due to carbapenemase producing Enterobacteriaceae (CPE) and the lack of new antibiotic developments has led to the reintroduction of colistin for treating patients with CPE-associated infections. Therefore, colistin (also known as Polymyxin E) has been referred to as “a last-resort antimicrobial.” Increased colistin use for treatment of carbapenem resistant bacteria in human patients has led to a rise in colistin resistance due to chromosomal point mutations leading to changes of the lipid A of lipopolysaccharides, the primary target of colistin (Baron et al., 2016). In clinical human use, colistin resistance has been shown to emerge during colistin monotherapy or combination therapy (Mammuna et al., 2012; Matheeussen et al., 2019). Furthermore, colistin has been used worldwide for decades in livestock, especially pig production for prevention or treatment of infections associated with Enterobacteriaceae, as well as growth promoters in countries such as China, India, Vietnam (Kempf et al., 2016). Therefore, it is unsurprising that the extensive use of colistin in food animal production has contributed to further development of colistin resistance (Kempf et al., 2016). Consequently, the discovery of plasmid mediated resistance to colistin via carriage of *mcr-1* in both *Escherichia coli* and *Klebsiella pneumoniae* in human and animal isolates in 2015 (Liu et al., 2016) triggered world-wide concern about the prospect of horizontal transfer of this gene amongst human and animal isolates. This discovery was followed by the investigation of numerous bacterial isolates or DNA sequence collections for the presence of the gene, which revealed *mcr-1* to be widespread in isolates from human, animal and environmental sources from countries on all five continents (Rebelo et al., 2018). This retrospective analysis of existing collections led to the identification of plasmid mediated colistin resistance in isolates collected from as far back as 1980, although most *mcr-1* carriers were identified in gram-negative isolates from 2011 to 2012 onward (Schwarz and Johnson, 2016).

Soon after the discovery of *mcr-1*, other *mcr*- variants such as *mcr-2* and *mcr-3* were identified in bovine and swine *E. coli* isolates from Belgium and China, followed by *mcr-4* in *E. coli* and *Salmonella* spp. from pigs in Italy, Belgium and Spain, and *mcr-5* which was identified in *Salmonella* Paratyphi B from poultry in Germany (Rebelo et al., 2018). Recently, *mcr-6* was described in fecal *Moraxella* spp. from healthy pigs whilst a novel colistin resistance gene (*mcr-7.1*) was described in *K. pneumoniae* isolates recovered from chickens in China (Wang X. et al., 2018; Zhang et al., 2018). Finally and very concerning, the coexistence of *mcr-8* and the carbapenemase-encoding gene *bla<sub>NDM</sub>* was demonstrated in *K. pneumoniae* isolates of livestock origin in

China (Wang X. et al., 2018). Worldwide, there are more reports of *mcr*-mediated resistance in livestock isolates compared to human isolates, which indicates farm animals to be a potential reservoir of plasmid mediated colistin resistance and warrants increased surveillance of animal sources as part of a process to reduce the spread of colistin-resistance. The 2014 report of the (European Centre for Disease Prevention and Control [ECDC], 2015), shows that in Romania and Greece approximately 20% of carbapenem-resistant *K. pneumoniae* isolates from blood cultures were resistant to colistin. However, there is a general lack of surveillance on antimicrobial resistance (AMR) data from animal isolates in Romania and a gap in the knowledge regarding the extent of AMR spread in livestock in this country.

The aim of this study was to investigate the prevalence of plasmid-mediated colistin resistance in a collection of cephalosporin resistant *E. coli* isolates from poultry and colistin/carbapenem resistant human clinical *E. coli* isolates from Romania, a country where antibiotic consumption in livestock is not well monitored. We also aimed to resolve the genomic structure of *mcr*-positive *E. coli* isolates and to identify whether colistin resistance spreads through clonal expansion or acquisition by different isolates. Finally, we aimed to decipher the phylogenetic relatedness and genotype characteristics of isolates obtained from poultry abattoir workers using whole genome sequencing (WGS).

## MATERIALS AND METHODS

### Bacterial Isolates and Colistin Resistance Screening

Third generation cephalosporin resistant (3GCR) *E. coli* isolates obtained from 92 chickens during a previous study (Maciuca et al., 2015) and confirmed to harbor plasmid-mediated AmpC  $\beta$ -lactamase (pAmpC) and/or extended spectrum  $\beta$ -lactamase (ESBL) production were screened for colistin resistance. The isolates were obtained during October 2011–October 2012, from broiler chicken caecal samples collected from two geographically separate abattoirs (A1 and A2) in the North-East (NE) of Romania. In that study, staff working at one broiler abattoir (A1) were also screened for fecal carriage of multidrug resistant (MDR) *E. coli* to determine the potential for ESBL and AmpC producing *E. coli* transmission between the abattoir workers and the poultry they handle. Fifteen 3GCR human *E. coli* isolates obtained from abattoir workers at abattoir A1 were included for colistin-resistance screening. In addition, to determine the prevalence of *mcr*-mediated colistin resistance amongst human clinical isolates from Romanian hospitals, a total of 543 colistin and/or carbapenem resistant isolates, obtained from clinical



specimens analyzed between 2014 and 2017 were included in the study. These isolates consisted of Enterobacteriales, mainly *K. pneumoniae* ( $n = 223$ ), *E. coli* ( $n = 105$ ), *Serratia marcescens* ( $n = 50$ ), *Enterobacter cloacae* ( $n = 3$ ), *Morganella morganii* ( $n = 3$ ) and gram-negative non-fermentative bacteria (*Acinetobacter baumannii*,  $n = 28$  and *Pseudomonas aeruginosa*,  $n = 131$ ) and were obtained from four large national human hospitals (H1-Bucharest, H2-Târgu Mureș, H3-Cluj-Napoca and H4-Timișoara) and two hospitals from Iași, in North-East Romania (H5 and H6). The bacterial isolates from the Iași hospitals were from the same geographical area (same county) as the poultry isolates (Figure 1).

For the current study, colistin resistance screening was performed on MacConkey agar with colistin sulfate (Sigma-Aldrich, United Kingdom) included in the media at the (Eucast The European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2018) breakpoint concentration ( $2 \mu\text{g/ml}$ ). Where growth occurred on colistin sulfate MacConkey agar, two individual colonies (from light growth) or three individual colonies (from moderate-heavy growth) were subcultured onto 5% sheep blood agar (all media from Oxoid, Basingstoke, United Kingdom). Cell lysates from all subcultured colonies were used for DNA extraction and PCR detection of *mcr-1* and *mcr-2* gene as previously described (Liu et al., 2016; Xavier et al., 2016). The resulting amplicon DNA sequences were analyzed by Sanger sequencing and compared using BLASTn against sequences in GenBank.

## Antimicrobial Susceptibility Testing

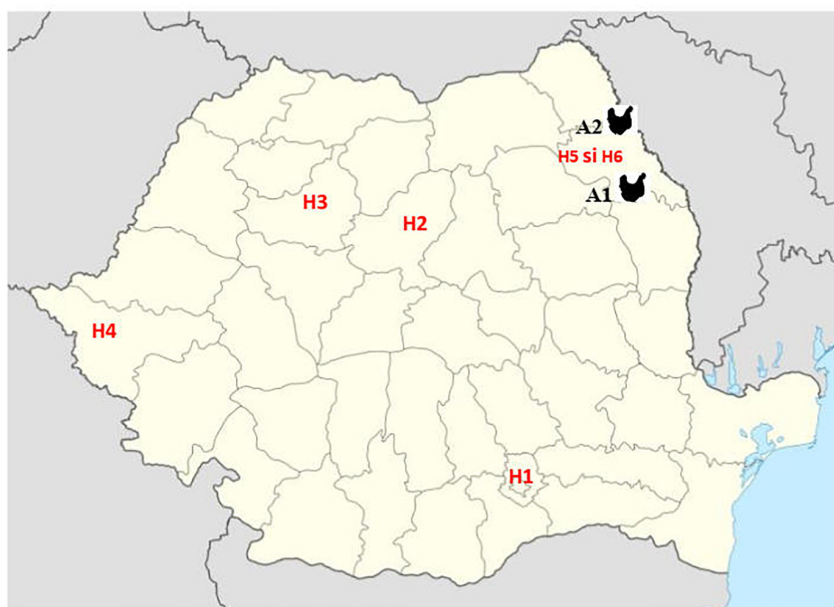
All human clinical isolates were included in the study based on their characterization as colistin or carbapenem resistant

following susceptibility testing which was performed as part of the clinical investigation in the participating hospitals. Antimicrobial susceptibility testing was performed by disk diffusion for all *mcr*-positive isolates with a panel composed of ampicillin ( $10 \mu\text{g}$ ), amoxicillin/clavulanic acid ( $30 \mu\text{g}$ ), ceftiofur ( $30 \mu\text{g}$ ), cefepime ( $10 \mu\text{g}$ ), ceftazidime ( $30 \mu\text{g}$ ), ciprofloxacin ( $5 \mu\text{g}$ ), gentamicin ( $10 \mu\text{g}$ ), streptomycin ( $10 \mu\text{g}$ ), tetracycline ( $30 \mu\text{g}$ ), trimethoprim/sulfamethoxazole 1:19 ( $25 \mu\text{g}$ ) (all disks and media from Oxoid, United Kingdom) following EUCAST methodology and interpretation guidelines. Clinical and Laboratory Standard Institute (CLSI, 2011) interpretation guidelines were used for nalidixic acid, streptomycin, ceftiofur and tetracycline resistance. The minimal inhibitory concentration (MIC) of colistin was determined for all *mcr*-positive isolates by broth dilution. Colistin sulfate (Sigma-Aldrich Company Ltd., Dorset, United Kingdom) was used for broth microdilution and performed in accordance with the EUCAST MIC method with dilutions ranging from  $0.125 \mu\text{g/ml}$  to  $64 \mu\text{g/ml}$  which were performed in untreated 96-well polystyrene microplates (Greiner, Frickenhausen, Germany) according to the current recommendations of the joint CLSI-EUCAST Polymyxin Breakpoints Working Group<sup>1</sup>.

## Conjugation and Molecular Characterization of Isolates

To ascertain whether colistin resistance was transferable, conjugation was performed by broth mating. Conjugation assays were attempted for all isolates found to carry the

<sup>1</sup>[http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/General\\_documents/Recommendations\\_for\\_MIC\\_determination\\_of\\_colistin\\_March\\_2016.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf)



**FIGURE 1 |** Geographic position of samples from Romanian human hospitals [H1 – Bucharest, H2 – Târgu Mureș, H3 – Cluj-Napoca, and H4 – Timișoara and Iași (H5 and H6)] and poultry abattoirs (A1 and A2).

*mcr-1* gene, using a streptomycin-resistant *E. coli* HB101 strain as a recipient as previously described (Oliver et al., 2002). Transconjugants were selected on nutrient agar (Oxoid, United Kingdom) supplemented with streptomycin (50 µg/ml) and colistin (2 µg/ml).

## Whole Genome Sequencing and Genome Assembly

*Escherichia coli* from poultry ( $n = 97$ ) and human abattoir workers ( $n = 15$ ) displaying reduced susceptibility to third generation cephalosporins (3GCR) were characterized by WGS to determine the genetic features and phylogenetic relationships shared among the collection. Specifically, we determined phylogroup, multi-locus sequence type (MLST), antibiotic resistance and virulence gene carriage and plasmid incompatibility marker carriage.

DNA was purified from *mcr-1* containing isolates using QIAGEN-QiaAmp DNA Mini kit (Qiagen, United Kingdom). DNA was quantified using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, United Kingdom) and standardized to 30 ng/µL before being prepared into sequencing libraries using Nextera® DNA Library Preparation kits. Sequencing was performed with an Illumina HiSeq® 2500, generating 150-bp paired end reads. Read quality was assessed using FastQC version 0.11.5 before *de novo* assembly using the A5 assembly pipeline version A5-miseq 20150522 (Darling et al., 2014a).

## Phylogenetic Classification, Genotyping, and Fluoroquinolone Resistance-Associated Single Nucleotide Polymorphism Analysis

Determination of e-serotype, phylogroup, MLST and carriage of genes of interest, including carriage of SNPs associated with fluoroquinolone resistance (FQR), was undertaken using the read-mapping tool ARIBA (Hunt et al., 2017). Nucleotide sequences were sourced from various public databases including VirulenceFinder, PlasmidFinder, and ResFinder from the Center for Genomic Epidemiology (Siguier et al., 2006; Zankari et al., 2012; Joensen et al., 2014) and the SRST2 serotype database (Inouye et al., 2014). Additional sequences of interest not contained therein were sourced from the NCBI nucleotide database, ISfinder (Carattoli et al., 2014) and Virulence Factor Database (Chen et al., 2005). Processing of ARIBA output files was undertaken using a custom script described in 2019 (Cummins et al., 2019). Specific versions of nucleotide databases used in the analysis, as well as workflows detailing all *in silico* analyses and the dependencies and software versions utilized to this end, are available on GitHub<sup>2</sup>.

## Phylogenetic Trees

Maximum-likelihood phylogenetic tree analyses were produced using the PhyloSift pipeline version 1.0.1 (Darling et al., 2014b) and FastTree 2.1.8 (Price et al., 2010), modified to resolve

short branches as previously described (Wyrsh et al., 2015). Phylogenetic SNP trees were generated using Snippy version 4.3.60, Gubbins version 2.3.4 (Croucher et al., 2015), SNP-sites version 2.4.1 (Page et al., 2016) and FastTree 2.1.8. Trees were visualized using iTOL (Letunic and Bork, 2006) or the R package ggtree version 1.8.2. SNP counts were generated using SNP phylogeny<sup>3</sup>. Details of these workflows are also available at <https://github.com/maxlcummins/Romanian-mcr-1-Escherichia-coli/>.

## Global Phylogenetic Comparisons

The EnteroBase Backend Pipeline v1.1.2 (Alikhan et al., 2018) was used to determine the core-genome MLST (cgMLST) of strains found to carry *mcr-1*, according to their carriage of allelic variants of 2512 core genetic loci. Subsequently, a collection of the 116,610 strains accessible via EnteroBase (at time of writing, August 14, 2019) were compared to the *mcr-1* positive lineages under analysis, to provide lineage-specific epidemiological insights. Specifically, the most closely related strains, and the most closely related strains carrying *mcr-1*, were identified. Generally, default settings were used, except for selected reference strains for SNP analyses involving ST57, ST744, ST10, and ST156 being Liv111, Liv111M, Liv37M and Liv30MB, respectively. These analyses were facilitated by EnteroBase SNP analysis pipelines and Hierarchical Clustering of CgMLST (HierCC), which are described at <https://enterobase.readthedocs.io/>. See specific workflows at <https://github.com/maxlcummins/Romanian-mcr-1-Escherichia-coli/>.

## Determination of *mcr-1* Gene Context

Exploratory analysis of the genetic contexts of *mcr-1* in relevant assemblies was undertaken using megablast<sup>4</sup> on default settings with the NCBI nucleotide collection database. Subsequent analysis involved the use of Blast Ring Image Generator (BRIG) version 0.95 (Alikhan et al., 2011) in combination with PATRIC<sup>5</sup> (Wattam et al., 2017) and Snapgene<sup>6</sup>, to provide insight into the potential genetic context of the *mcr-1* genes. More detailed methodology is available at <https://github.com/maxlcummins/Romanian-mcr-1-Escherichia-coli/>.

## RESULTS

### Bacterial Isolates, Antimicrobial Susceptibility Testing, and PCR

Initially, PCR testing identified plasmid mediated colistin resistance associated with *mcr-1* amongst 3GCR *E. coli* isolates obtained from eleven chickens (11/92, 11.9%) from both abattoirs (A1 and A2). Susceptibility testing of individual colonies obtained on the screening medium, indicated that four chickens were likely to carry two different *mcr-1* positive *E. coli* (isolates 37 and 37M; 40 and 40M; 95 and 95M; 111 and 111M),

<sup>3</sup>[https://github.com/bogemad/snp\\_phylogeny](https://github.com/bogemad/snp_phylogeny)

<sup>4</sup><https://blast.ncbi.nlm.nih.gov/Blast.cgi>

<sup>5</sup><https://www.patricbrc.org/>

<sup>6</sup><https://www.snapgene.com>

<sup>2</sup><https://github.com/maxlcummins/Romanian-mcr-1-Escherichia-coli/>

whilst *mcr-1* positive *E. coli* from another chicken showed three different antibiotypes (isolates 30, 30MA and 30MB). In total, 17 *mcr-1* positive *E. coli* isolates were obtained from 11 chickens and were characterized further. In all cases, Sanger sequencing demonstrated 100% sequence identity with *mcr-1* gene sequences deposited in the NCBI database (GenBank: KU743384.1). Eleven *mcr-1* positive *E. coli* isolates were obtained from 7 chickens from abattoir A1 and the remaining 6 isolates were obtained from 4 birds from abattoir A2. The *mcr-2* gene was not detected in any of the poultry isolates. In addition, *mcr-1* and *mcr-2* genes were not detected in any fecal isolates from abattoir workers or in any of the 543 colistin and/or carbapenem resistant human clinical isolates (both Enterobacteriales and gram-negative non-fermentative bacteria) investigated across the hospital populations. The MIC of colistin for *mcr-1* positive isolates varied between 4–8 µg/ml and disk diffusion susceptibility testing indicated resistance to all tested beta-lactam and quinolone antimicrobials and variable resistance to chloramphenicol and tetracyclines. However, all isolates were fully susceptible to carbapenem agents.

Conjugation experiments were successful in only one isolate (40M) where PCR identified *mcr-1* but not *bla<sub>CIT-M</sub>* or *bla<sub>TEM</sub>* genes in the transconjugant.

### WGS of the *mcr-1* Positive *E. coli* Isolates

Genomic data sets were obtained through short-read sequencing and deposited in the NCBI Sequence Read Archive (SRA) under the BioProject PRJNA560337. Individual Accession numbers can be found in **Supplementary Table S1**. Four sequence types were determined to carry *mcr-1*; ST744 ( $n = 7$ ), ST57 ( $n = 7$ ), ST156 ( $n = 2$ ), and ST10 ( $n = 1$ ). Carriage of genes and the sequence types, serotypes and phylogroups of *mcr-1*-positive samples can be seen in **Figure 2**. Within sequence type serotypes were conserved, and notably, all but one of the *mcr-1*-positive isolates, Liv37M:ST10:A:O16:H48, were found to carry FQR associated SNPs in both *gyrA* and *parC* (S83L and D87N in the former and S80I in the latter).

ST744:A:O89-O186:H9 isolates were determined to be rich in resistance gene determinants, with all isolates found to carry *aac-3-IIa*, *aph-3-Ia* aminoglycoside modifying enzymes; *strAB* streptomycin resistance gene; *sul2*-sulfonamide resistance gene; *bla<sub>TEM-1</sub>* narrow-spectrum β-lactamase resistance gene; *bla<sub>CMY-2</sub>* giving resistance to 3rd gen cephalosporins and *mcr-1* colistin resistance gene; six of seven isolates also carried the tetracycline resistance gene *tetA*. This same sequence type also consistently carried *repA* genes associated with incompatibility types IncF, IncX, and IncI, indicating the presence of three plasmid types. Carriage of extra-intestinal pathogenic *E. coli* (ExPEC) virulence associated genes (VAGs) including *cvABC/cvi*, *etsA*, *hylF*, *iroN*, *iss*, *iucD/iutA*, *ompT*, *sitA*, *traT*, and *tsh* was also widespread across this sequence type.

Within isolates of ST57:D:O86:H25, all isolates were found to carry *aac-3-IIa*, *aph-3-Ia*, *bla<sub>CMY-2</sub>*, *bla<sub>TEM-1</sub>*, *mcr-1*, and *sul2*. Similarly, while there was variability between samples in carriage of Col plasmid associated replicons, there was consistency in these isolates with regard to the carriage of IncF, IncX and p0111 *repA* genes. Additionally, all isolates carried a repertoire

of ExPEC-associated VAGs including *cvaA/cma*, *etsA*, *hylF*, *iroN*, *iss*, *iucD/iutA*, *ompT*, *traT*, and *tsh*, while 4/7 ST57:D:O86:H25 isolates carried *sitA*.

While there were variations in genotype between ST156:A/B1:O4:H28 isolates, they exhibited high similarity in carriage of virulence, resistance and plasmid associated genes. Both isolates carried p0111, IncI and IncX *repA* genes, as well as the AMR genes *aph-3-Ia*, *bla<sub>CMY-2</sub>*, *bla<sub>TEM-1</sub>*, *mcr-1*, *mef(B)* encoding for macrolide resistance, *sul3* and *tet(B)*. VAGs were relatively low in abundance within this lineage, with *fimH*, *hek*, *iss* and *lpfA* and *yeeT* the only VAGs detected within both isolates.

Lastly, a singular isolate of ST10:A:O16:H48, Liv37M, was found to carry *mcr-1*. This isolate also carried an IncX4 *repA* gene; however, no other plasmid replicons were detected according to ARIBA. Similarly, apart from carrying *mcr-1*, this Liv37M carried no AMR genes, and did not exhibit extensive carriage of ExPEC-associated VAGs.

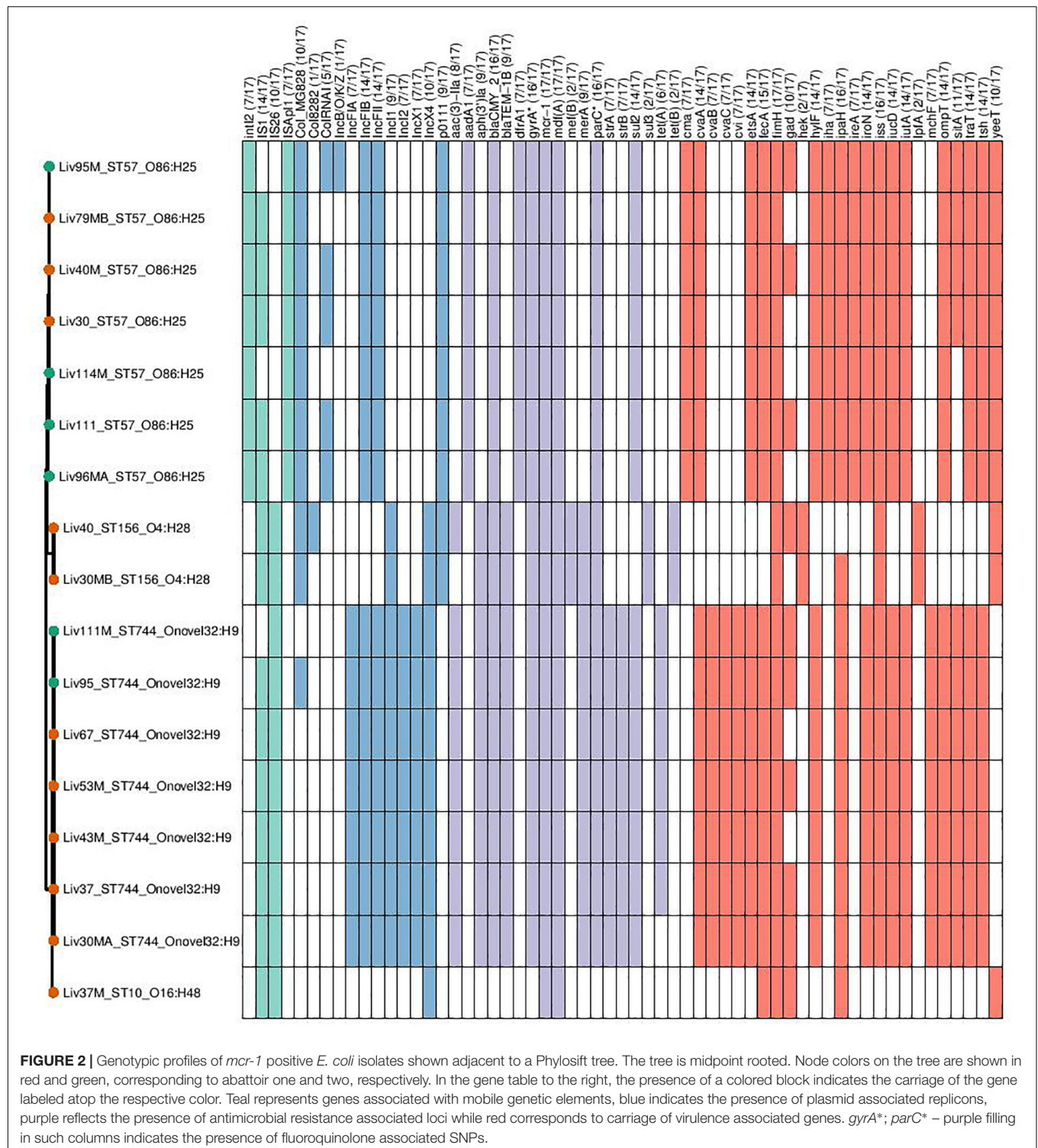
### Phylogenetic Relationship of the Poultry Isolates (Abattoirs A1 and A2) and Human Isolates From Abattoir A1

Phylogenetic overlap between human and poultry isolates was detected only superficially at the ST level, with ST10 isolates being common between both such sources. These samples differed in their serotypes and genotypes, however (**Figure 3**). In regard to the *mcr-1* positive lineages belonging to ST57 and ST744 among poultry from abattoir 1 and 2, it was found that within sequence types, fewer than 10 SNPs were identified. Additionally, samples Liv111:ST57 and Liv40M:ST57, despite being collected from different abattoirs, were found to differ by only 1 SNP. The core genomes of isolates Liv111M:ST744 and Liv30MA:ST744, which were also sourced from different abattoirs, were found to be indistinguishable by our SNP analysis [**Supplementary Table S2** (ST57 SNP counts) and **Supplementary Table S3** (ST744 SNP counts), respectively]. It should be noted, however, that both such pairs of isolates exhibited differences in their genotypes (**Figures 4, 5**). Nonetheless, the extent of sequence homology in the core genomes of both pairs of isolates, as indicated by SNP analysis, suggests they have a recent shared origin. Analysis using cgMLST was also in support of close inter-sequence type relatedness, indicating that all *mcr-1* positive strains of ST744 other than Liv37 differ only by two or less of alleles across a 2506 core genomic loci. Similarly, all but three of the *mcr-1* positive ST57 strains (Liv30, Liv40M and Liv95M) were of the same degree of relatedness, the latter of which differed by five or fewer cgMLST alleles.

### Phylogenetic Characteristics of *mcr-1* Positive *E. coli* Isolates

Overall, MLST and phylogroup (PG) typing data showed that there was evidence of clonal dissemination with some STs/PGs (i.e., ST57/D, ST744/A) being present across isolates from both abattoirs (**Figure 3**). In addition, MLST and PG typing has confirmed that one chicken from abattoir A1 was colonized with *mcr-1* *E. coli* isolates belonging to three different STs/PGs (i.e., isolates 30, 30MA and 30MB were typed to ST57/D,





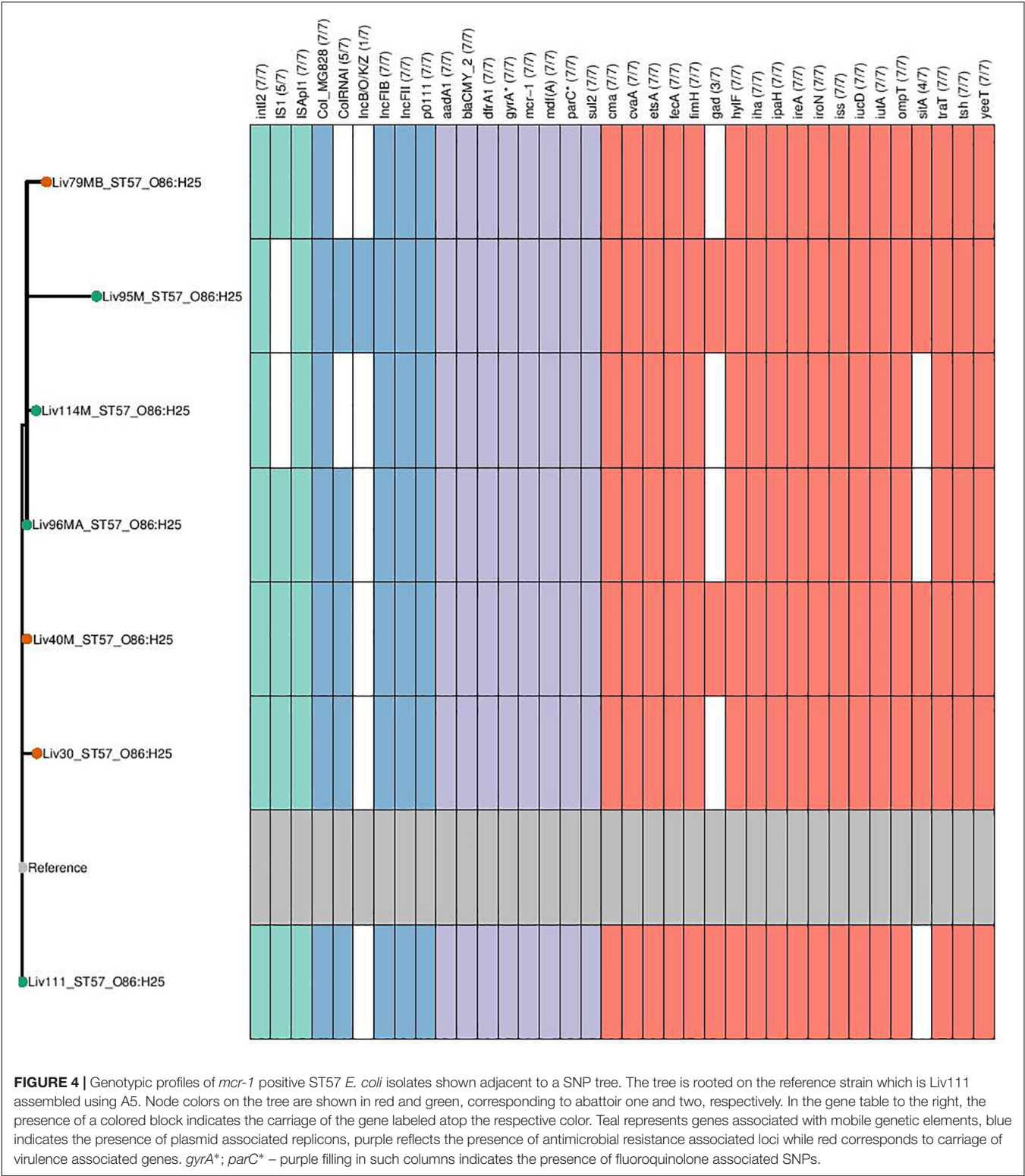
ST744/A and ST156/B1) whilst four chickens (two from each abattoir) were colonized with *mcr-1* positive *E. coli* isolates belonging to two different STs and in three cases, also different phylogroups (Table 1).

On EnteroBase, the most closely related strains to the ST744 lineage were found to differ by  $\leq 20$  of 2512 cgMLST alleles,

with a single strain (SRA Accession No. ERR712576) isolated in 2013 from a hospital patient in Muenster, Germany, being found to exhibit just 19 SNPs relative to Liv111M:ST744. This strain was found by BLASTn to be *mcr-1* negative. The most closely related *mcr-1* carrying strain to Liv111M:ST744 was sourced from Italy in 2014 (additional metadata is lacking, EnteroBase



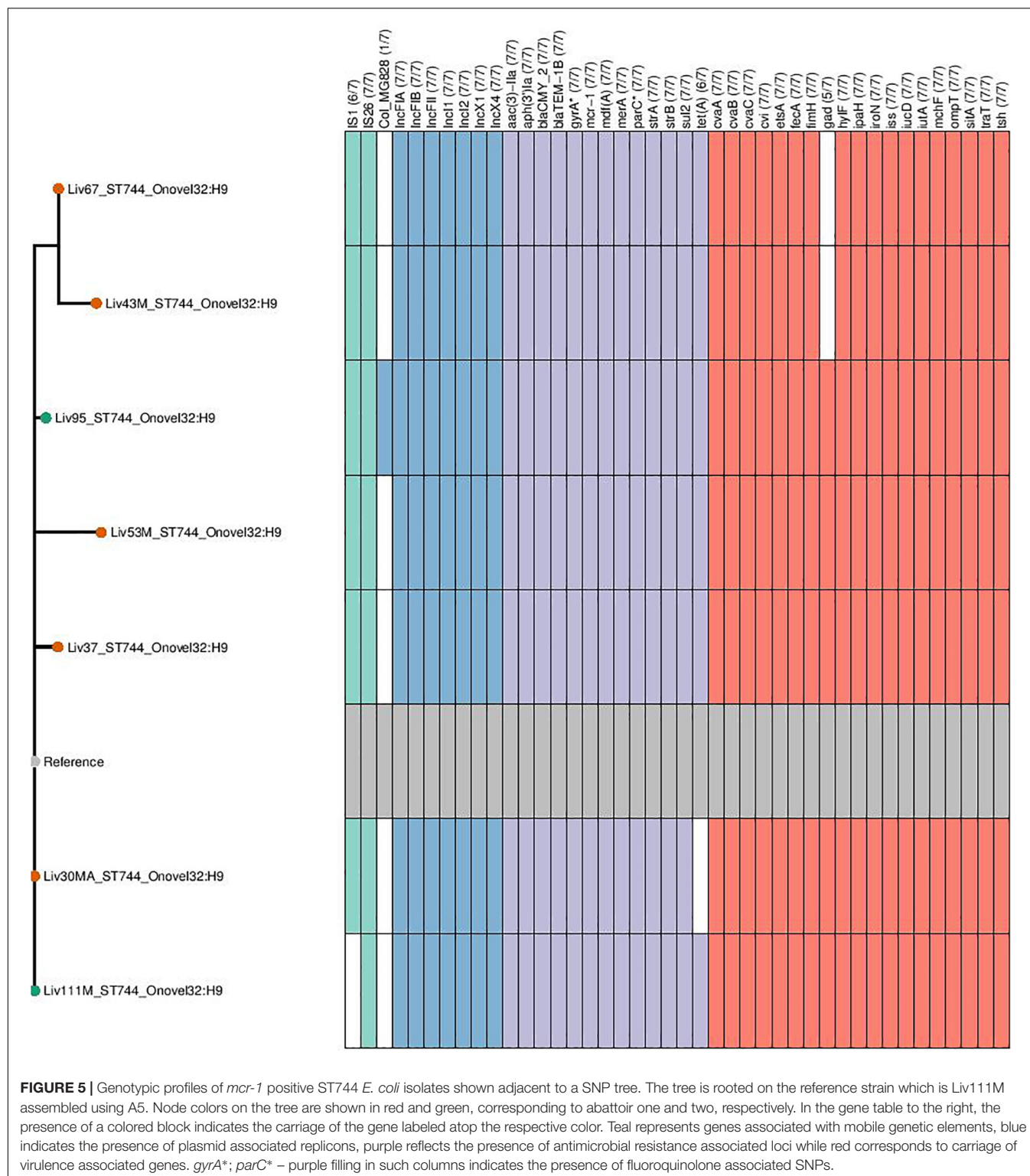




**Investigation Into the Genetic Contexts of *mcr-1* Genes**

Exploratory BLAST analysis revealed two genomic contexts of *mcr-1* within the collection. ST57 isolates carrying *mcr-1* were

found to have this gene localized between two copies of *ISAp11*, a composite transposon known as Tn6330. Scaffold breaks occurred within these IS elements, as is commonly the case with short-read sequencing and subsequent assembly, preventing their



linkage to plasmid or chromosomally associated scaffolds, and limiting the determination of their context.

However, ST744, ST156, and ST10 isolates carrying *mcr-1* did not carry this insertion sequence proximal to the *mcr-1* gene. Further analysis using BLAST revealed that the *mcr-1* containing

scaffolds in these samples, some greater than 30kb in length, exhibited extensive sequence homology with publicly available IncX type *mcr-1*-bearing plasmids such as pWI2-mcr. Presence of *mcr-1* on a plasmid similar to pWI2-mcr was investigated using BRIG, as shown in **Figure 6**. All of these latter samples were

**TABLE 1 |** Summary of epidemiological data and genotypic typing of the 17 *mcr-1* positive *Escherichia coli* isolates obtained from 11 poultry fecal samples collected from both abattoirs (A1 and A2).

	Sample no.	Time/date of isolation	Susceptibility profile	Colistin MIC $\mu$ g/ml	ST	O:H	PG	IncX	Tn6330
Abattoir A1	30	09/10/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP	4	57	O86:H25	D	–	+
	30MA	09/10/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP, CN, S	8	744	O32:H9	A	+	–
	30MB	09/10/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP, STX, CN, TE	4	156	O4:28	B1	+	–
	37	03/04/2011	AMP, AMC, FOX, EFT, CPD, NA, CIP, CN, S	4	744	O32:H9	A	+	–
	37M	03/04/2011	AMP, AMC, CPD	4	10	O16:H48	A	+	–
	40	11/12/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP, STX, CN, TE	4	156	O4:28	B1	+	–
	40M	11/12/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP	4	57	O86:H25	D	–	+
	43M	09/10/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP, CN, S	4	744	O32:H9	A	+	–
	53M	09/10/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP, CN, S	4	744	O32:H9	A	+	–
	67	09/10/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP, CN, S	4	744	O32:H9	A	+	–
	79MB	09/10/2010	AMP, AMC, FOX, EFT, CPD, NA, CIP, CN, S	4	57	O86:H25	D	–	+
Abattoir A2	95M	03/04/2011	AMP, AMC, FOX, EFT, CPD, NA, CIP, TE	4	57	O86:H25	D	–	+
	95	03/04/2011	AMP, AMC, FOX, EFT, CPD, NA, CIP, S, TE	4	744	O32:H9	A	+	–
	96M	05/06/2011	AMP, AMC, FOX, EFT, CPD, NA, CIP, S, TE	4	57	O86:H25	D	–	+
	111	05/06/2011	AMP, AMC, FOX, EFT, CPD, NA, CIP, C, TE	4	57	O86:H25	D	–	+
	111M	05/06/2011	AMP, AMC, FOX, EFT, CPD, NA, CIP, C, S	4	744	O32:H9	A	+	–
	114M	05/06/2011	AMP, AMC, FOX, EFT, CPD, NA, CIP	4	57	O86:H25	D	–	+

shown to exhibit high coverage and sequence homology with the reference plasmid.

DISCUSSION

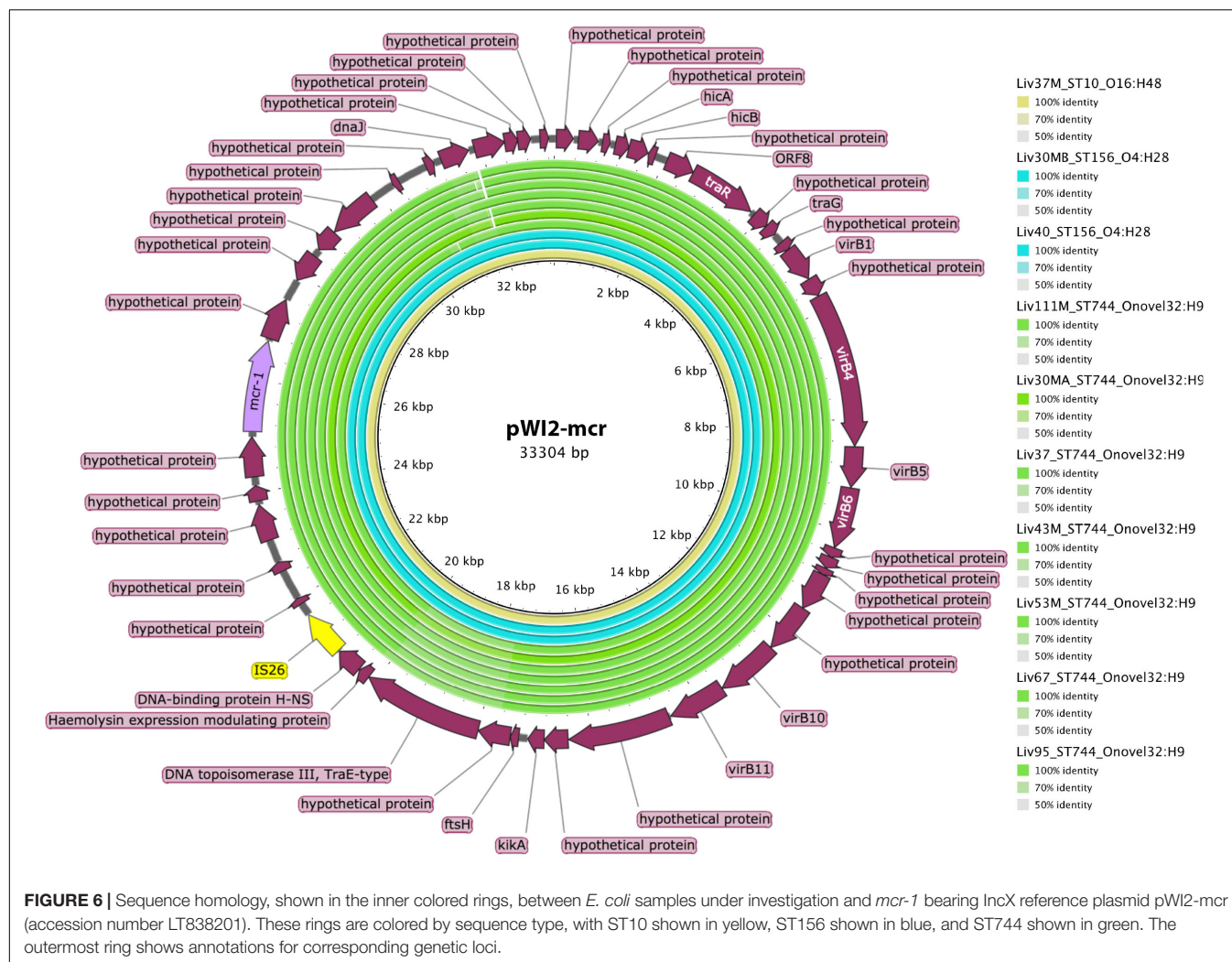
Here, we report a high prevalence (11.9%) of *mcr-1* plasmid mediated colistin resistance in commensal 3GCR AmpC producing *E. coli* from poultry sampled in two abattoirs in North-Eastern Romania in 2011/2012. Although we could not obtain data on antimicrobial usage on the farms where the samples originated, this prevalence may suggest frequent use of colistin and possibly other antimicrobials in the poultry industry in this region. Reports of the *mcr-1* gene in food production species are increasingly emerging worldwide, although in Europe they are still sparse and mainly originate from countries where large collections of isolates from national surveillance studies, or large datasets obtained by WGS, are readily available (Hasman et al., 2015; Kluytmans-Van Den Bergh et al., 2016; Perrin-Guyomard et al., 2016). The fact that the collection of poultry *E. coli* isolates investigated in this study is relatively small and comes from one region of Romania, makes the findings quite remarkable and could represent just a snap-shot of the general situation in the farmed poultry population in this country. Although is difficult to make direct *mcr-1* prevalence comparison between this and other studies due to different selection criteria for the isolates investigated, this is still likely to be one of the highest prevalences of *mcr-1* encoded colistin-resistance in commensal 3GCR *E. coli* reported for poultry in Europe and is certainly concerning. An overall prevalence of 1.2% *mcr-1* positive *E. coli* was found in a pan-European surveillance study investigating poultry isolates from 11 European countries (El Garch et al., 2018); a higher prevalence of this gene has been reported in turkey isolates from

Italy (25%) as well as in poultry from China and Tunisia (Skov and Monnet, 2016; Alba et al., 2018).

Contrarily, we did not find evidence of *mcr*-mediated colistin resistance in phenotypically colistin and/or carbapenem resistant Enterobacteriales or gram-negative non-fermentative bacteria human clinical specimens from Romania. The human clinical isolates originated from six hospitals across the country, including two hospitals from the same area were the poultry samples originated. This may indicate that, at least up to 2017, when the last human isolates were analyzed, there is no evidence of over spilling of *mcr*-mediated resistance into the human population via the food chain or other routes. These findings are in agreement with other studies which show that the occurrence of *mcr* in human clinical isolates is still rare (Kluytmans-Van Den Bergh et al., 2016).

In this study, *mcr-1* was also not identified in 3GCR *E. coli* from abattoir workers fecal samples. However, interpretation of the role that the high prevalence of *mcr-1* identified in *E. coli* from poultry samples may have in the transmission of colistin-resistance to humans in this region or to the workers from the food chain, has to be tempered by the relatively low number of human clinical isolates and fecal samples from abattoir workers available in this study. While overlap was detected between poultry and abattoir workers at a sequence type level, this overlap was only seen in isolates belonging to ST10. This sequence type (ST10) is a globally distributed lineage associated with a wide breadth of environments including swine (Reid et al., 2017) and also human and poultry fecal carriage (Reid et al., 2019). Comparisons of the serotypes of the ST10 isolates between humans and poultry (within this collection) did not reveal any overlap, nor high phylogenetic relatedness of these isolates. However, other studies have shown that ST10 can play a major role in the





dissemination of *mcr-1* *E. coli* isolates from European farm animals (El Garch et al., 2017).

Most of the *mcr-1* positive *E. coli* identified in this study also carried plasmid mediated AmpC  $\beta$ -lactamase (CMY-2 type) which affords resistance to extended-spectrum cephalosporins and beta-lactam inhibitor combinations. Although *bla*<sub>CMY-2</sub> and *bla*<sub>TEM-1</sub> were not present in the single transconjugant obtained in our experiments, other similar studies have demonstrated that genes encoding colistin and ESC resistance (ESBL/AmpC) are often co-located on the same plasmids (Grami et al., 2016), which raises concerns about co-selection of these genes when either colistin or cephalosporins are used prophylactically in food production animals.

In our study, the *mcr-1* gene was identified in a diverse population of *E. coli* where two STs (ST57 and ST744) predominated. In addition, *mcr-1* was also identified in ST57 and ST744 isolates across both abattoirs, providing evidence for clonal transmission, also evidenced by low ( $\leq 10$ ) SNP counts observed within sequence these sequences types. For instance, ST57/D was identified in seven isolates from both abattoir A1 and A2. This ST has been previously identified in ESBL-producing *E. coli*

isolates from chickens in the United Kingdom, Germany, and Canada as well as in healthy human and food-producing isolates demonstrating its potential for zoonotic transmission (Wang et al., 2013; Lemma et al., 2014). Similarly, ST744/A was identified in both abattoirs and its potential for clonal dissemination was recently demonstrated by its involvement in the epidemic spread of *mcr-3/bla*<sub>CTX-M-55</sub>-positive *E. coli* collected in diseased veal calves from France (Haenni et al., 2018).

Investigations using EnteroBase revealed that while some strains housed in public databases that were closely related to *mcr-1* positive samples under investigation, these publically available strains were determined not to carry *mcr-1*, and that the most closely related strains to those under investigation that do carry *mcr-1* exhibit many (>1500) SNPs relative to any *mcr-1* strain identified within the collection. While only a single reference strain from each sequence type was used for this analysis, the SNP counts within STs from the two abattoirs indicate that the strains are clonal and therefore significantly higher SNP counts would not be expected if a different reference were used from within the *mcr-1* positive lineages. These data indicate that clonal expansion has taken place between sources

prior to the acquisition of *mcr-1* by the Romanian strains of ST744, ST57, ST10. High resolution SNP and cgMLST analysis of publicly available genomes on EnteroBase showed no evidence of clonal associations with Liv30MB or Liv40:ST156.

The fact that *mcr-1* positive ST744 lineages differ by upwards of 1500 SNPs indicates it is likely that this sequence type has undergone multiple *mcr-1* acquisition events. This hypothesis is supported by preliminary analysis of an ST744 *mcr-1* positive *E. coli* isolate that was obtained from a human blood stream infection in Denmark (, which also carried *bla*<sub>CMY-2</sub> (amongst other resistance genes) (Hasman et al., 2015). This strain (EnteroBase Barcode: ESC\_FA2130AA) exhibited 2527 SNPs compared to Liv111M, with preliminary BLAST analysis (data not shown) indicating that the *mcr-1* gene it carries is localized to an IncI plasmid similar to that of ZE36 (Accession No.: KY802014).

The ST57 lineages under investigation seemed relatively distinct from most samples present in EnteroBase, with the closest strains differing by 1687 SNPs and up to 200 cgMLST alleles. Notably, while metadata on public repositories is often lackluster, the most closely related strains (those that differed by 200 or fewer cgMLST alleles) were predominantly sourced ( $n = 25/50$ ) from poultry-associated environments. While 7 of these correspond to ST57 isolates from the present study, removal of these strains still results in 42% (18/43) of strains deposited from seven separate Bioprojects across three countries (Australia, The United States, and Germany) being of poultry-associated origin. Note that while none of these strains other than the Romanian isolates under study carried *mcr-1*, this evidence is in support of the literature that suggests lineages of ST57 are common in poultry (Alonso et al., 2017; Cummins et al., 2019). It is also worth noting that 14% (6/43) of these strains were associated with blood or urine infections in humans, indicating the potential zoonotic role of this sample of strains, a hypothesis strengthened by the rich extraintestinal virulence gene carriage of the subset of ST57 strains in the present study. Analysis of samples on EnteroBase did not indicate that the *mcr-1* strains in the present study are part of a larger, potentially international outbreak, as the closest related strains to Liv30MB that carried *mcr-1* differed by more >6000 SNPs. As online databases are increasingly populated by whole genome sequence data, a different trend may become apparent, however.

Samples Liv30MB:ST156 and Liv40:ST156 were found to be relatively distinct at a genomic level to other isolates present in EnteroBase, differing by more than 2500 SNPs relative to the most closely related isolates, regardless of *mcr-1* carriage status. Similarly, Liv37M:ST10 also did not seem part of a larger clonal outbreak of *mcr-1* carrying strains, however, there were three strains found to differ by  $\leq 10$  SNPs and  $\leq 5$  cgMLST alleles from the strain under discussion. Unfortunately, due to poor metadata pertaining to such strains, little insight can be gained into the origins of these samples, highlighting the importance of researcher diligence in uploading metadata sufficient to facilitate epidemiological investigations; such situations are commonplace when utilizing online sequence repositories. It does, however, indicate a potential shared origin of these strains which, by metrics used in other research, may constitute clonal outbreaks

(Woksepp et al., 2017). This data highlights the value of EnteroBase, and other similar tools such as BacWGSTdb (Ruan and Feng, 2016), in the undertaking of investigations involving en-masse genotypic and phylogenomic characterization; an increasingly important area of research as AMR prospects continue to worsen at a global level.

Investigations into the genetic contexts of *mcr-1* genes revealed two main genomic contexts of *mcr-1* within the collection. Firstly, all ST57 isolates harbored the *mcr-1* gene between two copies of IS*ApI1* (or the Tn6330 transposon) which is consistent with findings from a recent study looking at the global epidemiology of *mcr-1* and which showed that a single mobilization event of the *mcr-1* gene by an IS*ApI1* transposon occurred first in 2006 (Wang R. et al., 2018). According to this study, in some lineages the flanking IS was then lost and the transposon was imported on several plasmid backgrounds which contributed to its spread. This may be the case in the second group of our *mcr-1 E. coli* isolates which belonged to the ST744, ST156 and ST10 which did not carry IS*ApI1*; instead, they harbored a common *mcr-1* containing scaffold highly similar to IncX type *mcr-1*-bearing plasmids such as pWI2-mcr, a plasmid isolated from a clinical *E. coli* isolate from France in 2016 (Beyrouthy et al., 2017). It is also worth noting that while the above IncX associated *mcr-1* isolates were of varying phylogenetic backgrounds (ST10, ST744 and ST156), those carrying Tn6330 appear to be clonal, all being of the same sequence type (ST57) and serotype (ST57:O86H25) and being of almost identical genotype as per the virulence, plasmid and resistance genes analyzed. However, the short read sequencing performed in the current study was insufficient to localize the Tn6330 elements to a chromosomal or extra-chromosomal context. Instead, long read sequencing will be required to gain insight in this regard. Location of *mcr-1* within a composite transposon is consequential however, as it enhances the mobility and recombination of this gene through processes of replicative transposition and also through homologous recombination events involving the IS*ApI1* genes (Snesrud et al., 2016).

It is well recognized that use of antimicrobials in food animals may contribute to development and spread of resistant organisms, particularly so in countries like Romania where antimicrobial use in both human and animals may be poorly regulated. In Romania, this situation is reflected in the high prevalence of ESBL and carbapenemase producing *E. coli* invasive human isolates reported to the EARS-Net, or by the high prevalence of CTX-M-15 ESBL enzymes in commensal *E. coli* from poultry as shown in the original study from where the current isolates were obtained (Maciucă et al., 2015). We could not obtain specific data regarding use of antimicrobials at the local farm level either for treating infections or for prophylaxis, as data on veterinary antimicrobial agents sales is completely lacking from this country. Moreover, Romania has the third highest antibiotic consumption in man in Europe whilst no data is available for consumption of antimicrobials in food-producing animals (European Centre for Disease Prevention and Control, 2014; European Medicine Agency, 2015).

The high prevalence of the *mcr-1* gene in poultry *E. coli* isolates with co-resistance to cephalosporins and quinolones, in a country which exports chicken meat to the global food market,

is certainly concerning. The findings from this study suggest that rigorous surveillance of AMR in food-production animals in Romania is critical for reducing the burden of resistance genes circulating through the food chain and the associated risks for local or European (through export) food industries and markets. Finally, the recent emergence of plasmid-mediated resistance to colistin (Hasman et al., 2015; Falgenhauer et al., 2016; Hu et al., 2016; Kluytmans-Van Den Bergh et al., 2016; Liu et al., 2016; Mulvey et al., 2016; Perrin-Guyomard et al., 2016; Ruppe et al., 2016; Stoesser et al., 2016) has triggered an international review and recommendations for restrictions for colistin use in farm animals (Liu and Liu, 2018). Identifying strategies for implementing these restrictions in countries where antimicrobial use is less strictly regulated is critical for preserving the efficacy of last resort antimicrobials like colistin, for treating human and veterinary infections.

## CONCLUSION

The high prevalence of *mcr-1* plasmid mediated colistin resistance in commensal AmpC producing *E. coli* from poultry in North-Eastern Romania suggests selection of these isolates by prophylactic and/or therapeutic farm use of colistin and/or cephalosporins. This level of AMR contamination in food products will undoubtedly lead to human exposure through the food chain, representing a serious public health risk. At very least, this report provides further substantial evidence for the need to review the extensive use of colistin in food production animals in this country and also emphasizes the need to safeguard or restrict its use as a last resort antimicrobial for treating human and animal infections, especially those associated with gram-negative carbapenemase-producing bacteria.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/Supplementary Files.

## ETHICS STATEMENT

This study uses strains obtained from four human hospitals in Romania. For isolates from two hospitals (Grigore T. Popa University of Medicine and Pharmacy and University of Medicine, Pharmacy, Science and Technology of Târgu Mureș), ethics approval was not required as per local procedures which state that samples/isolates resulted from the routine diagnostic process do not require further ethics approval for their use

in research. The remaining two hospitals (Timișoara and Cluj-Napoca) have generic ethics approvals for research use of isolates derived from the routine diagnostic investigations: Ethics Approval No. 130/13.09.2017 issued by the Ethics Committee of Victor Babes University of Medicine and Pharmacy, Timișoara and Ethics Approval No. 10536/12.06.2018 issued by the Ethics Committee of Clinical Hospital of Infectious Diseases, Cluj-Napoca; both of these approvals include retrospective isolates obtained since 2000.

## AUTHOR CONTRIBUTIONS

IM and AC performed the preliminary molecular testing, analyzed the data, and wrote the manuscript. MC and SD performed the whole genome sequencing analysis, analyzed the data, and wrote the manuscript. IM, CR, and EG collected and performed the phenotypic characterization of poultry isolates. CP, ML, ES, and MF collected and performed the phenotypic analysis of human clinical isolates. SD and DT planned and coordinated the study, analyzed the data, and wrote the manuscript. All authors revised and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02267/full#supplementary-material>

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# Emergence of Genetic Diversity and Multi-Drug Resistant *Campylobacter jejuni* From Wild Birds in Beijing, China

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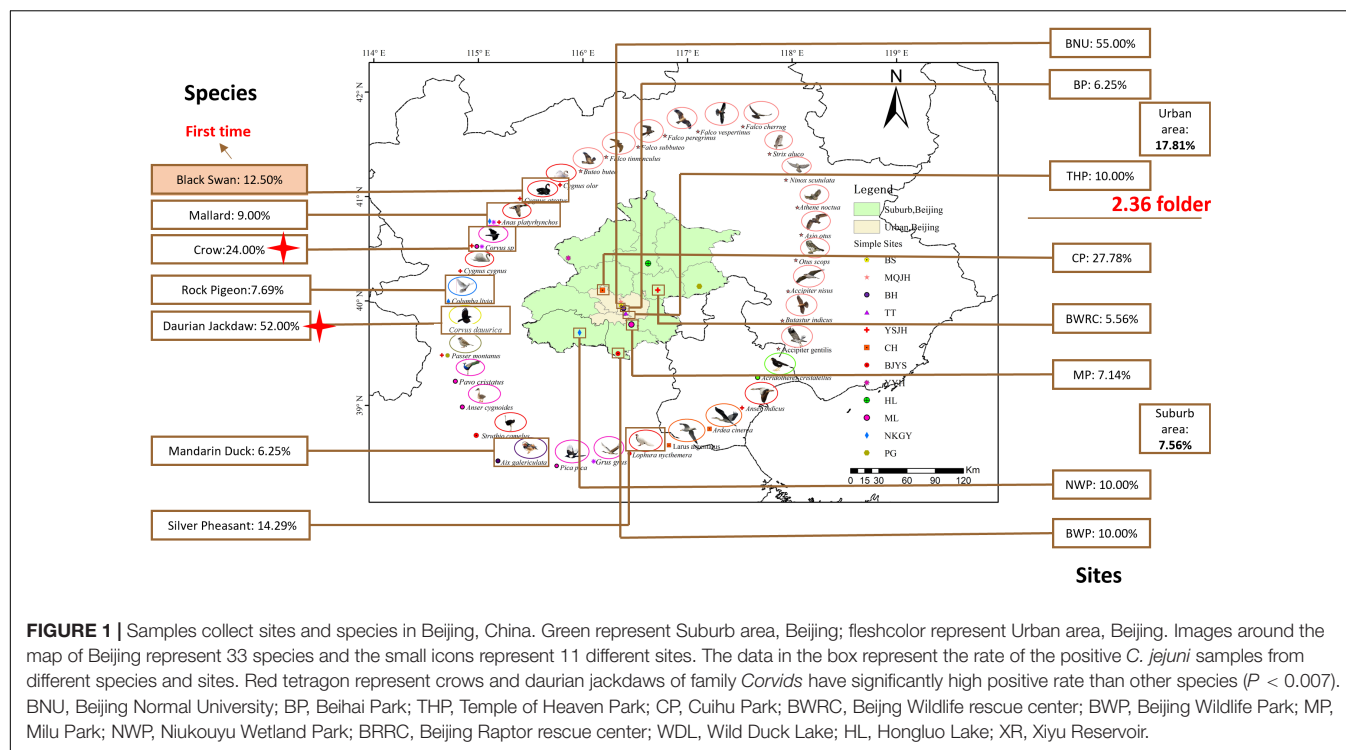
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*Campylobacter jejuni* (*C. jejuni*) is considered as an opportunistic zoonotic pathogen that may cause gastroenteritis in humans and other animals. Wild birds may be as potential vectors of *C. jejuni* around urban and suburban areas. Here, 520 samples were collected from 33 wild bird species in urban and suburban areas, Beijing. In total 57 *C. jejuni* were isolated from seven species. It was found that Nineteen (33.33%, 19/57) isolates were resistant to at least one of 11 antibiotics, especially streptomycin (36.84%) and four isolates resistant to all. Nineteen (33.33%, 19/57) isolates were multi-drug resistance. Multilocus sequence typing (MLST) analysis of the isolates showed that 36 different sequence types (STs) belonged to four Clonal complexes and unassigned. Twenty STs (55.56%) and six alleles among them were first detected. Virulence genes including *flaA*, *cadF*, and the cytolethal distending toxin (CDT) gene cluster, were detected in all isolates, but truncated *cdt* gene clusters only detected in the isolates from the crow, daurian jackdaw and silver pheasant. In conclusion, it was the first detection of *C. jejuni* involved truncated *cdt* gene clusters from the silver pheasant. These wild birds around urban and suburban areas may pose potential public health problems as reservoir vectors of *C. jejuni*.

**Keywords:** emergence, *Campylobacter jejuni*, wild birds, MLST, multi-resistance, CDT gene cluster

## INTRODUCTION

*Campylobacter jejuni* is a gram-negative spiral rod bacterium that causes gastroenteritis in humans and other animals. In 2016, a total of 246,307 confirmed cases of human campylobacteriosis were reported in the European Union (EU), representing almost 70% of all the reported human cases of zoonoses (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2017). There was a



significantly increasing trend over the period 2008–2016. Compared with European countries, reports on campylobacteriosis in Asian countries, including China are limited, and the campylobacteriosis prevalence in humans is generally low (Wang et al., 2015).

The clinical presentation of campylobacteriosis includes watery or bloody diarrhea lasting for a median duration of 6 days, with 80% of patients having cramps and fever (Friedman et al., 2004). In some cases, the infection can lead to extra-intestinal complications and severe autoimmune disorders, such as pancreatitis, cholecystitis, obstructive hepatitis, Guillain-Barré syndrome (GBS), and Miller Fisher syndrome. Generally, the patients with campylobacteriosis are self-limited and disappear after 1 week without any specific treatment. However, in some relatively severe cases, antimicrobial chemotherapy is required. Macrolides and quinolones are commonly used as first-line therapies, and tetracycline, doxycycline, and chloramphenicol are alternative drugs (Ma et al., 2017).

Resistant strains and multi-drug resistance (MDR) strains are increasingly reported in humans and animals which may be induced by increasing use of antibiotics in humans, domestic animals and poultry (Franczek et al., 2012; Karaikos and Giamarellou, 2014; Alsan et al., 2015). *C. jejuni* has a high capacity to transfer genetic elements that lead to the combination of different strains. This characteristic may allow of *C. jejuni* transfer antibiotic resistance genes easily (de Boer et al., 2002; Wilson et al., 2003; Avrain et al., 2004). The abuse of antibiotics could increase the selection pressure and decrease the effectiveness of antibiotics further (Klena et al., 2004; Laxminarayan et al., 2013).

In *Campylobacter*, mutations in the 23S rRNA genes shown to contribute to macrolide resistance (Payot et al., 2006). *C. jejuni* resistance to tetracycline is usually associated with the *tet(O)* gene, which is carried on transmissible plasmids or located chromosomally. Ribosomal protein – Tet(O), which encoded by *tet(O)* gene can confer the resistance by displacing tetracycline from its primary binding site on the ribosome (Connell et al., 2003; Gibreel et al., 2004b). *C. jejuni* resistance to fluoroquinolones is mainly associated with mutations in the DNA gyrase gene (*gyrA*) (Smith and Fratamico, 2010). Accordingly, it is necessary to monitor the antibiotic resistance and research the antibiotic resistance mechanism of *C. jejuni* in wildlife.

The process of infection involved in adhesion, colonization, invasion and toxin production, especially, *flaA* genes, *cadF* genes, and *cdt* gene clusters are necessary for cell pathology and virulence in humans (Wei et al., 2018). These three virulence genes are frequently researched in isolates from various hosts, especially humans and poultry. However, little is known about these virulence genes in wild bird isolates (Wei et al., 2018).

The consumption of feces-contaminated raw or undercooked poultry has been identified as an important transmission vehicle for human campylobacteriosis (Wei et al., 2018). However, there is evidence that non-food-borne exposure of *C. jejuni* may contribute to the burden of illness as well. Thus contamination of the environment by domestic and wild birds feces may constitute an additional risk for human infection via environmental water or direct contact with them (French et al., 2009). Some studies suggested that *C. jejuni* is a commensal microorganism in the intestine of many wild and domestic animals, particularly avian species, and they can be natural reservoirs of *C. jejuni* (Oravcova et al., 2014). Furthermore, migratory birds may play a direct

**TABLE 1 |** Prevalence of *Campylobacter jejuni* isolates from different species.

Family	Species	Number	Isolation rate (%)	Family	Species	Number	Isolation rate (%)
Accipitridae	European Sparrowhawk ( <i>Accipiter nisus</i> )	3	0	Falconidae	Eurasian Kestrel ( <i>Falco tinnunculus</i> )	19	0
Accipitridae	Gray-faced Buzzard ( <i>Butastur indicus</i> )	1	0	Falconidae	Eurasian Hobby ( <i>Falco subbuteo</i> )	7	0
Accipitridae	Eurasian Goshawk ( <i>Accipiter gentilis</i> )	1	0	Falconidae	Peregrine Falcon ( <i>Falco peregrinus</i> )	2	0
Accipitridae	Common Buzzard ( <i>Buteo buteo</i> )	7	0	Falconidae	Red feet falcon ( <i>Falco amurensis</i> )	2	0
Anatidae	Mallard ( <i>Anas platyrhynchos</i> )	70	9%	Gruidae	Crane ( <i>Grus grus</i> )	22	0
Anatidae	Mandarin duck ( <i>Aix galericulata</i> )	16	6.25%	Laridae	Herring Gull ( <i>Larus argentatus</i> )	46	0
Anatidae	Black Swan ( <i>Cygnus atratus</i> )	8	12.50%	Paridae	Sparrow ( <i>Passer montanus</i> )	50	0
Anatidae	Swan Goose ( <i>Anser cygnoides</i> )	10	0	Phasianidae	Silver Pheasant ( <i>Lophura nycthemera</i> )	7	14.29%
Anatidae	Bar-headed Goose ( <i>Anser indicus</i> )	7	0	Phasianidae	Common Peafowl ( <i>Pavo cristatus</i> )	31	0
Anatidae	Mute Swan ( <i>Cygnus olor</i> )	4	0	Strigidae	European Scops Owl ( <i>Otus scops</i> )	12	0
Anatidae	Swan ( <i>Cygnus</i> sp.)	3	0	Strigidae	Long-eared Owl ( <i>Asio otus</i> )	2	0
Ardeidae	Gray Heron ( <i>Ardea cinerea</i> )	23	0	Strigidae	Little Owl ( <i>Athene noctua</i> )	1	0
Columbidae	Rock Pigeon ( <i>Columba livia</i> )	26	7.69%	Strigidae	Eagle owl ( <i>Ninox scutulata</i> )	2	0
Corvidae	Crow ( <i>Corvus</i> sp.)	62	24%	Strigidae	Tawny Owl ( <i>Strix aluco</i> )	1	0
Corvidae	Daurian Jackdaw ( <i>Corvus dauurica</i> )	60	52%	Struthionidae	Common Ostrich ( <i>Struthio camelus</i> )	3	0
Corvidae	Magpie ( <i>Pica pica</i> )	1	0	Sturnidae	Crested Myna ( <i>Acridotheres cristatellus</i> )	7	0
Falconidae	Saker falcon ( <i>Falco cherrug</i> )	4	0				

or indirect role in the zoonotic transmission of *Campylobacter* through the foods with fecal contamination (Kapperud et al., 2003; Gardner et al., 2012). *C. jejuni* has been isolated from wild birds such as pigeons, crows, geese, ducks, gulls, and cranes (Broman et al., 2002; Chen et al., 2011). Isolates from black-headed gulls (*Chroicocephalus ridibundus*), Sandhill cranes (*Grus canadensis*), and European starlings (*Sturnus vulgaris*) have been implicated in human disease (Palmer et al., 1983; Broman et al., 2002, 2004; Waldenstrom et al., 2002; Pearson et al., 2007; Karaïskos and Giamarellou, 2014).

Although *C. jejuni* is the main cause of bacterial diarrhea, there are still limited data on *C. jejuni* in China, especially for a wide range of free-living and migrating birds in various parts of cities, and the threat to public health cannot be ignored. Here, we investigated the prevalence, genetic diversity (Multilocus sequence typing), antimicrobial resistance patterns and virulence genes (*flaA* genes, *cadF* genes, and *cdt* gene cluster) of *Campylobacter* in wild birds in Beijing, China.

## RESULTS

### The Prevalence of *Campylobacter jejuni*

In total, 520 samples were collected from 33 species and 12 sites during the 4 months in Beijing, China (Figure 1).

*C. jejuni* was isolated from 57 samples (10.96%, 57/520) including seven species. In the positive *C. jejuni* samples, 24.19% (15/62) were from crow (*Corvus* sp.), 51.67% (31/60) were from daurian jackdaw (*Corvus dauurica*), 14.29% (1/7) were from silver pheasant (*Lophura nycthemera*), 8.57% (6/70) were from mallard (*Anas platyrhynchos*), 6.25% (1/16) were from mandarin duck (*Aix galericulata*), 12.5% (1/8) were from black swan (*Cygnus atratus*) and 7.69% (2/26) were from rock pigeon (*Columba livia*). Crows (*Corvus* sp.) and Daurian jackdaws (*Corvus dauurica*) had significantly higher positive rates than other species ( $P < 0.007$ ). Mallard (*Anas platyrhynchos*), mandarin duck (*Aix galericulata*) and black swan (*Cygnus atratus*) showed a relatively low positive rate between 6.25 and 12.5%. Silver Pheasant (*Lophura nycthemera*), family *Phasianidae* showed a similar positive rate as chicken, which is one of the most important reservoirs of *C. jejuni*. Rock pigeon (*Columba livia*), had relatively lower positive rate than others. This is the first time that *C. jejuni* has been isolated from black swan (*Cygnus atratus*) (Table 1).

The 12 collection sites were divided into two groups, including urban area and suburban area. Totally, all the positive samples of *C. jejuni* were from 8 sites, including Beijing Normal University (BNU) (55.00%), Beihai Park (BP)



(6.25%), Temple of Heaven Park (THP) (10.00%), Cuihu Park (CP) (27.78%), Beijing Wildlife Rescue Center (BWRC) (5.56%), Beijing Wildlife Park (BWP) (10.00%), Milu Park (MP) (7.14%), and Niukouyu Wetland Park (NWP) (10.00%). The highest was BNU (55%) which located in an urban area, and the lowest was BWRC (5.46%) which located in the suburb area. The average positive rate in urban areas (17.81%) was 2.36 times higher than in suburb areas (7.66%) (Table 2).

## Multilocus Sequence Typing for *Campylobacter jejuni*

To discover the genetic diversity of *C. jejuni* in wild birds and explore the role of wild birds in disease transmission, the genotype of *C. jejuni* isolates from wild birds were tested by multilocus sequence typing (MLST). MLST, which uses seven genes to build a classification system, is a common way to reveal genetic diversity. Fifty-seven isolates were divided into 36 different sequence types (STs) that clustered into four clonal complexes (CCs) and unassigned.

The same bird species could carry a variety of STs from different individuals (Table 3). Twenty-three STs were identified from 31 *C. jejuni* isolates in daurian jackdaws, and 7 STs from 15 isolates in crows. Overall, 52.6% of novel STs were first discovered in the present study (Table 3), especially more than 10 novel STs were from one or more new allelic genes, and six novel alleles were found (aspA484, aspA485, glnA673, glyA754, tkt718, tkt719) (Supplementary Table S1). Moreover, 84.5% (49 strains) of the STs did not belong to any clonal complex (CC). Three STs (ST-692, ST-991, ST-9191) belonged to ST-692 clonal complex and ST-52, ST-952, and ST-1275 complex comprised three STs, respectively (Table 3).

**TABLE 2 |** Prevalence of *Campylobacter jejuni* isolates from different sites.

Location	Site	Sample number	Isolation rate (%)	Average isolation rate (%)
Urban area	BNU	20	55.00	17.81
	BRRC	64	0.00	
	BP	16	6.25	
	THP	40	10.00	
	BWRC	72	5.56	
	CP	90	27.78	
Suburb area	BWP	10	10.00	7.56
	WDL	22	0.00	
	HL	7	0.00	
	MP	98	7.14	
	NWP	40	10.00	
	XR	41	0.00	

<sup>1</sup>BNU, Beijing Normal University; BP, Beihai Park; THP, Temple of Heaven Park; CP, Cuihu Park; BWRC, Beijing Wildlife rescue center; BWP, Beijing Wildlife Park; MP, Milu Park; NWP, Niukouyu Wetland Park; BRRC, Beijing Raptor rescue center; WDL, Wild Duck Lake; HL, Hongluo Lake; XR, Xiyu Reservoir.

## Phylogenetic Analysis of *Campylobacter jejuni* Strains

The minimum spanning tree for 57 *C. jejuni* isolates and other reference isolates from the PubMLST database was constructed (Supplementary Figure S1). The genetic diversity of the *C. jejuni* isolates showed that nine STs (ST-995, ST-991, ST-2367, ST-3938, ST-692, ST-52, ST-1540, ST-448, ST-951,

**TABLE 3 |** Clonal complex (CC) and sequence type (ST) distribution of *Campylobacter* in wild bird species.

CC	ST	Number	Avian species (No. of isolates)	Site
52	52	1	Black Swan (1)	BERC
692	692	1	Mandarin Duck (1)	BP
	991	2	Mallard (2)	NWP
	<b>9191</b>	1	Mallard (1)	BWSC
952	<b>9176</b>	2	Crow (2)	THP
1275	1540	1	Daurian Jackdaw (1)	CP
U	448	4	Daurian Jackdaw (4)	MP
	951	1	Daurian Jackdaw (1)	CP
	953	2	Daurian Jackdaw (2)	CP
	995	1	Mallard (1)	CP
	999	1	Daurian Jackdaw (1)	CP
	2367	2	Pigeon (2)	NWP
	3938	1	Daurian Jackdaw (1)	CP
	4069	1	Daurian Jackdaw (1)	CP
	4382	2	Crow (2)	BNU
	4571	1	Crow (1)	BNU
	6168	1	Daurian Jackdaw (1)	CP
	7805	1	Daurian Jackdaw (1)	CP
	<b>9175</b>	4	Crow (4)	BNU
	<b>9177</b>	2	Daurian Jackdaw (2)	MP/CP
	<b>9178</b>	2	Daurian Jackdaw (1)	MP
	<b>9179</b>	1	Daurian Jackdaw (1)	CP
	<b>9180</b>	2	Daurian Jackdaw (2)	CP
	<b>9181</b>	1	Daurian Jackdaw (2)	CP
	<b>9182</b>	1	Daurian Jackdaw (2)	CP
	<b>9183</b>	2	Daurian Jackdaw (2)	CP
	<b>9184</b>	1	Daurian Jackdaw (2)	CP
	<b>9185</b>	2	Daurian Jackdaw (2)	CP
	<b>9186</b>	1	Daurian Jackdaw (2)	CP
	<b>9190</b>	2	Mallard (2)	BWRC
	<b>9192</b>	2	Crow (2)	BNU
	<b>9194</b>	1	Daurian Jackdaw (2)	CP
	<b>9195</b>	1	Daurian Jackdaw (3)	CP
	<b>9196</b>	2	Crow (2)	THP/BNU
	<b>9197</b>	2	Crow (3)	THP
	<b>9222</b>	2	Daurian Jackdaw (1)	CP
			Silver Pheasant (1)	BWP

<sup>1</sup>U, Unassigned clonal complex. <sup>2</sup>Novel STs are indicated in bold. <sup>3</sup>In parentheses, the number of isolates from each bird. <sup>4</sup>BNU, Beijing Normal University; BP, Beihai Park; THP, Temple of Heaven Park; CP, Cuihu Park; BWRC, Beijing Wildlife rescue center; BWP, Beijing Wildlife Park; MP, Milu Park; NWP, Niukouyu Wetland Park; BRRC, Beijing Raptor rescue center; WDL, Wild Duck Lake; HL, Hongluo Lake; XR, Xiyu Reservoir.

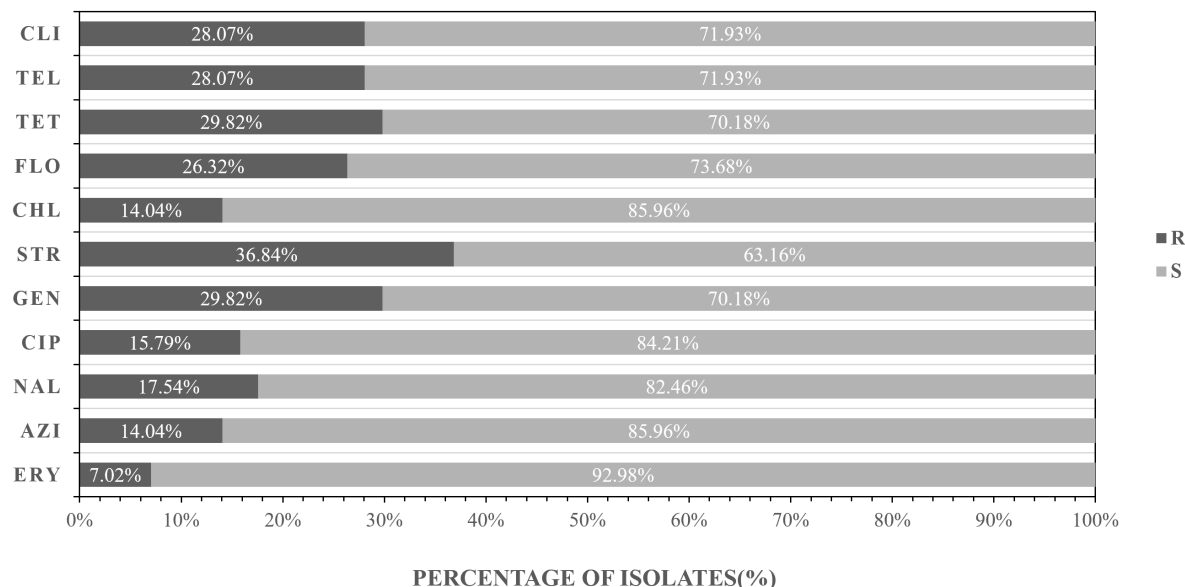
ST4069) were found in wild birds and human, 5 STs (ST-995, ST-991, ST-2367, ST-52, ST692, ST1540) in wild birds, chicken and humans, 2 STs (ST-448 and ST-995) in monkeys, wild birds and humans, and 2 STs (ST-52 and ST-995) in 4 hosts (wild birds, humans, chicken, dogs/monkeys). Researches previously reported showed that humans and other animals

have the same predominant STs, which was suggested that these animals are important reservoirs of human domestically acquired infections (Olkola et al., 2016). In this study, the isolates from black swan, mandarin duck, mallard, daurian jackdaw, and rock pigeon were found in humans (Table 4). Therefore these results above indicated that the *C. jejuni*

**TABLE 4 |** Sequence types (STs) and their association certain species and area.

STs	Species	Area	STs	Species	Area
52	Human stool	United Kingdom/United States/Brazil/Israel/Botswana/ Germany/Sweden/Luxembourg/ Switzerland/Canada/Japan/ The Netherlands/Australia/Greece	995	Human stool	Sweden
	Sheep	United Kingdom		Wild birds	Sweden/Finland
	Chicken	United Kingdom /United States/New Zealand/ Spain/Luxembourg/ Senegal/Switzerland/Uruguay		Chicken	Canada/Sweden/ United Kingdom
				Duck	New Zealand
	Cattle	United Kingdom		<b>Mallard</b>	<b>China</b>
	Xiangjiang River	<b>China</b>	3938	Human stool	Sweden
	Monkey	<b>China</b>		<b>Daurian Jackdaw</b>	<b>China</b>
	<b>Black Swan</b>	<b>China</b>	4069	Human stool	Canada
692	Human stool	United Kingdom		<b>Daurian Jackdaw</b>	<b>China</b>
	Goose	United Kingdom	953	Wild birds	United Kingdom
	Chicken	The Netherlands/ Luxembourg/China		<b>Daurian Jackdaw</b>	<b>China</b>
	Wild birds	Sweden	999	Starling	United Kingdom
	Environmental waters	United States/New Zealand		Wild birds	United Kingdom
	<b>Mandarin Duck</b>	<b>China</b>		<b>Daurian Jackdaw</b>	<b>China</b>
991	Human stool	United Kingdom /Luxembourg/ New Zealand/Germany	4382	Wild birds	Canada
	Environmental waters	Canada		<b>Crow</b>	<b>China</b>
	Human stool	United Kingdom /United States	4571	/	Finland
	Wild birds	Sweden/New Zealand/Finland		<b>Crow</b>	<b>China</b>
	Chicken	United Kingdom	6168	Environmental waters	Luxembourg
	Sheep	New Zealand		<b>Daurian Jackdaw</b>	<b>China</b>
	Environmental waters	Canada	7805	Wild birds	Finland
	<b>Mallard</b>	<b>China</b>		<b>Daurian Jackdaw</b>	<b>China</b>
1540	Human stool	United Kingdom		<b>9175 Crow</b>	<b>China</b>
	Chicken	United Kingdom		<b>9176 Crow</b>	<b>China</b>
	Environmental waters	Luxembourg		<b>9177 Daurian Jackdaw</b>	<b>China</b>
	Wild birds	United States/Japan		<b>9178 Daurian Jackdaw</b>	<b>China</b>
	<b>Daurian Jackdaw</b>	<b>China</b>		<b>9179 Daurian Jackdaw</b>	<b>China</b>
448	Human stool	United Kingdom /Switzerland/Sweden		<b>9180 Daurian Jackdaw</b>	<b>China</b>
	Wild birds	United Kingdom /Japan/United States		<b>9181 Daurian Jackdaw</b>	<b>China</b>
	Environmental waters	Canada/The Netherlands/France		<b>9182 Daurian Jackdaw</b>	<b>China</b>
	<b>Daurian Jackdaw</b>	<b>China</b>		<b>9183 Daurian Jackdaw</b>	<b>China</b>
951	Human stool	United Kingdom		<b>9184 Daurian Jackdaw</b>	<b>China</b>
	Wild birds	United Kingdom		<b>9185 Daurian Jackdaw</b>	<b>China</b>
	<b>Daurian Jackdaw</b>	<b>China</b>		<b>9186 Daurian Jackdaw</b>	<b>China</b>
2367	Human stool	United Kingdom		<b>9190 Mallard</b>	<b>China</b>
	Chicken	Germany		<b>9191 Mallard</b>	<b>China</b>
	<b>Pigeon</b>	<b>China</b>		<b>9192 Crow</b>	<b>China</b>
				<b>9194 Daurian Jackdaw</b>	<b>China</b>
				<b>9195 Daurian Jackdaw</b>	<b>China</b>
				<b>9196 Crow</b>	<b>China</b>
				<b>9197 Crow</b>	<b>China</b>
				<b>9222 Daurian Jackdaw/</b>	<b>China</b>
				<b>Silver Pheasant</b>	<b>China</b>

<sup>1</sup> Novel STs are indicated in bold. <sup>2</sup> Species isolated campylobacter jejuni in this study are indicated in bold. <sup>3</sup> Areas in china are indicated in bold. <sup>4</sup> BNU, Beijing Normal University; BP, Beihai Park; THP, Temple of Heaven Park; CP, Cuihu Park; BWRC, Beijing Wildlife rescue center; BWP, Beijing Wildlife Park; MP, Milu Park; NWP, Niukouyu Wetland Park; BRRC, Beijing Raptor rescue center; WDL, Wild Duck Lake; HL, Hongluo Lake; XR, Xiyu Reservoir.



**FIGURE 2 |** Frequency of resistance to 11 antibiotics among the 57 *Campylobacter jejuni* isolates. R represents resistant, I represents intermediate, S represents sensitive. AZI, azithromycin; NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin, STR, streptomycin; CHL, streptomycin, FLO, florfenicol; TET, tetracycline, TEL, telithromycin, CLI, clindamycin; ERY, erythromycin.

could transmit between different species (Tables 3, 4 and Supplementary Figure S1).

## Antibiotic Resistance of *Campylobacter jejuni*

The antibiotic resistance profile of *C. jejuni* isolates was evaluated by 11 antibiotics according to the recommendations of the Clinical Laboratory Standards Institute (CLSI, 2015). Antibacterial resistance revealed that streptomycin (36.84%) was most common, followed by tetracycline (29.82%), gentamicin (29.82%), clindamycin (28.07%), telithromycin (28.07%), florfenicol (26.32%), nalidixic acid (17.54%), and ciprofloxacin (15.79%), azithromycin (14.04%), chloramphenicol (14.04%), erythromycin (7.02%) (Figure 2 and Table 5).

The isolates from different locations and sites showed different antimicrobial profile. In the urban area, the antimicrobial efficiency of three antibiotics commonly used in humans and animals are as follows: streptomycin (62.50%), gentamicin (62.50%), and telithromycin (50.00%) (Table 6). In the suburb area, the rate of antibiotic resistance was low, ranging from 7.32 to 26.83% (Table 6).

Multi-drug resistance of bacteria is also common in this study. Nineteen (33.33%, 19/57) isolates were MDR. In detail, The main antibiotics producing drug resistance were streptomycin, gentamicin, and clindamycin (26.32%) (Figure 3 and Supplementary Table S2).

## Antibiotic Resistance Mechanism of *Campylobacter jejuni*

The characteristics of macrolide resistance associated with the genes were analyzed in 4 resistant isolates and 53 susceptible

isolates, and this helps investigate the molecular mechanisms of the macrolide-resistant isolates. The A2075G mutation in the 23S rRNA gene, which is responsible for high-level resistance to macrolide, was not detected in all of the resistant strains and susceptible isolates (Supplementary Figure S2A).

All *Campylobacter* isolates were also investigated for the presence of the *tet(O)* gene associated with the resistance to tetracycline. The *tet(O)* marker was detected in all but three resistant strains (3/17) (Supplementary Figure S2B).

To investigate the molecular mechanisms of the fluoroquinolones resistant isolates, Multiplex PCR was designed, which uses three primers in a single reaction. Only the isolates with the gene mutation generated a product with the reverse primer with mutation and the conserved forward primer, whereas all 57 strains generated the *gyrA* PCR product with reverse and forward conserved primers. 87.50% (7/8) *C. jejuni* resistant isolates generated the specific product in MAMA-PCR that indicated the mutation Thr-86-to-Ile (ACA→ATA for *C. jejuni*), while none of the susceptible strains gave positive results (Supplementary Figure S2C).

All *Campylobacter* isolates were also investigated for the presence of the *aphA-3* gene associated with the resistance to Aminoglycosides. The *aphA-3* marker was detected in all but one resistant strains (1/17) (Supplementary Figure S2B).

## Determination of the Presence of Virulence Genes

To determine whether virulence differences exist among isolates from different wild birds, we tested the virulence genes including the *cdt* gene cluster, *flaA* gene and *cadF* gene, the gene

**TABLE 5 |** Prevalence of antibiotic resistance among different species.

Species (Number)	Aminoglycosides		Tetracyclines	Lincomides	Macrolides Ketolides			Quinolones		Chloramphenicols	
	GEN	STR	TET	CLI	ERY	AZI	TEL	NAL	CIP	CHL	FLO
Crow (15)	60.00% (9/15)	60.00% (9/15)	26.67% (4/15)	60.00% (9/15)	6.67% (1/15)	26.67% (4/15)	26.67% (14/15)	13.33% (2/15)	20.00% (3/15)	26.67% (4/15)	33.33% (5/15)
Daurian Jackdaw (31)	0.00% (0/31)	6.45% (2/31)	6.45% (2/31)	6.45% (2/31)	0.00% (0/31)	0.00% (0/31)	3.23% (1/31)	9.68% (3/31)	6.45% (2/31)	0.00% (0/31)	6.45% (2/31)
Balck Swan (1)	100.00% (1/1)	100.00% (1/1)	100.00% (1/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)
Sliver Pheasant (1)	100.00% (1/1)	100.00% (1/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)
Mallard (6)	50.00% (3/6)	50.00% (3/6)	83.33% (5/6)	50.00% (3/6)	16.67% (1/6)	16.67% (1/6)	50.00% (3/6)	33.33% (2/6)	33.33% (2/6)	33.33% (2/6)	50.00% (3/6)
Mandarin duck (1)	100.00% (1/1)	100.00% (1/1)	100.00% (1/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)
Rock Pigeon (2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)	100.00% (2/2)
Total (57)	29.82% (17/57)	36.84% (21/57)	29.82% (17/57)	28.07% (16/57)	7.02% (4/57)	14.04% (8/57)	28.07% (16/57)	17.54% (10/57)	15.79% (9/57)	14.04% (8/57)	26.32% (15/57)

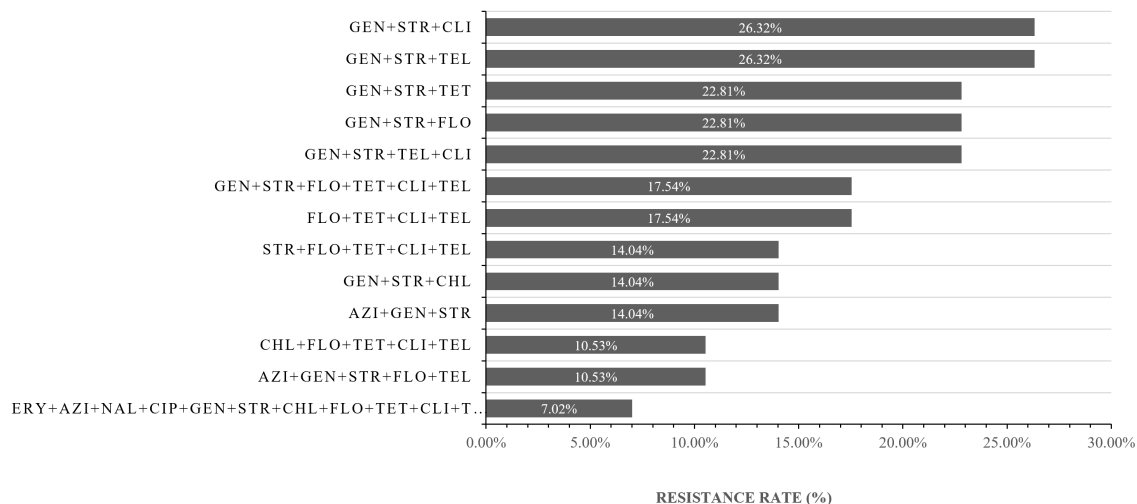
<sup>1</sup>In parentheses, (A), (B/A), A represent the number of isolates from each bird, B represent the number of isolates from each bird resistance to each antibiotic. <sup>2</sup>AZI, azithromycin; NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; CHL, chloramphenicol; FLO, florfenicol; TET, tetracycline; TEL, telithromycin; CLI, clindamycin; ERY, erythromycin.



**TABLE 6 |** Prevalence of antibiotic resistance among different sites.

Location (Number)	Site (Number)	Aminoglycosides		Tetracyclines	Lincomides	Macrolides Ketolides			Quinolones		Chloramphenicols	
		GEN	STR	TET	CLI	ERY	AZI	TEL	NAL	CIP	CHL	FLO
Urban (16)	BNU (11)	72.73% (8/11)	72.73% (8/11)	36.36% (4/11)	54.55% (6/11)	9.09% (1/11)	27.27% (3/11)	63.64% (7/11)	18.18% (2/11)	27.27% (3/11)	36.36% (4/11)	36.36% (4/11)
	BP (1)	100.00% (1/1)	100.00% (1/1)	100.00% (1/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)
	THP (4)	25.00% (1/4)	25.00% (1/4)	25.00% (1/4)	25.00% (1/4)	100.00% (0/4)	25.00% (1/4)	25.00% (1/4)	0.00% (0/4)	0.00% (0/4)	0.00% (0/4)	25.00% (1/4)
	Subtotal (16)	62.50% (10/16)	62.50% (10/16)	37.50% (6/16)	50.00% (8/16)	6.25% (1/16)	25.00% (4/16)	56.25% (9/16)	18.75% (3/16)	18.75% (3/16)	25.00% (4/16)	37.50% (6/16)
	BWRC (4)	50.00% (2/4)	50.00% (2/4)	100.00% (4/4)	50.00% (2/4)	0.00% (0/4)	0.00% (0/4)	50.00% (2/4)	25.00% (1/4)	25.00% (1/4)	0.00% (0/4)	50.00% (2/4)
	CP (25)	4.00% (1/25)	12.00% (3/25)	4.00% (1/25)	4.00% (1/25)	0.00% (0/25)	0.00% (0/25)	4.00% (1/25)	8.00% (2/25)	8.00% (2/25)	4.00% (1/25)	4.00% (1/25)
Suburb (41)	BWP (1)	100.00% (1/1)	100.00% (1/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)	0.00% (0/1)	0.00% (0/1)	0.00% (0/1)	0.00% (0/1)	100.00% (1/1)
	MP (7)	0.00% (0/7)	14.29% (1/7)	28.57% (2/7)	28.57% (2/7)	0.00% (0/7)	0.00% (0/7)	14.29% (1/7)	14.29% (1/7)	0.00% (0/7)	0.00% (0/7)	28.57% (2/7)
	NWP (4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)	75.00% (3/4)
	Subtotal (41)	17.07% (7/41)	26.83% (11/41)	26.83% (11/41)	19.51% (8/41)	7.32% (3/41)	9.76% (4/41)	17.07% (7/41)	17.07% (7/41)	14.63% (6/41)	9.76% (4/41)	21.95% (9/41)
Beijing (57)	Total (57)	29.82% (17/57)	36.84% (21/57)	29.82% (17/57)	28.07% (16/57)	7.02% (4/57)	14.04% (8/57)	28.07% (16/57)	17.54% (10/57)	15.79% (9/57)	14.04% (8/57)	26.32% (15/57)

<sup>1</sup>In parentheses, (A), (B/A), A represent the number of isolates from each site, B represent the number of isolates from each site resistance to each antibiotic. <sup>2</sup>AZI, azithromycin; NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; CHL, chloramphenicol; FLO, florfenicol; TET, tetracycline; TEL, telithromycin; CLI, clindamycin; ERY, erythromycin. <sup>3</sup> BNU, Beijing Normal University; BP, Beihai Park; THP, Temple of Heaven Park; CP, Cuihu Park; BWRC, Beijing Wildlife rescue center; BWP, Beijing Wildlife Park; MP, Milu Park; NWP, Niukouyu Wetland Park; BRRC, Beijing Raptor rescue center; WDL, Wild Duck Lake; HL, Hongluo Lake; XR, Xiyu Reservoir.



**FIGURE 3 |** The resistance spectrum of strains of *Campylobacter jejuni* to various antibiotic combinations. The X-axis represents the resistance rate of *Campylobacter jejuni*, The Y-axis represents a series of combination of antibiotics. Thirty-five (61.4%, 35/57) isolates were multi-drug resistance (resistant to more than two antibiotics respectively). AZI, azithromycin; NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; CHL, chloramphenicol; FLO, florfenicol; TET, tetracycline; TEL, telithromycin; CLI, clindamycin; ERY, erythromycin.

that encodes an adhesin that involves in colonization for all *C. jejuni* isolates.

The results showed that all isolates contained *flaA* genes, *cadF* genes, and *cdt* gene cluster. Further analysis indicated that the truncated *cdt* gene clusters (approximately 1400 bp) only existed in the isolates from crows, daurian jackdaws and silver pheasants. In particular, the truncated *cdt* gene clusters were first detected in *C. jejuni* isolates from silver pheasant. *C. jejuni* isolates from *Corvidae* are more likely to carry truncated *cdt* gene clusters, other species belong to family *Corvidae* and *Phasianidae* may also have deletions within the *cdt* Gene Cluster and still need a further survey. These results above indicated that bacteria in the family of *Anatidae* and *Columbidae* might have complete *cdt* gene clusters, but further research is still needed for two families species (Table 7).

## DISCUSSION

*Campylobacter jejuni* carried by wild birds has been identified to be potentially pathogenic to humans and other animals (Kapperud et al., 2003; Gardner et al., 2012). The majority of studies have focused on food sources that may cause human campylobacteriosis, while studies on wild birds are rare, especially in China. In order to get a better understanding of the distribution and transmission of *C. jejuni* in wild birds, we analyzed the prevalence, genetic diversity, antimicrobial resistance and virulence genes of *C. jejuni* in Beijing, China.

In the present study, we found that 10.96% of fecal samples collected from 33 species carried with *C. jejuni*. Previous studies demonstrated that the prevalence of *C. jejuni* among different countries and wild birds species varied from 1.4 to 72.7% (Ramonaite et al., 2015). Among these species we found that the highest prevalence of *C. jejuni* was daurian jackdaw

**TABLE 7 |** Prevalence of CDT isolates from different species.

Family	Species	CDT character (%)
Corvidae	Crow ( <i>Corvus</i> sp.)	Truncated 100% (15/15)
	Daurian Jackdaw ( <i>Corvus dauurica</i> )	Truncated 100% (31/31)
Phasianidae	Silver Pheasant ( <i>Lophura nycthemera</i> )	Truncated 100% (1/1)
Anatidae	Mallard ( <i>Anas platyrhynchos</i> )	WT 100% (6/6)
	Mandarin duck ( <i>Aix galericulata</i> )	WT 100% (1/1)
	Black Swan ( <i>Cygnus atratus</i> )	WT 100% (1/1)
Columbidae	Rock Pigeon ( <i>Columba livia</i> )	WT 100% (2/2)

<sup>1</sup>In parentheses, the positive number of isolates from each species of bird.

51.67%, followed by crow 24.19%, silver pheasant 14.29%, black swan 12.5%, mallard 8.57%, rock pigeon 7.69%, and mandarin duck 6.25%. *C. jejuni* isolated from different wild birds with different prevalence which in agreement with other studies that the prevalence of *Campylobacter* spp. in different taxonomic families wild birds were diverse (Waldenstrom et al., 2007; French et al., 2009).

It is believed that the variation of *C. jejuni* prevalence in wild bird species is due to ecological factors including feeding habits, habitat preferences, and migration patterns (Waldenstrom et al., 2002; Colles et al., 2009; Waldenstrom et al., 2010; Colles et al., 2011). Located in urban, BNU had the highest prevalence of *C. jejuni*. Although the proportion of human disease attributable to environmental sources is relatively low, we could not ignore the effect on humans (Wilson et al., 2008; Sheppard et al., 2009; Colles et al., 2011). Most importantly, to our knowledge, it is the first detection of *C. jejuni* carried by black swans in China. These data strengthen the hypothesis that the high prevalence of *C. jejuni* in wild birds might provide evidence of wild birds being a natural reservoir of *C. jejuni* (Oravcova et al., 2014).

Multilocus sequence typing was the golden standard to use for comparison between isolates from different sources because of its high reproducibility and accessible to comparability amongst laboratories worldwide (Dingle et al., 2001). Previous studies have shown that human patients and other animals have the same predominant STs suggesting that these animals are important reservoirs of human domestically acquired infections (Olkkola et al., 2016). From our results, 36 different STs belonging to 4 CCs and unassigned, among which 20 were novel. Of the 20 novel STs, 25% were from one or more new allelic sequences, and a total of 6 novel alleles were found (aspA484, aspA485, glnA673, glyA754, tkt718, tkt719). No new allele sequences were found for another 75% new STs, and these STs resulted from novel combinations of alleles already existed in the PubMLST database. These results indicated that mutation frequency in the MLST alleles is substantially lower than the recombination frequency, which is in agreement with previous research (Schouls et al., 2003). ST448, ST951, and ST52 these isolates from daurian jackdaw and black swan, however other researches indicated that all of these three strains isolated from other animals, poultry, and humans (Griekspoor et al., 2010; Olkkola et al., 2016). So wild birds as a potential source of known and novel multilocus sequence types of *C. jejuni* may have the potential to transmit to other animals, poultry, and humans.

The antibiotic resistance profile of *C. jejuni* isolates from these animals was determined using 11 antibiotics. The results of antimicrobial susceptibility testing in this study indicated that the isolates were in general resistant to the tested antibiotics at rates ranging from 7.02 to 36.84%. The high rate of resistance to streptomycin and gentamicin was seen among *C. jejuni* isolates from these birds. All *Campylobacter* isolates were also investigated for the presence of the *aphA-3* gene associated with the resistance to Aminoglycosides. The most common form of resistance to Aminoglycosides related antibiotics involves the synthesis of 3'-aminoglycoside phosphotransferases [APH(3')](Gibrel et al., 2004a). The *aphA-3* marker was detected in all but one resistant strains (1/17), which is consistent with previous research results.

From the different locations and sites, the antibiotic resistance profile performed differently. In urban areas, the isolates from wild birds have high antibiotic resistance, which might be due to contaminated environment water. The reasonable interpretation for this difference may be human activities, such as antibiotic abuse, are more active in urban areas than in the suburbs, resulting in birds in different habitats getting different resistant bacteria from the environment. However, the relationship between high antibiotic resistance and contaminated environment water needs further study. As previous study indicated that *C. jejuni* isolates detected in crows and pigeons as potential infection sources to humans (Ramonaite et al., 2015), it was worth noting that four strains from wild birds (crow, mallard, and rock pigeon) are resistant to all 11 antibiotics which may be an important indicator of public health safety.

All wild birds isolates had *flaA* genes, *cadF* genes, and *cdt* gene clusters, which in agreement with the previous study (Sen et al., 2018). The results suggest that these three genes are conserved amongst different sources. As before, *C. jejuni* isolates

from crows had a truncated gene cluster of about 1400 bp (Sen et al., 2018), *C. jejuni* isolates from daurian jackdaw also had a truncated gene cluster (Kovanen et al., 2019). However, the most unexpected results were isolates from silver pheasant which also have a truncated gene cluster of about 1400 bp. To our knowledge, it was the first detection of the isolates from silver pheasant also have a truncated gene cluster of about 1400 bp. The CDT toxin is a tripartite protein formed by the expression of three tandem genes, *cdtA*, *cdtB*, and *cdtC* where *cdtB* encodes the active component of the toxin, while *cdtA* and *cdtC* are responsible for binding and internalization of the toxin (Pickett et al., 1996). Some research confirmed that *C. jejuni* 81-176 *cdtB*<sup>-</sup> strains were significantly attenuated in HeLa cytotoxicity assays, while still holding some toxigenicity. However, *C. jejuni* NCTC 11168 *cdtB*<sup>-</sup> strains produced no detectable cytotoxicity in HeLa cell (Purdy et al., 2000). So, if these isolates with a truncated *cdt* gene cluster still retaining the toxigenicity required further verification.

## CONCLUSION

Wild birds as a reservoir of potentially pathogenic *C. jejuni* strains and can be a vector of disease transmission. However, further studies are needed to link the high occurrence of *Campylobacter* in wild birds to human campylobacteriosis cases and transmission to other animals.

## EXPERIMENTAL PROCEDURES

### Samples Collection and *Campylobacter* Isolation

Five hundred and twenty fecal samples were collected from 33 species in 12 sites, Beijing, China, between January 2018 and April 2018 (Figure 1 and Table 1). Samples were collected in sterile tubes and stored at 4 to 7°C for 2 to 6 h before culturing in Lab (Sen et al., 2018). Fecal samples were inoculated onto Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) containing Cefoperazone, Rifampicin, and Amphotericin B (Qingdao Hope Bio-Technology, Co., Ltd.), with incubation at 37°C under microaerophilic conditions (CampyGen; Oxoid Limited, Hampshire, United Kingdom) for 48 to 168 h. Then picked white to translucent colonies subculture onto mCCDA for further characterization.

### *Campylobacter* Identification

All positive samples were used to extract DNA by adding 100 µl 0.25% SDS and boiling in a heater block at 95°C for 10 min, followed by centrifugation at 12,000 g for 5 min. Template DNA was stored at -20°C until used for PCR and at least 1 year without any degradation (Sen et al., 2018). Then *Campylobacter* spp. identified by a qPCR method based on the 16S rRNA gene and primers used as previously described (Lund and Madsen, 2006) (Table 8). The qPCR conditions as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, with a final cycle of 72°C for

5 min. For determining the presence of *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari*, and *Campylobacter upsaliensis*, a multiplex PCR method was performed with the isolates using the lipid A gene (*lpxA*) as previously described (Klena et al., 2004), PCR conditions were as follows: 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 5 min. The presence of *flaA*, *cadF* and CDT gene cluster in 57 isolates were determined by PCR using the primer sets described in Table 8 (Nachamkin et al., 1993; Konkel et al., 1999; Bang et al., 2003). The thermocycling conditions which can be found in the respective references in Table 8. The PCR products were detected on 1% Agarose gels and verified by sequencing.

## Multilocus Sequence Typing for *Campylobacter jejuni*

Multilocus sequence typing was performed by amplifying and sequencing seven housekeeping genes loci, *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*, and primers used for *C. jejuni* are listed in Table 9 (Dingle et al., 2001). The PCR reaction conditions were as follows: initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 2 min, 50°C for 1 min and 72°C for 1 min; last denaturation at 72°C for 7 min. The PCR products were detected on 1% Agarose gels and verified by sequencing. Allele numbers and STs were assigned using the *Campylobacter* MLST database<sup>1</sup>.

## Antimicrobial Susceptibility Testing for *Campylobacter jejuni*

Antimicrobial resistance analysis was performed on 57 *Campylobacter* isolates. All isolates were cultured overnight before testing. The *C. jejuni* strains were tested against phenotypic resistance to 11 antimicrobial agents (erythromycin, azithromycin, nalidixic acid, ciprofloxacin, gentamicin,

streptomycin, chloramphenicol, florfenicol, tetracycline, telithromycin, and clindamycin) (Zhongchuan biology technology Company, Qingdao, China) by the agar dilution method according the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015). Mueller-Hinton agar (Oxoid) with dilutions ranging from 64 to 0.5 µg/mL for erythromycin, azithromycin, nalidixic acid, ciprofloxacin, gentamicin, streptomycin, chloramphenicol, florfenicol, tetracycline, telithromycin, and clindamycin was prepared. Subcultured colonies were harvested and suspended in sterile water to a standardized cell density (0.5 McFarland), and 2 µL of approximately 10<sup>4</sup> CFU of bacteria was pipetted into each well (after diluting in PBS). The plates were incubated under a microaerophilic atmosphere at 42°C for 24 h. The MIC values were defined as the lowest concentration that produces complete inhibition of *C. jejuni* growth. For quality control, the reference strain *C. jejuni* ATCC 33560 was included. The *C. jejuni* isolates were considered resistant to chloramphenicol (CHL), erythromycin (ERY), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), streptomycin (STR), and telithromycin (TEL) at MICs of  $\geq 32$ ,  $\geq 32$ ,  $\geq 4$ ,  $\geq 64$ ,  $\geq 16$ ,  $\geq 16$ , and  $\geq 16$  µg/ml, respectively. For gentamicin (GEN), florfenicol (FLO), clindamycin (CLI) and azithromycin (AZI), the isolates with MICs  $\geq 8$  µg/ml were considered resistant.

## Determination of Mechanisms of Antimicrobial Resistance

All strains tested for the antibiotic resistance were examined for the presence of molecular background of the appearing resistance. For the determination of macrolide resistance, the 23S rRNA genes mutations were detected by the use of PCR for *C. jejuni* (Zhou et al., 2016). For the determination of fluoroquinolone resistance, the *gyrA* mutations were detected by the use of the Mismatch Amplification Mutation Assay – PCR (MAMA-PCR) suitable for *C. jejuni* (Wieczorek, 2011). The presence of *tet(O)* gene associated with the resistance to

<sup>1</sup><http://pubmlst.org/campylobacter/>

**TABLE 8 |** *Campylobacter* specific primers in this study.

Primer	Sequence (5'–3')	Gene	References
CampF2	5'-CACGTGCTACAATGGCATAT-3'	<i>Campylobacter</i> spp. 16S rRNA	Lund and Madsen, 2006
CampR2	5'-GGCTTCATGCTCTCGAGTT-3'		
Camp P2 (Probe)	<b>FAM</b> -5'-CAGAGAACAATCCGAAGCTGGGACA		
LYA-F	5'-CTTTATGCATGTTCTTCTAAATTT-3'	<i>cdt</i> gene cluster	Bang et al., 2003
MII-R:	5'-GTTAAAGGTGGGGTTATAATCATT-3' (25)		
<b>Forward</b>			Klena et al., 2004
<i>lpxA C. coli</i>	5'AGA CAA ATA AGA GAG AAT CAG-3'	<i>C. coli</i> <i>lpx</i> (391bp)	
<i>lpxA C. jejuni</i>	5'ACA ACT TGG TGA CGA TGT TGT A-3'	<i>C. jejuni</i> <i>lpx</i> (331 bp)	
<i>lpxA C. lari</i>	5'TRC CAA ATG TTA AAA TAG GCG A-3'	<i>C. lari</i> <i>lpx</i> (233 bp)	
<i>lpxA C. upsaliensis</i>	5'AAG TCG TAT ATT TTC YTA CGC TTG TGTG-3'	<i>C. upsaliensis</i> <i>lpx</i> (206 bp)	
<b>Reverse</b>			
<i>lpxAARKK2M</i>	5'CAATCATGDGCDATATGASAATAHGCCAT-3'	<i>cdt</i> gene cluster	
<i>flaA-F</i>	5'-GGATTTTCGTATTAACACAAATGGTGC-3	<i>fla</i> (1728 bp)	Nachamkin et al., 1993
<i>flaA-R</i>	5'-CTGTAGTAATCTTAAACATTTTG-3		
<i>cadFU</i>	5'-TTGAAGGTAATTTAGATATG-3'	<i>cadF</i> (400 bp)	Konkel et al., 1999
<i>cadFR</i>	5'-CTAATACCTAAAGTTGAAAC-3'		



**TABLE 9 |** Oligonucleotide primers for *Campylobacter* MLST.

Dideoxyligonucleotide primer				
Name and sequence				
Loucs	Function	Forward	Reverse	Amplification size (bp)
asp	Amplification	asp-A9, 5'-AGT ACT AAT GAT GCT TAT CC-3'	asp-A10, 5'-ATT TCA TCA ATT TGT TCT TTG C-3'	899
	Sequencing	asp-S3, 5'-CCA ACT GCA AGA TGC TGT ACC-3'	asp-S6, 5'- TTC ATT TGC GGT AAT ACC ATC-3'	
gln	Amplification	gln-A1, 5'-TAG GAA CTT GGC ATC ATA TTA CC-3'	gln-A2, 5'-TTG GAC GAG CTT CTA CTG GC-3'	1262
	Sequencing	gln-S1, 5'- GCT CAA TTC ATG GAT GGC-3'	gln-S4, 5'- GCA TAC CAT TGC CAT TAT CTC CG-3'	
glt	Amplification	glt-A1, 5'-GGG CTT GAC TTC TAC AGC TAC TTG-3'	glt-A2, 5'-CCA AAT AAA GTT GTC TTG GAC GG-3'	1012
	Sequencing	glt-S3, 5'-CTT ATA TTG ATG GAG AAA ATG G-3'	glt-S8, 5'- TGC TAT ACA GGC ATA AGG ATG-3'	
gly	Amplification	gly-A1, 5'-GAG TTA GAG CGT CAA TGT GAA GG-3'	gly-A2, 5'-AAA CCT CTG GCA GTA AGG GC-3'	816
	Sequencing	gly-S5, 5'- GCT AAT CAA GGT GTT TAT AT-3'	gly-S4, 5'-AGG TGA TTA TCC GTT CCA TCG C-3'	
pgm	Amplification	pgm-A7, 5'-TAC TAA TAA TAT CTT AGT AGG-3'	pgm-A8, 5'-CAC AAC ATT TTT CAT TTC TTT TTC-3'	1150
	Sequencing	pgm-S5, 5'- GGT TTT AGA TGT GGC TCA TG-3'	pgm-S2, 5'- TCC AGA ATA GCG AAA TAA GG-3'	
tkf	Amplification	tkf-A3, 5'-GCA AAC TCA GGA CAC CCA GG-3'	tkf-A6, 5'-AAA GCA TTG TTA ATG GCT GC-3'	1102
	Sequencing	tkf-S5, 5'- GCT TAG ACG ATA TTT TAA GTG-3'	tkf-S6, 5'- AAG CCT GCT TGT TCT TTG GC-3'	
unc	Amplification	unc-A7, 5'-ATG GAC TTA AGA ATA TTA TGG C-3'	unc-A8, 5'-ATA AAT TCC ATC TTC AAA TTC C-3'	1120
	Sequencing	unc-S3, 5'- AAA GTA CAG TGG CAC AAG TGG-3'	unc-S4, 5'- TGC CTC ATC TAA ATC ACT AGC-3'	

tetracyclines was also detected (Wieczorek, 2011). The presence of *aphA-3* gene associated with the resistance to aminoglycosides was also detected (Gibreel et al., 2004a) primers used for determination of Mechanisms of Antimicrobial Resistance are listed in **Table 10**.

The 23S *rRNA* genes PCR reaction conditions were as follows: initial denaturation at 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; last denaturation at 72°C for 7 min. The *tet(O)* gene PCR reaction conditions were as follows: initial denaturation at 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; last denaturation at 72°C for 7 min. The *gyrA* gene PCR reaction conditions were as follows: initial denaturation at 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s; last denaturation at 72°C for 7 min. The *tet(O)* gene PCR reaction conditions were as follows: initial denaturation at 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30 s; last denaturation at 72°C for 7 min. The primers used for these three genes are listed in **Table 10**. The PCR products were detected on 1% Agarose gels and verified by sequencing, and the sequences were analyzed to identify mutations using the BLAST program of the GenBank sequence database.

## Phylogenetic Analysis

The MLST profiles were clustered with the Bionumerics software, version 7.6 by using a categorical coefficient and a graphing method called the minimum spanning tree as described before (Schouls et al., 2004). The minimum spanning tree showing the relatedness of 57 *C. jejuni* strains and other *C. jejuni* strains from the PubMLST database, which was based on the STs. Each ST is represented by a circle that is proportional to the number of isolate species comprising that ST. Circles (STs) are linked by lines indicating allelic variation. The different color

**TABLE 10 |** *Campylobacter* specific primers in this study.

Primer	Sequence (5'–3')	Gene	References
F1-campy-23S	5'-AAGAGGATGTATAG GGTGTGACG-3'	23S <i>rRNA</i>	Zhou et al., 2016
R1-campy-23S	5'-AACGATTTC AACCGTTCTG-3'		
DMT 1	5'-GGCGTTTTGTTT ATGTGCG-3'	<i>tet(O)</i>	Wieczorek, 2011
DMT 2	5'-GTAAAGGTGGGGTTAT AATCATT-3'		
CampyMAMAgryA1	5'-TTTTTAGCAA AGATTCTGAT-3'	<i>gyrA</i>	Wieczorek, 2011
GZgyrA4	5'-CAGTATAACGCATC GCAGCG-3'		
CampyMAMAgryA5	5'-CAAAGCATCA TAAACTGCAA-3'		
AphA-3 F	5'-GGGACCACTATGATG TGGAACG-3'	<i>AphA-3</i>	Gibreel et al., 2004a
AphA-3 R	5'-CAGGCTTGATCC CCAGTAAGTC-3'		

of each ST indicates the animal host from which each isolate was recovered (red-human, green -wild bird, blue-chicken, yellow-dog, sky blue-monkey). Thick and short lines connect single-locus variants, thin and longer lines connect double-locus variants and dashed lines represent three or more allele differences. For MLST, a maximum neighbor difference of 2 was used to create complexes. Background shading highlights clonal complexes.

## Accession Number(s)

Multilocus sequence typing sequences of the bird isolates that represented novel STs were deposited in the PubMLST database (see footnote 1) to assign new sequence types and allelic profiles.

## Statistical Analysis

Statistical analysis of the prevalence of positive rate in the *C. jejuni* isolates was performed using the chi-squared test with SPSS version 16.0. A value of  $p < 0.05$  was considered to be statistically significant.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## AUTHOR CONTRIBUTIONS

JD, HH, JL, and CW designed the project. JD and JH performed the main experiments. JD, JL, CW, and ML wrote and revised the manuscript. BJW, BW, HC, JJ, and KS conducted part of the experiments.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02433/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.

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# Petting Zoo Animals as an Emerging Reservoir of Extended-Spectrum $\beta$ -Lactamase and AmpC-Producing Enterobacteriaceae

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Extended spectrum beta-lactamases and AmpC-producing Enterobacteriaceae (ESBL/AmpC-E) have become a great concern in both human and veterinary medicine. One setting in which this risk could be particularly prominent is petting zoos, in which humans, especially children, directly and indirectly interact with the animals. Yet, while the zoonotic transmission of various Enterobacteriaceae has been reported previously in petting zoos, reports on ESBL/AmpC-E shedding in this setting is currently lacking, despite the high potential risk. To fill this knowledge gap, we conducted a prospective cross-sectional study to explore the prevalence, molecular epidemiology, and risk for shedding of ESBL/AmpC-E in petting zoos. We performed a prospective cross-sectional study in eight petting zoos. Altogether, we collected 381 fecal and body-surface samples from 228 animals, broth-enriched them, and then plated them onto CHROMagar ESBL-plates for ESBL/AmpC-E isolation. Next, we identified the isolated species and tested their susceptibility to various antibiotics using the Vitek-2 system, determined bacterial relatedness by multilocus sequence typing (MLST), and identified ESBL/AmpC genes by using PCR and sequencing. Finally, we asked petting zoo owners and veterinarians to complete questionnaires, which we then analyzed to evaluate risk factors for ESBL/AmpC-E shedding. We found that ESBL/AmpC-E shedding is an important, currently overlooked risk in petting zoos, as the overall shedding rate was 12% (35 isolates, including 29% ESBL-producers, 34% AmpC-producers, and 37% ESBL and AmpC-producers). The isolated bacteria included *Enterobacter cloacae* (55%), *Escherichia coli* (31%), and *Citrobacter freundii* (14%), with diverse ESBL genes. MLST revealed diverse sequence types (STs), including the highly virulent Enterotoxigenic ST656 and the Uropathogenic ST127 *E. coli* strains, indicating complex



epidemiology with inter-animal bacterial transmission. Shedding was associated with petting permission and antibiotic treatment in the petting zoo (OR = 7.34), which were identified as risk factors for ESBL/AmpC shedding. Our findings highlight petting zoos as a source for antibiotic-resistant ESBL/AmpC-producing bacteria, including highly virulent, disease-associated MDR *E. coli* strains. As this risk has not been previously described in detail, it calls for the implementation of infection control and active surveillance programs in petting zoos and raises the need for a comprehensive guideline to restrain this emerging concern.

**Keywords:** petting zoos, animals, ESBL, AmpC, environmental shedding, Enterobacteriaceae, risk factors

## INTRODUCTION

Petting zoos – either permanent or temporary – are popular attraction sites that allow both direct and indirect exposure of children and adults to diverse animals (Steinmuller et al., 2006). Despite the educational and entertainment value of such interactions, petting zoos raise a significant concern regarding the zoonotic transmission of pathogens due to contact with animals, mainly through the oral–fecal route (Conrad et al., 2016). Indeed, the US Centers for Disease Control and Prevention (CDC) has published recommendations on “how to stay healthy at animal exhibits”<sup>1</sup>. Previous reports of public health risks and zoonosis originating from petting zoos, mostly in North America, typically describe either the transmission of highly virulent bacterial pathogens, including *Escherichia coli* and *Salmonella* outbreaks in petting zoos (Friedman et al., 1998; Goode et al., 2009), or risk behaviors for disease transmission (Weese et al., 2007; Erdozain et al., 2013).

In the past two decades, the global incidence of plasmid-mediated AmpC and extended spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae (ESBL/AmpC-E) has increased constantly in both humans and animals (Schwaber et al., 2006; Barco et al., 2015; Leonard et al., 2015). Environmental shedding of ESBL/AmpC-E by farm animals, such as cattle, poultry, and swine has been widely investigated in the past (Horton et al., 2011), but it is alarmingly understudied in petting zoos. In a single study, ESBL-producing *E. coli* were isolated from feces of petting zoo animals (Conrad et al., 2018), but larger studies on the incidence and risk factors for ESBL/AmpC-E shedding in petting zoos are lacking. Due to the direct contact between visitors (mainly children) and animals, identifying and characterizing the presence of these antibiotic-resistant bacteria on the body surface of the animals and the possible environmental shedding is of great public importance. Accordingly, in this prospective study, our aim was to determine the prevalence of ESBL/AmpC-E shedding in various petting-zoo animal species, characterize the molecular epidemiology of the isolates, and define the risk factors for shedding.

Addressing the emerging threat of antibiotic-resistant bacteria in petting zoo animals requires a “One Health” perspective and, therefore, the data from this study are crucial to the fight against the spread of resistance.

<sup>1</sup> <https://www.cdc.gov/features/animalexhibits/index.html>

## MATERIALS AND METHODS

### Petting Zoos and Study Design

We conducted a prospective cross-sectional study in eight permanent petting zoos across Israel (December 2016–May 2017), chosen randomly. The study was approved by the Internal Ethics Committee of the Koret School of Veterinary Medicine, Israel (Protocol KSVM-VTH/25\_2016), and was made possible through a respectable collaboration with the facility owners and veterinarians. We recruited petting zoos based on owners’ cooperation, considering the appropriate sample size. In order to examine diverse risk factors for shedding, we chose petting zoos which differed in characteristics (schools, exhibition, in a zoo property and ambulatory). In interviews conducted with the owners, we collected demographic and medical data, throughout owners’ questioners. Data included the total number of animals in each facility, the number of animal species, the type of veterinary care, petting and feeding policies by visitors and employees, the number of employees, and the average daily number of visitors. Data on each sampled animal included its class, species, diet, and sex, and antibiotic treatments that had been received during the past year, according to owners’ questioner.

### Bacteria Sampling, Isolation, Identification, and Antibiotic Susceptibility Testing

In each petting zoo, we sampled the maximum number of animals from diverse species. The goal was to sample all animals housed in the petting zoo, whereas in practice we sampled the animals which the owners approved to sample, mainly due to safety considerations. Sampling was performed during morning–noon hours. We collected fecal specimens from the close vicinity of the animal and analyzed them only if we could link them, through direct observation, to a specific animal. According to the decision of the owners and the ability to safely approach the animal, we also collected surface specimens from the skin, fur, or feathers by rubbing a sterile cotton swab, pre-moistened with saline, on the surface of the animal, in the back area, for at least 10 s. The sampling area was proportional to the size of each sampled animal.

Samples were stored at room temperature, in the commercial transport gel and were processed within 24 h of sampling. All samples were inoculated into 2-mL of a brain–heart infusion

enrichment broth, so as to increase the sensitivity of ESBL and AmpC-E detection (Murk et al., 2009). After incubation of 18–24 h at 37°C, the enriched cultures were plated (10 µL) onto Chromagar ESBL plates (Hy-Labs, Rehovot, Israel). Colonies that appeared after an overnight incubation at 37°C were recorded, and one colony of each distinct color was re-streaked onto a fresh Chromagar ESBL plate to obtain a pure culture. Next, the pure ESBL/AmpC-E suspected isolates were stored at –80°C stocks for further workup. All isolates were subjected to Vitek-2 for species identification and antibiotic susceptibility testing (AST-N270 Vitek2 card, BioMérieux, Inc., Marcy-l'Étoile, France). The identification of *Enterobacter* and *Citrobacter* species was verified using 16S rRNA gene sequencing and an RDP database comparison (Cole et al., 2005). ESBL and AmpC production were confirmed using combination disc diffusion confirmatory assays and interpreted according to the CLSI guidelines (CLSI, 2017, 27th edition). We adopted the guidelines for *E. coli* ESBL confirmatory assay by disc diffusion, and implemented it for *Enterobacter cloacae* and for *Citrobacter freundii*. All *E. cloacae* and for *C. freundii* isolates were automatically defined as AmpC producers, due to intrinsic resistance. Plasmid mediated AmpC production was tested via cefoxitin and resistance to second generation cephalosporin.

## Genotyping and Detection of $\beta$ -Lactamase Genes of ESBL/AmpC-E

To determine the genetic relatedness between isolates belonging to the same species, genotyping was performed using an Enterobacterial repetitive intergenic consensus (ERIC) PCR amplification with the following primer: 5'-AAG TAAGTGACTGGGGTGAGCG-3' (Versalovic et al., 1991). Results were analyzed using GelJ software (Heras et al., 2015) and all strains exhibiting a distinct ERIC PCR pattern were subjected to multilocus sequence type (MLST) using schemes for *E. coli*, *E. cloacae*, and *C. freundii*, as described previously (Diancourt et al., 2005; Bai et al., 2012; Miyoshi-Akiyama et al., 2013). Sequences of new gene alleles and sequence types (STs) were submitted to and assigned by PubMLST<sup>2</sup>.

Extended spectrum beta-lactamases and AmpC  $\beta$ -lactamase genes were identified by PCRs and sequencing. Isolates were examined for the presence of *bla*<sub>CMY-1</sub>, *bla*<sub>CMY-2</sub> (Kim et al., 2005), *bla*<sub>CTX-M</sub> group (Woodford et al., 2006), *bla*<sub>OXA-1</sub>, *bla*<sub>OXA-2</sub>, *bla*<sub>OXA-10</sub> (Lin et al., 2012), *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub> groups (Tofteland et al., 2007). Genes identified as *bla*<sub>CTX-M-1</sub> and *bla*<sub>CTX-M-9</sub> groups were sequenced to identify the specific gene allele, using the following primers (designed in this study): *bla*<sub>CTX-M-1F</sub> ATGGTTAAAAAATCACTGCG and *bla*<sub>CTX-M-1R</sub> TTACAAACCGTTGGTGACG, *bla*<sub>CTX-M-9F</sub> ATGGTGACAAAGAGAGTGCAAC and *bla*<sub>CTX-M-9R</sub> TTACAGCCCTTCGGCGATGA, respectively.

## Statistical and Risk Factor Analyses

The minimal sample size (number of animals sampled) was calculated using WinPepi, based on an estimated shedding rate of 10% for ESBL-E in community companion animals in

Israel (A. Shnaiderman-Torban, unpublished), with a confidence level of 95% and an acceptable difference of 5%, resulting in  $n = 139$ . Statistical analyses were performed using the IBM STATISTICS SPSS software (SPSS Version 24; SPSS Inc., Chicago, IL, United States). Data distribution was examined by testing whether the Skewness and Kurtosis equal zero and by performing the Shapiro–Wilk's test. Continuous variables were analyzed using *t*-tests or Mann–Whitney *U*-tests, according to the distribution of the variable. Categorical variables were analyzed using the Fisher's exact test or the Pearson chi-square test, as appropriate. In all statistical analyses,  $p \leq 0.05$  indicated significance. A multiple logistic regression model, using the ENTER method, was applied for ESBL/AmpC-E shedding using variables with  $p \leq 0.2$ .

## RESULTS

### Characterization of Petting Zoos and Animal Populations

The study population included animals in eight petting zoos in Israel, which were diverse in type, size, and other characteristics (Table 1). Overall, 228 animals (42 species) were sampled for ESBL/AmpC gut-shedding, including 161 mammals (71%, 23 species), 47 reptiles (20%, 12 species), and 20 avian species (9%, 7 species). Altogether, 381 specimens were collected from these animals, including fecal samples from 52 animals, surface samples (skin/fur/feathers) from 23 animals, and both fecal and surface samples from 153 animals.

### Prevalence of ESBL and/AmpC-Producing Enterobacteriaceae (ESBL/AmpC-E)

Of the 228 sampled animals, 12% ( $n = 28$ , CI 95% 8–17%) carried at least one strain of ESBL/AmpC-E and 25% co-carried more than one antibiotic-resistant strain (Figure 1, Table 2, and Supplementary Table S1). Carriage rates within different petting zoos varied significantly, from 0 to 22% (Table 2). Overall, 35 ESBL/AmpC-E isolates were recovered from 28 animals, of which 77% were from feces samples and 23% were from surface samples ( $n = 27$  and eight samples, respectively; Figure 1, Table 2, and Supplementary Table S1).

Of the 153 animals that were sampled from both feces and body surface, 15 animals were positive for ESBL/AmpC-E only in fecal samples, four animals were positive only in surface samples, and two animals were positive in both fecal and surface samples: a turtle from petting zoo #8, which carried two different *E. cloacae* strains on the skin (ST102) and in the feces (ST1152), with different resistance phenotypes (isolates p151.2 and p152.2; Supplementary Table S1); and a meerkat from petting zoo #6, which carried the same ESBL-producing *E. coli* strain, ST648, on its fur and in its feces (isolates p381.2 and p382.2; Supplementary Table S1).

Of the 42 animal species that were sampled, 19 species carried ESBL/AmpC-E (11 mammals, 7 reptiles, and one avian species). To the best of our knowledge, for 13 of these animal species, this

<sup>2</sup><https://pubmlst.org/>

**TABLE 1** | Characteristics of the eight petting zoos included in this study.

Petting zoo	Type of facility	per zoo per zoo	No. of animal species	No. of employees	Average daily visitors	Permitted policy		No. of animals treated with antibiotics at sampling (%) <sup>a</sup>
						Petting	Eating	
1	Zoo property	<50	15	6	50	+	—	0
2	Exhibition <sup>b</sup>		20	3	50	—	+	8/19 (42)
3	Zoo property		10	8	150	+	—	0
4	Ambulatory <sup>c</sup>		30	2	10	+	—	4/24 (17)
5	School property	50–100	20	3	50	+	+	2/46 (4)
6	School property		30	2	10	+	—	2/53 (4)
7	Private		20	9	70	+	+	0
8	Zoo property		35	14	>100	+	—	5/38 (13)

<sup>a</sup>Percent of antibiotic-treated animals at the sampling day, out of the total sampled animals at the respective petting zoo. <sup>b</sup>Restricted only for exhibitions with a petting prohibition policy. <sup>c</sup>Used for celebrations of children parties and festivals.

is the first report describing ESBL/AmpC-E shedding (**Figure 1** and **Supplementary Table S1**).

More than half (55%,  $n = 19$ ) of the 35 ESBL/AmpC-producing isolates belonged to the genera *E. cloacae* complex, while the rest were identified as *E. coli* (31%,  $n = 11$ ) and *C. freundii* (14%,  $n = 5$ ). The isolates encompassed strains that produce both ESBLs and AmpC (37%,  $n = 13$ ), AmpC alone (34%,  $n = 12$ ), or ESBL alone (29%,  $n = 10$ ). *E. cloacae* was the only species that was associated with gut shedding ( $p = 0.019$ ; **Figure 1**, **Table 2**, and **Supplementary Table S1**).

## Antibiotic Susceptibility Profiles of ESBL/AmpC-E

The antibiotic susceptibility profiles of the ESBL/AmpC-E isolates were diverse (**Supplementary Table S1**). For isolates producing both ESBLs and AmpC ( $n = 13$ ), resistance rates were 100% to amoxicillin/clavulanate, 38% to fosfomycin, and 31% to nitrofurantoin (intermediate susceptibility). All isolates were susceptible to carbapenems, gentamicin, and trimethoprim/sulfamethoxazole. For AmpC-E ( $n = 12$ ), resistance rates were 100% to amoxicillin/clavulanate, 33% to fosfomycin, 17% to ofloxacin, 8% to ciprofloxacin, and 25% to nitrofurantoin (intermediate susceptibility). All AmpC-E isolates were susceptible to gentamicin and trimethoprim/sulfamethoxazole. For ESBL-E ( $n = 10$ ), resistance rates were 50% to trimethoprim/sulfamethoxazole, 20% to ofloxacin, 33% to ciprofloxacin, and 10% to gentamicin. All ESBL-E isolates were susceptible to amoxicillin/clavulanate (one isolate had an intermediate susceptibility), piperacillin/tazobactam, fosfomycin, and nitrofurantoin (**Supplementary Table S1**).

## Genotyping of the ESBL/AmpC-E Isolates and Resistance Genes

To understand the genetic relatedness between ESBL/AmpC-E strains within and between different petting zoos, we performed ERIC PCR, followed by MLST analysis. We performed ERIC PCR on 30 isolates that were kept and stored successfully. *E. cloacae* revealed 14 isolates with 13 different clusters, *E. coli* revealed

11 isolates with six different clusters and *Citrobacter freundii* revealed five isolates with three different clusters (**Supplementary Figure S1**). MLST analysis performed on *E. cloacae* complex demonstrates that isolates belonged to multiple STs, of which seven are known STs, four (ST1151–ST1154) were assigned as new STs possessing new allele combinations, and one (ST1189) was assigned as a new ST possessing five new alleles: dnaA-329, fusA-215, gyrB-356, leuS-402, and rplB-153 (**Figure 1**).

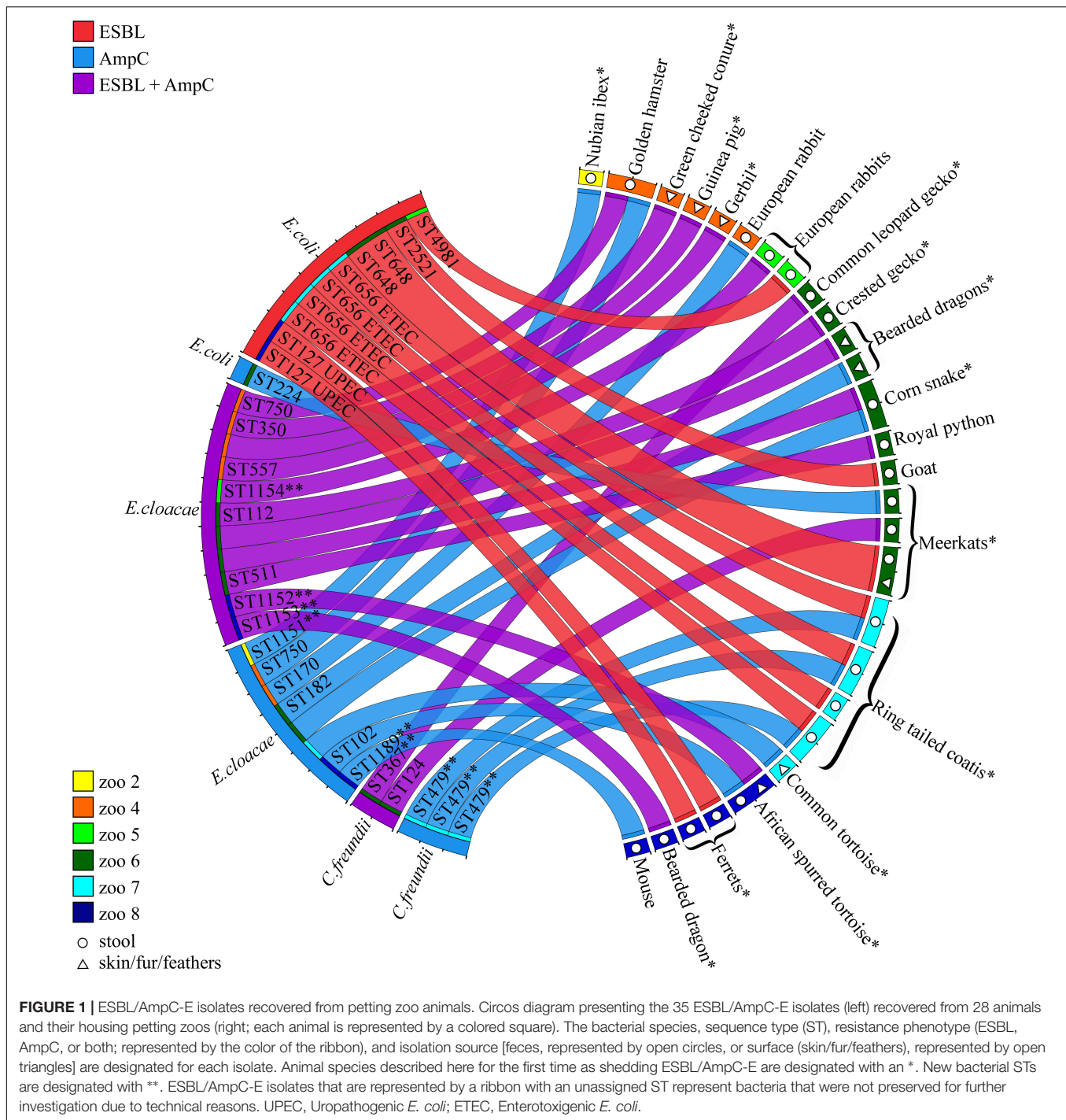
*Escherichia coli* was the second most prevalent  $\beta$ -lactamase-producing species (31%,  $n = 11/35$ ), in which the majority of the isolates (90%,  $n = 10/11$ ) were ESBL-producers and only one strain (ST224) was an AmpC-producer carrying a *bla*<sub>CMY-2</sub>. The ESBL genes detected in this species belonged to either the *bla*<sub>CTX-M-1</sub> group (three isolates: *bla*<sub>CTX-M-28</sub>), the *bla*<sub>CTX-M-9</sub> group (one isolate: *bla*<sub>CTX-M-14</sub>), and the *bla*<sub>SHV</sub> group (*bla*<sub>SHV-12</sub>, *bla*<sub>SHV-31</sub>, *bla*<sub>SHV-2</sub>, and *bla*<sub>SHV-2a</sub>, each detected in a different, single isolate). The 11 ESBL-producing *E. coli* isolates belonged to six known STs, including ST656 (four ring-tailed coatis from petting zoo #7), ST648 (two meerkats from petting zoo #6), ST127 (two ferrets from petting zoo #8), and three single-isolate STs: ST4981, ST2521, and ST224 (**Figure 1**). Neither of the *E. coli* isolates belonged to the worldwide ESBL-producing *E. coli* ST131 lineage.

The third species recovered from animals was the AmpC-producer *C. freundii* (five isolates; **Figure 1** and **Supplementary Table S1**). Three isolates carried the *bla*<sub>CMY-2</sub> gene; two of these isolates were also ESBL-producers, one produced *bla*<sub>CTX-M-28</sub>, and the other ESBL gene was not identified. Genotyping revealed the presence of two different strains shed by different animal species in zoo #6 (ST124 and ST367), and one *C. freundii* strain, assigned with a new ST, ST479, encoding two new alleles: aspC-177 and dnaG-167, shed by three individual coatis housed together in petting zoo #7 (**Figure 1**), suggesting inter-animal spread.

## Risk Factor Analysis for ESBL/AmpC-E Shedding

In a Univariable analysis, the shedding of an ESBL-E or an AmpC-E or ESBL/AmpC-E by an individual animal was





found to be significantly associated with antibiotic treatment ( $p = 0.038$ ,  $p = 0.011$ , and  $p = 0.029$ , respectively; **Table 3**). Overall, 11% of the sampled animals ( $n = 25/228$ ) were treated with antibiotics, including trimethoprim/sulfamethoxazole, cephalosporins, doxycycline, metronidazole, chloramphenicol, and the veterinarian quinolones enrofloxacin and marbofloxacin. ESBL/AmpC-E shedding was not associated with any specific antibiotic agent. AmpC-E and ESBL-E shedding

were associated with antibiotic treatment, the permitted petting policy, and the petting zoo sampled (**Table 3**). These factors were included in a logistic regression model, which revealed that antibiotic therapy is a risk factor for ESBL/AmpC-E shedding (OR = 7.34, 95% CI 1.88–28.56). In addition, petting zoo #2 was found to be a protective factor against ESBL/AmpC-E carriage (OR = 0.078, 95% 0.007–0.92).



**TABLE 2 |** Shedding rates of ESBL and AmpC-producing *Enterobacteriaceae* in petting zoos.

Petting zoo	No. of sampled animals	Number of positive ESBL/AmpC shedding animals (%) and bacterial species			Total No. of ESBL/AmpC carriers (%)
		Mammals	Reptiles	Avian species	
1	10	0/10 (0)	Not sampled	Not sampled	0
2	19	1/14 (7) <i>E. cloacae</i> complex (1)	0/2 (0)	0/3 (0)	1/19(5)
3	15	0/15 (0)	Not sampled	Not sampled	0
4	24	4/17 (24) <sup>a</sup> <i>E. cloacae</i> complex (5)	0/4 (0)	1/3 (30) <i>E. cloacae</i> complex (1)	5/24(20)
5	46	2/33 (6) <i>E. cloacae</i> complex (1); <i>E. coli</i> (1)	0/5 (0)	0/8 (0)	2/46(4)
6	53	4/25 (16) <sup>a</sup> <i>C. freundii</i> (1); <i>E. coli</i> (4)	6/23 (30) <sup>a</sup> <i>E. cloacae</i> complex (6); <i>C. freundii</i> (1)	0/5 (0)	10/53(19)
7	23	4/16 (25) <sup>b</sup> <i>C. freundii</i> (3); <i>E. coli</i> (4)	1/6 (17) <i>E. cloacae</i> complex (1)	0/1 (0)	5/23(22)
8	38	3/31 (10) <i>E. cloacae</i> complex (1); <i>E. coli</i> (2)	2/7 (29) <i>E. cloacae</i> complex (3)	Not sampled	5/38(13)
Total	228	18/161 (11)	9/47 (19)	1/20 (5)	28/228(12)

<sup>a</sup>One animal shed two bacterial species. <sup>b</sup>Three animals shed two bacterial species each, and one animal shed one bacterial species.

**TABLE 3 |** Associations and risk factor analysis for ESBL-E/AmpC shedding.

Risk factor/ Sampling site	AmpC shedding			ESBL shedding			Overall resistance shedding	Logistic regression OR
	Gut	Skin/fur/feathers	Total	Gut	Skin/fur/feathers	Total		
Antibiotic treatment <sup>a</sup>	0.012 <sup>e</sup>	0.128	0.011 <sup>e</sup>	0.069	0.457	0.038 <sup>e</sup>	0.029 <sup>e</sup>	7.34
Permitted petting policy	0.002 <sup>e</sup>	0.6	0.015 <sup>e</sup>	0.012 <sup>e</sup>	0.58	0.057	0.021 <sup>e</sup>	Not included
Petting zoo	0.186	0.191	0.017 <sup>e</sup>	0.073	0.073	0.039 <sup>e</sup>	0.077	0.078 <sup>b</sup>
Animal class <sup>c</sup>	0.255	0.024 <sup>e</sup>	0.055	0.25	1	0.269	0.215	Not included <sup>f</sup>
Animal diet <sup>d</sup>	0.802	0.251	0.5	0.629	0.159	0.26	0.302	Not included <sup>f</sup>
Animal gender	0.914	0.351	1	0.641	1	0.927	1	Not included <sup>f</sup>

<sup>a</sup>Any antibiotic treatment given to a specific animal during the past year. <sup>b</sup>Petting zoo #2 was found as a protective factor. <sup>c</sup>Mammal/reptile/avian species. <sup>d</sup>Diet as described by the owner and categorized as various plant materials (hay, vegetables, or concentrated vegetative feed), insects, or other prey (mice). <sup>e</sup> $p < 0.05$ . <sup>f</sup>Not included: excluded from the logistic regression model due to  $p > 0.2$ .

## DISCUSSION

This prospective study investigated the shedding of ESBL/AmpC-producing *Enterobacteriaceae* in a large and highly diverse sample of petting zoo animal species. To the best of our knowledge, this is the first study that focuses specifically on ESBL/AmpC-E shedding and defines the related risk factors. Of the 228 animals sampled throughout the country, 12% shed ESBL/AmpC-E. The prevalence of animal shedding varied significantly between different petting zoos, which may be explained by the diverse facilities that were sampled and that represent various animal–visitor interfaces. Because data regarding the prevalence of ESBL/AmpC-E in petting zoos in other countries are unavailable, these data are incomparable with other studies.

Although ESBL/AmpC-E shedding was previously reported in various mammals, reptiles, and avian species (Vittecoq et al., 2016), we screened a highly diverse population of new animal species. We report, for the first time, ESBL/AmpC-E shedding in 13 new host species of mammals, reptiles, and avian species (Figure 1). We found that ESBL/AmpC-E gut shedding was independent of the type of animal species, possibly due to

the small number of individual animals sampled within each species. The study included both fecal and surface sampling of diverse animal species, from smooth-skin reptiles to feathered birds or large furred animals, such as sheep or deer. Therefore, ESBL/AmpC-E recovery could be influenced by the type and area of the sampled surface. Supporting this claim is the finding that, in seven animals from four petting zoos, we recovered two different ESBL/AmpC-E strains from the feces and body surface of the same animal, indicating that ESBL/AmpC-E surface shedding may be due to fecal or environmental contamination, rather than gut shedding. In spite of these obstacles, the presence of MDR bacteria on animal surfaces highlights the potential risk of ESBL/AmpC transmission from healthy shedding animals to children and other visitors due to the possible close contact through petting and animal holding.

Importantly, all 35 ESBL/AmpC-E isolates recovered from animals belonged to only three different genera – *Enterobacter cloacae*, *E. coli*, and *C. freundii* – with *E. cloacae* being the most prevalent species, and which was found to be significantly associated with gut shedding. *Enterobacter* was previously reported to be a commensal bacteria shed by animals in zoos (Ahmed et al., 2007) and a pathogen causing infections in

animals (Gibson et al., 2010), but it is less frequently reported as a major ESBL/AmpC-producing genus in animals. The main investigated and reported ESBL species in the literature is *E. coli*. However, in this study we sampled a variety of different species, representing a variety of environmental conditions and interfaces. *E. cloacae* is ubiquitous in terrestrial and aquatic environments, such as water, sewage, soil, and food (Davin-Regli and Pagès, 2015). We hypothesize that due to the heterogeneous study population, we detected a high prevalence of *E. cloacae*. Previous reports on zoonotic bacterial outbreaks in petting zoos focused on highly transmissible virulent pathogens, such as *E. coli* O157:H7 (Stirling et al., 2007; Conrad et al., 2016), *Salmonella* spp., *Campylobacter* (Bender and Shulman, 2004), and *Shigella* (Stirling et al., 2007). Although *Enterobacter* and *Citrobacter* are known to be AmpC-producing human pathogens (Santos et al., 2015), they were not described previously as potential zoonotic bacteria. In light of our findings, these genera should be recognized as a possible source for ESBL/AmpC and it is recommended that they be actively monitored in petting zoo facilities.

Bacterial genotyping revealed multiple sequence types, which varied both between and within the same facility. Alarming, among the different ESBL-producing *E. coli* STs recovered, we identified three distinct pathogenic *E. coli* strains: ETEC ST656 (Oh et al., 2014) and UPEC ST127 (Gibreel et al., 2012), which have both been described as highly virulent, and ST648, which has previously been reported in humans and in domestic and wild animals (Ewers et al., 2014). Each of these three highly virulent *E. coli* STs was recovered from the same mammalian species, housed in the same cage, demonstrating inter-animal clonal transmission that could be explained by animal-to-animal contact or by environmental shedding. These strains were found in two petting zoos (#7 and #8) that had a permitted petting policy; thus, transmission may be a relevant risk and could be mediated, e.g., via workers and environmental shedding. In addition, we identified five new *E. cloacae* STs and one new *C. freundii* ST, which may suggest that these are commensal/environmental strains that may have acquired the ESBL/AmpC resistance via plasmid or gene acquisition. The high diversity of bacterial STs and resistance genes indicates the complex transmission mechanisms and the possible involvement of horizontal transfer of ESBL/AmpC genes and plasmids among petting zoo animals.

We found antibiotic treatment to be a risk factor for ESBL/AmpC-E shedding, similar to previous data on ESBL shedding in both animals (Belas et al., 2014) and humans (Ben-Ami et al., 2009). Assuming that human-animal contact is a risk factor for bacterial transmission in petting zoos, antibiotic-treated animals may constitute a high-risk population for resistant bacterial transmission. In light of these findings, it may be beneficial to consider the interactions between antibiotic-treated animals and visitors. In addition, we found that such animals are treated with a variety of antimicrobials, including third-generation cephalosporins and quinolones. An appropriate guideline for antimicrobial use in petting zoos is currently lacking, and our findings call for the establishment of such a guideline. A recommendation for

isolation of antibiotic-treated animals should further studied, since the duration of ESBL/AmpC shedding in animals was not established.

Another important finding was that ESBL/AmpC-E shedding was significantly associated with the petting permission policy. Strong support for this correlation resides in our findings that petting zoo #2, which had a petting prohibition policy, was found to be a protective factor for ESBL/AmpC-E carriage. We also found ESBL/AmpC-E carriage on the surfaces of animals (skin, fur, or feathers) – an exceptionally important finding in petting zoos, where direct contact is the main interaction between visitors (mostly children) and animals. These findings emphasize the connection between human-animal contact and ESBL/AmpC-E shedding, and they further highlight the importance of implementing strict hygiene and prevention guidelines.

In summary, the data reported in the current study raise new concerns regarding petting zoos as possible sources for ESBL/AmpC-E due to environmental shedding. Considering the valuable contribution of animal-associated activities to physical, social, and psychological aspects of human health (Friedmann et al., 2015), the educational and psychological importance of petting zoos is unequivocal. Therefore, we highly recommend promoting appropriate guidelines and interventions, some of which may include immediate actions and should be further investigated and implemented in the future. Immediate recommendations for petting zoo owners constitute the implementation of hygiene guidelines, including accessible means for hand washing and disinfection, as well as restricted refreshment areas for visitors. Long-term recommendations may comprise improved antibiotic stewardship and the implementation of active surveillance programs. Our findings emphasize the need of additional national and international surveillance studies, which, together, should facilitate the establishment of a standard comprehensive guideline for petting zoo operators and visitors, so as to minimize the associated public health risks.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the <https://pubmlst.org/>. Accession numbers: ST1151–ST1154 and ST1189.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Internal Ethics Committee of the Koret School of Veterinary Medicine, Israel (Protocol KSVM-VTH/25\_2016).

## AUTHOR CONTRIBUTIONS

AS-T and GM collected and analyzed the specimens and petting zoo owners' questioners. AS-T performed the molecular

analysis. YP performed the bacterial identification. AS-T and WA performed the statistical analysis. AS-T, AS, and SN-V contributed to the conception and design of the study. AS and SN-V wrote the manuscript. All authors read and approved the submitted manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02488/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.

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# Resistance Reservoirs and Multi-Drug Resistance of Commensal *Escherichia coli* From Excreta and Manure Isolated in Broiler Houses With Different Flooring Designs

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Carriage of resistant bacteria and spread of antimicrobial resistance (AMR) in the environment through animal manure pose a potential risk for transferring AMR from poultry and poultry products to the human population. Managing this risk is becoming one of the most important challenges in livestock farming. This study focused on monitoring the prevalence of multi-drug resistance (MDR) bacteria and development of AMR depending on flooring. In two experiments (2 × 15,000 birds), broilers were always divided in two different stables. In the control group, the entire floor pen was covered with litter material and in the experimental group, the flooring system was partly modified by installing elevated slat platforms equipped with water lines and feed pans. Over the whole fattening period, excreta and manure samples were taken (days 2, 22, and 32). In total, 828 commensal *E. coli* isolates were collected. The development and prevalence of resistance against four different antibiotic classes (quinolones,  $\beta$ -lactams, tetracyclines, and sulfonamides) were examined by using broth microdilution. At the end of the trials, the amount of manure per square metre was twice as high below the elevated platforms compared to the control group. Approximately 58% of *E. coli* isolates from excreta showed resistance against at least one antibacterial agent at day 2. During and at the end of the fattening period, resistant *E. coli* isolates at least against one of the four antibacterial agents were observed in excreta (46 and 46%, respectively), and manure samples (14 and 42%, respectively), despite the absence of antibacterial agent usage. In spite of less contact to manure in the experimental group, the prevalence of resistant *E. coli* isolates was significantly higher. Birds preferred the elevated areas which inevitably led to a local high population density. Animal-to-animal contact seems to be more important for spreading antimicrobial resistant bacteria than contact to the

litter-excreta mixture. Therefore, attractive areas in poultry housing inducing crowding of animals might foster transmission of AMR. In poultry farming, enrichment is one of the most important aims for future systems. Consequently, there is a need for keeping birds not carrying resistant bacteria at the start of life.

**Keywords:** antibiotic resistance, multi-drug resistance, commensal *E. coli*, housing system, flooring design, slatted floor, broilers

## INTRODUCTION

Important resistance problems are the spread of resistant bacteria within and between food-producing animals and humans (Schwarz et al., 2001). Understanding the development as well as transmission of resistant bacteria in intensive poultry production is necessary to implement effective risk management strategies of antibiotic resistance from food-producing animals to humans (GERMAP, 2016; Mehdi et al., 2018; WHO, 2018). Antimicrobial resistance (AMR) is recognised as one of the “One Health” issue that involves links along the potential human-animal-environment infection chain (Aidara-Kane et al., 2018; Collignon and McEwen, 2019). The occurrence of AMR can be caused by horizontal and vertical transmission (Schwarz and Chaslus-Dancla, 2001; McEwen and Collignon, 2018). Multi-drug resistance (MDR) is increasingly reported in poultry worldwide, and due to the impact of these organisms on public health, there is increasing interest in the origin and spread of these organisms in the poultry production chain (Dandachi et al., 2018; Amador et al., 2019).

Antibiotic resistance is a rapidly growing problem in *Enterobacteriaceae*; particularly *E. coli* found in intensive broiler production are developing resistance to multiple antibacterial agents that are important to human health (Hanon et al., 2015; Werckenthin, 2016; Nhung et al., 2017; EFSA and ECDC, 2018). Moreover, it has been reported that the degree of multi-resistance in *E. coli* isolates was highest in broiler chickens compared to other livestock categories (Hanon et al., 2015). *E. coli* are a commensal in the gut microbiome of both humans and animals (Hammerum and Heuer, 2009). Additionally, human and animal gut can be a reservoir of transferable AMR (Rolain, 2013). Commensal *E. coli* are frequently tested for their resistance to antibiotics as they are considered a good indicator of antibiotic exposure of their host (van den Bogaard et al., 2001).

Possible transmission of AMR from poultry products to the human population and additional links to the spread of antimicrobial resistant bacteria in the environment through animal manure have been one of the most important livestock farming challenges for years (van den Bogaard et al., 2001; Kemper, 2009). The poultry industry must develop innovative techniques along the production chain that guarantee high quality and safe consumer products through good management in animal health and welfare as well as in food safety (Windhorst, 2017). Preventive actions to decrease the risk of AMR in order to reduce the need for antimicrobial treatment have to be implemented (De Jong et al., 2014).

Poultry are kept on littered concrete floors for commercial poultry production in Europe (Windhorst, 2017). As a result,

the birds spend most of their productive life in close contact with excreta and manure (Kamphues et al., 2011). Thus, these materials can be recognised as a possible reservoir of antibiotic-resistant bacteria (Furtula et al., 2010) and act as a potential reservoir for spreading these organisms to humans via the food chain (Kemper, 2009; OIE, 2018; Amador et al., 2019; Thakur and Gray, 2019).

Information has been reported concerning the effects on the development of AMR in commensal *E. coli* in fattening poultry by separating animals from their excreta under experimental conditions (Chuppava et al., 2018a,b). However, so far, studies have mainly focused on the development of antibiotic-resistant bacteria alongside with antibiotic use (Schwarz and Chaslus-Dancla, 2001; Chantziaras et al., 2013).

The aim of the present study without antibiotic usage was to examine the occurrence and development of AMR and prevalence of multi-drug resistant *E. coli* isolated from excreta and manure samples from large-scale broiler housings with special consideration of the effects of different flooring designs in areas of a stable, which are highly frequented due to their attractiveness.

## MATERIALS AND METHODS

### Design of Experiment

The broiler chickens in this study were raised under standardised husbandry conditions on the Farm for Education and Research in Ruthe, University of Veterinary Medicine Hannover, Foundation, Germany. The animals were housed in two adjacent buildings, one for the control and one for the experimental groups. The poultry stable was equipped with a gas air-heating system and an automatic temperature, humidity and weight control assembly. The feeding and drinking areas were equipped with a common feed pan system (Big Dutchman International GmbH, Vechta, Germany). A common drinking water system with nipple drinkers for broilers was used (Big Dutchman International GmbH, Vechta, Germany). Dust-free wood shavings were used as litter material and sanitised straw bundles as perching material. Before beginning with the trials, stable floors and all materials had been disinfected as well as screening tests for *Enterobacteriaceae* had been carried out to confirm that they were free of contamination.

Two consecutive trials were conducted with 15,000 birds each. The birds were reared with a stocking density in accordance with German regulations. The experiments started with 1-day-old broiler chickens (as hatched; Ross 308;  $N = 30,000$ ). Broilers had been obtained from a commercial hatchery. The birds in the

control and experimental groups each came from the identical hatch for each trial and were randomly distributed to the stables. The growing period lasted about 32 days (d) and was carried out under controlled environmental housing conditions. Animals had *ad libitum* access to fresh, clean water. A commercial pelleted growing diet was offered (MEGA Tierernährung GmbH & Co. KG, Visbek-Rechterfeld, Germany). The feeding programme was divided into three phases. First, a starter feed was offered [12.4 MJ ME/kg, 224 g crude protein (CP)/kg, non-genetically modified organisms (GMO), d 0–11]. From d 12 onward, a grower I followed (12.2 MJ ME/kg, 194 g CP/kg, non-GMO, d 12–19) and after d 20 the feed was changed to a grower II (12.0 MJ ME/kg, 193 g CP/kg, non-GMO, d 20–32). Birds were not given any antibacterial agents throughout the rearing period.

The first group represented the conventional broiler housing and served as a control. Birds were reared on litter material [Litter (L) – entire stable floor covered with wood shavings; **Figure 1A**]. In the second group [Partial-slats (PS) – stable floor with wood shavings and partially slatted flooring; **Figure 1B**], plastic slatted flooring was installed at the west side of the stable (about one-quarter of the total flooring area; legally classified as not belonging to the actual usable flooring area). In this area, there was also the possibility to access feed and water. Plastic slatted-flooring composed of plastic-coated steel slats consisting of holes (15 × 10 mm) and bridges (plastic-covered steel, 3.5 mm wide, Big Dutchman International GmbH, Vechta, Germany). The excreta were stored at a depth of approximately 15 cm below the slatted flooring without removing any material during the entire fattening period. The birds in this study had contact with the litter-excreta mixture during the whole study period in the littered area of the control and the experimental groups.

## Collection of Excreta and Manure Samples

Excreta ( $n = 720$ ) and manure samples ( $n = 108$ ) were collected at three different points of time during the experiment (d2, 22,

and 32 of the growing period). Samples were taken from nine defined places on each sampling day (**Figure 1**). The sample points were distributed over the whole stable area and included drinking areas, feeding areas and the aisle between the drinking and feeding lines. At each area, fresh excreta were sampled from single animals. Manure samples (50 g) were taken with a plastic cup (6 cm in diameter). Material was punched out from the full depth of the litter manure. At the end of each trial, total amounts of manure were replaced, weighed and the dry matter (DM) content was determined so as to calculate the amount of manure per square metre (**Supplementary Table S3**). Samples were oven-dried over 24 h at 103°C.

## Isolation and Confirmation of *E. coli*

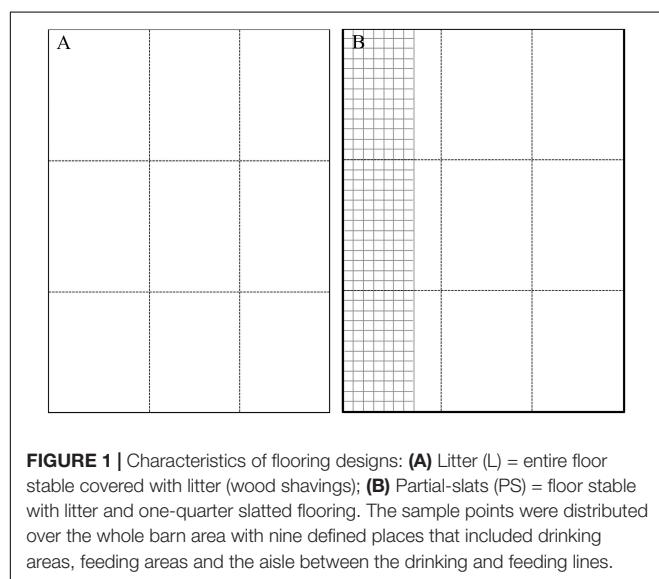
The bacteriological screening was conducted as previously described (Chuppava et al., 2018b). In short: Swab samples with excreta material were processed by streaking on Gassner agar plates (Oxoid Deutschland GmbH, Wesel, Germany), and incubated overnight at 37°C. Twenty-five grammes of each manure sample were suspended in 50 mL of peptone water (Oxoid Deutschland GmbH, Wesel, Germany) and added to a sterile Whirl-Pak® Bag (Nasco International Inc., Fort Atkinson, Wisconsin, United States). The bags were mixed with a Bag Mixer® 400 VW (Interscience International, St Nom la Bretèche, France) for 3 min. Of each mixed-sample, 10 µL was streaked on Gassner agar (Oxoid Deutschland GmbH, Wesel, Germany) and afterward incubated at 37°C for 18–24 h. With the same procedure, one single blue colony from each plate was chosen and spread onto Columbia blood agar (Oxoid Deutschland GmbH, Wesel, Germany) and Tryptone Bile X-glucuronide (TBX) agar (Oxoid Deutschland GmbH, Wesel, Germany). Incubation took place overnight at 37°C. Glucuronidase activity is indicated by blue-green colonies on TBX agar. To confirm the test result, the positive indole test was used with Kovac's indole reagent (Merck KGaA, Darmstadt, Germany).

## Antibiotic Susceptibility Testing

Minimal inhibitory concentrations (MICs) of enrofloxacin (ENR), ampicillin (AMP), tetracycline (TET) and trimethoprim/sulfamethoxazole (SXT) were examined by using broth microdilution and determined by using Micronaut plates (Merlin®, Merlin Diagnostika GmbH, Bornheim-Hersel, Germany) with Mueller-Hinton Broth (Merlin®, Merlin Diagnostika GmbH, Bornheim-Hersel, Germany). Dried antibacterial agents in serial dilutions of ENR (0.015625 – 16 µg/mL), AMP (0.125 – 256 µg/mL), TET (0.0078125 – 16 µg/mL), and SXT (0.02/0.30 – 32/608 µg/mL) were placed in wells of these plates. For each isolate, a growth control was added to one well. The manufacturer's guidelines and those of the Clinical and Laboratory Standards Institute (CLSI) formed the basis for the evaluation of the results. Reference strain, *E. coli* ATCC 25922, was tested concurrently on each testing day.

## Classification Using Clinical Breakpoints

The results were categorised as susceptible (S), intermediate (I) or resistant (R) in accordance with the CLSI veterinary breakpoints available for *Enterobacteriaceae* (CLSI, 2014). The prevalence of



**FIGURE 1 |** Characteristics of flooring designs: **(A)** Litter (L) = entire floor stable covered with litter (wood shavings); **(B)** Partial-slats (PS) = floor stable with litter and one-quarter slatted flooring. The sample points were distributed over the whole barn area with nine defined places that included drinking areas, feeding areas and the aisle between the drinking and feeding lines.

antibiotic resistance was defined as the percentage of resistance. MIC distributions for four antibacterial agents were evaluated and summarised as a percentage of frequency. If an isolate with resistance to three or more different antibacterial agents was found, it was defined as MDR (Magiorakos et al., 2012).

## Statistical Analysis

The SAS statistical software package version 7.15 (SAS Inst., Cary, NC, United States) was used to analyse the collected of AMR data. Significant differences were verified among the prevalence of AMR and MDR in isolates acquired from birds from different sampling times by using Pearson's chi-square test or Fisher's exact test when expected frequency values were below five. This test was also used to determine the significant differences in the frequency of resistance between the two groups of flooring designs. The statistical significance was set at a  $p$ -value of  $p < 0.05$ .

## RESULTS

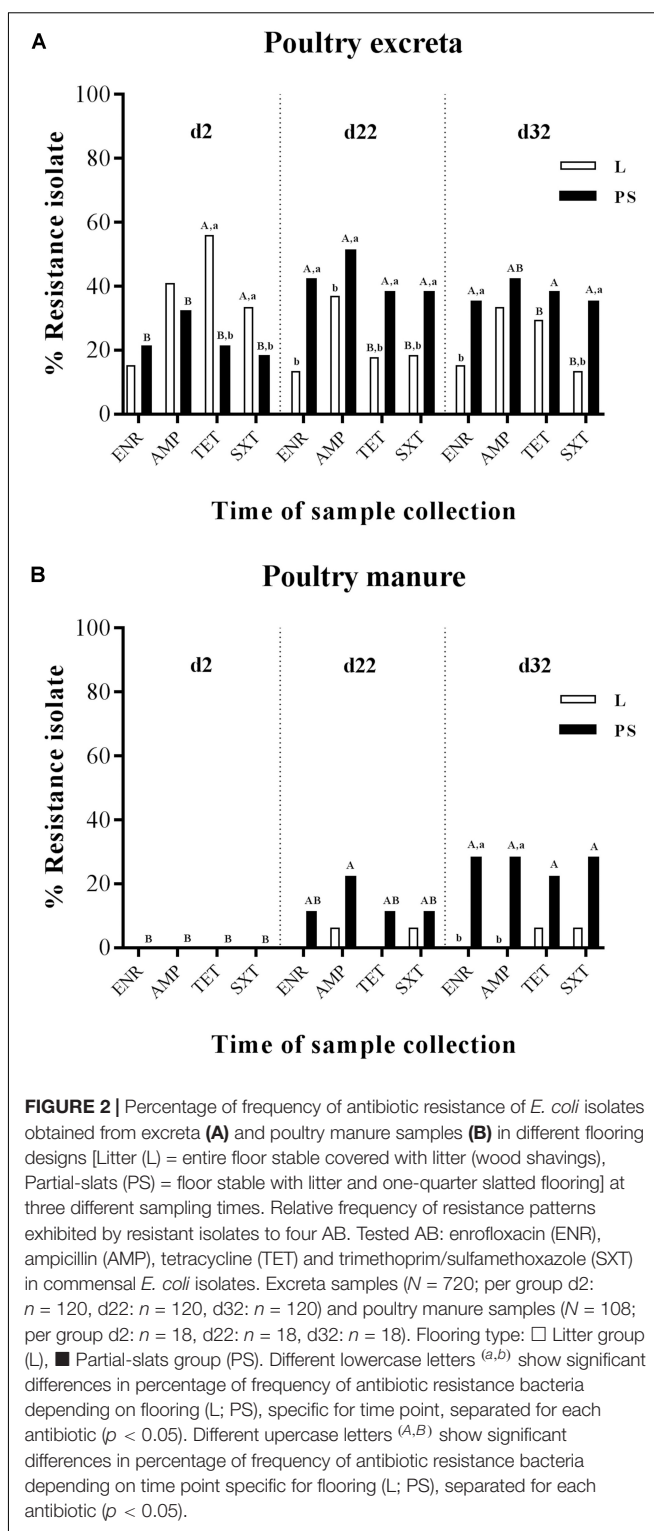
All 828 *E. coli* isolates (720 excreta samples and 108 manure samples) were tested against four antibiotics (ENR, AMP, TET, and SXT); the results are summarised in **Figures 2A,B**. The prevalence of resistant *E. coli* was defined as the percentage of excreta (**Figure 2A**) and manure samples (**Figure 2B**) for each of the two different flooring designs [Litter (L) and Partial-slats (PS)]. Collection was done at three different times (d2, d22, and d32). Details of the percentage values of resistance to four antibacterial agents in *E. coli* isolates between sampling times and flooring designs are described in **Supplementary Tables S1A,B**.

### Prevalence of Resistance to Antibacterial Agents in *E. coli* Depending on Sampling Times

When comparing the sampling times (**Figures 2A,B**; the percentages values of resistant *E. coli* for each collection time are described in detail in **Supplementary Tables S1A,B**), it became apparent that *E. coli* resistance to ENR, AMP, TET, and SXT isolated from fresh excreta material was already detectable at the beginning of the experiment (d2; **Figure 2A**). In contrast, at d2, all of the *E. coli* isolates from manure samples were susceptible to all four antibacterial agents (**Figure 2B**). During the experiment at d22, a significant difference occurred in isolates from the excreta samples (**Figure 2A**). At the end of the rearing period (d32), the significance between d22 and d32 could not be found in isolates from excreta swabs and manure samples (**Figures 2A,B**).

Regarding *E. coli* isolated from the fresh excreta material (**Figure 2A**), the prevalence of resistance to ENR in the PS group significantly increased from the start to the middle of the experiment at d22 (21–42%, respectively; **Supplementary Table S1A**). Nevertheless, the ENR-resistant *E. coli* isolates from the excreta samples (**Figure 2A**) and manure samples (**Figure 2B**) demonstrated no significant differences in resistance between d22 and d32.

Ampicillin-resistant *E. coli* isolates were detected from fresh excreta swabs (**Figure 2A**) at the start of fattening. The highest tested prevalence of resistance to AMP was detected in excreta



**FIGURE 2 |** Percentage of frequency of antibiotic resistance of *E. coli* isolates obtained from excreta (**A**) and poultry manure samples (**B**) in different flooring designs [Litter (L) = entire floor stable covered with litter (wood shavings), Partial-slats (PS) = floor stable with litter and one-quarter slatted flooring] at three different sampling times. Relative frequency of resistance patterns exhibited by resistant isolates to four AB. Tested AB: enrofloxacin (ENR), ampicillin (AMP), tetracycline (TET) and trimethoprim/sulfamethoxazole (SXT) in commensal *E. coli* isolates. Excreta samples ( $N = 720$ ; per group d2:  $n = 120$ , d22:  $n = 120$ , d32:  $n = 120$ ) and poultry manure samples ( $N = 108$ ; per group d2:  $n = 18$ , d22:  $n = 18$ , d32:  $n = 18$ ). Flooring type: □ Litter group (L), ■ Partial-slats group (PS). Different lowercase letters ( $a, b$ ) show significant differences in percentage of frequency of antibiotic resistance bacteria depending on flooring (L; PS), specific for time point, separated for each antibiotic ( $p < 0.05$ ). Different uppercase letters ( $A, B$ ) show significant differences in percentage of frequency of antibiotic resistance bacteria depending on time point specific for flooring (L; PS), separated for each antibiotic ( $p < 0.05$ ).

swab and manure samples at d22 in the PS group (51 and 22%, respectively). In addition, the percentage of samples with isolation of resistant *E. coli* among excreta swabs and manure samples significantly increased from d2 to d22; 32% to 51% and 0% to 22%, respectively (**Figures 2A,B**).



The results of percentage of tetracycline-resistant *E. coli* isolates from fresh excreta swabs showed a significant decrease from the beginning to the middle of the experiment in group L (56% to 18%; **Figure 2A**). On the contrary, the percentage of TET-resistant *E. coli* isolates in the PS group increased from 21% to 38% during the experiment (**Figure 2A**).

Significant differences between the sampling times could also be found in trimethoprim/sulfamethoxazole-resistant *E. coli* isolates (**Figures 2A,B**). The prevalence of resistance in fresh excreta swabs significantly decreased from the beginning to the middle of the experiment in group L (33% to 18%; **Figure 2A**). On the other hand, in the PS group, the percentage of excreta samples with isolation of resistant *E. coli* significantly increased from d2 to d22 (18% to 38%; **Figure 2A**). In manure samples, there were no significant differences between d2 and d22 (**Figure 2B**).

### Depending on Flooring Designs

At the beginning of the experiment (d2), there was no significant difference in percentage of isolates from excreta resistant against ENR and AMP between the L and PS groups, whereas isolates were more often resistant against TET and SXT in the L group at start (**Figure 2A**). TET-resistant *E. coli* isolates showed the significantly highest prevalence (**Supplementary Table S1A**). There were significant differences between flooring systems in this collection time. The prevalence of TET-resistant isolates was significantly different between the L and PS groups, 56 and 21%, respectively ( $p$ -value < 0.05; **Figure 2A** and **Supplementary Table S1A**). At the same point in time, SXT-resistant isolates in the L group also showed significantly higher resistance rates compared with the isolates collected from the PS group, 33 and 18%, respectively ( $p$ -value < 0.05; **Figure 2A** and **Supplementary Table S1A**). On the other hand, none of the *E. coli* isolates from manure samples showed any differences between the groups at d2 (**Figure 2B**). The resistance rates in the L and PS groups were similar.

During the experimental period (d22), a significant difference between the two flooring systems was found only in isolates from fresh excreta swabs (**Figure 2A**). At this point, resistant *E. coli* isolates of all four antibacterial agents in the L group showed significantly lower percentages than in the PS group; ENR: 13 and 42%, AMP: 37 and 51%, TET: 18 and 38% and SXT: 18 and 38%, respectively ( $p$ -value < 0.05; **Supplementary Table S1A**). For manure samples, there were no significant differences in the resistance between the L and PS groups during this sampling time (**Figure 2B**).

At the end of the fattening period (d32), the *E. coli* isolates in L group also showed significantly lower percentages in resistance to ENR and SXT acquired from fresh excreta swabs than in the PS group (ENR: 15 and 35% and SXT: 13 and 35%, respectively; **Figure 2A** and **Supplementary Table S1A**). However, the resistant *E. coli* isolates acquired from manure samples (**Figure 2B** and **Supplementary Table S1B**) at d32 showed significant differences between the L and PS groups (ENR: 0 and 28% and AMP: 0 and 28%, respectively;  $p$ -value < 0.05). Regarding TET and SXT resistance in isolates from the manure samples (**Figure 2B**), there were no significant differences between the groups.

## MIC Distribution of the Commensal *E. coli* Isolates

Minimal inhibitory concentration distribution of the 720 commensal *E. coli* isolates (percentage of frequency) from fresh excreta was tabulated separately for each antibacterial agent depending on sampling time points and flooring design according to age (two, 22 and 32 days of age, respectively) in **Figures 3A–D**. Categorisation was done by using CLSI breakpoints [ENR  $\geq 2$   $\mu\text{g/mL}$ , AMP  $\geq 32$   $\mu\text{g/mL}$ , TET  $\geq 16$   $\mu\text{g/mL}$  and SXT  $\geq 4/76$   $\mu\text{g/mL}$ ] for *Enterobacteriaceae* (CLSI, 2014).

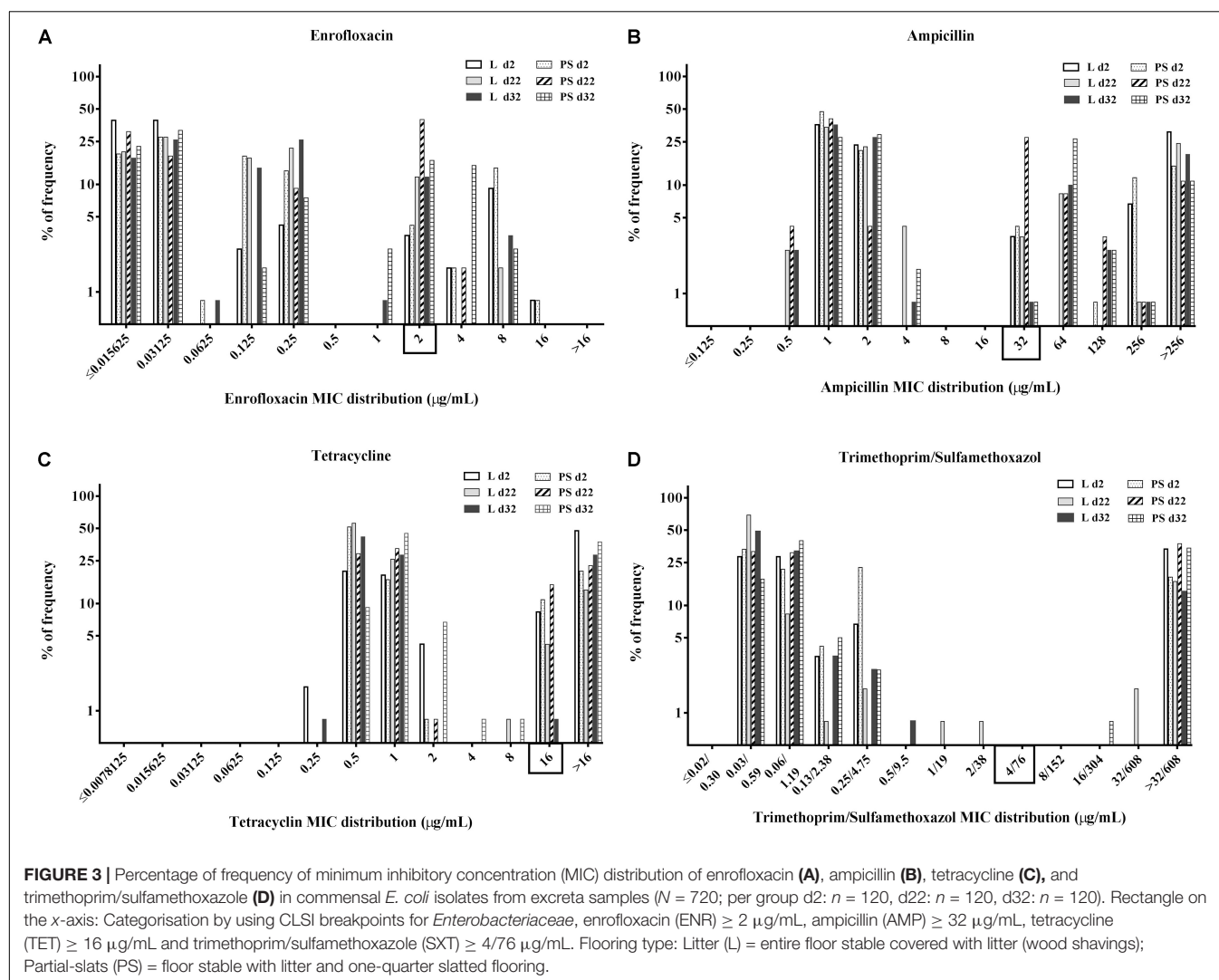
When isolates were compared based on the flooring types in the two groups, the overall percentages of resistant *E. coli* isolates in the PS group were higher than those in the L group (**Figures 3A–D**). When isolates were grouped according to time of collection, the overall resistance percentages were higher at d22 compared with the others sampling times (**Figures 3A–D**).

Regarding the MICs distribution for ENR (**Figure 3A**), a high percentage of resistant *E. coli* isolates was found at d22 in the PS group, with 42% isolates ( $n = 120$ ) having enrofloxacin MICs of 2  $\mu\text{g/mL}$ . Approximately 37 and 21% of isolates from fresh excreta samples ( $N = 720$ ) had ampicillin MICs of 1 and 2  $\mu\text{g/mL}$ , respectively (**Figure 3B**), with 18% of isolates ( $N = 720$ ) having AMP MICs of  $>256$   $\mu\text{g/mL}$ . Concerning the percentage of isolates with tetracycline MICs (**Figure 3C**), approximately 66% of *E. coli* isolates from fresh excreta samples ( $N = 720$ ) had MIC-values below the clinical breakpoint (MIC < 16  $\mu\text{g/mL}$ ). Approximately 26% of *E. coli* isolates from fresh excreta samples ( $N = 720$ ) had SXT MIC-values above the clinical breakpoint (MIC  $\geq 4/76$   $\mu\text{g/mL}$ ; **Figure 3D**).

## Prevalence of Multi-Drug Resistance and Resistance Pattern

Isolate resistance to at least three antibacterial agents was defined as MDR (Magiorakos et al., 2012), belonging to different antibiotic classes: enrofloxacin (fluoroquinolones (FQ) class), ampicillin ( $\beta$ -lactams class), tetracycline (tetracyclines class), trimethoprim/sulfamethoxazole (sulphonamides class). The values of multi-resistance in *E. coli* for each collection time and group are given in detail in **Supplementary Table S2**. At the beginning of the study, the MDR rates were significantly higher in L group samples (34 and 19% in L and PS group, respectively;  $p$ -value < 0.001; **Figure 4**). The highest levels of MDR were found at d 22 in the PS group (38%; **Figure 4**). The prevalence of MDR continually increased in *E. coli* isolates in the PS group from d2 to d22 (19% to 38%; **Supplementary Table S2**).

Overall prevalence of AMR in the present study showed a high percentage of *E. coli* isolates resistant to at least one antibiotic agent from day-old chicks' excreta in both the L and PS groups, 66 and 51%, respectively, (**Supplementary Table S2**) as well as 33% isolated from the paper inlays from the transport boxes (data not shown). During and at the end of the fattening period (**Supplementary Table S2**), *E. coli* isolates resistant to at least one antibiotic agent were observed in fresh excreta samples in both the L and PS groups (d22: 42 and 51%; d32: 46 and



46%, respectively), despite the absence of antibacterial agent usage in this study.

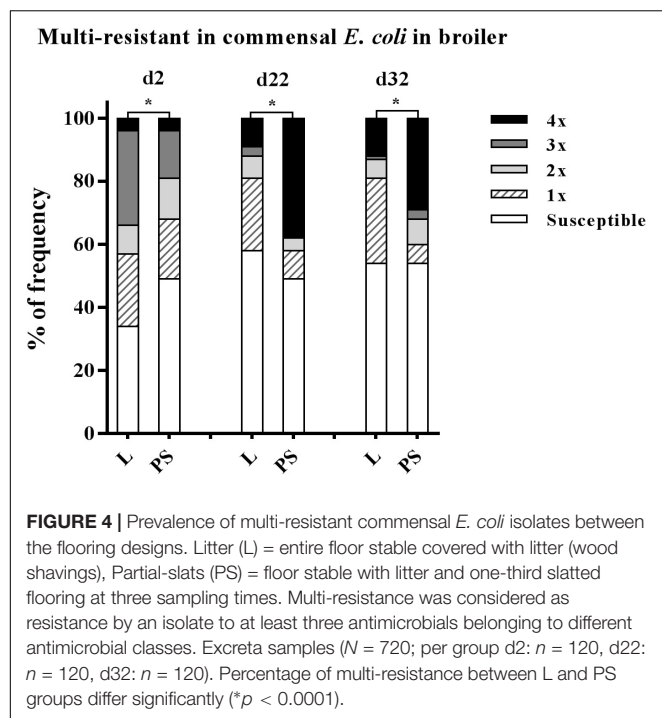
The 720 resistant *E. coli* isolates from fresh excreta samples in this study were grouped into fourteen different resistance patterns (Table 1). Approximately fifty percent of all isolates from these swabs (359 out of 720) show resistance to at least one of the tested antibacterial agents (Table 1), 19% were individually resistant (130 out of 720) and of these 130 isolates, 64 of them showed resistance to AMP. Seven per cent were resistant to two antibacterial agents and 24% (176 out of 720) were defined as multi-resistant (having at least three resistance determinants). The most common resistance patterns in this study were ENR-AMP-TET-SXT (119 isolates out of 720). The highest number of broad-spectrum resistance pattern (ENR-AMP-TET-SXT) was found at d22 in isolates from the PS group (45 isolates; Table 1). Regarding other additional resistance patterns, the prevalence of MDR was observed in excreta samples ( $N = 720$ ; Table 1); AMP-TET-SXT (56 isolates), AMP-TET (26 isolates), ENR-AMP (11 isolates), ENR-TET (eight isolates), TET-SXT (six isolates), AMP-SXT (two isolates), and ENR-AMP-SXT (one isolate).

## DISCUSSION

Antibiotic resistance is a global health threat (WHO, 2018). Concerns due to the emergence of AMR in humans are justified by the occurrence of AMR in animals and their environment (Schroeter and Kaesbohrer, 2010; DART, 2015). AMR in poultry production is one of its contributing sources. This has been the major topic of a large number of studies in recent years on single drug resistance as well as MDR in poultry (Furtula et al., 2013; Diarra and Malouin, 2014; Nhung et al., 2017; Chuppava et al., 2018a; McEwen and Collignon, 2018; Roth et al., 2018; Thakur and Gray, 2019).

## Resistance to Antibacterial Agents Found in Day-Old Chicks

*E. coli* isolated from 2-day-old chicks' excreta in the present study showed resistance to enrofloxacin, ampicillin, tetracycline and trimethoprim/sulfamethoxazole, despite the birds not having been previously in contact with antibacterial agents as well as



stable floors and all materials were free of *Enterobacteriaceae* contamination before beginning with the trials. Similar results were reported in previous studies (Jiménez-Belenguer et al., 2016; Chuppava et al., 2018a,b) finding *E. coli* from 1-day-old chicks resistant to ENR, AMP, TET and SXT; and also from chicks on laying hen farms (Moreno et al., 2019).

The high resistance rates found in our study including from the paper inlays from the transport boxes, could be associated with vertical transmission of resistant isolates from parent flocks as described in literature (Petersen et al., 2006; ECDC et al., 2017). Contamination during incubation in the hatchery or during transport seems to be the most probable explanation (Dierikx et al., 2013; Projahn et al., 2018). Hatchery-related factors can generally influence the occurrence of resistance to antibacterial agents (Persoons et al., 2011). In newly hatched chicks, the bacteria found in the environment, whether they are susceptible or resistant, colonise the gut and become part of the normal intestinal flora (Persoons et al., 2011). Therefore, the possible explanation for the resistance rates found in our study could be due to contamination of chickens by vertical transmission.

According to previous findings (Petersen et al., 2006; Bortolaia et al., 2010; Moreno et al., 2019), resistance to FQ,  $\beta$ -lactams, tetracycline and sulphonamides in *E. coli* was related to parent chickens. Therefore, it is possible to have vertical transmission of AMR of commensal *E. coli* that were selected for AMR long ago and remain as commensal populations within the hatchery or in stable, whether acquired resistance or natural. However, in every single case, other possible sources of contamination such as the antibiotic usage upstream the hatchery cannot be ruled out.

The findings regarding young animals as a potential reservoir of AMR in this study suggest that besides the effects of the management practices known from previous studies, the focus of reduction approaches should be on implementing poultry hatcheries and sources along the distribution chain to control the spread of AMR. Consequently, further research is strongly recommended, paying particular attention to analysing the genetic basis of resistance in the isolates. This should be done on as many isolates as possible to avoid bias from sample

**TABLE 1 |** Antibiotic resistance pattern for *E. coli* isolates obtained from excreta samples ( $N = 720$ ) in different flooring designs at the age of 2, 22, and 32 days.

No. antibacterial agents	Antibiotic resistance pattern*	Number of isolates						Total
		d2 (n = 240)		d22 (n = 240)		d32 (n = 240)		
		L	PS	L	Ps	L	PS	
1	Susceptible to all	41	60	71	59	66	64	361
	ENR	12	17	1	0	2	1	33
	AMP	0	7	25	11	16	5	64
	TET	16	3	0	0	13	1	33
	SXT	0	0	0	0	0	0	0
2	ENR-AMP	0	1	0	5	1	4	11
	ENR-TET	5	2	1	0	0	0	8
	ENR-SXT	0	0	0	0	0	0	0
	AMP-TET	9	8	0	0	6	3	26
	AMP-SXT	0	0	2	0	0	0	2
3	TET-SXT	0	0	3	0	0	3	6
	ENR-AMP-TET	0	0	0	0	0	0	0
	ENR-AMP-SXT	0	0	0	0	0	1	1
	AMP-TET-SXT	33	17	3	0	1	2	56
	4	ENR-AMP-TET-SXT	4	5	14	45	15	36

\*ENR, enrofloxacin; AMP, ampicillin; TET, tetracycline; SXT, trimethoprim/sulfamethoxazole.

selection. This should be done in order to understand the origin, development as well as transfer of the resistance mechanism.

## Broiler Chickens Excreta and Manure Harboured Antibiotic Resistant *E. coli*

The occurrence of resistant and multi-resistant *E. coli* isolates was shown in this study. The observed high prevalence of resistance in *E. coli* to four antibacterial agents in isolates from excreta and manure, particularly during the fattening period, may be a consequence of intensive animal farming (Nhung et al., 2017). Therefore, it has been hypothesised that animals as well as manure may be the reservoir of these resistant bacteria. Similar findings of prevalence of *E. coli* isolates from broilers and their products, particularly meat, resistant to FQ,  $\beta$ -lactam, tetracyclines, and sulfonamides were frequently found in other studies (Furtula et al., 2010; Ozaki et al., 2011; Rugumisa et al., 2016; EFSA and ECDC, 2018). Regarding development of AMR over time, our data show increasing trend, unlike other studies (Diarra et al., 2007). Diarra et al. (2007) had an insignificantly decreasing trend in the occurrence of AMR noted in *E. coli* isolates as the birds aged.

A relatively high prevalence of *E. coli* isolates (50%) was found to be resistant to multiple antibiotics. Interestingly, approximately 17% of *E. coli* isolates showed a resistance pattern to four antibiotics (ENR-AMP-TET-SXT), in spite of antibiotics not having been used in our study. Our findings agree with previous reports from Germany (GERMAP, 2016), from European countries (Chaslus-Dancla et al., 1987; Amador et al., 2019) and global ones (Nhung et al., 2017) that found resistance in *E. coli* isolated from poultry farms to many classes of antibiotics, including FQ,  $\beta$ -lactams, tetracyclines and sulfonamides. Similarly, Ozaki et al. (2011) reported that the amount of resistance in *E. coli* detected among isolates from excreta increased during the growth of chickens. Therefore, it was not possible to determine where the resistant bacteria originated from even in the absence of antibiotic administration. However, our study demonstrates that these bacteria carry out to other animals in the same stable despite the absence of selection pressure related to the non-use of antimicrobial agents. Due to the increasing prevalence of resistant bacteria during fattening, one can assume that a transfer of resistances or resistant bacteria did occurred in this study.

Similarities among these antibiotics (Poirel et al., 2018) show a need for further studies to analyse whether these resistances may have developed during the growing phase or whether the explanation for the difference in terms of prevalence of AMR in each sampling time is possibly due to intensive animal-to-animal contact transmission. Horizontal transmission greatly contributes to the widespread dissemination of AMR (Khan et al., 2005). In our study, at the beginning of the experiment birds carried resistant bacteria. These bacteria might be spread from the digestive tracts via shedding and persist in the environment (Diarra and Malouin, 2014). This could result in rapid contamination

of the other individuals in the same flock and in the stable environment (Jiménez-Belenguer et al., 2016). Nevertheless, the role of contamination of the animal's direct environment through dust in the stable should be taken into account. Dust formation (litter, feather/skin particles, excreta, etcetera) is common in practice, so that a possible particle-related transmission of resistant bacteria between animals could not be ruled out because particles could also be transported by air (Schulz et al., 2016).

There are various possible explanations for an increase or a decrease in antibiotic resistance in *E. coli*. More importantly, however, it constitutes a major and shared reservoir of resistance determinants to most families of antibacterial agents transmitted by animals. Despite this, the different transmission pathways of resistant *E. coli* isolates in this study remain to be clarified. The transmission may include direct contact with animals or indirect transfer through the environment. As no further genetic analyses were carried out, the reason for this difference remains unknown. Development of resistance is very complex. We cannot regard all interactions when we only obtain one isolate from a sample and then by way of example, try to conclude the complexity of resistance development. Therefore, more research is required to find possible explanations concerning the mechanism behind the shedding of antibacterial agent resistant *E. coli* in broilers.

## Relationship Between Flooring System and the Occurrence of Resistance in the Isolates

Few studies have examined reducing the development of resistance to antibacterial agents by applying different flooring designs simulating different contact intensity between animals and their manure. Despite the fact that the prevalence of resistant *E. coli* depending on flooring design has been documented (Chuppava et al., 2018a,b), information is lacking concerning the MDR pattern to antibacterial agents in large-scale broiler farming.

Wright (2010) and Furtula et al. (2010) stated that the environment, including dirty litter, could also be a potential reservoir of resistant bacteria. Nevertheless, our study in the PS group, the animals had less contact to litter material, the development of AMR still occurred in these animals, or rather, it was even more protracted. Not only the prevalence of resistant *E. coli* isolates from excreta material was significantly higher, but also the highest percentage of multi drug-resistant *E. coli* was found in the partially slatted flooring group (PS) compared to the litter group during the experimental period. Interestingly, the results were contrary to our expectations, as significantly higher AMR was shown in the PS group where the animals in about one-quarter of the stable had no direct contact to the excreta because of using elevated levels with slatted flooring. In contrast, Chuppava et al. (2018b) concluded from their experimental model that a lower exposure to resistant bacteria in manure might lead to a lower percentage of resistant *E. coli* isolates in their study animals. However, their research was an



experimental study with very small animal groups in which the effects of crowding were irrelevant. Therefore, additional focus is needed elsewhere.

The animals in the PS group in our study preferred the elevated areas (areas with the possibility of simultaneous intake of water and feed) and thus, a high population density in these areas was observed. The amount of manure in the respective areas allows this conclusion to be drawn. Below the slatted areas, the amount of manure per square metre was higher than in the littered area of the same stable or in the control stable in general. While between 12.6 and 13.3 kg (on DM-basis) of litter per square metre was found in the control group housing, up to 24.2 kg was found below the slats (**Supplementary Table S3**). High stocking density is common in poultry farming. Crowding can be an important factor inducing AMR in bacteria. This may be an explanation for the high prevalence and multi-resistance in faecal *E. coli* of poultry in this and other studies (van den Bogaard et al., 2001; Nhung et al., 2017).

When analysing the results from the final litter quality and litter moisture content in the poultry manure between the L and PS groups (**Supplementary Table S3**), the quality of the manure in the PS group was characterised by the lowest values of DM content (highest moisture content with loose structure) in the slatted flooring area, compared with the other areas in the same stable. Although there was no direct contact with the animals, this may have been a good reservoir for viable bacteria. Further studies should therefore clarify whether this directly affects the development of AMR or whether the higher temporary animal density described above is relevant. The first question could be clarified by permanently removing the excrements below the elevated levels.

## CONCLUSION

According to our findings, animals carrying resistance at the start of the fattening period can be a reservoir and the starting point for the transmission of bacterial resistance to the other animals in the same flock. Elevated areas, particularly if there is a possibility of accessing feed and drinking water, seem to be very attractive, which is reflected in high amounts of manure beneath these areas. This can induce crowding of animals. The resulting animal-to-animal contact seems to be more important for spreading of resistance than contact to the litter material. This might foster transmission of AMR within the whole flock. To resolve the problems with AMR, one important requirement is still that of obtaining animals not carrying resistance at the start of life. The absence of resistance is all the more important the more the environment of the animals leads to an intensive animal-to-animal contact (high density overall, attractive stable areas with partially high animal density, etcetera).

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The University of Veterinary Medicine Hannover, Foundation, has an Animal Protection Office. This is the local committee dealing with ethical questions regarding animal experiments. The experiments were carried out in accordance with German regulations (Animal Protection Act). No direct interventions were carried out on animals which could be associated with pain, suffering or damage to these animals. Solely excreta samples and litter material were tested for antimicrobial resistance. This sample material was collected during common fattening of broiler chickens. For this reason, these examinations required no announcement or permission with regard to the animal protection law (§7, paragraph 2) since no measures inflicting pain, suffering or damage on these animals were carried out.

## AUTHOR CONTRIBUTIONS

CV and CS conceptualised the study and acquired funding. CV and BK designed the methodology. BC performed the experiments. BC and AA collected the samples. BC and CV analysed the data. The original manuscript draft was prepared by BC and CV. CV reviewed and edited the manuscript and supervised the project. All authors contributed to reading the manuscript and approving the submitted version.

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## SUPPLEMENTARY MATERIAL

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# Multidrug-Resistant and Clinically Relevant Gram-Negative Bacteria Are Present in German Surface Waters

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Water is considered to play a role in the dissemination of antibiotic-resistant Gram-negative bacteria including those encoding Extended-spectrum beta-lactamases (ESBL) and carbapenemases. To investigate the role of water for their spread in more detail, we characterized ESBL/Carbapenemase-producing bacteria from surface water and sediment samples using phenotypic and genotypic approaches. ESBL/Carbapenemase-producing isolates were obtained from water/sediment samples. Species and antibiotic resistance were determined. A subset of these isolates ( $n = 33$ ) was whole-genome-sequenced and analyzed for the presence of antibiotic resistance genes and virulence determinants. Their relatedness to isolates associated with human infections was investigated using multilocus sequence type and cgMLST-based analysis. Eighty-nine percent of the isolates comprised of clinically relevant species. Fifty-eight percent exhibited a multidrug-resistance phenotype. Two isolates harbored the mobile colistin resistance gene *mcr-1*. One carbapenemase-producing isolate identified as *Enterobacter kobei* harbored *bla<sub>VIM-1</sub>*. Two *Escherichia coli* isolates had sequence types (ST) associated with human infections (ST131 and ST1485) and a *Klebsiella pneumoniae* isolate was classified as hypervirulent. A multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolate encoding known virulence genes associated with severe lung infections in cystic fibrosis patients was also detected. The presence of MDR and clinically relevant isolates in recreational and surface water underlines the role of aquatic environments as both reservoirs and hot spots for MDR bacteria. Future assessment of water quality should include the examination of the multidrug resistance of clinically relevant bacterial species and thus provide an important link regarding the spread of MDR bacteria in a One Health context.

**Keywords:** ESBL, surface water, WGS (whole genome sequencing), MCR-1, clinical isolate



## INTRODUCTION

The recent emergence of antibiotic-resistant Gram-negative bacteria, in particular those encoding extended-spectrum beta-lactamases (ESBL) and/or carbapenemases, poses a significant threat for human and animal health. Nowadays, they account for a large proportion of the global pandemic spread of antimicrobial resistance (World Health Organization [WHO], 2017). ESBL/Carbapenemase-producers have been isolated from various sources including both animal- and human- populations, as well as from environmental sources including water bodies (Woodford et al., 2014; Grundmann et al., 2017; Hölzel et al., 2018; Huijbers et al., 2015; Madec et al., 2017; Muraleedharan et al., 2019).

An understanding of the causes for the emergence of ESBL/Carbapenemase-producing isolates and probable pathways of their spread would be aided by a deep genetic characterization of isolates from the different sources to detect those clones/antibiotic resistance determinants that are drivers of antibiotic resistance spread. Isolates from humans, animals and food have been studied for a long time, and whole-genome-based approaches have identified identical clones circulating among these compartments (Falgenhauer et al., 2016a,b). Far less is known for aquatic environments, where analyses are mostly based on the phenotypic analysis of antibiotic susceptibility or quantification of resistance genes (Hembach et al., 2017; Müller et al., 2018). Whole-genome-based studies have been performed on isolates from raw and treated wastewater (Gomi et al., 2018; Mahfouz et al., 2018), but are scarce for other water bodies, in particular recreational water.

To gain more insight into this topic, an investigation was performed in collaboration with investigative journalists in Northern Germany (Baars and Lambrecht, 2018). This resulted in the detection of antibiotic-resistant Gram-negative isolates in surface water bodies, partly used for recreational purposes that depicted multilocus sequence types found previously in hospital settings and are representative of relevant human pathogens. Here we report on these findings.

## MATERIALS AND METHODS

### Sampling and Detection of ESBL-Producing Isolates

Water ( $n = 12$ ) and sediment ( $n = 10$ ) samples were taken from different water bodies located in northern Germany (Supplementary Figure S1 and Supplementary Table S1) in autumn 2017. Sampling sites were selected for their relevance to resistance spread (e.g., neighborhood to human clinics, recreational water bodies or food processing plants). All samples were processed within 24 h. For detection of viable ESBL/Carbapenemase-producing bacteria in the water samples, four different volumes (exact volumes differed from sample to sample depending on the expected “contamination” level) were filtered (two technical replicates per sample; pore size 0.2  $\mu\text{m}$ , cellulose acetate filters, Sartorius; Göttingen, Germany). The filters were put onto CHROMagar ESBL<sup>®</sup> plates

(MAST Diagnostica, Reinfeld, Germany) and incubated for  $18 \pm 2$  h at 42°C. Sediment samples were supplemented with sodium pyrophosphate solution (20 mL 0.1% solution/100 g wet sediment) and shaken (460 rpm, room temperature, 30 min). Two-hundred microliters of the supernatant were subsequently plated on CHROMagar ESBL<sup>®</sup> plates and incubated as described above.

### Species Determination and Antibiotic Susceptibility Testing

A subset of bacterial isolates ( $n = 134$ ) growing on selective agar were characterized by VITEK<sup>®</sup> MS (BioMérieux, Nüertingen, Germany) for the determination of species. Antibiotic susceptibility testing was performed using the VITEK<sup>®</sup>2 system following to the manufacturer's instructions (BioMérieux, Nüertingen, Germany). Minimal inhibitory concentrations were interpreted according to EUCAST, 2017 and CLSI, 2016 guidelines (internal VITEK<sup>®</sup>2 adjustment) The multidrug-resistance (MDR) status was evaluated for seven clinically relevant species (*Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter cloacae* complex, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) using the classification system of Magiorakos et al. (2012), with resistance to  $\geq 3$  classes of antibiotics defining the MDR status.

### Whole Genome Sequencing and Genome-Based Analysis

Thirty-three isolates out of the 134 phenotypically characterized isolates (Table 1) were subjected to whole-genome sequencing and subsequent analysis. These isolates included seven different species (*A. baumannii*, *Enterobacter cloacae* complex, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *P. aeruginosa*, and *Pseudomonas resinovorans*). If more than one isolate of an identical species was present at one sampling site, isolates representative of different antibiotic resistance phenotypes were chosen.

DNA was isolated from overnight cultures using the PureLink<sup>®</sup> Pro 96 Genomic DNA Purification Kit (ThermoFisher Scientific, Dreieich, Germany). For short-read sequencing, an Illumina Nextera XT library was prepared and sequenced on a NextSeq 500 device (Illumina, Eindhoven, Netherlands) using the NextSeq 500/550 Mid Output v2 kit ( $2 \times 150$  nt read length). Sequence data analysis (coverage, mean read length, quality clipping, assembly, determination of multilocus sequence types and virulence genes using the database of VFDB (Chen et al., 2016) was performed using the in-house pipeline ASA<sup>3</sup>P (Schwengers et al., 2019). A mean coverage of  $103\times$  and a mean read length of 130 nt was achieved. Quality controlled raw reads were assembled using Spades v3.10.1 (Bankevich et al., 2012) implemented in the ASA<sup>3</sup>P pipeline, with a mean N50 value of 103,031 nt. The sequencing data was deposited in the ENA database under the accession number PRJEB29745.

Antibiotic resistance genes and plasmid incompatibility groups were analyzed using the bacterial analysis pipeline at the Center for Genomic Epidemiology (Thomsen et al., 2016). *E. coli* *fimH*-types were determined using the Fimtyper tool (Roer et al., 2017). Virulence genes were additionally analyzed

**TABLE 1** | Characteristics of the sequenced isolates.

Isolate	Species	MLST	Beta-lactamase	Colistin	Tetracycline	Phenicol	Aminoglycoside	Fosfomycin	Macrolide	Sulfonamide	Fluoroquinolone	Trimethoprim	Lincosamide
1w-12	<i>A. baumannii</i>	203%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-78</sub>										
2w-4	<i>A. baumannii</i>	155%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-51</sub>										
3w-14	<i>A. baumannii</i>	1017%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-203</sub>										
6s-1	<i>A. baumannii</i>	1321%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-51</sub>										
6w-10	<i>A. baumannii</i>	1322%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-69</sub>										
7w-12	<i>A. baumannii</i>	647%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-208</sub>										
8s-2	<i>A. baumannii</i>	1112%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-88</sub>										
8s-4	<i>A. baumannii</i>	1112%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-88</sub>										
8w-16	<i>A. baumannii</i>	690%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-71</sub>										
8w-19	<i>A. baumannii</i>	1323%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-93</sub>										
10w-8	<i>A. baumannii</i>	1324%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-208</sub>										
3w-16	<i>A. pittii</i>	249%	<i>bla</i> <sub>ADC-25</sub> , <i>bla</i> <sub>OXA-421</sub>										
12w-5	<i>Enterobacter kobei</i>	910	<i>bla</i> <sub>VIM-1</sub>				<i>aadA1</i> , <i>aadB</i> , <i>aacA4</i>	<i>fosA</i>		<i>sul1</i>	<i>aac(6')/lb-cr</i>		
1w-4	<i>E. coli</i>	2064\$	<i>bla</i> <sub>CTX-M-1</sub>										
1w-5	<i>E. coli</i>	648\$	<i>bla</i> <sub>CTX-M-14</sub>										
2w-3	<i>E. coli</i>	167\$	<i>bla</i> <sub>CTX-M-1</sub>		<i>tet(Y)</i>	<i>floR</i>	<i>aac(3)-IIa</i> , <i>aadA1</i> , <i>aph(4)-la</i> , <i>strA</i> , <i>strB</i>		<i>mph(A)</i>			<i>dfrA1</i>	
3w-1	<i>E. coli</i>	101\$	<i>bla</i> <sub>CTX-M-1</sub>				<i>aadA5</i>			<i>sul2</i>		<i>dfrA17</i>	
3w-4	<i>E. coli</i>	10\$	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>TEM-1</sub>	<i>mcr-1</i>	<i>tet(B)</i>	<i>catB3</i>	<i>aadA17</i>				<i>aac(6')/lb-cr</i>		<i>lnu(F)</i>
6w-2	<i>E. coli</i>	9417\$	<i>bla</i> <sub>CTX-M-1</sub>				<i>aadA5</i>			<i>sul2</i>		<i>dfrA17</i>	
7w-2	<i>E. coli</i>	542\$	<i>bla</i> <sub>CTX-M-1</sub>				<i>aadA1</i>						
7w-4	<i>E. coli</i>	69\$	<i>bla</i> <sub>CTX-M-15</sub>		<i>tet(B)</i>				<i>mph(A)</i>			<i>dfrA14</i>	
8w-1	<i>E. coli</i>	1485\$	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>TEM-1</sub>								<i>qnrS1</i>		

(Continued)

TABLE 1 | Continued

Isolate	Species	MLST	Beta-lactamase	Colistin	Tetracycline	Phenicol	Aminoglycoside	Fosfomycin	Macrolide	Sulfonamide	Fluoroquinolone	Trimethoprim	Lincosamide
8w-3	<i>E. coli</i>	155 <sup>\$</sup>	<i>bla</i> <sub>CTX-M-1</sub> , <i>bla</i> <sub>TEM-1</sub>	<i>mcr-1</i>	<i>tet(A)</i>		<i>aac(3)-IIa</i> , <i>aadA1</i> , <i>aph(3')-Ic</i> , <i>strA</i> , <i>strB</i>			<i>sul2</i>		<i>dfrA1</i>	
9w-1	<i>E. coli</i>	131 <sup>\$</sup>	<i>bla</i> <sub>CTX-M-1</sub>						<i>mph(A)</i>				
10w-3	<i>E. coli</i>	10 <sup>\$</sup>	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>OXA-1</sub>		<i>tet(B)</i>	<i>catB3</i>					<i>aac(6')Ib-cr</i>		
1w-9	<i>K. oxytoca</i>	241	<i>bla</i> <sub>OXY-2-2</sub>										
3w-10	<i>K. oxytoca</i>	34	<i>bla</i> <sub>OXY-2-8</sub>				<i>strA</i> , <i>strB</i>		<i>mph(A)</i>	<i>sul2</i>			
1w-10	<i>K. pneumoniae</i>	3681	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>SHV-28</sub>		<i>tet(D)</i>	<i>catB3</i>	<i>strA</i> , <i>strB</i>	<i>fosA</i>		<i>sul2</i>	<i>oqxA</i> , <i>oqxB</i> , <i>aac(6')Ib-cr</i>	<i>dfrA14</i>	
3w-9	<i>K. pneumoniae</i>	307	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>SHV-28</sub> , <i>bla</i> <sub>TEM-1</sub>			<i>catB3</i>	<i>strA</i> , <i>strB</i>	<i>fosA</i>		<i>sul2</i>	<i>oqxA</i> , <i>oqxB</i> , <i>aac(6')Ib-cr</i>	<i>dfrA14</i>	
7w-11	<i>K. pneumoniae</i>	268	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>SHV-11</sub>					<i>fosA</i>	<i>ere(A)</i>	<i>sul1</i>	<i>oqxA</i> , <i>oqxB</i>	<i>dfrA5</i>	
8w-11	<i>K. pneumoniae</i>	2155	<i>bla</i> <sub>SHV-2</sub>		<i>tet(D)</i>		<i>aac(3)-IId</i> , <i>strA</i> , <i>strB</i>	<i>fosA</i>		<i>sul2</i>	<i>oqxA</i> , <i>oqxB</i> , <i>qnrS1</i>	<i>dfrA14</i>	
9w-9	<i>P. aeruginosa</i>	3304	<i>bla</i> <sub>OXA-50</sub> , <i>bla</i> <sub>PAO</sub>			<i>catB7</i>	<i>aph(3')-IIb</i>	<i>fosA</i>					
10w-9	<i>P. resinovorans</i>	ND	<i>bla</i> <sub>POM-1</sub>										

ND: not determined due to the lack of a known MLST scheme. <sup>%</sup> MLST determined using Pasteur Scheme (Diancourt et al., 2010), <sup>\$</sup> MLST determined using Achtman scheme (Wirth et al., 2006). The first number in the name of the isolates depicts, from which sampling site they were isolated (as described in **Supplementary Table S1**); s, source soil, w, source water.

using the bacterial analysis pipeline at the Center for Genomic Epidemiology (Thomsen et al., 2016) and BIGSdb (scheme “virulence genes”) for *K. pneumoniae* isolates<sup>1</sup>.

Assignment of the *Enterobacter* species was performed using the genome sequence of the type strains referred in **Supplementary Table S2** and the online GGDC tool (Meier-Kolthoff et al., 2013) applying a threshold of 70% sequence similarity for taxonomic identification.

The determination of the source of different multilocus sequence types (ST) was performed using Enterobase for *E. coli* (Alikhan et al., 2018) and PubMLST database for *Acinetobacter baumannii* isolates<sup>2</sup> [Pasteur scheme (Diancourt et al., 2010)].

For a detailed analysis of the source, BacWGSTdb (Ruan and Feng, 2016) was used to determine the closest relative with the function “Single genome analysis” and a core genome MLST (cgMLST) allele threshold based on 200 alleles. The analysis was performed for *E. coli*, *K. pneumoniae*, and *A. baumannii* isolates ( $n = 27$ ), because the BacWGSTdb included schemes for these species.

## RESULTS AND DISCUSSION

The role of aquatic environments and wastewaters for the dissemination of antibiotic-resistant Gram-negative bacteria is an increasing source of concern worldwide and has not yet been investigated in detail so far (Berendonk et al., 2015). The presence of both contaminated wastewater and other anthropogenic selection pressures on aquatic environments are likely to create hotspots and reservoirs for the selection of antibiotic-resistant bacteria and the transfer of antibiotic resistance genes. Despite these facts, most water samples are routinely monitored only for the concentration of fecal indicators (*E. coli* and *Enterococcus* sp.; EU Directive 2006/7/EC). It is not mandatory for authorities to store or characterize the detected isolates further. Consequently, no systematic information on the presence of antibiotic resistance genes or the clinical relevance of the isolates is collected.

Several studies have been performed to estimate the role of surface waters for the spread of antibiotic resistance. qPCR-based studies are still the standard procedure in many countries, including Germany (Hembach et al., 2017). The disadvantage of these studies is that they do not allow the direct association of antibiotic resistance genes to a given bacterium. Studies implementing the analysis of viable antibiotic-resistant bacteria for antibiotic resistance phenotype and antibiotic resistance genes do not allow to determine the relationship of the bacteria in water to bacteria from other sources. This is only possible using genome-based studies that are still an exception (Jorgensen et al., 2017). The only genome-based study of water in Germany employs the analysis of wastewater (Mahfouz et al., 2018).

In our study, we combined phenotypic and genotypic analyses of antibiotic-resistant bacteria isolated from water and sediment samples to determine their relationship to bacteria from other sources and to determine their possible role on human health.

## Clinically-Relevant Multidrug-Resistant Gram-Negative Bacteria Are Present in Recreational Water Samples

In 10 out of 12 water samples, ESBL/Carbapenemase-producing Gram-negative bacteria were detected by a culture-based approach, with concentrations varying between  $2.7$  and  $8.1 \times 10^3$  colony forming units per 100 mL. In 20% of the analyzed sediment samples, viable ESBL/Carbapenemase-producers were detected (**Supplementary Table S3**). The lack of positive sediment samples should not be the result of the method used, as it has been shown in an earlier study that the number of cells derived by this method was not increased by ultrasonic treatment (Heß et al., 2018).

One hundred-thirty-four isolates growing on selective agar plates were selected for species determination and antibiotic susceptibility testing. The most common species detected were *E. coli* and members of the *A. baumannii* complex (**Figure 1**, 39%, respectively), followed by *K. pneumoniae* (6%). Other clinically relevant species (*C. freundii*, *E. cloacae*, *P. aeruginosa*) were less abundant.

The multidrug-resistance (MDR) status of seven clinically relevant species (**Figure 2**) of the isolates was evaluated and varied between 3.8% of a respective species (*A. baumannii* complex) to 100% (*C. freundii*, *E. cloacae*, *E. coli*, *K. oxytoca*, and *K. pneumoniae*, **Figure 2**). The low number of MDR *A. baumannii* is concordant to data from Austria (Kittinger et al., 2017) where only 4.4% of the *A. baumannii* isolates were MDR. These MDR-isolates were mostly third generation cephalosporin resistant, but also Carbapenem non-susceptible isolates were present within the characterized isolates ( $n = 7$ ) including two isolates of *P. aeruginosa* (9w-9, 10w-5), *A. baumannii* complex (6w-8, 8s-4), *K. oxytoca* (11w-2, 12w-10) and one *E. cloacae* complex isolate (12w-5) (**Supplementary Table S4**).

## Water Isolates Harbor Antibiotic Resistance Genes Toward Eleven Antibiotic Classes

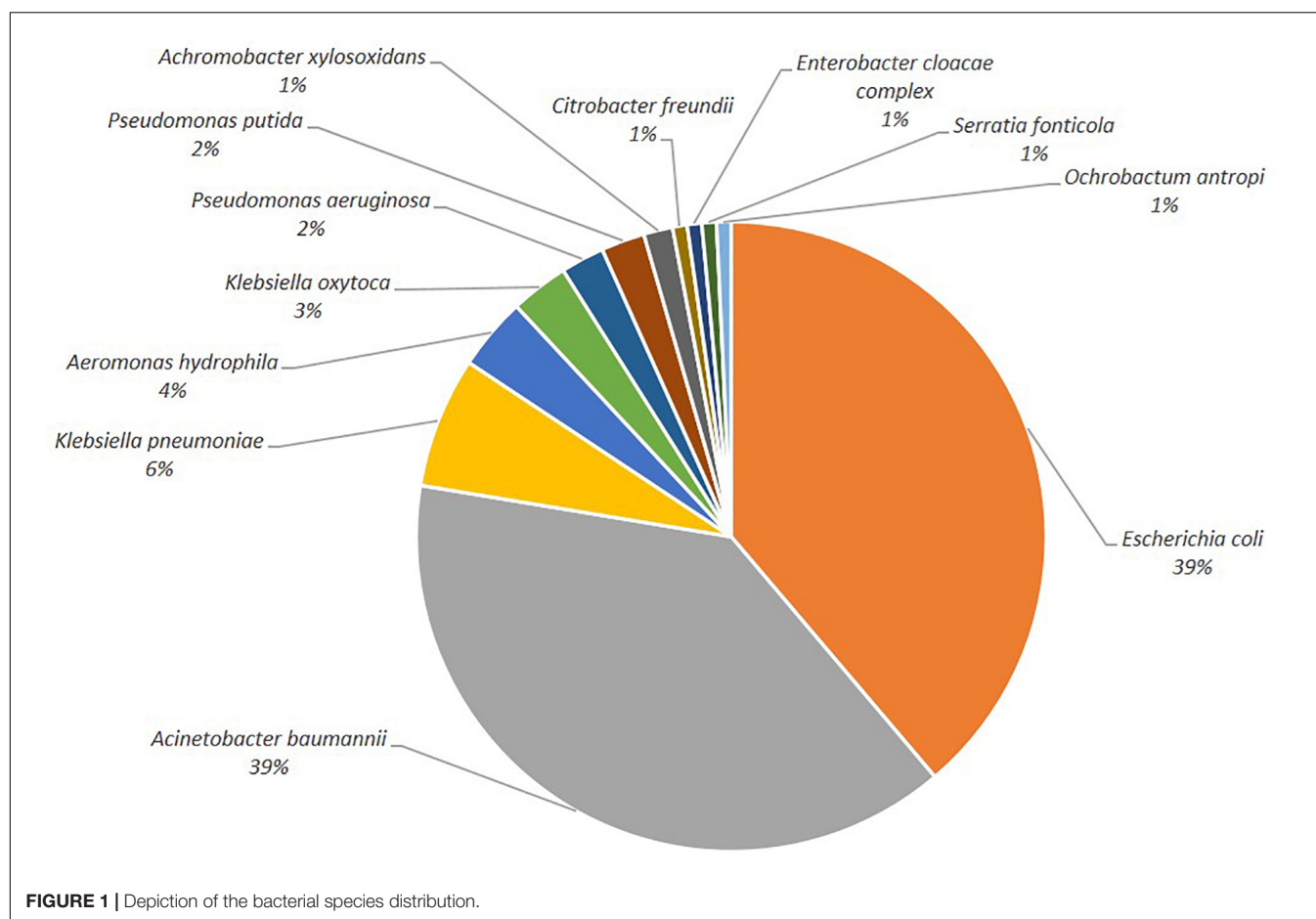
Genome-based analysis revealed that genes encoding resistance to eleven different antibiotic classes were present in the sequenced isolates (**Table 1**). All sequenced isolates harbored at least one beta-lactamase gene, accompanied by different phenotypes. The *P. aeruginosa* isolate 9w-9 encoded the *bla*<sub>OXA-50</sub> gene (carbapenemase) and *bla*<sub>PAO</sub> (AmpC gene) beta-lactamase genes, while the *P. resinovorans* isolate (10w-9) harbored the beta-lactamase gene *bla*<sub>POM-1</sub>. The *A. baumannii* isolates ( $n = 11$ ) harbored the chromosomally-encoded AmpC gene *bla*<sub>ADC-25</sub> in combination with a *bla*<sub>OXA</sub>-like beta-lactamase conferring phenotypic ertapenem resistance (**Table 1** and **Supplementary Table S4**). The *Enterobacter kobei* isolate (12w-5) harbored the carbapenemase gene *bla*<sub>VIM-1</sub>.

The most common ESBL genes detected (**Table 1**) were *bla*<sub>CTX-M-1</sub> ( $n = 7$ ) and *bla*<sub>CTX-M-15</sub> ( $n = 7$ ). The ESBL gene *bla*<sub>CTX-M-1</sub> was found exclusively in *E. coli* isolates, whereas the *bla*<sub>CTX-M-15</sub> gene was also detected in *K. pneumoniae*. Less frequent ESBL genes were *bla*<sub>SHV-28</sub> ( $n = 2$ ), *bla*<sub>CTX-M-14</sub> ( $n = 1$ )

<sup>1</sup> [https://bigsd.b.pasteur.fr/cgi-bin/bigsd/bigsd.pl?db=pubmlst\\_klebsiella\\_seqdef\\_public](https://bigsd.b.pasteur.fr/cgi-bin/bigsd/bigsd.pl?db=pubmlst_klebsiella_seqdef_public)

<sup>2</sup> <https://pubmlst.org/abaumannii/>





and *bla*<sub>SHV-2</sub> ( $n = 1$ ). The ESBL gene *bla*<sub>SHV-28</sub> was found only in *K. pneumoniae* and always in combination with *bla*<sub>CTX-M-15</sub>. The ESBL genes are similar to those found in other studies (Jorgensen et al., 2017), but the distribution was different, as the *bla*<sub>CTX-M-1</sub> gene was detected more frequently ( $n = 7$ ) than *bla*<sub>CTX-M-15</sub> ( $n = 4$ ). The prevalence of these genes also reflect the situation found in hospital settings in Germany, together with the very low incidence of acquired carbapenemase genes.

With the exception of *A. baumannii*, isolates harbored additional antibiotic resistance genes conferring resistance to ten antibiotic classes (Table 1). The most commonly found resistance genes were those encoding aminoglycoside resistance (12/33, e.g., *strA/strB*, *aadA1*) followed by genes conferring resistance against sulfonamides ( $n = 9$ , *sul1*, *sul2*), trimethoprim ( $n = 9$ , *dfrA1*, *dfrA14*, *dfrA17*) and Fluoroquinolones ( $n = 8$ , *qnrS1*, *oqxA*, *oqxB*, *aac(6')Ib-cr*).

Most strikingly, the mobile colistin-resistance gene *mcr-1* was detected in 2/33 isolates, all being *E. coli* isolates. In the isolate 3w-4, the *mcr-1* gene was located on an IncX4 plasmid, while 8w-3 it was located on an IncHI2 plasmid. The isolates harboring *mcr-1* differed from those found in Switzerland (Zurfuh et al., 2016) both by multilocus sequence type and the encoded ESBL gene. The *mcr-1*-encoding isolates were only found in the neighborhood of a meat processing plant (sampling site 3)

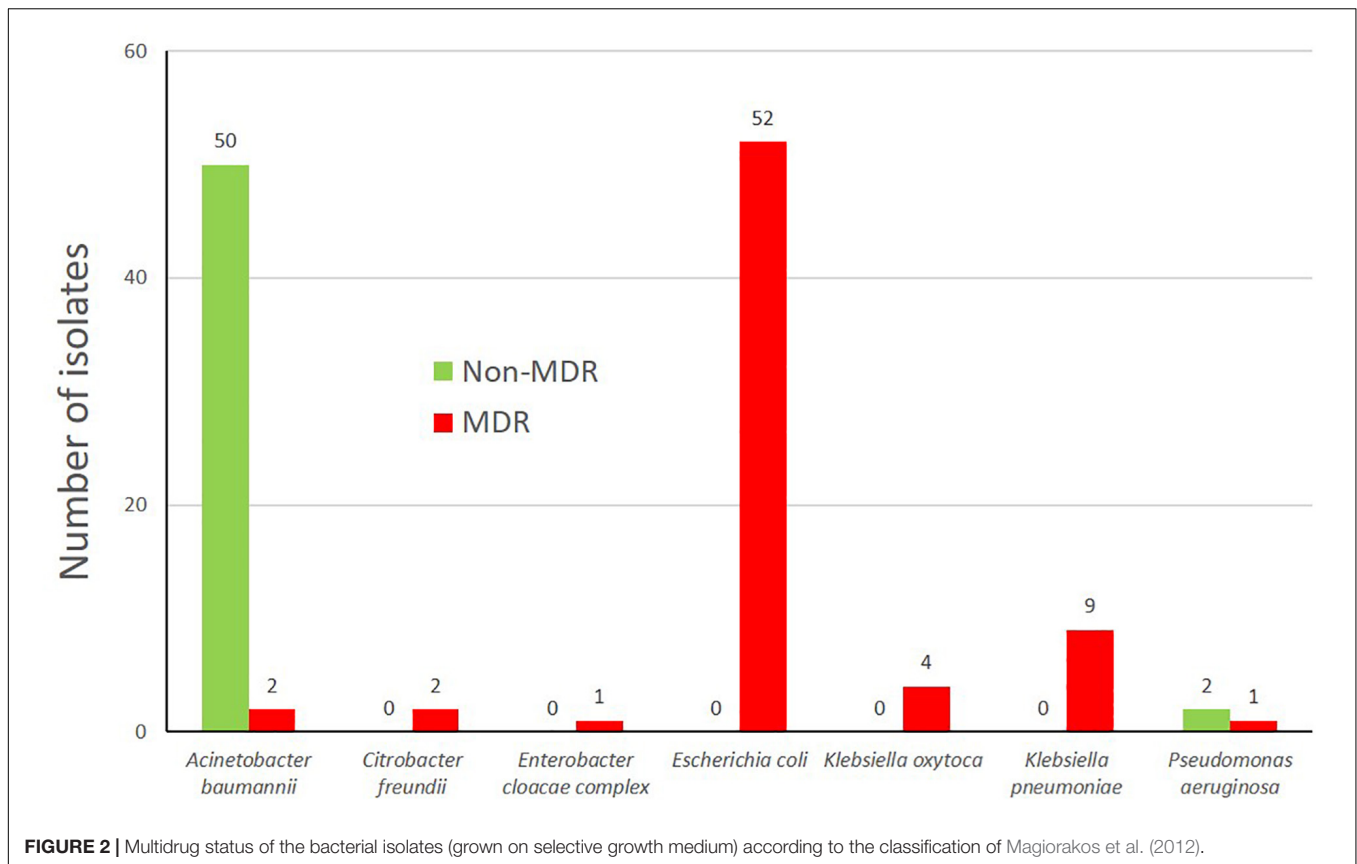
and a slaughterhouse (sampling site 8), concordant with the finding that *mcr-1* is more prevalent in livestock than in humans (Schwarz and Johnson, 2016).

### Detection of Plasmid Incompatibility Groups Present in the Isolates

The Enterobacteriaceae isolates harbored a number of different plasmid incompatibility groups (Supplementary Table S5 and Supplementary Figure S2). Col-type plasmids were the most frequent plasmids, followed by IncI1 and IncF-type plasmids. IncI1 and IncF plasmids are frequently encoding ESBL genes (García-Fernández et al., 2008; Villa et al., 2010).

### Detection of Virulence Determinants and Phylogeny of the Isolates

The sequenced isolates were subjected to detailed analysis to determine a clinical relevance or possible transmission from/to hospital settings. *K. pneumoniae* isolates belonged to four different multilocus sequence types (Table 1), of which two (ST268 and ST307) have previously been described in human clinical specimens (Zhang et al., 2016; Wyres et al., 2019). The present isolates were isolated at water treatment plants, but also in the neighborhood of a slaughterhouse



indicating a possible spread of these into the animal population. ST307 *K. pneumoniae* isolates have emerged worldwide only recently and are supposed to have a high transmission potential, so they are able to quickly move between different countries and even continents (Wyres et al., 2019). They are almost exclusively associated with the carriage of the ESBL gene *bla*<sub>CTX-M-15</sub>. The *K. pneumoniae* isolate 7w-11 (ST268) harbored determinants discriminatory for hypervirulent *Klebsiella* isolates of the sequence type ST268 (pLVPK plasmid, Integrative conjugative element, **Supplementary Figure S3**; Zhang et al., 2016). Hypervirulent *K. pneumoniae* are associated with severe infections in younger and healthier persons than other *Klebsiella* (Lee et al., 2017).

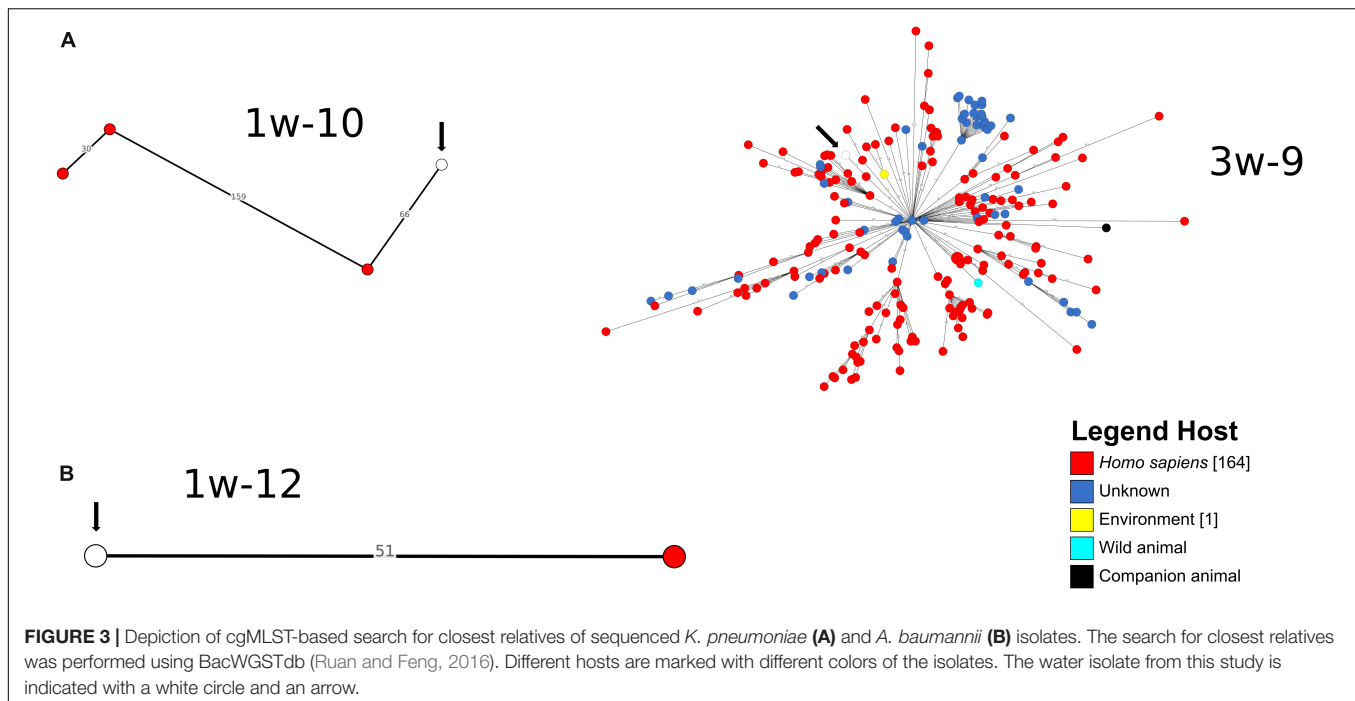
For the *E. coli* isolates, eleven different multilocus sequence types were detected (**Table 1**) of which four (ST10, ST131, ST155, and ST1485) are associated with human clinical infections (Pietsch et al., 2015; Skurnik et al., 2016; Gilrane et al., 2017; Schaufler et al., 2019). For the isolate 9w-1 with the sequence type ST131, *fimH*-typing was performed in order to investigate its relationship among the ST131 lineages. It harbors the *fimH*22 allele, therefore placing it into the ST131-H22 lineage which is associated with both human clinical and animal/environmental isolates (Liu et al., 2018).

Other STs have been previously isolated from animal sources [ST167, ST542 (Maamar et al., 2016; Irrgang et al., 2017)]. The sequence types ST10 ( $n = 2$ ), ST101 and ST648 have been isolated from different sources (humans, animals -wild animals

and livestock- and food) (Pietsch et al., 2015; Michael et al., 2017; Irrgang et al., 2018; Schaufler et al., 2019)<sup>3</sup>. ST155 is mainly found in association with livestock and only recently emerged in humans (Skurnik et al., 2016). These results indicate that water samples harbor isolates from different sources. They may even be able to spread throughout different compartments as their relatives. Five of the above mentioned STs have already been detected in recreational water and/or wastewater samples (ST101, ST10, ST69, ST155, and ST131) in Norway (Jorgensen et al., 2017).

All *E. coli* isolates harbored between two and nine virulence determinants, mostly involved in adherence and iron acquisition/transport (**Supplementary Table S6**). The three most common virulence determinants were *fdeC* (adherence,  $n = 11$ ), enterobactin (iron acquisition/transport,  $n = 10$ ) and the ECP operon (adherence,  $n = 10$ ). According to the virulence gene content, the pathotypes were determined for 7/12 *E. coli* isolates following the schemes presented by Kaper et al. (2004) and Johnson and Russo (2005). All of these were classified as extra-intestinal pathogenic *E. coli* (ExPEC). Within these, two ExPEC subtypes could be determined, including neonatal meningitis-causing *E. coli* (NMEC, 2/12) and uropathogenic *E. coli* (UPEC, 1/12), respectively. In contrast to the study of Jorgensen et al. (2017) enteropathogenic *E. coli* (EPEC) were not detected.

<sup>3</sup><https://enterobase.warwick.ac.uk/species/index/ecoli>



The *P. aeruginosa* isolate 9w-9 was classified as a multidrug-resistant (MDR) isolate. It was of the new ST3304 (one-allele variant of ST274). *P. aeruginosa* ST274 isolates are of high clinical relevance, as they are known to be associated with severe lung infections in cystic fibrosis patients (Fernández-Olmos et al., 2013). The 9w-9 isolate was found in close proximity to a senior residence, which is concordant with its predominant association with immune-compromised patients. The virulence genes found in this *P. aeruginosa* isolate were previously described disease-associated genes (Gellatly and Hancock, 2013) for the production of flagella, pili, alginate, pyoverdine, pyocyanin, and the effector proteins ExoS, ExoT, ExoY. Flagella and pili enable adherence to host cells and movement, while the production of alginate is crucial for biofilm formation. Through the use of pyoverdine and pyocyanin, these bacteria can grow in iron-depleted environments, as e.g., blood, and can cause oxidative stress to the host. The type III secretion system is required for the successful excretion of the virulence effector proteins ExoS, ExoT and ExoY that cause host cell death. The presence of all these virulence determinants qualified this particular isolate as a successful pathogen indicating that water may also be a source for these.

The *A. baumannii* isolates are representative members of ten different sequence types, of which all but one (ST1112) were present only once among the isolates. Five STs have been found in a specific source (Supplementary Table S7). The *A. baumannii* isolates of the sequence types ST690 and ST1017 have been isolated previously from animals only, while ST155 has been isolated predominantly from animals, but also from humans. ST203 was detected in human isolates and ST647 in environmental isolates. These results indicate that there is a transmission of human and animal *A. baumannii* isolates to

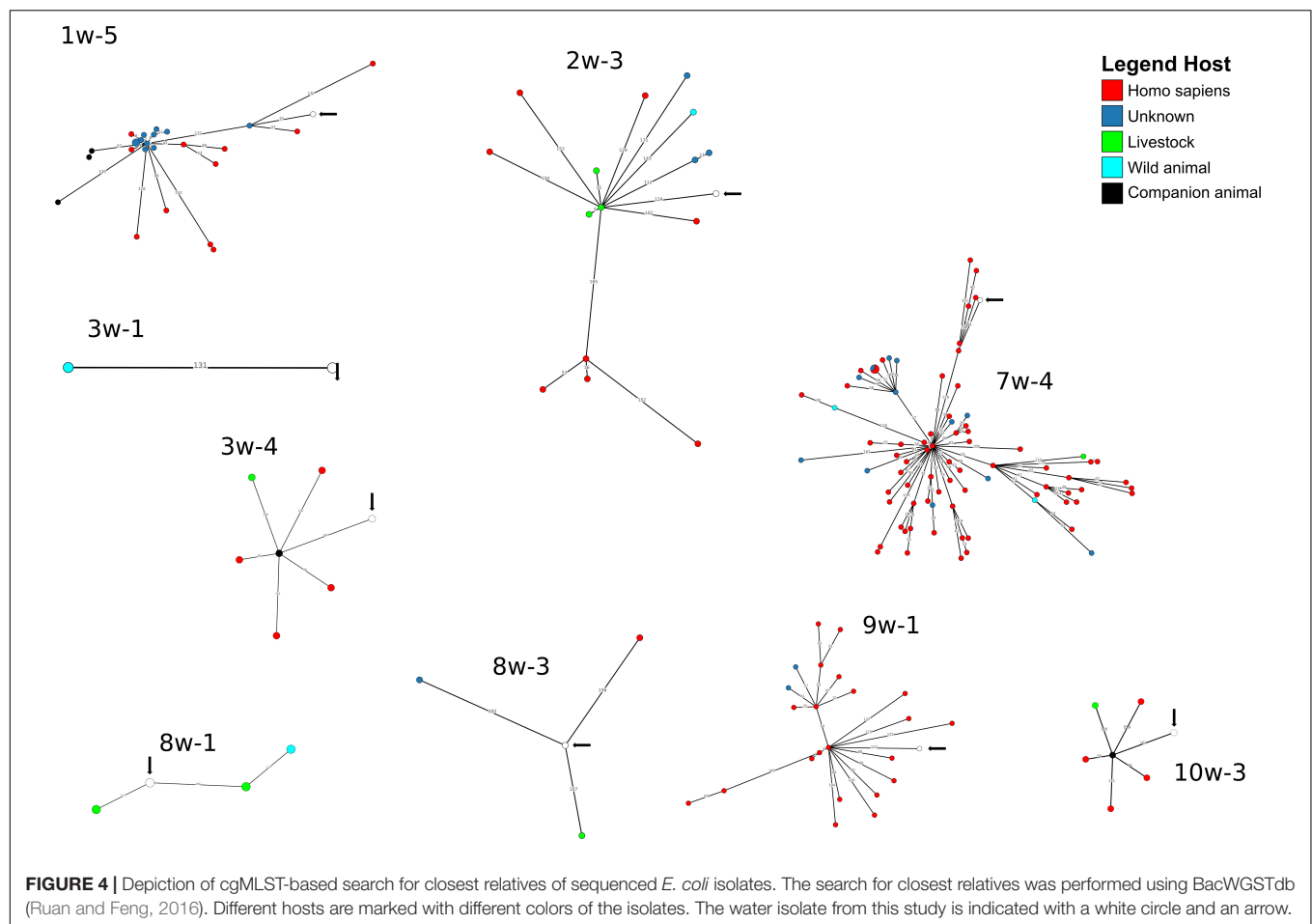
the water bodies. The *A. baumannii* isolates did not harbor any known virulence genes.

The *E. kobei* isolate belonging to the *E. cloacae* complex harbored a *bla*<sub>VIM-1</sub> carbapenemase and had the sequence type ST910. *E. cloacae* complex isolates are nosocomial pathogens often associated with high resistance and also virulence, especially in immunocompromised patients (Mezzatesta et al., 2012).

A cgMLST-based analysis was performed for *A. baumannii*, *E. coli* and *K. pneumoniae* isolates ( $n = 27$ ) using BacWGSTdb. For 12/27 isolates (*E. coli*  $n = 9$ , *K. pneumoniae*,  $n = 2$ ; *A. baumannii*,  $n = 1$ ), close relatives were detected within the threshold of 200 cgMLST allele differences (Figures 3, 4 and Supplementary Table S8). Of the *E. coli* isolates, 6/9 were closely related to animal isolates, while two isolates were related to *E. coli* from human sources. The *A. baumannii* isolate was closely related to an isolate from a human sample, while both *K. pneumoniae* isolates exhibited a high similarity to isolates of human origin. These results are concordant with data from other European countries that have shown similarity of human isolates with water isolates (Jorgensen et al., 2017).

Our study should raise the awareness of the public and the regulatory bodies on the problem of the presence of clinically relevant antibiotic-resistant bacteria in water samples and their impact on human health. Indeed, an associated documentary based on this data (Baars and Lambrecht, 2018) has raised social and political discussions regarding the possible role of water for the dissemination of multidrug resistance in a One health context, and its implication on human health.

The aim of this study was to examine the presence of ESBL/carbapenemase-producing isolates in different water bodies, including recreational water, and to compare their genetic content and relationship to isolates from humans and animals.



We were not only able to show that ESBL-, carbapenemase- and mobile colistin resistance gene-encoding isolates are present in water samples, but also that they harbored clinically relevant species displaying high rates of multidrug-resistance. These MDR bacteria were frequently associated with human disease, as for e.g., a hypervirulent *K. pneumoniae* ST268.

These findings suggest that there is already presence and dispersion of clonal isolates of clinically relevant bacteria in surface waters. In addition, the presence of isolates/antibiotic resistance genes of human and animal origin in water bodies (e.g., *bla<sub>VIM-1</sub>*, human; *mcr-1*, animal) suggests that water itself may be regarded as a reservoir for the transmission and the exchange of isolates and antibiotic resistance genes originating from both human clinical sources and animal husbandry.

The prevalence and the real impact of MDR clinically relevant isolates derived from water bodies on human health is hard to estimate. However, there are reports on outbreaks caused by Carbapenemase-producing Enterobacterales/Enterobacteriaceae deriving from water samples (Carstens et al., 2014) as well as highly-related isolates from water sources and patient samples (Lepuschitz et al., 2019). Thus, there exists a risk of being colonized or, depending on the individual health status and the intensity of exposure, even infected through contact with contaminated water.

Our study has some limitations. Firstly, only ESBL/Carbapenemase-producers in the water samples were determined but not the overall number of isolates. Thus, the prevalence of ESBL/Carbapenemase-producers cannot be determined. Secondly, the study was designed as an observational study; therefore, the number of sequenced isolates was too low to perform statistical analyses.

## CONCLUSION

As clinically relevant antibiotic-resistant Gram-negative isolates were detected in the water samples examined, it is important to increase our knowledge concerning water sources as reservoirs and disseminators of such isolates. Additional investigations to quantify the transmission between the different environmental compartments are now highly warranted.

## DATA AVAILABILITY STATEMENT

The datasets generated and analyzed for this study can be found in the European Nucleotide Archive (ENA) under the project accession number PRJEB29745.



## AUTHOR CONTRIBUTIONS

LF, CB, OL, TB, SH, TC, and CI designed the study. CB and OL collected the data and samples. SH and TB processed the samples. LF performed antibiotic resistance and species determination. LF, JF, TC, and CI performed WGS of the isolates. LF, OS, JS, CB, OL, JF, TC, and CI analyzed the data. LF, TC, and CI wrote the manuscript that was critically reviewed and approved by all authors.

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## SUPPLEMENTARY MATERIAL

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# Gene Transmission in the One Health Microbiosphere and the Channels of Antimicrobial Resistance

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Antibiotic resistance is a field in which the concept of One Health can best be illustrated. One Health is based on the definition of communication spaces among diverse environments. Antibiotic resistance is encoded by genes, however, these genes are propagated in mobile genetic elements (MGEs), circulating among bacterial species and clones that are integrated into the multiple microbiotas of humans, animals, food, sewage, soil, and water environments, the One Health microbiosphere. The dynamics and evolution of antibiotic resistance depend on the communication networks linking all these ecological, biological, and genetic entities. These communications occur by environmental overlapping and merging, a critical issue in countries with poor sanitation, but also favored by the homogenizing power of globalization. The overwhelming increase in the population of highly uniform food animals has contributed to the parallel increase in the absolute size of their microbiotas, consequently enhancing the possibility of microbiome merging between humans and animals. Microbial communities coalesce might lead to shared microbiomes in which the spread of antibiotic resistance (of human, animal, or environmental origin) is facilitated. Intermicrobiome communication is exerted by shuttle bacterial species (or clones within species) belonging to generalist taxa, able to multiply in the microbiomes of various hosts, including humans, animals, and plants. Their integration into local genetic exchange communities fosters antibiotic resistance gene flow, following the channels of accessory genome exchange among bacterial species. These channels delineate a topology of gene circulation, including dense clusters of species with frequent historical and recent exchanges. The ecological compatibility of these species, sharing the same niches and environments, determines the exchange possibilities. In summary, the fertility of the One Health approach to antibiotic

resistance depends on the progress of understanding multihierarchical systems, encompassing communications among environments (macro/microaggregates), among microbiotas (communities), among bacterial species (clones), and communications among MGEs.

**Keywords:** one health, accessory genes, resistance genes, gene flow channels, microbiome merging

## INTRODUCTION: ONE HEALTH AS A COMMUNICATION SPACE

A recent reformulation of the classic One Health approach emphasize the role of interconnected (and hence geographically close) ecosystems in the emergence and dissemination of traits that influence local human, animal, plant, and integrated environmental health (ecosystem health), such as antibiotic resistance (Knapp et al., 2009; Dandachi et al., 2019; Hernando-Amado et al., 2019; Scott et al., 2019; van Bruggen et al., 2019). In fact, antibiotic resistance has been considered the quintessential One Health issue (Robinson et al., 2016). One Health is an ecological concept, and antibiotic resistance is a trait linked to microbiotas, microbial assemblages that are organized and evolve by fundamental processes of community ecology (Costello et al., 2012). Community ecology is a science of environmental communication. As with any communication process, the success of antibiotic resistance transmission is based on three aspects: the communication space, the vehicle for the communication, and the interpretation by the recipient of the message (Baquero, 2017, 2018).

We can consider three communication spaces in the One Health dimension: (1) communication networks between humans, animals, and plants environments, and also with the external environments; (2) communication networks between microbiomes belonging to the above environments, and their sub-environments; and (3) communication networks between various bacterial species within these microbiomes (resulting from microbiome merging). The edge density (density of interconnecting links) in these networks should be proportional to the possibility of the spread of antibiotic resistance genes in this One Health ensemble. In addition to the communication networks, the elements of transmission are also relevant to defining in depth the process of transmission that, in the case of antibiotic resistance, largely relies on the hierarchical organization of antibiotic resistance elements (Baquero, 2004), which allows a selection space with various levels. Selection is then a critical element for the success of the communication because it provides for interpretation of the transmitted message. Antibiotic-resistant mutants are present in all bacterial populations, and, of course, antibiotic resistance is very ancient in biological times (D'Costa et al., 2011). The current mobile genetic elements (MGEs) carrying antibiotic-resistance genes (as plasmids, transposons, or integrons) were already circulating in Enterobacteriaceae long before the use of antibiotics (Datta and Hughes, 1983; Rowe-Magnus et al., 2001); these elements were rapidly colonized with antibiotic resistance genes, in part evolving from pre-resistance genes, at the time of anthropogenic antibiotic use and selection. However, it is this utilization that

provides a meaning to antibiotic resistance, which allows for communication and hence the spread of the message, in this case antibiotic resistance.

## COMMUNICATION BETWEEN HUMANS, ANIMALS, PLANTS, AND LOCAL EXTERNAL ENVIRONMENTS

Communication is proportional to the density and connectivity (capacity for interconnection) of such entities. The coincidence of dense human populations with a high density of terrestrial vertebrate animals (those with a higher probability of microbiome merging), both sharing a common environment, provides a strong opportunity for frequent biological interactions, particularly microbiome **merging** (Ley et al., 2008). Frequent interactions between human (and pre-human) and other animal microbiomes started by hunting and scavenging meat activities, but were significantly increased during the Neolithic period, with the invention of farming and the associated increase in the size of human populations stably coexisting with animals in the same habitat (Armelaos et al., 2005; Fournie et al., 2017; Roughgarden et al., 2018). However, this interaction has greatly increased in the last century, with sociodemographic changes in population, dietary habits, particularly the increase in animal production and meat consumption in low and middle income countries, and the green revolution in agriculture (Tilman, 1998; Tilman et al., 2011; Van Boeckel et al., 2019).

## Communication and Population Sizes

Agriculture currently uses 11% of the world's land surface for crop production. Since 1961, while total cultivated land has shown a net increase of 12 percent to 2009, land under irrigation has more than doubled (FAOSTAT)<sup>1</sup>. Farming activity has escalated since World War I to reach massive proportions. The world cattle inventory in 2018 is at one billion heads, with half of these animals in India and Brazil, and the third-most in China<sup>2</sup>. Data from the Food and Agriculture Organization of the United Nations indicates that the world's average stock of chickens is estimated at almost 23 billion, and pigs account for 770 million (Lawrence, 2019; Metcalfe, 2019). Interestingly, such an "animal invasion" has frequently occurred in combination with a decline in animal diversity due to anthropogenic selection of a limited range of animal varieties of economic interest. The predictable

<sup>1</sup><http://faostat3.fao.org/faostat-gateway/go/to/download/E/EL/E>

<sup>2</sup><https://beef2live.com/story-world-cattle-inventory-ranking-countries-0-106905>



effect is the increased possibility of interactions among large numbers of a few animal types with large numbers of humans.

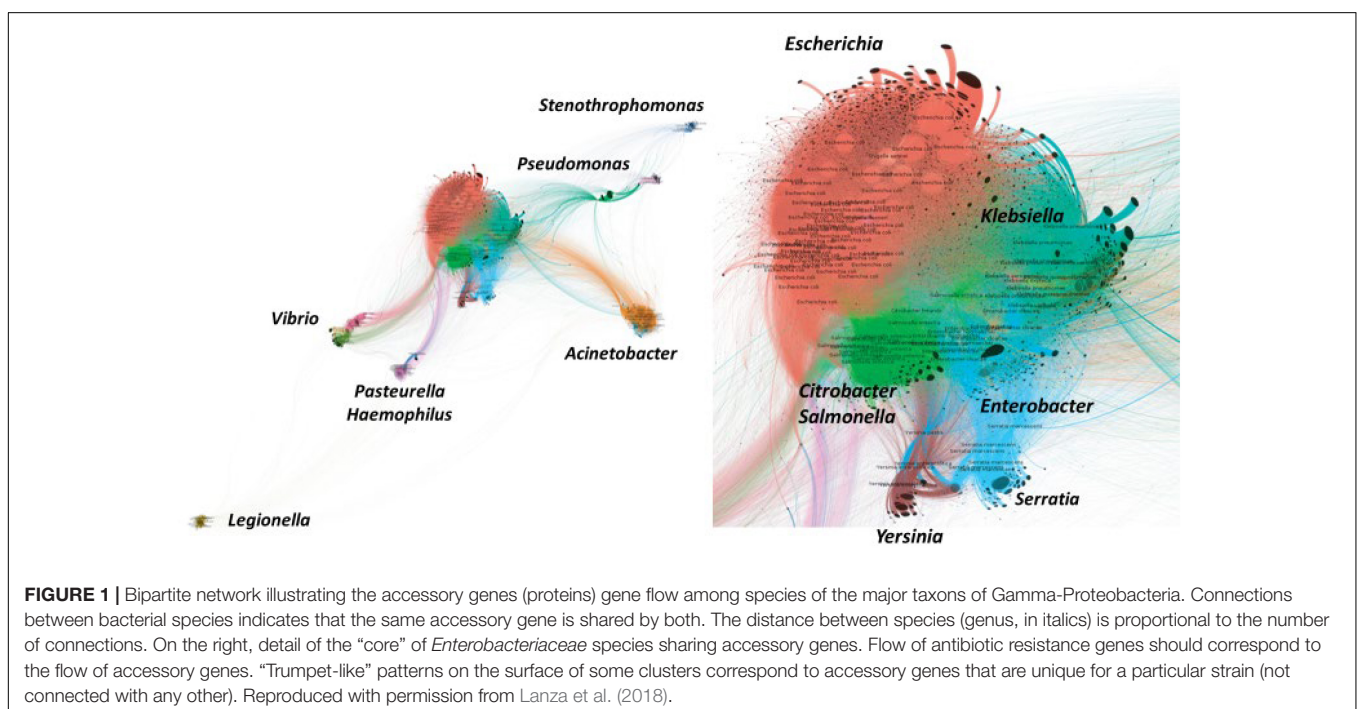
There has also been a spectacular increase in the population of particular crop plants due to the technologically driven “Green Revolution” starting in 1950s and 1960s (Matson et al., 1997), which has recently intensified by intercropping, growing two or more crops in proximity (Martin-Guay et al., 2018). We should not forget frequent animal co-culturing (interbreeding), or animal and plants co-production. The net result is the enlargement of fields promoting a mixing ecology of vegetables, animals and humans. For instance, intensively managed rice farming paddy soils might constitute unique agroecosystems, providing opportunities for mixing different microbiomes (Tanskul et al., 1998). As we will state in the next section, the dimensions of this mix are and will be critical in shaping the problem of antibiotic resistance.

## Connectivity Facilitates Microbiome Merging and Hybridization

However, the density of contacts also depends on anthropic local interventions, which are deeply influenced by sociology and economics. Urban ecosystems have established barriers to exclude contact with rural (farming) areas dominated by animals and plants. Such barriers are still weak in some developing countries; thus, the number of interactions is high. However, the challenge of feeding 11 billion people in 2050 implies an increase in contact between humans and animals in the agricultural use of water, antibiotics, and fertilizers, all of which are risk factors for the development of infectious diseases and antibiotic resistance (Rohr et al., 2019). In summary, if some general trends occur at a global scale, such as the increase in the

population of humans, food animals, and plants, the investigation of local conditions influencing One Health dynamics will become essential to shaping the dimension of the local risk of interactions based on **microbiome merging** (Ley et al., 2008; Pehrsson et al., 2016; Flandroy et al., 2018; Trinh et al., 2018). Note that microbiome merging is expected to occur preferentially among hosts sharing some basic common features. For instance, the intestinal microbiota of most vertebrates is dominated (in various proportions) by the same taxa, typically Firmicutes, Bacteroidetes, and Proteobacteria, regardless of whether the host is herbivorous or omnivorous, including marine mammals; a quite different pattern is obtained in invertebrates (Ley et al., 2008; Nelson et al., 2015; Colston and Jackson, 2016). A more detailed work is waited for lower taxa, more dependent on the habitat conditions. However, at the level of species, the intestine-adapted microorganism *Escherichia coli* is ubiquitous in mammals (Gordon and FitzGibbon, 1999). In fact, the coincidence of taxa reflects the fact that evolution of animals has occurred in parallel to the evolution of their microbiomes (McFall-Ngai et al., 2013). The terms “phylosymbiosis” was in fact coined to refer to the concordance between a host phylogeny and microbial community (microbiome) dendrogram (Theis et al., 2016). Horizontal accessory gene transfer, and the potential spread of antibiotic resistance genes, occurs preferentially among phylogenetically related bacteria (as is illustrated in **Figure 1**), even though transfer might bridge different taxonomic levels (Boto, 2009).

It is also worth mentioning that most probably one of the first barriers to preventing colonization by bacteria originated in other hosts (eventually antibiotic resistant) is the presence of a dissimilar microbiome able to outcompete the alien novel microorganisms. Gut microecology is based on ensembles of



bacteria that have evolved in proto-cooperation or synergy; if the “right partners” are absent, alien organisms are difficult to establish, or might produce dysbiosis and reduction in diversity. For instance, because of the influence of pig’s microbiota, the microbiota of farm workers is less diverse than in neighbor villagers (Sun et al., 2017). Such reduction in microbial diversity decreases colonization resistance (Van der Waaij et al., 1971; Buffie and Pamer, 2013) facilitating acquisition of antibiotic resistant strains. That has a correspondence in the host-occupied environments, as it was observed in built environments (as private homes, workplaces, hospitals) where a loss of microbiomes’ diversity following interventions to decrease microbial load correlates with an increase of antibiotic resistance (Mahnert et al., 2019).

## COMMUNICATION BETWEEN MICROBIOMES OF DIFFERENT SPECIES

### Microbiome Communication in Virgin and Stressed Habitats

The increasing density and connectivity of individuals of different species (humans, animals, plants) produces a major increase in the total size of a limited number of particular microbiomes, facilitating microbiome merging, a condition for the interbacterial spread of antibiotic resistance genes (Hernando-Amado et al., 2019; van Bruggen et al., 2019). This effect is possibly modified by the **short lifespan of farm animals**, reaching their slaughter age much earlier than their potential maximum life expectancy, approximately 10 times earlier for cattle, 50 for pigs, and 100 times for chickens<sup>3</sup>. On one hand, the slaughtering process eliminates the microbiota, including antibiotic-resistant populations (except in the case of slaughterhouse wastewater contamination). On the other hand, the replacement with newborn animals implies an intensive buildup of new microbiotas (high rate of microbiota reproduction). During the construction of the microbiota in each new individual, not only the access of microorganisms from other hosts and environments is facilitated, but the higher bacterial (and community) replication rate in a underexploited nutrient-rich habitat provides more opportunities for genetic exchange, and if antibiotics are present in the environment, selection of resulting resistant populations.

The emergence of novel opportunistic pathogens of non-human environmental origin, particularly among particular species or clones in Enterobacterales, *Acinetobacter*, *Pseudomonas*, and *Enterococcus* associated with the use of antimicrobial agents, might be linked to this process. When involved in clinical infections, these organisms necessarily colonize the mucosal surface of the host and interact with the local microbiota, most probably with phylogenetically close organisms sharing similar Hutchinsonian niches. An Hutchinsonian niche an imaginary space with many dimensions (hypervolume), in which each dimension or axis represents the

range of some environmental condition or resource required for the optimal growth of a sublineage or genotypic group (Holt, 2009).

Environments under stress (natural or anthropogenic, including antimicrobial agents) tend to reflect reduced microbial alpha diversity, the number of species/clones harbored in particular microbiota (Rocca et al., 2018). To a certain extent, stress tends to produce species-deficient habitats, providing the opportunity for alien colonizers to invade. Stress-driven reduction of species and clones reduces the diversity of microniches that are dependent on their functions. Empty niches (in terms of resources that are not exploited) act as attractors for members of neighboring organisms. Many of these free-microbe-specific microniches are now occupied by more stress-resistant, less niche-specific organisms, which originated from other microbiotas. As in the case of the naturally bacterial-free intestinal habitat of newborns, the opportunity arises for microbiome collisions and hybridizations. Reduction in alpha diversity could be expected to reduce the possibility of asymmetric dispersion of a variety of rare organisms in neighboring habitats (reduction in beta diversity). However, the collision of microbiotas creates new configurations in which rare taxa could emerge (Rocca et al., 2018, 2019). The application of zeta diversity metrics (measuring the degree of overlap in the type of taxa present between a set microbiotas) will be most useful to illustrate the spatial structure of multispecies distributions in various environments, and therefore the dimensions of microbiome merging (Hui and McGeoch, 2014).

### The “Shared Microbiome” in the Communities of Hosts

Certainly, the case of mother-child microbiome transmission illustrates the importance of shared microbiomes among closely related families of hosts (Arrieta et al., 2014). In fact, this is a case of “microbial community reproduction,” given that not only individual cells, but communities such as microbiotas also reproduce and evolve (Baquero, 2014; Gordo, 2019). As in other cases of biological reproduction, the reproducibility of the original microbiota pattern is imperfect, that is, not maintained in its integrity; thus, differences can be detected among individuals (Vallès et al., 2014). Most importantly, the reproduction of a recently acquired microbiota among closely related newborns maintains a highly conserved composition; with time, however, “microbial evolution within hosts” both in terms of species genetic evolution and migration of strains, takes place (Theis et al., 2016), increasing differences between them, which is beta diversity (Garud et al., 2019; Gordo, 2019). Thus, animals that are slaughtered early should have a more homogeneous microbiota, and the amount of this particular type of microbiota should increase in the environment.

In a shared environment, the dispersion of microbiota in highly related hosts, for instance in the same or closely related species and under similar dietary regimens (Moeller et al., 2013; David et al., 2014) ensures that the more advantageous variants emerging in a particular individual (Garud et al., 2019) can be spread to the other members of the host’s community. To a

<sup>3</sup><https://www.four-paws.us/campaigns-topics/topics/farm-animals/life-expectancy>

certain extent, there is a “collective microbiota optimization” favoring the health of the herd. This phenomenon is the other side of the coin of that giving rise to deleterious epidemics. Indeed, microbiota homogenization in ensembles of hosts is a major factor facilitating specific interactions and genetic transfer.

## Microbiota Community Coalescence

The process of microbiome merging and hybridization that might give rise to (at least partially) novel assemblies of bacteria originated in different environments is a phenomenon known in ecology as “**community coalescence**” (Rillig et al., 2015; Rillig, 2017). The combined increase in number and collapse in diversity of animals interacting with humans should facilitate reiterative coalescence events between the same microbiotic types, and thus interbacterial gene transfer.

The dynamics of microbiome merging are insufficiently understood (Roughgarden et al., 2018). New observations, suggesting the **modular structure of microbiota** (Earle et al., 2015; Tropini et al., 2017), indicate the possibility of a “**recombinational merging**” within and between microbiomes, eventually resulting in emerging taxa and emerging communities (Rocca et al., 2018, 2019). In the soil, specific microbial aggregate communities can be considered “microbial villages,” periodically connected through wetting events, where soil moisture is increased as a result of rainfall infiltration, allowing for the transfer of bacterial organisms and genetic material (Wilpiszeski et al., 2019).

Fecal transplantation provides an excellent case study for microbiota coalescence, at least in closely related types of hosts. In fact, bacterial species that might graft in the receptor host could probably be predicted by the abundance and phylogeny of bacteria in the donor and the pre-transplant receptor (Smillie et al., 2018). In the immediate period after transplantation, the invading microbiota from the donor tends to prevail, however, the before-transplant microbiota tends to progressively be restored, typically after 3 months (Seekatz et al., 2014). This is a type of “resilience effect,” where the recovery of the remaining minorities of substituted populations, or the reacquisition of the lost strains, or their functional equivalents from the environment, reconstruct the original pattern of the microbiota (Allison and Martiny, 2008). However, novel strains or species introduced by transplantation procedures can colonize without necessarily replacing the indigenous strains or species of the recipient; in fact, the presence of a kin-strain might facilitate colonization (Li et al., 2016). Thus, for a prolonged period of time there is a transient hybrid microbiota in which new consortia can be established, facilitating genetic interactions.

## Generalist Bacterial Shuttles

Intermicrobiome communication can be facilitated by shuttle bacterial species (or clones within species) belonging to generalist taxa, able to multiply in the microbiomes of various hosts. In fact, a part of these taxa can be considered “colonizing opportunistic pathogens” (Price et al., 2017). The specialist-generalist paradigm predicts that specialists will have local advantages (narrow resource utilization but high performance), and should predominate in specific microbiotas; whereas

generalists, which are probably less abundant locally (broader resource utilization but lower performance), are able to colonize diverse microbiotas (Mariadassou et al., 2015). Generalist taxa are identified by a wide Levin’s niche width index, detecting a broad range of niche conditions that a species could inhabit and successfully survive and reproduce, which can be obtained considering the proportion of operational taxonomic units (reflecting the bacterial diversity) in various microbiomes (Pandit et al., 2009). These taxa act as “microbial hubs” in scale-free networks, linking diverse microbiotas. Eventually, these shuttle taxa could have a deeper effect on the architecture of the recipient microbiota than could be expected by their abundance (Muller et al., 2018), thus representing “**keystone species**” (whose presence facilitate the establishment of many others) such that these species disappear or have reduced fitness, and the health (optimal composition) of several apparently unrelated microbiomes might be impaired (Berry and Widder, 2014).

## Niche Overlap and Metabolic Landscapes

Incoming bacteria might compete for those organisms in the recipient microbiota sharing the same function (functional redundancy) (Koskella et al., 2017). The complexity of most natural habitats likely frequently precludes the extinction of competing organisms, following a kind of “imperfect exclusion principle.” Indeed, microbiome merging depends on the local metabolic landscape, which largely determines the “Hutchinsonian niche” of bacterial species and communities (Holt, 2009).

Certainly, the degree of microbiome merging could be positively influenced by bacterial species **niche overlap**, which occurs when incoming and recipient organisms share the same resources and other ecological factors (Cornell, 2012; Moeller et al., 2013). Local microecological similarities between some areas (habitats) in the colonized hosts or between hosts and external environments should facilitate merging. However, available data regarding the microecology of colonizable habitats, such as the intestine of humans and animals, remain scarce (Baquero, 2015). Progress in metabolomics and metabolic reconstruction will soon remediate this important gap (Abubucker et al., 2012). In fact, the structure of microbiomes is ecologically determined by their metabolic networks (Muller et al., 2018). The field termed “metabobiomics” has been suggested to study the correlations between the composition of the intestinal microbiome and the metabolome (Xu et al., 2015).

## Ecological Microbiota Mixing in Gradients

An important but insufficiently explored issue is the role of ecological gradients, containing a series of partially overlapping niches, in the bridging process of microbiome merging. For instance, members belonging to different host microbiomes (including animals, plants, soils, humans) might transiently coexist in inland water sewage, wastewater treatment plants, in contaminated agricultural puddled areas, or simply in the soil of farms and human habitats



in regions with poor sanitation (Baquero et al., 2008; Berendonk et al., 2015; Pärnänen et al., 2019). Transient, but reiterated coexistence between various microbiotas (or microbiotic modules) might provide the opportunity for new associations of bacteria originated in different hosts, led by generalist taxa. In areas with poor sanitation, such associations could be introduced by continuous exposure to contaminated water or food in the microbiota of humans or animals, providing the opportunity for microbiome evolution, eventually reaching a generalist-like, “broader spectrum microbiota” (Fondi et al., 2016).

## COMMUNICATION BETWEEN BACTERIAL SPECIES: MOBILITY OF ANTIBIOTIC RESISTANCE GENES

### Connectivity of Microbial Genetic Networks

Ecological connectivity is certainly the basis of the formation of gene exchange communities, as well as of common mechanisms of niche construction or even of task distribution (Smillie et al., 2011; Fondi et al., 2016). However, gene exchange might favor the members of the community in an asymmetrical manner, particularly if the transmitted element is of great profit, as occurs in the case of antibiotic resistance. This asymmetry could have consequences for increasing the population sizes of the organism under selection, and thus for connectivity with other populations. In this regard, it would be important to document whether the recent spread of MGEs carrying antibiotic resistance genes across various microbiotas is contributing to remodeling (maybe expansion) of the borders of gene exchange communities, by recruiting novel partners able to communicate with new potential gene receptors. This possibility is in line with the concept of cumulative genetic evolution or “genetic capitalism,” in which the more adapted organisms increase in population size and consequently in connectivity and genetic interactions (Baquero, 2004).

One aspect that is not yet fully understood is the construction of gene exchange communities, particularly when the microbiome emerges *de novo* in virgin habitats, as in newborns (Skippington and Ragan, 2011; Boon et al., 2014; Mansfeldt et al., 2019). An important aspect to be considered is whether these communities “replicate” in newborns, maintaining an identical member composition, or whether new members (originated in other hosts or environments) are accepted in the genetic exchange club in these early stages of microbiota construction. A model based on puzzle construction (Baquero and Nombela, 2012) suggests that the building of the microbiota depends (as in the pieces of a puzzle) on the successive mutual recognition of the components of the community, which is independent from the order of accession. However, this is a “degenerated puzzle,” so that various pieces (different but functionally related species) can occupy the same space and establish the same, or very similar, interactions with the other pieces. This situation provides an opportunity to create variant genetic exchange communities.

Under natural circumstances, transenvironmental colonizers are probably a minority among those that are transferred. However, exposure to high inocula and/or population amplification by antibiotic selection can facilitate local adaptation, successful colonization, and integration into new genetic exchange communities (Baquero, 2018; Sheppard et al., 2018).

Communication between microbiomes is a condition for the propagation of genes between bacterial populations. Let us first clarify that the concept of antibiotic resistance genes is extremely anthropocentric. With few exceptions, possibly antibiotic producers (Davies, 1990), antibiotic resistance genes were not born to resist antibiotics (Linares et al., 2006; Martínez, 2012). They simply belong to a large pool of genes encoding paraphysiological adaptive functions. In this section, we will focus on trans-specific mobile antibiotic resistance genes: those that can be detected, with a high degree of nucleotide identity, in various bacterial species.

### The Mobile Accessory Genome

The pangenome is the gene repertoire of a given bacterial species, that is, the ensemble of all genes contained in all individuals within the species (Tettelin et al., 2008). In these studies, the notion of “species” should be defined in robust way, the ensemble of organisms with at least 95% of average nucleotide identity, as obtained in all-versus-all sequences comparisons. In most species, the pangenome is much larger than the “core genome,” accounting for genes that are contained in every individual of the species, involved in the basic machinery of cell functioning. The difference between the pangenome and the core genome is due to a collection of genes that can be present or not in a given population inside the species, unique genes responsible for functions that are adaptive, niche-specific, and eventually of a contingent nature. The genes are considered “dispensable” (at least in basic culture conditions) or “accessory” (complementing the core genome). The fact that they are not present in all individuals of a species means that they can be gained or lost; in addition, the same genes (with high sequence homology) can be found across various species, indicating interspecific mobility (Segerman, 2012). Interspecific gene mobility can be neutral if the genes are transported and acquired unspecifically only because they are hosted in MGEs, but do not provide a current benefit for the recipient bacteria. These genes can, however, be “markers” to trace genetic transfer. In many cases, interspecific accessory gene flow has an adaptive function, and the transmitted genes are critical for survival in particular environments and contribute to bacterial eco-specific diversification (Wiedenbeck and Cohan, 2011; Rouli et al., 2015). Of course, acquired antibiotic resistance genes are accessory genes, and are transferred among microorganisms by using the same MGEs than other pre-antibiotic accessory genes encoding inhibitors-resistance, including those determining resistance to heavy metals (van Hoek et al., 2011).

The “convergence of adaptive needs” among bacterial species should foster interspecific communication. Antimicrobial exposure is forcing many different organisms to survive, and there are a limited number of genes able to provide resistance.



If these genes are carried by the mobilome of a particular microbiota where these species can coexist, an increased possibility of interspecific genetic transfer is expected to occur. This transfer suggests the interesting possibility that antibiotic exposure could trigger interspecific gene flow. Certainly, the accessory genes (constituting most of the pangenome) reflect the ecological needs of organisms and might be useful to redefine species and subspecies (Laing et al., 2010; Caputo et al., 2015).

Species located in very stable, reduced, highly specialized niches are less exposed to the gene-traffic circuit; thus, their pangenome is close to their core genome (Martínez et al., 2017; McNerney et al., 2017). Significant examples are *Listeria monocytogenes*, or *Legionella pneumophila*, able to exploit intracellular (stable, isolated) niches, which have larger core genomes (“closed genomes”) than other members of their phylogenetic relatives, indicating less exposure to horizontal gene transfer (Gomez-Valero et al., 2011; Collins and Higgs, 2012). On the contrary, many bacterial organisms of importance in public health, and particularly those able to colonize different environments, have an “open pangenome” that is open to the immigration (capture) of a wide variety of genes. In the case of *E. coli*, a recent study has estimated a pangenome of 15,950 genes in 60 strains, 13,076 for the accessory genome and 2874 for the core genome (Her and Wu, 2018). In one of the largest available studies of *E. coli* (more than 2000 genomes), the authors estimated 3188 core gene families (defined as being present in 95% of genomes) and approximately 90,000 unique gene families (Land et al., 2015). The discovery of a cumulative number of new genomes in species with “open pangenomes” suggests that the number of potential accessory genes has no real limit (Lapierre and Gogarten, 2009). Why does this massive amount of horizontal genetic flow not cause a significant phylogenetic disruption in bacterial species? Probably because the preservation of the species’ “core genome” in different circumstances and environments is assured by the acquired accessory genome (Ochman et al., 2005). On the other hand, in most cases, the recent origin of these accessory genes can be traced in organisms sharing a common or convergent eco-evolutionary history with the receptor (Smillie et al., 2011; Fondi et al., 2016). This asymmetrical pattern of gene transfer allows us to identify highways of gene sharing (Beiko et al., 2005).

## Antibiotic Resistance Genes in the Mobile Accessory Genome

The ensemble of antibiotic resistance genes is the resistome (D’Costa et al., 2006; Fajardo et al., 2008; Wright, 2010). The term can be applied to the resistance genes of a given bacterial population, a species or any higher taxa, or to the whole microbiota. However, the estimated size of the resistome is highly dependent on the definition of the resistance gene (Martínez et al., 2015). A mutation in a chromosomal gene might result in a resistance phenotype, but this mutated gene is rarely transferred to other bacterial species. In fact, the majority of the resistance genes detected in metagenomes are permanently associated with the same microorganisms (Fondi et al., 2016); i.e., they are intrinsic resistance genes (Olivares-Pacheco et al.,

2013; Forsberg et al., 2014; Ruppé et al., 2019). However, most of antibiotic resistance genes of importance in public health are located into MGEs (Martínez et al., 2015). Any type of genetic interaction based on horizontal gene transfer favors the spread of antibiotic resistance (Huddleston, 2014). High-risk, transtaxa antibiotic resistance genes are prone to horizontal gene transfer by being included in structures such as plasmids, integrative and conjugative elements, conjugative islands, phages, and phage-like elements. To calculate the real proportion of antibiotic resistance genes among accessory genes transmitted by MGEs is presently a difficult task, given these elements are over-represented in the available databases, which are enriched with antibiotic-resistant organisms.

## Accessory Genome Interspecific Flow Channels and the Spread of Antibiotic-Resistance Genes in Gammaproteobacteria

The spread of accessory genes, antibiotic resistance genes being a fraction of these, occurs asymmetrically between bacterial species (Hu et al., 2016). The antibiotic resistance gene flow between species can be envisioned as interbacterial roads and highways, which are used by the mobile elements serving as “vessels of the communal gene pool” (Beiko et al., 2005; Norman et al., 2009). Note that antibiotic resistance function (phenotype) might depend on the horizontal co-transfer of neighbor (clustered) non-resistance genes, when are part of an operon. The operon organization is beneficial as enables the transfer of functionally coupled genes (Lawrence, 1999). Knowing the roads and highways by which the accessory genome flows should help us predict the itineraries that will be used by antibiotic resistance genes. In other words, antibiotic resistance genes circulate in the same channels as the accessory genome, comprising most genes involved in cell-environment adaptive interactions (Paquola et al., 2018).

The accessory gene flow among Gammaproteobacteria has been represented as a bipartite network, where the edges (links) connect two independent sets of entities, in our case bacterial genomes and antibiotic resistance proteins (genes) (Lanza et al., 2018; **Figure 1**). The distance between two bacterial species is proportional to the number of connections, that is, the number of shared proteins. This representation was based on the study of 21 Gammaproteobacterial species, represented by 47,885 genomes, analyzed using the Porous material Analysis Toolbox<sup>4</sup> platform, based on AcCNET software (Lanza et al., 2017). As mentioned above, the bipartite network includes nodes belonging to different categories, in our case genomes and proteins (gene sequences were translated into proteins), so that each genome has links with their corresponding proteins. A statistical module allows inferring both genome clustering and protein clustering. Genome clustering arranges the genomes into groups (units) that share specific antibiotic resistance proteins. Protein clustering illustrates the possibility of the co-occurrence of specific proteins that are found in the same group of genomes.

<sup>4</sup><https://github.com/irycisBioinfo/PATO>

This representation suggests that the accessory genome gene flow circulates in gamma proteobacteria favored by a phylogenetic neighborhood. In Enterobacterales, the flow is preferential between a “flow core” constituted by *Escherichia*, *Klebsiella*, *Salmonella*, *Citrobacter*, and *Enterobacter*, linked with the closer genera *Serratia* and *Yersinia*. Frequently transited pipelines linking this flow core to other distant species occur between *Vibrio* and *Salmonella*; *Escherichia*-*Salmonella* and *Pasteurella* and *Haemophilus*; *Klebsiella*-*Serratia* with *Acinetobacter* and *Pseudomonas*. However, many links occur outside these high roads, including a few reaching the far-located *Legionella*. With the precaution of considering the biased composition of available genetic databases, these roads correspond well with the history of recent antibiotic resistance events.

These gene flow highways are highly consistent with the genome-based phylogeny of the bacterial organisms. Seven phylogenetic groups or clades have recently been proposed in Enterobacterales (Adeolu et al., 2016). The first, the *Escherichia*-*Enterobacter* clade, comprises *Escherichia*, *Klebsiella*, *Enterobacter*, *Raoultella*, *Kluyvera*, *Citrobacter*, *Salmonella*, *Leclercia*, and *Cronobacter*, and corresponds to the organisms more involved in gene flow between human and animal microbiomes (Hu et al., 2016, 2017). Other clades, such as *Erwinia*-*Pantoea*, *Pectobacterium*-*Dickeya*, *Serratia*-*Yersinia*, *Hafnia*-*Edwardsiella*, *Proteus*-*Xenorhabdus*, and *Budvicia* can certainly be considered as candidates in transenvironmental and transmicrobiome genetic transfer of antibiotic resistance genes to the species of the *Escherichia*-*Enterobacter* clade.

## ONE HEALTH COMMUNICATION AND THE ECOLOGY OF BACTERIAL SPECIES

An important corollary to the above is that by knowing the species composition and their relative frequency in a particular location, we could probably predict the local risks for communication and eventual dissemination of antibiotic resistance. Note that for such a purpose we should consider all relevant species in the various microbiotas converging in the One Health microbiosphere. However, the taxa-area relationship of bacteria, which is a critical aspect for understanding interspecies communication in One Health studies, remains difficult to establish (Horner-Devine et al., 2004). These studies should be oriented to acquire data about four main relevant issues. First, to localize the preferential or primary “reproductive sites” of the various organisms (species/clones), i.e., the natural locations where they reach the highest growth rates and population densities. Second, to identify secondary multiplication sites where they reproduce less efficiently but can reach significant population sizes. Third, to examine other environments, the “tolerated environments” where they can survive during significant periods of time, probably under very slow multiplication or persistence conditions. Fourth, to identify the “excluded environments” where these populations are unable to survive.

Connectivity of bacterial species depends on the overlapping of sites where their multiplication or persistence is possible, and thus the possibility of acquiring resistance genes or accessory genes at large. Sites where bacteria can meet and evolve resistance have been named “genetic exchange reactors” (Baquero et al., 2008). To illustrate this point, and with the awareness that this is only a partial view (excluding, for example, antibiotic gene flow in Gram-positives), in the following paragraphs we summarize the main ecological traits of the main genera of the *Escherichia*-*Enterobacter* clade that might explain transenvironmental One Health antibiotic resistance gene flow.

The genus *Escherichia*, and particularly *E. coli*, is by far the deadliest type of bacterial organism influencing human health; consequently, the control of antibiotic resistance acquisition is a critical issue (Vila et al., 2016). Most probably, the preferential reproductive site is the lower intestinal tract of warm-blooded animals. However, *E. coli* can also integrate and multiply into indigenous microbial communities in the environment (Jang et al., 2017), which might constitute secondary multiplication sites. Ecological barriers have prevented gene flow between environmental and intestinal *E. coli* lineages (Luo et al., 2011), but such hurdles are collapsing in an increasingly polluted environment. Sewage water, including water from treatment plants, allows the persistence of many related Enterobacteriaceae, predominantly *E. coli* (Vilanova et al., 2004). In addition, *E. coli* populations can persist and maintain growth potential in the soil (Byappanahalli et al., 2009). In proportion to their relative population size and replicative potential, *E. coli* can acquire resistance genes from donors at these sites. Note that most relevant antibiotic resistance genes in *E. coli* originated in environmental (non-intestinal) bacteria (Hernando-Amado et al., 2019).

*Klebsiella* is a pivotal organism in the transfer of antibiotic resistance determinants from environmental (note that *Klebsiella* is a nitrogen-fixing type of organism) to intestinal microbes. *K. pneumoniae* is ubiquitous in the environmental microbiotas surrounding humans and animals, including in water, soil, and plants. Copper-resistance is probably a good marker for soil-water versus intestinal habitat, being *Klebsiella* much more frequently resistant than the more intestinal-adapted *E. coli* (Sánchez-Valenzuela et al., 2017). There are no significant differences between environmental and clinical strains, with the possible exception of capsular antigens. Interestingly, there is a possible shift in the *K. pneumoniae* accessory genome toward human and animal adaptation (Martin and Bachman, 2018), increasing the possibility of genetic interactions with more human-animal adapted bacteria, such as *E. coli*. In fact, most of the currently threatening mechanisms of resistance, including extended-spectrum beta-lactamases (Valverde et al., 2007) and carbapenemases, as well as colistin-resistance, were introduced in the intestinal microbiota via *K. pneumoniae* (Holt et al., 2015; Rolain et al., 2016; Hadjadj et al., 2017). In fact, carbapenemase-producing *K. pneumoniae* gut colonization precedes *E. coli* acquisition of resistance (Hernández-García et al., 2019). Once undistinguished from *K. pneumoniae*, *K. variicola*

has been mostly found in soil and plants (as sugar cane stems, maize shoots, and banana leaves), but has also been associated with human infections (Martínez-Romero et al., 2015). *K. quasipneumoniae* probably has an intermediate position between *K. pneumoniae* and *K. variicola* with respect to human and animal colonization. *Klebsiella oxytoca* (probably a complex genetic group of related bacteria) is now part of the consortium of environmental microorganisms that has likely contributed to the spread among human strains of antibiotic resistance, including carbapenemase genes (Khan et al., 2018), as with the related species *K. michiganensis* and *Klebsiella grimontii* (Liu et al., 2018) or the *K. huaxensis*-*K. spallanzani* group (Merla et al., 2019). However, a deeper study of the ecology of *Klebsiella* species is warranted. In general, as in the case of *E. coli*, this study should be based on the recognition of species ecotypes colonizing specific microhabitats where they can overlap with potential donors of antibiotic resistance (Koeppel et al., 2008). Each ecotype presents different opportunities for horizontal gene transfer.

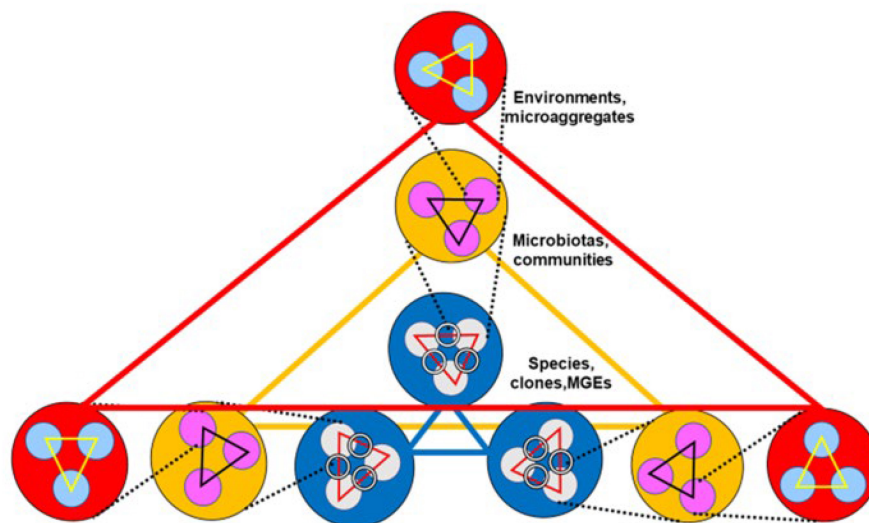
Within *Enterobacter*, the *E. cloacae* complex, an environmental-animal-human genus (endophytic symbionts) that includes the cluster *E. xianfangensis* (an organism of the plant rhizosphere) and *E. hormaechei*, harbors transferable carbapenemases, suggesting an important role in resistance gene flow (Peirano et al., 2018). *E. aerogenes* is much more closely related than *E. cloacae* to the *Klebsiella* genus (*Klebsiella aerogenes*).

In the genus *Serratia*, *S. marcescens* was considered an “environment-only” organism until the 1950s and 1960s,

producing pink colonies. In recent decades, many strains have been isolated from the clinical environment, all of them non-pigmented. *S. marcescens* is widespread in nature and is a frequent food colonizer, particularly in starchy foods. Strains isolated from patients are frequently antibiotic-resistant, however, many strains from the environment, including the hospital environment, are much more susceptible (Ehrenkranz et al., 1980). Resistant clinical strains are carriers of extended-spectrum beta-lactamases and carbapenemases (Yang et al., 2012).

Other environmental organisms, probably rare in the intestine, such as *Kluyvera*, in which the first CTX-M extended-spectrum beta-lactamases probably originated, might have played a key role in its early transmission to the *Klebsiella* genus and from there to *E. coli*. *Kluyvera* has been shown to belong to a resistance gene exchange community in the intestine of patients, together with *Raoultella ornithinolytica*, *K. pneumoniae*, and *E. coli* (Hernández-García et al., 2018). In fact, *Raoultella* is usually found in water and plants, but is not infrequent in human-associated isolates (Seng et al., 2016).

Finally, *Citrobacter* and *Salmonella* should also be included among shuttle species able to colonize humans, animals, plants, and the environment. High-risk transferable resistance genes, such as carbapenemases, have been consistently found in *Citrobacter* (Pepperell et al., 2002; Arana et al., 2017). The genus *Salmonella* has a known association with human and animal pathogenicity, but it also interacts with the surfaces and tissues of plants and their associated microbiota, including protists (Brandl et al., 2013).



**FIGURE 2 |** Multilevel communication between environments influencing antibiotic resistance. Communication occurs among environments (red circles and large red “communication” triangle), among the microbiotas contained in these environments (yellow circles y large yellow triangle), and among the species and clones contained in these microbiotas (blue circles and blue triangle). Inside environments there are spatially defined subenvironments or macroaggregates (light blue small circles). They contain microbiotas, bacterial community microaggregates (pink circles), which at their turn contain bacterial species and clones (light gray circles), which contain mobile genetic elements (rings, representing plasmids). At each one of these levels, communications (small triangles) are established. One Health emphasized that merging of environments, microbiotas, and bacterial communities, favors communications and consequently the spread of antibiotic resistance genes.



## ONE HEALTH MULTILEVEL DYNAMICS OF ANTIBIOTIC RESISTANCE

As presented in the preceding sections, the dynamics of antibiotic resistance is a multi-hierarchical phenomenon (Baquero et al., 2013; Hernando-Amado et al., 2019). In a highly simplified way, the first level in the One Health hierarchy (large red triangle in **Figure 2**) is constituted by the interactions among environments (environmental merging); typically, human, animal, plant, soil, and water environments (Thanner et al., 2016). Indeed, environmental merging occurs by gradient formation, so that a multiplicity of hybrid environments is expected to occur. In fact, such a process occurs by merging sub-environments. The matrix of many environments is composed (as it is expressed in soil ecology) by sub-environments as macroaggregates, spatially differentiated structures, containing microaggregates, typically smaller than 250  $\mu\text{m}$ , composed of diverse inorganic, organic and biotic materials, where assemblies of microbial organisms (microbiota, and sub-microbiota assemblies) are located (Wilpiszeski et al., 2019). Such spatial organizations of bacterial communities and populations also occur in the lumen of the intestine (Earle et al., 2015).

The second level (large yellow triangle in **Figure 2**) is formed by the interactions among the microbiotas of these environments (microbiota merging), which occur by blending the microbial communities and subcommunities that compose the microbiota. The third level (large blue triangle in **Figure 2**) is composed of the interactions among bacterial species or clones, either of an ecological nature, such as cross nutrition, synergies, or antagonistic effects, or by being linked in genetic exchange communities (Skippington and Ragan, 2011). Genetic exchanges, including antibiotic resistance genes, are facilitated by MGEs. Of course, we can consider further levels of interaction, including the interactions among MGEs and ultimately, interactions between genes, including gene fusion or gene recombination. The modification of the conditions at each one of these levels should influence (up and down) the other hierarchical levels; for instance, the variable chemical composition of the gut lumen (the local chemosphere) influences bacterial interactions and probably microbiome merging (Baquero et al., 2019). All these interactive

levels shape the emergence, spread, and maintenance of antibiotic resistance.

A problem to be addressed in research on multihierarchical systems is how to predict to which extent the changes in a given level of the hierarchy might alter the composition of the neighboring levels. This key problem in ecology, and generally in One Health, has been approached recently by computational sciences, including the application of membrane computing modeling technologies, a biologically inspired methodology that has been recently applied to the prediction of antibiotic resistance (Campos et al., 2019).

The highly integrative concept of One Health (and the highly related concept of Global Health) has provided a holistic image of the problem of antibiotic resistance, far beyond the historical consideration as a “hospital-based problem.” At the same time, the One Health approach opens the door for the investigation and development of the new biochemical, microbiological, ecological, bioinformatic, and computational tools required to understand and control the problem of antibiotic resistance on a planetary scale.

## AUTHOR CONTRIBUTIONS

FB wrote the review. TC, J-LM, VL, and SA-G contributed with paragraphs, and provided a deep intellectual contribution of the concepts exposed.

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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.

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# Characterization and Bio-Typing of Multidrug Resistance Plasmids From Uropathogenic *Escherichia coli* Isolated From Clinical Setting

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Urinary tract infection is primarily caused by *Escherichia coli*. Multidrug resistance and their rapid dissemination in this pathogenic microbe complicate therapeutic strategies and threaten public health. Conjugation systems responsible for interbacterial transmission of antibiotic resistance are plasmid-encoded and can be classified as the P, F, and I types. Specific pili types and pili associated proteins were related to the transfer among this gram-negative organism and were thought to depend on contacts created by these structures at the time of DNA transport. In this study, conjugation system types of the plasmids that harbor multidrug resistant genes (*aac-1b-cr*, *oqxAB*, *qnrB*, *qnrS*, *bla<sub>TEM</sub>*, *bla<sub>OXA</sub>*) amongst 19 *E. coli* uropathogenic isolates were characterized under ciprofloxacin/ceftazidime selection individually by pili and pili associated gene types. Investigations indicated incidence of single plasmid of multiple replicon type amongst the transconjugants. *bla<sub>TEM</sub>*, *bla<sub>CTX-M</sub>*, *bla<sub>OXA</sub>*, *aac-1b-cr*, *oqxAB*, *qnrB*, *qnrS* genes in varied combination were observed to be successfully co-transmitted against ceftazidime/ciprofloxacin selection. Seven primer pair sets were selected that encodes pili and pili associated genes (*traF*, *trwJ*, *traE*, *trhE*, *traG*, *pilM*, *pilx4*) by nucleotide database search tools using annotated plasmids of different incompatibility types to assign the conjugation system type of the transmissible resistant plasmids by PCR. *traF* was predominant irrespective of drug selection that indicated F-type conjugation system was responsible for transmission of resistant plasmids which results in the rapid dissemination of antibiotic resistance in the isolates screened. Therefore this is a first report of its kind that investigated pili and pili associated genes to bio-type multidrug resistant plasmids and their transmission in clinical settings amongst uropathogenic *E. coli* circulated in the eastern part of India.

**Keywords:** uropathogenic *E. coli*, MDR, PBRT, co-transmission, T4SS, pili

## INTRODUCTION

Urinary tract infection (UTI) is known to cause significant levels of morbidity and mortality in developed countries and has become a public health concern. *Escherichia coli* is reported to be one of the primary etiologic agents that cause UTI. Emergence of antibiotic resistance in this bacterial pathogen is recognized as one of the greatest threats to global healthcare management system (reviewed in Hadifar et al., 2017).

Multidrug resistant (MDR) isolates causing UTI have serious implications for the empiric therapy against pathogenic isolates and for the possible co-selection of antimicrobial resistant pathogens. There are several factors responsible for dissemination of antimicrobial resistance genes among these pathogens, and plasmid-mediated transfer has been considered one of the most important mechanisms for the horizontal transfer of multidrug resistance (Akingbade et al., 2014). The most widely used plasmid classification scheme is PCR based replicon typing (PBRT) which exploited loci encoding plasmid replication machinery (Carattoli et al., 2005, 2014) which provided insights into resistance plasmid epidemiology, such as whether resistance dissemination involves diverse plasmids or one dominant “epidemic” type (Valverde et al., 2009).

A recent review summarized the major plasmid families that are currently emerging in MDR *Enterobacteriaceae* strains isolated in several parts of the world including those conferring resistance to important antibiotics such as extended-spectrum cephalosporins, fluoroquinolones, and aminoglycosides (Carattoli, 2009). Certain replicon types were found to be associated with MDR as well as with bacterial disease outbreaks (Huang et al., 2012). In turn, this can be useful for epidemiologic surveillance and the development of strategies to prevent their spread (Huang et al., 2012; Dehbanipour et al., 2016). However, evidence of multiple replicon types among individual resistant plasmid questioned the fidelity of the classification technique and demands further characterization of the resistant plasmids to address strategies to prevent their spread.

Conjugation systems play key role for interbacterial transfer of antibiotic resistance genes, pathogenicity, and genes encoding other traits of potential benefit to the bacterial host. The plasmid encoded type IV secretion systems (T4SSs) that belongs to IncP, IncF, and IncI identified in *E. coli* and other species of *Enterobacteriaceae* function exclusively in conjugative DNA transfer and had established its role in organizing bacterial genomes and transmission of antibiotic resistance in clinical settings (Christie, 2016). In IncI plasmid systems, which have both, type IV pili (T4P) and conjugative pili, the former involved in binding of the donor to the recipient cells and after binding, the F-pilus retracts and a stable association between donor and recipient cells initiated by a process of mating pair stabilization (Mps) that involves the translocation of a structure containing TraG into the recipient cell periplasm which further signals the commencement of conjugative DNA transport and replication by the donor cell (Hazes and Frost, 2008). Therefore specific pili types and pili associated proteins were related to the transfer of IncP, IncF and IncI plasmids among this gram-negative organism which was thought to depend on contacts created by these structures at the time of DNA transport (Christie, 2016).

In this study, we characterized multidrug resistant plasmids obtained from uropathogenic *E. coli* isolates collected from hospitalized patients with respect to  $\beta$ -lactamase and quinolone resistant gene acquisition and their co-transmission by conjugation, a common phenomenon in the natural habitat. Moreover the type of conjugation system followed by the clinical

plasmids was explored based on pili and pili associated gene types to understand and evaluate potential of horizontal gene transfer among these gram negative pathogen.

## MATERIALS AND METHODS

### Bacterial Culture

A total of 80 urine samples were collected from Carmichael Hospital for Tropical Diseases, Kolkata from patients suffering from UTI. *E. coli* were detected in the urine culture positive samples by standard biochemical tests and cultured in Luria Bertani Broth (Hi-Media Laboratories, India). The study protocol was approved by the institutional ethical committee.

### Antibiotic Susceptibility Testing

*Escherichia coli* isolates were tested by Kirby Bauer disk diffusion method on Muller Hinton agar plates using following antibiotic disks; amikacin (AK; 10  $\mu$ g), ceftazidime (CAZ; 30  $\mu$ g), cefotaxime (CTX; 30  $\mu$ g), cefoxitin (CX; 30  $\mu$ g), ciprofloxacin (CIP; 5  $\mu$ g), levofloxacin (LE; 5  $\mu$ g), cotrimoxazole (COT; 25  $\mu$ g), nitrofurantoin (NIT; 300  $\mu$ g), and imipenem (IPM; 10  $\mu$ g) (Hi-Media laboratories, India). *E. coli* ATCC 25922 was used as quality control strain. Isolates resistant, intermediate resistant and sensitive to individual antibiotics were determined by the zone of inhibition that was interpreted following CLSI (2016) guidelines (CLSI, 2016). An isolate was considered as multidrug resistant if it was resistant to  $\geq 3$  classes of antibiotics. Extended spectrum  $\beta$ -lactamase production was determined in isolates that were resistant to cephalosporin (Sharma et al., 2013).

### Plasmid Isolation, Bacterial Conjugation, and PCR Analysis

Plasmid DNA was prepared by alkaline lysis method and electrophoresed on 0.8% agarose gels and visualized by Gel documentation system (BioRad) (Khadgi et al., 2013). Plasmid bands of varied size (approximate) were detected using molecular weight marker, lambda/*Hind*III double digest. Conjugal transfer of plasmid to *E. coli* J53AzideR recipient strain was performed by broth mating assay (Ghosh and Mukherjee, 2016). Transconjugants were screened by double selection method on MacConkey agar plates containing sodium azide (100  $\mu$ g/ml) and ceftazidime (30  $\mu$ g/ml), or ciprofloxacin (5  $\mu$ g/ml), respectively. Plasmids from the clinical isolates and the transconjugants were screened by PCR for the detection of  $\beta$ -lactamase genes; *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub> (Tayebi et al., 2016; Bandyopadhyay and Mukherjee, 2017) and PMQR genes; *oqxAB*, *qnrA*, *qnrB*, *aac(6')-Ib-cr*, *qepA* genes with gene specific primers (Tayebi et al., 2016). Purity of the plasmid preparations isolated from transconjugants was ascertained by PCR with chromosomal gene (*fimH*, *papC*, *cnf1*) specific primers (Supplementary Table S1) and RFLP analysis. Discrete difference in the RFLP pattern was observed amongst the plasmid DNA which was absent in genomic DNA isolated from the clinical counterpart that was used as a control (Supplementary Figures S1, S2, S3).

## Plasmid Replicon Typing

Incompatibility groups of the plasmids isolated from the transconjugants were determined by PCR-based replicon typing (PBRT) method using IncFrep, F1B, N, I1, A/C, H1, X, Y, L/M, W by specific PCRs as described previously (Carattoli et al., 2005).

## In silico Analysis

An *in silico* analysis was carried out using GenBank BLAST<sup>1</sup> on *E. coli* annotated plasmids of IncF, IncI1, IncN, IncW, IncHI, IncA/C, IncX replicon types. However, due to insufficient data on the various incompatibility types from *E. coli*, the *in silico* analysis was extended to some plasmids of other *Enterobacteriaceae* family. For the seven Inc groups of plasmids incident in this study pili and pili associated genes involved in conjugation event was used as template for nucleotide-Search<sup>2</sup> to identify candidate genes which were specific for individual incompatibility types (Bousquet et al., 2015). The target genes were further validated by blastN (see footnote 2) and ClustalW2 software<sup>3</sup>. Primer pairs covering most sequences in each family were designed using FastPCR<sup>4</sup> (Table 1 and Supplementary Figure S4).

## RESULTS

### Bacterial Isolates

In the present study 50 urine samples yielded significant growth out of 80 samples collected from patients suffering from UTI. 19 *E. coli* isolates were identified from the 50 urine culture positive samples by routine biochemical analysis.

### Antibiotic Resistance

All 19 *E. coli* isolates were resistant toward ciprofloxacin, ceftazidime, cefotaxime, cefipime, amikacin, levofloxacin,

nitrofurantoin, and imipenem except 2, 3, 4, 8, and 8 out of the 19 which showed intermediate resistance against amikacin, levofloxacin, nitrofurantoin and imipenem, respectively.

## Acquired Plasmids and Resistant Gene Profiles

Plasmid profiling of all 19 isolates showed presence of one to seven plasmids of approximate sizes ranging from ~12 to ~1 kb. Prevalence of a plasmid of an approximate size in the range of ~12 kb was common in all isolates. 8 out of 19 isolates exhibited three plasmid bands while one, two, four and five/seven plasmid bands were detected in 2/19, 1/19, 4/19, and 4/19 of the isolates, respectively (Figure 1). Plasmids from all 19 isolates indicated presence of  $\beta$ -lactamase genes *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub> and the PMQR genes *aac*(6')*1b-cr*, *oqxAB*, *qnrB* in varied combinations. *bla*<sub>TEM</sub> and *bla*<sub>OXA</sub> was observed in all 19 isolates in combination with *bla*<sub>CTX-M-15</sub> type (13/19), and the PMQR genes; *aac*(6')*1b-cr* (14/19), *oqxAB*(2/19), *qnrB*(7/19), and *qnrS* (1/19), respectively (Table 2). Therefore it may be assumed that *bla*<sub>TEM</sub> and *bla*<sub>OXA</sub> genes harbored by the clinical isolates do not encode ESBLs and exhibited  $\beta$ -lactam-  $\beta$ -lactamase inhibitor resistant phenotype (data not shown).

## Conjugal Transfer

Transfer of resistance determinants from all 19 clinical *E. coli* donors to the *E. coli* J53AziR recipients strain was observed against  $\beta$ -lactam antibiotics (ceftriazone, ceftazidime, cefotaxime) and fluoroquinolones (ciprofloxacin, levofloxacin). The  $\beta$ -lactamase genes *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub> and the PMQR genes *aac*(6')*1b-cr*, *oqxAB*, *qnrB*, respectively were transferred in various combinations (Table 3).

Selection of transconjugants against ceftazidime and ciprofloxacin independently showed acquisition of resistant plasmids with both single as well as multiple replicon types with universal presence of IncF type plasmid. Single replicon type plasmid which belonged to IncF type (IncFrep) was observed in four transconjugants selected against ciprofloxacin and 10 (IncFIB; 6, IncFrep; 4) selected against ceftazidime respectively.

<sup>1</sup><http://blast.ncbi.nlm.nih.gov/>

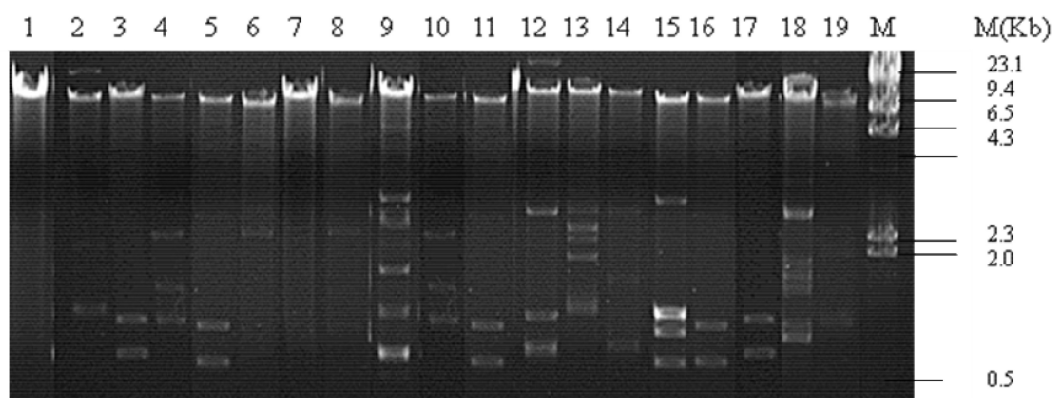
<sup>2</sup><https://www.ncbi.nlm.nih.gov/nucleotide/>

<sup>3</sup><http://www.ebi.ac.uk/Tools/msa/clustalw2/>

<sup>4</sup><http://primerdigital.com/fastpcr.html>

TABLE 1 | Primers used in this study.

Annotated plasmids	Primer name	Sequence (5'-3')	Length (bp)	Amplicon size (bp)
R100	<i>traF</i> -fp	CCGGCTGCAGAATTACTGGAC	21	114
	<i>traF</i> -rp	CTGCAGTACAGCCATTACAACGG	23	
R64	<i>pilM</i> -fp	ATGGGATGGCTGGTCATGGCC	21	232
	<i>pilM</i> -rp	CCCTGTCACTCCGGACTCCC	20	
R46	<i>traE</i> -fp	GCTAATAAAAAACAGGGC	18	624
	<i>traE</i> -rp	AACCCGGAAGTTAACTGA	18	
R388	<i>trwJ</i> -fp	TGAAGAAGCTGGTTATGAC	19	560
	<i>trwJ</i> -rp	TATCCAGAACGAAACGAC	18	
R27	<i>trhE</i> -fp	ATGAACTTCTCAGCAGGT	19	442
	<i>trhE</i> -rp	AACATCGGTTTTGTTACAAC	20	
RA1	<i>traG</i> -fp	TTGTTGTTGATGCTCTAAACA	21	350
	<i>traG</i> -rp	ATTGCTTTTATTATCGTGATG	21	
R6K	<i>pilx4</i> -fp	CGAGTTTTCTCAGATCAGC	19	358
	<i>pilx4</i> -rp	CTAAATCCCTCCTTGCTG	18	



**FIGURE 1 |** Plasmid pattern of clinical uropathogenic *E. coli* isolates. Lanes 1–19, plasmid extracted from the clinical isolates ( $n = 19$ ) and electrophoresed on 0.8% agarose gel. M, lambda/*Hind*III DNA ladder.

Additionally presence of multiple replicon Inc type plasmids (I1, W, X, Y, A/C, L/M, HI) were detected in transconjugants obtained under ciprofloxacin compared to IncF and IncI1 obtained under ceftazidime selection (Table 3).

## Identification of Pili and Pili Associated Genes

Genes encoding pili and pili associated proteins were members of the bacterial T4SS system and actively participates facilitating the IncF and IncP bacterial conjugation machinery. However, pili

associated with IncI conjugation system enhances the process of conjugation without directly playing a role in the transfer mechanism. Nucleotide sequences of genes encoding pili and pili associated proteins were identified in the annotated plasmids R100 (Ac No. NC\_002134.1), R64 (Ac.No. AP005147.1.), R46 (Ac.No. NC\_003292.1.), R388 (Ac.No. NC\_028464.1), R27 (Ac.No. NC\_002305.1), pRA1 (Ac.No. NC\_012885.1), R6K (Ac No. AJ006342.1) assigned to IncF, IncI, IncN, IncW, IncH, IncA/C, and IncX plasmid incompatibility types, respectively (Table 4) to characterize the conjugation system type in the transconjugants that harbor plasmid of multiple replicon types.

Plasmid isolated from the 16 and 3 transconjugants obtained under ceftazidime selection showed presence of *traF* and *pilM*, respectively (Table 5). Overall similar results were observed in transconjugants selected under ciprofloxacin pressure, however, the transconjugants that exhibited presence of *pilM* amongst the later group were *traF* positive amongst the former respectively (Table 5). Moreover combination of *traF* and *pilM* or other pili associated genes *trwJ*, *traE*, *trhE*, *traE*, and *pilx4* were not detected. Presence of *traF* and *pilM* amongst the transconjugants were further confirmed by southern blot hybridization using the *traF* and *pilM* cloned amplicons as probes (data not shown).

## DISCUSSION

In this study, we focused on the genetic classification of the transmissible plasmids isolated from multidrug resistant uropathogenic *E. coli* which has an impact on dissemination of drug resistance amongst this uropathogen. Multidrug resistance is an emerging threat propagated in this pathogenic microbe. Worldwide and nationwide studies showed different resistance rates against several antibiotic groups (reviewed in Hadifar et al., 2017). Moreover recent reports from India indicated an alarming rise in the incidence of multidrug resistance (i.e., simultaneous resistance to various class of antibiotics, such as aminoglycosides, cephalosporins, fluoroquinolones) amongst the uropathogenic *E. coli* isolates that varied from 76.51 to 100%

**TABLE 2 |** Resistance genes in the clinical uropathogenic *E. coli* isolates ( $n = 19$ ).

Clinical isolates	Resistance genes						
	$\beta$ -lactamase				PMQR		
	<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>CTX-M</sub>	<i>bla</i> <sub>OXA</sub>	<i>aac-1b-cr</i>	<i>oqxAB</i>	<i>qnrB</i>	<i>qnrS</i>
EC175	+	+	+	+	+	–	–
EC 176	+	+	+	+	–	–	–
EC 177	+	–	+	+	–	–	–
EC 178	+	–	+	+	–	+	–
EC 180	+	+	+	–	–	–	+
EC183	+	+	+	+	–	–	–
EC 185	+	+	+	+	–	+	–
EC 187	+	–	+	+	–	–	–
EC 194	+	–	+	+	–	–	–
EC 195	+	+	+	+	–	–	–
EC 197	+	+	+	+	–	–	–
EC 207	+	+	+	+	+	+	–
EC 211	+	+	+	–	–	+	–
EC 215	+	+	+	–	–	+	–
EC 216	+	–	+	–	–	+	–
EC 217	+	+	+	–	–	+	–
EC 219	+	+	+	+	–	–	–
EC 222	+	–	+	+	–	–	–
EC 224	+	+	+	+	–	–	–



**TABLE 3 |** Plasmid replicon types and resistance gene distribution among the transconjugants.

Drug selection	Transconjugants	$\beta$ -lactamase genes			Replicon(s)	PMQR genes			
		<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>CTX-M</sub>	<i>bla</i> <sub>OXA</sub>		<i>aac-1b-cr</i>	<i>oqxAB</i>	<i>qnrB</i>	<i>qnrS</i>
C	TCa175	+	+	–	Frep, I1	+	+	–	–
E	TCa176	+	+	+	Frep	–	–	–	–
F	TCa177	+	–	+	Frep, I1	+	–	–	–
T	TCa178	+	–	+	F1B	–	–	–	–
A	TCa180	+	+	+	F1B	–	–	–	–
Z	TCa183	+	+	+	Frep,F1B	+	–	–	–
I	TCa185	+	+	–	Frep, I1	–	–	–	–
D	TCa187	+	–	+	Frep	–	–	–	–
I	TCa194	+	–	+	Frep,F1B	–	–	–	–
M	TCa195	+	+	+	Frep,F1B	+	–	–	–
E	TCa197	+	+	+	F1B	+	–	–	–
	TCa207	+	+	+	Frep	+	+	–	–
	TCa211	+	+	–	Frep	+	–	–	–
	TCa215	+	+	–	Frep, I1	+	–	–	–
	TCa216	+	–	+	Frep, I1	–	–	–	–
	TCa217	+	+	+	F1B	–	–	–	–
	TCa219	+	+	+	F1B	–	–	–	–
	TCa222	+	–	+	F1B	–	–	–	–
	TCa224	+	+	+	Frep,N	+	–	–	–
	TCi175	+	–	–	Frep,W,X	+	–	–	–
C	TCi175	+	–	–	Frep,W,X	+	–	–	–
I	TCi176	+	+	+	Frep,A/C,F1B,W	+	–	–	–
P	TCi177	+	–	+	Frep,W	+	–	–	–
R	TCi178	+	–	–	Frep,I1,L/M,W	+	–	–	–
O	TCi180	+	+	–	Frep,A/C,F1B,Y,W,X	–	–	–	+
F	TCi183	–	+	+	Frep,F1B,W	+	–	–	–
L	TCi185	+	–	–	Frep	+	–	+	–
O	TCi187	+	–	–	Frep,I1	+	–	–	–
X	TCi194	+	–	+	Frep,F1B	+	–	–	–
A	TCi195	+	–	+	Frep	+	–	–	–
C	TCi197	+	+	+	Frep	+	–	–	–
I	TCi207	+	–	–	Frep,F1B,W	+	–	+	–
N	TCi211	+	+	+	Frep,A/C,F1B,W	–	–	+	–
	TCi215	+	–	+	Frep,F1B,W	–	–	+	–
	TCi216	+	–	+	Frep,L/M,W,X	–	–	+	–
	TCi217		+	+	Frep	–	–	+	–
	TCi219	+	–	+	Frep,F1B,W,X	+	–	–	–
	TCi222	+	–	–	Frep,A/C,F1B, F11S,H1,W,X	+	–	–	–
	TCi224	+	–	+	I1,W	+	–	–	–

**TABLE 4 |** Pili and pili associated genes in annotated plasmids assigned to specific plasmid replicon types.

Replicon	Annotated plasmids	Accession No.	Genes encoding	
			Pili	Pili associated proteins
IncF	R100	NC_002134.1		<i>traF</i>
IncI1	R64	AP005147.1	<i>pilM</i>	
IncN	R46	NC_003292.1		<i>traE</i>
IncW	R388	NC_028464.1		<i>trwJ</i>
IncH	R27	NC_002305.1		<i>trhE</i>
IncA/C	pRA1	NC_012885.1		<i>traG</i>
IncX	R6K	AJ006342.1	<i>pilx4</i>	

(Annapurna et al., 2014; Niranjana and Malini, 2014; Ranjini et al., 2015) that was very similar to our study which cause difficulty in clinical management especially in a resource poor country, India.

It is of particular concern that resistance to  $\beta$ -lactams and fluoroquinolones is frequently driven by plasmid-borne extended spectrum  $\beta$ -lactamase genes and plasmid mediated quinolone resistance (PMQR) genes in this uropathogen (Tayebi et al., 2016). Various reports suggested acquisition of plasmids of varied size and numbers associated with multiple drug resistant genes among pathogenic *E. coli* worldwide and nationwide (Sharma et al., 2010; Khadgi et al., 2013) with incidence of a plasmid band at 26 or 21 kb, respectively (Jan et al., 2009;

**TABLE 5 |** Conjugal plasmid types associated with transmissible resistant plasmids in transconjugants.

Drug selection	Transconjugants	Pili and pili associated genes							Conjugal plasmid types
		<i>traF</i>	<i>pilM</i>	<i>traE</i>	<i>trwJ</i>	<i>trhE</i>	<i>traG</i>	<i>pilx4</i>	
C	TCa175	+	–	–	–	–	–	–	F
E	TCa176	+	–	–	–	–	–	–	F
F	TCa177	–	+	–	–	–	–	–	I
T	TCa178	+	–	–	–	–	–	–	F
A	TCa180	+	–	–	–	–	–	–	F
Z	TCa183	+	–	–	–	–	–	–	F
I	TCa185	+	–	–	–	–	–	–	F
D	TCa187	+	–	–	–	–	–	–	F
I	TCa194	+	–	–	–	–	–	–	F
M	TCa195	+	–	–	–	–	–	–	F
E	TCa197	+	–	–	–	–	–	–	F
	TCa207	+	–	–	–	–	–	–	F
	TCa211	+	–	–	–	–	–	–	F
	TCa215	–	+	–	–	–	–	–	I
	TCa216	–	+	–	–	–	–	–	I
	TCa217	+	–	–	–	–	–	–	F
	TCa219	+	–	–	–	–	–	–	F
	TCa222	+	–	–	–	–	–	–	F
	TCa224	+	–	–	–	–	–	–	F
C	TCi175	+	–	–	–	–	–	–	F
I	TCi 76	+	–	–	–	–	–	–	F
P	TCi177	+	–	–	–	–	–	–	F
R	TCi178	–	+	–	–	–	–	–	I
O	TCi 80	+	–	–	–	–	–	–	F
F	TCi183	+	–	–	–	–	–	–	F
L	TCi185	+	–	–	–	–	–	–	F
O	TCi187	–	+	–	–	–	–	–	I
X	TCi194	+	–	–	–	–	–	–	F
A	TCi195	+	–	–	–	–	–	–	F
C	TCi197	+	–	–	–	–	–	–	F
I	TCi207	+	–	–	–	–	–	–	F
N	TCi211	+	–	–	–	–	–	–	F
	TCi215	+	–	–	–	–	–	–	F
	TCi 216	+	–	–	–	–	–	–	F
	TCi217	+	–	–	–	–	–	–	F
	TCi219	+	–	–	–	–	–	–	F
	TCi222	+	–	–	–	–	–	–	F
	TCi224	–	+	–	–	–	–	–	I

Khadgi et al., 2013). In this study the number and size of the clinical plasmids varied with predominance of a ~12 kb band. Incidence of two or more plasmid borne  $\beta$ -lactamase (*bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub>) and either one or two PMQR (*aac-1b-cr*, *oqxAB*, *qnrB*, *qnrS*) genes was observed. The resistant genes were successfully transmitted by conjugation in an environment irrespective of ceftazidime/ciprofloxacin selection. Successive co-existence and co-transmission (Dhawde et al., 2018) of these resistant determinants were previously reported that poses difficulty in health care management. Random administration of the respective group of drugs facilitates mobilization of plasmids that carry resistance genes by horizontal gene transfer (HGT) through natural mechanisms such as conjugation or

transformation (Davies and Davies, 2010). Additionally resistant plasmids confer positively selectable phenotypes with the presence of antimicrobial resistance genes. Other essential regions of plasmid include their replication origin involved in replication and genes that are involved in the formation of *trans*-envelope machinery and pili structure that includes Type IV secretion system (T4SS), a large macromolecular machinery which control conjugation and dissemination of antibiotic resistance (Fillox, 2010; Cabezón et al., 2015). T4SSs are implicated not only in bacterial conjugation but also in the secretion of virulence factors to the host. IncF, IncP, and IncI conjugation systems were reported in *E. coli* that were further related to plasmid incompatibility groups

(Hazes and Frost, 2008) which were ascertained to understand resistance plasmid epidemiology, as well as strain epidemiology (Edwards and Holt, 2013). Additionally the T4SSs encoded in plasmids with IncI conjugation system have a number of physical and functional features that distinctly distinguish them from the IncF and IncP systems in the *Enterobacteriaceae* strains (Hazes and Frost, 2008).

In this study predominance of multiple replicon types were observed among individual plasmid screened from the transconjugants irrespective of ceftazidime/ciprofloxacin selection although the number increased in the latter. Acquisition of resistant plasmid with multiple replicon types were reported (Huang et al., 2012) that may be attributed to frequent recombination events under antibiotic selection which creates difficulty in typing the resistant plasmids and their mode of dissemination. The random recombination events may also result in re-shuffling of resistant genes under antibiotic pressure during selection of transconjugants. However, conjugative-plasmid transfer in the gram-negative bacteria was observed to be related to contacts created by the conjugative pilus machinery which was constitutively expressed and independent of antibiotic selection (Lopatkin et al., 2016). Pili and pili associated gene types assisting in conjugal transfer of resistant plasmids were thought to be universally present amongst these plasmids that disseminated by IncF, IncP, and IncI conjugation system, respectively. A variety of mechanisms and structures involved in the transport of DNA amongst bacteria was dependent on the assembly of a pilus, be it an F, P, I, or T pilus, which brings cells together and provides an interface to exchange macromolecules directly from cell to cell (Hazes and Frost, 2008; Shintani et al., 2015).

Nucleotide sequences of genes encoding pili and pili associated genes identified from annotated plasmids R100, R64, R46, R388, R27, RA1, R6K assigned to IncF, IncI, IncN, IncW, IncH, IncA/C, and IncX plasmid replicon types, respectively (Burmølle et al., 2012; Carattoli et al., 2014) revealed patterns of association that corresponds to those of the constituent single family replicons like IncF types (IncFrep, IncF1B, IncF11S), IncI, IncN, IncW, IncH, IncA/C, IncX associated with *traF* (Arutyunov et al., 2010), *pilM* (Kim and Komano, 1997), *traE* (Dolejska et al., 2013), *trwJ* (Liosa et al., 1991; Yeo et al., 2003; Revilla et al., 2008), *trhA* (Rooker et al., 1999; Sherburne et al., 2000), *traG* (Fricke et al., 2009), and *pilX4* (Burmølle et al., 2012) genes related to the conjugation system types respectively. In this study absence of *traE*, *trwJ*, *trhA*, *traG*, *pilX4* and presence of *traF* and *pilM* types among the resistant transmissible plasmids selected individually against ceftazidime and ciprofloxacin from all 19 transconjugants indicated that either IncF or IncI conjugation system was involved in the dissemination of the resistant determinants respectively. Conjugation system followed during the transfer of individual plasmid with multiple replicon type to the recipient strain was identical (F-type) in 13 out of 19 of the donor strains irrespective of the drug selection pressure. Moreover variation in the type of conjugation system (IncF/IncI) with respect to drug selection was observed in six clinical donors during conjugal transfer of genetic material to the recipient strain. Hence our study indicated that plasmid isolated from MDR uropathogenic *E. coli* obtained from hospitalized patients from the eastern part of India showed successful

transmissibility by IncF and IncI conjugation system, the former being predominant. As bacteria have developed sophisticated ways to select, attach and infect their target cells, so adhesive and secretion pili serves as key protagonists during these events (Craig et al., 2004; Burrows, 2005; Hazes and Frost, 2008). Therefore pili and pili associated genes in transmissible resistance gene-carrying plasmids irrespective of their incompatibility types drives the success of recipient strains toward dissemination of drug resistance. Furthermore the results of this study also indicated that the type of conjugation system was independent of the associated resistance genes and the drugs used for selection that implied acquisition of resistance genes to be random, however, their dissemination was dependent upon the pili type related to T4SS of the transmissible plasmid that harbored the resistant determinants. Therefore the strength of this study relies on introduction of a novel technique of bio-typing plasmids of multiple replicon types in uropathogenic *E. coli* isolated from clinical settings. However, there were limitations as this study was conducted on a small pool of uropathogenic *E. coli* isolates. Moreover the conjugation system of plasmids without Inc group (non-typeable) could not be addressed by this technique due to their absence in the limited pool of isolates investigated.

## CONCLUSION

In summary, this is the first study of its kind which addressed novel technique of bio-typing plasmids of multiple replicon types in uropathogenic *E. coli* based on pili and pili associated genes. These plasmids were multidrug resistant and transmissible by conjugation, although the dissemination was independent of the associated resistance genes.

## ETHICS STATEMENT

Ethical approval was obtained from the Institutional Ethical Committee of the Calcutta School of Tropical Medicine (Kolkata, India) constituted under Order No. 1006 dated May 23, 2009. According to the standard guidelines of the ethical committee, the participants were duly informed and a written consent was obtained from them.

## AUTHOR CONTRIBUTIONS

MM and SM conceived and designed the experiments, wrote the manuscript, analyzed the data, and read and approved the final manuscript. SM performed the experiments.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02913/full#supplementary-material>

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# One Health Approach Reveals the Absence of Methicillin-Resistant *Staphylococcus aureus* in Autochthonous Cattle and Their Environments

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Antimicrobial resistance represents one of the greatest challenges of the twenty-first century, and it is globally recognized that addressing this problem requires a concerted One Health approach involving humans, animals, and the environment. Methicillin-resistant *Staphylococcus aureus* (MRSA) currently represents a global burden; it is resistant to almost all beta-lactams and some MRSA strains are highly multiresistant. *S. aureus* infection in cattle results in major economic losses in the food industry. Moreover, cases of livestock-associated MRSA strains responsible for invasive life-threatening infections have been reported among human patients in contact with infected or colonized animals. The autochthonous Maronesa cattle breed is a threatened rustic traditional Portuguese breed of mountain cattle of high importance for the Vila Real region. It has been used for centuries as motive power in all kinds of agricultural work and also for meat production, which is its current dominant use and the main source of economic value, being the Maronesa meat commercialized with PDO - Protected Designation of Origin. This study aimed to determine the prevalence and transmission of MRSA in cattle of the Maronesa breed, through a concerted One Health approach comprising human, water, and soil samples of the animals' handlers and environments. In a total of 195, 63, 40, and 43 cattle, human, water, and soil samples screened in selective ORSAB media supplemented with 2 mg/L oxacillin; only one human sample harbored a MRSA isolate which was ascribed to *spa*-type t9413 and to ST30, one of the most common genetic lineages associated with community-acquired MRSA. Considering the increasing reports of MRSA isolation from cattle and handlers in Europe, the absence of this major human and animal

pathogen in Maronesa cattle and their production systems represents a serendipitous result, valuing this important autochthonous breed. To our knowledge, this is the first study to determine MRSA prevalence and transmission in Maronesa cattle. Through a concerted One Health approach, this study revealed that the Maronesa cattle and their surrounding environments do not represent reservoirs for Methicillin-resistant *Staphylococcus aureus*.

**Keywords:** antimicrobial resistance, One Health, methicillin-resistant *Staphylococcus aureus*, MRSA, autochthonous Maronesa cattle

## INTRODUCTION

Antimicrobial resistance (AMR) is one of the greatest challenges of the twenty-first century (Tacconelli and Pezzani, 2019). According to the World Health Organization (WHO), addressing the rising threat of AMR requires a holistic and multisectoral One Health approach involving humans, animals, and the environment since resistant bacteria may spread from one to the other, without recognizing human-animal or geographic borders. *Staphylococcus aureus* is a robust and versatile opportunistic pathogen that can survive in a diversity of environments (Sergelidis and Angelidis, 2017). It is frequently present in the natural flora of the nose and skin of both humans and animals, being also isolated from foods, food production systems and the environment, causing a range of illnesses from minor skin infections and food poisoning to life-threatening diseases (Cuny et al., 2015; Dweba et al., 2018; Gajdacs, 2019). Methicillin-resistant *Staphylococcus aureus* (MRSA) is considered the first representative of multidrug-resistant bacteria and currently represents a global burden since it is resistant to almost all beta-lactams and can also show resistance to other major antimicrobial classes such as the fluoroquinolones (Gajdacs, 2019). After being well established in the healthcare setting, MRSA has emerged in the community and subsequently in animals and food of animal origin, revealing new reservoirs for MRSA (Aires-de-Sousa, 2017; Boswihi and Udo, 2018). *S. aureus* is currently a leading cause of infection of livestock such as cows, resulting in major economic losses in the food industry (Spoor et al., 2013; Aires-de-Sousa, 2017; Boswihi and Udo, 2018). Cases of livestock-associated MRSA (LA-MRSA) strains have also been reported among human patients in contact with infected or colonized animals, which is the major risk factor for LA-MRSA colonization, being the causing agent of invasive infections in humans such as endocarditis, osteomyelitis, and ventilator-associated pneumonia (Hetem et al., 2013; Goerge et al., 2017; Boswihi and Udo, 2018). The autochthonous Maronesa cattle breed is a threatened rustic traditional Portuguese breed of mountain cattle of high importance for the Vila Real region that has been used for centuries as the motive power par excellence in all kinds of agricultural work (García, 2000). In parallel, this breed has always been used for meat production, which is its current dominant use and the main source of economic value, being the Maronesa meat commercialized with PDO - Protected Designation of Origin (García, 2000; DGAV, 2013). This study aimed to determine the prevalence and

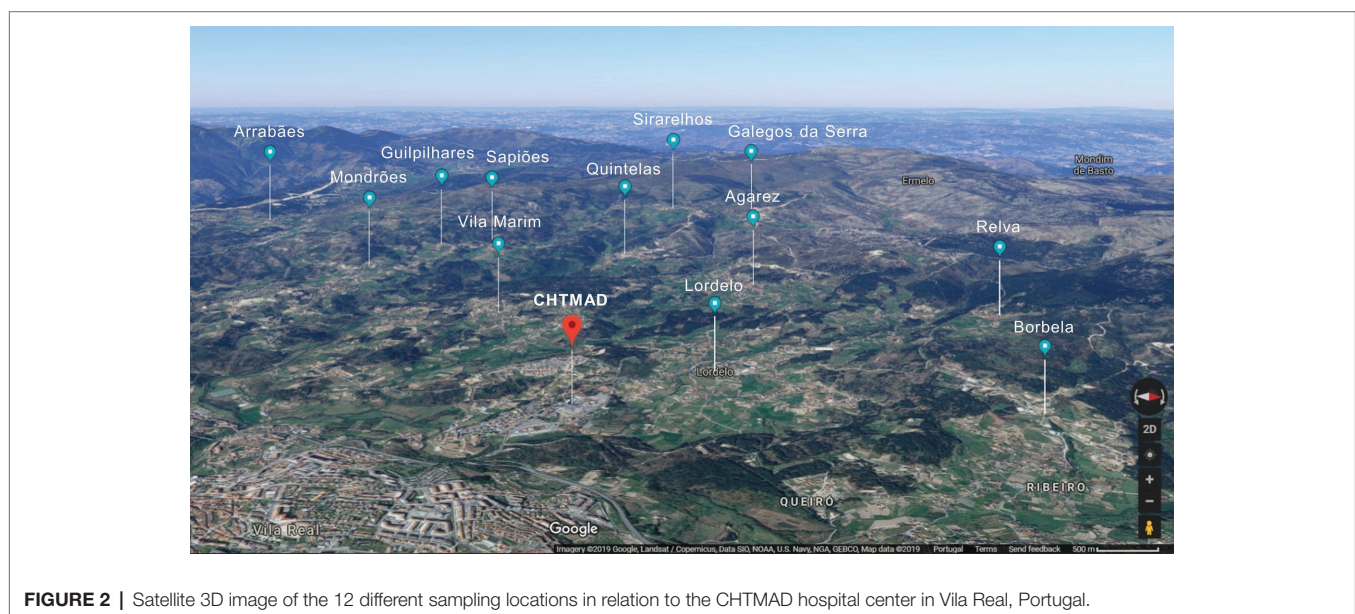
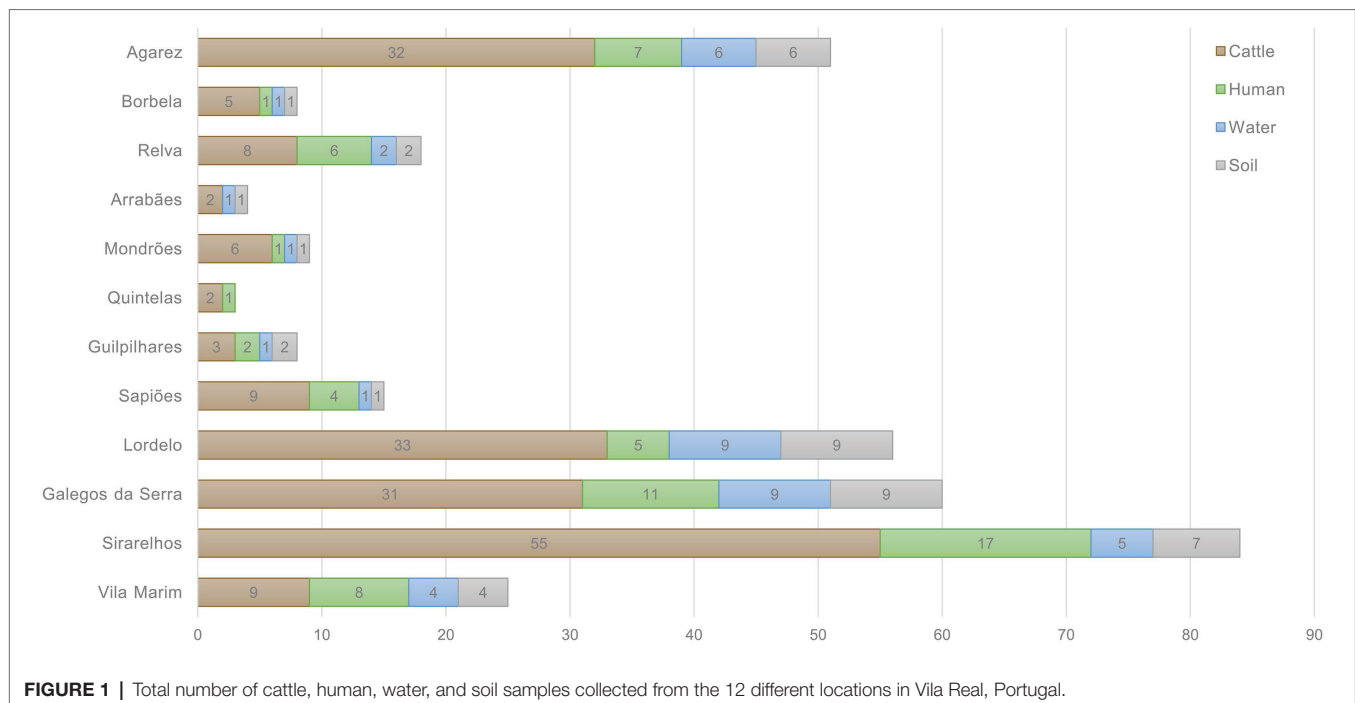
transmission of MRSA in cattle of the autochthonous Maronesa breed, through a concerted One Health approach comprising human, water, and soil samples of the animals' handlers and environments. From February to April 2019, a total of 195 and 63 mouth and nose swabs were collected from healthy cows and their handlers, respectively. Additionally, 40 samples were collected from the cattle's drinking water and 43 soil samples were obtained from the animals' surroundings. Sampling was performed in 12 different locations in a radius of approximately 6 km from the CHTMAD hospital center in Vila Real, Portugal (Figures 1, 2). Mouth and nose swabs were recovered into Stuart transport media; water samples were collected into 500 ml PET flasks with sodium thiosulphate (60 mg/L); and soil was gathered into zipper seal sample bags. Samples were processed on the same day or stored at 4°C for a maximum of 24 h. Mouth and nose swabs and soil samples were incubated into Brain Heart Infusion broth containing 6.5% (w/v) NaCl for 48 h at 37°C and after seeded on Oxacillin Resistance Screening Agar Base (ORSAB) supplemented with 2 mg/L oxacillin and 50,000 IU/L of Polymyxin B. Water samples were filtrated through 47 mm 0.2 µm filters that were further placed on ORSAB plates with 2 mg/L oxacillin and 50,000 IU/L of polymyxin B. All plates were incubated for 24–48 h at 37°C and after screened for presumptive MRSA colonies. Quality of sampling was assured by observed growth of non-MRSA colonies on ORSAB and simultaneously cultured Mannitol Salt Agar plates. Confirmation of presumptive MRSA isolates was carried out by multiplex PCR of the 16S rDNA, *nuc* and *mecA* genes. Characterization by spa-typing and multilocus sequence typing (MLST) was performed using specific primers and compared with the Ridom SpaServer<sup>1</sup> and MLST<sup>2</sup> databases, respectively (Silva et al., 2019).

## DISCUSSION

MRSA strains have been shown to colonize and infect a wide range of species such as livestock, companion animals, and wildlife; cases with particularly significant economic impact include bovine mastitis, poultry lameness, and severe and lethal infections in farmed rabbits (Bardiau et al., 2013; Paterson et al., 2014; Aires-de-Sousa, 2017; Gopal and Divya, 2017).

<sup>1</sup><http://spaserver.ridom.de>

<sup>2</sup><http://saureus.mlst.net>



Besides the importance from an animal welfare and economic perspective, MRSA in animals can also act as a reservoir for zoonotic infection of humans (Bardiau et al., 2013; Paterson et al., 2014; Aires-de-Sousa, 2017). Particularly, the abundance of clonal complex (CC)398 among livestock (mostly in mainland Europe), and the infection of humans which are often in close contact with these animals, such as farmers or veterinarians, has led to the recognition of LA-MRSA as a new MRSA epidemiological form (Bardiau et al., 2013; Paterson et al., 2014; Aires-de-Sousa, 2017). Some MRSA lineages described in cattle including CC130, CC1943, and ST425 are

thought to be bovine-specific; however, certain animal lineages have been shown by molecular typing to be able to colonize or infect a wide variety of animals and also humans (Bardiau et al., 2013; Aires-de-Sousa, 2017). Moreover, the *mecC* gene, a new *mecA* gene homolog conferring methicillin resistance in *S. aureus*, has been described among bovine and human isolates and *mecC* MRSA strains reported to date are referred to mostly belong to common cattle lineages suggesting a zoonotic reservoir (Paterson et al., 2014; Aires-de-Sousa, 2017). Reports of *mecC* MRSA are presently uncommon and have been restricted to Europe; however, *mecC* MRSA represent



a potential diagnostic problem due to the reliance on *mecA* or PBP2a/2' detection for MRSA diagnosis (Paterson et al., 2014). Hence, livestock and livestock production systems have been reported to act as potential reservoirs for the emergence of new MRSA clones with the capacity to cross the species barrier and endure host-adaptive evolution, showing potential to become established in human populations worldwide as successful epidemic lineages (Spoor et al., 2013; Dweba et al., 2018). However, among the 195 cattle, 63 human and 83 environmental samples recovered from the 12 different farms included in this study and only one human sample was positive for presumptive MRSA colonies. This MRSA isolate harbored the *nuc* and *mecA* genes, confirming the *Staphylococcus* species and methicillin resistance and was ascribed to ST30 and *spa*-type t9413. ST30 is one of the most common genetic lineages associated with community-acquired MRSA (Ramundo et al., 2016) and ST30 associated with methicillin-susceptible *S. aureus* (MSSA) was one of the most prevalent clones circulating in the hospital and community in Portugal between 1992 and 2011 (Conceição et al., 2017). As far as we know, the ST30-MRSA clone has only been found among livestock animals in Portugal in healthy pigs (Pomba et al., 2009) and the presence of *spa*-type t9413 was only previously reported associated with ST22 in a comparative genomic analysis performed on food-borne *S. aureus* CC30 strains from Russia (Abaev et al., 2017). Hence, the MRSA strain detected is common among human isolates which alienates the possibility of being transmitted from cattle, reinforcing the safety of the Maronesa breed from a zoonotic point of view. Moreover, considering the increasing reports of MRSA isolation from cattle and handlers in Europe (Paterson et al., 2014; Aires-de-Sousa, 2017; Goerge et al., 2017), the absence of this major human and animal pathogen in Maronesa cattle and their production systems represents a serendipitous result, valuing this important autochthonous breed. Although the closeness to the main hospital center of the region (which according to the 2018 activity report has a MRSA prevalence of about 30%, with nearly 50% MRSA in the total *S. aureus* isolates recovered), samples were collected in higher mountain rural areas from extensive production systems that mainly use natural resources (García, 2000) and do not routinely use antimicrobials in subtherapeutic doses. This would result in lower levels of antibiotic pressure selecting for MRSA. Cattle management in the study area is characterized by farms with low head number, usually operated by both women and men, with over 55 years old on average, that work together, indicating family-type management with low economic profit. Veterinary management is scarce due to lack of literacy and low profit thus neither prophylactic treatments (e.g., deworming or vaccination schedules) nor biosecurity measures are usually implemented. Contact with the veterinarian is scarce and usually associated with the compulsory surveillance of bovine tuberculosis and brucellosis. In the case of sick cattle, assistance of the veterinarian occurs only when requested by the farmer. Antimicrobials such as procaine benzylpenicillin plus dihydrostreptomycin, ceftiofur, florfenicol and tulathromycin are administered to Maronesa cattle in cases of bovine respiratory

disease (BRD), the most common disease affecting this breed. However, a lower prevalence of BRD is observed when compared to other fattening cattle breeds such as crossbreed, Charolais or Limousine since the Maronesa breed is well adapted to the local environment (extensive grazing at the mountain) (Diez et al., 2015). To our knowledge, this is the first study to determine the prevalence and transmission of MRSA in Maronesa cattle. Through a concerted One Health approach, this study revealed that the Maronesa cattle and their surrounding environments do not represent reservoirs for methicillin-resistant *Staphylococcus aureus*. Nonetheless, it would still be interesting to study the prevalence and transmission of different lineages of methicillin-susceptible *S. aureus* which can possibly evolve to MRSA and also carry resistance determinants for other major antimicrobial drug classes. Moreover, it would also be interesting to extend the study to other bacterial species that also represent major AMR threats such as carbapenem-resistant *Enterobacteriaceae* (CRE), which have a significant prevalence in the main hospital center of the region and have been increasingly detected from environmental, food, and animal sources, including cattle (European Centre for Disease Prevention and Control, 2018). It would also be noteworthy to develop similar studies in other different autochthonous breeds from extensive production systems in order to observe if similar results occur, which would give valuable insights that could lead to the implementation of new practices and policies to tackle AMR. In our perspective, many studies may have been performed with similar results that are kept undivulged due to the absence of antimicrobial-resistant strains detected and, from our point of view, the current publication principles, and incentives should encourage more the divulgation of such studies.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## ETHICS STATEMENT

This work was approved Institutionally by University of Trás-os-Montes and Alto Douro Board and written informed consent was obtained from all subjects.

## AUTHOR CONTRIBUTIONS

SC, JG-D, KP, VS, and SO carried out the sample collection. SC, VS, KP, and SO developed the microbiology work. PT and JG-D were responsible for the collaboration that allowed sample recovery and provided information regarding the Maronesa cattle. VS performed MRSA confirmation experiments. CM and JR carried out the MLSA and *spa*-type analyses. SC wrote the manuscript. JP, GI, and PP provided critical feedback in shaping the research and manuscript.

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# Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in Patients Admitted to Kuwait Hospitals in 2016–2017

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Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) has been reported to colonize and cause infections in animals as well as in humans. LA-MRSA isolates have only recently been identified in patients admitted to Kuwait hospitals. This study was conducted to characterize LA-MRSA isolates obtained from patients admitted to Kuwait hospitals. A total of 202 (7.1%) of 2,823 MRSA isolates obtained from clinical samples in 2016 and 2017 in 11 public Kuwait hospitals were assigned to lineages previously known to be associated with livestock. They were characterized using antibiogram, *spa* typing, and DNA microarray for the assignment of clonal complexes (CCs) and detection of antibiotic resistance and virulence determinants. Identification as putative LA-MRSA clones was based on the molecular definition inferred from DNA microarray. The LA-MRSA isolates consisted of CC96 ( $N = 31$ ), CC97 ( $N = 169$ ), and CC398 ( $N = 2$ ). Isolates belonging to CC96 and CC398 were resistant to erythromycin and clindamycin mediated by *erm(A)* and *erm(C)*. CC97 isolates were multiresistant to gentamicin, kanamycin, erythromycin, clindamycin, tetracycline, chloramphenicol, fusidic acid, trimethoprim, and ciprofloxacin and harbored *aacA-aphD*, *erm(A)*, *erm(C)*, *msr(A)*, *tet(K)*, *cat*, *fusC*, and *dfcS1*. In total, 35 *spa* types were identified among the isolates. CC398 isolates consisted of t899 and t034. Ten *spa* types were identified among CC96 with t11822 ( $N = 13$ ) as the most prevalent. CC97 consisted of 26 *spa* types with most belonging to t267 ( $N = 73$ ) followed by t359 ( $N = 39$ ). CC398 was composed of CC398-MRSA-IV and CC398-MRSA-V (PVL<sup>+</sup>). CC96 belonged to CC96-MRSA-IV and CC96-MRSA-IV (PVL<sup>+</sup>) Central Asian caMRSA/WA MRSA-119. CC97 consisted of six strains including CC97-MRSA-V (*fusC*<sup>+</sup>), CC97-MRSA-IV WA MRSA-54/63, CC97-MRSA-V, CC97-MRSA-(V+*fus*), CC97-MRSA-(*mec* VI+*fus*), and CC97-MRSA (*mecV/V<sub>T</sub>*+*fus*+*ccrAB2*). Whereas CC96 and CC97 isolates were identified in 2016 and 2017, CC398 isolates were detected only in 2016. This study identified four LA-MRSA clones among MRSA isolated from patients in Kuwait hospitals in 2016–2017 with CC97-MRSA-V (*fusC*<sup>+</sup>) as the dominant clone. The presence of LA-MRSA with different genetic backgrounds suggests its independent acquisition from different sources.

**Keywords:** livestock-associated methicillin-resistant *Staphylococcus aureus*, molecular typing, DNA microarray, antibiotic resistance genes, virulence factors

## INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an opportunistic pathogen that colonizes humans as well as animals (Kluytmans et al., 1997). In humans, MRSA causes a wide range of infections including skin and soft tissue infections (SSTIs), and invasive infections such as pneumonia and endocarditis (Gordon and Lowy, 2008). Since it was first reported in the United Kingdom (Jevons, 1961), MRSA has evolved into three types, including health-care-associated MRSA (HA-MRSA), which was isolated from patients in health-care settings (Campanile et al., 2010); community-acquired MRSA (CA-MRSA), which was initially isolated from healthy individuals with no previous exposure to health-care facilities (Udo et al., 1993); and the new strains that were associated with livestock in the early 2000s (Armand-Lefevre et al., 2005; Voss et al., 2005), which were designated as livestock-associated MRSA (LA-MRSA). Initially, livestock-associated *S. aureus* isolates causes major problems in agriculture and are the leading cause of bovine mastitis (Fluit, 2012). In addition, MRSA isolates associated with livestock (called LA-MRSA) belonging to specific lineages such as ST398 have been reported to cause infections in animals and animal handlers (Armand-Lefevre et al., 2005; Voss et al., 2005). In recent years, these LA-MRSA lineages were able to break the species barrier to colonize and cause infections in humans with or without contact with livestock (Fitzgerald, 2012; Hetem et al., 2013).

Several molecular typing methods including staphylococcal protein A (*spa*) typing, multilocus sequence typing (MLST), pulsed-field gel electrophoresis, staphylococcal cassette chromosome *mec* (SCC*mec*) typing, DNA microarray, and whole genome sequencing (WGS) have been used to characterize and identify *S. aureus* lineages including LA-MRSA. Several LA-MRSA lineages have been identified including CC398 and CC9, which are predominant in Europe and Asia, respectively (Lo et al., 2012; Chuanga and Huang, 2015). Other clones associated with livestock include ST72, ST97, ST5, ST1, and ST433 (Lo et al., 2012; Chuanga and Huang, 2015).

The SCC*mec* genetic element is a mobile genetic element that confers methicillin resistance and resistance to other beta-lactam antibiotics to susceptible strains following its acquisition. The SCC*mec* element is variable in structural organization and the carriage of additional genetic structures such as transposons and insertion sequence elements. The high diversity in its structural organization and composition has formed the basis of SCC*mec* typing of MRSA strains (Hiramatsu et al., 2013). HA-MRSA isolates carry SCC*mec* types I, II, and III; CA-MRSA isolates carry SCC*mec* types IV, V, and VI; and LA-MRSA can carry any of the SCC*mec* types associated with CA-MRSA or HA-MRSA. For example, CC9 isolated from pigs in Asia were reported to harbor SCC*mec* III, SCC*mec* V, or SCC*mec* IX element (Cui et al., 2009; Neela et al., 2009). Similarly, CC398 isolates have been reported to carry different SCC*mec* types including SCC*mec* IV, SCC*mec* V, and SCC*mec* IX (van Loo et al., 2007).

*Staphylococcus aureus* is endowed with multiple virulence factors, such as toxins, enzymes, hemolysins, and leukocidins including Pantone–Valentine leukocidin (PVL), that enhance the

capacity of the bacterium to cause disease in humans. Genomic studies have revealed that LA-MRSA clones such as CC398 lack or rarely carry specific virulence factors including PVL and toxic shock syndrome toxin (TSST), which are considered major contributors in *S. aureus* infections (Jamrozny et al., 2012; Price et al., 2012). PVL is a pore-forming cytotoxin that plays a major role in *S. aureus* infections by targeting leukocytes (Maltezou and Giamarellou, 2006).

Although HA-MRSA and CA-MRSA have been widely reported in patients attending Kuwait hospitals (Boswihi et al., 2016), LA-MRSA has only recently been detected among MRSA isolates obtained from patients admitted to Kuwait hospitals. This paper reports the molecular characterization of LA-MRSA isolates obtained from patients in Kuwait hospitals in 2016–2017. LA-MRSA isolates selected for this study were identified based on their clonal complex (CC), which was determined by DNA microarray.

## MATERIALS AND METHODS

### Ethical Approval

The study did not require ethical approval, because all the MRSA isolates were obtained as part of routine diagnostic microbiology investigations.

### Methicillin-Resistant *Staphylococcus aureus* Strains

A total of 4726 MRSA isolates were obtained from clinical samples in 2016 ( $N = 2305$ ) and 2017 ( $N = 2421$ ) in 11 public Kuwait hospitals. MRSA isolates were obtained from clinical samples submitted to the clinical microbiology diagnostic laboratory in the 11 hospitals. The isolates were identified using biochemical tests and tube coagulase at the diagnostic microbiology laboratory. Once it was identified as MRSA in the diagnostic laboratories, the isolates were sent to MRSA Reference Laboratory located in the Department of Microbiology, Faculty of Medicine, Kuwait University, for molecular typing. The information accompanying the submitted MRSA isolates was sample ID, date of isolation, patient location, patient ID, and clinical source. The isolates were subcultured twice on brain–heart infusion agar (BHIA) plates to obtain pure colonies and incubated at 35°C for 18 h. Pure cultures were preserved in beads and stored at  $-20$  and  $-80^{\circ}\text{C}$ . They were recovered on BHIA and incubated at 35°C prior to testing.

### Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed using the disc diffusion method according to the Clinical Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standard Institute, 2015). The susceptibility testing was performed with 13 antibiotics including benzyl penicillin (10 U), cefoxitin (30  $\mu\text{g}$ ), kanamycin (30  $\mu\text{g}$ ), mupirocin (200 and 5  $\mu\text{g}$ ), gentamicin (10  $\mu\text{g}$ ), erythromycin (15  $\mu\text{g}$ ), clindamycin (2  $\mu\text{g}$ ), chloramphenicol (30  $\mu\text{g}$ ), tetracycline (10  $\mu\text{g}$ ), trimethoprim (2.5  $\mu\text{g}$ ), fusidic acid (10  $\mu\text{g}$ ), rifampicin (5  $\mu\text{g}$ ), and ciprofloxacin



(5 µg). Minimum inhibitory concentration (MIC) for cefoxitin, vancomycin, and teicoplanin were determined with Etest strips (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. *S. aureus* strains ATCC25923 and ATCC29213 were used as quality control strains for the disc diffusion and MIC determination, respectively. The *D*-test was used to test for inducible resistance to clindamycin. Susceptibility to fusidic acid was interpreted according to the British Society to Antimicrobial Chemotherapy (BSAC) (British Society to Antimicrobial Chemotherapy [BSAC], 2013).

## DNA Isolation for Amplification

Three to five identical colonies of an overnight culture were picked using a sterile loop and suspended in a microfuge tube containing 50 µl of lysostaphin (150 µg/ml) and 10 µl of RNase (10 µg/ml) solution. The tube was incubated at 37°C in the heating block (ThermoMixer, Eppendorf, Hamburg, Germany) for 20 min. To each sample, 50 µl of proteinase K (20 mg/ml) and 150 µl of Tris buffer (0.1 M) were added and mixed by pipetting. The tube was then incubated at 60°C in the water bath (VWR Scientific Co., Shellware Lab, United States) for 10 min. The tube was transferred to a heating block at 95°C for 10 min in order to inactivate proteinase K activity. Finally, the tube was centrifuged, and the extracted DNA was stored at 4°C till used for PCR.

## Spa Typing

Amplification of *spa* gene was performed using synthetic primers published previously (Harmsen et al., 2003) in a total volume of 25 µl. The PCR protocol consisted of an initial denaturation at 94°C for 4 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension for 3 min at 72°C, and a final cycle with a single extension for 5 min at 72°C. Five microliters of the PCR product was analyzed by 1.5% agarose gel electrophoresis to confirm amplification. The PCR product was purified using MicroElute Cycle-Pure Spin kit (Omega Bio-Tek Inc., United States) according to the manufacturer's protocol. The purified DNA was used for sequencing PCR. A total of 10 µl of the sequencing reaction mixture containing 2 µl of big dye terminator mix, 2 µl of 5× sequencing buffer, 3 µl of nuclease-free water, 1 µl of 3.2 pM primer (forward and reverse), and 2 µl of purified DNA were prepared. The sequencing PCR protocol consisted of initial denaturation for 1 min at 94°C, followed by 25 cycles of denaturation for 10 s at 96°C, annealing at 55°C for 5 s, and extension for 4 min at 66°C. Ultra-Sep Dye Terminator Removal kit (Omega Bio-Tek Inc., United States) was used to purify DNA. Purified DNA was sequenced in an automated 3130 × 1 genetic analyzer (Applied Biosystems, United States) in accordance with the manufacturer's protocol. The sequence of *spa* gene was analyzed using the Ridom Staph Type software (Ridom GmbH, Wurzburg, Germany). The software detected the *spa* repeats and assigned the *spa* type for each isolate.

## DNA Microarray

The *S. aureus* Genotyping kit 2.0 (Alere GmbH, Germany) was used for clonal assignment and the detection of genes encoding

antibiotic resistance and virulence factors for MRSA isolates representing each *spa* type identified using the protocol provided by the manufacturer (Monecke et al., 2008).

## RESULTS

### Detection of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates

A total of 4,726 MRSA isolates collected from patients in 11 hospitals in 2016–2017 were investigated by *spa* typing. From this, 2,823 isolates selected on the basis of *spa* types were analyzed by DNA microarray to assign the isolates into CCs. The 2,823 isolates included isolates from all clinical samples in all hospitals with the same *spa* types. The DNA microarray results identified 202 (7.1%) of the 2,823 isolates as LA-MRSA isolates. The isolates were defined as LA-MRSA solely on the basis of molecular rather than epidemiological definition. The LA-MRSA isolates that belonged to CC96 (31 isolates), CC97 (169 isolates), and CC398 (2 isolates) were identified as LA-MRSA. The CC96 isolates were obtained in 2016 (*N* = 21) and 2017 (*N* = 10). Eighty-three and 86 CC97 isolates were isolated in 2016 and 2017, respectively. CC398 isolates were only detected in 2016. Of the non-LA-MRSA isolates, the dominant CCs were CC5 (*N* = 796), CC22 (*N* = 397), CC8 (*N* = 304), CC1 (*N* = 239), CC6 (*N* = 223), CC30 (*N* = 179), CC80 (*N* = 178), and CC88 (*N* = 88). This report focuses on the characteristics of the LA-MRSA isolates.

The characteristics of the LA-MRSA are summarized in **Table 1**. The results for each isolate is presented in the **Supplementary Table S1**. The LA-MRSA isolates were obtained from clinical samples of patients treated in Kuwait hospitals. The samples were collected from 137 inpatients and 50 outpatients. The locations of 17 patients were not specified. The isolates were obtained from nasal swab (47; 23.2%), skin and soft tissue (47; 23.2%), groin (13; 6.4%), high vaginal swab (HVS) (13; 6.4%), blood (12; 5.9%), sputum (12; 5.9%), throat (10; 4.9%), eye (5; 2.4%), trachea (4; 1.9%), urine (4; 1.9%), axilla (2; 0.9%), ear (2; 0.9%), and fluid (1; 0.5%). The source of 30 (14.8%) isolates was unspecified.

The CC398 belonged to two genotypes including PVL-negative CC398-MRSA-IV/t899 and PVL-positive CC398-MRSA-V/t034.

Thirty CC96 isolates belonged to CC96-MRSA-IV, and one isolate belonged to CC96-MRSA-IV (PVL<sup>+</sup>), also known as the Central Asian caMRSA/WA MRSA-119.

Seven different subtypes were identified among the CC97 isolates. These included CC97-MRSA-V (*fusC*<sup>+</sup>) (139 isolates), CC97-MRSA-V (14 isolates), CC97-MRSA-IV WA MRSA-54/63 (11 isolates), CC97-MRSA-(*mec* VI+*fus*) (2 isolates), CC97-MRSA-(*mec* V/V<sub>T</sub>+*fus*+*ccrAB2*) (1 isolate), CC97-MRSA-(V+*fus*) (1 isolate), and CC97-MSSA (1 isolate).

The LA-MRSA isolates belonged to 35 *spa* types. CC398 isolates consisted of two *spa* types, t899 and t034. Ten *spa* types were identified among CC96 isolates including t11822 (*N* = 13), t4955 (*N* = 4), t1028 (*N* = 2), t8154 (*N* = 2), t8731 (*N* = 2), t9867

( $N = 2$ ), t14838 ( $N = 2$ ), t1234 ( $N = 1$ ), t1198 ( $N = 1$ ), and t203 ( $N = 1$ ). *Spa* type was not determined (ND) for one isolate.

Twenty-six *spa* types were identified among CC97 isolates, with t267 ( $N = 67$ ), t359 ( $N = 39$ ), and t2297 ( $N = 12$ ) as the common *spa* types in this lineage. The other *spa* types were detected less frequently. *Spa* types t203, t1234, and t4955 were identified in both CC96 and CC97 isolates (Table 1).

## Antibiotic Susceptibility Testing and Antibiotic Resistance Genes

All LA-MRSA isolates were tested for susceptibility to antimicrobial agents. The results for the disk susceptibility testing and the genetic resistance determinants are summarized in Table 2. Antibiotic susceptibility testing showed that all LA-MRSA isolates were resistant to cefoxitin and were positive for *mecA*. A total of 187 (92.5%) isolates were resistant to penicillin mediated by *blaZ*. Sixteen CC97 isolates were phenotypically resistant to penicillin by disc diffusion method but lacked *blaZ* (Haveri et al., 2005).

Gentamicin resistance and kanamycin resistance were detected in 120 CC97 isolates, with only 118 isolates positive for *aacA-aphD*. One CC96 and two CC97 isolates were phenotypically resistant to gentamicin and kanamycin but

lacked any of the aminoglycoside resistance genes in the DNA microarray panel (Table 2).

Macrolide–lincosamide–streptogramin-B (MLS-B) resistance was detected in 20.7% of the LA-MRSA. All CC96 isolates resistant to erythromycin and clindamycin carried *erm(C)*. MLS-B resistance in the CC398 isolates was mediated by *erm(A)* and *erm(C)*. Four CC97 erythromycin- and clindamycin-resistant isolates carried *erm(C)*, three isolates were resistant only to erythromycin carried *msr(A)*, and five isolates phenotypically resistant to erythromycin and clindamycin lacked any of the MLS-B resistance genes in the microarray panels.

Fusidic acid resistance genes *fusB* and *fusC* were identified in CC96 and CC97 isolates. Two CC96 isolates were phenotypically resistant to fusidic acid, but only one isolate carried *fusB*. *fusB* was also detected in one isolate belonging to CC97, whereas 142 isolates carried *fusC* (Table 2).

Tetracycline resistance was detected in 30 CC97 isolates with 27 isolates carrying *tet(K)*. Tetracycline resistance gene, *tet(M)*, was found in one CC398 isolate (Table 2). Trimethoprim resistance gene *dfrS1* was detected in one CC398 isolate and in two of the four phenotypically resistant CC97 isolates. Chloramphenicol resistance mediated by *cat* was detected in a single CC97 isolate (Table 2).

*vga(A)*, which confers resistance to streptogramin A compound (not tested phenotypically in this study), was found

TABLE 1 | Genotypic characterization of LA-MRSA isolates.

Clonal complex (CC)	Specimen	LA-MRSA strain	Number of isolates	<i>Spa</i> type
CC398	Nasal	CC398-MRSA-IV	1	t899 (1)
	Swab	CC398-MRSA-V (PVL <sup>+</sup> )	1	t034 (1)
CC96	Nasal (11), sputum (1), throat (2), groin (2), wound (4), skin (6), blood (1), unspecified (3)	CC96-MRSA-IV	30	t1028 (2), t11822 (13), t1198 (1), t1234 (1), t14838 (2), t203 (1), t4955 (4), t8154 (2), t8731 (1), t9867 (2), ND (1)
	Pus	CC96-MRSA-IV (PVL <sup>+</sup> ), Central Asian caMRSA/WA MRSA-119	1	t8731 (1)
CC97	Groin	CC97-MRSA- ( <i>mec</i> V/V <sub>T</sub> + <i>fus</i> + <i>ccrAB2</i> )	1	t2297 (1)
	Unspecified	CC97-MRSA- ( <i>mec</i> VI+ <i>fus</i> )	2	t359 (2)
	Wound (4), nasal (2), pus (1), urine (1), swab (1), trachea (1), unspecified	CC97-MRSA-IV, WA MRSA-54/63	11	t267 (2), t359 (5), t521 (1), t1234 (1), t693 (1), ND (1)
	Wound (1), throat (1), HVS (2), groin (2), urine (1), nasal (2), ear (2), unspecified (3)	CC97-MRSA-V	14	t1234 (5), t203 (1), t267 (4), t359 (1), t4955 (1), t2297 (1), t693 (1)
	Unspecified (17), HVS (10), fluid (1), wound (17), nasal (31), skin (5), swab (2), groin (8), pus (8), blood (11), axilla (2), throat (7), eye (5), sputum (11), trachea (3), urine (2)	CC97-MRSA-V ( <i>fusC</i> <sup>+</sup> )	139	t1814 (2), t1965 (1), t267 (67), t2734 (1), t359 (30), t376 (2), t521 (4), t527 (1), t189 (4), t16486 (1), t2802 (1), t2297 (10), t044 (1), t15069 (1), t16606 (2), t17281 (1), t17282 (1), t17330 (1), t2770 (1), t701 (1), t9638 (1), ND (5)
	HVS	CC97-MSSA (1)	1	t16903 (1)

ND, not determined; LA-MRSA, livestock-associated methicillin-resistant *Staphylococcus aureus*; PVL, Pantone–Valentine leukocidin; HVS, high vaginal swab.

in three CC97 isolates and one isolate belonging to CC398. Fosfomycin was not tested in this study. However, two isolates belonging to CC97 carried *fosB*, which confers fosfomycin resistance (Table 2).

## Virulence Encoding Genes

DNA microarray demonstrated that all LA-MRSA isolates carried genes for virulence factors including genes for adhesions, accessory gene regulators (*agr*), capsular polysaccharides (*cap*), and enzymes but varied in their carriage of genes for exotoxins (Table 2). All isolates were positive for *agr* and *cap* but differed in the types of *agr* and *cap* alleles. CC398 and CC97 carried *agr* I and *cap* 5, whereas CC96 isolates carried *agr* III and *cap* 8.

Of the 204 LA-MRSA isolates, 80 (39.6%) isolates carried enterotoxins. Two CC97 isolates carried *sed*, *sek*, and *seq*, whereas CC96 isolates variably carried *sec*, *sea*, and *sel* (Table 2). No enterotoxins were detected among CC398 isolates (Table 2).

Gene for TSST-1, *tst*, was detected in one CC97 isolate. PVL was only found in two CC96 isolates.

All isolates carried microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). However, they varied in the carriage of collagen adhesion (*cna*). The *cna* gene was detected in CC96 and CC398 isolates but not in CC97 isolates (Table 2).

The immune evasion cluster (IEC) genes (*scn*, *chp*, and *sak*) were identified in all LA-MRSA isolates. CC398 carried *scn*, *chp*, and *sak* encoding genes (IEC type B). All CC97 and one CC96 isolates lacked *chp* and carried *scn* and *sak* (IEC type E).

## DISCUSSION

Livestock-associated methicillin-resistant *S. aureus* was initially isolated from livestock and later in isolates from humans who were in contact with livestock (Kinross et al., 2017). Subsequently, LA-MRSA isolated from individuals with no contact with livestock was reported in different places including Italy (Pan et al., 2009), Spain (Lozano et al., 2011), Australia (Monecke et al., 2011), and Saudi Arabia (Monecke et al., 2012). In this study, we characterized LA-MRSA obtained from human patients in Kuwait hospitals. The result of the study revealed that LA-MRSA constituted 7.1% of MRSA isolated from patients in hospitals in Kuwait in 2016–2017. The low prevalence of LA-MRSA reported in this study is similar to results reported in patients in Europe including the United Kingdom (Harrison et al., 2017); Luxembourg, Poland, and Norway (Kinross et al., 2017); and Asian countries such as China, Taiwan, Japan, and Malaysia (Chuang and Huang, 2015). Human colonization with LA-MRSA is more common in areas with high density of livestock (Cuny et al., 2013; Kinross et al., 2017). Nevertheless, colonization with LA-MRSA isolates was also reported in people with no contact with livestock. A study published in 2013 in the United States reported CC398 MSSA as the dominant strain among detainees in the Dallas County Jail (David et al., 2013), which showed the transmission of these isolates in the absence of an animal source.

The LA-MRSA isolates belonged to three CCs, including CC96, CC97, and CC398, in this study. CC97 was found in 169 isolates, making CC97 the dominant LA-MRSA clone in Kuwait hospitals in 2016–2017. Prior to this report, CC97 isolates were reported to cause an outbreak in a neonatal intensive care unit (ICU) in a Kuwait hospital in 2007 (Udo et al., 2011; Udo and Al-Sweih, 2017) and was detected in four isolates in another hospital in 2010 (Boswihi et al., 2016). The increase in the prevalence of CC97 in recent years in Kuwait may suggest an increased transmission among patients in hospitals. CC97 has also been sporadically reported in patients in Saudi Arabia (Monecke et al., 2012), Spain (Lozano et al., 2011; Reynaga et al., 2018), and Australia (Monecke et al., 2011). Although CC97 is rarely reported in humans (Grundmann et al., 2010; Schaumburg et al., 2012), it is commonly isolated from cattle, and it is considered one of the most common causes of bovine mastitis (Smith et al., 2005; Aires-de-Sousa et al., 2007; Sung et al., 2008) and one of the most common in the Italian pig industry (Battisti et al., 2010; Feltrin et al., 2016). The increase in the prevalence of CC97 among human patients observed in this study is of concern, as it highlights the increasing transmission of LA-MRSA among human patients. The CC97 isolates consisted of 26 *spa* types and six genotypes, revealing the diversity of the isolates. CC97-MRSA-V (fusC<sup>+</sup>)-t267 was the dominant strain carrying few enterotoxin genes (*sed*, *sek*, and *seq*) and bovine PVL (P83), which is similar to the bovine CC97 isolates reported in Italy (Feltrin et al., 2016). *Spa* type t267 was also reported in isolates obtained from bovine in Portugal (Conceição et al., 2017), Switzerland (Boss et al., 2016), and Brazil (Rabello et al., 2007). The other *spa* types associated with CC97 isolates in this study (t359, t521, t2297) have also been reported in patients as well as animals in other studies (Albrecht et al., 2015; Feltrin et al., 2016), making them successful zoonotic subtypes.

Few reports have described the virulence profiles of CC97 isolates. A study in South Africa (Schmidt et al., 2017) showed that CC97 isolated from bovine and humans carried few enterotoxins genes including *sec* and *sel*. Similarly, the CC97 isolates in this study harbored *sec* and *sel*, suggesting that *sec* and *sel* may be common features of CC97 MRSA isolates.

CC97 isolates in this study were multiresistant to antibiotics, including resistance to gentamicin, kanamycin, erythromycin, clindamycin, and tetracycline. Similarly, multiresistant strains of CC97 were isolated from human patients in Saudi Arabia (Monecke et al., 2012) and in bovine in Spain (Gómez-Sanz et al., 2010). In contrast, non-multiresistant isolates of CC97 were obtained from dairy milk in China (Wang et al., 2018). We observed differences between the antibiotic susceptibility patterns and the carriage of antibiotic resistance genes in CC97 isolates obtained in this study. Penicillin resistance was detected in 157 of the CC97 isolates, but only 141 carried *blaZ*, which could be due to a lack of signal to *blaZ*, *blaI*, and *blaR*. A similar observation was reported by Williamson et al. (2014) in two CC398-t034 isolates in which *blaZ* could not be detected by DNA microarray, although they were resistant to penicillin and the resistance was confirmed by the detection of penicillinase with nitrocefin. A similar pattern was also observed in isolates

**TABLE 2 |** Phenotypic and genotypic characteristics of LA-MRSA isolates.

Locus	CC96 (N = 31)	CC97 (N = 169)	CC398 (N = 2)	Total (N = 202)
<b>Virulence factors</b>				
<i>Sea</i>	30			30
<i>Sec</i>	20			22
<i>Sed</i>		2		2
<i>Sel</i>	22			22
<i>sek</i>		2		2
<i>seq</i>		2		2
<i>tst</i>		1		1
PVL	1		1	2
PVL (P83)		4		4
<i>chp-scn-sak</i> (Type B)	30		2	32
<i>scn-sak</i> (Type E)	1	196		197
<i>can</i>	31		2	33
<b>Antibiogram/antibiotic resistance genes</b>				
<b>Penicillin resistance</b>				
Penicillin (phenotypic)	28 (90.3%)	157 (92.8%)	1 (50%)	188 (92.1%)
<i>blaZ</i>	31	141	1	175
<b>Methicillin-resistance</b>				
Cefoxitin (phenotypic)	31 (100%)	169 (100%)	2 (100%)	204 (100%)
<i>mecA</i>	31	169	2	204
<b>MLS-B resistance</b>				
Erythromycin (phenotypic)	26 (83.8%)	12 (7.1%)	2 (100%)	42 (20.5%)
Clindamycin (phenotypic)	26 (83.8%)	7 (4.1%)	2 (100%)	35 (17.1%)
<i>erm(A)</i>			1	1
<i>erm(C)</i>	26	4	1	31
<i>msr(A)</i>		3		5
<b>Aminoglycoside resistance</b>				
Gentamicin (phenotypic)	1 (3.2%)	120 (71.0%)		121 (59.3%)
Kanamycin (phenotypic)	1 (3.2%)	119 (70.4%)		120 (58.8%)
<i>aacA-aphD</i>		118		118
<b>Trimethoprim resistance</b>				
Trimethoprim (phenotypic)		4 (2.3%)	1 (50%)	5 (2.4%)
<i>dfrS1</i>		2	1	3
<b>Fusidic resistance</b>				
Fusidic acid (phenotypic)	2 (6.4%)	138 (81.6%)		140 (68.6%)
<i>fusC</i>		142		142
<i>fusB</i>	1	1		2
<b>Tetracycline resistance</b>				
Tetracycline (phenotypic)		30 (17.7%)	2 (100%)	32 (15.6%)
<i>tet(K)</i>		27		27
<i>tet(M)</i>			1	1
<b>Chloramphenicol resistance</b>				
Chloramphenicol (phenotypic)		1 (0.59%)		1 (0.49%)
<i>cat</i>		2		2
<b>Streptogramin A resistance</b>				
<i>vga(A)</i>		3	1	4
<b>Fosfomycin resistance</b>				
<i>fosB</i>		2		4

LA-MRSA, livestock-associated methicillin-resistant *Staphylococcus aureus*; PVL, Panton–Valentine leukocidin.



resistant to erythromycin, gentamicin, kanamycin, trimethoprim, and tetracycline, in which the corresponding resistance genes could not be detected by the arrays. This could be due to the presence of other resistance mechanisms that are not in the DNA microarray panel or due to intrinsic resistance in these isolates. Intrinsic resistance was documented in *S. aureus* with penicillin-binding protein 2a (PBP2a), which renders the effectiveness of the beta-lactam antibiotics.

CC96 was the second most common LA-MRSA clone detected in this study. Isolates from this lineage were first detected in Kuwait in 2016 (21 isolates) and then in 2017 (10 isolates). CC96 MRSA isolates are rare in humans with only single isolates reported previously from Russia (Mendes et al., 2012) and Saudi Arabia (Senok et al., 2016; Mat Azis et al., 2017). However, ST96-MSSA is a common pathogen of rabbits, where it causes different infections (Mendes et al., 2012; Viana et al., 2015; Merz et al., 2016; Moreno-Grúa et al., 2018). An isolate of ST96-MRSA belonging to *spa* type t1190 was isolated from a rabbit meat sample that could not be characterized as either CA-MRSA or HA-MRSA (European Food Safety Authority, 2017), suggesting that a previously ST96-MSSA/t1190 had acquired the *mecA* determinant. Although the ST96-MRSA in this study belongs to different *spa* types, we argue that they are probably related to the rabbit ST96 lineage. Furthermore, the CC96-MRSA in this study belonged to *agr* III and *cap8* and were resistant to erythromycin mediated by *ermC* similar to ST96-MSSA isolates isolated from rabbits (Merz et al., 2016). The lack of information on the epidemiology and the genetic characteristics of ST96-MRSA in the literature warrants further studies to describe the origin, prevalence, and molecular characteristics of the emerging CC96-MRSA.

Since its discovery in the Netherlands in the early 2000s, CC398 has become the most common LA-MRSA clone circulating in Europe (Butaye et al., 2016). This is the first report of CC398 in human patients in Kuwait hospitals and as far as we know in the Gulf Cooperation Council (GCC) countries. CC398 is the most prevalent lineage in pigs (Butaye et al., 2016; Feltrin et al., 2016), but it has also been reported in horses, poultry, cattle, and companion animals (Butaye et al., 2016). The CC398-MRSA was classified into two strains carrying SCC*mec* IV and V each and belonged to *spa* types t899 and t034, respectively. The CC398-IV-t899 isolate was PVL negative, similar to isolates obtained from animals in United Kingdom (Bortolami et al., 2017) and human patients in Spain (Lozano et al., 2012), Italy (Pan et al., 2009), and Denmark (Larsen et al., 2016). MRSA belonging to *spa* type t899 has been described as a hybrid LA-MRSA of CC9 and CC398 (Larsen et al., 2016; Sharma et al., 2018).

Apparently, CC398-MRSA of animal origin usually lacks enterotoxins and PVL (Feltrin et al., 2016), whereas the early-branching East Asian strain is positive for PVL (Yu et al., 2008). In this study, one CC398-MRSA-V isolate was positive for PVL, as has also been reported in human patients in China (Yu et al., 2008), Sweden (Welinder-Olsson et al., 2008), Finland (Salmenlinna et al., 2010), and New Zealand (Williamson et al., 2014), suggesting that our strain may belong to the

human branch of CC398. The lack of enterotoxins in these two CC398-MRSA isolates is consistent with previous results suggesting that absence of enterotoxins may be a characteristic feature of isolates from this lineage (Lozano et al., 2012; Feltrin et al., 2016).

CC398 strains of animal origin usually lack the IEC genes that facilitate the colonization and invasion of MRSA in human hosts (Price et al., 2012). The two CC398 isolates detected in this study carried the IEC genes *scn*, *chp*, and *sak* (IEC type B). Similarly, recent studies by Cuny et al. (2015) and Pérez-Moreno et al. (2017) also reported CC398 isolates of human origin carrying the IEC genes. The presence of IEC genes in these isolates may explain the ability of these strains to jump from livestock and successfully adapt and colonize human beings. One of the CC398 isolates belonged to *spa* type t899.

The major limitation of this study is the lack of information on the patients' travel history or contact with animals. Therefore, it is difficult to determine if these LA-MRSA strains were acquired by contact with livestock or by household members who are in contact with livestock. Nevertheless, the detection of these clones in human patients is significant because it shows their expansion beyond the usual livestock hosts, which may pose new problems for infection control.

## CONCLUSION

In conclusion, this study described the characteristics of LA-MRSA strain belonging to CC96, CC97, and CC398 in patients in Kuwait hospitals. Genotyping showed that our isolates are diverse and belonged to different lineages including CC398, which are prevalent in Europe. This is the first report of CC398 LA-MRSA in Kuwait. The study also revealed that most of the isolates belonging to CC97 expressed resistance to multiple classes of antibiotics. The CC97 and CC398 MRSA isolates shared characteristics similar to those obtained from bovine and human patients in Europe. These observations suggest that LA-MRSA isolates were introduced to Kuwait via different routes. Further surveillance studies are required to monitor future transmission patterns of these isolates. Although LA-MRSA may have the same virulence potential and causes similar infections as *S. aureus*, identifying these isolates can inform on their origin, which will help in controlling the spread of MRSA in the clinical settings.

## DATA AVAILABILITY STATEMENT

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

## AUTHOR CONTRIBUTIONS

SB, BM, BN, TV, and ST carried out the laboratory work. SB performed the data analysis. EU performed the experimental design. SB and EU carried out the manuscript writing and editing. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02912/full#supplementary-material>

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# Bacteria From the Multi-Contaminated Tinto River Estuary (SW, Spain) Show High Multi-Resistance to Antibiotics and Point to *Paenibacillus* spp. as Antibiotic-Resistance-Dissemination Players

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Bacterial resistance to antibiotics is an ever-increasing phenomenon that, besides clinical settings, is generally assumed to be prevalent in environmental soils and waters. The analysis of bacteria resistant to each one of 11 antibiotics in waters and sediments of the Huelva's estuary, a multi-contaminated environment, showed high levels of bacteria resistant mainly to Tm, among others. To further gain knowledge on the fate of multi-drug resistance (MDR) in environmental bacteria, 579 ampicillin-resistant bacteria were isolated tested for resistance to 10 antibiotics. 92.7% of the isolates were resistant to four or more antibiotic classes, indicating a high level of multi-resistance. 143 resistance profiles were found. The isolates with different MDR profiles and/or colony morphologies were phylogenetically ascribed based on 16S rDNA to phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*, including 48 genera. Putative intrinsic resistance was detected in different phylogenetic groups including genera *Altererythrobacter*, *Bacillus*, *Brevundimonas*, *Erythrobacter*, *Mesonía*, *Ochrobactrum*, and *Ponticaulis*. Correlation of the presence of pairs of the non-intrinsic-resistances in phylogenetic groups based on the kappa index ( $\kappa$ ) highlighted the co-habitation of some of the tested pairs at different phylogenetic levels. Maximum correlation ( $\kappa = 1.000$ ) was found for pairs Cz<sup>R</sup>/Tc<sup>R</sup> in Betaproteobacteria, and Cc<sup>R</sup>/Tc<sup>R</sup> and Em<sup>R</sup>/Sm<sup>R</sup> in Sphingobacteriia at the class level, while at the genus level, was found for Cc<sup>R</sup>/Tc<sup>R</sup> and Nx<sup>R</sup>/Tm<sup>R</sup> in *Mesonía*, Cz<sup>R</sup>/Tm<sup>R</sup> and Em<sup>R</sup>/Km<sup>R</sup> in *Paenibacillus*, and Cc<sup>R</sup>/Em<sup>R</sup> and Rp<sup>R</sup>/Tc<sup>R</sup> in *Pseudomonas*. These results could suggest the existence of intra-class and intra-genus-transmissible genetic elements containing determinants for both members of each pair. Network analysis based on  $\kappa$  values higher than 0.4 indicated the sharing of paired resistances among several genera, many of them centered on the *Paenibacillus* node and raising the hypothesis of inter-genera

transmission of resistances interconnected through members of this genus. This is the first time that a possible hotspot of resistance interchange in a particular environment may have been detected, opening up the possibility that one, or a few, bacterial members of the community could be important promoters of antibiotic resistance (AR) dissemination in this environment's bacterial population. Further studies using the available isolates will likely give insights of the possible mechanisms and genetic elements involved.

**Keywords:** antibiotic resistance, estuary, environmental bacteria isolates, multi-drug resistance, *Paenibacillus*, tinto river, resistance dissemination, contaminated environment

## INTRODUCTION

The antibiotic resistance (AR) of bacterial pathogens is currently a worldwide problem with severe consequences for the treatment of infectious diseases (World Health Organization [WHO], 2018). However, AR existed in prehistoric (Perry et al., 2016) and more recent times (Devault et al., 2017); and its origin is not directly related to the clinical use of antibiotics. It was probably triggered by the appearance of antibiotics in the environment when their producers needed to be protected from their effects (Jiang et al., 2017). The environmental role of these compounds is not yet clear and for a long time was considered only ecological. This function was demonstrated only in a few cases (Currie et al., 1999; Neeno-Eckwall et al., 2001; Haas and Défago, 2005). Antibiotic concentrations in nature may not be enough for this purpose (Fajardo et al., 2009), while low concentrations have pleiotropic effects that are not related to competition (Davies et al., 2006; Linares et al., 2006; Yim et al., 2007; Fajardo and Martinez, 2008; Sengupta et al., 2013; Andersson and Hughes, 2014; Goneau et al., 2015; Martínez, 2017; Xiong et al., 2017). However, low individual concentrations of a cocktail of antibiotics could have a combined effect [reviewed by Danner et al. (2019)]. Additionally, sub-lethal concentrations select resistant phenotypes from mutations or horizontal gene transfer (HGT) that sometimes use mobile genetic elements (MGEs) (Kohanski et al., 2010; Baharoglu and Mazel, 2011; Gutierrez et al., 2013; Haaber et al., 2017; Lekunberri et al., 2017). The AR in the environment is a natural process but is also promoted by anthropogenic pollutants like antibiotics, biocides, heavy metals, hydrocarbons, pesticides, and nanomaterials, among others (Baker-Austin et al., 2006; Dealtry et al., 2014; Pal et al., 2015, 2017; Chen et al., 2017; Poole, 2017; Wang et al., 2018; Nguyen et al., 2019). Therefore, naturally antibiotic-resistant bacteria (ARB) could be a prime source for antibiotic resistance genes (ARGs) found in pathogens as certain findings may indicate (Rodríguez et al., 2004; Poirel et al., 2005, 2012; Yang J. et al., 2013; Jiang et al., 2017), but may later have evolved and be selected.

The interaction of microorganisms from natural, clinical, and farming sources could select multi-resistant bacteria (MRB), severely affecting environments, change biodiversity, and modify evolution paths in favor of resistants (Ding and He, 2010; Gupta, 2011; Proia et al., 2013; Laverman et al., 2015; Hiltunen et al., 2017; Grenni et al., 2018). The major challenge posed

by ARB is their acquisition of MDR. Several mechanisms for the accumulation of resistances are known (Cambray et al., 2010; Michael et al., 2012; Brown-Jaque et al., 2015; Carraro et al., 2016, 2017; Ramsay and Firth, 2017). However, MDR spreads in the environment and the resistance patterns that are passed-on together or transferred to pathogenic bacteria, or how these bacteria can model their environments remain unknown (Chamosa et al., 2017). Studies to estimate the risk of HGT between environmental and pathogenic bacteria have identified hotspots of transfer including wastewater treatment plants (WWTPs), which are recognized as one of the main sources of antibiotics pollution in surface waters (Guo et al., 2017; Manaia et al., 2018). Few experimental studies on the specific transmissible elements and their dissemination capabilities have been conducted. The structure, function, and inter-relationships of the environmental microbiomes are complex phenomena only partially studied and their comprehension needs to use multidisciplinary approaches and methodologies, including different culture-dependent and independent approaches, to analyze in a “one health” approach how the AR affects environmental microbial biodiversity and limits the use of antibiotics (Stefani et al., 2015; Ellington et al., 2017; Hiltunen et al., 2017; Tripathi and Cytryn, 2017).

Recent studies have shown that many rivers worldwide contain relevant concentrations of antibiotics (Boxall and Wilkinson, 2019; Wilkinson and Boxall, 2019) up to 50 µg/L in some African countries or up to 10 µg/L in European ones (Danner et al., 2019). The prescriptions of antibiotics in Spain has risen from 2012 to 2018 (European Centre for Disease Prevention and Control [ECDC], 2017)<sup>1</sup> and several studies on the concentration of antibiotics in Spain's surface waters have shown concentrations of 1.3 µg/L on average [reviewed in Danner et al. (2019)]. Running surface waters such as rivers and their estuaries in anthropogenically influenced areas are a means for antibiotic resistance dissemination through their courses and receiving waters. Some rivers receive effluents from WWTPs facilities, which are not able in most cases to completely eliminate pharmaceuticals such as antibiotics, ARBs, or ARGs, even with the implementation of disinfection protocols (Loos et al., 2012; Suzuki et al., 2017; Barancheshme and Munir, 2018; Ju et al., 2019). So, these bacteria and chemicals carried by the treated

<sup>1</sup><https://www.ecdc.europa.eu/en/antimicrobial-consumption/database/country-overview>

wastewater can affect the environment by selection of antibiotic resistant bacteria, changing biodiversity and increasing the levels of resistant and MRB (Sanseverino et al., 2018). Also soils and sediments can receive antibiotics and other pollutants that are not completely degraded and can impact their microbial activity and diversity (Cycon et al., 2019). In addition, other substances can be contaminating rivers and estuaries when they receive effluents from industries established within their borders.

The Huelva estuary (SW, Spain) receives waters from two acidic and heavy metal-rich rivers that mix with seawater from the Atlantic Ocean. The WWTP of Huelva, a city with about 145,000 inhabitants, processes urban wastewaters about 240,000 population equivalents<sup>2</sup>; and emits its effluents into the estuary. Also, effluents from a number of industries around the estuary, including oil refineries, metallurgic industries, fertilizers plants, etc., (**Figure 1**) arrive in the estuary to create a multi-contaminated environment (Davis et al., 2000; Sainz et al., 2004; Pérez-López et al., 2011; Amils, 2016; Papaslioti et al., 2018). Moreover, some studies have reported the presence of radioactivity in the estuary that is related to the fertilizer production that uses uranium-containing phosphogypsum, which accumulates in sediments (Martínez-Aguirre and García-León, 1997; Elbaz-Poulichet et al., 1999). As stated above, several studies have reported that the appearance of antibiotic resistance can be facilitated by the presence in the environment of heavy metals that are abundant in the Huelva estuary; hydrocarbons that come from oil refineries' management or accidental spills; and for antibiotics that are likely provided by the WWTP. Due to the prevalence of and magnitude of the metals pollution, previous studies have been limited to this aspect.

This study reports our conclusions as to the prevalence of culturable ARB in the surface water and sediments of the Huelva estuary with an aim to contribute to the current knowledge on the diversity and fate of MDR of culturable environmental bacteria as well as to collect biological materials for further study on the transmission of antibiotic resistance and the MGEs involved. 579 ampicillin-resistant bacteria were isolated and their MDR levels and resistance profile diversity were determined. The analysis of these data identified putative intrinsic resistances in different phylogenetic groups as well as correlations of the presence of pairs of acquired resistances mainly at the genus level. A network analysis of the best correlations suggests that members of the *Paenibacillus* genus could be important players in the dissemination of resistance in this environment.

## MATERIALS AND METHODS

### Huelva's Estuary Sampling

Samples were collected in areas named H1 and H2 (**Figure 1**). H1 locates at 37° 16' 57.81'' N, −6° 50' 59.68'' W, and H2 was an aliquot mix from samples taken at: H2-I (37° 12' 48.92'' N, −6° 56' 15.23'' W), H2-II (37° 12' 41.30'' N, −6° 56' 27.21'' W), and H2-III (37° 12' 41.51'' N, −6° 55' 49.97'' W). Surface water (H1L, H2L) and sediments (H1S, H2S) were collected.

<sup>2</sup><https://www.iagua.es/data/infraestructuras/edar/huelva>

## Physicochemical Parameters of Huelva's Estuary

They were measured *in situ* using the multi-parametric portable sensor Thermo Orion 290A (Thermo Fisher Scientific, United States). The elemental compositions were measured by the Servicio Interdepartamental de Investigación (SIDI) of the Universidad Autónoma de Madrid<sup>3</sup>, using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for water samples and Total X-Ray Reflection Fluorescence (TXRF) for sediments.

## Culture and Isolation of ARB

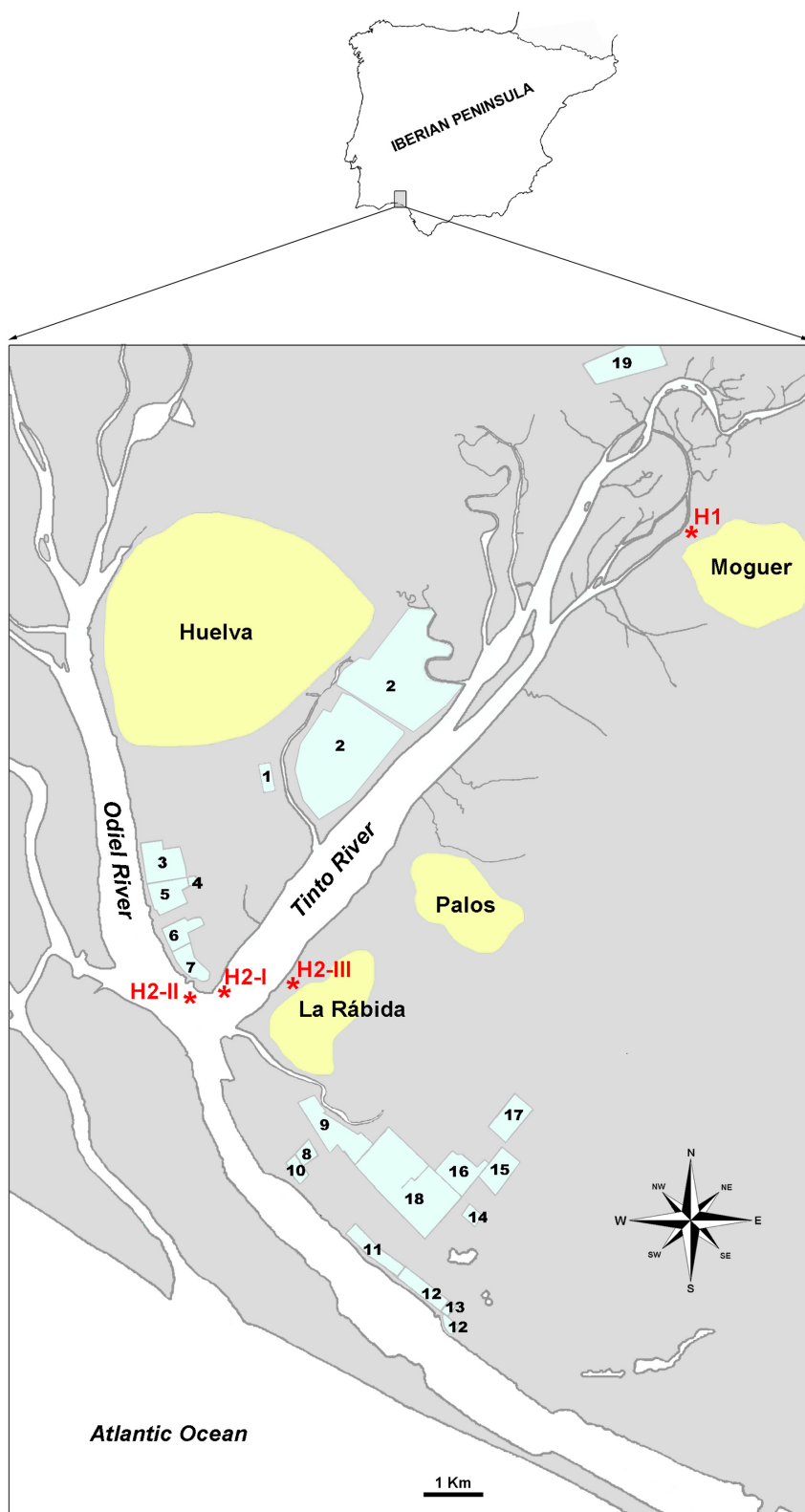
In order to quantify total culturable and resistant bacteria, dilutions in the culture media ( $10^0$ – $10^{-6}$ ) of the liquid samples and culture-media-suspended sediments were inoculated by extension in Petri dishes containing autoclaved solid media. Two culture medias were used: marine [55.1 g/l Difco Marine agar (BD, Sparks, MD, United States)] and nutritive [3 g/l meat extract (Merck, Darmstadt, Germany), 5 g/l NaCl (Merck), 10 g/l bactopectone (Labs. Conda, Madrid, Spain), 15 g/l agar (Labs. Conda)]. After autoclaving (121°C, 20', 1 atm), media were left to cool down to 50°C and when needed, supplemented with one out of 11 antibiotics at the following final concentrations: 100 µg/ml for streptomycin (Sm) (Duchefa Biochemie, Haarlem, Netherlands); 50 µg/ml for ampicillin (Ap) and vancomycin (Vm) (Duchefa), ceftazidime (Cz), kanamycin (Km), and nalidixic acid (Nx) (Sigma-Aldrich, St. Louis, MO, United States); 25 µg/ml for chloramphenicol (Cc) and erythromycin (Em) (Duchefa), and tetracycline (Tc) (Sigma); 16 µg/ml for trimethoprim (Tm) (Duchefa); and 8 µg/ml for rifampicin (Rp) (Duchefa). To all the media, cycloheximide (Duchefa) was added at the final concentration of 75 µg/ml. Three dishes per dilution were inoculated and cultured at 30°C for 5 days before the colony counts were done. Bacteria growing on Ap-containing media (Ap<sup>R</sup>) and showing different colony morphologies were selected for isolation. When many colonies with the same morphology appeared from a sample, one out of every three was collected. Isolation from the initial plating colonies was performed by successive passages on solid media containing the same selective agent until isolated colonies of the same morphology were obtained during three consecutive replatings. Isolates were stored at −80°C in 50% (v/v) of glycerol (87%, Merck) and the corresponding liquid culture medium.

Multi-resistance of the isolates was tested on the solid medium used for their isolation containing individual antibiotics at the concentrations indicated above. Dishes inoculated with Ap served as growth control.

## DNA Extraction

DNA was isolated from individual bacterial colonies according to Kai et al. (2006), or using the Ultraclean Microbial DNA Isolation Kit (MOBIO, Carlsbad, CA, United States) according to the manufacturer's instructions [except for a pretreatment with lysozyme 10 mg/ml (Sigma) for 1 h at 37°C].

<sup>3</sup><http://www.uam.es/UAM/SIDI/1242663052047.htm?language=es>



**FIGURE 1 |** Situation map of sampling sites. Sampling points are labeled as H1 and H2-I, H2-II and H2-III. 1. WWTP of Huelva. 2. Phosphogypsum deposits. 3., 6., and 15. Fertilizers plants. 4. Liquefied gases plant. 5. Copper metallurgy plant. 7. Thermic energy plant. 8. Chemicals production plant. 9. Hydrocarbons storage. 10. Petroleum coke storage. 11. Natural gas plant. 12. Petrochemical products storage. 13. Palm oil refinery. 14. Biodiesel plant. 15. Pharmaceuticals and food supplements plant. 17. Pigments plant. 18. Oil refinery. 19. Biomass thermic plant (old cellulose plant).



## Phylogenetic Analysis

Conventional PCR amplification of 16S rDNA was performed using 27F and 1492R universal primers (Weisburg et al., 1991). Reactions (50  $\mu$ l) contained: 3  $\mu$ l DNA sample, 0.5  $\mu$ M each primer (Sigma), 0.5  $\mu$ M dNTPs (Invitrogen, Carlsbad, CA, United States), 5  $\mu$ l 10X buffer (Promega, Fitchburg, WI, United States), 3 mM MgCl<sub>2</sub> (Promega) and 2.5 U Taq polymerase (Promega). Amplifications [2720 thermocycler (Applied Biosystems, Waltham, MA, United States)] were done using the following program: 94°C 5 min; 30 cycles: 94°C 1 min, 54°C 1 min, 72°C 1.30 min; 72°C 10 min. Amplicons were purified by precipitation by mixing the reaction solution with equal volume of 20% (w/v) PEG [polyethylene glycol 6000 (Merck) in 2.5 M NaCl (Merck)], incubating for 30 min at 37°C with shaking every 10 min, centrifuging at 2850  $\times$  g for 15 min and washing the pellet twice with 80% (v/v) ethanol (Merck). After vacuum drying the DNA was resuspended in MilliQ water and sequenced by Macrogen Inc., (Seoul, South Korea). Sequences were deposited at the European Molecular Biology Laboratory (EMBL) (Acc. No.: LT601033-LT601378).

Isolates were initially assigned to the genus or species with the highest sequence similarity using NCBI BLAST (Altschul et al., 1990); and were further identified compared with type strains in the phylogenetic trees built with the sequences at the Ribosomal Database Project (RDP) (RRID: SCR\_006633) (Cole et al., 2014). Clustal X<sup>4</sup> was used for alignments and building of neighbor-joining trees, with 1000 repetitions for bootstrapping.

## Statistical Analysis

Data were analyzed using SPSS Statistics V21.0 software (IBM Corporation, New York, NY, United States) and Microsoft Excel 2013. The normality of the distribution of the variables was evaluated by using the Kolmogorov–Smirnov test.

To characterize MDR level the multiple-antibiotic resistance index (MAR) was calculated for each isolate and for the groups of them considered as the median ( $\bar{x}$ ) of the isolates' MARs (Krumperman, 1983).

The non-parametric tests of Mann-Whitney and Kruskal–Wallis were performed to compare the MARs median. Statistical correlations between the sampling areas or culture media with the observed resistance to each antibiotic were analyzed using contingency tables and contrasting Chi-square.

The kappa indexes ( $\kappa$ ) for association of resistance pairs were calculated for groups with eight or more isolates. Networking among pairs with  $\kappa \geq 0.4$  by class and genus were represented using gephi<sup>5</sup>.

For all analysis, results were considered statistically significant for  $p < 0.05$ .

Distributions of resistants among zones or phylogenetic groups are presented by using the Circos online application<sup>6</sup>.

## RESULTS

### Physicochemical Parameters

Physicochemical parameters of water samples were: pH 6.2–6.5, temperature: 17.5–20.6°C, salinity: 27.1–31.9, conductivity: 42.1–48.6 mS/cm, and redox potential: 159.0–208.0 mV (Supplementary Table S1). Elemental composition of samples is reported in Supplementary Tables S2, S3.

### Abundance of Antibiotic-Resistant Culturable Bacteria

Total culturable bacteria reported as CFU/mL for water and as CFU/g for sediments were, respectively: in marine medium:  $1.1 \pm 0.042 \times 10^6$  and  $4.7 \pm 2.2 \times 10^7$  for H1, and  $6.8 \pm 1.1 \times 10^4$  and  $3.0 \pm 1.1 \times 10^7$  for H2, while in nutritive one:  $5.1 \pm 0.13 \times 10^4$  and  $9.6 \pm 1.2 \times 10^5$  for H1 and  $1.4 \pm 0.037 \times 10^3$  and  $1.1 \pm 0.4 \times 10^5$  for H2.

Relative abundances of resistance to each antibiotic (as% of resistants/total culturable bacteria) (Figure 2) were, in general, higher for bacteria tested on the marine rather than on the nutritive medium. The highest was obtained for Tm-resistants, irrespective of the sampling zone or the culture medium. However, Tc-resistants were common among the bacteria grown on marine medium but scarce among those from nutritive one. Percentages of resistants to Cc, Em, or Rp, were low, but higher in the nutritive than in the marine medium.

### Ampicillin-Resistant Bacteria

579 ampicillin-resistant bacteria were isolated corresponding to 286 and 293 colonies from H1 and H2 areas, respectively. From each zone, 70.63 and 74.06%, respectively, were isolates from the marine medium. Table 1 summarizes the distribution of isolates per sample.

### MDR of Ampicillin-Resistant Isolates

Figure 3 summarizes the prevalence of resistances in the different groups of isolates considered and Table 2 shows the order of prevalences. Distributions of resistants to each antibiotic were significantly different among the samples (H1L, H2L, H1S, and H2S) and with respect to the culture media for almost all antibiotics ( $p < 0.05$ ).

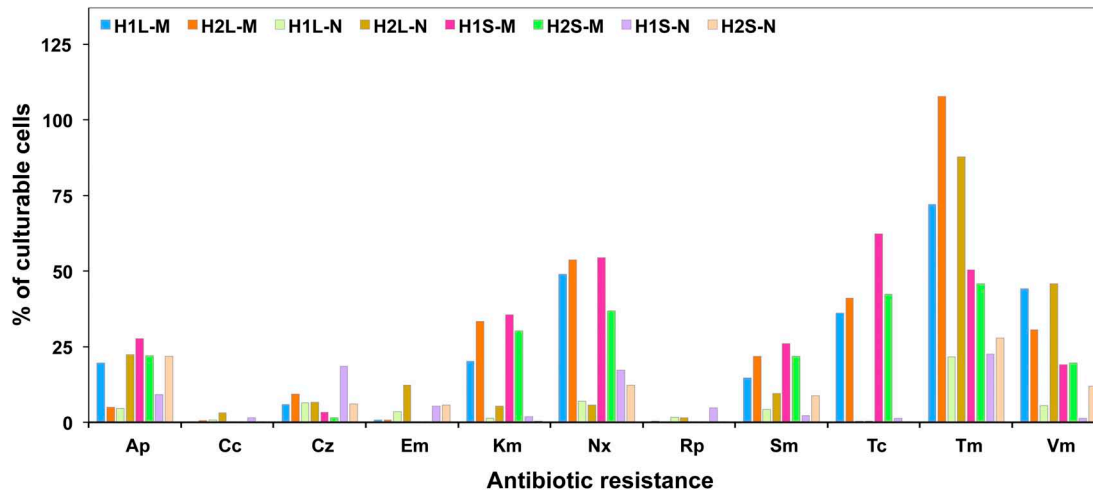
A total of 143 different resistance profiles were identified (Supplementary Table S4), with 2.1% of resistants only to Ap, and 2.6% to all the antibiotics. The most frequent profiles included resistance to eight antibiotics (128 isolates and 22 profiles), while the most diverse included resistance to 6 or 7 antibiotics (26 profiles each). 92.7% of the isolates were MRB [resistance to 4 or more antibiotic classes (Narciso-da-Rocha and Manaia, 2016)]. Table 1 summarizes the profiles/isolates ratios in each group.

The calculated MAR index medians ( $\bar{x}$ ) for considered groups of isolates (Table 1) were 0.636 for the whole set of isolates, H1 and H2. There was no statistically significant difference between water samples (HL, H1L, H2L) ( $\bar{x} = 0.545$ ,  $p > 0.05$ ); however, sediments showed significantly higher values than the corresponding water: HS ( $\bar{x} = 0.727$ ,  $p < 0.001$ ), H1S ( $\bar{x} = 0.636$ ,  $p = 0.001$ ), and H2S ( $\bar{x} = 0.727$ ,  $p < 0.001$ ). Distributions of MAR

<sup>4</sup><http://www.clustal.org/clustal2/>

<sup>5</sup><http://circo.ca/>

<sup>6</sup><https://gephi.org/>



**FIGURE 2 |** Relative abundance of culturable resistant bacteria in waters and sediments of the sampling sites.

**TABLE 1 |** Characteristics and distribution of Ap<sup>R</sup> isolates among the samples tested on nutritive and marine media.

	Samples								
	H			H1			H2		
	H	HL	HS	H1	H1L	H1S	H2	H2L	H2S
<b>Culture medium</b>									
<b>M</b>									
Median	0.545	0.545	<b>0.636</b>	0.545	0.545	<b>0.636</b>	0.545	0.545	<b>0.682</b>
% MAR <sub>≥0.636</sub>	45.8	39.7	<b>64.7</b>	47.5	42.6	<b>61.1</b>	44.2	37.3	<b>68.8</b>
N° profiles (% total)	<b>85 (59.4)</b>	<b>75 (52.4)</b>	34 (23.8)	57 (39.9)	49 (34.3)	25 (17.5)	64 (44.8)	54 (37.8)	22 (15.4)
N° isolates (% total)	419 (72.4)	317 (54.7)	102 (17.6)	202 (34.9)	148 (25.6)	54 (9.3)	217 (37.5)	169 (29.2)	48 (8.3)
Ratio (%) profiles/isolates	20.3	23.6	33.3	28.2	33.1	46.3	29.5	32.0	45.8
<b>N</b>									
Median	<b>0.727</b>	0.545	<b>0.727</b>	<b>0.636</b>	0.545	<b>0.636</b>	<b>0.727</b>	0.364	<b>0.727</b>
% MAR <sub>≥0.636</sub>	<b>66.9</b>	40.4	<b>79.6</b>	<b>58.3</b>	44.1	<b>68.0</b>	<b>76.3</b>	33.3	<b>89.7</b>
N° profiles (% total)	64 (44.8)	35 (24.5)	43 (30.1)	50 (35.0)	26 (18.2)	35 (24.5)	22 (15.4)	13 (9.1)	11 (7.7)
N° isolates (% total)	160 (27.6)	52 (9.0)	108 (18.7)	84 (14.5)	34 (5.9)	50 (8.6)	76 (13.1)	18 (3.1)	58 (10.0)
Ratio (%) profiles/isolates	40.0	67.3	39.8	59.5	76.5	70.0	28.9	72.2	19.0
<b>Total</b>									
Median	<b>0.636</b>	0.545	<b>0.727</b>	<b>0.636</b>	0.545	<b>0.636</b>	<b>0.636</b>	0.545	<b>0.727</b>
% MAR <sub>≥0.636</sub>	<b>51.6</b>	39.8	<b>72.4</b>	<b>50.7</b>	42.9	<b>64.4</b>	<b>52.6</b>	36.9	<b>80.2</b>
N° profiles (% total)	<b>143 (100)</b>	<b>107 (74.8)</b>	<b>73 (51.0)</b>	<b>104 (72.7)</b>	<b>73 (51.0)</b>	58 (40.6)	<b>82 (57.3)</b>	66 (46.2)	30 (21.0)
N° isolates (% total)	579 (100)	369 (63.7)	210 (36.3)	286 (49.4)	182 (31.4)	104 (18.0)	293 (50.1)	187 (32.3)	106 (18.3)
Ratio (%) profiles/isolates	24.7	29.0	34.8	36.4	40.1	55.8	28.0	35.3	28.3

Numbers in bold correspond to values greater than 50% of the maximum possible ones.

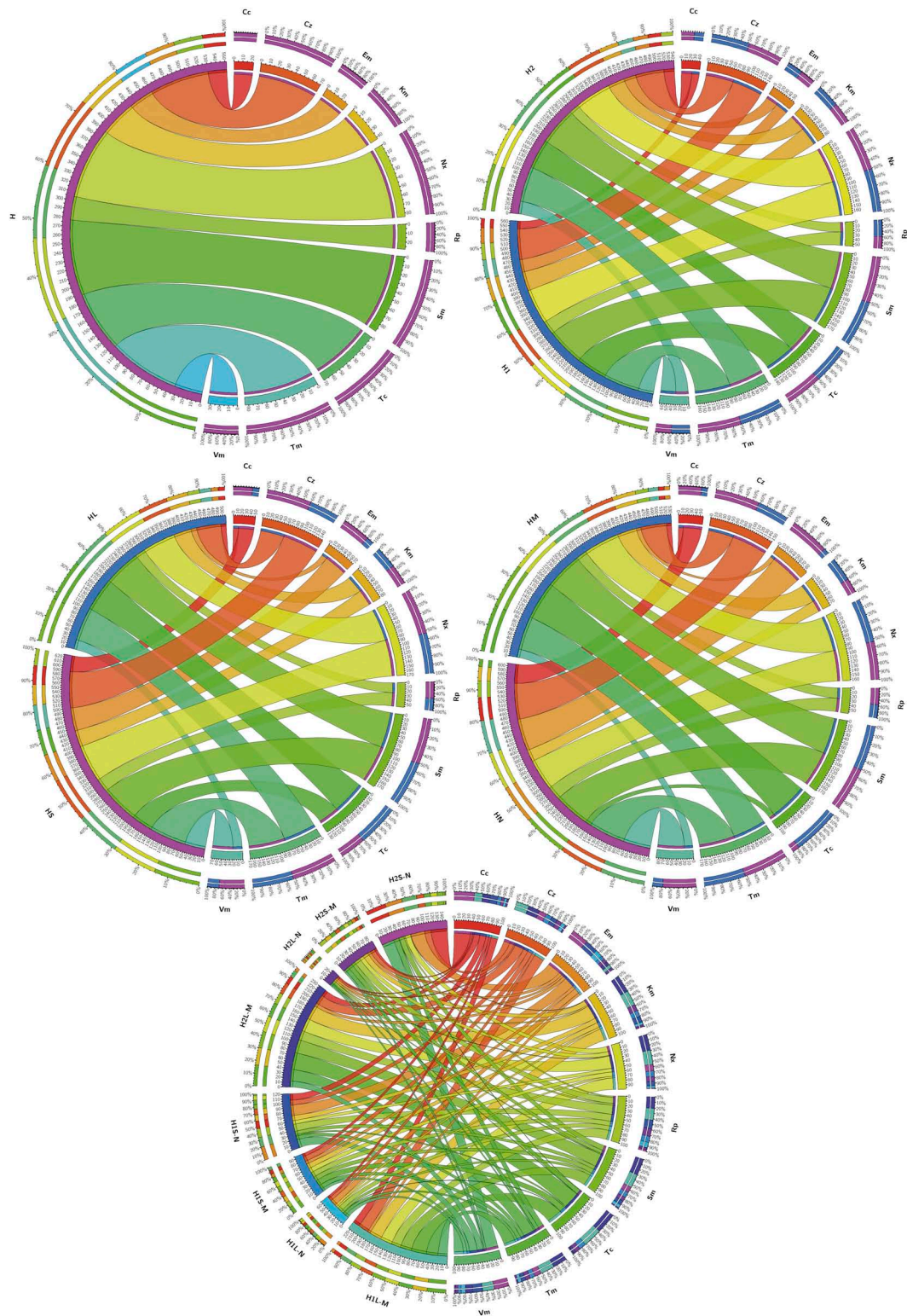
values of isolates from water and sediments of both sampling sites (H1L, H1S, H2L, H2S) were significantly different ( $p < 0.001$ ) as well as when considering culture media (H1-M, H1-N, H2-M, H2-N) ( $p < 0.001$ ).

## Phylogenetic Identification of Bacterial Isolates

Partial 16S rRNA sequences of the 345 isolates considered different by their MDR profiles and colony morphology

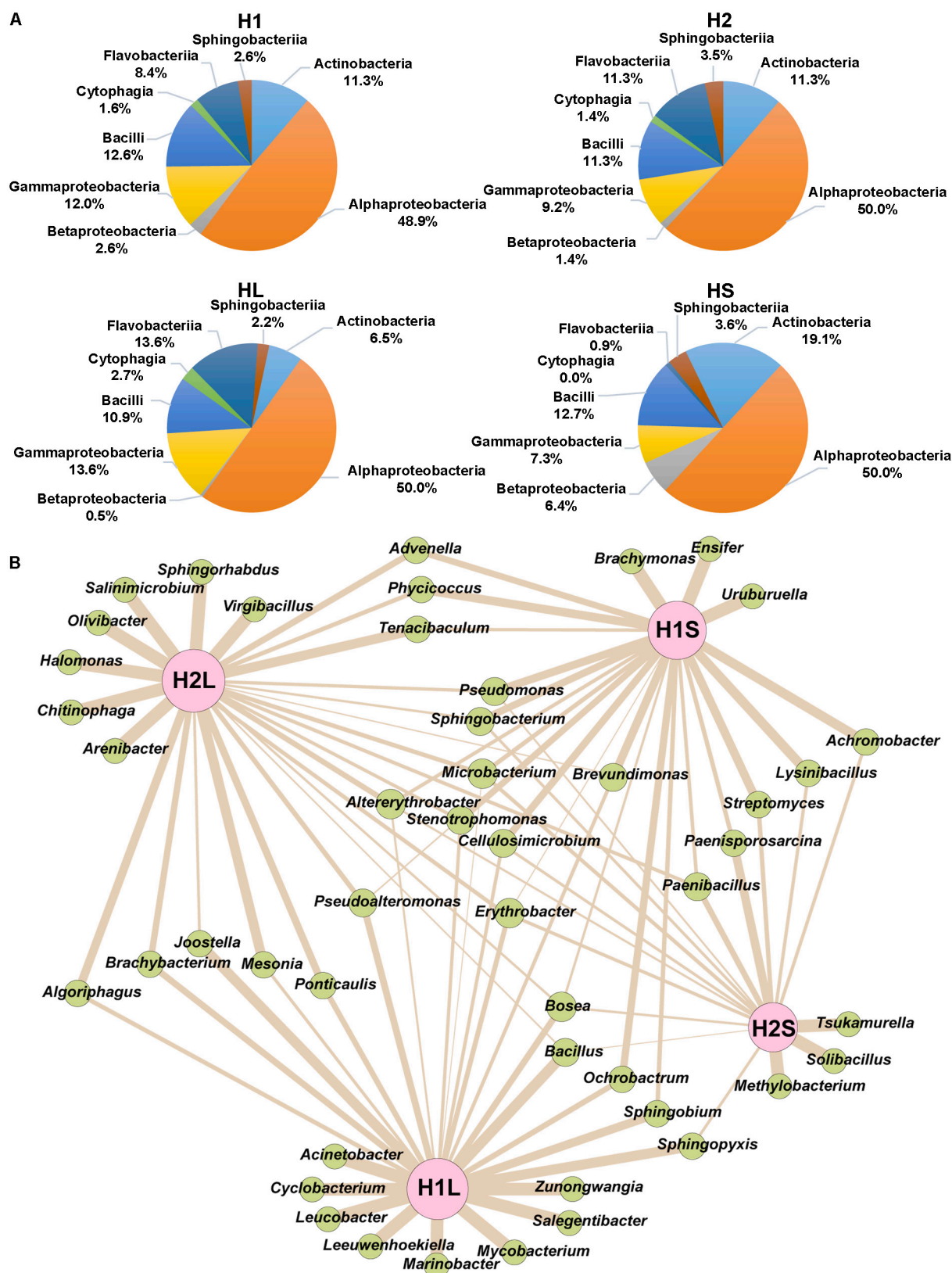
were obtained, but phylogenetic assignation of some of them sometimes gave different results, mostly at the species level, when comparisons were made using either sequences at GenBank or phylogenetic trees (**Supplementary Figures S1–S7**); however, most affiliations were coincident at the genus level (**Supplementary Table S5**).

From H1 and H2 areas, 168 and 141 isolates, respectively, were considered to be different based on sample origin, multi-resistance profiles and 16S rDNA sequences. These



**FIGURE 3 |** Prevalence of isolates resistant to the different antibiotics tested in the indicated groups of isolates. Considered groups of isolates were H: all, H1 and H2: all from both sampling sites independently, HL and HS: all from waters and sediments, respectively, HM and HN: all obtained from marine and nutritive media, respectively, and H1L-M, H1L-N, H1S-M, H1S-N, H2L-M, H2L-N, H2S-M, and H2S-N: groups considering independently sampling zones, phases and media. Ribbons connect antibiotic to which resistant isolates have been found and the groups of isolates in which they were found. Ribbons wideness represents percentages of bacteria resistant to the corresponding antibiotic among the isolates of the corresponding considered group.





**FIGURE 4 |** Distribution of phylogenetically identified isolates by class and genus in the indicated samples. **(A)** Classes, **(B)** Genera; wideness of each connector bar of the network is proportional to the number of isolates in each genus.



**TABLE 2 |** Order of prevalence of resistance to individual antibiotics in the indicated groups of isolates.

Group of isolates	Order of prevalence
H	Sm > Tm = Nx > Tc > Cz > Km > Vm > Em > Rp > Cc
HL	Sm > Tc > Tm > Nx > Cz > Km > Rp > Vm > Em > Cc
HS	Sm > Nx > Tm > Cz > Em > Tc > Vm > Km > Cc > Rp
H1	Sm > Tm > Nx > Cz > Tc > Km > Vm > Rp > Em > Cc
H2	Sm > Nx > Tm > Tc > Cz > Km > Vm > Em > Cc > Rp
H1L	Sm > Tm > Nx > Tc > Cz > Km > Vm > Rp > Em > Cc
H1S	Sm > Nx > Cz > Tm > Tc > Km > Em > Vm > Rp > Cc
H2L	Sm > Tc > Nx > Tm > Cz > Km > Rp > Vm > Cc > Em
H2S	Tm > Nx > Sm > Cz > Em > Vm > Cc > Tc > Km > Rp

isolates were assigned to phyla Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria and classes Alphaproteobacteria, Bacilli, Gammaproteobacteria, Actinobacteria, Flavobacteriia, Betaproteobacteria, Sphingobacteriia, and Cytophagia (Figure 4A). Moreover, 48 different genera were identified, 52.1% were ubiquitous, 27.1% specifically of H1, and 20.8% of H2 (Figure 4B). Alphaproteobacteria showed the greatest diversity in genera and Cytophagia the least.

### Levels of MDR Among Phylogenetically Ascribed Isolates

Based on MAR values the level of MDR was: Actinobacteria = Bacteroidetes ( $\bar{x} = 0.727$ ) > Proteobacteria ( $\bar{x} = 0.636$ ) > Firmicutes ( $\bar{x} = 0.455$ ). At the Class level: Betaproteobacteria ( $\bar{x} = 0.909$ ) > Sphingobacteriia ( $\bar{x} = 0.773$ ) > Actinobacteria = Flavobacteriia ( $\bar{x} = 0.727$ ) > Alphaproteobacteria = Gammaproteobacteria ( $\bar{x} = 0.636$ ) > Bacilli = Cytophagia ( $\bar{x} = 0.455$ ). At the genus level, the highest was for *Stenotrophomonas* ( $\bar{x} = 1.000$ ) and the lowest was for *Pseudomonas* ( $\bar{x} = 0.273$ ). All *Ochrobactrum*, *Stenotrophomonas*, and *Streptomyces* were resistant to at least six antibiotics.

Sm<sup>R</sup> was the most frequently observed and Cc<sup>R</sup> the least observed. The prevalent resistances (Figure 5) for the phylum/class Actinobacteria were Km<sup>R</sup> and Tm<sup>R</sup>, and the less common were Em<sup>R</sup> and Vm<sup>R</sup>. For Bacteroidetes, Km<sup>R</sup> and Sm<sup>R</sup> were the most frequent, while Em<sup>R</sup> was the least. For Firmicutes, Tm<sup>R</sup> and Vm<sup>R</sup> were the most and least frequently found, respectively. For Proteobacteria, the most prevalent was Sm<sup>R</sup> and the least was Cc<sup>R</sup>.

### Putative Intrinsic or Acquired Resistance of the Isolates

Resistance to a specific antibiotic was considered as putatively intrinsic in a particular phylogenetic group given the high majority ( $\geq 90\%$ ) of isolates that belong to that group were resistant (Figure 5). Thus, at the phylum level (Figure 5A), Bacteroidetes were intrinsically resistant to Km, Proteobacteria to Sm, and InR was neither observed in Firmicutes down to class nor in Actinobacteria down to genus.

At the class level (Figure 5B), Alphaproteobacteria showed InR only to Sm, and Betaproteobacteria to Em,

Nx, and Sm, but for Gammaproteobacteria no InR was detected at this level or down to genus. Class/Order Flavobacteriia/Flavobacteriales showed InR to Km, Nx and Sm and class Sphingobacteriia to Cz and Km.

At the order level (Figure 5C), Caulobacteriales showed InR to Sm, Rhizobiales to Nx, Sm and Tm, and Sphingomonadales to Nx and Sm.

At the family level (Figure 5D), considering only those with more than one genus or species, Bacillaceae isolates appeared intrinsically resistant to Tm; Brucellaceae to Cz, Em, Sm, Tm, and Vm; Caulobacteriaceae to Cz, Nx and Sm; Erythrobacteriaceae to Nx and Sm; and Sphingomonadaceae to Sm.

Five genera of Alphaproteobacteria showed InR: *Altererythrobacter* to Sm and Tm; *Brevundimonas* to Cz, Nx, and Sm; *Erythrobacter* to Nx, Sm, and Tc; *Ochrobactrum* to Cz, Em, Sm, Tm, and Vm; *Ponticaulis* only to Sm; all *Bacillus* isolates were resistant to Tm; and all *Mesonina* isolates to Sm. No genus showed InR to Cc, Km, or Rp; and all isolates of the genera *Altererythrobacter*, *Bacillus*, and *Mesonina* were susceptible to Cc, Vm, and Em, respectively (Figure 5E).

### Correlations of Resistances Pairs in Each Phylogenetic Group

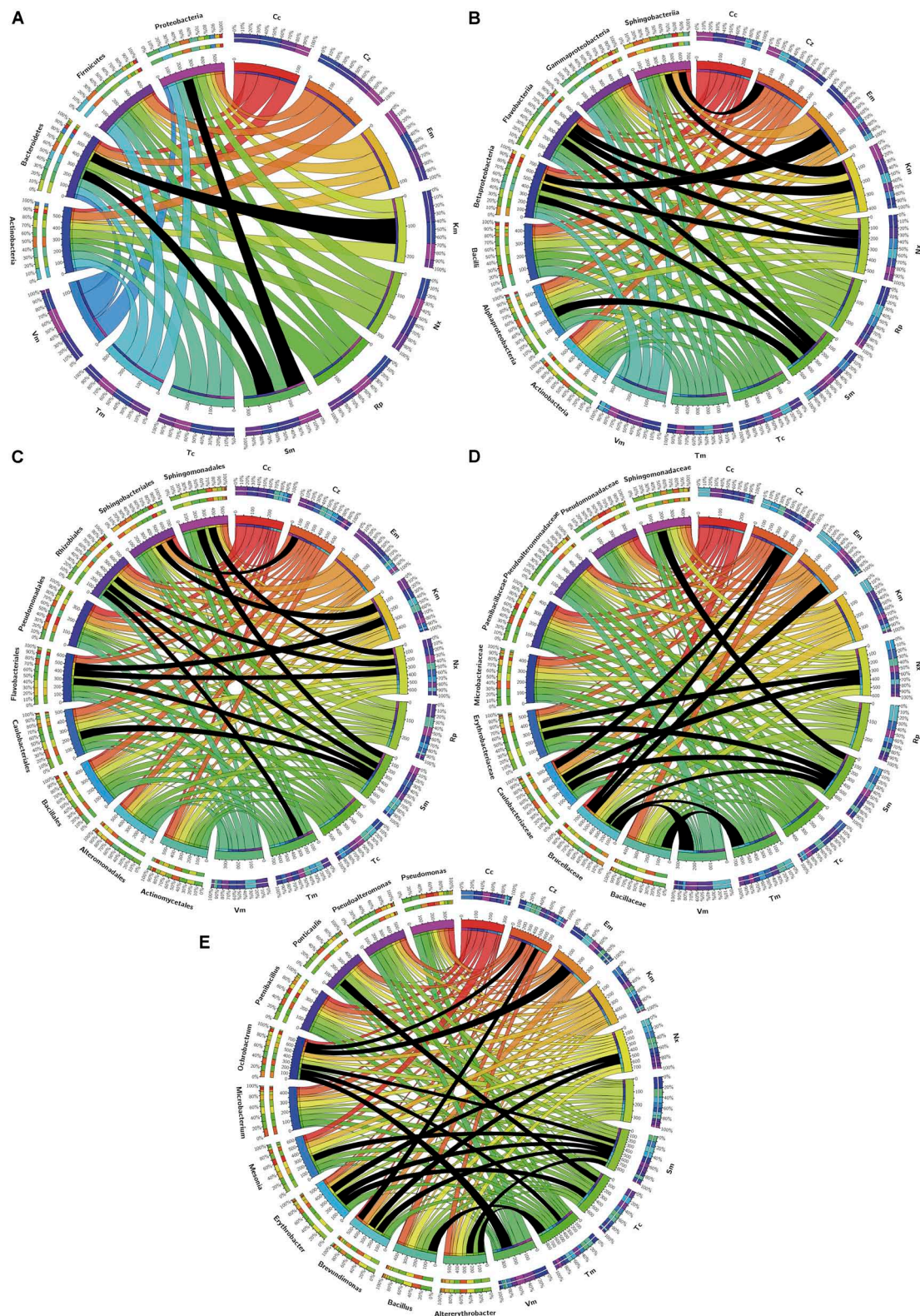
The kappa correlation index ( $\kappa$ ) for pairs of antibiotic resistances was determined for the whole set of isolates and for the phylogenetic groups that included at least eight isolates.

Considering all the isolates, no significant correlation appeared, but specific phylogenetic groups showed several positive and negative correlations. Only some of the positive ones reached  $\kappa > 0.400$ , corresponding to moderate and up to perfect correlations ( $\kappa = 0.810$ – $1.000$ ) (Supplementary Table S6). Perfect correlations appeared for Cz<sup>R</sup>/Tc<sup>R</sup> in Betaproteobacteria and Cc<sup>R</sup>/Tc<sup>R</sup> and Em<sup>R</sup>/Sm<sup>R</sup> in Sphingobacteriia. At the genus level, these correlations were found in *Mesonina* for Cc<sup>R</sup>/Tc<sup>R</sup> and Nx<sup>R</sup>/Tm<sup>R</sup>, in *Microbacterium* for Cc<sup>R</sup>/Rp<sup>R</sup>, in *Paenibacillus* for Cc<sup>R</sup>/Em<sup>R</sup>, Cc<sup>R</sup>/Km<sup>R</sup>, Em<sup>R</sup>/Nx<sup>R</sup>, Km<sup>R</sup>/Nx<sup>R</sup>, Cz<sup>R</sup>/Tm<sup>R</sup> and Em<sup>R</sup>/Km<sup>R</sup>, in *Pseudoalteromonas* for Tm<sup>R</sup>/Vm<sup>R</sup>, and, finally, in *Pseudomonas* for Cc<sup>R</sup>/Em<sup>R</sup> and Rp<sup>R</sup>/Tc<sup>R</sup>. These correlations configure networks, which were represented for  $\kappa$  values higher than 0.4 (Figure 6). In these networks, some pairs of resistances with moderate or optimal correlations are shared by several genera while others appeared only in specific ones.

## DISCUSSION

### Environmental Conditions of the Sampling Zones

The samples used in this work contained heavy metals with a slightly higher level of the most abundant elements in the H1 area and with lower salinity and conductivity when compared to the H2 area. This result may be due to the effect of a higher influence from the Tinto River water, which constitutes the leading heavy metal source of the H1 station. The industrial plants around H2 also dump metals and metalloids into the



**FIGURE 5 |** Distribution among phylogenetic groups of isolates resistant to the tested antibiotics. Graphs correspond to (A) phylum, (B) class, (C) order, (D) family, and (E) genus. Ribbons connect the antibiotic to which resistance was detected with the phylogenetic group to which resistant isolates were assigned. The wideness of each ribbon represents the percentage of isolates within each phylogenetic group resistant to the antibiotic that it connects to. Black ribbons correspond to percentages of isolates  $\geq 90\%$ .





media. These results indicated that the population of bacteria growing on one or the other media are not the same and carry determinants for different ARs. The highest difference between media was for Tc<sup>R</sup>, common in isolates from the marine medium. Several studies have shown a great abundance of Tc<sup>R</sup> genes in different environments including marine sediments (Yang J. et al., 2013), river estuary (Lin et al., 2015), or wastewater and activated sludge (Auerbach et al., 2007; Zhang and Zhang, 2011; Yang Y. et al., 2013). Moreover, Tc has been reported to be non-biodegradable in activated sludge or different conditions (Prado et al., 2009; Cetecioglu et al., 2014), so it needs special treatment for degradation in water (Wang et al., 2011) and sediments (Chang and Ren, 2015); these factors could help with the selection of Tc<sup>R</sup> bacteria.

### Prevalence of Resistance to Individual Antibiotics Among Ap<sup>R</sup> Bacterial Isolates

Resistance to Sm was the most prevalent considering all isolates (H), those from liquid (HL) or sediment (HS) phases or H1 and H2 separately. Tc<sup>R</sup> was prevalent in most of the groups of isolates obtained on the marine medium except isolates from H2S-M and HS-M, which had a slightly higher prevalence of Nx<sup>R</sup>. Likewise, Tm<sup>R</sup> was prevalent on those on the nutritive medium except for the isolates from H1S-N and HS-N for which Cz<sup>R</sup> was more prevalent. These data show that, in general, Sm<sup>R</sup>, Tc<sup>R</sup>, and Tm<sup>R</sup> were the prevalent resistances among the isolates mostly in agreement with data from the direct plating of original samples. Thus, the isolates obtained are a good representation of the estuarine culturable ARB. The quantitative differential presence of each bacterium in the original samples was partially deconvoluted when colonies were selected in the isolation step and this might account for the differences observed.

Some studies have shown that streptomycin can persist in aquatic environments for some time and its degradation products still maintain certain antibacterial activity (Shen et al., 2017). On the other hand, selection of Sm<sup>R</sup> bacteria in *in vitro* evolution experiments with low concentrations of antibiotic has been observed even after only 96 h (Spagnolo et al., 2015). Moreover, some studies have shown a relationship between heavy metal and Sm resistances (Matyar et al., 2014) or aminoglycoside resistance genes (Zhao et al., 2019) and plasmids have been found to contain determinants for co-resistance to Sm and Cu (Sundin and Bender, 1996). So, one possible reason for the high prevalence of Sm<sup>R</sup> in the estuary's isolates could be the co-selection due to the presence of Cu or other heavy metals. Also, correlations of Tc<sup>R</sup> genes prevalences with the presence of heavy metals, also present in the Huelva estuary, such as Cu, Mn, Ni, and Zn have been reported (Knapp et al., 2017). Recently, in a paleontological study of sediments contaminated with Zn, bacteria were found to share Tm<sup>R</sup> with Zn resistance, which was related to Zn concentration (Dickinson et al., 2019). The co-selection for Zn<sup>R</sup> likely might be one of the causes for the prevalence of Tm<sup>R</sup> in bacteria from the Huelva estuary. In addition to possible effects by other contaminants, different

studies have shown the coexistence, and sometimes a correlation, of AR and heavy metals resistances in waters and sediments [reviewed by Nguyen et al. (2019)].

Further molecular studies on the genetics determinants for Sm<sup>R</sup>, Tc<sup>R</sup>, and Tm<sup>R</sup> in the isolates will give insights on the causes of such high prevalence and mechanisms of co-selection.

### MDR Levels and Profiles Diversity Among Ap<sup>R</sup> Bacterial Isolates

Multi-drug resistance levels were calculated for different groups of isolates using the MAR index median as the comparative parameter. The diversity of multi-resistance profiles were assessed as the ratio number-of-profiles/number-of-isolates. These parameters could give insights on the availability of resistance's determinants, some of which might be transmissible, as well as on the diversity of putative groups of them that might be transmitted together.

Based on the diversity of colony morphologies and sample origin, 579 Ap<sup>R</sup>-bacteria were isolated to determine the level and profiles of MDR. This is one of the highest numbers of isolates analyzed in similar studies using culturable bacteria. The test of resistance to the other 10 antibiotics carried out on this collection showed diverse resistance profiles. 92.7% of the whole set of isolates were resistant to 4 or more classes of antibiotics and the median of MAR indexes corresponded to resistance to 7/11 of the tested antibiotics. These results represent outstanding levels of multi-resistance and as far as we know, one of the highest measured up to now in a non-clinical setting.

Higher MDR levels were found in isolates from sediments than from those in waters when considering all isolates from the estuary (H), or each individual zone (H1 or H2). This difference between phases could be due to the lower mobility of sediments and the possible accumulation of antibiotics, metals, or other pollutants (including ARGs) that may facilitate the selection of resistant bacterial populations, as has been proposed for other systems (Rodgers et al., 2019). Furthermore, the accumulation of resistant bacteria in sediments could facilitate bacterial cell contact; and, therefore, to increase the efficiency of HGT (Zhang et al., 2018).

The diversity of MDR profiles is higher in sediments than in waters when considering all isolates (H) or those from H1 zone alone. When isolates were segregated by the culture medium used for isolation, only those on the marine medium showed higher profile diversity in sediments than in waters.

When comparing whole sets of isolates from the two sampling zones (H1 and H2), no difference was found in the MDR level. However, when isolates were segregated by the culture medium a significant difference appeared between the isolates on the nutritive medium, with the highest value for those of H2.

Thus, in general, the isolates on the nutritive medium from sediments were resistant to more antibiotics and showed less diverse profiles than those from water samples. However, those obtained on the marine medium showed higher levels of multi-resistance and also higher profiles diversity when considering the isolates obtained from sediments versus those isolated from water samples.



To try to explain the observed differences between the levels and diversity of MDR for the isolates from sediments of H1 and H2 zones on the nutritive medium, our primary hypothesis was the more direct influence of the effluents of the WWTP on H2. This might be responsible for the higher MDR in the isolates on the nutritive medium since the effects of WWTP effluents on bacterial resistance have been shown in several studies (Guo et al., 2017; Manaia et al., 2018). Nevertheless, we noticed that a number of isolates from the sample H2S-N had the same MDR profile and had similar colony morphology. They were initially selected because they appeared in high numbers in the original selection step. When only one of these isolates was considered, the MAR median for the H2S-N sample decreased to 0.636, with no difference with H1S-N. The ratio profiles/isolates became in this way 64.7 and 46.2% for H2S-N and H2S, respectively; and were more similar to the behavior of H1 isolates. Hence, the overrepresentation of a particular bacterium with a high MAR value was biasing the MAR value of the corresponding sample. This bias stresses the need for deconvolution of the isolates to detect the real levels of resistance and diversity of MDR. We thus concluded that not much difference existed between the MDR levels of isolates on the nutritive medium from the sediments of both sampling zones.

## Biodiversity of Culturable MRB From the Estuary

16S rDNA sequences from 345 isolates were firstly compared with those available in GenBank database and assigned to eight phylogenetic classes found in both sampling areas in similar proportions. Alphaproteobacteria constituted around 50% of the isolates in each zone. This percentage suggests that the tidal movement of water in the estuary could play an essential role in the homogenization of the bacterial composition at both sampling sites. However, analysis at the genus level showed higher diversity differences and stressed the need to phylogenetically ascribe down to the lowest possible phylogenetic level to obtain a more accurate picture of diversity distribution differences. A higher difference in the classes' distribution was observed between liquid and sediment phases: Actinobacteria and Betaproteobacteria were more abundant in the sediments; and Gammaproteobacteria and Flavobacteriia in waters.

A more detailed phylogenetic analysis using trees built with sequences at RDP identified 48 genera. Although most of the genera were common to both sampling zones, some were found only in one zone, which perhaps reflects physicochemical differences between them.

The phylogenetic composition of the culturable Ap<sup>R</sup> isolates found in this study is similar to the diversity of bacterioplankton detected in other estuaries or marine environments when compared at the phylum and class levels (Selje et al., 2005; Jamieson et al., 2012; Du et al., 2013; Sjöstedt et al., 2014; Vaz-Moreira et al., 2014). However, a deeper level of phylogenetic assignation is needed in most studies to be able to make further comparisons.

The majority of the identified species have not been described as pathogens. However, some of them may be considered as opportunistic pathogens or have been found in some sporadic cases of infections (**Supplementary Table S4**). So, some health concerns should be considered for the bacteria in the estuary due to their high level of multi-resistance. Since the phylogenetic diversity of MDR bacteria found in the estuary is high, the possible dissemination of AR or MDR to diverse other bacteria is also likely.

## Intrinsic and Acquired Resistance

Intrinsic resistance is the most common form of resistance in some bacteria (Cox and Wright, 2013). In this study, the putative InR of four phyla, seven classes, and 11 genera represented by eight or more isolates were analyzed and putative InR was found in Bacteroidetes and Proteobacteria. Different results have been found in other studies (Walsh and Duffy, 2013). Hence, a selection of bacteria that depends on the presence of different substances and physicochemical conditions should have occurred in the Huelva estuary.

At the class level, Gammaproteobacteria did not show any InR, while Alphaproteobacteria, Betaproteobacteria, and Flavobacteriia showed InR to Sm. These last two also shared InR to Nx. On the other hand, Flavobacteriia and Sphingobacteriia shared InR to Km. Moreover InR to Em was only found in Betaproteobacteria and Cz in Sphingobacteriia.

Among the genera that did not show any InR, the results for *Pseudomonas* contrast with the case of clinical *P. aeruginosa*, which is intrinsically resistant to several antibiotics (Alvarez-Ortega et al., 2011; Breidenstein et al., 2011; Vaz-Moreira et al., 2012). Prevalence of antibiotic resistance in environmental *Pseudomonas* is related to the phylogenetic species level rather than to the genus level (Vaz-Moreira et al., 2012), and for environmental *P. aeruginosa* is lower than for clinical isolates (Liew et al., 2019). In a recent study (Luczkiewicz et al., 2015), *Pseudomonas* spp. were isolated from influents, effluents, and receiving marine-water from a WWTP, recovering fewer isolates from marine water than from the water from the WWTP. Furthermore, *P. putida* was the predominant species in all samples whereas *P. aeruginosa* was the second major species in the WWTP effluents (enriched from the influents water), but the latter was found in a much lower proportion in receiving water. This effect could explain the lack of this species among our isolates. Additionally, in the above-referred report, *P. putida* and *P. aeruginosa* isolates were tested for AR to 14 antibiotics, but no InR would have been found under the criterion used by us. Our isolates of the remaining analyzed genera showed putative InR to one; as *Bacillus*, *Mesonnia*, and *Ponticaulis*; or several antibiotics. The genus with the highest numbers was *Ochrobactrum* that had five InRs.

None of the phylogenetic groups showed InR to Cc or Rp, and the number of isolates resistant to these antibiotics was generally low. The percentage of Cc<sup>R</sup>-isolates reached up to 50% only for *Pseudomonas*. These low levels of resistants may be a consequence of the limited use of Cc because of its toxicity (Ingebrigtsen et al., 2017); and, therefore, the

existence of low selective pressure for this antibiotic in the environment. It has been shown that  $Rp^R$  in pathogenic bacteria has a high metabolic cost, which significantly affects the fitness of the bacteria making their ARGs uncommon (Hall et al., 2011). The fitness cost might have conditioned most isolates, but more than 80% of the *Ochrobactrum* isolates showed  $Rp^R$  and seemed to be able to work around these fitness costs.

Non-intrinsic resistances should be considered as acquired independently of the mechanism by which they were obtained (mutation or HGT).

## Correlation of the Presence of Pairs of Antibiotic Acquired Resistances in Phylogenetic Groups of Isolates

In not considering the putative InRs discussed above, the correlations of resistances to pairs of antibiotics were low when all available isolates were considered. These data indicated a low probability of cross or co-resistance to any of the tested pairs of antibiotics in the whole bacterial population. However, some moderate to almost perfect correlations were found at the class level, more frequently in Beta or Gammaproteobacteria, and strong ones at the genus level.

Strong positive correlations were observed at the genus level, particularly in *Paenibacillus*. In the network built based on  $\kappa > 0.4$  values, *Paenibacillus* appears central sharing many correlated pairs of resistances with other genera suggesting possible sharing of MGEs. Some of these elements have been described in this genus (Pednekar et al., 2010; Soundarapandian et al., 2013). The presence of these elements indicates that this genus might act as an ARG exchanger among different genera, and perhaps, plays a crucial role in the resistance dissemination in the Huelva estuary. Apart from this, an accumulation of ARGs in this genus has been described but classified as intrinsic (Pawlowski et al., 2016). Our hypothesis about *Paenibacillus*' role in the ecosystem deserves to be studied in the future because specific published data about this genus suggest a great versatility among its members. First, this genus contains a high number of species and new ones are described frequently (Sáez-Niero et al., 2017), so this genus displays a high intra-genus genetic variability. In the second place, *Paenibacillus* species have been described in a variety of environments, some of them interacting with other organisms (Finkelshtein et al., 2015; Grady et al., 2016; Xie et al., 2016), which suggests their potential for affecting ecology. Also, strains of the same species showed numerous variations in genome sequences with a high rate of recombination, which makes them prone to exchange DNA fragments by horizontal genetic exchange (Xie et al., 2016; Xu et al., 2017). Likewise, several species of the genus produce diverse antibiotic substances (Zhao et al., 2018; Pasari et al., 2019), which could make them players in the shaping of their ecosystems. Furthermore, *Paenibacillus* genomes have a plethora of genes for adaptation, some of which considered to be acquired by HGT (Xie et al., 2016; Xu et al., 2017). Moreover, some studies have considered that the species of the genus evolve not as individual species, but inter-related in a

pan-genomic evolution model (Xu et al., 2017). Finally, the resistance elements found in genomes of some *Paenibacillus* species could be the origin of resistance elements found in other genera (Guardabassi et al., 2005; Liu et al., 2016); and, thus, they show mobilization potential. All this suggests that this genus may play a crucial role in any biological system because of its capabilities to adapt and interact with other organisms. Considering that some *Paenibacillus* species can infect humans, animals, and plants and are quite ubiquitous, they could be an essential and currently unconsidered threat when present in an ecosystem, either directly or by its putative ARGs exchanger role, which could affect the ecology of the systems where they are present. A recent study by Wright's group (Pawlowski et al., 2018) also suggested a role for Paenibacillaceae bacteria in environmental mobilization and dissemination of resistance genes.

## CONCLUSION

In conclusion, the culturable bacterial population from the Huelva estuary contains highly-multi-resistant bacteria some of which have been isolated and characterized for MDR profiles to 11 antibiotics, which show a diversity of combinations of resistances. The analysis of AR of the phylogenetically ascribed isolates has shown the existence of putative InR in most of the genera. However, some other ARs may be acquired and some are perhaps transferrable. Moderated to optimal correlations of pairs of resistances in certain phylogenetic groups suggests that these resistances could be transferred among their members. In particular, *Paenibacillus* showed a large number of these correlations, many of which are shared by other genera as a network study has shown. From these results, a hypothesis for future studies can be outlined in which *Paenibacillus* could have a role as a player in the dissemination of resistance in this environment.

## DATA AVAILABILITY STATEMENT

The 16S rRNA sequences generated for this study can be found in the GenBank (accession numbers LT601033-LT601378).

## AUTHOR CONTRIBUTIONS

JA contributed to the conception and design of the study, sampling, and wrote the manuscript. BE-C, HM-F, and JA performed the experiments and analyzed the data. BE-C and JA performed the statistical analysis. All authors contributed to the manuscript revision, read and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.03071/full#supplementary-material>



- ESAC-Net Surveillance Data. Available at: [https://www.ecdc.europa.eu/sites/default/files/documents/Final\\_2017\\_EAAD\\_ESAC-Net\\_Summary-edited%20-%20FINALwith%20erratum.pdf](https://www.ecdc.europa.eu/sites/default/files/documents/Final_2017_EAAD_ESAC-Net_Summary-edited%20-%20FINALwith%20erratum.pdf) (accessed December 24, 2019).
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# Heavy Metal Toxicity in Armed Conflicts Potentiates AMR in *A. baumannii* by Selecting for Antibiotic and Heavy Metal Co-resistance Mechanisms

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*Acinetobacter baumannii* has become increasingly resistant to leading antimicrobial agents since the 1970s. Increased resistance appears linked to armed conflicts, notably since widespread media stories amplified clinical reports in the wake of the American invasion of Iraq in 2003. Antimicrobial resistance is usually assumed to arise through selection pressure exerted by antimicrobial treatment, particularly where treatment is inadequate, as in the case of low dosing, substandard antimicrobial agents, or shortened treatment course. Recently attention has focused on an emerging pathogen, multi-drug resistant *A. baumannii* (MDRAB). MDRAB gained media attention after being identified in American soldiers returning from Iraq and treated in US military facilities, where it was termed “Iraqibacter.” However, MDRAB is strongly associated in the literature with war injuries that are heavily contaminated by both environmental debris and shrapnel from weapons. Both may harbor substantial amounts of toxic heavy metals. Interestingly, heavy metals are known to also select for antimicrobial resistance. In this review we highlight the potential causes of antimicrobial resistance by heavy metals, with a focus on its emergence in *A. baumannii* in war zones.

**Keywords:** *Acinetobacter baumannii*, bacteria, heavy metals, heavy metal tolerance, antimicrobial resistance, conflict, weapons

## INTRODUCTION

### Overview on Wars and Antimicrobial Resistance (AMR)

Over the past decades, *Acinetobacter baumannii* has emerged as a major driver of hospital-acquired Multi-Drug Resistant (MDR) infections (Manchanda et al., 2010; Williams et al., 2016; Mancilla-Rojano et al., 2019). In the decades following the Second World War, *A. baumannii* became one of the most prevalent pathogens during wars in Lebanon, Afghanistan, and Iraq, causing multiple outbreaks of MDR infections among combat casualties (Tong, 1972; CDC, 2004; Scott et al., 2007; Dallo and Weitao, 2010). According to The Centers for Disease Control and Prevention (CDC), *A. baumannii* was the single most isolated Gram-negative bacterium from war wounds during the recent wars in Iraq and Afghanistan and the number one causative agent of bloodstream infections among the US soldiers (CDC, 2004; Fournier et al., 2006). Moreover, the emergence of MDR, Extensively-Drug Resistant (XDR), and Pan-Drug Resistant (PDR) *A. baumannii* coincided with specific worldwide tension points such as the Lebanese Civil War (1975–1990) (Matar et al., 1992), Iraq-Iran war (1980–1988), Afghanistan war (2001–2014), Iraq war (2003–2011), and recently the Syrian war (2011–Present) (Tong, 1972; CDC, 2004; Scott et al., 2007). Warfare is associated with significant heavy metal contamination of the environment, due to destruction of built infrastructure and consequent release of HM and direct contamination from exploded ordnance and leakage from unexploded ordnance. HM resistance in bacteria is associated with antimicrobial resistance. In this review we investigate how heavy metal resistance can lead to antimicrobial resistance with a view to illuminating the emergence *A. baumannii*'s increased resistance in war regions.

### Living Systems and Heavy Metals

Heavy metals are a group of non-biodegradable metals and semi-metals (Metalloids) with high atomic weight and a density greater than 5 g/cm<sup>3</sup>. They have high electrical conductivity, malleability, metallic luster, and are capable of transferring electrons to form Cations (Jarup, 2003; Appenroth, 2010; Tchounwou et al., 2012). The major list of heavy metals includes: Cobalt (Co), Copper (Cu), Chromium (Cr), Zinc (Zn), Lead (Pb), Mercury (Hg), Arsenic (As), Cadmium (Cd), Nickel (Ni), Antimony (Sb), Boron (B), Barium (Ba), Silver (Ag), and Tungsten (W) (ATSDR, 2011). Essential heavy metals are required in many biological processes at low concentrations, where they serve as cofactors for metalloenzymes. For example, Cu serves as an integral cofactor for cytochrome c oxidase and superoxide dismutase required in mitochondrial electron transport and oxidative stress (Osredkar and Šuštar, 2011). Zn plays role in DNA and RNA polymerases catalytic functions (Markov et al., 1999). Ni and Cr are essential for the activity of urease and cytochrome P450 enzymes (Seiler and Berendonk, 2012). On the other hand, non-essential heavy metals such as Hg, Pb, Cd, and As have no known biological functions and are toxic even at low concentrations (ATSDR, 2011; Singh R. et al., 2011; Tchounwou et al., 2012).

Sources of heavy metals include water, soil, and rocks. Furthermore, they are present in the Earth's crust and date back to 4.5 billion years ago (Fru et al., 2016). The ecosystem is continuously flooded with high amounts of heavy metals due to volcanic eruptions, soil and rock erosion, industrial operations such as petroleum combustion and coal burning, in addition to agricultural activities that include pesticides and fungicides preparation (Seiler and Berendonk, 2012; Tchounwou et al., 2012; Jaroslawiecka and Piotrowska-Seget, 2014; Pal et al., 2017). Furthermore, heavy metals have been used in medical treatments as antimicrobial and anti-parasitic agents to treat skin infections and leishmaniasis respectively; anti-inflammatory compounds to treat itchiness, and in chemotherapy to treat cancer patients (Rizzotto, 2012; Ndagi et al., 2017; Pal et al., 2017).

### Heavy Metals-Mediated Bacterial Toxicity

Essential and non-essential heavy metals become toxic when exceeding specific concentrations that vary between different metals (Appenroth, 2010). In bacteria, toxic levels of heavy metals can accumulate in cells, altering cellular processes, inducing structural modifications, and ultimately leading to heavy metals-mediated damage (Rouch et al., 1995). Mechanisms of heavy metals toxicity include the production of Reactive Oxygen Species (ROS) that destroy essential biomolecules and sub-cellular organelles. When in excess, Pb, Cd, Cu, As, Ag, and Zn can induce oxidative damage in bacterial cells which leads to the production of free radicals that cause DNA damage and destabilize membranous integrity through lipid peroxidation (Szivák et al., 2009; Jaroslawiecka and Piotrowska-Seget, 2014; Williams et al., 2016). In addition, heavy metal ions can form complexes with thiol (R-SH)-containing enzymes to alter their functions. For example, Hg<sup>2+</sup>, Ag<sup>1+</sup>, and Cd<sup>2+</sup> can form covalent bonds with sulfhydryl functional groups (R-SH) present in enzymatic active sites thereby inducing structural conformational changes, and thus blocking their function (Rouch et al., 1995). Furthermore, heavy metals can act as competitive inhibitors leading to the displacement of essential ions from their target sites (Ianeva, 2009; Jan et al., 2015).

### Heavy Metals Resistance in Bacteria

Studies have shown that the presence of heavy metal resistance determinants is ubiquitous in almost all bacterial species (Silver and Phung le, 2005). For example, Farias et al. (2015), identified 35 bacterial strains from 8 different species harboring multi-metal resistant phenotypes from deep-sea hydrothermal vents. Moreover, *A. baumannii* isolated from agricultural soil and sediments, fuel-contaminated soil, and sewage water, have shown to exhibit resistance to various metals such as Hg, Ag, and As (Dhakephalkar and Chopade, 1994; Lima de Silva et al., 2012; Farias et al., 2015; Kim et al., 2015; El-Sayed, 2016; Huang et al., 2017). To date, six proposed mechanisms of heavy metal resistance have been elucidated: (1) Release of metal ions by extracellular barriers such as capsule, cell wall, and plasma membrane. (2) Extrusion of metal ions via efflux pumps or by diffusion. (3) Intracellular sequestration of metal



ions. (4) Extracellular sequestration of metal ions. (5) Bio-transformation/detoxification of toxic metal ions. (6) Decreased sensitivity of cellular targets to metal ions. In general, heavy metal resistance-encoding genes are carried on mobile genetic elements such as plasmids and transposons or on chromosomal DNA (Rouch et al., 1995; Silver and Phung le, 2005; Ianeva, 2009; Seiler and Berendonk, 2012; Hobman and Crossman, 2014). In the following sections, we will briefly discuss these mechanisms.

### Extrusion of Metal Ions via Efflux Pumps or Diffusion

Efflux pumps are the most prevalent bacterial tools conferring heavy metals resistance. This is achieved via Adenosine triphosphate (ATP) hydrolysis and/or through an electrochemical gradient of protons (Ianeva, 2009). Five major efflux system families are present in microorganisms: (1) ATP Binding Cassettes (ABC) family. (2) Resistance, Nodulation, Cell Division (RND) family. (3) Small Multi-Drug Resistance (SMR) family. (4) Multi-Drug and Toxic Compounds Efflux (MATE) transporters. (5) Major Facilitator Superfamily (MFS) family. These pumps differ in their amino acid sequence, substrate specificity, and energy consumption in pumping metal ions. For example, ATP Binding Cassettes (ABC) play a role in the efflux of metal ions and antimicrobial agents driven by ATP hydrolysis, while RNDs and SMRs pump out metal cations and antimicrobial agents via Chemiosmosis/Proton motive force (Silver and Phung le, 2005; Kourtesi et al., 2013; Abbas et al., 2017). Basal levels of efflux are usually not sufficient to confer heavy metal resistance in most bacterial species. However, changes in expression, either through mutations in promoter regions or efflux pump regulators, or inactivation of repressors, can lead to the over-expression of an efflux pump or confer resistance (Li et al., 2015; Blanco et al., 2016).

### Intracellular Sequestration of Metal Ions

One important resistance mechanism involves intracellular metal ions sequestration upon binding to metal ions binding proteins [Metallothioneins (MTs), Glutathione (GSH), and Metallochaperones] (Ianeva, 2009). For example, Ni can complex with  $\text{PO}_4^{3-}$ , leading to its intracellular precipitation. *Staphylococcus aureus*, *Providencia* spp., *Vibrio harveyi*, *Shewanella* spp., and *Bacillus* spp., can precipitate Pb as phosphate salts (Levinson et al., 1996; Smeaton et al., 2009; Shin et al., 2012). Cd, Hg, Ag, Pb, and Zn can be trapped on cysteine-rich MT polypeptides that provide tolerance to high concentrations of heavy metals. For example, *Synechococcus* spp., *Pseudomonas* spp., and *Anabaena* spp. tolerate high concentrations of heavy metals via MT trapping mechanisms (Olafson et al., 1988; Naik et al., 2012). Some bacteria use GSH as an alternative chelator to sequester metal ions. GSH scavenges and detoxifies metals via its thiol (R-SH) group. Lima et al. (2005), demonstrated the role of GSH in mediating tolerance to Cd in *Rhizobium leguminosarum*. Finally, metallochaperones like Cu chaperones ( $\text{Cu}^{1+}$  binding chaperone CusF,  $\text{Cu}^{1+}$ , and  $\text{Cu}^{2+}$  periplasmic chaperones PcoC and PcoE) can bind, trap, and transport metal ions to metalloenzymes and thus, decrease their toxic effects and protect cellular compartments (Yang et al., 2010; Pal et al., 2017).

### Extracellular Sequestration of Metal Ions

In addition to intracellular sequestration, extracellular sequestration of heavy metals is an additional mechanism conferring bacterial resistance. This strategy provides a “Pre-defense” strategy as it occurs outside the bacterial cell. It involves the secretion of extracellular chelating proteins such as siderophores, oxalateoxalate, phosphate, and sulfide. However, this mechanism is mainly active in static environments when constant concentrations of heavy metals are present (Cunningham et al., 1993; Rouch et al., 1995). For example, *Streptomyces acidiscabies* can sequester Ni via hydroxamate siderophores while *Clostridium thermoaceticum* use sulfide to sequester Cd (Cunningham et al., 1993; Dimkpa et al., 2008).

### Bio-Transformation/Detoxification of Toxic Metal Ions

Enzymatic detoxification reduces metal toxicity, which is accomplished via oxidation, reduction, and methylation reactions (Rouch et al., 1995; Silver and Phung le, 2005). For example,  $\text{Hg}^{2+}$  is reduced to a less toxic  $\text{Hg}^0$  form via Mercury(II) reductase encoded by *merA* gene (Seiler and Berendonk, 2012; Pal et al., 2017). In addition, upon bacterial entry  $\text{Cr}^{6+}$  is reduced to  $\text{Cr}^{3+}$  while  $\text{As}^{3+}$  is oxidized to  $\text{As}^{5+}$  thus, decreasing their toxicity (Silver and Phung le, 2005). Interestingly, *Pseudomonas* spp., and *Acinetobacter* spp., induce Pb methylation to minimize its toxic effects. To date, Hg methylation has only been only documented in anaerobic bacteria (Parks et al., 2013; Jaroslawiecka and Piotrowska-Seget, 2014).

### Decreased Sensitivity of Cellular Targets to Metal Ions

Reducing sensitivity of cellular targets to metal ions is a way to minimize heavy metals toxicity. This is fulfilled via several mechanisms: (1) Decreasing bacterial susceptibility to metals by introducing mutations in resistance genes or determinants, or increasing the expression of the metal target site. (2) Producing a more resistant form of the metal target site upon activating an alternative target encoded on a plasmid. (3) Repairing DNA damage upon the activation of an SOS response which is the case of Cr-induced DNA lesions (Frohlich, 2013; Maret, 2015).

### Heavy Metals in Weapons

Explosives harbor huge amounts of Pb and Hg [Mercury(II) fulminate] (Navy U. S., 2008; Gebka et al., 2016). Zn, Cu, Ni, Pb, and Cr are used to coat bullets, missiles, gun barrels, and military vehicles (Audino, 2006; Casey, 2009). Ba, Sb, and B are weapon priming compounds, (Fitchett, 2019) and W is a kinetic bombardment due to its high density ( $19.3 \text{ g/cm}^3$ ) (Rowlatt, 2014). In general, the use of heavy metals in weapons has increased since the end of World War II (Gebka et al., 2016).

### Known Resistance Mechanisms to Heavy Metals Frequently Found in Ordnance

#### Copper (Cu)

Copper (Cu) exists in nature as a free metallic element or alternates between 2 oxidative states  $\text{Cu}^{1+}$  and  $\text{Cu}^{2+}$ . In

humans, Cu is essential for blood vessel elasticity, brain development, maintenance of immune responses, and neurotransmitter production (Dorsey et al., 2004; Copper Development Association, 2018). Excess concentrations of Cu lead to kidney and liver damage, neurological, and immune diseases (Dorsey et al., 2004). In wars, Cu, Ni, Pb, and Cr are heavily used as coatings for bullets, missiles, gun barrels, and in military vehicles (tanks, trucks, and aircrafts). This could increase exposure to Cu in wartime and might explain increased observation of *A. baumannii* in studies conducted in these settings (Davis et al., 2005; Calhoun et al., 2008; Williams et al., 2016). Extensive studies on *Escherichia coli* and *Pseudomonas* spp. reveal four Cu homeostatic resistance systems: Cue, Cus, Pco, and Cop. Cue and Cus are chromosomally encoded efflux systems while Pco and Cop are plasmid encoded resistance systems (Pal et al., 2017).

### Cue System

The Cue system (Copper Efflux) is active at low Cu concentrations and under aerobic conditions. It consists of an inner membranous  $\text{Cu}^{1+}$  exporting P-type ATPase (CopA) and a periplasmic multi-Cu Oxidase (CueO). *copA* and *cueO* are activated by a cytoplasmic transcriptional Cu-responsive Regulon (CueR) upon sensing increased Cu concentrations (Hobman and Crossman, 2014; Delmar et al., 2015; Pal et al., 2017; **Figure 1**).

### Cus System

Unlike the Cue system, the Cus efflux system is active at high Cu concentrations, is strictly anaerobic, and pumps out Cu and Ag cations (Silver and Phung le, 2005; Singh S.K. et al., 2011; Delmar et al., 2015). It detoxifies Cu in the periplasmic compartment, unlike the Cue system that extrudes periplasmic and cytoplasmic Cu (Jaroslawska and Piotrowska-Seget, 2014). The Cus system consists of 4 genes forming the *cusCFBA* operon, which is regulated by a two-component regulatory system (CusS/CusR) (Franke et al., 2003; Silver and Phung le, 2005; Pal et al., 2017). CusS, a histidine kinase, is activated upon Cu/Ag stimulation, while CusR is a DNA-binding transcriptional activator that activates *cusCFBA* expression. CusC, CusB, and CusA form a multi-Cu/Ag efflux pump (CusCBA) that functions as a proton-antiporter.  $\text{Cu}^{1+}/\text{Ag}^{1+}$  are transported to CusCBA via the periplasmic metallo-chaperone, CusF (Franke et al., 2003; Silver and Phung le, 2005; Pal et al., 2017). Cu resistance via the Cue and Cus systems are detailed in **Figure 1**.

### Pco System

The *E. coli*-resistant Pco system (Plasmid-borne-Copper Resistance) found in Cu-fed pigs consists of two operons, *pcoGFE*, and *pcoABCD* that are encoded by a 9-10 gene cluster (Brown et al., 1995). Like the Cus system, the Pco system is regulated by a two-component regulatory system (PcoR/PcoS) (Brown et al., 1995; Pal et al., 2017). To actively function, the Pco system requires the action of CopA from the Cue system in addition to PcoA and PcoC. PcoA is a multi-Cu Oxidase that oxidizes  $\text{Cu}^{1+}$  to  $\text{Cu}^{2+}$ , while PcoC is a periplasmic Cu-binding protein that acts as a chaperone which delivers

$\text{Cu}^{1+}$  to CopA during oxidation and to PcoD. PcoD is an inner membrane Cu transporter that is involved in Cu uptake. PcoB and PcoE are an outer membrane transporter and a metallo-chaperone, respectively (**Figure 2**; Lee et al., 2002; Bondarczuk and Piotrowska-Seget, 2013; Pal et al., 2017).

### Cop System

The Cop system is encoded by a cluster of 6 plasmid-borne genes arranged in two operons, *copABCD* and *copRS* (Pal et al., 2017). *copABCD* and *copRS* are homologs of *pcoABCD*. The Cop determinants are genetically associated with the Cop system and have similar roles. *copABCD* is under the regulation of CopR/CopS. Protein products are associated with Cu sequestration in the periplasm and outer membrane (Bondarczuk and Piotrowska-Seget, 2013; Pal et al., 2017). In *Cupriavidus metallidurans* CH34 and *E. coli*, Cu ions can be sequestered by CusF in the periplasm, exported by the RND-driven CusCBA efflux pump, or oxidized to  $\text{Cu}^{2+}$  (Nies, 2016).

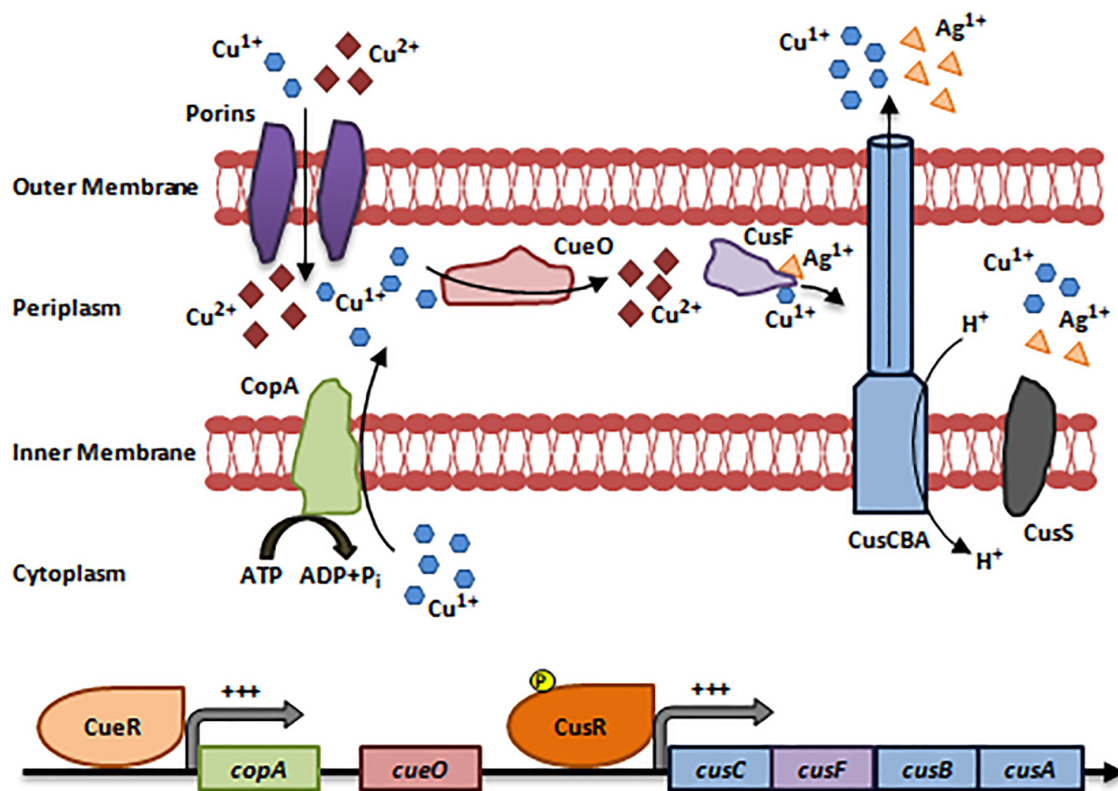
### Mercury (Hg)

Mercury (Hg) is released into the environment via geological and human activities such as soil and rock erosion, volcanic eruptions, mining, and fuel combustion (Tchounwou et al., 2012). In the wake of recent conflicts in Lebanon, Syria, Iraq, Yemen, and Afghanistan, the Middle East has become one of the most polluted regions with Hg (Gworek et al., 2017). This may have led to an increased bacterial tolerance to this metal. In humans, Hg has no biological role and at very low concentrations it is fatal, leading to brain, lung, and kidney failure (Risher and Rob, 1999).

Hg can access bacteria in 2 forms: organic ( $\text{CH}_3\text{-Hg}^+$ ) and inorganic ( $\text{Hg}^{2+}$ ), both of which are toxic (Hobman and Crossman, 2014). Despite this toxicity, several bacterial species have developed resistance mechanisms to  $\text{CH}_3\text{-Hg}^+/\text{Hg}^{2+}$  via the *mer* operon, and are found mainly in war zone regions (Mirzaei et al., 2008; Pérez-Valdespino et al., 2013; Hobman and Crossman, 2014). The *mer* operon is present on plasmids and transposons and consists of a cluster of 8 genes *merTPCAGBDE* regulated by MerR (Silver and Phung le, 2005; Hobman and Crossman, 2014). This operon encodes a chain of proteins that bind  $\text{CH}_3\text{-Hg}^+/\text{Hg}^{2+}$  and oxidize them, such as MerA [Mercury(II) Reductase], the key player in  $\text{Hg}^{2+}$  detoxification (**Figure 3**).

### Arsenic (As)

Arsenic (As) is released into the environment from soil and rock erosion, volcanic eruptions, mining, and crops treated with pesticides and herbicides (Paez-Espino et al., 2009; Tchounwou et al., 2012). In 1918, two organic As compounds, Lewisite ( $\text{C}_2\text{H}_2\text{AsCl}_3$ ) and Adamsite ( $\text{C}_{12}\text{H}_9\text{AsClN}$ ) were developed by the US army as chemical weapons; both are classified as potential bioterrorism agents by CDC (2013). Agent Blue, an arsenical mixture of cacodylic acid and sodium cacodylate was sprayed by the United States on crops as part of “resource deprivation” strategies in the Vietnam war beginning in 1962 (Radke et al., 2014). The use of chemical weapons in the Syrian Civil War has been confirmed by the United Nations. This resulted in increased bacterial resistance to As via oxidation, reduction,



**FIGURE 1 |** Resistance to Copper via the Cue and Cus systems. The Cue system is activated at low Cu concentrations and under aerobic conditions.  $\text{Cu}^{1+}/\text{Cu}^{2+}$  enter the bacterial cells via non-specific porin proteins. CueR senses an increase in intracellular Cu concentrations and activates the expression of *copA* and *cueO*. Then, CopA translocate  $\text{Cu}^{1+}$  ions into the periplasm thus, protecting Cu-sensitive cytoplasmic compartments. In the periplasm, CueO oxidizes  $\text{Cu}^{1+}$  ions to the less toxic form  $\text{Cu}^{2+}$ . The Cus efflux system is activated at high Cu concentrations, is strictly anaerobic, and pumps out Cu and Ag ions.  $\text{Cu}^{1+}/\text{Ag}^{1+}$  ions enter the periplasm and induce the activation of CusS, which in turn phosphorylates and activates CusR. CusR induces the expression of *cusCFBA* operon. The protein products CusC, CusB, and CusA form a multi-Cu/Ag efflux pump (CusCBA) that pumps out  $\text{Cu}^{1+}/\text{Ag}^{1+}$  ions after being transferred by the CusF metallochaperone (Franke et al., 2003; Delmar et al., 2015; Pal et al., 2017). Adapted and modified with permission from Pal et al. (2017).

methylation, efflux, and intracellular sequestration on cysteine-rich peptides (Silver and Phung le, 2005; Paez-Espino et al., 2009), and was associated with detrimental health effects ranging from cardiovascular disease, respiratory disorders, gastro-intestinal symptoms, hematological disorders, diabetes, neurological, and developmental anomalies (Chou et al., 2007; Tchounwou et al., 2012).

As exists in two chemical forms: inorganic and organic. Inorganic As occurs as pentavalent Arsenate ( $\text{As}^{5+}$ ), trivalent Arsenite ( $\text{As}^{3+}$ ), elemental Arsenic ( $\text{As}^0$ ), and Arsenide ( $\text{As}^{3-}$ ) with  $\text{As}^{3+}$  and  $\text{As}^{5+}$  being the most toxic inorganic forms and most prevalent in nature (Nies, 1999; Paez-Espino et al., 2009; Tchounwou et al., 2012). Organic As is less toxic than inorganic arsenicals (Chou et al., 2007). Bacterial resistance to As is mainly encoded by efflux via the *ars* operon, which can be plasmid or chromosomally driven, even though it can also be encoded by other genetic determinants such as *arr* genes and *aox* genes (Figure 4; Silver and Phung le, 2005; Paez-Espino et al., 2009).

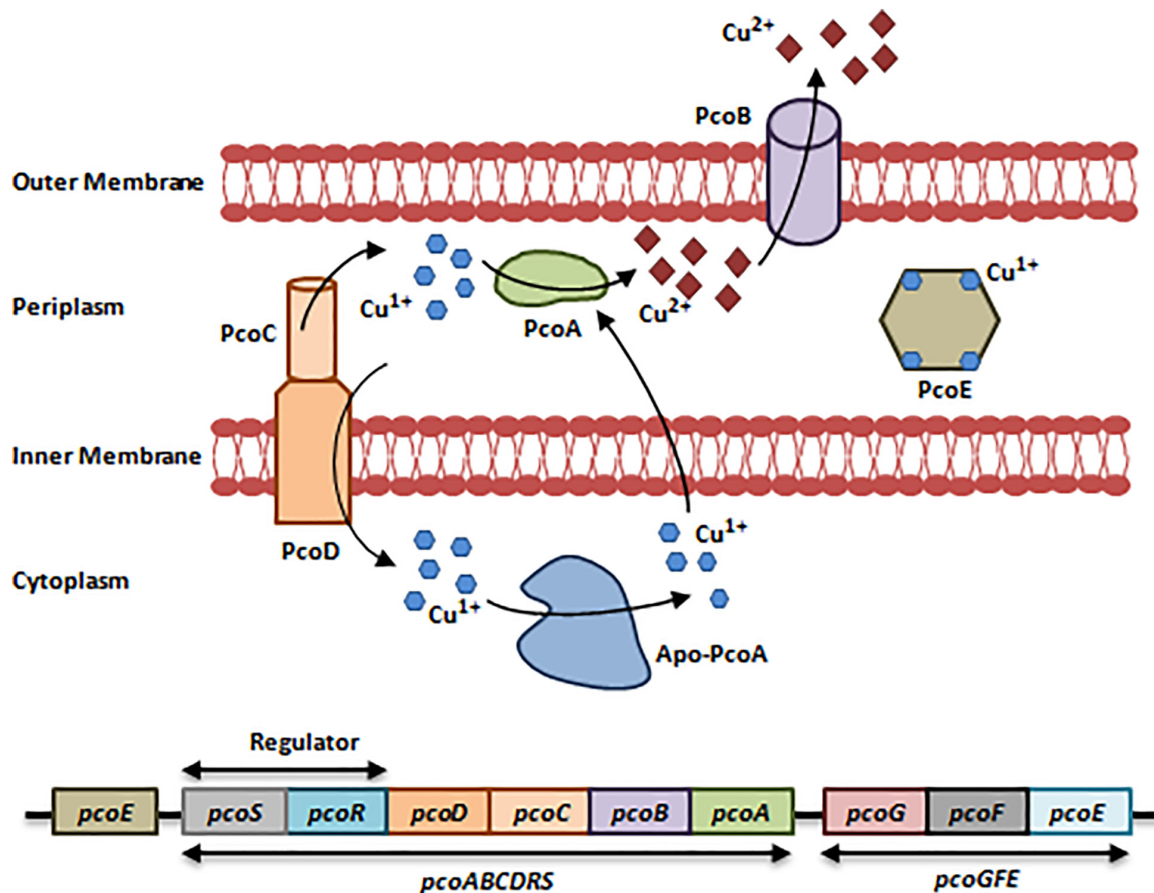
## Chromium (Cr)

Chromium (Cr) is the 7th most abundant heavy metal in the earth's crust and is present in nature in several oxidation states

ranging from divalent (+2) to hexavalent (+6)  $\text{Cr}^{3+}$  and  $\text{Cr}^{6+}$  are the most stable. While  $\text{Cr}^{3+}$  is naturally present in the environment,  $\text{Cr}^{6+}$  is mostly produced by industrial processes such as mining, electroplating, dye production, and leather tanning (Ahemad, 2014; Joutey et al., 2015; Pradhan et al., 2016). In weapons, Cr was initially used by the Chinese to coat metal weapons (Dunham, 2019). Nowadays, Cr is heavily used to coat gun barrels, where it is used as a bore protection (Audino, 2006). Moreover, Cr levels were highest in deciduous teeth from Iraqi patients during the Iraqi war, which highlights the heavily polluted Middle Eastern region with heavy metals (Savabieasfahani et al., 2016). The solubility and oxidizing potential of  $\text{Cr}^{6+}$  makes it 1000× more toxic to humans as compared to  $\text{Cr}^{3+}$ , and this makes it a strong factor associated with nasal and bronchogenic carcinomas (International Agency for Research on Cancer [IARC], 1990; Ahemad, 2014).

In bacteria, Cr has no metabolic role and thus, it is toxic in several species such as *Pantoea* spp., *Aeromonas* spp., *Acinetobacter* spp., and *E. coli* (Nies, 1999). However, many bacteria developed Cr resistance via 5 reported mechanisms that are mostly plasmid encoded (Camargo et al., 2005; Joutey et al., 2015). (1) Reduction of  $\text{Cr}^{6+}$  uptake. (2)  $\text{Cr}^{6+}$  efflux.





**FIGURE 2 |** Resistance to Copper via the Pco system. The Pco system consists of 2 operons, *pcoGFE* and *pcoABCDRS* in addition to a single gene *pcoE*. This system cannot function independently; it requires the activity of the Cue system and CopA in specific to induce resistance to Cu, which is heavily present in bullets, missiles, gun barrels, and in military vehicles. First,  $\text{Cu}^{1+}/\text{Cu}^{2+}$  enter the bacterial cell via non-specific porin proteins. PcoD transports  $\text{Cu}^{1+}$  into the cytoplasm.  $\text{Cu}^{1+}$  is toxic in the cytoplasm; Apo-PcoA transports  $\text{Cu}^{1+}$  back to the periplasm and PcoR/PcoS senses an increase in Cu concentrations and in turn induces the expression of *pcoGFE* and *pcoABCDRS*. In addition to Apo-PcoA, the periplasmic Cu-chaperone PcoC transports  $\text{Cu}^{1+}$  to the periplasm, where CopA from the Cue system and PcoA oxidize  $\text{Cu}^{1+}$  to the less toxic form  $\text{Cu}^{2+}$ .  $\text{Cu}^{2+}$  ions are expelled out via the PcoB efflux pump. Finally, PcoE is a metallochaperone, which is believed to provide initial bacterial resistance to Cu upon its entry through sequestering  $\text{Cu}^{1+}$  ions until the activation of the Pco system is fulfilled (Bondarczuk and Piotrowska-Seget, 2013; Pal et al., 2017). Adapted and modified with permission from Pal et al. (2017).

(3) Activation of oxidative stress related enzymes. (4) Repairing DNA damage induced by  $\text{Cr}^{6+}$  and its derivatives. (5)  $\text{Cr}^{6+}$  reduction (Figure 5; Branco et al., 2008; Panda and Sarkar, 2012; Ahemad, 2014; Joutey et al., 2015; Pradhan et al., 2016).

### Lead (Pb)

Lead (Pb) is predominantly released into the environment from human activities such as manufacturing pipes, X-ray shields, lead-acid storage batteries, munitions, and bullets (Abadin et al., 2007; Jaroslawska and Piotrowska-Seget, 2014). It exists in two main oxidative states ( $\text{Pb}^{2+}$  and  $\text{Pb}^{4+}$ ). In addition to bullets, Pb is present in explosives that ignite gunpowder. It usually vaporizes upon firing and thus, Pb fumes and dust are inhaled, leading to brain damage, anemia, and high blood pressure (Abadin et al., 2007; Dermatas and Chrysochoou, 2007).

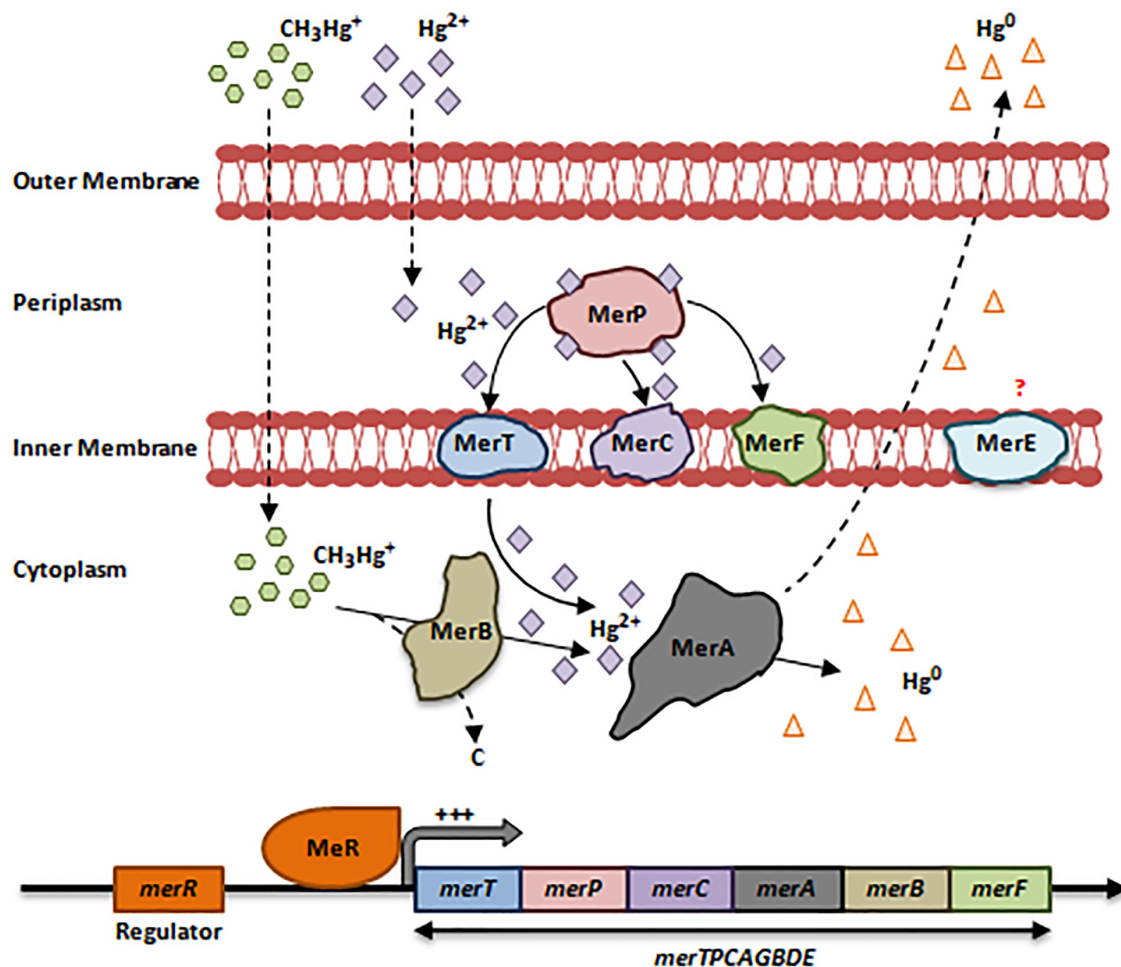
Pb toxicity involves inducing cellular damage through ROS formation, disrupting enzymatic conformations, and interfering in calcium (Ca) metabolism (Tchounwou et al., 2012). Due

to the widespread Pb contamination, bacteria have developed Pb resistance mechanisms (Levinson et al., 1996; Jaroslawska and Piotrowska-Seget, 2014). (1) Adsorption of Pb on EPS and bacterial cell wall. (2) Reducing Pb accumulation via intracellular and extracellular precipitation. (3) Pb sequestration via intracellular proteins. (4) Pb detoxification via methylation. (5) Pb extrusion via efflux pumps (Figure 6; Levinson et al., 1996; Silver and Phung le, 2005; Jaroslawska and Piotrowska-Seget, 2014; Subramanian, 2018).

## Association Between Heavy Metals and AMR

Worldwide concerns about heavy metal contamination, resistance, and its ability to induce AMR are increasing. These concerns are associated with the heavy metals used in manufacturing weapons; most heavy metals are non-biodegradable and persist in the environment. Moreover, many





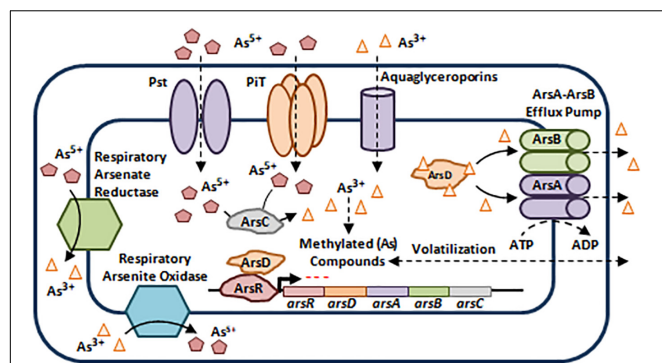
**FIGURE 3 |** Bacterial resistance to mercury. The *mer* operon consists of a cluster up to 8 genes *merTPCAGBDE*. Upon the entry of the inorganic form of Hg ( $\text{Hg}^{2+}$ ) via non-specific porin proteins, the first protein to bind it is MerP, a small periplasmic chaperone. MerP transports  $\text{Hg}^{2+}$  to MerT, MerC or MerF, which are inner membranous mercuric ions-binding proteins that in turn transport  $\text{Hg}^{2+}$  to the cytoplasm. *merT* is the most prevalent gene within the *mer* operon as compared to *merC* and *merF*. In the cytoplasm, MerA detoxifies  $\text{Hg}^{2+}$  ions through reduction-catalyzed volatilization process to a non-toxic elemental form  $\text{Hg}^0$ . This form is volatile at room temperature; it diffuses outside the membranes allowing the bacterial cell to escape Hg toxicity (Nies, 1999; Silver and Phung le, 2005; Boyd and Barkay, 2012; Hobman and Crossman, 2014). MerE is an inner membranous protein of unknown function (Silver and Phung le, 2005). In Gram-negative bacteria, the *mer* operon is regulated by MerR, which is in turn activated by increased  $\text{Hg}^{2+}$  levels in the cytoplasm. This induces the expression of the whole *merTPCAGBDE* operon (Boyd and Barkay, 2012; Hobman and Crossman, 2014). Resistance to the organic form of Hg ( $\text{CH}_3\text{-Hg}^+$ ) is achieved by *merB*, which encodes an Organomercurial Lyase (MerB) located in the cytoplasm. When  $\text{CH}_3\text{-Hg}^+$  enter the cytoplasm via non-specific porin proteins, MerB cleaves the Mercury-Carbon bond and releases  $\text{Hg}^{2+}$  in the cytoplasm. At this point,  $\text{Hg}^{2+}$  is reduced to  $\text{Hg}^0$  that diffuse outside the bacterial cell (Nies, 1999; Boyd and Barkay, 2012; Hobman and Crossman, 2014; Pal et al., 2017). Adapted and modified with permission from Silver and Phung le (2005), Boyd and Barkay (2012) and Pal et al. (2017).

bacterial species evolved resistance mechanisms to combat metals toxicity (Seiler and Berendonk, 2012; Yu et al., 2017). These mechanisms are encoded by resistant genes to heavy metals and antimicrobial agents that are physically linked on mobile genetic elements (Seiler and Berendonk, 2012; Yu et al., 2017). More importantly, heavy metals can induce selective pressure on microbial populations leading to antimicrobial resistance through a mechanism called “co-selection” which occurs via 3 major ways (Seiler and Berendonk, 2012).

## Co-resistance

Co-resistance occurs when genes encoding resistance to heavy metals and antimicrobial agents are physically linked/located

in close proximity to each other on mobile genetic elements such as plasmids, genomic islands (GIs), transposons, or integrons (Trevors and Oddie, 1986; Seiler and Berendonk, 2012; Yu et al., 2017). For example, in Cu-resistant *Enterococcus faecium* isolated from pigs, *tcvB* and genes encoding resistance to erythromycin and vancomycin are encoded on the same conjugative plasmid (Hasman and Aarestrup, 2002; Silveira et al., 2014). Moreover, in *Serratia marcescens*, plasmid-borne resistance to chloramphenicol, kanamycin, and tetracycline is genetically linked to As, Cu, Hg, and Ag resistance genes (Gilmour et al., 2004). Interestingly, Whole-Genome Sequencing (WGS) analysis in *Salmonella typhi* reveal a genetic association between Hg resistance and several unrelated antimicrobial agents



**FIGURE 4 |** Bacterial resistance to arsenic. (As) enter the bacterial cell using Phosphate-Specific Transporters (Pst) and Type III Transporters (PIT) in the case of  $\text{As}^{5+}$  and Aquaglyceroporins in the case of  $\text{As}^{3+}$  (Paez-Espino et al., 2009). The *ars* operon harbors 3 co-transcribed core genes that confer resistance not only to  $\text{As}^{3+}$  and  $\text{As}^{5+}$ , but also to Antimony ( $\text{Sb}^{3+}$ ). *arsR* encodes a Transcriptional Repressor, *arsC* encodes a Cytoplasmic Arsenate Reductase, and *arsB* encodes a membrane bound Arsenite Efflux Pump. Two additional genes may be present within the *ars* operon, *arsA* and *arsD*. The former encodes an intracellular ATPase which binds *ArsB* to form an *ArsA-ArsB* ATPase Efflux Pump, while the latter is a metallochaperone that binds and delivers  $\text{As}^{3+}$  and  $\text{Sb}^{3+}$  to *ArsA-ArsB* complex for efflux, in addition to its role as a trans-activating co-repressor of the *ars* operon along with *ArsR* (Silver and Phung le, 2005; Paez-Espino et al., 2009; Hobman and Crossman, 2014). Moreover, some microorganisms escape As toxicity by methylation thus, leading to the production of less toxic and volatile derivatives that diffuse outside the bacterial cell (Paez-Espino et al., 2009). Besides As toxicity, bacteria belonging to the *Shewanella* spp., *Sulfurospirillum* spp., *Clostridium* spp., and *Bacillus* spp., use  $\text{As}^{5+}$  as a final electron acceptor during anaerobic respiration by reducing it to  $\text{As}^{3+}$ , while other bacteria use  $\text{As}^{3+}$  as an electron donor and oxidize it to  $\text{As}^{5+}$  during aerobic oxidation (Paez-Espino et al., 2009). The oxidation/reduction processes are mediated by the *arsAB* operon and *asoAB* genes respectively (Silver and Phung le, 2005; Paez-Espino et al., 2009). Adapted and modified with permission from Paez-Espino et al. (2009).

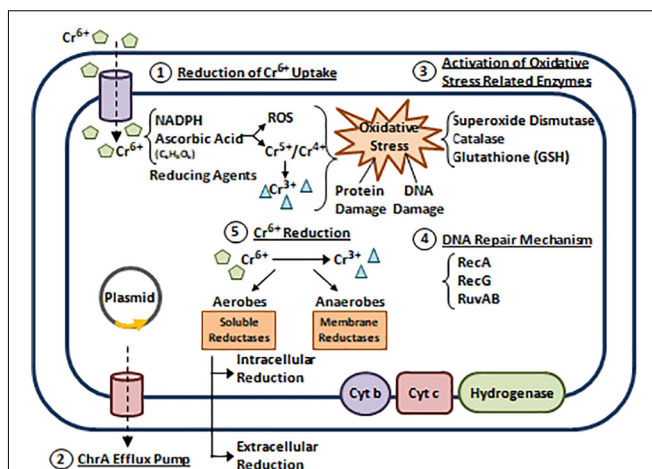
resistance genes (chloramphenicol, ampicillin, streptomycin, sulfonamide, and trimethoprim) (Wireman et al., 1997).

## Cross-Resistance

Cross-resistance occurs when one resistance mechanism confers resistance to heavy metals and antimicrobial agents simultaneously. This is mainly achieved via multi-drug efflux pumps (Baker-Austin et al., 2006; Pal et al., 2017). The MdrL efflux pump in *Listeria monocytogenes* encodes resistance to Zn, Co, Cr, erythromycin, josamycin, and clindamycin (Mata et al., 2000). Moreover, the DsbA-DsbB (Disulfide Bond) multi-drug efflux system in *Burkholderia cepacia* induces cross-resistance to  $\beta$ -lactams, kanamycin, erythromycin, novobiocin, ofloxacin, and  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  metal ions (Hayashi et al., 2000). In addition, resistance to antimicrobial agents, Co, and Cu is mediated via the CmeABC multi-drug efflux pump in *Campylobacter jejuni* (Lin et al., 2002).

## Co-regulatory Resistance

Co-regulation is the least common mechanism of co-selection. It is fulfilled when resistant genes to antimicrobial agents and

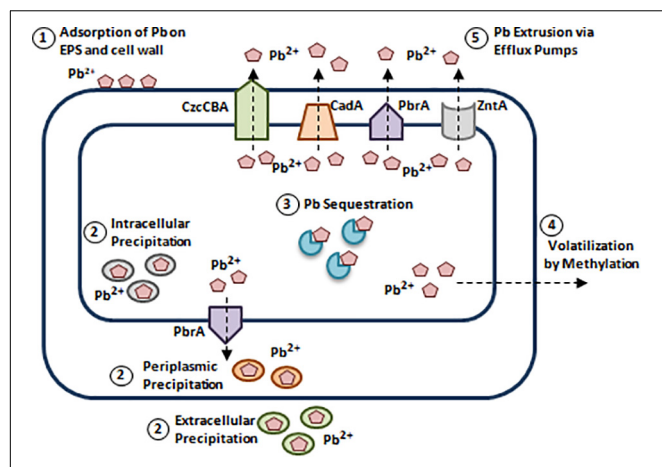


**FIGURE 5 |** Bacterial resistance to chromium. Five Cr resistance mechanisms are reported (Camargo et al., 2005; Joutey et al., 2015). (1) Reduction of  $\text{Cr}^{6+}$  uptake.  $\text{Cr}^{6+}$  exists in the form of Oxyanions Chromate ( $\text{CrO}_4^{2-}$ ) and Dichromate ( $\text{Cr}_2\text{O}_7^{2-}$ ). Bacterial cells can reduce  $\text{Cr}^{6+}$  uptake via the sulfate transport system (Nies, 1999; Ahemad, 2014; Joutey et al., 2015). (2)  $\text{Cr}^{6+}$  efflux. Studies reveal that *P. aeruginosa* and *Alcaligenes eutrophus* can extrude  $\text{Cr}^{6+}$  by active efflux through *ChrA* (Chromate) pump (Collard et al., 1994; Alvarez et al., 1999). In 2008, a plasmid encoded operon (*chrBACF*) was identified in *Ochrobactrum tritici* responsible for Cr efflux, where *chrB* and *chrA* are the main genes involved (Branco et al., 2008). (3) Activation of oxidative stress related enzymes. When  $\text{Cr}^{6+}$  enter the bacterial cell, it interacts with reducing agents such as Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and Ascorbic Acid to produce free radicals and unstable Cr intermediates ( $\text{Cr}^{4+}$  and  $\text{Cr}^{5+}$ ) that are further reduced to  $\text{Cr}^{3+}$ . End products of these reactions cause oxidative stress leading to protein and DNA damage. This induces the up-regulation of antioxidants enzymes that scavenge ROS and protect cellular compartments (Ahemad, 2014; Joutey et al., 2015; Pradhan et al., 2016). (4) Repairing DNA damage induced by  $\text{Cr}^{6+}$  and its derivatives. This is achieved via SOS response activation. Several studies highlight the roles of RuvAB, RecA, and RecG (helicases) in mediating Cr resistance through repairing  $\text{Cr}^{6+}$  induced DNA damage (Miranda et al., 2005; Morais et al., 2011). (5)  $\text{Cr}^{6+}$  reduction.  $\text{Cr}^{6+}$  can be reduced aerobically or anaerobically to a less toxic form  $\text{Cr}^{3+}$ . Aerobic reduction uses cytoplasmic soluble reductases and NADPH, while anaerobic reduction uses membrane reductases belonging to the electron transport chain (cytochromes b and c, and hydrogenases) (Morais et al., 2011; Ahemad, 2014; Joutey et al., 2015). Adapted and modified with permission from Ahemad (2014).

heavy metals are controlled by a mutual regulatory protein (Pal et al., 2017). A very well characterized co-regulatory resistance system is the CzcS-CzcR two component regulatory system of *P. aeruginosa*. This system induces resistance to  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Co}^{2+}$  by activating the expression of *czcCBA* (Cobalt Zinc Cadmium) efflux pump and to the carbapenem imipenem by suppressing expression of the *OprD* porin encoding gene (Perron et al., 2004).

## Whole-Genome Sequencing and Heavy Metal Resistance

Many reports highlighted the importance of WGS as an effective tool to detect genome-wide modifications and the emergence of heavy metal resistance genes. Nowadays, it is feasible to assess the entire bacterial genome at low costs and in a timely manner, making it an ideal method for AMR surveillance. Therefore, WGS



**FIGURE 6 |** Bacterial resistance to lead. Bacterial species such as *Pseudomonas* spp., and *Acinetobacter* spp., developed Pb resistance mechanisms. (1) Adsorption of Pb on EPS and bacterial cell wall. Structures like cell wall and extracellular polymers can adsorb Pb<sup>2+</sup> due to the presence of negatively charged functional groups [Carboxyl (C(=O)OH), Hydroxyl (R-OH), and Phosphate groups (PO<sub>3</sub><sup>3-</sup>)] (Jaroslawska and Piotrowska-Seget, 2014). (2) Reducing Pb accumulation via intracellular and extracellular precipitation. *S. aureus*, *Providencia* spp., and *Pseudomonas* spp., can precipitate Pb intracellularly in the form of Lead(II) phosphate [Pb<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>], while in *Citrobacter freundii*, extracellular Pb precipitation is mediated by phosphatase. In addition to intracellular and extracellular precipitation, periplasmic precipitation of Pb involves adsorption to polymers present in the cell wall (al-Aoukaty et al., 1991; Levinson et al., 1996; Jaroslawska and Piotrowska-Seget, 2014). (3) Pb sequestration via intracellular proteins. Pb binding-MTs were reported in Pb resistant *P. aeruginosa* strain WI-1 and *Providencia vermicola* strain SJ2A. This is mediated by a plasmid-borne MT encoding gene, *bmtA* responsible for Pb sequestration (Naik et al., 2012; Sharma et al., 2017). (4) Pb detoxification via methylation. Methylation of Pb is documented in *Acinetobacter* spp., *Pseudomonas* spp., *Aeromonas* spp., and others. Arctic marine bacteria convert inorganic Pb to tri-methyl-lead (C<sub>3</sub>H<sub>3</sub>Pb), while *Acinetobacter* spp., convert it to tetra-methyl derivatives (Wong et al., 1975; Jaroslawska and Piotrowska-Seget, 2014). (5) Pb extrusion via efflux pumps. Pb efflux is mediated by P-type ATPases such as, CadA of *S. aureus*, ZntA of *E. coli*, and PbrA of *Cupriavidus metallidurans* and to a lower extent by RND/CBA chemiosmotic transporters. CadA, ZntA, and PbrA are homologous P-type ATPases that can pump out Pb<sup>2+</sup>, Zn<sup>2+</sup>, and Cd<sup>2+</sup> (Leedj  r et al., 2007; Jaroslawska and Piotrowska-Seget, 2014). Adapted and modified with permission from Jaroslawska and Piotrowska-Seget (2014).

provides a practical solution to evaluate genomes and determine resistant genes for compounds that are not frequently assessed. Moreover, this tool allows scientists to discover novel resistance mechanisms and provides valuable information to researchers and clinicians in antimicrobial prescriptions (Khromykh and Solomon, 2015; Jagadeesan et al., 2019). For example, the arsenic resistance cassette, *arsRCDAB*, present on a class 1 integron and mobilized on a conjugative plasmid was detected in two *Salmonella enterica* isolates from Singapore with high tolerance to arsenate (Wilson et al., 2019). Moreover, heavy metal resistant genes to Manganese (Mn<sup>2+</sup>) and Cd<sup>2+</sup> in addition to exopolysaccharides production (EPS) were documented upon WGS analysis in *Pseudaminobacter manganicus* isolated from a manganese mine. This aspect sheds light on metal removal/adsorption and reflects bioremediation capabilities in

contaminated regions (Xia et al., 2018). In *A. baumannii*, WGS analysis revealed the role of two integrases in the excision and circularization of heavy metal resistance (GIs) in *E. coli* (Al-Jabri et al., 2018). Bacterial WGS and typing databases such as BacWGSTdb (PMID: 26433226)<sup>1</sup> serve as easy, rapid, powerful, and convenient tools to assess AMR and provide valuable information to WGS analysis and daily use in the clinical microbiology laboratories (Ruan and Feng, 2016). Moreover, “BacMet: Antibacterial Biocide and Metal Resistance Genes Database” provides an accurate and high quality resource of bacterial genes associated with heavy metal resistance present in literature<sup>2</sup>. These available databases serve as important tools to help understand bacterial resistance mechanisms to heavy metals by linking various factors and parameters involved.

## CONCLUSION

The reason for the rapid emergence of drug resistant *A. baumannii* in war-wounded patients remains unclear. Heavy metal contaminated areas may be driving the increase in antimicrobial resistance. This may explain an observed increase in bacterial resistance to both heavy metals and antimicrobial agents and lead to the development of novel mechanisms of resistance. The role of this pathway in *A. baumannii* is poorly understood.

Until now antimicrobial resistance has been largely attributed to poor antimicrobial stewardship in humans and in animals. The mechanisms described above, whereby heavy metals may produce antimicrobial resistance in their absence, identifies a potential pathway driving global antimicrobial resistance that would not be addressed through improved antimicrobial stewardship. This pathway would be facilitated in wartime, and could explain the emergence of previously little reported pathogens as possible amplification points along this pathway.

Further research into the role of heavy metals driving antimicrobial resistance, its influence in war zones, and the contribution of *A. baumannii* as a reservoir, are therefore warranted given the implications for addressing the global AMR crisis.

## AUTHOR CONTRIBUTIONS

AGA, WB, AN, L-PH, MZ, and PH contributed to reviewing the literature and the write up of the manuscript. V-KN, HL, OD, GA-S, MM, NK, AA, CK, and GM contributed to the editing of the manuscript.

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<sup>1</sup><http://bacdb.org/BacWGSTdb>

<sup>2</sup><http://bacmet.biomedicine.gu.se/>



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# Genome-Based Analysis of Extended-Spectrum $\beta$ -Lactamase-Producing *Escherichia coli* in the Aquatic Environment and Nile Perch (*Lates niloticus*) of Lake Victoria, Tanzania

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Extended-spectrum  $\beta$ -lactamase (ESBL)-producing bacteria constitute an emerging global health issue with food products being vehicles of transmission and the aquatic environments serving as potential reservoirs. This study aimed to characterize ESBL-producing *Escherichia coli* in Nile perch and water from Lake Victoria in Tanzania. A total of 180 samples of Nile perch and 60 water samples were screened for ESBL-producing *E. coli* on MacConkey agar supplemented with 2  $\mu$ g/ml of cefotaxime and confirmed by *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> PCR. Antimicrobial resistance was determined by the disk diffusion method, and the ESBL-producing isolates were whole genome sequencing (WGS). ESBL-producing *E. coli* were detected in eight of the 180 analyzed Nile perch samples, and only one water sample was positive (1.7%,  $n = 60$ ). Isolates were resistant to sulfamethoxazole–trimethoprim (100%), ampicillin/cloxacillin (100%), erythromycin 72.7% (8/11), tetracycline 90.9% (10/11), and nalidixic acid 63.6% (7/11). This mostly corroborates the resistance genes that they carried for sulfonamides (*sul1* and *sul2*), trimethoprim (*dfrA* and *dfrB*), aminoglycosides [*aac(3)-IId*, *strA*, and *strB*], tetracycline [*tet(B)* and *tet(D)*], and fluoroquinolones (*qepA4*). They harbored plasmid replicon types IncF, IncX, IncQ, and Col and carried *bla*<sub>CTX-M-15</sub> and *bla*<sub>TEM-1B</sub> genes generally found on the same contigs as the IncF plasmid replicon. Although epidemiologically unrelated, the strains formed three separate sequence type–phylogroup–serotype-specific clusters: C1, C2, and C3. Cluster C1 included five strains (3 to 13 SNPs) belonging to ST167, phylogroup A, and serotype O9:H21; the two C2 strains (11 SNPs) belong to ST156, phylogroup B1, and serotype ONT:H28; and C3 was made up of four strains (SNPs ranged from 4 to 17) of ST636, phylogroup B2, and serotype O45:H7. The common virulence gene *gad* was reported in all strains. In addition, strains in C2 and C3 possessed *iss*, *lpfA*, and *nfaE* virulence genes, and the *vat* gene was found only in C3. The present study reports the occurrence of multidrug-resistant ESBL-producing

*E. coli* carrying plasmid-mediated ESBL genes in offshore water and Nile perch in Lake Victoria. Strains formed three clonal clusters of unknown origin. This study reveals that the Lake may serve as reservoir for ESBL-producing bacteria that can be transmitted by fish as a food chain hazard of One-Health concern.

**Keywords:** nile perch, food safety, extended-spectrum  $\beta$ -lactamase, antimicrobial resistance, *Escherichia coli*

## INTRODUCTION

*Escherichia coli* and other related bacteria can produce extended-spectrum  $\beta$ -lactamase (ESBL) enzymes that hydrolyze a broad spectrum of  $\beta$ -lactam drugs such as cephalosporins (e.g., ceftazidime and cefotaxime) and monobactams (e.g., aztreonam and nocardicin), classes of antimicrobials that are critical in human medicine (Lavilla et al., 2008; Shaikh et al., 2015; Adelowo et al., 2018). ESBL-producing Gram-negative bacteria especially those producing cefotaximase-Munich (CTX-M) enzymes have emerged as important pathogens causing healthcare- and community-associated infections worldwide.

Studies in many different countries increasingly document how foods may be important sources of ESBL-producing *E. coli*, such as vegetables, poultry, pork, and other animal foods. In particular, poultry meat has been reported commonly to be associated with ESBL-producing *E. coli* globally (Overdevest, 2011; Chishimba et al., 2016; Nguyen et al., 2016; Falgenhauer et al., 2019; Projahn et al., 2019). Possible horizontal transfer of resistance genes from urban sewage and clinical isolates to bacteria associated with fish and aquatic recipients of wastewater has been documented (Kümmerer, 2009; Martinez, 2009; Jiang et al., 2013; Blaak et al., 2014).

The  $\beta$ -lactamase enzymes are derived from mutations in temoneira (TEM), sulfhydryl variable (SHV), and CTX-M genes located on bacterial plasmids or chromosomes (Ben Said et al., 2015; Legese et al., 2017; Adelowo et al., 2018). These genes can easily be horizontally transferred from one bacterial strain to another including across bacterial species (Lavilla et al., 2008). The SHV enzyme seems more dominant in *Klebsiella pneumoniae* among the Enterobacteriaceae (Rupp and Fey, 2003). The CTX-M enzymes are often the most common ESBLs produced by *E. coli* and have been isolated in human clinical, animal food, and environmental samples (Egea et al., 2012; Boonyasiri et al., 2014; Dib et al., 2018). On the other hand, the TEM enzymes especially of TEM-1 and TEM-2 are predominantly reported in *E. coli* and other members of Enterobacteriaceae, although TEM-1 is not considered as ESBLs. These TEM enzymes have been reported from *Klebsiella* spp. and *Enterobacter* spp. isolated in food, clinical, and environmental samples around the world as previously described (Smet et al., 2010; Delgado et al., 2016).

The reservoirs of ESBL-producing *E. coli* are warm-blooded animals including humans, which can transmit the bacteria to different environments, for example, aquatic environments, through fecal pollution (Jiang et al., 2012). Recently, studies have reported ESBL-producing *E. coli* in seafood such as shrimps, sardines, farmed fish, Nile tilapia (*Oreochromis niloticus*) as well as in frozen mackerel (Jiang et al., 2012; Moremi et al., 2016b; Nasreldin and Khaldoun, 2015; Dib et al., 2018). It has been

documented that urban sewage, for example, from hospitals, is an important source of ESBL-producing enteric bacteria (Ojer-Usoz et al., 2013; Abgottspon et al., 2014). Thus, ESBL-producing bacteria in sewage and in runoff water from agricultural soil fertilized with livestock manure can enter the aquatic environment where antimicrobial resistance genes may be transferred horizontally to the ubiquitous bacterial flora, although the rate and health importance of such transfer are unknown.

In Tanzania, ESBL-producing *E. coli* have been isolated from different sources such as human specimens, foods, and aquatic environments (Moyo et al., 2010; Moremi et al., 2016b). Although ESBL-producing *E. coli* have been reported in humans in different regions of Tanzania (Moyo et al., 2010; Seni et al., 2016; Katakweba et al., 2018) and also in livestock and poultry (Katakweba et al., 2018), the importance of livestock and their meats as sources of ESBL-producing *E. coli* is unknown. ESBL-producing Enterobacteriaceae recovered from the aquatic environments and tilapia from Lake Victoria were previously characterized (Moremi et al., 2016b). This study was carried out with tilapia, which is a fish species commonly found in shallow water, which often is polluted by discharged human wastes of different sources mostly of fecal origin. Thus, a variety of ESBL-producing bacteria were found in the samples analyzed by Moremi et al. (2016b). Our study investigated the occurrence of ESBL-producing Enterobacteriaceae spp. along the chain from capture to market including water and Nile perch at offshore deep water fishing areas. We further applied whole genome sequencing (WGS), which provides important information about antimicrobial resistance and virulence genes in bacterial pathogens and is a powerful molecular tool in investigations of disease outbreaks.

The aim of this study was to determine the occurrence and genomic characteristics of ESBL-producing *E. coli* isolated in water at offshore fishing grounds and in Nile perch (*Lates niloticus*) from Lake Victoria, Tanzania.

## MATERIALS AND METHODS

### Study Design, Sample Collection, and Processing

The study employed a cross-sectional design and was conducted from February to July 2017. A total of 240 samples of water and Nile perch were collected and analyzed for ESBL-producing Enterobacteriaceae, and the isolates were tested for antimicrobial resistance. This sample size was calculated based on the estimated prevalence of ESBL-producing Enterobacteriaceae spp. detected



in other fish spp. from Lake Victoria reported by Moremi et al. (2016b) using the following formula:

$$n = (Z_{\alpha})^2 \times P(1 - P) / d^2$$

Out of 240 samples, 180 were Nile perch and were collected from fishing grounds ( $n = 60$ ), landing sites ( $n = 60$ ), and domestic fish markets ( $n = 60$ ). In addition, offshore water samples ( $n = 60$ ) were collected and analyzed from fishing grounds. The size of fish sampled ranged between 1 and 2 kg by weight. The selection of Nile perch samples at markets was based on the availability of fish from vendors. At each market, 10 samples were collected from five retailers, with two fish purchased from each vendor. We did not register how many Nile perch were on sale at each market. Details on sample collection, transport, and processing are provided by Baniga et al. (2019). Nile perch samples were prepared by weighing intestines, gills, flesh, and fish surface mucus in a ratio of 1:9 to Buffered Peptone Water (BPW) (Oxoid Ltd., Hampshire, United Kingdom). Fish intestines, gills, and flesh (15- to 25-g volumes) were homogenized for 60 s in a stomacher (Seward 400, United Kingdom), whereas fish mucus was obtained and analyzed by massaging the fish surface in a sterile stomacher bag containing 225 ml of BPW. The weight of fish gills and intestine samples was not always the same because the weight varies based on the size of the fish; for example, a fish weighing 1 kg will have gills and intestines each weighing below 25 g and not less than 15 g. Stomacher bags used to obtain the surface mucus of fish were of 16063/0606 size, that is, Stomacher® Bags, Seward Genuine 16063/0606.<sup>1</sup> The collection of fish mucus sample was done while fish was still intact before other subsamples, that is, flesh, gills, and intestines, were collected. All fish samples were processed following standard method (ISO 6887-3, 2003). Twenty-five milliliters of each water sample was filtered using a standard filter membrane of 0.45 µl of pore size (Thermo Fisher Scientific, Waltham, MA, United States). After filtration, the filter membrane was torn into pieces using sterile forceps and then placed into 225 ml of BPW, which were shaken rigorously and then incubated at 37°C overnight for enrichment before plating out on the MacConkey agar. Thus, the limit of detection was 4 colony-forming units (cfu) ESBL-producing *Escherichia coli* per 100 ml. Water sample handling and processing were done according to ISO and Tanzanian standards incorporated with some modifications (ISO 9308-1, 2000; Tzs 117, 1981).

## Study Site

The study was conducted along the Tanzanian basin of Lake Victoria in Mwanza. Sampling points included fishing grounds (open water), landing sites, and domestic fish markets located in Ilmela and Nyamagana districts in the Mwanza region. At the fishing grounds, there were no other activities besides fishing, and there were no toilet facilities on board. At some of the landing sites, there were households nearby and a range of different human activities including agriculture and scavenging animals,

for example, cattle, goats, and dogs, around the landing sites. Also, there were several migratory fish-eating birds around the shore of the lake. At every landing site, there were toilet facilities, but it is uncertain whether these discharged into the lake. In the domestic markets, a variety of goods were sold, and toilets were present at all markets visited during sampling. However, there was poor waste management around the markets owing to accumulation and access of wastes in the garbage bins and also lack of proper wastewater discharge. Processing of samples and bacteriological analysis were done at the National Fish Quality Control Laboratory, Mwanza, and at the laboratory of the Department of Microbiology, Parasitology and Biotechnology, Sokoine University of Agriculture (SUA). The WGS of ESBL-producing *E. coli* was done at the Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark.

## Identification of Extended-Spectrum $\beta$ -Lactamase-Producing Enterobacteriaceae

ESBL-producing Enterobacteriaceae in Nile perch and water were screened on MacConkey agar (Oxoid Ltd.) supplemented with 2 mg/L of cefotaxime (Moremi et al., 2016b). Briefly, 1 ml of an overnight enriched sample in BPW at 37°C was added to the MacConkey agar plates by the pour plate method and incubated overnight at 37°C (CLSI, 2016). Then, colonies with characteristics of Enterobacteriaceae were selected for ESBL confirmation using the double disk synergy method (Drieux et al., 2008; CLSI, 2016). The disks used were ceftazidime (30 µg) and cefotaxime (30 µg) (HiMedia Laboratories Pvt. Ltd., Mumbai, India), which were placed 20 mm apart from the center of a Mueller–Hinton agar (MHA) plate (Oxoid Ltd.) where amoxicillin/clavulanic acid (20 µg/10 µg) disk was placed. Plates were incubated at 37°C for 24 h. An inhibition zone around any of the disks under test increasing toward amoxicillin/clavulanic acid disk was interpreted as a presumptive ESBL-producing isolate, and the bacterial species were subsequently identified using API 20E (bioMérieux, France) based on the analytical profile index. The confirmed ESBL-producing *E. coli* isolates were further analyzed by PCR targeting  $\beta$ -lactamases genes – *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub> – in single multiplex reactions using primer sequences described by Nasreldin and Khaldoun (2015) to confirm their ESBL-producing status. The confirmed ESBL-producing isolates were used for further analyses.

## Antimicrobial Disk Susceptibility Testing

The ESBL-producing *E. coli* isolates were subjected to antimicrobial susceptibility testing using standard disk diffusion method on MHA following the protocol and recommended antimicrobials by CLSI (2016). The following antimicrobials were selected: ampicillin/cloxacillin (AX; 10 µg), amoxicillin/clavulanic acid (AMC; 30 µg), gentamicin (GEN; 10 µg), sulfamethoxazole–trimethoprim (SXT; 25 µg), ciprofloxacin (CIP; 5 µg), cefotaxime (CTX; 30 µg), chloramphenicol (CHL; 30 µg), nalidixic acid (NAL; 30 µg), ceftazidime (CAZ; 30 µg), erythromycin (ERY; 15 µg),

<sup>1</sup> www.seward.co.uk

imipenem (IMP; 10 µg), and tetracycline (TET; 30 µg) (HiMedia Laboratories Pvt.). Although CLSI does not recommend the inclusion of erythromycin and ampicillin/cloxacillin, these antibiotics are commonly prescribed in Tanzania and were therefore included in the testing. *E. coli* ATCC 25922 was used as a quality control.

## DNA Extraction and Whole Genome Sequencing

DNA was extracted from the 11 ESBL-producing *E. coli* isolates using Maxwell RSC culture cell's DNA kit following the manufacturer's protocol in the automated Maxwell RSC instrument (Promega, Fitchburg, WI, United States). Genomes were sequenced at the 250-bp paired-end-read format using Nextera XT kit and the MiSeq instrument (Illumina, Inc., San Diego, CA, United States). The average coverage of WGS and read length are indicated in **Supplementary Table 3**. Raw sequence reads have been submitted to the European Nucleotide Archive (ENA) under the project number PRJEB34642 with the accession numbers of each sample available in **Supplementary Tables S1, S3**.

## Data Analysis

Data collected were entered and stored into Microsoft Excel version 2010 (Microsoft Ltd., United States), and the prevalence of ESBL-producing *E. coli* in water and Nile perch at each sampling point was determined. *E. coli* genomes were *de novo* assembled using SPAdes 3.9.0 (Bankevich et al., 2012). Further analyses were performed using various web bioinformatics tools from the Center for Genomic Epidemiology (CGE),<sup>2</sup> Enterobase,<sup>3</sup> and BLASTn at National Centre for Biotechnology Information (NCBI).<sup>4</sup> Plasmid replicons were detected using PlasmidFinder v2.0 (Carattoli et al., 2014), whereas virulence genes were determined using VirulenceFinder v2.0 (Joensen et al., 2014), and *In Silico* analysis of resistance genes using ResFinder v2.2 (Zankari et al., 2012) from CGE using default settings.

Resistance to heavy metals and detergents was assessed using MyDbFinder 1.2 where our genomes were analyzed against plasmid-mediated genes such as the tellurite resistance gene *tehA* (NC\_000913.3), the detergent-resistant phospholipase A, *pldA* (NC\_003198.1), and the quaternary ammonium compound efflux *qacEdelta* (NG\_048042.1) (Moremi et al., 2016b). Further heavy metal resistance operons encoding resistance to copper, cobalt, zinc, cadmium, magnesium, mercury, and chromium were analyzed through the subsystem annotation in RAST<sup>5</sup> according to Brettin et al. (2015). The contigs harboring these genes and their positions on the contigs were compared with contigs harboring plasmids in the strains to confirm their location in the genomes. The location of β-lactamase genes on plasmids or chromosomes was determined by analyzing the contigs harboring the

*bla<sub>CTX-M-15</sub>* and *bla<sub>TEM-1B</sub>* using BLASTn in NCBI (Zhang et al., 2000).

Sequence types of the isolates were detected using *In Silico* MLST typing tool based on the seven housekeeping genes—*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*—for *E. coli* in Enterobase v.1.1.2 (Wirth et al., 2006). The phylogenetic analysis was performed using CSI Phylogeny v1.4 (Kaas et al., 2014) with the default options. The phylogenetic analysis included strains from this study and publicly available genomes of *E. coli* isolated from humans, foods, and the environments of the same sequence types as those identified for our strains. In addition to sequence type similarities, strains with the same serotype as our isolates were an added criterion in the selection of public genomes for phylogeny analysis. *E. coli* strain K12 sub-strain MG1655 (accession number SAMN02604091) was used as reference to root the tree. The list of *E. coli* strains used in phylogenetic analysis with their accession numbers is indicated in the table (**Supplementary Table S1**). The newick file of the tree was downloaded and edited using iTOL v4<sup>6</sup> (Letunic and Bork, 2016). The pairwise SNP difference data can be found in a table (**Supplementary Table S2**).

## RESULTS

### Prevalence of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* in Nile Perch and Water

The overall prevalence of ESBL-producing *Escherichia coli* in Nile perch was 4.4% (8/180), whereas only one water sample collected offshore in the lake contained ESBL-producing *E. coli*. In this study, no other ESBL producers except for *E. coli* were detected. One Nile perch sample 1.7% (1/60) caught at fishing grounds offshore contained ESBL-producing *E. coli*, whereas two fish samples from landing sites contained ESBL-producing *E. coli* 3.3% (2/60) as did five fish samples purchased in domestic fish markets 8.3% (5/60) (**Table 1**).

### Genomic Characteristics and Phylogenetic Analysis of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* From Nile Perch

Analysis of the genome sequences from the 11 ESBL-producing *E. coli* revealed three clusters (C1, C2, and C3) separated by unique sequence type, serotype, phylogroup and also virulence and resistance genes patterns (**Tables 2, 3**). The cluster C1 included five clonal related strains (Z1 to Z5) with a SNP difference ranging from 3 to 13, all belonging to ST167, phylogroup A, and having serotype O9:H21. These strains possessed only one virulence gene (*gad*) and were isolated from fish and water samples collected at offshore fishing grounds, as well as from fish purchased at the landing sites. Subsample Z2 of mucus from the fish surface and Z3 (fish intestine) was from

<sup>2</sup><https://cge.cbs.dtu.dk/services/>

<sup>3</sup><http://enterobase.warwick.ac.uk/>

<sup>4</sup><https://www.ncbi.nlm.nih.gov/>

<sup>5</sup><http://rast.nmpdr.org/rast.cgi>

<sup>6</sup><https://itol.embl.de/>

**TABLE 1** | Prevalence of ESBL-producing *Escherichia coli* in water and Nile perch from Lake Victoria, Tanzania.

Origin	Sample type	Subsample type
Fishing grounds	Nile perch 1/60 (1.7%)	Fish gills 1/60 (1.7%)
	Water 1/60 (1.7%)	N/A
Landing sites	Nile perch 2/60 (3.3%)	Fish intestines 1/60 (1.7%)
		Fish surface mucus 2/60 (3.3%)
Markets	Nile perch 5/60 (8.3%)	Fish intestines 4/60 (6.7%)
		Fish gills 2/60 (3.3%)

N/A, not applicable; ESBL, extended-spectrum  $\beta$ -lactamase.

the same fish sampled at a landing site, which harbored two ESBL producers. Likewise, subsamples Z9 and Z10 from the same fish collected at a market harbored two different ESBL producers isolated from fish intestines and gills. Two clonal strains (Z8 and Z9) formed cluster C2 with 11 SNPs apart. They belong to ST156, phylogroup B1, and showed serotype O157:H28 and were both isolated from Nile perch obtained at local fish markets. These two strains contained additional virulence genes including *gad*, *iss*, *lpfA*, and *nfaE*.

Strains Z6, Z7, Z10, and Z11 isolated from Nile perch from fish markets formed the phylogenetic cluster C3 and had a SNP difference ranging from 4 to 17. These four strains belong to ST636, phylogroup B2; have serotype O45:H7; and harbor the *vat* gene in addition to all the virulence genes present in C2 strains. Among these isolates, we noted that one fish sample harbored two different ESBL producers (ST156 and ST636) in different sample subtypes, that is, from fish intestines and gills.

In a comparison with *E. coli* isolated from humans, animals, and the environment globally, our strains showed a sequence type-based clustering where the publically available ST167 genomes clustered with our C1 strains, as did the publically available ST156 and ST636 genomes with our C2 and C3 strains, respectively. However, we recorded some wide within-clade variations between our strains and the public genomes of the same STs with up to 5,104, 2,440, and 1,391 SNP

differences in C1, C2, and C3, respectively, (Figure 1 and Supplementary Table S2).

## Antimicrobial Resistance, Plasmid Replicon Profiles, Heavy Metal, and Detergent Resistance

Overall, the genotype was consistent with the phenotypic resistance patterns, as well as occurrence of resistance genes and plasmid replicon types in the 11 ESBL-producing *E. coli*, which showed a similar clustering pattern as shown above (Tables 3, 4; Supplementary Table S3). The isolates showed phenotypic antimicrobial resistance to sulfamethoxazole–trimethoprim (100%), ampicillin/cloxacillin (100%), erythromycin (72.7%; 8/11), tetracycline (90.9%; 10/11), nalidixic acid (63.6%; 7/11), and chloramphenicol (63.6%; 7/11) (Table 3). C1 strains showed phenotypic and genotypic resistances to aminoglycosides [*aadA2* and *aac(3)-IId*], sulfonamide–trimethoprim (*sul1*, *sul2*, and *dfrA12*), fluoroquinolone (*qepA4*), tetracycline (*tetB* and *tetD*), macrolides (*mphA* and *mdfA*), chloramphenicol (*catA1*), and the  $\beta$ -lactams ampicillin/cloxacillin and cefotaxime (*bla<sub>CTX-M-15</sub>* and *bla<sub>TEM-1B</sub>*). However, strains Z1, Z2, and Z4 of C1 did not show phenotypic resistance to erythromycin despite the presence of *mphA* and *mdfA* conferring resistance to macrolides. All C1 strains have the plasmid replicon types IncFIA, IncFIB, IncFII, IncX1, Col8282, and Col156. Isolates contained IncF plasmids with the pMLST type [F48:A1:B49]. Strains Z8 and Z9 of clade C2 showed resistance to sulfonamide–trimethoprim (*sul1* and *dfrB4*), fluoroquinolone (*qepA4*), tetracycline (*tetB*), macrolides (*mphA* and *mdfA*), chloramphenicol (*catA1*), and the  $\beta$ -lactams ampicillin/cloxacillin and cefotaxime (*bla<sub>CTX-M-15</sub>* and *bla<sub>TEM-1B</sub>*). They were susceptible to aminoglycosides and only possessed one plasmid replicon type Col440I harboring the  $\beta$ -lactam genes.

The C3 strains were susceptible to fluoroquinolones and chloramphenicol but showed resistance to aminoglycosides (*aadA1*, *strA*, and *strB*), sulfonamide–trimethoprim (*sul2* and *dfrA1*), tetracycline (*tetB*), macrolides (*mphA* and *mdfA*), and the

**TABLE 2** | Genomic characterization of ESBL-producing *Escherichia coli* isolated in Nile perch.

Code	Origin	Sample type <sup>a</sup>	MLST	Serotype	Phylogroup	Virulence genes <sup>b</sup>
Z1	Landing site	Fish surface	167	O9:H21	A	<i>gad</i>
Z2	Landing site	Fish surface	167	O9:H21	A	<i>gad</i>
Z3	Landing site	Fish intestines	167	O9:H21	A	<i>gad</i>
Z4	Fishing grounds	Water	167	O9:H21	A	<i>gad</i>
Z5	Fishing grounds	Fish gills	167	O9:H21	A	<i>gad</i>
Z6	Markets	Fish intestines	636	O45:H7	B2	<i>gad</i> , <i>nfaE</i> , <i>iss</i> , <i>vat</i>
Z7	Markets	Fish intestines	636	O45:H7	B2	<i>gad</i> , <i>nfaE</i> , <i>iss</i> , <i>vat</i>
Z8	Markets	Fish gills	156	ONT:H28	B1	<i>gad</i> , <i>iss</i> , <i>lpfA</i>
Z9	Markets	Fish gills	156	ONT:H28	B1	<i>gad</i> , <i>iss</i> , <i>lpfA</i>
Z10	Markets	Fish intestines	636	O45:H7	B2	<i>gad</i> , <i>nfaE</i> , <i>iss</i> , <i>vat</i>
Z11	Markets	Fish intestines	636	O45:H7	B2	<i>gad</i> , <i>nfaE</i> , <i>iss</i> , <i>vat</i>

ESBL, extended-spectrum  $\beta$ -lactamase. <sup>a</sup>Subsamples Z2 and Z3 from a landing site harbored two ESBL producers in mucus from the fish surface and fish intestine. Likewise, subsamples Z9 and Z10 from a market harbored two different ESBL producers isolated from fish intestines and gills. <sup>b</sup>*gad*, glutamate decarboxylase; *nfaE*, diffuse adherence fimbrial adhesin gene; *iss*, increased serum survival; *vat*, vacuolating autotransporter toxin; and *lpfA*, long polar fimbriae.

TABLE 3 | Phenotypic and genotypic antimicrobial resistance of ESBL-producing *Escherichia coli* isolates.

Code	Aminoglycoside	Sulfonamide-trimethoprim	Fluoroquinolone	Tetracycline	Macrolide	$\beta$ -Lactamases	Chloramphenicol
Z1	GEN/+; aadA2, aac(3)-IId	SXT/+; sul1, sul2, dfrA12	CIP, NAL/+; qepA4	TET/+; tetB, tetD	-/-; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	CHL/+; catA1
Z2	-/-; aadA2, aac(3)-IId	SXT/+; sul1, sul2, dfrA12	CIP, NAL/+; qepA4	TET/+; tetB, tetD	-/-; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	CHL/+; catA1
Z3	-/-; aadA2, aac(3)-IId	SXT/+; sul1, sul2, dfrA12	CIP, NAL/+; qepA4	TET/+; tetB, tetD	ERY/+; mphA, mdfA	AX/+; CTX-M-15, TEM-1B	CHL/+; catA1
Z4	-/-; aadA2, aac(3)-IId	SXT/+; sul1, sul2, dfrA12	CIP, NAL/+; qepA4	TET/+; tetB, tetD	-/-; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	CHL/+; catA1
Z5	-/-; aadA2, aac(3)-IId	SXT/+; sul1, sul2, dfrA12	CIP, NAL/+; qepA4	TET/+; tetB, tetD	ERY/+; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	CHL/+; catA1
Z6	GEN/+; aadA1, strA, strB	SXT/+; sul2, dfrA1	ND	-/-; tetB	ERY/+; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	ND
Z7	GEN/+; aadA1, strA, strB	SXT/+; sul2, dfrA1	ND	TET/+; tetB	ERY/+; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	ND
Z8	ND	SXT/+; sul1, dfrB4	CIP, NAL/+; qepA4	TET/+; tetB	ERY/+; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	CHL/+; catA1
Z9	ND	SXT/+; sul1, dfrB4	CIP, NAL/+; qepA4	TET/+; tetB	ERY/+; mphA, mdfA	AX/+; CTX-M-15, TEM-1B	CHL/+; catA1
Z10	GEN/+; aadA1, strA, strB	SXT/+; sul2, dfrA1	ND	TET/+; tetB	ERY/+; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	ND
Z11	GEN/+; aadA1, strA, strB	SXT/+; sul2, dfrA1	ND	TET/+; tetB	ERY/+; mphA, mdfA	AX, CTX/+; CTX-M-15, TEM-1B	ND

AX, ampicillin/cloxacillin; AMC, amoxicillin/clavulanic acid; CAZ, ceftazidime; CIP, ciprofloxacin; CHL, chloramphenicol; CTX, cefotaxime; ERY, erythromycin; GEN, gentamicin; IMP, imipenem; NAL, nalidixic acid; SXT, sulfamethoxazole-trimethoprim; and TET, tetracycline; ESBL, extended-spectrum  $\beta$ -lactamase. ND, no phenotypic and genotypic resistance; +, phenotypic resistance; -, no phenotypic resistance.

$\beta$ -lactams ampicillin/cloxacillin and cefotaxime (*bla*<sub>CTX-M-15</sub> and *bla*<sub>TEM-1B</sub>). Despite the presence of *tetB*, strain Z6 did not show phenotypic resistance to tetracycline. All C3 strains have plasmid replicon types IncFIA, IncFIB, IncFII, IncQ1, ColRNAI, and Col (BS512) with the IncF harboring the *bla*<sub>TEM-1B</sub> gene, and they carried the *bla*<sub>CTX-M-15</sub> on their chromosome. Isolates in this cluster contained IncF plasmids with the pMLST-type IncF [F1:A1:B1]. Strains in all three clusters were sensitive to imipenem.

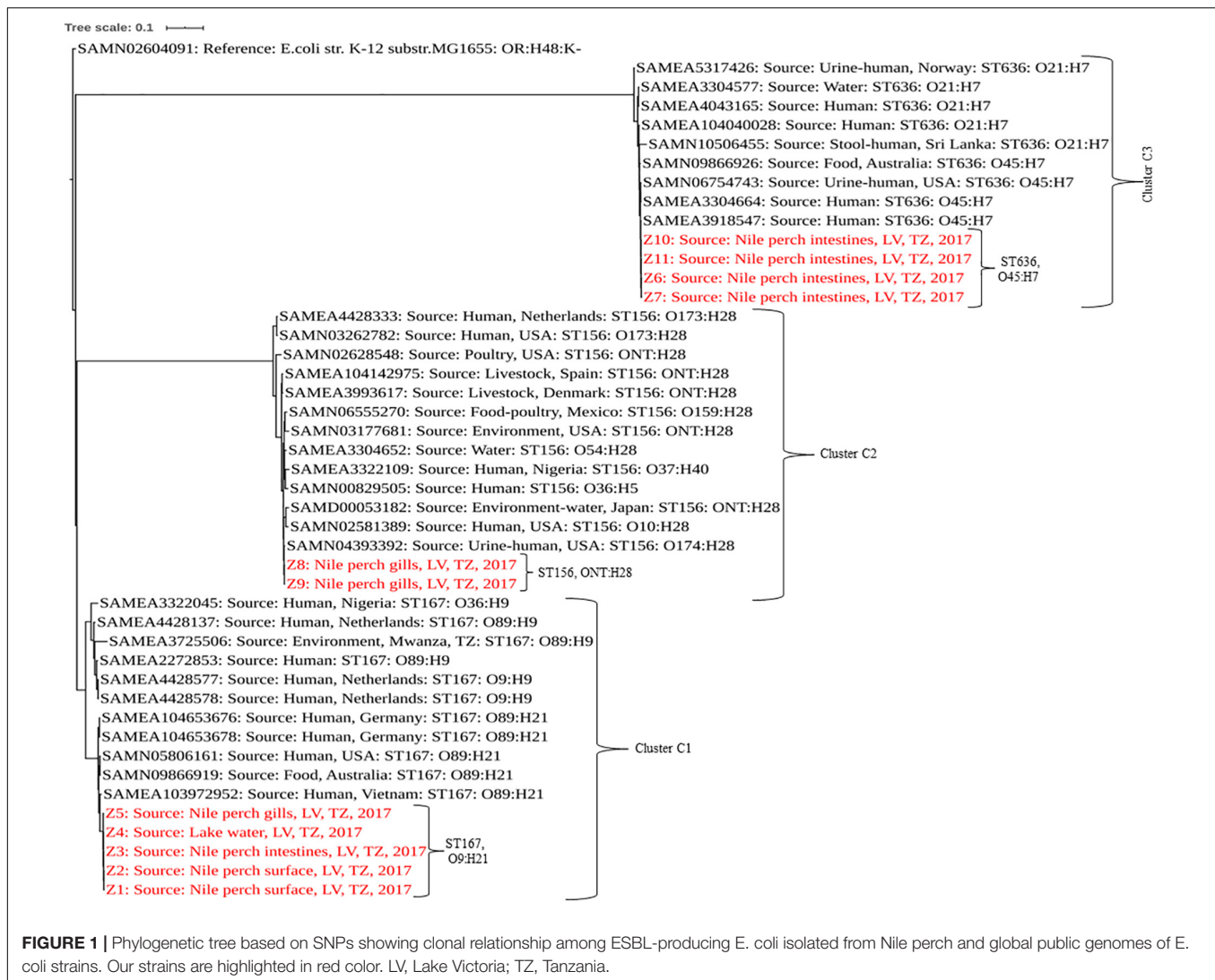
All cluster C1 and C2 strains harbored the plasmid-mediated detergent resistance gene, *qacEdelta*, encoding resistance to quaternary ammonium compounds. All the strains contained resistance genes to copper, cobalt, zinc, cadmium, magnesium, mercury, and tellurite, which were chromosomally located (Supplementary Table S4). However, chromium resistance was only recorded in strains of cluster C1 isolated from fishing grounds and landing sites.

## DISCUSSION

The occurrence of ESBL-producing *Escherichia coli* was low in Nile perch and water samples collected at offshore fishing grounds and at landing sites of Lake Victoria but higher in fish from local fish markets in Mwanza. ESBL-producing *E. coli* have been reported in different wild-caught fish species in Switzerland, Algeria, and India (Abgottspon et al., 2014; Singh et al., 2017; Dib et al., 2018), as well as in farmed fish in China (Jiang et al., 2012) and Egypt (Ishida et al., 2010). Moremi et al. (2016b) reported a slightly higher prevalence of ESBL-producing bacteria in wild-caught tilapia sold at markets in Mwanza (13.3%) as compared with our findings in Nile perch (8.3%). Diverse bacterial species were found in the tilapia (Moremi et al., 2016b), whereas only *E. coli* was isolated in our Nile perch. This is probably because tilapia is usually found in shallow water with relatively high levels of fecal pollution and hence higher possibility of finding a variety of Enterobacteriaceae than finding these bacteria in the Nile perch, which are caught in deep water with lower pollution levels. Thus, these studies suggest that the aquatic environment and fish are likely sources and dissemination routes for ESBL-producing bacterial species and their resistance genes, although their relative importance from a public health perspective is uncertain.

The low prevalence of ESBL-producing *E. coli* in Nile perch from fishing grounds is likely due to the fact that these fish are commonly found and caught in deep water far away from the lake shore where fecal pollution is low compared with the shallow water near the shore of the lake (Baniga et al., 2019). Any occurrence of ESBL-producing *E. coli* in Nile perch caught offshore could be attributed to their eating habits feeding on other fish species, for example, tilapia and sardines, which are found in shallow water with higher fecal contamination levels. This hypothesis is corroborated by the findings of Moremi et al. (2016b) where gut intestinal samples from tilapia contained ESBL-producing bacteria. Because boats of artisanal small-scale fishers do not have toilet facilities, the fishermen have to defecate into the lake, with a potential introduction of ESBL-producing *E. coli* into the lake environment.





ESBL-producing *E. coli* isolated in Nile perch from markets belonged to clusters C2 and C3 and seem to harbor more virulence genes than do isolates originating from fish caught offshore. This higher pathogenicity indicates a human origin, and their occurrence in fish sold at markets is likely caused by fecal cross-contamination from fish handlers and vendors. This is further supported by the fact that most of the market strains of C3 belong to phylogroup B2, which is usually composed of extra-intestinal pathogenic *E. coli* strains (Abgottspoon et al., 2014; Huang et al., 2016; Sánchez-Benito et al., 2017). It is important to note that our prevalence of ESBL-producing *E. coli* are generally very low, not allowing for any statistical comparison between fish of different origins. Moreover, possible associations between occurrence of ESBL-producing *E. coli* in fish and human handling activities can be better studied through comparative genomic investigations including clinical human isolates and our environmental strains.

The virulence gene *gad* harbored by C1 strains is an important gene for both commensal and pathogenic enteric bacteria

especially *E. coli*, as the reaction products of *gad* are essential for survival in an acidic environment and for successful colonization of the host cell (Tramonti et al., 2002). The additional virulence genes shown by C2 and C3 strains included *nfaE*, which is essential for diffuse adherence fimbriar adhesin; *iss* plays a significant role in increased serum survival; and the *vat* gene is important for vacuolating autotransporter toxin in host cells for pathogenicity processes. These virulence genes were previously reported from *E. coli* in humans as well as from pigs and bovine (Szmolka et al., 2012; Madoshi et al., 2016; Ahmed et al., 2017). The *lpfA* gene is important for long polar fimbriae and has been commonly associated with the ability to invade epithelial cells in animals and humans (Dogan et al., 2012).

Our strains were epidemiologically unrelated, but their grouping into three clusters with further clustering with other *E. coli* strains of the same STs isolated from human, food, and environmental samples around the globe underlines the transmission potential of the ESBL genes across various niches and locations favored by plasmids that they harbor. Our strains

of cluster C1 (ST167) belong to the phylogroup A known to contain commensal strains (Abgottsson et al., 2014; Huang et al., 2016; Sánchez-Benito et al., 2017), and this is supported by the presence of only one virulence gene (*gad*). Moremi et al. (2016b) reported a single environmental sample from Mwanza containing an ESBL-producing *E. coli* of ST167, belonging to the newly defined phylogroup E harboring *bla*<sub>CTX-M-15</sub> and *bla*<sub>OXA-1</sub>. This ST167 strain of phylogroup E differed from our strains of ST167 of phylogroup A with up to 5,104 SNPs. The variation among strains of the same STs can be attributed to the differences in serotype as well as their genetic content such as the differences in the ESBL genes that they contained. Our study corroborates earlier findings that *bla*<sub>CTX-M-15</sub> is the predominant ESBL gene in Enterobacteriaceae in Tanzania (Moremi et al., 2016b), yet the sources are still unknown. ESBL-producing *E. coli* ST167 have been previously reported isolated in humans in China and Spain carrying *bla*<sub>CTX-M-15</sub> and *bla*<sub>TEM-1</sub> genes (Huang et al., 2016; Sánchez-Benito et al., 2017). This substantiates the possibilities of horizontal gene transfer of the ESBL genes from food products or the environment to humans where bacterial pathogens can acquire them. Such transmission is favored by plasmids on which the resistance genes are located (Anjum et al., 2019) as is the case for the *bla*<sub>CTX-M-15</sub> and *bla*<sub>TEM-1</sub> genes, which were located on the IncF plasmid (Table 4). We observed the plasmid IncF [F1:A1:B1] within all four C3 strains, which had the same ST636 and the same pMLST and showed a SNP difference ranging from 4 to 17. The C3 strains all came from intestinal samples, and we cannot explain their clonal nature as the origin and previous exposures of the fish purchased at the markets are unknown. The IncF [F48:A1:B49] was shown in all five C1 strains originating from intestinal and surface mucus samples of fish from one landing site as well as from fish (gills) and water collected offshore. Similarly to C3 strains, the C1 strains were epidemiologically unrelated, and we are not able to explain the clonal nature of the C1 strains. In contrast to our study, the ESBL-producing bacteria reported in tilapia from the Mwanza region by

Moremi et al. (2016b) included a much more diverse population of bacterial species, a variety of ESBL genes, and no indication of a clonal relationship. The occurrence of genes encoding for resistance to metals such as copper, cobalt, zinc, cadmium, and mercury, and also genes encoding resistance to detergents is similar to previous findings in fish and aquatic environment of the Lake Victoria; the genes have been reported to play a role in environmental persistence support in bacterial survival (Moremi et al., 2016b; Hounmanou et al., 2019). Most of metal resistance genes were located on the chromosome, whereas ESBL genes were mostly located on the plasmids, except that all four ST636 isolates harbored a chromosomally located *bla*<sub>CTX-M-15</sub> gene, and it is uncertain to what extent exposure to metals, for example, used as livestock feed additives and as pollutants in aquatic environments, may play a role as co-selectors of ESBL resistance.

In addition to the ESBL genes, our strains are all multidrug resistant despite their relatively low virulence (Magiorakos et al., 2012). The resistance genes found have been frequently reported in Gram-negative bacteria, and because the genes are often plasmid mediated, they may be circulated horizontally among different bacterial species (van Hoek et al., 2011). These additional resistances shown by the ESBL-producing *E. coli* may also reflect the frequent use of antimicrobials reported in human and veterinary medicine in the Mwanza region (Moremi et al., 2016a; Seni et al., 2016). The location of the additional resistance genes requires further analysis and depiction of the genetic environment of *bla*<sub>CTX-M15</sub> genes in the *bla*<sub>CTX-M-15</sub>-encoding isolates (Moremi et al., 2016b).

Some strains in C1 and C3 did not show phenotypic resistance to antimicrobials that they harbored resistance genes for, that is, erythromycin and tetracycline. This is an increasing observation in genomic studies and could be due to various factors including the concentration and quality of the antimicrobial disks (Eze et al., 2014) or intrinsic factors inhibiting the expression of the resistance genes (Weill et al., 2017). This has also been associated with random mutations, which could be accumulated

**TABLE 4 |** Plasmid profiles and location of the  $\beta$ -lactam genes in ESBL-producing *Escherichia coli*.

Node	Plasmid replicon types	pMLST	Location of ESBL and other $\beta$ -lactamase genes
Z1	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF [F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z2	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF [F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z3	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF [F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z4	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF [F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z5	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF [F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z6	IncFIA; IncFIB; IncFII; IncQ1; ColRNAI; Col (BS512)	IncF [F1:A1:B1]	Chromosome (CTX-M-15) Plasmid (TEM-1B)
Z7	IncFIA; IncFIB; IncFII; IncQ1; ColRNAI; Col (BS512)	IncF [F1:A1:B1]	Chromosome (CTX-M-15) Plasmid (TEM-1B)
Z8	Col440I	N/A	Plasmid (CTX-M-15: TEM-1B)
Z9	Col440I	N/A	Plasmid (CTX-M-15: TEM-1B)
Z10	IncFIA; IncFIB; IncFII; IncQ1; ColRNAI; Col (BS512)	IncF [F1:A1:B1]	Chromosome (CTX-M-15) Plasmid (TEM-1B)
Z11	IncFIA; IncFIB; IncFII; IncQ1; ColRNAI; Col (BS512)	IncF [F1:A1:B1]	Chromosome (CTX-M-15) Plasmid (TEM-1B)

ESBL, extended-spectrum  $\beta$ -lactamase.

in gene sequences encoding resistance to some antimicrobials (Davis et al., 2011; Hussain et al., 2014).

## CONCLUSION

In conclusion, we report a very low prevalence of ESBL-producing *E. coli* in Nile perch from Lake Victoria. Our data suggest that as far as ESBL-producing enteric bacteria are concerned, the consumption of Nile perch represent limited food safety risks compared with other human exposures to ESBL-producing *E. coli*, for example, through direct human-to-human fecal transmission and consumption of livestock meat products. The grouping of the 11 ESBL-producing *E. coli* into three clades each showing identical characteristics, for example, STs, phylogroup, antimicrobial resistance, and virulence genes, is surprising, and we are not able to explain the clonal nature of these clades as the *E. coli* strains were epidemiologically unrelated. All isolates harbored *bla*<sub>CTX-M-15</sub> and *bla*<sub>TEM-1</sub> genes together with additional antimicrobial and detergent resistance genes carried by the common plasmids replicon types IncF, IncX, IncQ, and Col. Further studies are needed to determine the role of fish and aquatic environments as sources of ESBL-producing bacteria and resistance genes including the importance of fecal pollution sources, for example, discharge of sewage and runoff water from fertilized agricultural soil, as well as the ecology of such resistant bacteria in aquatic environments.

Our study has some limitations, including failure to obtain other Enterobacteriaceae spp. than *E. coli*. This is likely because of the selective isolation procedure used with supplement of cefotaxime to obtain ESBL-producing strains; thus, most of other bacteria present both in deep water and in fish from markets were likely sensitive to cefotaxime and did therefore not grow on the MacConkey agar. Although *E. coli* isolated from Nile perch at fish markets were of different sequence types and harbored more virulence genes than did isolates from deep water, a comparison study with ESBL-producing *E. coli* from clinical specimens with environmental strains may provide further information about transmission with the aquatic environment, fish, and human compartments.

## DATA AVAILABILITY STATEMENT

Raw sequence reads have been submitted to the European Nucleotide Archive (ENA) under the project number PRJEB34642.

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## ETHICS STATEMENT

The present study required no ethical approval because we did not manipulate any live fish in the study. Fish samples used in the study were fished by fishermen and constitute dead but fresh fish products ready for marketing and consumption. We purchased these fish from the fishermen, in boats at landing sites and in the markets from fish retailers as indicated in the manuscript like any other fish consumer, and then we brought them to the laboratory for analyses.

## AUTHOR CONTRIBUTIONS

ZB collected samples, carried out laboratory and data analysis, and wrote the draft manuscript. YH participated in laboratory analysis and performed genomic data analysis and editing and critical review of the manuscript. EK participated in genomic data analysis and reviewed the manuscript. LK and RM supervised the study and were involved in reviewing the manuscript and facilitation of resources for the study. AD supervised the study and provided guidance and resources, contributed in genomic data analysis, revised the manuscript, and gave the final approval of the manuscript. All authors read and approved the final manuscript for submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.00108/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.

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# Point Deletion or Insertion in CmeR-Box, A2075G Substitution in 23S rRNA, and Presence of *erm*(B) Are Key Factors of Erythromycin Resistance in *Campylobacter jejuni* and *Campylobacter coli* Isolated From Central China

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*Campylobacter jejuni* and *Campylobacter coli* are major food-borne pathogens that cause bacterial gastroenteritis in humans, and poultry is considered as their most important reservoir. Macrolides, such as erythromycin, are the first-line choice for treatment of campylobacteriosis. In this study, of the 143 *Campylobacter* isolates recovered from poultry in central China during 2015–2017, 25.2% were erythromycin resistant. A2075G substitution in 23S ribosomal RNA (rRNA) and ribosomal methylase encoded by *erm*(B) were found in 4.2 and 4.9% isolates, respectively, and correlated with erythromycin resistance. The polymorphisms of CmeR-Box were also analyzed in our isolates. Among them, 9.1% isolates harbored a point deletion or insertion within the CmeR-Box, and we first showed that point deletion or insertion, but not substitution, in CmeR-Box led to high expression of *cmeABC*, which was significantly associated with erythromycin resistance ( $p < 0.05$ ). These results suggest that point deletion or insertion in CmeR-Box, A2075G substitution in 23S rRNA, and presence of *erm*(B) are three main factors to erythromycin resistance in *C. jejuni* and *C. coli*.

**Keywords:** *Campylobacter*, erythromycin resistance, 23S rRNA, *erm*(B), CmeR-Box

## INTRODUCTION

*Campylobacter* species, especially *Campylobacter jejuni* and *Campylobacter coli*, are major food-borne pathogens that cause bacterial gastroenteritis in humans (Huang et al., 2009; Kaakoush et al., 2015). *Campylobacter* is widespread in animals, with poultry as the most important reservoir (Kaakoush et al., 2015). Contaminated poultry products are recognized as the main source of human infection (Zhang T. et al., 2016). In view of the high incidence of fluoroquinolone resistance, including in China (Zhang et al., 2017b), macrolides, such as erythromycin, are the first-line choice for treatment of campylobacteriosis (Bolinger and Kathariou, 2017). Although erythromycin

has been limited for use in animal production in China in 2000, the incidence of erythromycin resistance in *Campylobacter* continues to increase (Zhang A. et al., 2016; Du et al., 2018). Therefore, the surveillance of erythromycin-resistant *Campylobacter* is important not only for animal breeding but also for public health.

Macrolides are antibiotics that act by binding to the bacterial 50S ribosomal subunit to obstruct the ribosomal exit tunnel, resulting in inhibition of protein synthesis in bacteria (Dinos, 2017). The A2075G and A2074C/G substitutions in the 23S ribosomal RNA (rRNA) are the most common mechanism for erythromycin resistance in *Campylobacter* (Hao et al., 2009; Perez-Boto et al., 2010; Zhao et al., 2016). Recently, the *erm*(B) gene, which encodes a horizontally transferable ribosomal methylase, was identified in *Campylobacter* (Qin et al., 2014). *erm*(B) can dimethylate a single adenine in the 23S rRNA, leading to the inhibition of the binding of macrolides to the 50S subunit of bacterial ribosomes (Qin et al., 2014; Wang et al., 2014). In addition, various native efflux pumps are encoded in bacteria, which provide baseline resistance levels (Trastoy et al., 2018). *cmeABC* is an important efflux system in *C. jejuni* and *C. coli* (Lin et al., 2002), and the inactivation of *cmeB* results in a significant decrease in the minimum inhibitory concentrations (MICs) of various antibiotics (Ge et al., 2005). CmeR acts as transcriptional repressor by binding to the promoter of *cmeABC* operon to control its expression (Lin et al., 2005a). Mutations in the regulatory region of *cmeABC* promoter (CmeR-Box) have been reported to confer fluoroquinolone resistance in *Campylobacter* (Zhang et al., 2017a; Du et al., 2018), but the effect of these mutations on macrolide resistance has not been investigated.

In this study, to uncover the prevalence and the underlying molecular basis of erythromycin-resistant *Campylobacter* in central China, resistance analysis was conducted, and the mutations on macrolide targets and the present of *erm*(B) were screened. In addition, the polymorphisms of CmeR-Box in the promoter of the *cmeABC* efflux pump were also investigated.

## MATERIALS AND METHODS

### Ethics Statement

All animal studies were conducted in strict accordance with the animal welfare guidelines of the World Organization for Animal Health. The protocols were approved by the Hubei Provincial Animal Care and Use Committee (approval number SCXK 2015-0021).

### Bacterial Isolation

From 2015 to 2017, 143 *Campylobacter* isolates were collected from chickens or chicken meats in central China (three farms and four markets in Hubei, two farms and three markets in Henan, two farms and two markets in Jiangxi, one farm and two markets in Anhui, and one farm in Hunan), and all the chickens were from commercial broiler flocks. In brief, freshly collected anal and meat swabs were kept into Cary-Blair modified transport media (AMRESCO, United States) and transported to the laboratory for *Campylobacter* isolation.

The samples were resuspended in phosphate-buffered saline (PBS) first, and then inoculated in Bolton broth containing *Campylobacter* growth supplement (Oxoid, United Kingdom) and *Campylobacter* Bolton broth selective supplement (Oxoid, United Kingdom) for 24 h at 42°C under microaerobic condition. After inoculation, 100 µl of the culture was spread onto a modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid) plate containing *Campylobacter* CCDA-selective supplement (Zhang A. et al., 2016). The suspected *Campylobacter* colonies were identified by polymerase chain reaction (PCR) targeting 16S ribosomal DNA (rDNA) and sequencing as described (Weisburg et al., 1991), and the primers were as follows: 27F, 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R, 5'-TACGGYTACCTTGTTACGACTT-3'. *C. jejuni* and *C. coli* were differentiated by hippuric acid hydrolysis test and PCR test targeting *C. jejuni*-specific *hipO* gene and *C. coli*-specific *asp* gene (Persson and Olsen, 2005; Keller and Shriver, 2014). To generate a microaerobic environment, all the bacterial culture processes were carried out at 42°C in air tight jars containing the AnaeroPack (Mitsubishi, Japan).

### Antimicrobial Susceptibility Test

According to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2018), erythromycin resistance was first determined by the disk diffusion method on Mueller–Hinton agar (Oxoid, United Kingdom) using erythromycin disks with 15 µg. After incubation for 40 h at 42°C, the diameters (in mm) of the inhibition zones were measured, and <20 mm (*C. jejuni*) or <24 mm (*C. coli*) was determined to be resistant. Then, the MICs of erythromycin-resistant strains were measured by the broth dilution method. In brief, twofold serial dilutions of erythromycin was used at the concentrations of 2–2,048 µg/ml, and  $5 \times 10^5$  CFU/ml of each isolate was incubated in Mueller–Hinton broth containing serial dilutions of erythromycin under microaerobic condition at 42°C for 24 h. The MICs were determined as the lowest concentration of the agent that completely inhibits visible growth. The antibiotic disks and powder were purchased from Oxoid, United Kingdom. The *Escherichia coli* ATCC 25922 was used as a quality control strain.

### MLST

Multilocus sequence typing (MLST) was carried out in all erythromycin-resistant isolates. In brief, genomic DNA of the *Campylobacter* isolates was extracted using MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. MLST analysis was conducted by sequencing seven *Campylobacter* housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*) as previously described (Dingle et al., 2001). Allele numbers, sequence types (STs) and clonal complexes (CCs) were assigned using the *Campylobacter* MLST database<sup>1</sup>. The calculated tree of the erythromycin-resistant isolates was constructed using the SliptsTree 4 version 1.2 based on the ST types.

<sup>1</sup><http://pubmlst.org/campylobacter>

## Detection of *erm(B)* and Mutations in 23S rRNA and CmeR-Box

The presence of *erm(B)* was screened by PCR as previously reported (Wang et al., 2014), and the primers used were as follows: *ermB*-F, 5'-GGGCATTTAACGACGAACTGG-3'; *ermB*-R, 5'-CTGTGGTATGGCGGGTAAGT-3'. Polymorphisms present on the amplified fragment of the 23S rRNA and on the promoter of *cmeABC* operon (CmeR-Box) were investigated using PCR and double-stranded DNA sequencing as previously described (Corcoran et al., 2005; Perez-Boto et al., 2010). The primers for amplifying the fragment of the 23S rRNA and CmeR-Box were as follows: 23S-F, 5'-GCTCGAAGGTTAATTGATG-3' and 23S-R, 5'-GCTCTTGGCAGAACAAC-3'; *Cbox1*-F, 5'-GGTTGTACAGGTTGAGGC-3' and *Cbox1*-R, 5'-AGCTTACGCAAAGGATAATG-3' for *C. jejuni*; and *Cbox2*-F, 5'-GGTTGTACAGGTTGAGGC-3' and *Cbox2*-R, 5'-AGCTTACGCAAAGGATAATG-3' for *C. coli*.

## Electrophoresis Mobility Shift Assay

Binding of recombinant CmeR protein to the promoters of the *cmeABC* operon, which contained different CmeR-box sequences, was performed by electrophoresis mobility shift assay (EMSAs) as previously described (Zhang et al., 2012; Grinnage-Pulley and Zhang, 2015). Briefly, the DNA fragments containing CmeR-Box sequences were amplified from genomic DNA of *Campylobacter* of the *Campylobacter* isolates. To obtain the CmeR protein, the coding sequence of *cmeR* was amplified and cloned into vector pET-28a (Novagen, Shanghai, China). *Escherichia coli* BL21(DE3) was transformed with the recombinant plasmid pET28a-*cmeR*, and then, the expression was induced by the addition of 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at 18°C for 12 h. The recombinant CmeR protein was purified with Ni-NTA agarose (Bio-Rad, Shanghai, China) under native conditions, according to the manufacturer's instructions. Binding reactions were carried out in a 20  $\mu$ l volume containing 0.1  $\mu$ g promoter DNA and different amounts of purified recombinant CmeR protein (0, 2, 4, and 8  $\mu$ g), and incubated at room temperature for 30 min. Electrophoresis was carried out with 5% native polyacrylamide gels at 100 V for 1 h. The gels were stained with 1  $\mu$ g/ml of ethidium bromide. To compare the bonding abilities, the optical densities of bound DNA and free DNA were measured using ClinX Image Analysis software (ClinX Science Instruments Co., Ltd., Shanghai, China). The proportions of bound DNA were calculated according to the optical densities values (0% was "–"; >0 to  $\leq$ 50% was "+"; >50 to  $\leq$ 95% was "++"; >95% was "+++").

## Real-Time RT-PCR

To analyze the effect of CmeR-Box polymorphism on the expression of *cmeABC*, five *C. jejuni* isolates (QCJ3, JSJ27, XZJ48, WHJ54, and XTJ10) were chosen. The QCJ3 strain contained a wild-type CmeR-Box, the JSJ27 strain contained a point substitution in CmeR-Box, the XZJ48 (ST-7510) and WHJ54 (ST-7512) strains contained the same point deletion in CmeR-Box, and the XTJ10 (ST-7508) strain contained a

point insertion in CmeR-Box. None of the isolates contain the *erm(B)* and/or A2075G substitution in the 23S rRNA. Total RNAs of the selected *Campylobacter* isolates were isolated as follows: overnight cultured bacteria were diluted 1:100 in fresh Mueller–Hinton broth and then incubated to mid-log phase ( $OD_{600} = 0.5$ ) at 42°C under microaerobic condition. Eight micrograms per milliliter (two times of MIC breakpoint by EUCAST, and the breakpoint of erythromycin is 4  $\mu$ g/ml) of erythromycin was added, and the bacteria were collected at 0, 5, and 120 min post-treatment. Total RNA was isolated and purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Then, the isolated RNA was reverse transcribed to complementary DNA (cDNA), and the expression levels of the *cmeA* gene were assessed by real-time reverse transcription PCR (RT-PCR) using SYBRGreen detection (TAKARA BIO INC., Dalian, China) in an ABI7500 system (Thermo Fisher Scientific, CA, United States). The primers for *cmeA* were as follows: *qcmeA*-F, 5'-CTGACAAGTTTAGCAGGGTA-3', *qcmeA*-R, 5'-GCAGCAAAGAAGAAGCACCA-3'. The 16S rRNA and *gapdh* genes were used as the internal control. The primers were as follows: *q16S*-F, 5'-TACCTGGGCTTGATATCCTA-3', *q16S*-R, 5'-GGACTTAACCAACATCTCA-3'; *qgapdh*-F, 5'-AGGCAGTGTGATAGTGAAGG-3', *qgapdh*-R, 5'-CAATTTGTGCGCCGTGTT-3'. The expression level of *cmeA* before treatment of the strain harboring wild-type CmeR-Box (*C. jejuni* strain QCJ3) was used as control condition. Each assay was carried out with at least three biological replicates. Differences in relative transcript abundance level were calculated using the  $2^{-\Delta\Delta CT}$  method (Pfaffl, 2001), and  $\geq 2$ -fold changes were considered as differentially expressed.

## Statistical Analysis

The Fisher test was used to identify the correlation between resistance and mutations, and the Student's *t*-test was used to compare the expression levels of target genes of different strains. The analysis was carried out by SPSS 19.0. A probability (*p*) value of <0.05 was considered statistically significant.

## RESULTS

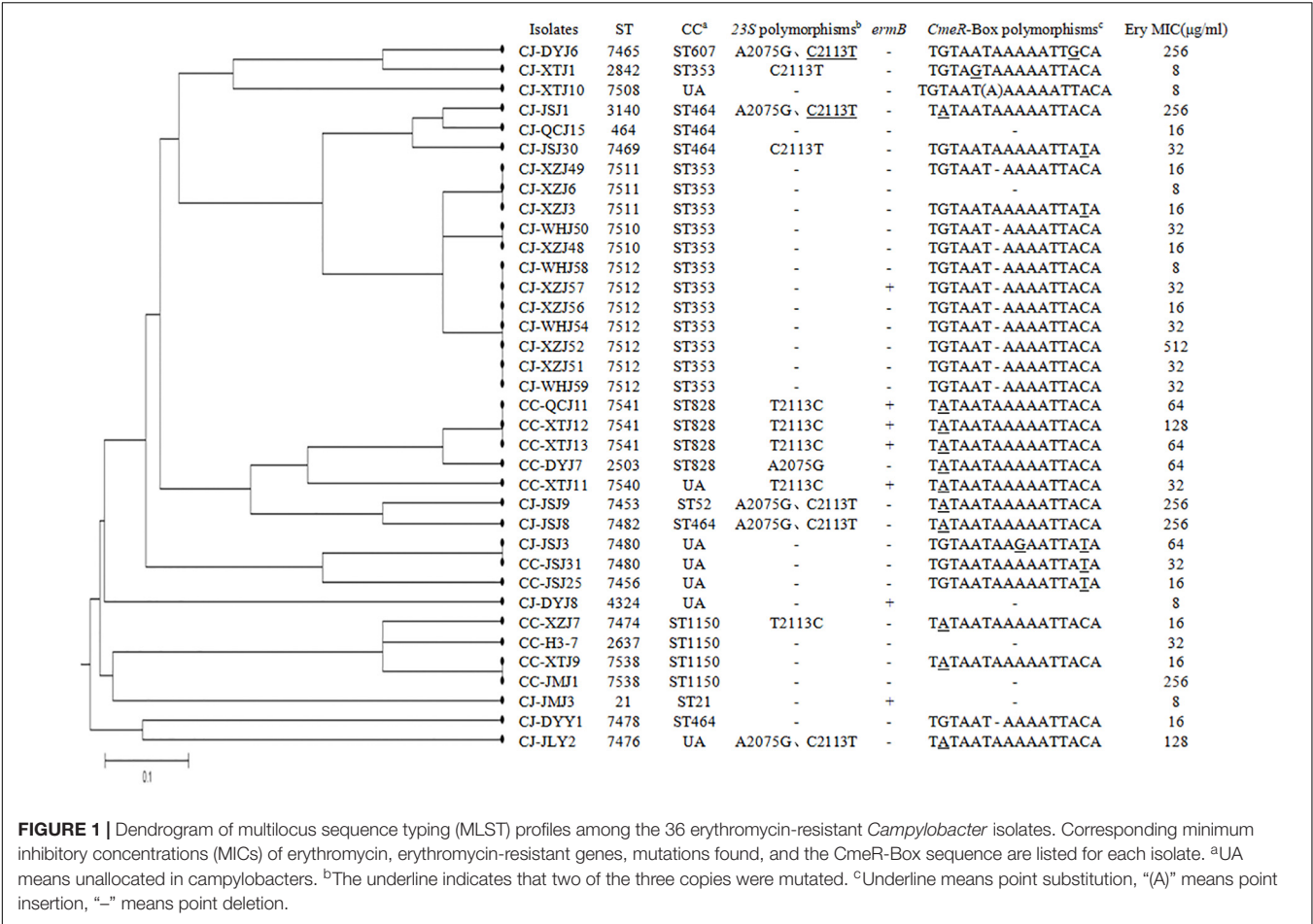
### Prevalence of Erythromycin-Resistant *Campylobacter*

A total of 143 *Campylobacter* strains, including 83 *C. jejuni* and 60 *C. coli* isolates, were isolated and tested for erythromycin susceptibility. Among them, 36 (25.2%) *Campylobacter* isolates were erythromycin resistant, including 25 (30.1%, MIC > 4 mg/l) *C. jejuni* and 11 (18.3%, MIC > 8 mg/l) *C. coli* (Figure 1). These results showed high erythromycin resistance rates of *C. jejuni* and *C. coli* isolates in our study.

### Mutations in 23S rRNA of *Campylobacter* Isolates

Polymorphisms on the 23S rRNA were analyzed in all *Campylobacter* isolates. As shown in Table 1, Figure 1, and





**Supplementary Material**, point substitutions on 23S rRNA were found in 23 isolates (8 *C. jejuni* and 15 *C. coli* isolates). According to published sequences, the *Campylobacter* genome encodes three copies of 23S rRNA (Sheppard and Maiden, 2015). Except for the isolates that contained A2075G substitution, 16 isolates contained the C2113T (*C. jejuni*) or T2113C (*C. coli*) substitution in all three copies of 23S rRNA, while one *C. jejuni* contained this substitution in two of three copies (double peak and the peak for guanine was two times higher than the peak for adenine), and among them, 7 of the 17 isolates were erythromycin resistant; thus, the correlation was not significant ( $p > 0.05$ ). The A2075G substitution was observed in 6 (5 *C. jejuni* and 1 *C. coli* isolates) of the 23 isolates, and all of them were erythromycin resistant. These results suggested that the A2075G, but not the C2113T or T2113C, substitution in 23S rRNA was responsible for erythromycin resistance in *Campylobacter*.

**Presence of *erm(B)* in *Campylobacter* Isolates**

The ribosomal methylase *erm(B)* gene was found in seven *Campylobacter* isolates (4.9%), including three *C. jejuni* and four *C. coli*. These seven isolates were also erythromycin resistant, and none of them contained an A2075G substitution in 23S rRNA (Figure 1; Wang et al., 2014). Although the incidence of *erm(B)*

was not high, the seven *erm(B)*-harboring strains were isolated from five different regions.

**Polymorphism Analysis of CmeR-Box in *Campylobacter* Isolates**

Polymorphisms of CmeR-Box were analyzed in all isolates, and seven CmeR-Box variants were identified (Figure 1 and Table 2). Among them, 13 (9.1%) isolates contained a point deletion/insertion within the inverted sequences (Figure 2A), and 12 out of 13 were resistant to erythromycin. Among the 12 resistant isolates, 11 did not contain *erm(B)* or a mutation on 23S rRNA. Statistical analysis showed that the occurrence of point deletion/insertions significantly correlated with erythromycin resistance ( $p < 0.05$ ), suggesting an important role in the erythromycin resistance phenotype. In contrast, point substitutions in CmeR-Box were found in 41 (49.4%) *C. jejuni* isolates and 36 (60.0%) *C. coli* isolates, but among them, only 9 (22.0%) *C. jejuni* and 9 (25.0%) *C. coli* isolates were erythromycin resistant, and among the 18 resistant isolates, most of them also harbor mutations in 23S rRNA and/or the *erm(B)* gene (Figure 1). Statistical analysis showed that nucleotide substitution was not correlated with erythromycin resistance ( $p > 0.05$ ). Our results suggested that point deletions

**TABLE 1** | Polymorphisms on the 23S rRNA gene of *C. jejuni* and *C. coli* isolates.

	Position <sup>c</sup>	Number of isolates	Number of resistant isolates	Resistance frequency (%)
<b><i>C. jejuni</i></b>				
Mutations in 23S rRNA <sup>a</sup>	A2075G(3/3), C2113T(2/3)	2	2	100
	A2075G(3/3), C2113T(3/3)	3	3	100
	C2113T(2/3)	1	0	0
	C2113T(3/3)	2	2	100
Without mutation	–	75	18	24.0
<b><i>C. coli</i></b>				
Mutations in 23S rRNA <sup>b</sup>	A2075G(3/3)	1	1	100
	T2113C(3/3)	14	5	35.7
Without mutation	–	45	5	11.1

<sup>a</sup>The mutations were determined based on the sequence of the reference strain *C. jejuni* NCTC 11168 (accession number: AL111168.1). <sup>b</sup>The mutations were determined based on the sequence of the reference strain *C. coli* OR12 (accession number: CP013733.1) (O’Kane and Connerton, 2017). <sup>c</sup>The numbers in parentheses represent the number of mutations in the three copies of the 23S rRNA gene.

**TABLE 2** | CmeR-Box polymorphisms in *C. jejuni* and *C. coli* isolates.

	Number of isolates	Number of resistant isolates <sup>a</sup>	Resistance frequency (%)	vs. None <sup>b</sup>
<b><i>C. jejuni</i></b>				
Point substitution in CmeR-Box	41	9(5)	22.0	$p > 0.05$
Point deletion or insertion in CmeR-Box	13	12(1)	92.3	$p < 0.01$
Without mutation	29	4(2)	13.8	–
<b><i>C. coli</i></b>				
Point substitution in CmeR-Box	36	9(5)	25.0	$p > 0.05$
Without mutation	24	2(0)	8.3	–

<sup>a</sup>The numbers in brackets indicate the strains containing A2075G substitution and/or *erm*(B). <sup>b</sup>The correlation between resistance and mutations were calculated with the Fisher test.

or insertions, but not nucleotide substitution, in the CmeR-Box was significantly associated with erythromycin resistance.

## EMSAs and Expression Analysis of *cmeABC*

To further understand the effect of point deletion/insertion in the CmeR-Box on erythromycin resistance, the binding of CmeR to the CmeR-Box with a point deletion/insertion was confirmed with the EMSAs. As shown in **Figure 2B**, the binding

of CmeR to CmeR-Box with a point deletion/insertion was significantly decreased, suggesting that the *cmeABC* operon in these isolates was highly derepressed and associated with erythromycin resistance. Analysis of the expression levels of *cmeA* at the time point 0 min further confirmed these results (**Figure 2C**). Although *cmeA* expression could be induced by the addition of erythromycin in all the isolates, as compared with the isolate without a mutation in the CmeR-Box, higher expression was observed in the isolates harboring a point deletion/insertion before and after erythromycin treatment ( $p < 0.05$ ). It is worth noting that, in the isolates harboring a point deletion/insertion, *cmeA* expression was originally high before erythromycin treatment and only increased by approximately twofold at 5 min post-treatment and recovered at 2 h post-treatment. In contrast, in the wild-type isolate and isolate with a point substitution, although *cmeA* expression was upregulated by more than fourfold at 5 min post-treatment, their expression levels were still lower than in isolates with a point deletion/insertion.

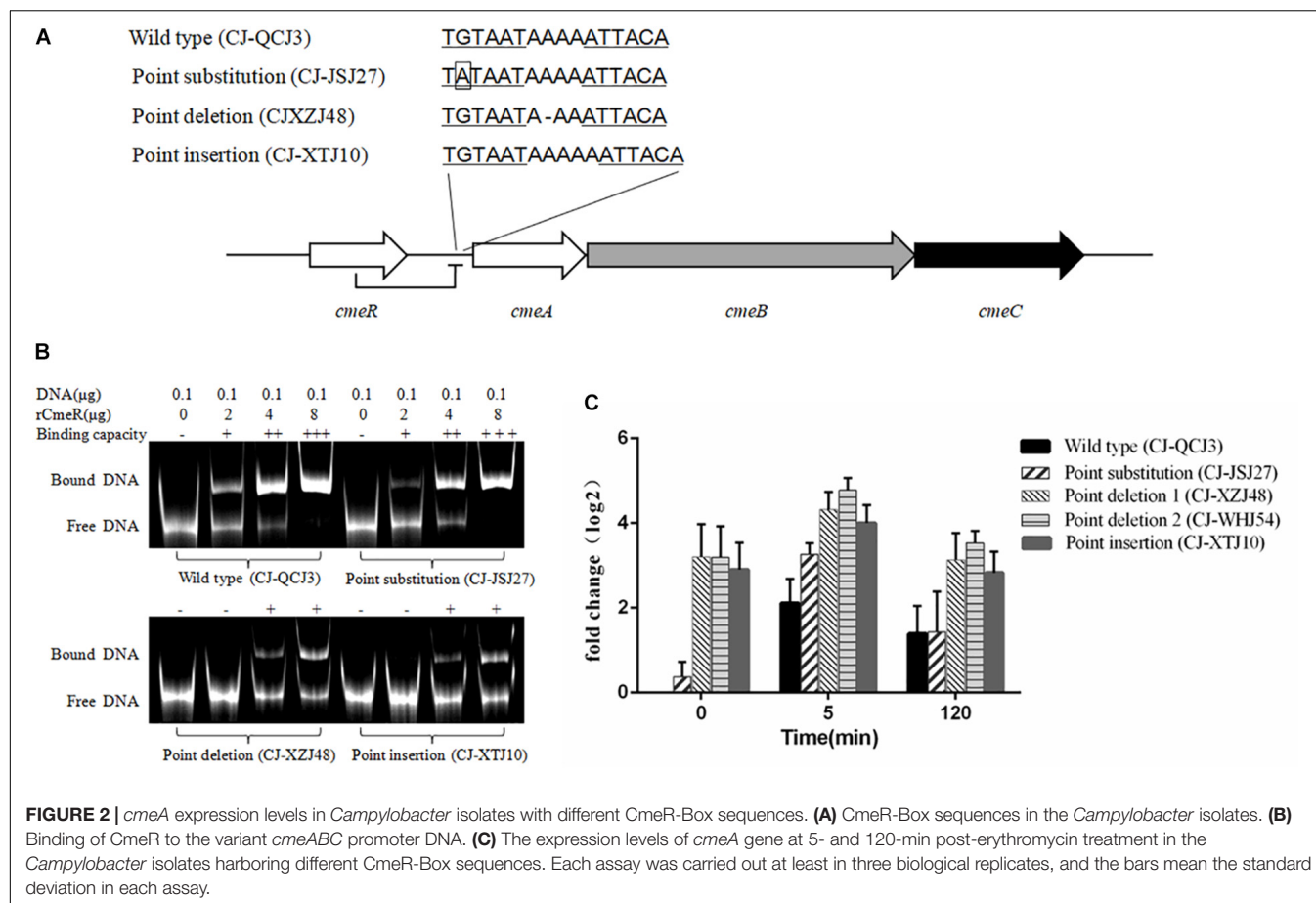
## Genetic Diversity Analysis

The genetic diversity of erythromycin-resistant *Campylobacter* isolates was analyzed with MLST, as previously described (Dingle et al., 2001). As shown in **Figure 1**, the 36 erythromycin-resistant *Campylobacter* isolates were distributed along 23 STs and 8 CCs. The dominant CC was CC353, and seven isolates could not be assigned to a CC. Five *C. jejuni* strains with an A2075G substitution in the 23S rRNA belonged to three CCs (CC607, CC464, and CC52), and one could not be assigned to a CC. Three *C. jejuni* *erm*(B)-harboring strains belonged to two different CCs (CC353 and CC21), and one was not assigned. These results suggest a diverse distribution of genotypes in *C. jejuni* resistant to erythromycin. In contrast, 10 out of 11 erythromycin-resistant *C. jejuni* with a point deletion in the CmeR-Box were distributed between two STs both belonging to the dominant CC353, suggesting a common ancestor. In *C. coli* isolates, three *erm*(B)-harboring isolates shared the same CC (CC828) clustered with an unallocated (UA) strain, suggesting a common ancestor.

## DISCUSSION

*Campylobacter jejuni* and *Campylobacter coli* are major food-borne pathogens worldwide, and poultry is recognized as the most important reservoir of these pathogens (Crawshaw, 2019; Thomas et al., 2019). Usually, erythromycin is the first-line choice for treatment of campylobacteriosis (Bolinger and Kathariou, 2017). Therefore, to investigate the prevalence and erythromycin resistance mechanisms of *C. jejuni* and *C. coli* is not only important for the poultry industry but also for public health.

In our study, 30.1% *C. jejuni* and 18.3% *C. coli* isolates were erythromycin resistant. As previously reported, the prevalence of macrolide resistance is common in *C. coli* but remains low in *C. jejuni*. For example, data from the National Antimicrobial Resistance Monitoring System (NARMS) report of the U.S. Food and Drug Administration on human clinical isolates and food-producing animal isolates indicated that the resistance rate in *C. jejuni* was under 4% in 2014 (FDA, 2014). In



a previous investigation in China, the global resistance rate of *Campylobacter* to erythromycin was 18.4%, and among the resistant strains, 97.5% were *C. coli* and 2.5% were *C. jejuni* (Zhang A. et al., 2016). However, in this study, high erythromycin resistance rates were found in both *C. jejuni* and *C. coli* isolated from central China, which pressed us to further understand the molecular mechanisms of erythromycin resistance in these isolates.

Mutations in 23S rRNA, and single adenine methylation in the 23S rRNA by ribosomal methylase *erm(B)*, are two well-known mechanisms for erythromycin resistance in *Campylobacter* (Zhao et al., 2016). In this study, A2075G substitution in 23S rRNA and ribosomal methylase encoded by *erm(B)* were found in 4.2 and 4.9% isolates, respectively, and all of them were erythromycin resistant. As previously reported, the A2075G substitution is one of the most prevalent genetic events conferring high-level resistance to erythromycin (Zhao et al., 2016), as further evidenced by our results. Substitutions at 2113 site were found in our isolates. Based on previous reports and the sequenced reference strains, including *C. jejuni* NCTC 11168 and *C. coli* OR12 (Parkhill et al., 2000; Perez-Boto et al., 2010; O'Kane and Connerton, 2017), the dominant nucleotide at 2113 site is "C" in *C. jejuni* and "T" in *C. coli*, which were defined as wild type in both species in this study. Correlation analysis showed that this substitution was not correlated with erythromycin

resistance ( $p > 0.05$ ). We infer that this substitution exists extensively in *Campylobacter* and does not affect erythromycin resistance. Some of the resistant isolates with this substitution could be explained by other resistance factors, such as *erm(B)* in strains QCJ11, XTJ11, XTJ12, and XTJ13. However, the causes of resistance on the part of isolates are still unknown and require further study.

*erm(B)*-harboring *Campylobacter* isolates were first reported in 2014 in China (Qin et al., 2014) and then detected in Spain and the United States (Florez-Cuadrado et al., 2016). In this study, although the incidence of *erm(B)* was not high, seven *erm(B)*-harboring strains were isolated from five different regions. Our results suggested that *erm(B)* might be widespread at the regional distribution in central China.

Drug efflux is another important resistance mechanism conferred by native efflux systems in bacteria. The *cmeABC* operon, which encodes a drug efflux pump and plays an important role in drug resistance in *Campylobacter* (Lin et al., 2005a), is negatively regulated by CmeR by binding to a 16-base inverted repeat sequence [CmeR-Box, TGTAATA(or T)TTTATTACA] in the promoter region (Yan et al., 2006). In our isolates, a couple of CmeR-Box variants were identified (Figure 2A and Table 2), including point substitutions and deletion/insertion within the inverted sequences. It is interesting that point deletions/insertions were

found in 13 (9.1%) isolates, and 12 out of 13 isolates were resistant, which was correlated to erythromycin resistance ( $p < 0.05$ ). However, *Campylobacter* harboring point deletions in CmeR-Box were seldom reported and not well characterized (Abd El-Tawab et al., 2019). In contrast, point substitutions were not correlated to erythromycin resistance ( $p > 0.05$ ). Substitutions in CmeR-Box have been reported to cause overexpression of *cmeABC* although not affecting the susceptibility of *C. jejuni* to most tested antimicrobials including erythromycin (Grinnage-Pulley and Zhang, 2015). It is worth noting that all of the mutations identified in CmeR-Box were point substitutions in that report, so their result was consistent with our finding that there was no correlation between point substitutions in CmeR-Box and erythromycin resistance. In our study, point deletion/insertion in CmeR-Box was identified in *C. jejuni* isolates, and our results suggested that point deletion or insertion, but not substitution, in CmeR-Box was significantly associated with erythromycin resistance.

To further investigate the effect of point deletion/insertion in CmeR-Box on resistance, the binding of CmeR to the various CmeR-Box and the expressional levels of *cmeABC* were detected. It is well-known that CmeR can interact with bile salts and transduce this signaling, but little is known about the capacity of antibiotics to induce *cmeABC* expression (Lin et al., 2005b). In this study, the expression of *cmeA* was induced after erythromycin treatment. We found that the expression of *cmeR*, which encodes the repressor of *cmeABC*, was slightly reduced 5 min post-treatment, and there were no significant difference between these tested isolates (data not shown). We inferred that the upregulated expression of *cmeABC* after erythromycin treatment was due to the reduced expression of its repressor CmeR. Comparing with the wild-type CmeR-Box and CmeR-Box with point substitution, an obvious decrease in binding ability of CmeR to CmeR-Box with a point deletion/insertion, and subsequent higher overexpression of *cmeABC*, was showed. These results support why point deletion or insertion, but not substitutions, in CmeR-Box was significantly associated with erythromycin resistance. Functional CmeR is a dimer, and each monomer binds to one half of the inverted repeat of the CmeR-Box (Gu et al., 2007; Lei et al., 2011). We inferred that, although point substitutions reduced the bonding strength of CmeR to CmeR-Box, the bonding strength was still moderate (Figure 2B). Therefore, the expression of *cmeABC* was only slightly increased (Figure 2B) in these isolates harboring CmeR-Box with point substitutions. The slightly increased expression of *cmeABC* might result in increased MICs, but the MICs did not reach the breakpoint in these isolates. In contrast, a point deletion/insertion changed the distance between two consecutive binding sequences, leading to an obvious decrease in binding ability and subsequent overexpression of *cmeABC* (Figure 2), which conferred high level of erythromycin resistance. With regard to that one susceptible isolate carrying point deletion in CmeR-Box, we infer that there might be some unknown mutations, such as the mutations in the *cmeABC* proteins, which affect its resistant ability. CC-JMJ1 is a specific highly resistant isolate,

which does not contain any mutations in CmeR-Box and 23S rRNA, and for *erm(B)*, we infer that it may contain a previously reported resistance-enhancing variant of the efflux pump *cmeABC*, or due to other unknown mechanisms (Yao et al., 2016).

## CONCLUSION

In summary, 25.2% of *Campylobacter* isolates in central China were erythromycin resistant. The A2075G substitution in 23S rRNA and the presence of *erm(B)* were identified as two important factors that lead to erythromycin resistance. Furthermore, this is the first study to report that a point deletion/insertion, but not substitution, in CmeR-Box could significantly increase the expression of *cmeABC*, which plays important roles in erythromycin resistance. These findings will help us to further understand the mechanism of erythromycin resistance in *Campylobacter*.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

All animal studies were conducted in strict accordance with the animal welfare guidelines of the World Organization for Animal Health. The protocols were approved by the Hubei Provincial Animal Care and Use Committee (approval number SCXK 2015-0021).

## AUTHOR CONTRIBUTIONS

YC, TZ, and HS conceived and designed the experiments and wrote the manuscript. YC, QL, QLo, and ZZ performed the experiments. GW and WZ analyzed the data.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.00203/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.

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# Characterizing Antimicrobial Resistant *Escherichia coli* and Associated Risk Factors in a Cross-Sectional Study of Pig Farms in Great Britain

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Combating antimicrobial resistant (AMR) using a One-Health approach is essential as various bacteria, including *Escherichia coli*, a common bacteria, are becoming increasingly resistant and livestock may be a reservoir. The AMR gene content of 492 *E. coli*, isolated from 56 pig farms across Great Britain in 2014–2015, and purified on antibiotic selective and non-selective plates, was determined using whole genome sequencing (WGS). The *E. coli* were phylogenetically diverse harboring a variety of AMR profiles with widespread resistance to “old” antibiotics; isolates harbored up to seven plasmid Inc-types. None showed concurrent resistance to third-generation cephalosporins, fluoroquinolones and clinically relevant aminoglycosides, although ~3% harbored AMR genes to both the former two. Transferable resistance to carbapenem and colistin were absent, and six of 117 *E. coli* STs belonged to major types associated with human disease. Prevalence of genotypically MDR *E. coli*, gathered from non-selective media was 35% and that of extended-spectrum-beta-lactamase *E. coli* was low (~2% from non-selective). Approximately 72.6% of *E. coli* from ciprofloxacin plates and only 8.5% from the other plates harbored fluoroquinolone resistance due to topoisomerase mutations; the majority were MDR. In fact, multivariable analysis confirmed *E. coli* purified from CIP enrichment plates were more likely to be MDR, and suggested MDR isolates were also more probable from farms with high antibiotic usage, specialist finisher farms, and farms emptying their manure pits only after each batch. Additionally, farms from the South East were more likely to have MDR *E. coli*, whereas farms in Yorkshire and the Humber were less likely.

Future investigations will determine whether suggested improvements such as better biosecurity or lower antimicrobial use decreases MDR *E. coli* on pig farms. Although this study focuses on pig farms, we believe the methodology and findings can be applied more widely to help livestock farmers in the United Kingdom and elsewhere to tackle AMR.

**Keywords:** antimicrobial resistance, multidrug resistance, plasmids, epidemiology, risk factor analysis, pig farms, *Escherichia coli*, Great Britain

## INTRODUCTION

*Escherichia coli* is a key species associated with AMR, including multidrug resistance (resistance to 3 or more antimicrobial classes; MDR) (World Health Organisation [WHO], 2014), threatening delivery of effective healthcare and challenging basic procedures used in modern human and veterinary medicine (O'Neill, 2016).

In Europe, since 2006, non-therapeutic use of antimicrobials has been limited in livestock (Cogliani et al., 2011). However, concerns remain that antibiotic use in food animals for therapeutics increases the risk of selection and emergence of AMR bacteria; and animals are a reservoir (Argudin et al., 2017). Recent livestock studies in Great Britain (GB), suggest that *E. coli*, chosen as an indicator organism for monitoring AMR in commensal bacteria in the gut flora of livestock, may carry resistance to high priority critically important antibiotics (HP-CIAs) such as cefotaxime or colistin (Anjum et al., 2011; Randall et al., 2014; Card et al., 2016; Duggett et al., 2017; Kirchner et al., 2017), that may spread horizontally to pathogens. Harmonized monitoring of AMR across Europe has indicated resistance of *E. coli* to HP-CIAs varies from country to country in humans, animals, and food (European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC], 2017); but typically only phenotypic analysis of AMR is performed on isolate(s) from a single representative animal on farm, recovered from selective and/or non-selective media. Recently, the fecal resistome of pigs and poultry from nine European countries was assessed using metagenomics, to define the on-farm AMR load (Munk et al., 2018). The study reported a higher AMR load in pigs than poultry, however, the organisms harboring the AMR genes was not defined, possibly due to limits of Illumina short read sequencing. Also, mobile genetic elements such as plasmids that play a key role in AMR dispersal could not be classified further.

The aim of this work was to characterize, using WGS, the AMR genes present in 492 *E. coli* from multiple pigs on farm, collected in a cross-sectional study, across GB. WGS also enabled phylogenetic diversity of the host *E. coli* to be explored, as well as putative AMR plasmids, which may play a key role in the transferring of AMR genes between *E. coli*. Presence of MDR isolates was analyzed with on-farm risk factors to identify

features that affect their selection, as well as those that may help control their spread.

## MATERIALS AND METHODS

### Farm Recruitment and Sampling

Fifty-six farms in GB were recruited, from two different production types (38 Farrow-to-Finish and 18 Finisher-only farms). Farms were geographically diverse, and loosely representative of the finisher-only pig industry in GB; no farms were selected from the North West, Wales or Scotland (**Supplementary Table S1**) (Pig Health and Welfare Council, 2017). The farms selected were not random but an opportunistic selection of 53 farms participating in a *Salmonella* study, and these included 19 farms with low prevalence of *Salmonella* (Martelli et al., 2017; Smith et al., 2017, 2018). Three additional farms were recruited solely for this study. All farmers provided written consent to allow their pigs to be sampled and their laboratory results used for research purposes. Sampling of farms took place at 12 different abattoirs, between March 2014 and October 2015. A comprehensive questionnaire, completed by farmers, collected information on farming practices that included hygiene and disinfection of houses, biosecurity, as well as antibiotic usage. Ethical approval was not sought as sampling from carcasses is deemed outside of the Animal (Scientific Procedures) Act 1986.

### Sampling and Isolate Collection

At abattoirs, cecal content from 10 randomly selected healthy finishing pigs per herd/farm, were collected. For each herd, 0.5 g of pig cecal content from each pig was suspended into 22.5 ml of 0.1 M PBS (pH7.2) and the pooled pig cecal samples diluted up to  $10^{-5}$  in PBS.

Aliquots of 100  $\mu$ l were plated on to the following agar; Brilliance UTI agar (Oxoid, Basingstoke, United Kingdom) plates containing either 1 mg/L cefotaxime, 1 mg/L ciprofloxacin, and without antibiotics. Six morphologically distinct colonies were selected from each plate, however, if less than six colonies were isolated on either 1 mg/L cefotaxime or 1 mg/L ciprofloxacin plates, additional colonies from antibiotic-free plates were selected, to reach 18 isolates per farm. In addition, Brilliance carbapenem-resistant Enterobacteriaceae (CRE) Agar (Oxoid, Basingstoke, United Kingdom), was used. Purified colonies were subsequently stored at  $-80^{\circ}\text{C}$  in MicroBank beads (Pro-Lab Diagnostics, Neston, Cheshire,

**Abbreviations:** AMR, antimicrobial resistance; CRE, Brilliance carbapenem-resistant Enterobacteriaceae agar; HP-CIAs, highest priority critically important antibiotics; MDR, multidrug resistance (resistance to 3 or more antimicrobial classes); WGS, whole genome sequencing.



United Kingdom). The presumptive purified *E. coli* were identified to species level using Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-ToF) (Bruker, Coventry, United Kingdom) or 16S rRNA sequencing (Edwards et al., 2012). Antimicrobial susceptibility testing was performed using the BSAC agar dilution method on the four *E. coli* isolated from CRE agar to determine susceptibility to three carbapenem antibiotics (Doripenem, Imipenem, and Meropenem), for all *E. coli* isolated from ciprofloxacin and cefotaxime antibiotic plates (Andrews, 2001). The results were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off (ECOFF) and clinical breakpoint values.

## Whole Genome Sequence Analysis

DNA was extracted and Illumina HiSeq 4000 System used to perform WGS as described previously (Stoesser et al., 2013) on the 503 isolates identified as *E. coli* by MALDI-ToF. Kraken was used to confirm MALDI-ToF speciation results (Wood and Salzberg, 2014), which identified 11 isolates as *E. fergusonii*, a species that has been reported in livestock (Wragg et al., 2009), and excluded from further analysis. The presence of acquired AMR genes in the WGS of isolates was determined by mapping unassembled reads using APHA SeqFinder pipeline (Anjum et al., 2016). The criteria for determining gene presence using APHA SeqFinder pipeline was 100% gene mapping to the reference, and allowing between 1 and 10 non-synonymous SNP. The MIC results and the correlation between genotype and phenotype was >98% for *E. coli* isolates; these results have been presented elsewhere (Stubberfield et al., 2019), and due to the high correlation only the AMR genotype data is presented in this paper. Genotypic multidrug resistance was defined according to the European Food Safety Authority (EFSA) European surveillance reports (European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC], 2017) as an isolate harboring genes from the following 3 or more resistance classes: Extended spectrum  $\beta$ -lactamase (ESBL), Ampicillin (AMP), Tetracycline (TET), Gentamicin - clinically relevant aminoglycoside (GEN), Azithromycin (AZM), Chloramphenicol (CHL), Trimethoprim (TMP), Sulphonamide (SUL), and Fluoroquinolones (FQN).

Sequenced genomes were assembled using SPAdes 3.7.0 11 (Bankevich et al., 2012) and annotated using PROKKA 1.11.12 (Seemann, 2014). The Abricate<sup>1</sup> tool was used to determine which AMR and plasmid replicon genes were co-located on a single contig. BRIG was used to compare plasmids (Alikhan et al., 2011). The Multilocus Sequence Type (MLST) of *E. coli* isolates (Wirth et al., 2006) was determined using either SRTS2 (Inouye et al., 2014) or DTU pipeline<sup>2</sup> (Larsen et al., 2012). SNIPPY<sup>3</sup> was used to generate a whole genome single nucleotide polymorphisms (SNP) alignment produced from WGS data of

492 *E. coli* isolates using the default settings. A maximum-likelihood tree under the General Time Reversible model of nucleotide substitution with among-site rate heterogeneity model (GTR-G) and 100 bootstrap replicates was inferred from the whole genome of 272,385 SNP alignment using *E. coli* MG1655 (Accession number: U00096.2) as reference in RAXML-NG<sup>4</sup>. The tree was visualized and annotated in EvolView (He et al., 2016). The *E. coli* raw sequence data generated and analyzed in this work are available in the European Nucleotide Archive (ENA) under study accession number PRJEB26317.

## Statistical Analysis

The risk factor dataset contained 72 farm-level variables describing basic farm demographics, feeding and watering information including additive practices, and general farm management, which was provided to farmers for completion prior to cecal content sample collection and detection of the AMR gene content of isolates. Antimicrobial usage data were collected, however, due to difference in the detail of information provided by the farms, these responses were categorized subjectively into high, medium and low usage by the authors based upon answers to the total number of daily animal doses of antimicrobials given to pigs and the total cost of antimicrobials in a 12-month period. No standardized criteria existed to categorize the farms, but in general low usage farms were defined by an absence of prophylactic use of antibiotics and a rough cost of antibiotics under £0.5 per pig. Medium usage farms were defined by prophylactic use for short periods of time and a rough cost of antibiotics up to £2 per pig. High usage farms were generally either using in-feed or in-water treatment for multiple pig stages or using a combination of prophylactic/in-feed or in-water treatment and large quantities of injectables (e.g., routine injections to sows), with a rough cost of up to £3 per pig. The dataset also included a binary indicator of the MDR isolates gathered from WGS data analysis, for antimicrobials considered by EFSA (European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC], 2017).

A simple descriptive assessment was completed of each variable, excluding variables with little or no data and creating new categorical variables, where appropriate. Following this, a univariable analysis utilizing a mixed-effects logistic regression model, accounting for the non-independence of multiple isolates per farm, was carried out to determine variables for inclusion ( $p$ -value < 0.25) in the multivariable model. Finally, to assess the effect of including variables at the multivariable level, an iterative, forward step-wise approach was adopted with variables that improved the model fit [assessed via  $p$ -value and Akaike Information Criterion (AIC) (Akaike, 1974)], were selected at each step of the model. The categorical variable, related to antimicrobial usage on each farm over a 12-month period, was classed as *a priori* and automatically retained in the model as was a variable accounting for the use of the four different agar plates used in the study. A  $p$ -value of less than 0.05 was considered to indicate a statistically significant difference.

<sup>1</sup><https://github.com/tseemann/abicate>

<sup>2</sup><https://cge.cbs.dtu.dk/services/MLST/>

<sup>3</sup><https://github.com/tseemann/snippy>

<sup>4</sup><https://github.com/amkozlov/raxml-ng>

Potential confounding between variables were investigated by monitoring whether the addition of variables at each step inflated the Odds Ratio of the variables retained in the model. All statistical analyses were completed in Stata 12 (StataCorp, 2011).

## RESULTS

### *E. coli* AMR Genes and MDR

Four hundred and ninety-two *E. coli* were recovered from 56 pig farms; 51.6% were recovered on non-selective plates and the remainder on antibiotic selective plates (Table 1). The APHA SeqFinder pipeline (Anjum et al., 2016) identified 62 different AMR gene variants in 84.6% (416 of 492) of the *E. coli* isolated which were from 54 farms (Table 1). For the remaining two farms, which were both farrow-to-finish farms, the *E. coli* did not harbor any AMR genes. For the remaining farms, *E. coli* harbored between 1 and 15 AMR genes (Figure 1), and as expected, isolates from the antibiotic-free plate generally had less AMR genes (average = 4.9) than those from antibiotic containing plates (average = 7), indicating co-selection of multiple resistances. The number of AMR genes present in *E. coli* from each farm was variable with the mean number of AMR genes per isolate per farm being 5.1 (Figure 2); there was no association between farms and the presence of specific AMR genes. The most common AMR genes were the tetracycline resistance genes [*tet(A)*], followed by  $\beta$ -lactamases (*bla*<sub>TEM-1b</sub>) and the streptomycin resistance genes (*strAB*) (Table 1). Only 9.3% of isolates harbored genes conferring resistance to clinically relevant aminoglycosides (gentamicin, amikacin, netilmicin, and tobramycin), and no 16S rRNA methyltransferase enzyme (16S RMTase) genes were identified (Magiorakos et al., 2012; Public Health England, 2017). Fourteen farms had *E. coli* isolates harboring two *bla*<sub>TEM</sub> variants, with isolates from farm MSG54, harboring three different variants.

National (Public Health England, 2017) and European (European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC], 2017) surveillance programs monitor AMR in commensal and pathogenic bacteria, mainly at the phenotypic level. To facilitate comparison between these datasets with our AMR genotypes, we queried genes present in the AMR classes included in these programs (Supplementary Table S2). Using this criterion, 35% of *E. coli* recovered on non-selective plates, from 40 farms, were genotypically MDR (Supplementary Figure S1). A higher proportion of isolates (87%) recovered on antibiotic selective plates, from 49 farms, were genotypically MDR. Sixty-four MDR genotypes were found; the most common genotype (ampicillin, tetracycline, sulphonamide, trimethoprim, and fluoroquinolone) was present in isolates from 12 farms in both production systems (Supplementary Table S3). Forty-eight MDR profiles (221 isolates) included resistance classes reported in *E. coli* from human blood infections such as ESBL, fluoroquinolone or clinically relevant aminoglycosides (Public Health England, 2017); however, no isolate harbored all 3 classes. Fifteen isolates harboring ESBL and fluoroquinolone resistance were detected on five farms.

We considered the linkage of AMR genes and the plasmid incompatibility types (Inc-types) by observing the co-location of these genes on the same contigs, using the *de novo* assembled genomes of isolates. Most isolates harbored between 1 and 7 (average 3) Inc-types, with 90 Inc-types and AMR gene combinations being present (Supplementary Table S4). The most common contigs harboring an AMR gene and plasmid Inc-types ranged in size from ~4 to >100 Kb (Table 2); several of these contigs showed similarity to plasmids of diverse origins, mostly reported from *Salmonella* and *E. coli*. The number of farms and isolates in which these putative plasmid sequences were identified varied, with some (e.g., IncQ1, IncF, and IncI) present in isolates with different STs and farms. The putative IncQ1 plasmid harboring *strAB* and *sul2* was the most common and highly conserved, found in 48 isolates, but it only showed ~50% identity to the closest references available in databases (Supplementary Figure S2).

### Resistance to HP-CIAs in *E. coli*

Only 28 *E. coli* isolates (18 from cefotaxime plates) harbored genes, which mediate resistance to extended-spectrum cephalosporins (ESC) (Table 1); three farms had *E. coli* harboring two ESC resistance gene variants (*bla*<sub>CTX-M-1</sub> and *bla*<sub>CTX-M-15</sub>, *bla*<sub>CMY-2</sub> and *bla*<sub>CTX-M-15</sub>, or *bla*<sub>CMY-2</sub> and *bla*<sub>SHV-12</sub>). ESC resistance genes were found co-located on contigs with plasmid replicons, with some showing high homology to published plasmids (Table 2). For example isolates from farm MSG17 and MSG43 harbored *bla*<sub>CTX-M-1</sub> on a plasmid with high identity to the p369 IncI plasmid (Table 2 and Supplementary Figure S2). An additional 16 *E. coli*, isolated from cefotaxime plates, harbored mutations in the chromosomal AmpC promoter region associated with increased  $\beta$ -lactamase expression (Caroff et al., 2000).

Chromosomal mutations in quinolone resistance determining regions (QRDR) of *gyrA* and/or *parC* (Yoshida et al., 1991) were identified in 72.6% of *E. coli* (from 44 farms) recovered from ciprofloxacin plates (Table 1), with MICs ranging from 2 to 64 mg/L; 53.1% of these isolates were clinically resistant to ciprofloxacin (>4 mg/L). Only 8.5% of *E. coli* (from 18 farms) recovered from the other agar plates harbored mutations in QRDR, with MICs ranging between 2 and 128 mg/L; 48% of these were clinically resistant to ciprofloxacin (Table 1). Of the QRDR mutations identified, S83L/D87N double substitution (*n* = 80) was the most common for GyrA, and S80I substitution (*n* = 80) most common for ParC; 73 of these isolates harbored double-serine mutation (Fuji et al., 2017), and the majority were recovered on ciprofloxacin plates (Supplementary Table S5). In addition, three plasmid mediated quinolone resistant (PMQR) variants (Redgrave et al., 2014) were detected in 67 *E. coli* (13.6%) with the most common gene being *qnrS1* (Table 1). Majority of *E. coli* (*n* = 56) harboring PMQR genes did not harbor QRDR mutations; the MICs levels ranged between 0.06 and 8 mg/L, with only four isolate showing clinical resistance (>1 mg/L). Nineteen isolates had a contig encoding both *qnrS1* and IncI (Table 2), which showed ~79% sequence identity to the pSTM2 plasmid found in *Salmonella* Typhimurium (Supplementary Figure S2), which may be present in these isolates.

**TABLE 1** | Summary of antimicrobial resistance genes from different classes identified in *Escherichia coli* isolates.

Antimicrobial class	AMR gene	Percentage of positive isolates	Number of positive farms	Number of farms with isolates harboring AMR class (% of isolates)	Number of isolates harboring AMR gene recovered from different plates			
					1 mg/L cefotaxime	1 mg/L ciprofloxacin	No antibiotic	CRE
Clinically relevant aminoglycosides	<i>aac(3)-I/a</i>	4.70%	10	17 (30%)	0	11	12	0
	<i>aac(6')-IId</i>	0.20%	1		1	0	0	0
	<i>ant(2'')-Ia</i>	0.20%	1		0	1	0	0
	<i>aac(3)-IIId</i>	4.70%	9		0	21	2	0
Aminoglycosides	<i>aadA13</i>	0.40%	1	54 (96%)	0	2	0	0
	<i>aadA17</i>	0.20%	1		1	0	0	0
	<i>aadA2</i>	29.70%	43		3	81	62	0
	<i>aadA22_b</i>	0.40%	1		0	2	0	0
	<i>aadA3</i>	0.20%	1		0	0	1	0
	<i>aadA4</i>	0.60%	1		0	0	3	0
	<i>aadA5</i>	7.90%	18		2	32	5	0
	<i>aadA8</i>	5.70%	14		0	9	19	0
	<i>ant(3')-1a (s)</i>	5.70%	19		3	7	18	0
	<i>ant(3')-Ia (I)</i>	32.90%	48		6	80	76	0
	<i>aph(3')-Ia</i>	7.90%	15		4	27	8	0
	<i>strA</i>	39.00%	45		22	101	65	4
	<i>aph(3')-Ic</i>	1.20%	5		3	1	2	0
	<i>aph(3')-IIa</i>	1.00%	3		0	0	5	0
	<i>aph(4')-Ia</i>	4.70%	10		0	11	12	0
	<i>strB</i>	39.00%	46		21	101	66	4
Extended Spectrum Cephalosporins (ESC)	<i>bla<sub>CMY-2</sub></i>	2.00%	2	9 (16%)	7	0	3	0
	<i>bla<sub>CTX-M-1</sub></i>	1.80%	6		7	0	1	1
	<i>bla<sub>CTX-M-15</sub></i>	0.80%	2		1	0	0	3
	<i>bla<sub>SHV-12</sub></i>	1.00%	2		3	2	0	0
Penicillin	<i>bla<sub>TEM-1</sub></i>	1.20%	5	52 (93%)	0	6	0	0
	<i>bla<sub>TEM-135</sub></i>	1.60%	5		0	8	0	0
	<i>bla<sub>TEM-30</sub></i>	1.00%	2		4	1	0	0
	<i>bla<sub>TEM-1b</sub></i>	44.70%	49		13	126	77	4
	<i>bla<sub>TEM-1c</sub></i>	1.60%	4		0	7	1	0
	<i>bla<sub>TEM-1d</sub></i>	0.20%	1		0	1	0	0

(Continued)

TABLE 1 | Continued

Antimicrobial class	AMR gene	Percentage of positive isolates	Number of positive farms	Number of farms with isolates harboring AMR class (% of isolates)	Number of isolates harboring AMR gene recovered from different plates			
					1 mg/L cefotaxime	1 mg/L ciprofloxacin	No antibiotic	CRE
<b>Chloramphenicol/Florfenicol</b>	<i>catA1</i>	12.40%	21	43 (77%)	4	52	5	0
	<i>catA6</i>	0.20%	1		0	1	0	0
	<i>cml</i>	24.40%	36		3	58	59	0
	<i>floR</i>	5.50%	9		4	15	8	0
<b>Macrolide</b>	<i>ermB</i>	0.40%	1	18 (32%)	0	2	0	0
<b>(Azithromycin)</b>	<i>mefB</i>	5.30%	14		0	17	9	0
	<i>mphA</i>	2.20%	6		1	9	0	1
	<i>mphB</i>	1.40%	7		0	3	4	0
<b>Lincosamide</b>	<i>lnuF</i>	4.10%	8	8 (14%)	0	19	1	0
<b>Fluoroquinolone</b>	<i>qnrB19</i>	0.80%	2	26 (46%)	0	4	0	0
	<i>qnrB2</i>	0.20%	1		0	1	0	0
	<i>qnrS1</i>	12.60%	23		4	53	2	3
	<i>gyrA<sup>a</sup></i>	32.30%	45	45 (80%)	7	139	13	0
	<i>parC<sup>a</sup></i>	16.70%	29	29 (52%)	8	72	1	1
<b>Streptothricin</b>	<i>sat2A</i>	7.70%	24	24 (43%)	3	10	25	0
<b>Tetracycline</b>	<i>tet(A)</i>	44.90%	49	49 (88%)	15	100	103	3
	<i>tet(C)</i>	0.20%	1		0	0	1	0
	<i>tet(D)</i>	0.20%	1		0	0	1	0
	<i>tet(M)</i>	6.30%	13		2	18	11	0
	<i>tetA(B)</i>	34.30%	45		19	81	68	1
<b>Sulphonamide</b>	<i>sul1</i>	14.20%	32	48 (86%)	4	44	22	0
	<i>sul2</i>	34.80%	45		18	99	50	4
<b>Trimethoprim</b>	<i>dfrA1</i>	12.40%	27	53 (95%)	5	31	25	0
	<i>dfrA12</i>	18.30%	32		2	53	35	0
	<i>dfrA14</i>	7.90%	21		1	21	14	3
	<i>dfrA15</i>	1.60%	5		0	8	0	0
	<i>dfrA17</i>	10.40%	23		2	41	8	0

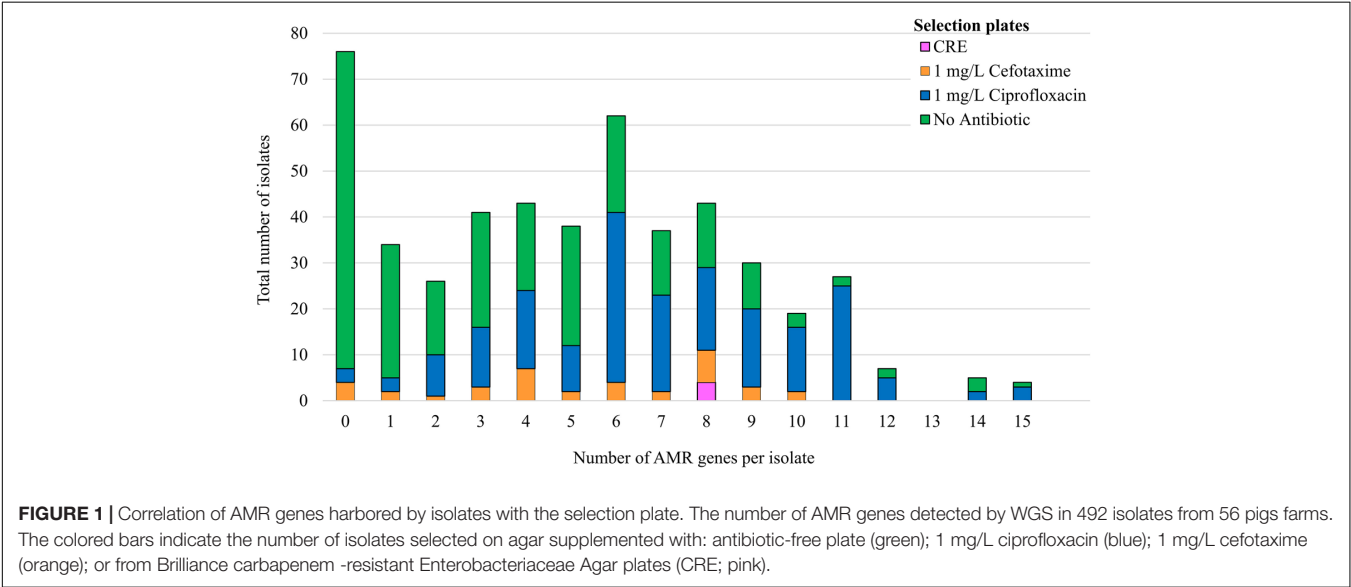
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TABLE 1 | Continued

Antimicrobial class	AMR gene	Percentage of positive isolates	Number of positive farms	Number of farms with isolates harboring AMR class (% of isolates)	Number of isolates harboring AMR gene recovered from different plates			
					1 mg/L cefotaxime	1 mg/L ciprofloxacin	No antibiotic	CRE
	<i>dfrA21</i>	0.60%	1		0	0	3	0
	<i>dfrA25</i>	0.20%	1		0	1	0	0
	<i>dfrA5</i>	4.70%	10		4	12	6	1
	<i>dfrA7</i>	0.20%	1		0	0	1	0
	<i>dfrA8</i>	0.60%	2		0	1	2	0
Number of gene types					34	50	44	13
Total number of genes					207	1603	915	33
Number of isolates					37	197	254	4
Ratio of genes to isolate					5.6	8.1	3.6	8.3

<sup>a</sup>SNPs within QRDR in *gyrA* or *parC* resulting in non-synonymous amino acid mutations which result in antibiotic resistance.



**FIGURE 1 |** Correlation of AMR genes harbored by isolates with the selection plate. The number of AMR genes detected by WGS in 492 isolates from 56 pigs farms. The colored bars indicate the number of isolates selected on agar supplemented with: antibiotic-free plate (green); 1 mg/L ciprofloxacin (blue); 1 mg/L cefotaxime (orange); or from Brilliance carbapenem-resistant Enterobacteriaceae Agar plates (CRE; pink).

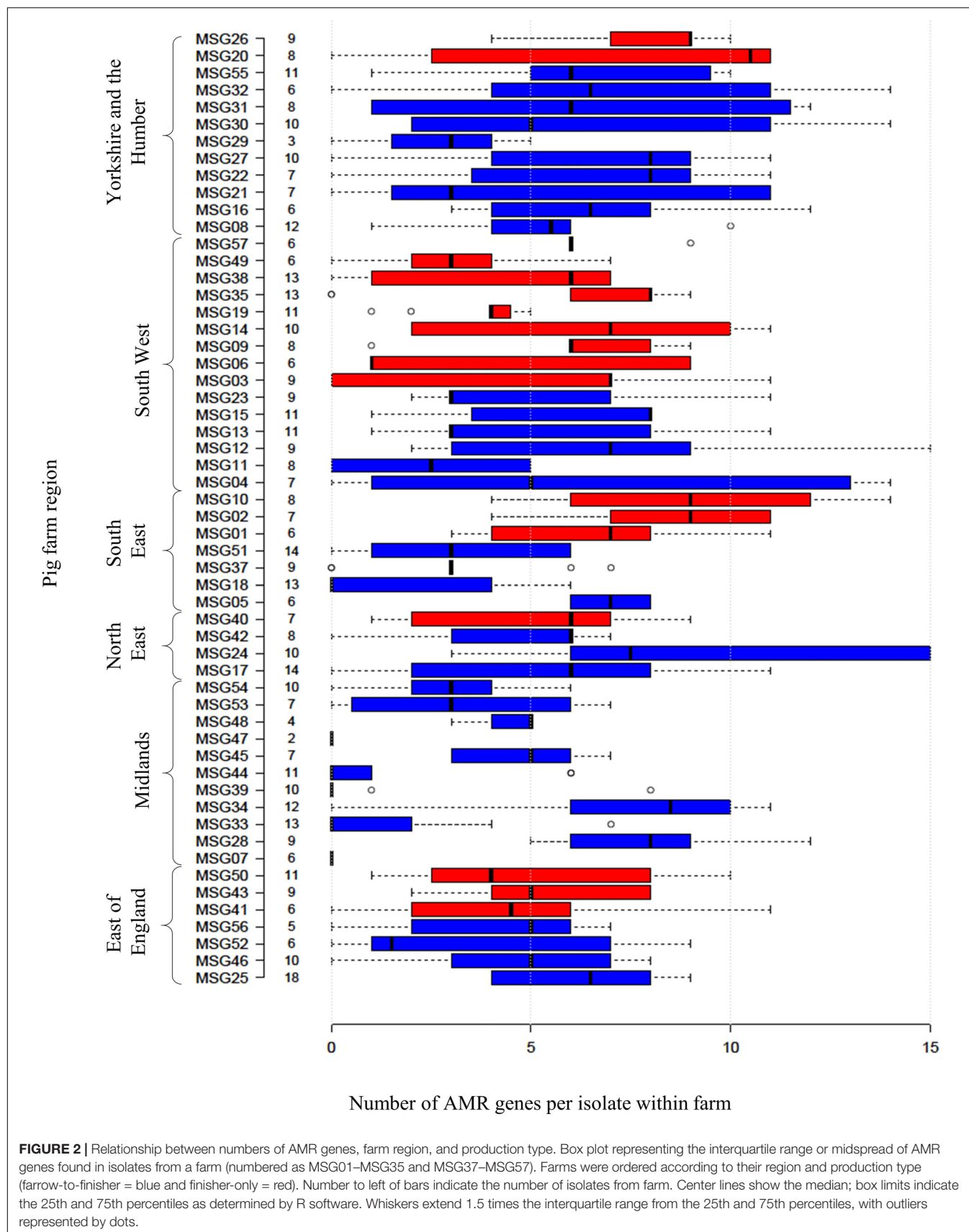
Four *E. coli* from two farms, recovered on carbapenem selection media, did not harbor transmissible carbapenem resistance genes and were phenotypically susceptible to carbapenems (**Supplementary Table S6**); these isolates were highly resistant to cefotaxime which may have contributed to the growth on CRE plates (MIC > 32 mg/L). Plasmid mediated colistin resistance genes were not detected.

### Phylogenetic Diversity

A whole genome SNP-based maximum-likelihood phylogenetic tree was constructed and separated isolates into two distinct clades (1 and 2) with the majority of isolates ( $n = 394$ ) clustering within clade 1 (**Figure 3**). There was little evidence of clustering by phylogeography, production type or farm,

with the only exception being farm MSG31, where all isolates clustered within S7.

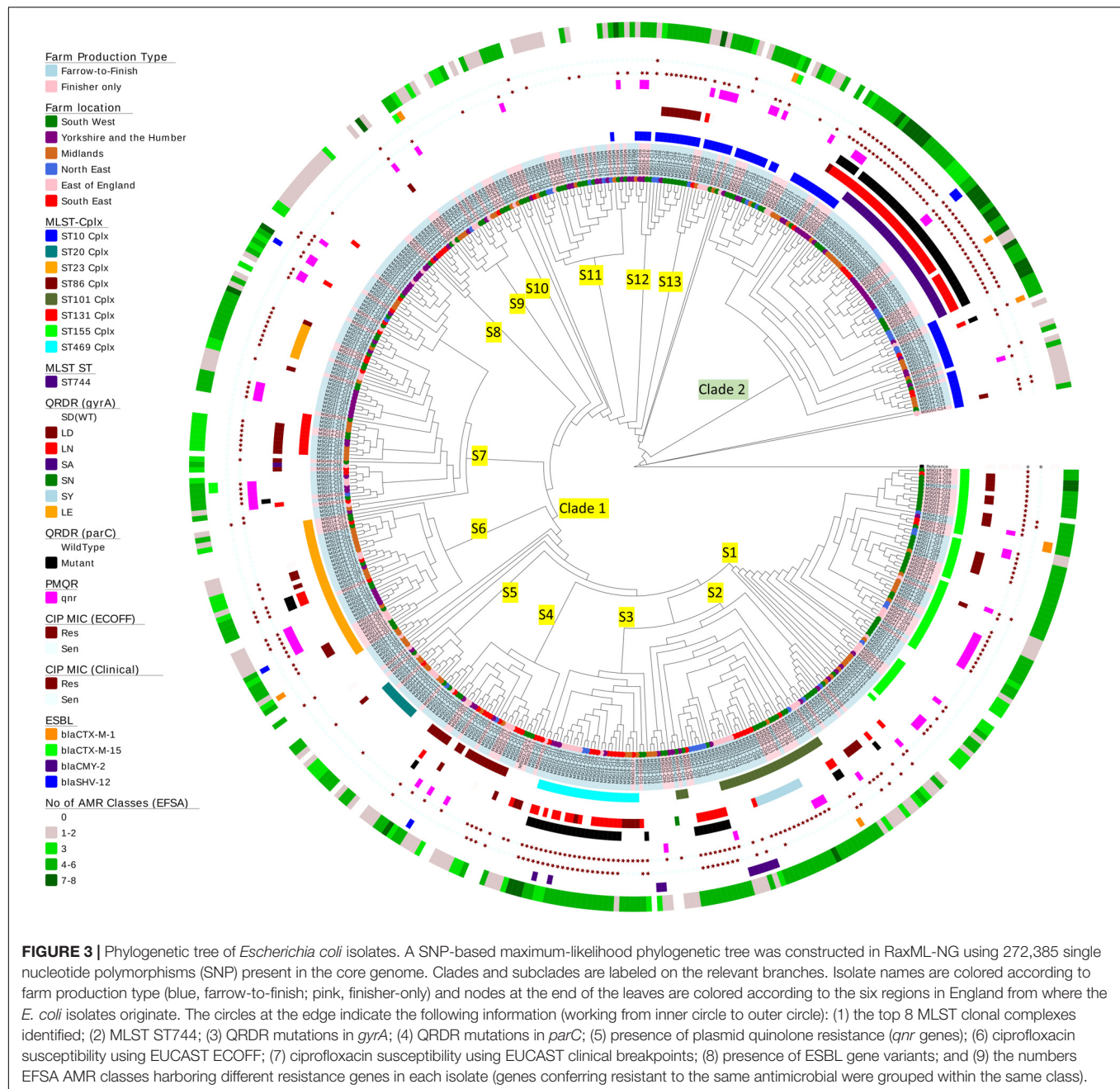
*Escherichia coli* isolates belonged to 117 multilocus sequence types (MLST) which included 19 clonal complexes and 17 new ST variants. Nearly half the isolates belonged to seven STs, but there were no apparent association between AMR profiles and ST (**Figure 3** and **Supplementary Table S7**). On average, farms had *E. coli* from five STs, with the exception of farm MSG17, which harbored nine STs. Fifty-eight percent of isolates, from different geographic regions, were located in clade 2; the majority were ST744, which has one SNP variation from ST10. The remainder clustered in three sub-clades within clade 1 and majority of isolates ( $n = 15$ ) belonged to ST162 (ST469 complex). Sixty-five percent of the STs ( $n = 76$ ) identified have



**TABLE 2 |** Plasmids involved in AMR gene dissemination.

Plasmid Inc type	AMR genes associated with replicon type <sup>a</sup>	Other genes found in isolates <sup>b</sup>	Contig size (Kb) <sup>c</sup>	Matches in NCBI database <sup>d</sup> (accession number)	Bacterial species for matched plasmid <sup>d</sup>	Sample origin <sup>e</sup>	% alignment <sup>f</sup>	Number of Isolates	Number of farms	Number of MLST STs
IncQ1	<i>strAB</i> , <i>sul2</i>	<i>repAC</i> <i>mobB</i>	4.4–4.7	pRSF1010 (NC001740)	<i>E. coli</i>	NK	53% (50–58%)	48	9	9
IncI1	<i>qnrS1</i>		18–106	pSTM2 (KF290378)	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Typhimurium</i>	Human	79% (75–82%)	19	6	7
IncFII	<i>strAB</i> , <i>bla</i> <sub>TEM-1</sub> , <i>sul2</i> , <i>tet(A)</i> , and <i>dfrA14</i>	<i>intI</i> , <i>tra</i> and <i>trb</i>	48	pM160133-p2 (CP022166)	<i>E. coli</i>	Human (urine)	95% (95–96%)	9	2	ST101
Unknown	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>qnrS1</i> , <i>strAB</i> , and <i>sul2</i>		21–36	pAR0162 (CP021681) pEco-CTXM15 (MF510423)	<i>E. coli</i>	NK Human (bile)	80% (73–95%) 92%	3	2	2
IncI1	<i>bla</i> <sub>CTX-M-1</sub> , <i>sul2</i>	<i>tet(A)</i>	107	p369 (IncI) (KT779550)	<i>E. coli</i>	Chicken	99.7%	1	1 (MSG43)	ST3205
unknown	<i>bla</i> <sub>CTX-M-1</sub> , <i>sul3</i> , <i>dfrA1</i>	<i>intI</i>	85				91%	1	1 (MSG17)	ST101
IncA/C2	<i>bla</i> <sub>CMY2</sub> , ( <i>sul2</i> )	<i>floR</i> , <i>tet(A)</i> , <i>strAB</i>	127–128	pSN254b (KJ909290)	<i>Aeromonas salmonicida</i>	Fish farm	92%	2	1 (MSG25)	ST162
IncHI2A	<i>strAB</i> , <i>bla</i> <sub>CMY2</sub>	Tellurite	124	pYD786-1 (KU254578)	<i>E. coli</i>	Human (urine)	82% (81–84%)	4	1 (MSG25)	ST101
IncI1	<i>bla</i> <sub>CMY2</sub>		86	p85 (CP023362)	<i>E. coli</i>	Canine	99%	1	1 (MSG53)	ST156

Summary of plasmids contigs present in isolates, as identified from WGS. <sup>a</sup>AMR genes identified using APHA SeqFinder from WGS. Genes that are variably present are in parenthesis. <sup>b</sup>Other genes found in isolate but not located on contigs harboring plasmid replicon. <sup>c</sup>Size of resolved contigs harboring AMR gene and plasmid replicon type. <sup>d</sup>Plasmid and bacteria with highest BlastN identity in NCBI database. <sup>e</sup>Sample origin of bacterial species harboring matched plasmid. <sup>f</sup>Percentage of aligned bases to reference calculated using Snippy.



previously been observed in human *E. coli* (Supplementary Table S7), however, due to lack of information in the MLST database it is not clear whether these are of clinical origin. Nevertheless, 89 isolates belonged to seven STs commonly associated with extra-intestinal pathogenic *E. coli* (ExPEC) from United Kingdom bacteraemia and UTI in man (Gibreel et al., 2012; Manges and Johnson, 2012; Horner et al., 2014; Kallonen et al., 2017; Day et al., 2019), which included ST10, ST117, and ST131 (Supplementary Table S7). Ten ST131 *E. coli*, from four farms, clustered within clade S7. However, unlike MDR human ST131 isolates (Nicolas-Chanoine et al., 2014) they were phenotypically susceptible at clinical breakpoints to

ciprofloxacin (Figure 3); although seven ST131-*fimH22* isolates from 3 farms harbored a single GyrA SNP (S83L), which led to resistances above the ECOFF and these isolates were MDR but not to any other HP-CIA; three ST131-*fimH298* isolates, all from the same farm, had no detectable AMR genes. Forty-seven of the 79 isolates (60%) from the remaining six STs showed a MDR genotype which included resistance to HP-CIAs (15 harbored resistance to gentamicin, 5 to ESBLs and 31 to fluoroquinolone, although none harbored resistance to all three classes to HP-CIAs).

Clustering was noted based on mutations in *gyrA* and *parC*. Half the isolates within clade 2 ( $n = 42$ ) harbored double *gyrA*



(S83L/D87N or LN) and a *parC* QRDR mutations, as did a number of isolates primarily from sub-clades 2 and 3 within clade 1 (7.6%,  $n = 30$ ). Seventy-one isolates harboring both LN and *parC* mutations were clinically ciprofloxacin resistant; 94% ( $n = 67$ ) of these were genotypically MDR (Figure 3).

## On-Farm Risk Factors

Multivariable risk factor analysis was performed at sample level rather than farm level using information gathered in a detailed questionnaire on farming practices, including hygiene and disinfection of houses, biosecurity, as well as antibiotic usage; the type of agar MDR isolates were selected on was also included. There were 29 potential risk factors ( $p < 0.25$ ) identified at univariable screening but the final multivariable logistic regression model retained only six risk factors associated with farms harboring genotypically MDR *E. coli* (Table 3). The retained variables included those identified as *a priori* (antimicrobial usage and ciprofloxacin enrichment plate) and four additional variables selected by forward stepwise selection that were significantly ( $p < 0.05$ ) associated with the outcome and provided the best model fit (indicated by a lower AIC). Not surprisingly, the greater the antibiotic usage on farms, the greater the risk of MDR *E. coli*. Farms with a medium usage of

antimicrobial products had almost three times the odds of MDR than farms with low usage ( $p = 0.050$ ), whereas farms with high usage of antimicrobial products had over five times the odds of MDR ( $p < 0.001$ ). Isolates recovered from ciprofloxacin plates had a higher probability of being MDR compared to isolates from the cefotaxime and non-selective plates (both  $p < 0.001$ ); the carbapenem-resistant Enterobacteriaceae plate had too few records to produce a credible result.

Specialist finisher farms were at a higher risk of MDR than breeder-finisher farms ( $p < 0.001$ ), while those farms that emptied their manure pit after every batch were at greater risk than those that emptied it weekly ( $p = 0.023$ ). Isolates from farms that on average, never cleaned and disinfected finisher pens between batches of pigs, were less likely to have MDR detected ( $p = 0.012$ ). This answer was only provided by breeder-finisher farms and might have been due to continuous production; variables related to this factor did not appear to effect the selection of this factor, indicating that they were unlikely to fully explain this outcome. The geographical region of the farm was also selected as a significant factor, with farms in the South East being at lower risk of MDR ( $p = 0.012$ ) and farms in Yorkshire and the Humber region at higher risk ( $p = 0.038$ ) than those in the South West.

**TABLE 3 |** Multivariable risk factor analysis, assessing for the association between genotypic multi-drug resistance and variables of interest ( $n = 492$ , of which 296 were multi-drug resistant).

Variable	Level	No. isolates	Odds ratio	P-value	95% confidence intervals	
Antimicrobial usage	Low	72	Baseline			
	Medium	116	2.84	0.050	1.00	8.08
	High	151	7.64	<0.001	2.73	21.35
Antibiotic selective or non-selective plate <sup>#</sup>	1 mg/L CIP	197	Baseline			
	1 mg/L CTX	37	0.15	<0.001	0.06	0.40
	CRE*	4	Undefined	0.985	–	–
	No antibiotic	254	0.03	<0.001	0.02	0.07
Production type	Breeder-finisher	339	Baseline			
	Finisher only	153	3.69	<0.001	1.79	7.58
How often is manure pit emptied	Weekly	65	Baseline			
	Monthly	108	2.09	0.114	0.84	5.20
	Per batch	123	3.57	0.023	1.20	10.65
	Half yearly	8	0.46	0.513	0.05	4.67
Region of farm	South West	137	Baseline			
	South East	63	3.65	0.012	1.32	10.07
	East of England	65	0.60	0.251	0.25	1.44
	Midlands	91	1.11	0.821	0.44	2.85
	Yorkshire and Humber	97	0.42	0.038	0.19	0.95
	North East	39	1.66	0.381	0.53	5.15
On average, how often are pens cleaned and disinfected	Always, sometimes or between batches	413	Baseline			
	Never	43	0.27	0.012	0.10	0.75
	Missing	36	1.24	0.708	0.40	3.80

Variable levels for 'not known' or missing answers have been omitted from the table. \*Small study population led to large uncertainty for odds ratio. <sup>#</sup>CRE, carbapenem-resistant Enterobacteriaceae; CIP, ciprofloxacin; CTX, cefotaxime.

## DISCUSSION

This study aimed to characterize the AMR genes, using WGS, present in 492 *E. coli* from pig farms in GB. The most common AMR genes harbored by these phylogenetically diverse *E. coli* of different STs were to “old” antimicrobials, including aminoglycosides (which were not within the clinically important group), tetracyclines, ampicillin, sulphonamides, and trimethoprim (Hopkins et al., 2007; Card et al., 2014, 2015; Kirchner et al., 2014; Ulstad et al., 2016). None of the porcine *E. coli* concurrently harbored resistance genes to ESBL, fluoroquinolone, and clinically relevant aminoglycosides, but 15 isolates from five farms carried AMR genes to the former two antimicrobials. This is in contrast to the AST data reported by Public Health England (PHE) for humans between 2012 and 2016 where ~5% of *E. coli* isolated from blood and cerebrospinal fluid showed resistance to third-generation cephalosporin, fluoroquinolones, and aminoglycosides, specifically gentamicin and/or tobramycin, which nevertheless have previously been reported from livestock (Szmolka et al., 2012). Also, the 16S RMTase genes reported from human clinical isolates (Public Health England, 2017), were absent. Importantly, several *E. coli* STs that have been isolated from humans were present, including seven collected from bacteraemia or feces of people admitted to UK hospitals, indicating some overlap between these compartments; many of these isolates were genotypically MDR and included resistance to HP-CIAs. Of the 10 ST131 isolates, 7 harbored the *fimH22* fimbriae, progenitor of *fimH30* associated with ST131 human pathogens (Ben Zakour et al., 2016). A previous study in retail meat also found that H22 strains were common and suggested the potential importance of *E. coli* ST131-*fimH22* as a foodborne pathogen (Liu et al., 2018). While this study only examined *E. coli*, the repertoire of AMR genes is likely to be greater due to other bacteria present in the pig intestine; for example *Moraxella* species harboring *mcr* (AbuOun et al., 2017) and three methicillin resistant *Staphylococcus aureus* (Sharma et al., 2016) has been reported from pig samples collected in this study. However, a thorough survey of the diverse bacterial population within the pig intestine was beyond the scope of this work.

In this study, 35% of *E. coli* isolates recovered from non-selective plates were genotypically MDR. This is lower than the average reported across Europe (38.1%) and United Kingdom (51%) from non-selective media from pigs during 2015 (European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC], 2017). Differences in the number of farms sampled, methods of collection and isolation, and numbers of isolates tested could account for the variations; this study included 18 isolates/farm compared to only one isolate/farm included in EU surveillance. In addition, we used WGS to determine presence of AMR genes, which is highly predictive of AMR presence and could be more sensitive than phenotyping (Ellington et al., 2017; Stubberfield et al., 2019).

Carbapenem usage is not permitted in food-producing animals in the United Kingdom (Grace et al., 2015; Broadfoot et al., 2016), therefore absence of plasmid mediated carbapenem resistance genes, a serious public health threat was reassuring (Logan and Weinstein, 2017). The sales/usage in animals of 3rd generation cephalosporins is low (Broadfoot et al., 2016), so the low percentage of farms (16%) positive for ESBL-harboring *E. coli* was expected; although it was lower than the figures reported from EU ESBL *E. coli* monitoring (EU prevalence = 31.9%, UK prevalence = 21.7%) (Randall et al., 2014; European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC], 2017). No isolates harboring a *bla*<sub>CTX-M</sub> group 1 enzyme belonged to ST73, ST131, or ST95, STs associated with human infections (Gibree et al., 2012; Horner et al., 2014; Kallonen et al., 2017).

The highest level of resistance to HP-CIAs was seen in fluoroquinolones and multivariable analysis indicated significantly higher likelihood of *E. coli* harboring MDR being isolated from ciprofloxacin compared to cefotaxime or non-selective plates. Thus, ciprofloxacin plates enabled detection not only of ciprofloxacin resistant *E. coli*, most often with QRDR mutations, but additional AMR genes. Furthermore, these MDR *E. coli* were less likely to be detected on other plates because they probably represented a small percentage of the total *E. coli*, as previously shown for *mcr*-harboring *E. coli* isolated from pig farms (Randall et al., 2018). QRDR mutations in *Salmonella* can confer protection against other antimicrobials (Webber et al., 2013) and the presence of double-serine QRDR mutations (GyrA83Ser and ParC80Ser) confers a selective advantage in lineages of several bacterial pathogens (Fuji et al., 2017). We speculate these mutations may also aid on-farm persistence of QRDR *E. coli* enabling them to gain MDR status by acquiring more AMR plasmids. It could then lead to increased antibiotic use due to possible treatment failures, as supported by multivariable analysis, which showed increased risk of MDR *E. coli* being present on farms with high antibiotic usage. Farmers should limit or cease in-feed or in-water antibiotic treatment of pigs at herd level in order to reduce the risk of MDR. More targeted treatment of individual pigs would be preferable, although this would only be suitable where any reduction in use did not impact upon the welfare of the pigs.

Additionally, improvements to biosecurity or more frequent emptying of the manure pit on farm may lead to reduced risk of MDR *E. coli* being present and hence less need for treatment. The frequent emptying of the pit below the flooring in pig housing has been recommended previously as a factor associated with reducing the prevalence of *Salmonella* on pig farms (Beloeil et al., 2004). Never using disinfectants on average when cleaning a pen was found to be protective, which may infer disinfectants are being diluted inappropriately. It is known that bacteria can become resistant to disinfection when exposed to sublethal concentrations (Zou et al., 2014). Finisher farms were shown to be at higher risk of MDR, which may reflect the differences in management (such as the number of incoming movements and sources used) and pig types present, when compared to

breeder-finisher farms. The region of the farm was also shown to be associated with MDR, which may reflect the density of pig farm production in those significant areas, as Yorkshire and Humber is a known high farm-density area, whereas the South East is sparsely populated. Although in this study each farm was sampled in more depth than many other cross-sectional national study, including for the EFSA harmonized monitoring of AMR. These results should nevertheless be treated with some caution as only 10 pigs were sampled per farm, and only from healthy slaughtered pigs, so it may not have been fully representative of these farms and appropriate for all farm types, such as those with only breeding pig stock.

As plasmids play a role in the mobilization of AMR genes between bacteria (Frost et al., 2005), putative plasmid contigs were used as surrogates for detecting circulating AMR plasmids. Isolates harbored between one and seven Inc-types and the most commonly circulating AMR plasmids belonged to the broad host range IncQ family (Loftie-Eaton and Rawlings, 2012), followed by IncI plasmids, which is frequently associated with AMR dissemination (Rozwandowicz et al., 2018). Future studies performed with hybrid short and long-read sequencing of isolates will enable a more complete resolution of plasmid genomes and other mobile genetic elements (MGEs) to accurately define the regions flanking AMR genes, and their transmission. However, high homology of plasmid genomes from this study with others present in the databases illustrate plasmids are part of a global network, cycling and disseminating AMR genes, as already made apparent by the *mcr*-plasmid phenomenon (Wyrsh et al., 2016; Duggett et al., 2017, 2018; Figueiredo et al., 2019).

## CONCLUSION

We used genomics to detect MDR *E. coli* present on pig farms and combined it with multivariable analysis to identify factors affecting their selection, and possible control measures to help mitigate their transmission. Future studies can define common circulating plasmid genomes and other MGEs harboring AMR genes more fully and verify whether measures recommended to control their dissemination were successful.

## Limitations

The farmer questionnaires were not completed fully by all farmers. Many of the questions were in a free format answer, which made it difficult to standardize and compare the results between farms, for example, it was difficult to gauge levels of antibiotics used by farmers. Classification of antimicrobial usage was subjective and may have led to some misclassification bias. For the risk factor analysis, the sample size was large enough to detect large associations (Odds Ratio >6.5) with 95% confidence and 80% power. However, as the number of pigs sampled on each farm was relatively low, and the number of *E. coli* isolates per farm in the analysis differed, the results may not be fully representative of the population on that farm. A random effect was utilized in the risk factor model to account for the

differing levels of clustering of isolates from farms and their non-independence.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the SRA PRJEB26317.

## ETHICS STATEMENT

APHA undertakes research using animals under the Animal (Scientific Procedures) Act 1986 (ASPA) which includes having its own Animal Welfare and Ethics Board (AWERB). The AWERB looks at all aspects of the Science Division's use of animals. As we had approval from the owners of the animals and the animals sampled were dead, this was outside ASPA (which only concerns live animals) and so it was considered that ethical approval from the committee was not required. Written informed consent was received from all participating farmers to allow their pigs to be sampled and their laboratory results and questionnaire data to be used for research purposes.

## AUTHOR CONTRIBUTIONS

MFA and RS conceived and directed the project and designed the work. MFA and DC funding acquisition. MA, ES, and EJS performed bacteriology laboratory work. MA, ES, EJS, and JN-G performed the gene and WGS analysis. MA, HO'C, EJS, and MFA interpreted the results. MA, RS, and MFA wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

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