ZOONOTIC MICROORGANISMS AND SPREAD OF ACQUIRED POLYMYXIN RESISTANCE DETERMINANTS

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ZOONOTIC MICROORGANISMS AND SPREAD OF ACQUIRED POLYMYXIN RESISTANCE DETERMINANTS

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EDITORIAL
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Editorial: Zoonotic Microorganisms and Spread of Acquired Polymyxin Resistance Determinants

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Keywords: colistin resistance, enterobacteria, zoonotic agent, antimicrobial resistance (AMR), MCR genes

Editorial on the Research Topic

Zoonotic Microorganisms and Spread of Acquired Polymyxin Resistance Determinants

Currently, the use of colistin as a last-resort antibiotic in human medicine is highly compromised due to the continuous emergence of resistant enterobacteria with acquired determinants like plasmid-mediated resistance genes (*mcr*), mutations that activate the PmrAB system, or still unknown mechanisms. On the other hand, the correlation between the individual, or combined activity of these mechanisms, with the functional expression of resistance to colistin is still poorly understood. Based on a One-Health approach, this topic aimed to gather knowledge in the transmission and expression of colistin resistance determinants in Gram-negative bacteria isolated from different sources.

Since the *mcr-1* plasmid-mediated colistin resistance gene was discovered in China in 2015 (Liu et al., 2016), numerous surveys have been performed worldwide to monitor the prevalence and spread of *mcr* in food animals. Industrial farming, particularly poultry and swine, have been highlighted as a zoonotic source of *mcr* (mainly *mcr-1*) *Escherichia coli* isolates (Irrgang et al., 2016; García-Meniño et al., 2018). Cheng et al. analyzed 200 fecal swabs collected from six swine farms in northeastern China between July 2016 and June 2017. A total of 176 *E. coli* strains were isolated and the prevalence of *mcr-1* in colistin-resistant *E. coli* was 53.33% (56/105). *mcr-1*-positive *E. coli* showed extensive antimicrobial resistance (AMR) profiles with the presence of additional resistance genes, increased expression of multidrug efflux pump-associated genes, and increased biofilm formation ability. The results of growth assay, competition experiment, and plasmid stability testing showed that acquisition of *mcr-1*-harboring plasmids could reduce the fitness of bacterial hosts, but *mcr-1* remained stable in the recipient strain.

The availability of accurate systems for the diagnosis of AMR is a cornerstone both for its clinical study and for the investigation of the propagation of clones with low susceptibility to colistin. Zhu et al. analyzed the performance of three mainstream commercial antimicrobial susceptibility testing (VITEK 2® COMPACT, Phoenix™ M50, and Bio-kont) in parallel to standard broth microdilution, finding out slight but significant differences among them, especially for enterobacteria like *Escherichia* spp., *Klebsiella* spp., and *Citrobacter* spp. Retrospective studies on frozen stored collections of samples are being used to investigate the evolution of new threats, like plasmid-mediated colistin resistance determinants. Miguela-Villoldo et al. showed that an SYBR Green qPCR assay designed to detect *mcr-1* in pig caecum samples is the best option to provide a highly representative frame of the initial population present in the sample, and although the freeze-thaw process affects bacterial viability, culture-based methods might be a useful complement to study colistin resistance levels.

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Poultry and poultry meat have also been highlighted as important contributors to the global antimicrobial burden with potential transmission risk for consumers (Díaz-Jiménez et al., 2021). In Lebanon, where the first *mcr-1*-positive *E. coli* found in poultry dates to 2015, Hiba Al-Mir et al. report a prevalence of 84.4% *mcr-1* positive poultry farms across three Lebanese governorates. The study was conducted on poultry samples collected in 2018 in one large slaughterhouse and originated from 32 individual farms. Furthermore, numerous associated resistances were identified, including the presence of bla_{CTX-M} or bla_{CMY} genes, and the *mcr-1* gene was mostly located in IncX4 ($n = 36$) and IncI2 ($n = 24$) plasmids.

The findings of Nakano et al. in Japan uncover the potential circulation of *mcr-1* mediated colistin-resistant *E. coli* among livestock and farmers. The authors analyzed fecal samples from 295 healthy livestock (202 cattle and 93 swine) and 62 healthy livestock farmers (53 cattle farmers and nine swine farmers) collected between 2013 and 2015, from 72 livestock farms. The prevalence of *mcr-1*-harboring *E. coli* was 25 (8.47%) of the 295 livestock from 11 farms, and 3.77% (2/53 strains) for cattle farmers, and 11.11% (1/9 strains) for swine farmers. Considering the isolates obtained from livestock and farmers, in four farms, nine isolates had the same genotypical characteristics (sequence types and pulsed-field gel electrophoresis band patterns), plasmid characteristics (incompatibility group and plasmid transferability), and minimum inhibitory concentrations.

Due to the high number of colonized animals, slaughterhouses might represent a significant source of the introduction of *mcr* genes into the food chain through possible contamination of carcasses and products. Furthermore, these bacteria might accumulate in process waters and wastewater from slaughterhouses, contributing to a broad spread of the resistance to other environmental ecosystems including surface waters. The study of Savin et al. supports the necessity of implementing advanced wastewater treatment technologies to limit the contamination of the environment with bacteria expressing resistances against last resort antimicrobials. The authors performed a study to evaluate the occurrence of colistin-resistant *Enterobacteriaceae* in process waters and wastewater from two poultry and two pig slaughterhouses in Germany. Their findings demonstrated a high occurrence of colistin-resistant *E. coli* and *Klebsiella pneumoniae* carrying *mcr-1* on transferable plasmids (incompatibility groups IncI1, IncHI2, IncX4, IncF, and IncI2) in poultry and pig slaughterhouses and indicate their dissemination into surface water. Environmental contamination with colistin-resistant enterobacteria carrying *mcr* genes is also described by Lopes et al., who detected the ST131 clone of *E. coli* isolated from a kale crop in Brazil. In addition to *mcr-1*, $bla_{CTX-M-15}$, and *qnrB19* genes were associated with IncH12, IncF, and ColE1 replicons, respectively, the two first harboring additional AMR determinants and being efficiently transferred by conjugation.

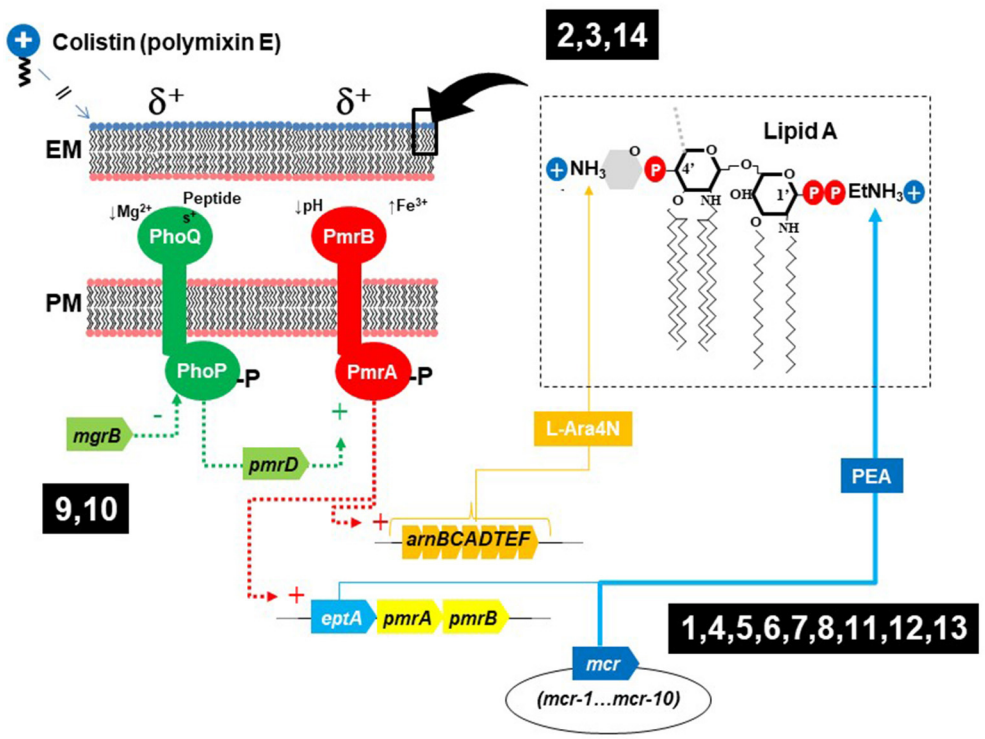

FIGURE 1 | Elements involved in colistin resistance in enterobacteria, biological context of the articles published in this research topic. (1, Cheng et al.; 2, Zhu et al.; 3, Miguela-Villoldo et al.; 4, Hiba Al-Mir et al.; 5, Nakano et al.; 6, Savin et al.; 7, Lopes et al.; 8, Zhang et al.; 9, Ngbede et al.; 10, Azam et al.; 11, Hu et al.; 12, Vázquez et al.; 13, Diaconu et al.; 14, Panta and Doerrler).

Wild birds play a key role in the spread of AMR due to their environmental exposure through ingested food or polluted water. In this respect, the results of Zhang et al. provide evidence that migratory birds are potential transmitters of AMR. The authors identified 22 *mcr-1*-positive *E. coli* from 303 *Anser indicus* fecal samples (7.3%) collected in the Guangdong province of China. Coexisting with 24 other types of AMR genes, *mcr-1* was located on IncX4, IncI2, and IncP plasmids.

Together with colistin, carbapenems are used as last-resort antibiotics. Ngbede et al. investigated carbapenem and colistin resistance in 583 non-duplicate Enterobacteriaceae isolates recovered from humans, animals, and the environment in Nigeria to find that 18.9% were resistant to at least one carbapenem and 9.1% exhibited concurrent carbapenem-colistin resistance. No carbapenem-resistant isolates carried any known carbapenemase-producing gene. Whole-genome sequencing supported that concurrent carbapenem-colistin resistance was mediated by novel or previously described alterations in chromosomal efflux regulatory genes, particularly *mgrB* (M1V), *ompC* (M1_V24del), *ompK37* (I70M, I128M), *ramR* (M1V), and *marR* (M1V). In addition, alterations/mutations were detected in the *etpA*, *arnT*, *ccrB*, *pmrB* in colistin-resistant bacteria and *ompK36* in carbapenem-resistant bacteria. In contrast, Azam et al. found that among 11 colistin-resistant *K. pneumoniae* isolated from humans in India, present non-synonymous potentially deleterious mutations in the *phoP*, *phoQ*, *pmrA*, *pmrB,* and *mgrB* genes, and amongst those, one clone carried the New Delhi metallo-beta-lactamase 1 gene (NDM-1) and expressed carbapenem resistance.

Salmonella is one of the leading causes of global bacterial food poisoning worldwide. However, the occurrence and transmission of *mcr* genes in *Salmonella* are still poorly understood. Hu et al. analyzed 755 foodborne *Salmonella* from 26 provinces in mainland China in 2016. As a result, 10% of the isolates were defined as multidrug-resistant (MDR), and two carried *mcr-1*: *S.* Derby CFSA231 and *S.* Typhimurium CFSA629. Both expressed an MDR phenotype and included a single circular chromosome and one plasmid. Among the 22 AMR genes identified in S. Derby CFSA231, only the *mcr-1* gene was localized on the IncX4 type plasmid pCFSA231 while 20 chromosomal AMR genes, including four plasmid-mediated quinolone resistance (PMQR) genes, were mapped within a 64 kb *Salmonella* genomic island (SGI) like region. *S.* Typhimurium CFSA629 possessed 11 resistance genes including an *mcr-1.19* variant and two ESBL genes. The contribution by Vázquez et al. shows that 2.2% of foodborne *Salmonella* isolated in Asturias, Spain, between 2014 and 2019 were resistant to colistin. Four of these isolates, belonging to the European monophasic ST34 clone of *S.* Typhimurium characterized by chromosomal genes conferring resistance to ampicillin, streptomycin, sulfonamides, tetracycline, heavy metals, and arsenic ± mercury, carried *mcr-1* in IncX4 or IncHI2 plasmids. Moreover, Diaconu et al. reported that 6.2% of AMR isolates from foodstuffs belonging to the same ST34 clone presented the *mcr-9* gene carried by IncHI2 megaplasmids of near 300 Kb, associated with increased resistance to colistin.

Previously unknown determinants for colistin resistance are starting to be revealed. In addition to chromosomal mutations affecting the two-component sensory systems (TCSS) PhoPQ or PmrAB, and the *mcr* genes, modification of lipopolysaccharide lipid A with aminoarabinose (L-Ara4N) is carried out by the enzymes encoded in *arn* operons (**Figure 1**). These modifications might confer colistin resistance upon up-regulation by chromosomal mutations of TCSS and can also be plasmid-mediated, similarly to *mcr* genes (Gallardo et al., 2021). Some Gram-negative bacteria are intrinsically resistant to very high levels of colistin, and the work by Panta and Doerrler evidenced that a DedA family protein in *Burkholderia thailandensis* (DbcA; DedA of *Burkholderia* required for colistin resistance) is a membrane transporter required for resistance to colistin, which function is partially restored by overexpression of *arn* genes or by the increase in membrane potential that can occur by lowering the pH.

Published contributions on this topic are indicated in **Figure 1** in relation to their context on colistin resistance (1, Cheng et al.; 2, Zhu et al.; 3, Miguela-Villoldo et al.; 4, Hiba Al-Mir et al.; 5, Nakano et al.; 6, Savin et al.; 7, Lopes et al.; 8, Zhang et al.; 9, Ngbede et al.; 10, Azam et al.; 11, Hu et al.; 12, Vázquez et al.; 13, Diaconu et al.; 14, Panta and Doerrler).

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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Colistin-Resistant *Enterobacteriaceae* Isolated From Process Waters and Wastewater From German Poultry and Pig Slaughterhouses

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Due to the high prevalence of colistin-resistant *Enterobacteriaceae* in poultry and pigs, process waters and wastewater from slaughterhouses were considered as a hotspot for isolates carrying plasmid-encoded, mobilizable colistin resistances (*mcr* genes). Thus, questions on the effectiveness of wastewater treatment in in-house and municipal wastewater treatment plants (WWTPs) as well as on the diversity of the prevailing isolates, plasmid types, and their transmissibility arise. Process waters and wastewater accruing in the delivery and unclean areas of two poultry and two pig slaughterhouses were screened for the presence of target colistin-resistant bacteria (i.e., *Escherichia coli*, *Klebsiella* spp., *Enterobacter cloacae* complex). In-house and municipal WWTPs (mWWTPs) including receiving waterbodies were investigated as well. Samples taken in the poultry slaughterhouses yielded the highest occurrence of target colistin-resistant *Enterobacteriaceae* (40.2%, 33/82), followed by mWWTPs (25.0%, 9/36) and pig slaughterhouses (14.9%, 10/67). Recovered isolates exhibited various resistance patterns. The resistance rates using epidemiological cut-off values were higher in comparison to those obtained with clinical breakpoints. Noteworthy, MCR-1-producing *Klebsiella pneumoniae* and *E. coli* were detected in scalding waters and preflooders of mWWTPs. A total of 70.8% (46/65) of *E. coli* and 20.6% (7/34) of *K. pneumoniae* isolates carried *mcr-1* on a variety of transferable plasmids with incompatibility groups IncI1, IncHI2, IncX4, IncF, and IncI2 ranging between 30 and 360 kb. The analyzed isolates carrying *mcr-1* on transferable plasmids ($n = 53$) exhibited a broad diversity, as they were assigned to 25 different *Xba*I profiles. Interestingly, in the majority of colistin-resistant

mcr-negative *E. coli* and *K. pneumoniae* isolates non-synonymous polymorphisms in *pmrAB* were detected. Our findings demonstrated high occurrence of colistin-resistant *E. coli* and *K. pneumoniae* carrying *mcr-1* on transferrable plasmids in poultry and pig slaughterhouses and indicate their dissemination into surface water.

Keywords: colistin resistance, *mcr* genes, slaughterhouse, wastewater, zoonotic microorganisms, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae* complex

INTRODUCTION

Since the 1950s, colistin (polymyxin E) has been extensively used in the European animal production (Koyama et al., 1950) to prevent and treat gastrointestinal infections caused by Gram-negative bacteria (e.g., diarrhea in pigs caused by *Escherichia coli* and *Salmonella* spp. as well as colibacillosis in poultry) (EMEA, 2002). Moreover, it was also used in a lower dosage as a feed additive until the ban of antimicrobial growth promoters in the European Union (EU) in 2006 (EMA, 2016).

Despite its nephrotoxicity and neurotoxicity, colistin was re-introduced into human therapy to treat infections caused by multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* or carbapenemase-producing *Enterobacteriaceae* (CPE) (Azzopardi et al., 2013). Due to its high impact, the World Health Organisation (WHO) included colistin into the group of the "highest priority critically important antimicrobials" for human medicine (World Health Organization, 2019). Alongside with other antibiotics of the last resort (e.g., tigecycline, amikacin and the new combinations of ceftazidime-avibactam and ceftozolane-tazobactam), its use is restricted to clinical cases for which no alternative options are available (Nation and Li, 2009). However, in 2016, colistin was also classified as a highly important antimicrobial (VHIA) in the veterinary sector by the World Organisation for Animal Health (OIE, 2018). Data from Germany indicate a reduction of colistin sales between 2011 and 2016 by 45.7% from 127 to 69 tons. However, from 2016 its sales have been slightly increasing and reached 74 tons in 2018 making up 10.2% of the total amount of antimicrobials sold for the veterinary use in Germany (BVL, 2019).

In Gram-negative bacteria, colistin interacts with lipopolysaccharide (LPS) and phospholipids in the outer cell membrane. Due to the competitive displacement of divalent cations Ca^{2+} and Mg^{2+} from the phosphate groups of membrane lipids (Falagas and Kasiakou, 2005), both cell membranes are disrupted leading to the leakage of intracellular contents and subsequent bacterial death.

Before 2015, colistin resistance in *Enterobacteriaceae* was assumed to be caused due to chromosomal mutations in genes (esp. *pmrA/B* and *phoP/Q* and *mgrB*) encoding regulatory proteins that influence transcription of enzymes that modify the lipopolysaccharide (Moskowitz et al., 2012; Quesada et al., 2015). But the description of the first plasmid-encoded, mobilizable colistin resistance gene (*mcr-1*) in *E. coli* from livestock in China and retail meat as well as in Chinese clinical *Klebsiella pneumoniae* isolates (Liu et al., 2016; Sun et al., 2018b) raised serious public health concern on the emergence of colistin-resistant bacteria.

Further studies on the genetic basis of colistin-resistant bacteria resulted in the discovery of nine additional *mcr* genes (*mcr-2* to *mcr-10*). However, *mcr-1* is the most prevalent worldwide (Elbediwi et al., 2019). *mcr*-occurrence is often associated with a variety of plasmids, including IncX4, IncF, IncHI1, IncHI2, IncI2, IncY, and broad host range (BHR) plasmids IncP (Zurfuh et al., 2016; Hadjadj et al., 2017; Poirel et al., 2017; Sun et al., 2018a). Furthermore, *mcr-1* is often bracketed by IS*Apl1* insertion sequence enabling their broad dissemination by transposition (Snesrud et al., 2016; Li et al., 2017).

Due to a high number of colonized animals, slaughterhouses might represent a significant source of introduction of *mcr* genes into the food chain, e.g., through possible contamination of carcasses and products (Irrgang et al., 2016; Inderbinen, 2017). Furthermore, slaughterhouse workers with occupational exposure to colonized animals and contaminated process water as well as employees of the wastewater treatment plants (WWTPs) might be exposed to an increased risk of colonization (Dohmen et al., 2017). Moreover, due to insufficient wastewater treatment by in-house and municipal WWTPs (mWWTPs), livestock wastewater might be an important route for dissemination of *mcr-1*-carrying bacteria into the environment (Hembach et al., 2017).

On the basis of the high prevalence of colistin-resistant *Enterobacteriaceae* in livestock feces, these bacteria might accumulate in process waters and wastewater from slaughterhouses. These waters might represent potential reservoirs that can contribute to a broad spread of the resistance to other environmental ecosystems including surface waters. So far, no data on the occurrence and characteristics of colistin-resistant *Enterobacteriaceae* in process waters and wastewater from German poultry and pig slaughterhouses have been reported. Furthermore, information on the impact of slaughterhouse wastewaters for the dissemination of this resistance is scarce and needs to be determined. Thus, this study aimed to evaluate their occurrence in the delivery and unclean areas of German poultry and pig slaughterhouses as well as in their in-house WWTPs. Moreover, their further spread into surface waters via municipal WWTPs was also investigated.

This hypothesis was tested using selective culture-dependent methods, followed by phenotypic and molecular characterization of the recovered isolates.

MATERIALS AND METHODS

Sampling and Sample Preparation

Sampling and sample preparation of process waters and wastewater taken in poultry and pig slaughterhouses, their in-house WWTPs as well as mWWTPs and on-site preflooders have been previously described (Savin et al., 2020a,b).

A total of 185 water samples were included in the study. Briefly, 82 samples of process waters and wastewater accruing in the delivery and unclean areas during slaughtering and cleaning operations were collected from two poultry slaughterhouses. Samples were taken at seven sampling sites: transport trucks (n = 5), transport crates (n = 10), stunning facilities (n = 10), scalders (n = 10), eviscerators (n = 10), production facilities (n = 5), influent (n = 16), and effluent (n = 16) of the in-house WWTPs. From each individual sample, one liter was collected using sterile Nalgene® Wide Mouth Environmental Sample Bottles (Thermo Fisher Scientific, Waltham, MA, United States). For more details please see Savin et al. (2020b).

Further 67 samples of process water and wastewater were collected from the delivery [animal transporters (n = 10), holding pens (n = 7)] and unclean areas [scalding and dehairing water (n = 10), aggregate wastewater from production facilities (n = 10)] as well as the in-house WWTPs (in- and effluent, each n = 15) of two pig slaughterhouses during slaughtering and cleaning operations. Additionally, 18 samples were collected from the influents (n = 9) and effluents (n = 9) of the mWWTPs receiving the wastewater from the investigated pig slaughterhouses for the final treatment. Their on-site preflooders upstream (n = 9) and downstream the discharge points (n = 9) were sampled as well. At each site, one liter was collected in sterile polyethylene Nalgene Wide Mouth Environmental Sample Bottles (Thermo Fisher Scientific, Waltham, MA, United States). For more details please see Savin et al. (2020a).

Cultivation, Identification and Susceptibility Testing of Target Polymyxin-Resistant Lactose-Fermenting *Enterobacteriaceae*

Water samples were screened for polymyxin-resistant lactose-fermenting *Enterobacteriaceae* (*E. coli*, *Klebsiella* spp., and *Enterobacter cloacae* complex) using SuperPolymyxin medium (Nordmann et al., 2016). For cultivation, aliquots of 100 µl and 1 ml of the original samples were applied onto SuperPolymyxin plates and incubated under aerobic conditions at 37°C for 18–24 h. When possible, up to three colonies of lactose fermenters were picked based on their morphology and sub-cultured on Columbia Agar with 5% sheep blood (MAST Diagnostica, Reinfeld, Germany) at 37°C for 18–24 h.

Identification of the isolates species was conducted by MALDI-TOF MS as previously described (Savin et al., 2020b).

The antimicrobial susceptibility testing of the isolates and transconjugants was performed by applying two different antibiotic susceptibility testing panels as well as epidemiological and clinical breakpoints. The first scheme (A) was based on broth microdilution according to CLSI guidelines (M07-A9) following application of epidemiological cut-off values of European Committee on Antimicrobial Susceptibility Testing (EUCAST) as recommended for isolates from livestock and food. The second one (B) was applied in order to assess the clinical relevance of recovered colistin-resistant isolates in human medicine. For this purpose, they were tested against clinically important antimicrobials for humans by microdilution method as previously described (Savin et al., 2020b). MICRONAUT MIC-Strips Colistin (MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany) were used to test the colistin concentrations of up to 64 mg/L.

Also, isolates of *E. coli*, *K. pneumoniae*, and *E. cloacae* complex that were cultivated from the same samples on CHROMagar™ ESBL plates (MAST Diagnostica, Reinfeld, Germany) as described previously by Savin et al. (2020b) and showed resistance to colistin, were included in this study.

Molecular Typing of Resistant Bacterial Isolates

Cell lysates prepared by boiling of bacterial suspensions (Aldous et al., 2005) were used as template for PCR. Determination of phylogenetic groups (A, B1, B2, C, D, E, F, clade I–V) of *E. coli* was conducted according to a previously published method (Clermont et al., 2013).

PCR Screening for *mcr-1* to *mcr-9* Genes and Sanger-Sequencing of the Amplicons

Isolates were screened for *mcr-1* to *mcr-5* as well as *mcr-6* to *mcr-9* genes using the multiplex PCR protocols as described by Rebelo et al. (2018) and Borowiak et al. (2020), respectively. As positive controls the isolates *E. coli* R2749 (*mcr-1*), *E. coli* KP37 (*mcr-2*), *Salmonella* Typhimurium SSI_AA940 (*mcr-3*), *S.* Typhimurium R3445 (*mcr-4*), *E. coli* 10E01066 (*mcr-5*), and *S.* Infantis 15-SA01028 (*mcr-9*) were used. The artificially synthesized positive controls for *mcr-6*, *mcr-7*, and *mcr-8* were kindly provided by the Department for Biological Safety of German Federal Institute for Risk Assessment (BfR) (Berlin, Germany) (Borowiak et al., 2020). PCR products were separated by electrophoresis on a 1.0% agarose-TBE gel and stained with midori green (Labomedic Medizin- und Labortechnik GmbH, Bonn, Germany). Sequence-based typing of *mcr-1* (Zhang et al., 2018) amplicons was performed at Microsynth Seqlab (Göttingen, Germany).

*Xba*I PFGE-Profiling of *mcr-1*-Positive *E. coli* and *K. pneumoniae* Isolates and *mcr-1* Localization

The phylogenetic relationship of the *mcr-1*-carrying *E. coli* and *K. pneumoniae* was assessed by *Xba*I macrorestriction via pulsed-field gel electrophoresis (PFGE) according to the PulseNet protocol (CDC, 2020). Plasmidal localization of the *mcr* genes was determined by S1-PFGE followed by Southern blotting and DNA-DNA hybridization against a digoxigenin-labeled PCR amplicon as previously described (Hammerl et al., 2018). The size of *mcr*-carrying plasmids was predicted on the basis of the

S1-PFGE pictures with Bionumerics (Applied Math, Sint Marten-Latem, Netherlands; version 7.5) using *Salmonella* Braenderup (H9812) as size marker.

Conjugation Assays and Plasmid Analyses

In vitro conjugation experiments were conducted in liquid medium using the plasmid-free rifampicin-resistant *E. coli* recipient strain CV601 GFP at a donor:recipient ratio of 1:1 as previously described (Blau et al., 2018). Transconjugants were selected after incubation at 37°C for 24–48 h under selective conditions on lysogeny broth (LB) agar (Sigma-Aldrich, St. Louis, MO, United States) containing colistin sulfate (1 μg/ml) and rifampicin (200 μg/ml) (w/v). Isolates that did not yield transconjugants were further subjected to filter mating assays with the rifampicin-resistant, lactose-negative *E. coli* recipient strain W3110 at a donor:recipient ratio of 10:1 (Kieffer et al., 2017). The selection of transconjugants was done on MacConkey agar (Sigma-Aldrich, St. Louis, MO, United States) containing colistin sulfate (1 μg/ml) and rifampicin (200 μg/ml) after incubation at 37°C for 24–48 h under selective conditions. Potential transconjugants were subjected to PCR to confirm the presence of the *mcr* genes. Those transconjugants obtained with *E. coli* CV601 as recipient were additionally examined for GFP fluorescence using fluorescence microscope Axio Scope.A1 (Carl Zeiss Microscopy GmbH, Jena, Germany).

Transformation Assays

mcr-1-positive isolates that did not generate any transconjugants were further submitted to transformation experiments using NEB® 10-beta electrocompetent *E. coli* cells (New England Biolabs, Ipswich, MA, United States) and MicroPulser Electroporator (Bio-Rad, Hercules, CA, United States) according to manufacturer's protocols. Plasmid DNA was extracted from overnight cultures of *mcr-1*-positive isolates using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, United States) according to manufacturer's protocol. The transformants were selected on LB agar (Sigma-Aldrich, St. Louis, MO, United States) containing colistin sulfate (1 μ g/ml).

The transconjugants and transformants were cryopreserved at −20°C using cryotubes (Mast Diagnostics, Reinfeld, Germany) until further analysis.

Plasmid Replicon Typing

Plasmid DNA was extracted from overnight cultures of *E. coli* CV601 and W3110 transconjugants using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, United States) according to manufacturer's protocol. The presence of IncF and IncI plasmids was tested by RT-PCR 5′-nuclease assays (TaqMan RT-PCR) as previously described (Blau et al., 2018). Plasmids from transconjugants that could not be detected by RT-PCR were further investigated by PCR-Based Replicon Typing (PBRT). Therefore, PCR amplification on plasmid DNA was performed using primers for the 30 different replicons (HI1, HI2, I1, I2, X1, X2, X3, X4, L, M, N, FIA, FIB, FIC, FII, FIIS, FIIK, FIB KN, FIB KQ, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M, and FIB-M), which are representative for the major plasmid incompatibility groups among *Enterobacteriaceae* (Carattoli et al., 2005; Villa et al., 2010).

Amplification and Sequencing of *pmrA* and *prmB* Genes in *mcr*-Negative *E. coli* and *K. pneumoniae* Isolates

The coding sequences of the *pmrA* and *pmrB* genes in *E. coli* and *K. pneumoniae* were amplified as previously described by Jayol et al. (2014), Quesada et al. (2015), and Haeili et al. (2017). PCR amplicons were purified using the innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany) and sequenced at Microsynth Seqlab (Göttingen, Germany). Genomic DNA from five randomly selected *mcr-1*-negative colistin-susceptible *E. coli* and *K. pneumoniae* isolates (colistin MIC < 2 mg/L) originating from the same samples were used as control. Sequence analysis was conducted with Chromas lite v.2.6.5 (Technelysium Pty. Ltd.) and BioEdit v.7.2.5 (Hall, 1999).

RESULTS

Detection of *Enterobacteriaceae* in Samples From Poultry and Pig Slaughterhouses as Well as From mWWTPs

Due to the growth of accompanying bacterial microbiota that belongs to intrinsically colistin-resistant genera (e.g., *Proteus, Providencia, Morganella*) and colistin-susceptible isolates on the selective agar plates as well as absence of sample replicates, it was not possible to perform accurate quantification of target bacteria. This could be considered as a limitation of this study.

Water samples collected in poultry slaughterhouses yielded the highest percentage of colistin-resistant *Enterobacteriaceae* (40.2%; 33/82) followed by mWWTPs (25.0%, 9/36) and pig slaughterhouses (14.9%, 10/67). Detailed information on species distribution is shown in **Figure 1**.

In the poultry and pig slaughterhouses the target bacteria were recovered at almost all sampling points as shown in **Figures 2**, **3**, respectively. Interestingly, only one out of nine samples taken in the effluent of the mWWTPs was positive for target colistin-resistant bacteria. Moreover, no colistin-resistant target bacteria were detected in the on-site preflooders upstream the discharge point (**Figure 3**).

Overall, 129 isolates were recovered from 185 samples. Of the isolates, 50.4% were determined as *E. coli*, 26.3% as *K. pneumoniae* and 23.3% as isolates of the *E. cloacae* complex. The most frequently isolated species in poultry and pig slaughterhouses was *E. coli*, whereas in mWWTPs *K. pneumoniae* was more abundant.

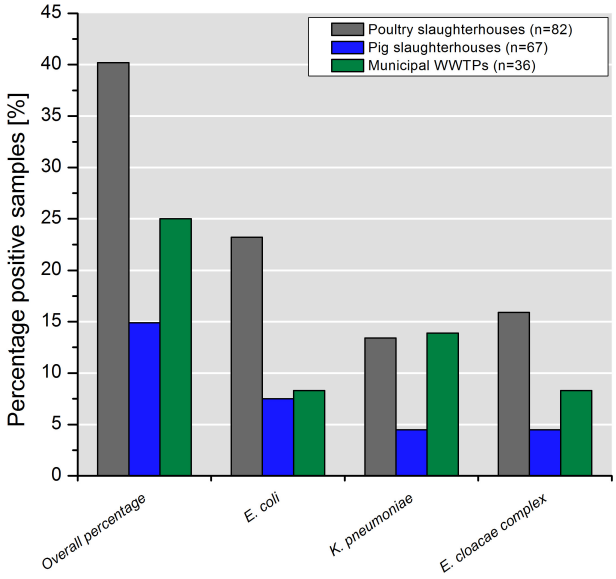

FIGURE 1 | Percentage of samples containing colistin-resistant target bacteria taken in poultry and pig slaughterhouses as well as in the municipal WWTPs.

Resistance Patterns [Scheme A (EUCAST) and Scheme B (KRINKO)] and MIC of Colistin per Species

Isolates of *E. coli*, *K. pneumoniae*, and *E. cloacae* complex exhibited various resistance patterns. The resistance rates using epidemiological cut-off values (**Figures 4A–C**) were higher and different in comparison to those obtained with clinical breakpoints (**Figures 5A–C**).

According to the scheme A, the recovered isolates were either susceptible or expressed low resistance rates to gentamicin, tigecycline and with exception of *E. cloacae* complex to carbapenems (IMP and MEM). The resistance rates to 3rd generation cephalosporins (CTX and CAZ) varied between isolated species and were in the range of 23.5% for *K. pneumoniae* and 46.7% for *E. cloacae* complex. The highest level of multiple drug resistance (MDR, combined resistance to CST, CIP, and TET) shown isolates of *E. coli* (49.2%), followed by *K. pneumoniae* (35.3%), and *E. cloacae* complex (33.3%). MICs of antimicrobials with undefined epidemiological cut-offs for *E. cloacae* complex (AMP, CHL, NAL, SMX, TMP, ETP, and FOX) are shown in **Table 1**. MIC values of colistin for recovered *E. coli* and *K. pneumoniae* isolates are shown in **Tables 2–4**. Among isolates of *E. cloacae* complex, MIC values of colistin varied between 16 and >64 mg/L.

According to the scheme B, the isolates with exception of *K. pneumoniae* had lower resistance rates to 3rd generation cephalosporins (CTX and CAZ). The differences varied between 12.3% for CAZ by *E. coli* and 26.6% for CTX by *E. cloacae* complex (**Figures 4**, **5**). Furthermore, they were susceptible to temocillin, ceftazidime-avibactam, imipenem, meropenem, amikacin and, with exception of some *E. cloacae* complex isolates, to tigecycline.

The highest 3MDRO rates (multidrug-resistant organisms with combined resistance to PIP, CTX, and CIP) were exhibited by *K. pneumoniae* (26.5%), followed by *E. cloacae* complex (20.6%) and *E. coli* (13.8%). However, if using piperacillin-tazobactam instead of piperacillin for determination of the MDR status, as recommended by Magiorakos et al. (2012), the 3MDRO rates were lower at 5.9% for *K. pneumoniae*, 3.3% for *E. cloacae* complex, and 3.1% for *E. coli*.

Phylogenetic Groups of *E. coli* (*n* = 65)

The majority of the *E. coli* isolates belonged to the most common phylogroups associated with commensal strains, such as A (32.3%), B1 (24.6%), C (16.9%), F (10.8%), Clade I, II (9.2%), and E (1.5%) (Clermont et al., 2000, 2013). Only two isolates (3.0%) recovered from the influent of the in-house WWTP of a poultry slaughterhouse were assigned to extraintestinal pathogenic (ExPEC) group D (Clermont et al., 2000, 2013). Furthermore, one isolate originating from the wastewater used for cleaning of poultry stunning facilities belonged to group B2.

Occurrence of *mcr* Genes

Of the *mcr* genes screened, only *mcr-1.1* was detected in 70.8% of *E. coli* and 20.6% of *K. pneumoniae* isolates. Colistin MICs of *mcr-1*-positive *E. coli* isolates ranged from 4 to 8 mg/L, whereas *mcr-1* carrying *K. pneumoniae* isolates expressed higher level of resistance from 4 to >64 mg/L.

In poultry and pig slaughterhouses the *mcr-1.1* carrying isolates of *E. coli* and *K. pneumoniae* were detected at almost all sampling points including scalding water and effluents of the in-house WWTPs. Furthermore, *mcr-1.1* positive isolates of *E. coli* were detected in on-site preflooders downstream the discharge point. Detailed information on the isolation source and phenotypic resistance of *mcr-1.1* carrying isolates of *E. coli* and *K. pneumoniae* is given in **Table 2**.

PFGE Patterns of Colistin-Resistant *mcr-1* Carrying Isolates, Location of *mcr-1* Gene

Overall, the analyzed isolates (*n* = 53, 46 *E. coli* and 7 *K. pneumoniae*) exhibited a broad diversity as they were assigned to 25 different *Xba*I profiles (20 for *E. coli* and 5 for *K. pneumoniae*). S1 nuclease PFGE, followed by Southern blot hybridization revealed the presence of *mcr-1* carrying plasmids ranging between 30 and 360 kb. Interestingly, the majority of the isolates exhibited a predominant plasmid type of 30 kb (**Table 2**). However, we had also determined a substantial number of isolates exhibiting the same *Xba*I macrorestriction patterns and/or plasmid profiles.

Conjugation and Transformation Experiments, Inc-Typing of Plasmids

In 67.4% (31/46) of *mcr-1* carrying *E. coli* isolates, the *mcr-1* gene was found to be encoded on plasmids of different Inc-groups that could be conjugated into recipient *E. coli* cells (**Table 1**). Plasmids were affiliated to IncI1 (41.9%), IncHI2, and IncX4 (each 22.6%), IncF (9.7%) as well as IncI2 (3.2%) types

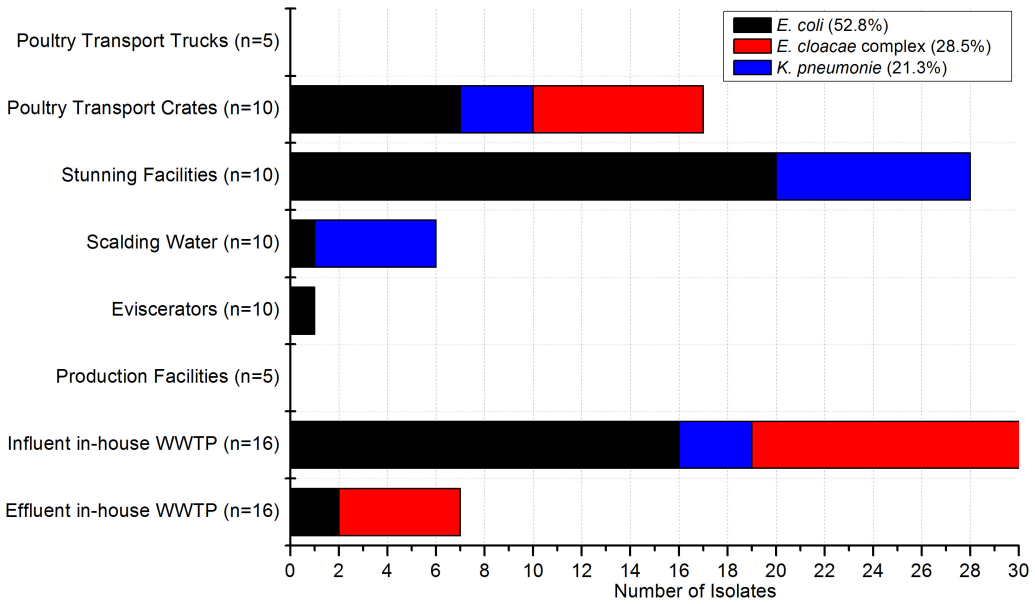

FIGURE 2 | Occurrence of target bacteria tested as colistin-resistant across the sampling points in poultry slaughterhouses (n = 82). Number of samples taken at each sampling point is stated.

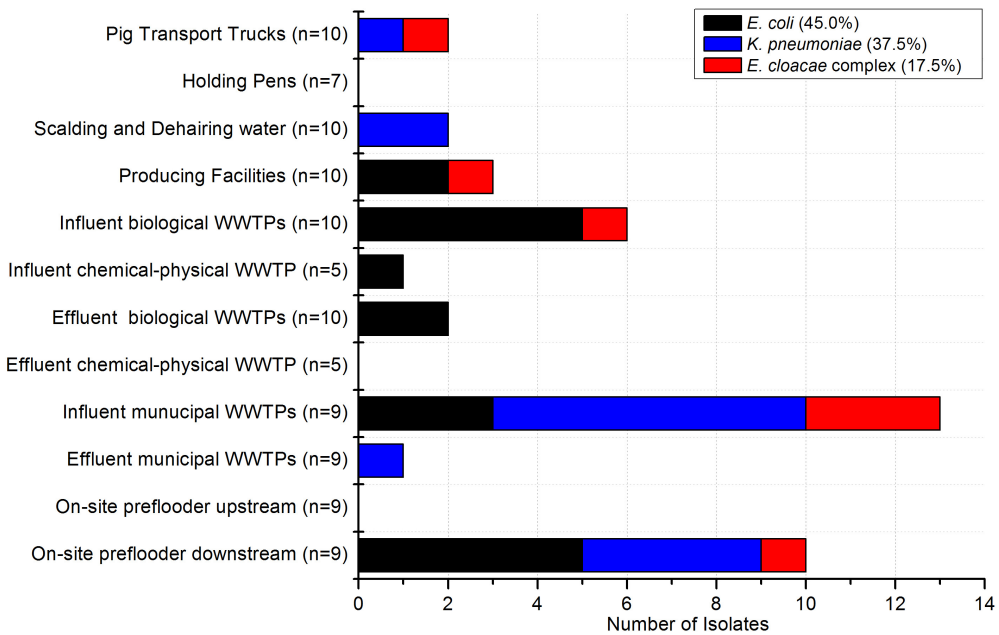

FIGURE 3 | Occurrence of target bacteria tested as colistin-resistant across the sampling points in pig slaughterhouses (n = 67) and in the municipal WWTPs receiving wastewater from the investigated pig slaughterhouses (n = 36). Number of samples taken at each sampling point is stated.

as demonstrated by TaqMan RT-PCR and PBRT method. All seven *mcr-1*-positive *K. pneumoniae* isolates carried the *mcr-1* on self-transmissible IncX4 (71.4%) and IncI1 (28.6%) plasmids. Of note, IncI1-type plasmids carrying *mcr-1* were predominant in all sampling sites. Colistin MICs of transconjugants were either identical or lower than those of the donor strains and ranged from 2 to 8 mg/L.

Conjugation experiments with the applied selection conditions resulted in diverse co-transferred resistance phenotypes. Using epidemiological cut-off values, 81.6% (31/38) of *E. coli* and *K. pneumoniae* transconjugants expressed resistance to further antimicrobials beside colistin. Among the isolates recovered in the poultry slaughterhouses the most frequently co-transferred resistance was to ciprofloxacin and

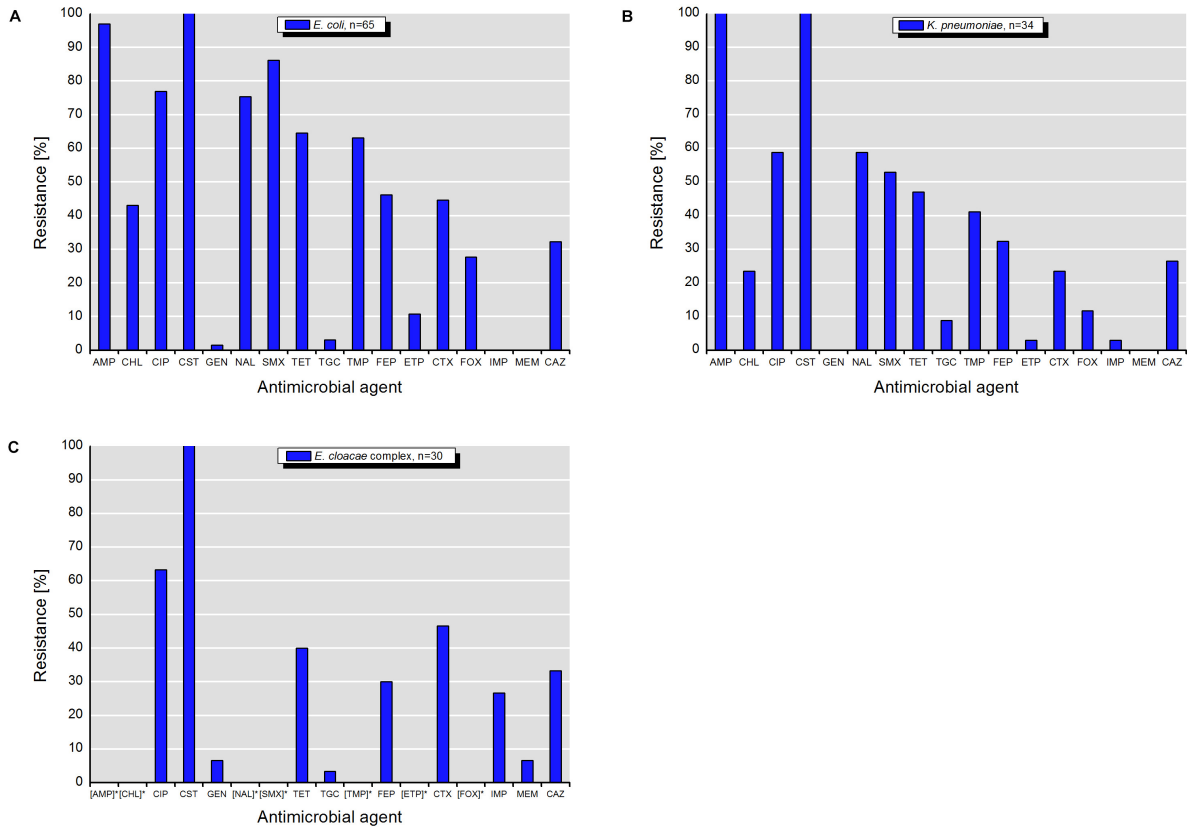

FIGURE 4 | Resistance to antimicrobial agents detected among target colistin-resistant isolates of **(A)** *E. coli*, **(B)** *K. pneumonia*, and **(C)** *E. cloacae* complex with MICs interpreted according to the epidemiological cut-off values (ECOFFs) of EUCAST (scheme A). MICs (mg/L) of antimicrobials with undefined epidemiological cut-offs for *E. cloacae* complex isolates are shown in **Table 1**. AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TMP, trimethoprim; FEP, cefepime; ETP, ertapenem; CTX, cefotaxime; FOX, cefoxitin; IMI, imipenem; MEM, meropenem; CAZ, ceftazidime. []* – antimicrobials with undefined ECOFFs.

nalidixic acid (70.8%, 17/24), followed by ampicillin (29.2%, 7/24) and trimethoprim/sulfamethoxazole (25.0%, 6/24). Only 8.3% (2/24) of the isolates co-transferred resistance against 3rd generation cephalosporins. In contrast, the majority of the isolates originating from the pig slaughterhouses co-transferred resistance against tetracycline (57.1%, 8/14). The resistance to ciprofloxacin and nalidixic acid was co-transferred by 35.7% (5/14) of the isolates. However, when applying scheme B based on clinical breakpoints, only 15.8% (6/38) of the colistin-resistant transconjugants expressed additional resistances, mostly to sulfamethoxazole-trimethoprim (5/6) and piperacillin (4/6).

The transformation experiments with 15 *E. coli* isolates carrying *mcr-1* gene (15/46) and for which no transconjugants could be obtained, did not yield any transformants.

Detailed information on Inc-types of *mcr-1* harboring plasmids, colistin MIC of the transconjugants and co-transferred resistance phenotypes of individual isolates is given in **Table 2**.

pmrAB

Sequences of Colistin-Resistant *E. coli* and *K. pneumoniae* Isolates Tested Negative for *mcr-1* to *mcr-9*

In 73.7% (14/19) of *E. coli* isolates non-synonymous polymorphisms at the protein level were detected in *pmrA* and *pmrB*. Nucleotide sequence polymorphisms that produce protein variants 15Gly→Arg, 80Ala→Val, 85Thr→Ala, 204Ala→X were found in *pmrA*. Furthermore, eleven variants, 2His→Arg, 10Leu→Arg, 12Gln→x, 14Leu→Pro, 29Ser→x, 44Phe→x, 94Pro→S, 285Ala→Thr, 312Asp→Asn, 333His→Gln, 360Ala→Val, were found in *pmrB*.

In 81.5% (22/27) of *K. pneumoniae* isolates the *pmrA* and *pmrB* genes revealed polymorphic positions that were non-synonymous at the protein level. Additionally, four non-synonymous polymorphisms were found in *pmrA* (37Ala→Thr, 57Glu→Gly, 147Ala→Glu, and 217Ala→Val) and six in *pmrB* (2Ala→Ser, 73Pro→x, 74Ser→x, 112Thr→Pro, 157Thr→Pro, 203Ser→Pro). In one *K. pneumoniae* isolate recovered from the on-site preflooder downstream the discharge point, a yet unknown insertion of nine amino acids (Gln-Leu-Gln-Gln-Leu-Ala-Arg-Val-Gly) between amino acid residues Glu-201 and Gln-202 of *pmrB* was identified. Detailed information on non-synonymous polymorphisms of individual *E. coli* and *K. pneumoniae* isolates, their origin and resistance phenotypes is given in **Tables 3**, **4**, respectively.

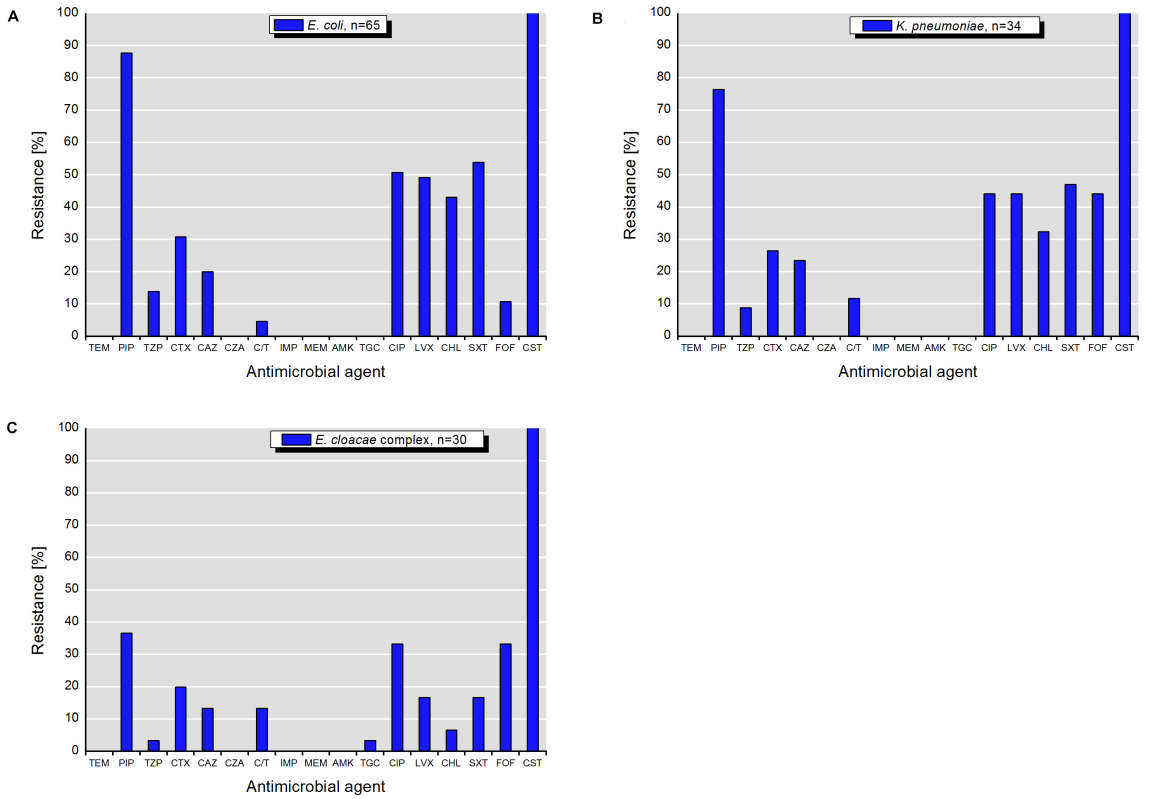

FIGURE 5 | Resistance to antimicrobial agents detected among target colistin-resistant isolates of **(A)** *E. coli*, **(B)** *K. pneumonia*, and **(C)** *E. cloacae* complex with MICs interpreted according to the clinical breakpoints of EUCAST (scheme B). TEM, temocillin; PIP, piperacillin; TZP, piperacillin-tazobactam; CTX, cefotaxime; CAZ, ceftazidime; CZA, ceftazidime-avibactam; C/T, ceftolozane-tazobactam; IMP, imipenem; MEM, meropenem; AMK, amikacin; TGC, tigecycline; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin.

DISCUSSION

Our study provides data on the occurrence of colistin resistant *Enterobacteriaceae* (*E. coli*, *K. pneumoniae*, and *E. cloacae* complex) in process waters and wastewater along the slaughtering processes in poultry and pig slaughterhouses, their in-house and mWWTPs as well as receiving waterbodies.

The highest prevalence of colistin-resistant bacteria was detected in poultry slaughterhouses. This is in accordance with other studies indicating frequent occurrence of colistin-resistant *Enterobacteriaceae* in the poultry production chain in Germany (Irrgang et al., 2016; Inderbinen, 2017). Current data from official bodies on antimicrobial usage in different animal species in Germany are not available. However, the Report of the Federal Ministry of Food and Agriculture on the Evaluation of the Antimicrobials Minimization Concept introduced with the 16th Act to Amend the Medicinal Products Act (16th AMG Amendment) indicates a higher usage of colistin in German poultry production in comparison to other livestock production chains (BMEL, 2019). Moreover, between 2014 and 2017 consumption of polypeptide antibiotics in broiler production in Germany slightly increased from 11 to 13 tons. Whereas in pig production chain polypeptide antibiotics are mostly used to treat piglets and for the treatment of fattening pigs a decrease from 4 tons in 2014 to 0.5 tons in 2017 was observed (BMEL, 2019). Thus, the higher use of colistin in poultry may coincide with the frequent occurrence of colistin-resistant bacteria in this production chain. Furthermore, in comparison to poultry, a longer life span and time gap between administration of antibiotic and slaughtering among pigs may result in a decrease of colistin resistance when selection pressure is absent. Moreover, the kind of antibiotic treatment, e.g., treatment of individual pigs or small groups thereof in comparison to the whole flock treatment, may also be responsible for the lower occurrence of colistin resistance among pigs and accordingly in the pig slaughterhouses (BMEL, 2010). Furthermore, our results are in line with the EU summary report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018 (ECDC, 2020) showing increased colistin resistance in *E. coli* isolates from broilers compared to those from pigs.

From nine *mcr* genes tested, *mcr-1* was the most prevalent one, which corroborates the study of Elbediwi et al. (2019) that emphasizes the global dissemination and high prevalence of *mcr-1* gene among colistin-resistant bacteria isolated from animals and food products worldwide. With prevalences of 0.04 to 20.3%, *mcr-1* is predominantly detected in *Enterobacteriaceae* isolates (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Salmonella* spp., and *Shigella* spp.) from livestock, retail meat (1.4–19%) and to a lesser

TABLE 1 | MICs (mg/L) of antimicrobials with undefined epidemiological cut-offs for *E. cloacae* complex isolates tested negative for *mcr-1* to *mcr-9*.

Isolate	Species	Origin	AMP	CHL	NAL	SMX	TMP	ETP	FOX
Poultry slaughterhouses									
C-04/10-01	*Enterobacter asburiae*	Effluent in-house WWTP	>64	≤8	>128	≤8	≤0.25	0.03	>64
C-04/02-01	*E. asburiae*	Transport crates	16	16	8	>1024	2	0.03	>64
C-04/02-03	*E. asburiae*	Transport crates	32	≤8	≤4	≤8	0.5	0.03	>64
C-04/02-16	*E. asburiae*	Transport crates	8	16	≤4	256	1	0.03	>64
C-04/02-22	*E. asburiae*	Transport crates	8	16	16	≤8	0.5	0.03	>64
C-04/05-23	*E. asburiae*	Influent in-house WWTP	16	≤8	>128	≤8	≤0.25	0.03	>64
C-04/05-24	*E. asburiae*	Influent in-house WWTP	16	≤8	64	≤8	0.5	0.03	>64
C-04/05-28	*E. asburiae*	Influent in-house WWTP	16	16	16	512	1	0.03	>64
C-04/05-31	*E. asburiae*	Influent in-house WWTP	8	≤8	>128	≤8	≤0.25	≤0.015	>64
C-04/05-33	*E. asburiae*	Influent in-house WWTP	4	≤8	>128	≤8	≤0.25	≤0.015	>64
C-04/05-35	*E. asburiae*	Influent in-house WWTP	8	≤8	>128	≤8	0.5	≤0.015	>64
C-04/05-41	*E. asburiae*	Influent in-house WWTP	8	≤8	>128	≤8	≤0.25	≤0.015	>64
C-04/05-42	*E. asburiae*	Influent in-house WWTP	16	≤8	16	≤8	≤0.25	≤0.015	>64
C-04/05-43	*E. asburiae*	Influent in-house WWTP	8	≤8	>128	≤8	≤0.25	0.06	>64
C-04/05-44	*E. asburiae*	Influent in-house WWTP	16	≤8	16	≤8	≤0.25	≤0.015	>64
C-04/06-04	*E. asburiae*	Effluent in-house WWTP	32	≤8	>128	≤8	≤0.25	0.03	>64
C-04/06-07	*E. asburiae*	Effluent in-house WWTP	32	≤8	≤4	≤8	≤0.25	0.12	>64
C-04/05-29	*Enterobacter hormaechei*	Influent in-house WWTP	>64	≤8	>128	≤8	≤0.25	0.03	>64
C-04/06-05	*E. hormaechei*	Effluent in-house WWTP	>64	≤8	>128	≤8	≤0.25	0.03	>64
C-04/06-01	*Enterobacter kobei*	Effluent in-house WWTP	32	16	≤4	≤8	≤0.25	0.03	>64
01/02-22	*E. asburiae*	Transport crates	>64	≤8	≤4	≤8	≤0.25	0.03	>64
04/02-02	*E. asburiae*	Transport crates	>64	≤8	>128	≤8	1	0.5	>64
04/02-04	*E. asburiae*	Transport crates	>64	16	16	32	1	1	>64
Pig slaughterhouses and mWWTPs									
C-03/02-01	*E. asburiae*	Influent biological WWTP	16	≤8	≤4	≤8	≤0.25	≤0.015	>64
C-05/10-24	*E. asburiae*	Influent municipal WWTP	8	≤8	≤4	≤8	≤0.25	0.03	>64
C-05/10-25	*E. asburiae*	Influent municipal WWTP	64	≤8	8	≤8	≤0.25	0.03	>64
C-03/09-03	*E. cloacae*	Producing facilities	16	≤8	≤4	≤8	≤0.25	0.03	>64
05/03-11	*Enterobacter aerogenes*	Pig Transport Trucks	>64	16	≤4	>1024	>32	0.06	>64
03/10-33	*E. asburiae*	Influent municipal WWTP	>64	≤8	>128	>1024	>32	2	>64
03/12-25	*E. asburiae*	On-site preflooder downstream	>64	≤8	>128	>1024	>32	0.12	>64

AMP, ampicillin; CHL, chloramphenicol; NAL, nalidixic acid; SMX, sulfamethoxazole; TMP, trimethoprim; ETP, ertapenem; FOX, cefoxitin.

extent in human clinical isolates (0.06–2%), worldwide (Hasman et al., 2015; Haenni et al., 2016; Kluytmans-van, den Bergh et al., 2016; Malhotra-Kumar et al., 2016; Webb et al., 2016). In Germany, colistin-resistant isolates from turkey and broilers food chains show the highest *mcr-1* prevalence in comparison to pigs and cattle (Irrgang et al., 2016; Borowiak et al., 2020). Thus, livestock and poultry are considered as an origin of *mcr-1* and is its important reservoir for transmission to humans (Liu et al., 2016). Based on the wide dissemination of *mcr-1*, EMA's (European Medicines Agency) Antimicrobial Advice *Ad Hoc* Expert Group (AMEG) advised to minimize sales of colistin for use in animals EU-wide to achieve a 65% reduction in 2016 (EMA, 2016).

The genes *mcr-2* to *mcr-9* have not been detected in our study. This could be due to their limited geographical distribution and bacterial host range (Borowiak et al., 2020) as well as substantially low prevalence compared with *mcr-1*. While *mcr-2* to *mcr-8* are being detected mostly in *E. coli* and *K. pneumoniae* isolates from pigs and poultry in China and South Europe (Xavier et al., 2016; AbuOun et al., 2017; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017; Wang et al., 2018; Yang et al., 2018), *mcr-9* and *mcr-10* were discovered in clinical strains of *Salmonella enterica* serotype Typhimurium (Carroll et al., 2019) and *Enterobacter roggenkampii*, respectively (Wang et al., 2020). Currently in Germany, *mcr-3* was detected in *Aeromonas* spp. isolates of fish origin (Eichhorn et al., 2018). Furthermore, *mcr-4* has been frequently identified in different *Salmonella* serovars from poultry meat and pork (Borowiak et al., 2020) as well as *mcr-5* has been detected in *E. coli* and *Salmonella* isolates of livestock origin (Hammerl et al., 2018; Borowiak et al., 2020). Kneis et al. (2019) reported high abundances of *mcr-3*, *mcr-4*, *mcr-5*, and *mcr-7* in German mWWTPs. However, the possible origin sources such as households, health care facilities, livestock farming sites or slaughterhouses as well as bacterial host ranges have not been identified (Kneis et al., 2019).

TABLE 2 | Characteristics of MCR-1–producing *E. coli* and *K. pneumoniae* isolates and their transconjugants.

			Isolates			Transconjugants			
Isolate	**Species**	**Origin**	**Colistin MIC, mg/L**	**Resistance phenotype (epidemiological cut-off values of EUCAST)[a]**	**Resistance phenotype (clinical breakpoints of EUCAST)[b]**	**Incompatibility group (kb) of *mcr-1* plasmids**	**Colistin MIC of transconjugants mg/L**	**Co-transferred resistance (epidemiological cut-off values of EUCAST)[a]**	**Co-transferred resistance (clinical breakpoints of EUCAST)[b]**
					Poultry slaughterhouses				
01/05-11	*E. coli*	Influent in-house WWTP	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncF (30)	8		
01/05-12	*E. coli*	Influent in-house WWTP	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncF (30)	8		
01/07-07	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncX4 (30)	4	CIP, NAL	
01/07-09	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncI1 (30)	8	CIP, NAL	
01/07-11	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, CHL, SXT, CST	IncX4 (30)	4	CIP, NAL	
01/07-12	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncX4 (30)	4	CIP, NAL	
01/08-08	*E. coli*	Eviscerators	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, CHL, SXT, FOF, CST	IncHI2 (30)	4	CIP, NAL	
04/05-12	*E. coli*	Influent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	IncHI2 (245)	4	AMP, CIP, CTX, CAZ	
C-01/05-03	*E. coli*	Influent in-house WWTP	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, SXT, CST	IncI1 (30)	4	AMP, SMX, TMP	SXT
C-01/05-04	*E. coli*	Influent in-house WWTP	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, CST	IncI2 (n.d.*)	4	CIP, NAL	
C-01/07-02	*E. coli*	Stunning facilities	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, CST	IncX4 (30)	4	CIP, NAL	PIP, CIP, LVX, SXT
C-01/07-04	*E. coli*	Stunning facilities	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, CST	IncX4 (30)	4	CIP, NAL	
C-01/07-06	*E. coli*	Stunning facilities	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, CST	IncX4 (30)	4	CIP, NAL	
C-04/02-02	*E. coli*	Transport crates	4	AMP, CIP, CST, NAL, SMX, TET	PIP, CIP, LVX, CST	IncX4 (30)	4	CIP, NAL	
C-04/05-10	*E. coli*	Influent in-house WWTP	4	AMP, CST, SMX, TET, TMP	PIP, SXT, CST	IncI1 (360)	4	AMP, SMX, TET, TMP	PIP, SXT
C-04/05-14	*E. coli*	Influent in-house WWTP	4	AMP, CST, SMX, TET, TMP	FOF, CST	IncI1 (360)	4	AMP, SMX, TET, TMP	
C-04/06-02	*E. coli*	Effluent in-house WWTP	8	AMP, CST, SMX, TMP	PIP, CIP, LVX, CST	IncHI2 (30)	8	AMP, SMX, TMP	PIP
C-04/07-04	*E. coli*	Stunning facilities	4	AMP, CIP, CST, SMX, NAL, TET, TMP	PIP, CIP, SXT, CST	IncF (215)	4	AMP, CIP, NAL, SMX, TMP	SXT

(Continued)

TABLE 2 | Continued

Isolate	Species	Origin	Isolates			Transconjugants			
			Colistin MIC, mg/L	Resistance phenotype (epidemiological cut-off values of EUCAST)[a]	Resistance phenotype (clinical breakpoints of EUCAST)[b]	Incompatibility group (kb) of *mcr-1* plasmids	Colistin MIC of transconjugants mg/L	Co-transferred resistance (epidemiological cut-off values of EUCAST)[a]	Co-transferred resistance (clinical breakpoints of EUCAST)[b]
Poultry slaughterhouses									
C-04/07-07	*E. coli*	Stunning facilities	4	AMP, CIP, CST, SMX, NAL, TET, TMP	PIP, SXT, CST	IncHI2 (215)	4	AMP, CIP, NAL, TET	PIP, SXT
04/07-04	*K. pneumoniae*	Stunning facilities	8	AMP, CIP, CST, CTX, NAL, SMX, CAZ, TET, TMP, FEP, FOX	PIP, CTX, CIP, LVX, CHL, SXT, FOF, CST	IncI1 (30)	4	CIP, NAL	
04/07-12	*K. pneumoniae*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, FEP	PIP, TZP, CTX, CAZ, CIP, LVX, CHL, SXT, FOF, CST	IncX4 (85)	8	CIP, CTX, NAL, CAZ, FOX	
04/07-14	*K. pneumoniae*	Stunning facilities	16[c]	AMP, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, SXT, FOF, CST	IncX4 (30)	4	CIP, NAL	
C-04/02-17	*K. pneumoniae*	Transport crates	>64[c]	AMP, CST	PIP, SMX, CST	IncI1 (30)	8		
C-04/03-01	*K. pneumoniae*	Scalding water	16[c]	AMP, CIP, CST, NAL	CIP, LVX, CST	IncX4 (30)	4	CIP, NAL	
Pig slaughterhouses and mWWTPs									
05/01-09	*E. coli*	Influent in-house WWTP	8	AMP, CHL, CIP, CST, CTX, FOX, TET, TMP, FEP	PIP, TZP, CTX, CIP, LVX, CHL, SMX, CST	IncI1 (30)	8	TET	
05/01-21	*E. coli*	Influent in-house WWTP	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	8	TET	
05/01-22	*E. coli*	Influent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
05/01-23	*E. coli*	Influent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
05/02-28	*E. coli*	Effluent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
05/02-29	*E. coli*	Effluent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CTX, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
05/06-69	*E. coli*	Aggregate wastewater from producing facilities	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
05/06-70	*E. coli*	Aggregate wastewater from producing facilities	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
C-03/01-04	*E. coli*	Influent in-house WWTP	8	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, CHL, CST	IncI1 (30)	4	CIP, NAL	
C-03/12-05	*E. coli*	On-site preflooder downstream	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, FEP	PIP, CST	IncHI2 (n.d.*)	2	CIP, NAL	
C-03/12-07	*E. coli*	On-site preflooder downstream	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, TGC, FEP	PIP, CST	IncHI2 (245)	2		

(Continued)

TABLE 2 | Continued

Isolate	Species	Origin	Isolates			Transconjugants			
			Colistin MIC, mg/L	Resistance phenotype (epidemiological cut-off values of EUCAST)[a]	Resistance phenotype (clinical breakpoints of EUCAST)[b]	Incompatibility group (kb) of *mcr-1* plasmids	Colistin MIC of transconjugants mg/L	Co-transferred resistance (epidemiological cut-off values of EUCAST)[a]	Co-transferred resistance (clinical breakpoints of EUCAST)[b]
Poultry slaughterhouses									
C-03/12-08	*E. coli*	On-site preflooder downstream	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, FEP	PIP, TZP, CST	IncHI2 (230)	4	CIP, NAL	
C-03/05-01	*K. pneumoniae*	Animal transporters	>64[c]	AMP, CHL, CIP, CST, NAL	PIP, CIP, LVX, CHL, CST	IncX4 (30)	4	CIP, NAL	
C-03/08-01	*K. pneumoniae*	Scalding water	>64[c]	AMP, CHL, CIP, CST, CTX, FOX, NAL, CAZ, FEP	FOF, CST	IncX4 (30)	4	CIP, NAL	

[a]*Antimicrobial Susceptibility Testing Plates of German Federal Institute for Risk Assessment (BfR) containing sulfamethoxazole (SMX), trimethoprim (TMP), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), tigecycline (TGC), ertapenem (ETP), meropenem (MEM), imipenem (IMI), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), colistin (CST), ampicillin (AMP), gentamicin (GEN). MIC were interpreted according to the epidemiological cut off values of EUCAST.*

[b]*Micronaut-S MDR MRGN-Screening system containing temocillin (TEM), piperacillin (PIP), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMI), meropenem (MEM), amikacin (AMK), tigecycline (TGC), chloramphenicol (CHL), fosfomycin (FOF), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LVX), and colistin (CST). MIC were interpreted according to the clinical breakpoints of EUCAST.*

[c]*Polymorphisms found for coding sequences for PmrA or PmrB in 04/07-14: 157T→P (PmrB) and C-04/03-01: 73P→x (PmrB), 74S→x (PmrB).*

**n.d., not detected.*

Escherichia coli isolates carrying *mcr-1* on transferable IncHI2 plasmids were detected in on-site preflooder downstream the discharge point of mWWTP. Possible entry sources could be run-offs from the fields fertilized with contaminated manure (Guenther et al., 2017) and feces of wild animals (birds) (Lin et al., 2020). Previously, (Zurfluh et al., 2016) and (Falgenhauer et al., 2019) detected *mcr-1* harboring *E. coli* in surface water and rivers in Switzerland and Germany, respectively. Moreover, in our study *mcr-1*-positive *K. pneumoniae* was recovered from poultry scalding water. This could be a possible source of contamination of carcasses and products and lead to the introduction of *mcr-1* carrying *K. pneumoniae* into the food chain. (Schrauwen et al., 2017) reported that 24.8% of retail chicken meat in Netherlands were positive for *mcr-1*, carried mostly by *E. coli* and to a lesser extent by *K. pneumoniae*. Furthermore, 40.6% of poultry meat samples originating from Germany were contaminated with *mcr-1* producing bacteria (Inderbinen, 2017). Some of the *mcr-1* carrying isolates recovered from wastewater used for cleaning of stunning facilities and influents of in-house WWTP from poultry slaughterhouses belonged to ExPEC groups B2 and D, which are known to harbor more virulence factors than commensal strains and pose a zoonotic risk (Johnson et al., 2012). This enables the transmission of *mcr-1*-positive ExPECs of poultry origin to humans and represents a potential vehicle of *mcr* genes for human diseases, e.g., bloodstream and urinary tract infections (Izdebski et al., 2016; Zhong et al., 2019). Moreover, study of Zhuge et al. (2019) shown that *mcr-1*-positive *E. coli* of phylogroups B1 and F also possessed high virulence in rodent models for ExPEC-associated human infections and could therefore pose an elevated risk of infections for humans.

According to the classification of Magiorakos et al. (2012) and applying epidemiological cut-off values, target isolates showed high percentage of multidrug resistance (combined resistance to CST, CIP, and TET) with the highest rate of 49.2% for *E. coli*. However, it is important to note that from a human clinical perspective, the antibiotic groups are not considered to be equally clinically relevant (Exner et al., 2017). Thus, from the point of view of KRINKO, the multidrug resistance rates (3MDRO rates, combined resistance to TZP, CTX, and CIP) were low with the highest percentage of 5.9% for *K. pneumoniae*. For this evaluation the combination of piperacillin/tazobactam instead of piperacillin is used, as in the clinical practice in Germany piperacillin is administered only in combination with β-lactamase inhibitors. Furthermore, applying clinical breakpoints, isolates were completely susceptible to reserve antibiotics ceftazidime-avibactam and tigecycline as well as carbapenems (IMP and MEM). Moreover, temocillin, which was introduced in 2019 for therapy of extended spectrum β-lactamase (ESBL) and AmpC producers, and amikacin, classified by WHO as reserve second−line drug, were also effective against all isolates. Thus, these substances could be still effective in antimicrobial therapy in case of infection.

It was already reported that *mcr-1* gene occurs frequently in isolates that are susceptible to most classes of antimicrobials (EMA, 2016). However, this contrasts with the findings of our study, as considerable percentage of colistin-resistant *mcr-1* positive isolates from our study showed resistance of up to eleven

TABLE 3 | PmrAB polymorphisms of colistin-resistant *E. coli* isolates tested negative for *mcr-1* to *mcr-9*.

Isolate	Species	Origin	Colistin MIC, mg/L	Resistance phenotype (epidemiological cut-off values of EUCAST)[a]	Resistance phenotype (clinical breakpoints of EUCAST)[b]	PmrAB[c]
			Poultry slaughterhouses			
C-01/07-07	*E. coli*	Stunning facilities	8	AMP, CIP, CST, NAL, SMX, TET	PIP, CST	2H→R (PmrB) 360A→V (PmrB)
C-01/07-08	*E. coli*	Stunning facilities	8	AMP, CIP, CST, NAL, SMX, TET	PIP, CST	2H→R (PmrB) 360A→V (PmrB)
C-01/07-11	*E. coli*	Stunning facilities	8	AMP, CIP, CST, NAL, SMX, TET	PIP, CST	2H→R (PmrB) 360A→V (PmrB)
C-01/07-12	*E. coli*	Stunning facilities	8	AMP, CIP, CST, NAL, SMX, TET	PIP, CST	2H→R (PmrB) 360A→V (PmrB)
04/02-15	*E. coli*	Transport crates	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TET, TGC, FEP, FOX	PIP, CTX, CAZ, CIP, LVX, CHL, CST	–
04/02-16	*E. coli*	Transport crates	4	AMP, CST, CTX, SMX, CAZ, TET, TMP, FEP, FOX	PIP, CTX, CAZ, CIP, LVX, CHL, SXT, CST	–
04/07-11	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TET, FEP, FOX	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	–
C-04/03-08	*E. coli*	Scalding water	8	AMP, CST, SMX	PIP, CIP, LVX, SXT, CST	14L→P (PmrB) 44F→x (PmrB)
C-04/05-08	*E. coli*	Influent in-house WWTP	8	AMP, CST, SMX, TMP	PIP, SXT, CST	14L→P (PmrB) 44F→x (PmrB)
C-04/05-13	*E. coli*	Influent in-house WWTP	8	AMP, CST, SMX	PIP, CST	10L→R (PmrB) 12Q→x (PmrB)
C-04/05-15	*E. coli*	Influent in-house WWTP	8	AM, CIP, CST, NAL, TMP	PIP, CIP, LVX	15G→R (PmrA) 85T→A (PmrA) 2H→R (PmrB)
C-04/05-17	*E. coli*	Influent in-house WWTP	8	AMP, CIP, CST, NAL	PIP, CIP, LVX, CST	312D→N (PmrB)
C-04/07-03	*E. coli*	Stunning facilities	4	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TET, FEP, FOX	PIP, CST	–
C-04/07-06	*E. coli*	Stunning facilities	4	AMP, CST, SMX, TET, TMP	PIP, CIP, LVX, SXT, FOF, CST	29S→x (PmrB)
C-04/07-08	*E. coli*	Stunning facilities	4	AMP, CST, SMX, TET, TMP	PIP, CIP, LVX, CHL, SXT, CST	–
			Pig slaughterhouses and mWWTPs			
C-03/02-02	*E. coli*	Effluent in-house WWTP	8	AMP, CIP, CST, CTX, NAL, SMX, CAZ, TET, TMP, FEP	PP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	204A→x (PmrA) 2H→R (PmrB)
C-03/10-10	*E. coli*	Influent municipal WWTP	4	CIP, CST, NAL	CST	80A→V (PmrA) 285A→T (PmrB) 333H→Q (PmrB)
C-03/10-14	*E. coli*	Influent municipal WWTP	4	CIP, CST, NAL	CST	80A→V (PmrA) 285A→T (PmrB) 333H→Q (PmrB)
03/10-43	*E. coli*	Influent municipal WWTP	16	AMP. CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, SXT, CST	44F→x (PmrB) 94P→S (PmrB)

[a] *Antimicrobial Susceptibility Testing Plates of German Federal Institute for Risk Assessment (BfR) containing sulfamethoxazole (SMX), trimethoprim (TMP), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), tigecycline (TGC), ertapenem (ETP), meropenem (MEM), imipenem (IMI), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), colistin (CST), ampicillin (AMP), gentamicin (GEN). MIC were interpreted according to the epidemiological cut off values of EUCAST.*
[b] *Micronaut-S MDR MRGN-Screening system containing temocillin (TEM), piperacillin (PIP), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMI), meropenem (MEM), amikacin (AMK), tigecycline (TGC), chloramphenicol (CHL), fosfomycin (FOF), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LVX), and colistin (CST). MIC were interpreted according to the clinical breakpoints of EUCAST.*
[c] *Polymorphisms found for coding sequences for PmrA or PmrB.*

antibiotics, including clinically relevant ones. This reinforces the theory that possible transmission of *mcr-1* gene to highly virulent bacteria carrying other antimicrobial resistance genes, e.g., ESBL or carbapenemases would narrow clinical therapeutic options (Forde et al., 2018). Zheng et al. (2017) and Zheng et al. (2016) reported on *E. coli* isolates from blood stream infections which co-produce NDM-1 and MCR-1.

In our study *mcr-1* gene was detected in a wide range of plasmid types such as IncI1, IncHI2, IncX4, IncF, and IncI2, which is in consent with other reports. *mcr-1* located on IncI1 plasmids was detected in *E. coli* recovered from pig manure (Guenther et al., 2017) and chicken feces (Hassen et al., 2020). *E. coli* isolates recovered from pigs in Portugal carried *mcr-1* on IncHI2 and IncX4 plasmids (Kieffer et al., 2017). In another study, *mcr-1* gene was located on IncX4 and IncHI2 plasmids in *E. coli* from broilers and veal calves in Netherlands (Veldman et al., 2016). Furthermore, Gelbíčová et al. (2019) isolated *E. coli*, *K. pneumoniae,* and *Citrobacter*

TABLE 4 | PmrAB polymorphisms of colistin-resistant *K. pneumoniae* isolates tested negative for *mcr-1* to *mcr-9*.

Isolate	Species	Origin	Colistin MIC, mg/L	Resistance phenotype (epidemiological cut-off values of EUCAST)[a]	Resistance phenotype (clinical breakpoints of EUCAST)[b]	PmrAB[c]
			Poultry slaughterhouses			
04/05-15	*K. pneumoniae*	Influent in-house WWTP	32	AMP, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, CIP, LVX, CHL, SXT, FOF, CST	112T→P (PmrB)
04/70-34	*K. pneumoniae*	Stunning facilities	16	AMP, CHL, CST, CTX, SMX, CAZ, TET, TMP, FEP	CHL, SXT, FOF, CST	157T→P (PmrB)
C-04/02-07	*K. pneumoniae*	Transport crates	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, CST	
C-04/02-08	*K. pneumoniae*	Transport crates	>64	AMP, CIP, CST, NAL, SMX, CAZ, TET, TMP	PIP, SXT, CST	73P→x (PmrB) 74S→x (PmrB)
C-04/03-09	*K. pneumoniae*	Scalding water	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, CST	73P→x (PmrB) 74S→x (PmrB)
C-04/03-11	*K. pneumoniae*	Scalding water	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, FOF, CST	73P→x (PmrB) 74S→x (PmrB)
C-04/03-12	*K. pneumoniae*	Scalding water	32	AMP, CIP, CST, NAL, SMX, CAZ, TET, TGC, TMP, FEP, FOX	PIP, CIP, LVX, SXT, FOF, CST	73P→x (PmrB) 74S→x (PmrB)
C-04/03-13	*K. pneumoniae*	Scalding water	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, CST	73P→x (PmrB) 74S→x (PmrB)
C-04/05-01	*K. pneumoniae*	Influent in-house WWTP	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, CST	73P→x (PmrB) 74S→x (PmrB)
C-04/05-19	*K. pneumoniae*	Influent in-house WWTP	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, FOF, CST	73P→x (PmrB) 74S→x (PmrB)
C-04/07-29	*K. pneumoniae*	Stunning facilities	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, COL	73P→x (PmrB) 74S→x (PmrB)
C-04/07-02	*K. pneumoniae*	Stunning facilities	>64	AMP, CIP, CST, NAL, SMX	CST	
C-04/07-25	*K. pneumoniae*	Stunning facilities	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CST	73P→x (PmrB) 74S→x (PmrB)
C-04/07-28	*K. pneumoniae*	Stunning facilities	32	AMP, CIP, CST, CTX, NAL, TET, TGC, FEP, FOX	PIP, CST	73P→x (PmrB) 74S→x (PmrB)
			Pig slaughterhouses and mWWTPs			
C-03/08-02	*K. pneumoniae*	Scalding water	16	AMP, CST, SMX, CAZ, TET, FEP	PIP, TZP, C/T, CST	
C-03/10-18	*K. pneumoniae*	Influent municipal WWTP	16	AMP, CST	FOF, CST	217A→V (PmrA)
C-03/10-19	*K. pneumoniae*	Influent municipal WWTP	16	AMP, CST	FOF, CST	217A→V (PmrA)
C-03/10-21	*K. pneumoniae*	Influent municipal WWTP	32	AMP, CST	PIP, FOF, CST	2A→S (PmrB)
C-03/10-22	*K. pneumoniae*	Influent municipal WWTP	32	AMP, CST	CST	2A→S (PmrB)
C-03/12-01	*K. pneumoniae*	On-site preflooder downstream	8	AMP, CIP, CST, NAL, CAZ, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	"Insertion" of QLQQLARVG between 201E and 202Q
C-03/12-02	*K. pneumoniae*	On-site preflooder downstream	16	AMP, CIP, CST, NAL, SMX, CAZ, TET, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	
C-03/12-06	*K. pneumoniae*	On-site preflooder downstream	16	AMP, CHL, CIP, CST, NAL, SMX, CAZ, TET, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	
C-03/12-10	*K. pneumoniae*	On-site preflooder downstream	>64	AMP, CHL, CIP, CST, NAL	PIP, CTX, CAZ, CIP, LVX, CHL, CST	217A→V (PmrA)
C-05/10-15	*K. pneumoniae*	Influent municipal WWTP	16	AMP, CST	PIP, CST	147A→E (PmrA) 217A→V (PmrA)
C-05/10-16	*K. pneumoniae*	Influent municipal WWTP	16	AMP, CST	CST	147A→E (PmrA) 217A→V (PmrA)
C-05/10-26	*K. pneumoniae*	Influent municipal WWTP	16	AMP, CST	FOF, CST	37A→T (PmrA)

(Continued)

TABLE 4 | Continued

Isolate	Species	Origin	Colistin MIC, mg/L	Resistance phenotype (epidemiological cut-off values of EUCAST)[a]	Resistance phenotype (clinical breakpoints of EUCAST)[b]	PmrAB[c]
				Poultry slaughterhouses		
05/11-29	*K. pneumoniae*	Effluent municipal WWTP	32	AMP, CHL, CIP, CST, CTX, NAL, CAZ, FEP, ETP, FOX, IMI	PIP, TZP, CTX, CAZ, CIP, LVX, CHL, FOF, CST	57E→G (PmrA) 203S→P (PmrB)

[a] *Antimicrobial Susceptibility Testing Plates of German Federal Institute for Risk Assessment (BfR) containing sulfamethoxazole (SMX), trimethoprim (TMP), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), tigecycline (TGC), ertapenem (ETP), meropenem (MEM), imipenem (IMI), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), colistin (CST), ampicillin (AMP), gentamicin (GEN). MIC were interpreted according to the epidemiological cut off values of EUCAST.*
[b] *Micronaut-S MDR MRGN-Screening system containing temocillin (TEM), piperacillin (PIP), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMI), meropenem (MEM), amikacin (AMK), tigecycline (TGC), chloramphenicol (CHL), fosfomycin (FOF), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LVX), and colistin (CST). MIC were interpreted according to the clinical breakpoints of EUCAST.*
[c] *Polymorphisms found for coding sequences for PmrA or PmrB.*

braakii from raw turkey meat and liver which harbored *mcr-1* gene on IncX4, IncHI2, and IncI2 plasmids. In addition to livestock and food products, MCR-1-producing *E. coli* which carry the resistance on IncX4, IncHI2, and IncI1 types of plasmids, were isolated from different environmental sources such as surface water in Germany (Falgenhauer et al., 2019) and public seawater beach in Norway (Jørgensen et al., 2017). The association of *mcr-1* gene with insertion sequence IS*Apl1* might play a major role in its mobilization, its further successful establishment in BHR plasmids and subsequent dissemination among *Enterobacteriaceae* (Snesrud et al., 2016; Poirel et al., 2017). On the other hand, without colistin exposure, IS*Apl1* is able to facilitate the deletion of resistance genes, as described by Zhang et al. (2019) for *mcr-1* and *mcr-3.19*.

The co-transfer of the decreased susceptibility to fluoroquinolones (MIC of CIP 0.25 mg/L) by the majority of the isolates recovered in the poultry slaughterhouses could be due to plasmid-mediated quinolone resistance (PMQR) genes. They are known to provide only low-level resistance that by itself does not exceed the clinical breakpoint of >0.5 mg/L for susceptibility (Jacoby et al., 2014). Furthermore, resistance to tetracyclines was co-transferred by the isolates from pig slaughterhouses, as tetracycline resistance genes are often located on mobile genetic elements such as plasmids, transposons, conjugative transposons, and/or integrons (Roberts, 2003). Thus, fluoroquinolones and tetracyclines, which make up 25.7% of the total antimicrobial usage in the veterinary medicine in Germany (BVL, 2019), may impose a selective pressure that could favor the selection of *mcr* genes, even without use of colistin and vice versa. Moreover, Savin et al. (2020a) reported on antimicrobial residues of ampicillin, ciprofloxacin, and ofloxacin detected in German mWWTPs which exceeded their PNECs (Predicted No Effect Concentration) (Bengtsson-Palme and Larsson, 2016). Ofloxacin exceeded its PNEC even after dilution of the treated wastewater with the recipient water. This may contribute to the co-selection of *mcr-1* carrying bacteria in surface water, whereas the residues of ampicillin may promote the dissemination of *mcr*-carrying strains of species with intrinsic resistance to this antimicrobial (e.g., *Klebsiella* spp., *E. cloacae* complex).

The great majority of colistin-resistant *E. coli* and *K. pneumoniae* which were tested negative for known *mcr* genes harbored chromosomal point mutations in the *pmrAB* coding regions. For *E. coli*, a mutation at the amino acid position 10 in *pmrB* has been detected by Cannatelli et al. (2017) leading to the substitution 10Leu→Pro that confers resistance to colistin. However, in our study, the polymorphisms at this position resulted in leucine to arginine substitution. One *K. pneumoniae* isolate recovered from the wastewater used for cleaning of poultry stunning facilities demonstrated mutation 157T→P (PmrB) that has been previously reported in *K. pneumoniae* from patients and healthy humans (Olaitan et al., 2014) as well as in clinical colistin-resistant *K. pneumoniae* carbapenemase (KPC)-producing isolates (Leung et al., 2017). Furthermore, a substitution 217A→V (PmrA) that has been already described in colistin-resistant isolates from clinical blood cultures (Esposito et al., 2018) was found in isolates recovered from the influent of mWWTPs and on-site preflooders. To determine whether other detected polymorphisms in *E. coli* and *K. pneumoniae* cause resistance to colistin, complementation assays are needed.

We are not aware of other studies in Germany that investigated such environmental samples (i.e., process waters and wastewater) which have been taken directly in the slaughterhouses and their on-site WWTPs that underlines the novelty of our study. In conclusion, our results indicate high prevalence of *E. coli* isolates which carry *mcr-1* on a wide variety of transferable plasmids in process water accruing along the slaughtering process in German poultry slaughterhouses. This may pose an elevated risk of colonization for slaughterhouse employees with occupational exposure to process water and wastewater. Furthermore, despite strict hygiene rules established in German slaughterhouses, *mcr-1* carrying bacteria could be introduced into the food chain through cross-contamination (e.g., scalding water). Moreover, due to insufficient treatment of wastewater, such strains were discharged into the environment. In order to determine the persistence of *mcr-1* carrying *E. coli* isolates in the receiving water bodies, further investigations are needed. Furthermore, besides colistin, overall reduction of the use of antibiotics in livestock is required, as it was shown that *mcr-1* can be also co-selected by fluoroquinolones and tetracyclines. In this way, the input of resistant bacteria into the slaughterhouses can be reduced. Additionally, as *mcr-1* carrying isolates were detected in the effluent of the WWTPs, a broad dissemination

the environment can be expected. Thus, this study supports the necessity of the implementing of advanced wastewater treatment technologies to limit the exposition of the environment with bacteria expressing resistances against last resort antimicrobials.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MS: project administration, conceptualization, methodology, investigation, writing – original draft, and visualization. GB, KS, and RS: writing – review and editing. KB, CH, and JH: investigation, writing – review and editing. MP and ES: investigation. JK: conceptualization, writing – review and editing, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Complementarity of Selective Culture and qPCR for Colistin Resistance Screening in Fresh and Frozen Pig Cecum Samples

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Retrospective studies involving the screening of frozen stored collections of samples are commonplace when a new threat emerges, but it has been demonstrated that the freeze-thaw process can affect bacterial viability. The study of colistin-resistant bacteria in human and animal samples is an example of this issue. In this study, we compared culture-based and PCR-based methods for analyzing relative occurrence and diversity of colistin-resistant bacteria in caecal samples to determine the most appropriate method for frozen samples. Thus, 272 samples from the caecal contents of healthy pigs were tested before and after a 6-month freezing period. A selective medium was used when traditional isolation of colistin-resistant bacteria was tested, while a real-time SYBR® Green I PCR assay was applied for *mcr-1* quantification. The number of samples with colistin-resistant isolates was higher in fresh samples (247/272) than in frozen ones (67/272) and showed a higher diversity of colistin-resistant genera. PCR identification of *mcr* colistin resistance genes evidenced that *mcr-1* was the most prevalent *mcr* gene and *mcr-2* was detected for the first time in pigs from Spanish animal production. The number of samples with *mcr-1*-carrying bacteria after a freezing period decreased, while real-time quantitation of the *mcr-1* gene showed similar values in frozen and fresh samples. Therefore, when frozen cecal samples need to be analyzed, molecular detection of DNA could be the best option to provide a highly representative frame of the initial population present in the sample, and culture-based methods might be a useful complement to study colistin resistance levels.

Keywords: freeze-thaw process, caecal samples, *mcr-1*, *mcr-2*, swine, antimicrobial resistance

INTRODUCTION

Retrospective studies involving the screening of stored frozen collections of isolates or, less frequently, biological samples are commonplace when a new microbiological threat emerges, and their aim is to test prior occurrences and features of the agent. One ongoing example is colistin-resistant bacteria in humans and animals. These isolates and samples usually belong to collections obtained in the past that have been kept frozen during a period of time. Temperature and time of storage are the most critical factors when a microbiological study is carried out with frozen material. In addition, the freeze-thaw process could be an important issue for bacterial recovery since it could dramatically affect bacterial survival because of the physical changes in the frozen material, assuming that at least a 74% decrease in cell viability may occur (Phalakornkule et al., 2017). This is of paramount importance when the aim is testing bacterial diversity of frozen samples, as previous studies have demonstrated that the freeze-thaw process can decrease bacterial diversity in the samples (Phalakornkule et al., 2017; Dorsaz et al., 2020). Therefore, the use of traditional methods, such as solid or liquid culture, could be challenging and may not provide representative data on the amount and diversity of bacteria in these samples prior to being frozen. On the other hand, DNA remains more stable after the freeze-thaw process as shown in different microbiome studies using Next Generation Sequencing (Bassis et al., 2017; Dorsaz et al., 2020). Similarly, the bacterial composition of stools was not dramatically affected after a freezing process using molecular methods, although this has been also associated with freezing conditions (Shao et al., 2012).

There are not many published studies that evaluate the effect of the freeze-thaw process comparing culture-based methods and molecular techniques as real-time PCR. Our work aims to evaluate how this process could affect the detection of colistin-resistant *Enterobacteriaceae* in frozen samples. Since the first description of a plasmid-mediated colistin resistance determinant in 2015 (*mcr-1*), up to ten *mcr*-related genes have been described. However, *mcr-1* still remains as the most common colistin resistance determinant carried by plasmids since it is present worldwide, especially in *Escherichia coli* of human and animal origin (Khedher et al., 2020; Wang et al., 2020); therefore, in this study we took *mcr-1* as an indicator of acquired colistin resistance. Many retrospective studies screened colistin resistance (both bacteria and genes) over bacterial strain collections (Carattoli et al., 2017; Garcia et al., 2018; Migura-Garcia et al., 2019), but they were rarely performed on biological samples (Miguela-Villoldo et al., 2019).

To provide more accurate insights on colistin resistance emergence and evolution, an analysis of samples from past years is required. For this reason, we tested and compared two different protocols, commonly used in routine bacterial screening to detect antimicrobial-resistant bacteria (Alba et al., 2018): selective culture (using a specific method to isolate colistin-resistant *Enterobaceriaceae*) and quantitative real-time PCR (targeting the *mcr-1* gene), in a collection of cecal samples from pigs. We aimed to determine the most appropriate method for testing these samples after the freezing period. In addition, we assessed the effect of the freeze-thaw process on the bacterial diversity of colistin-resistant bacteria in fresh and frozen samples using a selective culture medium.

MATERIALS AND METHODS

Sampling

A total of 272 isolates from cecal contents of healthy pigs were sampled at slaughterhouses in different Spanish regions from September to December 2018. Feces were taken from the cecum, transported on ice (+4°C) and analyzed within 24 h by culture-based and molecular methods. Then, samples were diluted (1:10) in buffered peptone water and stored with glycerol at −40°C. Each sample was kept frozen for 6 months and analyzed again by the same methods.

Culture-Based Methods

Samples were analyzed as described in Miguela-Villoldo et al. (2019) using ChromID Colistin R agar (ColR) (bioMérieux, France). Briefly, ColR plates were inoculated with 50 μL of a 500-fold BHI diluted sample with colistin (10 μg disk in BHI broth) and were incubated at 37^{o}C for 24 h. Three different-colored morphologies (pink-burgundy, green and white-colorless) could grow in ColR plates according to manufacturers' specifications. After incubation, one colony per morphology obtained in the ColR plate was subcultured in blood agar at 37^{o}C for 24 h. The isolates obtained were identified by mass spectrometry using a Bruker Daltonics UltrafleXtrem MALDI TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). Poorly identified isolates (score <2.3) were further analyzed by using the API® 20-E *Enterobacteriaceae* identification kit (bioMérieux) and the specific PCR (Cabal et al., 2013) for pink-burgundy colonies to confirm *E. coli* isolates (associated to this morphology). After identification, molecular characterization of the isolates was carried out by a conventional PCR to analyze the presence of colistin resistance *mcr* genes (*mcr-1* to 5) (Rebelo et al., 2018). As described in literature, *mcr-6* seems to be an endogenous gene from *Moraxella* spp., *mcr-7* was only described in *Klebsiella* spp., *mcr-8* was identified in *Klebsiella pneumoniae* and *Raoutella ornithinolitica* and *mcr-9* and *mcr-10* do not seem to confer resistance to colistin (Khedher et al., 2020; Wang et al., 2020). Therefore, we focused on genes *mcr-1* to 5 since they have been described in different bacterial genera and have not been linked to a particular bacterial group.

In this study, we differentiate two types of isolates depending on the nature of their resistance to colistin: the ACRB group that includes bacterial genera naturally sensitive to colistin that have acquired resistance mechanisms, and NCRB which is composed of those genera that have intrinsic resistance to colistin (Olaitan et al., 2014; Lepelletier et al., 2018).

Colistin MIC Determination From *mcr*-Carrying Bacteria

Colistin susceptibility testing was performed by the two-fold broth microdilution reference method according to ISO 20776–1:2019 (ISO 20776-1:2019, 2019) using Sensititre EUVSEC plates

(Trek Diagnostic Systems, United States) having a colistin range from 1 to 16 mg/L. Colistin resistant isolates where those having a minimal inhibitory concentrations (MIC) higher than 2 mg/mL (Spellerberg and Fedor, 2003).

DNA Extraction and Quantitative PCR

Direct DNA extraction from pig cecal samples was carried out using a commercial kit (FASTI001-1 FavorPrep Stool DNA Isolation Mini Kit, Favorgen-Europe, Vienna) and was coupled to a specific SYBR® Green I (Thermo Fisher Scientific, Vilnius) real-time PCR assay for quantitative detection of the *mcr-1* gene (qPCR) described previously by Li et al. (2017). PCRs were run on a thermal cycler CFX 96 (Bio-Rad). The specificity of the primers has been confirmed by melting curve analysis. Four μL of each DNA elute were run in triplicate. Samples were considered positive when quantitative values were higher than 1.00×10^2 per reaction (equivalent to 1.58×10^5 copies/100 mg cecal content).

Aiming to test the qPCR protocol, we conduct a recovery assay using spiked samples. We used a pool of 10 g of feces sampled from pigs that had never been treated with antimicrobials, which were confirmed as negative to *mcr-1* genes by qPCR. The sample was diluted 1:10 in saline water (0.085% NaCl) to a 100 mL final volume and then divided into four aliquots of 20 mL (named A1, A2, A3, and A4). Three of them were spiked using a *mcr-1* positive strain of *E. coli* at three different concentrations (A1: 10^6 CFU/mL; A2: 10^5 CFU/mL; A3: 10^4 CFU/mL), calculated from a 0.5 McFarland suspension (plate count of 5.3×10^7 CFU/mL), being the last (A4), used as negative control. Direct DNA extraction from all the samples was coupled with the *mcr-1* specific real-time PCR assay for quantitative detection of the *mcr-1* gene included in our protocol.

In addition, another quantitative PCR was performed to detect and quantify the amount of *E. coli* present in the sample using a published PCR that used as a target *uidA* gene (Cabal et al., 2013).

Statistical Analysis

The Shannon-Weaver index (H′) was calculated using results obtained after traditional culture to estimate genera diversity per sample (Spellerberg and Fedor, 2003):

$$H' = -\sum_{i=1}^{s} p_i ln\ p_i$$

Where H′ is the biodiversity index, i is the genera and $p_i = n_i/N$: where n_i is the total number of organisms of a particular genus and N is the total number of organisms of all genera. We used 95% confidence intervals (95% CI) to compare both fresh and frozen sample diversity indexes.

Statistical methods were used to analyze the data obtained by qPCR and the culture method; Fisher's exact test was applied to compare the proportion of samples where *mcr-1*-carrying bacteria were obtained using a traditional culture method. In this case, at least one positive *mcr-1* isolate in the sample was needed to consider it positive, both in fresh and frozen samples. It was assumed that the freeze-thaw process had a significant effect on our results if p-value was less than 0.05. T-Test for two related samples was applied to analyze qPCR results. Data was previously normalized transforming it into Log_{10}. A difference was considered significant when p-value was less than 0.05.

RESULTS

Culture-Based Methods

Detection of Colistin-Resistant Bacteria

Colistin-resistant isolates were detected in 91% of fresh samples (247/272) and 24% of frozen-thawed samples (67/272) (p-value < 0.001). ACRB isolates were detected in 222/272 of fresh samples, (82%) and 60/272 of frozen-thawed samples (22%) (p-value < 0.001), whereas NCRB isolates were obtained from 140/272 of fresh samples (51%) and 12/272 of frozen-thawed samples (4%) (p-value < 0.001).

Regarding fresh samples, ACRB isolated genera were *Aeromonas*, *Citrobacter*, *Enterobacter*, *Escherichia, Klebsiella* and *Raoutella*. On the other hand, NCRB genera were *Morganella*, *Myroides*, *Proteus*, *Providencia*, and *Vibrio.* The diversity of genera found in fresh samples was estimated using Shannon-Weaver Index, obtaining a value of 1.496 (95% CI = [1.393, 1.599]). In these samples, *Escherichia* spp. was the most frequent genus identified (50% of fresh samples with bacterial growth had at least one *Escherichia* spp. isolate) (**Table 1**).

ACRB obtained in frozen samples included genera such us *Citrobacter* spp., *Enterobacter* spp., *Escherichia* spp., *Klebsiella* spp., and *Raoutella* spp. Meanwhile, NCRB were represented by *Morganella* spp., *Proteus* spp. and *Providencia* spp. Shannon-Weaver Index, taking into account the genera observed, obtaining a value of 1.064 (95%CI = [0.782, 1.34]). In this case, *Escherichia* spp. was also the most frequent bacterial genus (72% of frozen samples with bacterial growth had at least one *Escherichia* spp. isolate) (**Table 1**).

TABLE 1 | Bacterial genera recovered by the culture method from fresh and frozen samples.

	Fresh Samples (n = 272)	Frozen Samples (n = 272)
Bacteria genera	*N*	*N*
ARCB group		
Escherichia spp.	212	56
Klebsiella spp.	20	2
Citrobacter spp.	9	1
Raoutella spp.	7	2
Enterobacter spp.	6	2
Aeromonas spp.	3	0
NCRB		
Providencia spp.	84	7
Proteus spp.	61	7
Morganella spp.	17	1
Myroides spp.	1	0
Vibrio spp.	1	0

Up to three different genera could be grown in the same sample, thus, the table represents the number of samples where each genus has been detected. N: number of samples with bacterial growth. n: number of studied samples.

The minimum overlapping between confidence intervals of Shannon-Weaver indexes from both fresh samples ([1.393, 1.599]) and frozen samples ([0.782, 1.346]) showed that diversity decreased in samples after the freezing process.

Interestingly, our results showed that the freeze-thaw process affected ACRB and NCRB differently. While NCRB experimented a 92% decrease in the number of isolates obtained, ACRB only decreased by 77%. Finally, the freeze-thaw process decreased viability to 84% (from 486 to 78 in fresh and frozen samples, respectively).

Molecular Detection of *mcr* Genes in Isolates Recovered by the Culture Method

From a total of 272 cecal samples, *mcr* genes were identified in 77% (209/272) and 21% (57/272) of fresh and frozen ones, respectively. Similarly, while the *mcr-1* gene was detected in 51.4% (250/486) of the isolates obtained from fresh samples, *mcr-4* was identified in four, one of them in co-occurrence with *mcr-1* (*E. coli*), and *mcr-2* in one *E. coli* isolate. The proportion of *mcr*-carrying isolates in the ACRB group was 86.4 and 92.1% for fresh and frozen samples, respectively. Finally, no *mcr* gene was found in 40 putative ACRB isolates; colistin resistance in those cases may thus be due to other mechanism(s). The remaining 192 isolates lacking *mcr-1-5* genes were NCRB.

However, *mcr-1* was detected in 73.07% (57/78) of the isolates from frozen samples (including all genera detected), while *mcr-4* was identified in one *E. coli*, all of them being ACRB. The remaining 21 isolates were PCR-negative for the *mcr* genes tested (6 ACRB and 15 NCRB) (**Table 2**).

Colistin Susceptibility of *mcr*-Carrying Isolates

Colistin MIC values were checked in the 312 *mcr*-carrying isolates from both fresh and frozen samples (**Table 3**). From a total of 254 *mcr*-carrying ACRB isolates obtained from fresh samples, 168 (66.2%) had a MIC value of 4 μg/mL, 79 isolates (31.0%) 8 μg/mL, two isolates 16 μg/mL and two more a MIC higher than 16 μg/mL. Three *mcr-1* isolates showed a MIC of 2 μg/mL.

Regarding frozen samples, from a total of 58 *mcr*-carrying ACRB isolates, 28 (48.3%) showed a MIC of 4 μg/mL, 29 (50%) 8 μg/mL and one isolate more than 16 μg/mL.

Detection of *mcr-1* Isolates in Cecal Samples

As summary, from the 272 fresh samples tested, 208 (76%) showed a growth of *mcr*-1 positive bacteria, all of them being ACRB. On the other hand, 56 frozen samples (20%) showed growth of *mcr*-1 positive bacteria, all being ACRB as well. The total number of isolates testing positive to the *mcr*-1 gene, obtained in both fresh and frozen samples, can be seen in **Table 2**, taking into account that in each sample up to three different colony morphologies could be detected. Fisher's exact test provided a *p*-value of 0.0001 comparing results of *mcr*-1 positive samples in fresh and frozen samples, the difference observed being statistically significant.

Quantification of *mcr-1* by Real-Time PCR

Regarding the recovery assay, qPCR reaction was carried out with 91.27% efficiency, r^2 value of 0.999 and 85°C melt

TABLE 2 | Molecular detection of *mcr* genes (*mcr-1* to *5*) by conventional PCR.

	Fresh samples (272 samples, 486 isolates)						Frozen samples (272 samples, 78 isolates)					
	mcr-1	***mcr-2***	***mcr-4***	***mcr-1/mcr-4***	**No *mcr***	**Total**	***mcr-1***	***mcr-2***	***mcr-4***	***mcr-1/mcr-4***	**No *mcr***	**Total**
ACRB group												
Escherichia spp.	236	1	1	1	10	249	54	0	1	0	1	56
Klebsiella spp.	7	0	1	0	12	20	2	0	0	0	0	2
Citrobacter spp.	5	0	1	0	3	9	1	0	0	0	0	1
Raoutella spp.	1	0	0	0	6	7	0	0	0	0	2	2
Enterobacter spp.	0	0	0	0	6	6	0	0	0	0	2	2
Aeromonas spp.	0	0	0	0	3	3	NA	NA	NA	NA	NA	0
Total	249	1	3	1	40	294	57	0	1	0	5	63
ACRB mcr%	85.70	0.34	1.02	0.34	13.60		90.50		1.59		7.94	
mcr-carrying ACRB proportion						86.40%						92.10%
NCRB group												
Providencia spp.	0	0	0	0	105	105	0	0	0	0	7	7
Proteus spp.	0	0	0	0	68	68	0	0	0	0	7	7
Morganella spp.	0	0	0	0	17	17	0	0	0	0	1	1
Myroides spp.	0	0	0	0	1	1	NA	NA	NA	NA	NA	0
Vibrio spp.	0	0	0	0	1	1	NA	NA	NA	NA	NA	0
Total	0	0	0	0	192	192	0	0	0	0	15	15
NCRB *mcr%*					100						100	
Total *mcr%*	51.2	0.21	0.62	0.21	47.74		73.08		1.28		25.64	

All isolates obtained from fresh and frozen samples are represented in this table, independently of the total number of samples. NA, "Not Applicable" values correspond to genera that have not been found in the sample, so no analysis could be applied. ACRB, Acquired colistin-resistance bacteria. NCRB, Natural colistin-resistance bacteria.

TABLE 3 | Distribution of colistin MIC values of *mcr*-carrying isolates obtained from both fresh and frozen samples.

		Number of isolates						
		Dilution test range: 1 – 16 μg/ml colistin MICs (μg/ml)						
	mcr pattern	≤1	2	4	8	16	>16	Total
Fresh samples	*mcr-1*	0	3	168	75	2	1	248
	mcr-2	0	0	0	1	0	0	1
	mcr-4	0	0	0	2	0	1	3
	mcr-1/mcr-4	0	0	0	1	0	0	1
	Total	0	3	168	79	2	2	254
Frozen samples	*mcr-1*	0	0	28	28	0	1	57
	mcr-2	0	0	0	0	0	0	0
	mcr-4	0	0	0	1	0	0	1
	mcr-1/mcr-4	0	0	0	0	0	0	0
	Total	0	0	28	29	0	1	58

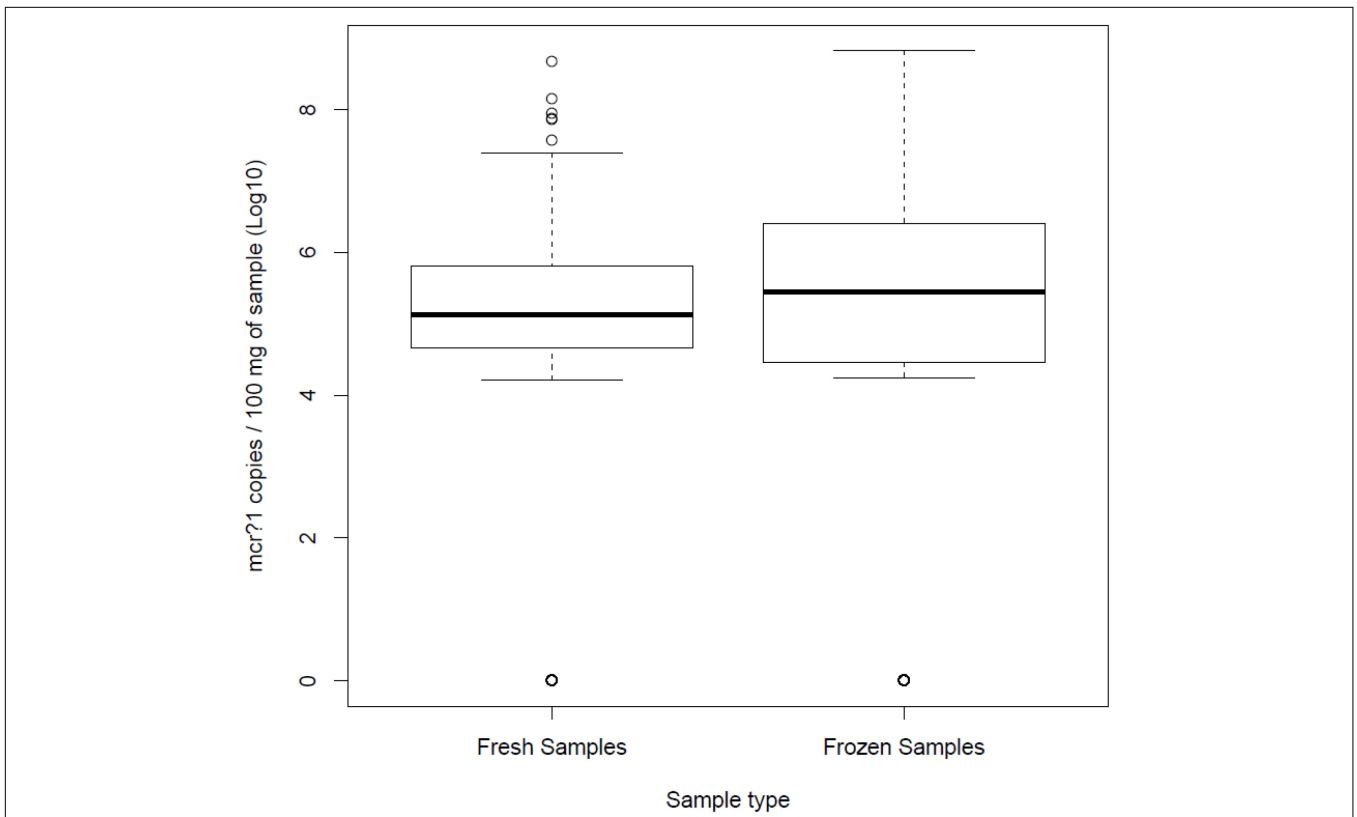

FIGURE 1 | Quantitative real-time PCR results for quantitation of the *mcr-1* gene in both fresh and frozen samples.

temperature. Cycle thresholds (cts) of the standard curve were 18.08 (sd = 0.113), 21.77 (sd = 0.174), 25.52 (sd = 0.135), 29.37 (sd = 0.266), and 32.98 (sd = 0.532) for standard 1 to 5, respectively. The results of the *mcr-1* qPCR were as follows: 7.22×10^9 copies/g, 8.08×10^8 copies/g, 9.22×10^7 copies/g and $< 10^1$ copies/g from A1, A2, A3, and A4, respectively, and a relative accuracy of 100.01%, 99.97% and 100.02% for A1, A2, and A3, respectively. These results agreed to those expected, taking into account that this test corresponds to a total *mcr-1* quantification.

The results of the *uidA* quantitative real-Time PCR were as follows: 1.60×10^{10} *uidA* copies/g, 6.43×10^9 *uidA* copies/g, 6.73×10^9 *uidA* copies/g, 3.96×10^9 *uidA* copies/g from A1, A2, A3, and A4, respectively.

From a total of 272 samples per group, the *mcr-1* gene was quantified in 128 fresh (47%) and 142 frozen samples (52%), with mean values of 4.75 Log_{10} copies/100 mg feces and 4.66 Log_{10} copies/100 mg feces, respectively (**Figure 1**), differences that were not statistically significant ($p = 0.508$, *T*-test).

Culture and PCR in Frozen Samples

From a total of 272 frozen samples analyzed, equivalent results were obtained from 176 samples (51 samples were positive, 125 were negative), whereas 96 showed discrepancies, mainly due to 91 samples that were quantified by qPCR but not detected by culture methods and only five samples were qPCR negative being positive by culture.

DISCUSSION

The freezing process could have a negative effect on the viability of bacteria present in cecal samples, which may affect retrospective studies based on the detection of bacteria using culture methods. In this study, we use freezing in glycerol to sample storage, a process involving physical and chemical changes that could affect their biological composition (Phalakornkule et al., 2017). Thawing-freezing is critical for bacterial viability and for decreasing their recoverability. The effect of this process must be considered in the study design, data analysis and interpretation of the results (Phalakornkule et al., 2017). In our study, fecal samples showed a higher diversity of colistin-resistant bacteria when tested prior to freezing, and a significant decrease was confirmed in both occurrence and diversity after freezing. Besides, the number of samples with colistin-resistant isolates was higher in fresh (247/272) than in frozen samples (67/272), according to the expected bacterial viability level after the freeze-thaw process previously described (Phalakornkule et al., 2017). Regarding both ACRB and NCRB groups, we noticed that the freeze-thaw process affected these two groups differently, since NCRB (192 in fresh samples vs. 15 in frozen samples) showed a higher decrease in the number of isolates obtained than ACRB (294 in fresh samples vs. 63 in frozen samples), although further analyses are needed to determine the cause. Our results showed that the freeze-thaw process drastically reduced bacterial viability, affecting some bacterial genera differently and altering the microbiological diversity of stored samples.

Regarding molecular detection of plasmid-mediated *mcr* genes, our results evidenced that *mcr-1* was the most prevalent colistin-resistance gene in our sample collection among the *mcr-1* to *mcr-5* group of determinants, in accordance with previous studies (Carattoli et al., 2017; Poirel et al., 2018; Migura-Garcia et al., 2020). We also identified *mcr-2* and *mcr-4* genes. To our knowledge, we report the first description of *mcr-2* in healthy pigs for food production in Spain and the second in Europe after its discovery in Belgium in 2016 (Xavier et al., 2016). This gene was identified in one *E. coli* (serotype O83:H42) belonging to ST-648 and showing 100% similarity to *mcr-2.1* described previously (Xavier et al., 2016). Most of these *mcr*-carrying isolates showed a colistin MIC value higher than 2, being 4 and 8 μg/mL the two most frequent MICs detected, as described in other studies (Xavier et al., 2016; Carattoli et al., 2017; Poirel et al., 2017; Lepelletier et al., 2018).

In this study, we compared both molecular and culture-based methods, focusing on those samples showing growth of ACRB carrying *mcr-1*, the main colistin-resistance determinant that has been quantified by SYBR® Green qPCR. The amount of *mcr-1* gene detected was similar between frozen and fresh samples, suggesting that the freeze-thaw process did not significantly modify the availability of functional DNA for the qPCR, in line with other studies that compared PCR results before and during different freezing periods (Bassis et al., 2017; Dorsaz et al., 2020). However, PCR may overestimate the content in viable cells showing colistin resistance of cecal samples, since dead cells could also be detected (Dorsaz et al., 2020).

Regarding samples with *mcr-1* positive isolates, freezing significantly decreases bacterial viability, while *mcr-1* DNA remained stable as detected by qPCR (**Figure 1**). This suggests that the use of culture-based methods for testing frozen samples might underestimate the occurrence of plasmidic colistin-resistance determinants in cecal samples, as it could be observed when comparing qPCR data with culture data. However, these methods provided information about bacterial diversity of biological samples which could not be achieved by using qPCR. Therefore, although culture and molecular methods differ in properties to detect colistin resistance, and mainly the specificity to detect viable cells or the sensitivity to quantify functional DNA molecules, both methods are complementary techniques used to characterize biological samples depending on the objectives of the study; taking into account that, while bacterial viability decreases as a result of the freezing, the DNA concentration remained stable.

CONCLUSION

Data obtained from selective culture showed that both bacterial viability and diversity of colistin-resistant genera were reduced after the freeze-thaw process, involving the loss of members of the bacterial population that was initially present in the sample. In contrast, *mcr-1* detection by qPCR directly on fresh or frozen samples produced similar results. Thus, detection of DNA by qPCR when frozen samples need to be analyzed would be the option of choice to provide a highly representative frame of the spectrum of resistance determinants initially present in the sample, and culture-based methods would be the best complement to detect the carriage of viable and colistin-resistant cells.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because no animal was manipulated in this study, only samples of cecal content taken at slaughterhouse.

AUTHOR CONTRIBUTIONS

PM-V, MM, LD, and MU-R: conceptualization, investigation, resources, data curation, writing – original draft preparation, project administration, and funding acquisition. PM-V, MH, and DR-L: methodology. MH, DR-L, and AQ: software and validation. PM-V, MH, MM, DR-L, AQ, LD, and MU-R: formal analysis, writing – review and editing, visualization, and supervision. All authors contributed to the article and approved the submitted version.

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A *Burkholderia thailandensis* DedA Family Membrane Protein Is Required for Proton Motive Force Dependent Lipid A Modification

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The DedA family is a conserved membrane protein family found in most organisms. A *Burkholderia thailandensis* DedA family protein, named DbcA, is required for high-level colistin (polymyxin E) resistance, but the mechanism awaits elucidation. Modification of lipopolysaccharide lipid A with the cationic sugar aminoarabinose (Ara4N) is required for colistin resistance and is dependent upon protonmotive force (PMF) dependent transporters. *B. thailandensis* Δ*dbcA* lipid A contains only small amounts of Ara4N, likely leading to colistin sensitivity. Two *B. thailandensis* operons are required for lipid A modification with Ara4N, one needed for biosynthesis of undecaprenyl-P-Ara4N and one for transport of the lipid linked sugar and subsequent lipid A modification. Here, we directed overexpression of each *arn* operon by genomic insertion of inducible promoters. We found that overexpression of *arn* operons in Δ*dbcA* can partially, but not completely, restore Ara4N modification of lipid A and colistin resistance. Artificially increasing the PMF by lowering the pH of the growth media also increased membrane potential, amounts of Ara4N, and colistin resistance of Δ*dbcA*. In addition, the products of *arn* operons are essential for acid tolerance, suggesting a physiological function of Ara4N modification. Finally, we show that Δ*dbcA* is sensitive to bacitracin and expression of a *B. thailandensis* UppP/BacA homolog (*BTH_I1512)* can partially restore resistance to bacitracin. Expression of a different UppP/BacA homolog (*BTH_I2750)* can partially restore colistin resistance, without changing the lipid A profile. This work suggests that maintaining optimal membrane potential at slightly alkaline pH media by DbcA is responsible for proper modification of lipid A by Ara4N and provides evidence of lipid A modification-dependent and -independent mechanisms of colistin resistance in *B. thailandensis.*

Keywords: proton motive force, membrane protein, lipid A modification, lipopolysaccharide, antibiotic resistance, colistin

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INTRODUCTION

Colistin (polymyxin E) is a polycationic and amphiphilic peptide which is a last resort antibiotic for treatment of many Gram-negative bacterial infections. Discovered in 1947 (Ainsworth et al., 1947), and isolated in 1949 from *Bacillus polymyxa* broth (Koyama, 1950), colistin was not widely used due to nephrotoxicity and neurotoxicity after intravenous administration

(Koch-Weser et al., 1970). However, clinical use of colistin is increasing due to limited alternative therapeutic options (Li et al., 2006). With increased colistin use, the emergence of colistin resistance has been documented in several human pathogens, such as *K. pneumoniae* (Bratu et al., 2005; Antoniadou et al., 2007; Farzana et al., 2020), *Salmonella enterica* serovar Newport (Elbediwi et al., 2020), and *Acinetobacter baumannii* (Marano et al., 2020; Papathanakos et al., 2020). More worrisome is the recent global spread of mobilized colistin resistance (*mcr*) genes (Elbediwi et al., 2019). Therefore, understanding the molecular mechanism of colistin resistance is of utmost importance at times when the antibiotic discovery pipeline is drying up.

Species belonging to the genus *Burkholderia* are intrinsically colistin resistant with minimal inhibitory concentrations (MIC) often exceeding 500 μg/ml (Loutet and Valvano, 2011). The molecular mechanisms behind this intrinsic colistin resistance of *Burkholderia* spp. is not yet fully understood. Electrostatic interaction of colistin with negatively charged lipopolysaccharide (LPS) lipid A in the outer membrane of Gram-negative bacteria and disruption of outer membrane is understood as the initial mode of action (Hancock and Chapple, 1999; Velkov et al., 2010). A common mechanism of colistin resistance among many Gram-negative bacteria is expression of biosynthetic pathways that result in the modification of LPS lipid A with cationic substituents aminoarabinose (Ara4N) or phosphoethanolamine (Raetz et al., 2007; Needham and Trent, 2013; Olaitan et al., 2014; Simpson and Trent, 2019). This reduces the electrostatic attraction between colistin and lipid A and thus results in colistin resistance.

The modification of lipid A with Ara4N begins with the enzymatic conversion of UDP-glucose to UDP-glucuronic acid in the cytoplasm by UDP-Glc dehydrogenase (*ugd*) (Breazeale et al., 2002). Then, several proteins ArnA, ArnB, ArnC, and ArnD convert UDP-glucuronic acid to undecaprenyl-P-Ara4N on the cytoplasmic leaflet of the inner membrane (Noland et al., 2002; Breazeale et al., 2003, 2005; Williams et al., 2005). Putative EmrE like transporters, ArnE and ArnF, flip undecaprenyl-P-Ara4N from the cytosolic leaflet to the periplasmic leaflet of the inner membrane (Yan et al., 2007) where another membrane protein ArnT transfers Ara4N to the lipid A (Trent et al., 2001). This is followed by the transport to the outer membrane by lipopolysaccharide transport pathway (Lpt) (Li et al., 2019; Owens et al., 2019). Previously, we found that a DedA family membrane protein, DbcA (DedA of *Burkholderia* required for colistin resistance) is required for lipid A modification with Ara4N and high level colistin resistance in *Burkholderia thailandensis* (Panta et al., 2019). The molecular mechanism behind this is unclear.

The DedA family is a highly conserved membrane protein family present within all three domains of life (Doerrler et al., 2013). There are currently 25,081 individual sequences in the protein database across 7172 species belonging to the "SNARE-associated PF09335" family of proteins (PFAM 33.1). An *E. coli* DedA family mutant (Δ*yqjA*, Δ*yghB*) was found to have defects in membrane potential and cell division, induction of extracytoplasmic stress responses, sensitivity to alkaline pH, temperature sensitivity, and sensitivity to several classes of antibiotics and membrane penetrating dyes (Thompkins et al., 2008; Sikdar and Doerrler, 2010; Sikdar et al., 2013; Kumar and Doerrler, 2014, 2015). Recently, a human DedA protein, TMEM41B, has been characterized as a novel ER-localized regulator of autophagosome formation and lipid mobilization (Moretti et al., 2018; Morita et al., 2018; Shoemaker et al., 2019).

B. thailandensis DbcA has been shown to be required for extreme colistin resistance, probably due to compromised proton motive force (PMF) (Panta et al., 2019). We proposed a model which suggests that the lower membrane potential observed in Δ*dbcA* is responsible for inefficient export of undecaprenyl-P-Ara4N by EmrE-like transporter BTH_I2194 (ArnEF homolog). We reasoned that overexpression of the *arn* genes in Δ*dbcA* could compensate colistin hypersensitivity of Δ*dbcA*. Based on Prokaryotic Operon Database (ProOpDB), putative *arn* genes in *B. thailandensis* are organized in two transcriptional units (**Figure 1A**), consistent with the finding in *Burkholderia cenocepacia* (Ortega et al., 2007; Taboada et al., 2012). Lipid A modification with Ara4N is essential for bacterial viability, LPS export, proper assembly of outer membrane, and intrinsic resistance to polymyxin B in *B. cenocepacia* (Ortega et al., 2007; Hamad et al., 2012). Absence of transposon mutants of aminoarabinose synthesis and transport genes suggest that *arn* genes are also essential in *B. thailandensis* (Gallagher et al., 2013). We therefore used a conditional mutagenesis technique where an inducible rhamnose promoter is inserted into the chromosome to drive the expression of targeted genes (Ortega et al., 2007). This enabled us to directly control the expression of *arn* gene clusters and study the role of lipid A modification in colistin resistance in both WT and Δ*dbcA* strains. We show evidence for a link between pH and membrane potential and how lipid A modification and colistin resistance in *B. thailandensis* is linked to pH homeostasis.

MATERIALS AND METHODS

Culture Conditions

The bacterial strains and plasmids used in this study are listed in **Supplementary Table S1**. *E. coli* and *B. thailandensis* were grown in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) unless otherwise indicated. Colistin (Col), trimethoprim (Tmp), rhamnose (rha), 2,6 diaminopimelic acid (DAP), bacitracin (Bac) were purchased from VWR or MilliporeSigma. Cultures were grown at 37°C shaking at 225 rpm.

E264B, E264T, Δ*dbcA*B, and Δ*dbcA*T strains were inoculated in 5 ml LB, 100 Tmp, and 0.1% rha and grown for ∼24 h. One milliliter culture was removed, washed with LB, and resuspended in fresh LB. 2.5×10^7 cells from all the strains were then inoculated in 5 ml fresh LB, 100 Tmp without any rhamnose and grown for 24 h. One milliliter of this O/N cultures without any rhamnose were again removed, washed with LB, and resuspended in fresh LB. Then 2.5×10^7 cells were inoculated in 50 ml LB, 100 Tmp with different concentrations of rhamnose and grown

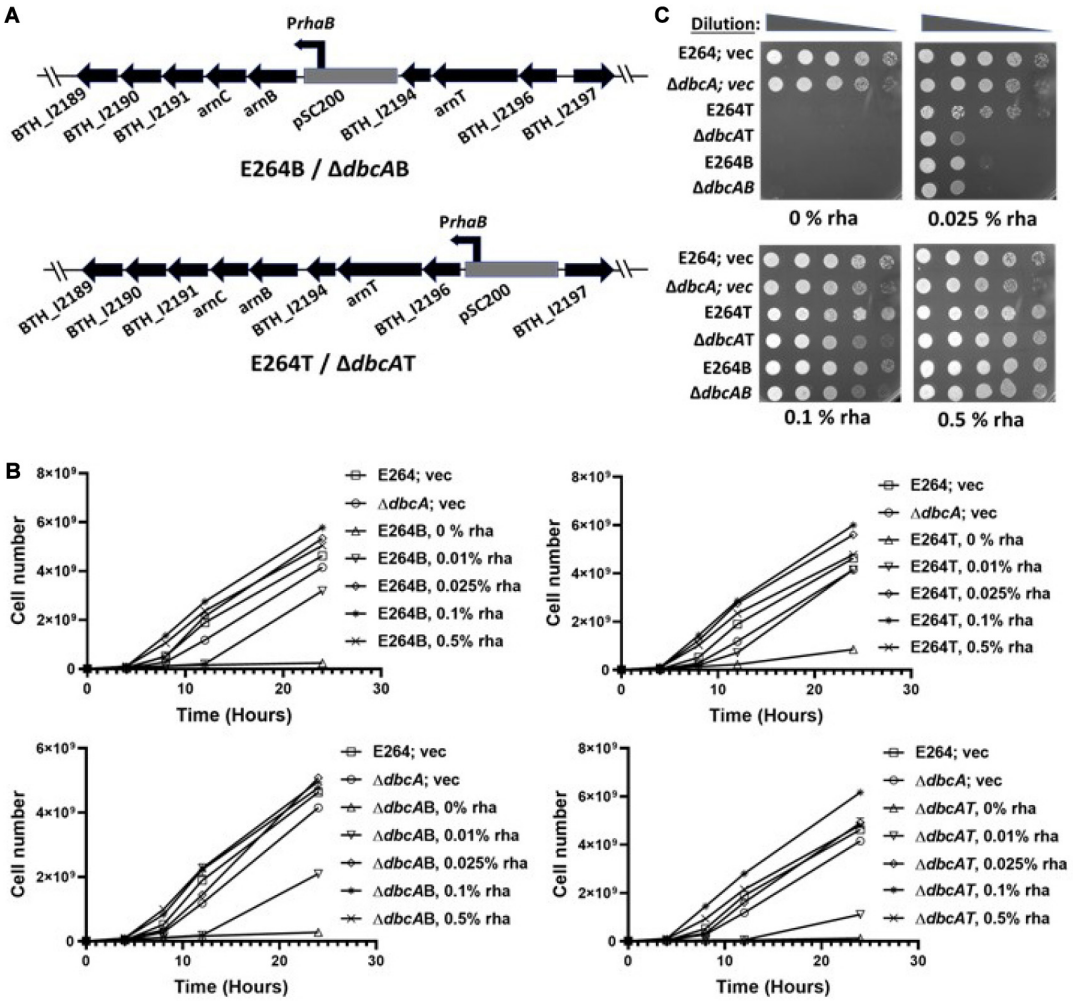

FIGURE 1 | Generation and characterization of Ara4N synthesis and transport conditional mutants. **(A)** Genetic organization of *arn* gene clusters and flanking regions of *B. thailandensis* E264. The gray box represents the plasmid, pSC200 with an inducible rhamnose promoter *(PrhaB)*. E264B/Δ*dbcA*B (B for biosynthesis) represents a strain of E264 or Δ*dbcA*:FRT with pSC200 insertion in front of the putative *arn* biosynthesis transcriptional unit. E264T/Δ*dbcA*T (T for transport) represents a strain of E264 or Δ*dbcA*:FRT with pSC200 insertion in front of the putative *arn* transport transcriptional unit. **(B)** Growth rate of E264; vec, Δ*dbcA*; vec, E264B, E264T, Δ*dbcA*B, and Δ*dbcA*T at times 0, 4, 8, 12, and 24 h in LB liquid media containing the indicated concentrations of rhamnose. The graph was created through GraphPad Prism 8.4.3. The error bars indicate standard deviations of three biological replicates. **(C)** Spot assay of E264; vec, Δ*dbcA*; vec, E264B, E264T, Δ*dbcA*B, and Δ*dbcA*T on LB agar media. 1:10 dilutions of indicated strains from 2nd overnight culture grown without any rhamnose were spotted on LB, 100 Tmp, and different amounts of rhamnose as indicated. Plates were analyzed after 24 h of growth at 37°C.

at 37°C and 225 rpm. Cell density was measured by optical density at 600 nm.

Generation of *Burkholderia thailandensis arn* Conditional Mutants

Creation of *B. thailandensis arn* conditional mutants was carried out by inserting the plasmid pSC200 upstream of either the *arn* biosynthetic cluster or *arn* transport cluster (**Figure 1A**) so that the expression of these operons could be regulated by the plasmid-borne inducible rhamnose promoter as previously described (Ortega et al., 2007; Wagley et al., 2014). PCR primers are listed in **Supplementary Table S2**. The plasmid, pSC200 was a generous gift by Dr. Miguel A. Valvano (Queen's University Belfast, United Kingdom). Two primers FWAra4NB and REVAra4NB were used to PCR amplify ∼290 bp portion of BTH_I2193 (*arnB*), the first gene in *arn* biosynthetic cluster (**Supplementary Figure S1**). Primers FWAra4NT and REVAra4NT were used to amplify ∼330 bp portion of BTH_I2196, the first gene in *arn* transport cluster (**Supplementary Figure S1**). The PCR fragments were ligated to pSC200 using Nde1 and Xba1 restriction sites, upstream of the rhamnose promoter, transformed into *E. coli* XL1-blue cells, and selected on LB agar with 50 μg/ml Tmp. The plasmids pSC200Ara4NB and pSC200Ara4NT were further transformed into *E. coli* RHO3 by electroporation (Tu et al., 2016). *E. coli* RHO3 is a generous gift of Dr. Erin C. Garcia (Department of Microbiology, Immunology, and Molecular Genetics, University

of Kentucky College of Medicine). *E. coli* RHO3 is a donor strain sensitive to all commonly used antibiotics and its DAP auxotrophy makes it easy to counter select on rich media by the exclusion of DAP from the selection media (Lopez et al., 2009). RHO3 transformants were then conjugated with *B. thailandensis* E264 and Δ*dbcA*:FRT (Garcia, 2017). Colonies were screened on LB agar with 100 μg/ml Tmp and 0.25% rhamnose. The Tmp^R transconjugants were further grown in Tmp and rhamnose and their extracted genomic DNA was used to confirm the integration of the plasmids pSC200Ara4NB or pSC200Ara4NT in E264 or Δ*dbcA*:FRT by using primers ConfirmAra4NB and ConfirmAra4NT as shown in the **Supplementary Figure S1**. The inability of these strains to grow on LB plates without rhamnose further confirmed the success of generation of conditional arn mutants in E264 and Δ*dbcA*:FRT. We named these strains E264B, E264T, Δ*dbcA*B, and Δ*dbcA*T as shown in **Figure 1A**.

Susceptibility Assays

For testing the susceptibility on solid medium, *B. thailandensis* strains were grown for 18–24 h in fresh LB broth supplemented with 100 μg/ml Tmp and rhamnose if required. 1 ml of an overnight culture was washed 1x with fresh LB, and resuspended with 1 ml fresh LB. The cell concentration was adjusted to 3×10^8 cells/ml. Five microliters of serially $\log_{10}$-diluted cells was then spotted on LB agar plates containing various concentrations of antibiotics and rhamnose as required. Growth was analyzed after incubation for 24–72 h at 37°C as indicated in figure captions. The colistin MIC was measured using Liofilchem® MIC Test Strips (Liofilchem, Inc.). Overnight cultures were adjusted to 1×10^8 cells/ml as described above and a sterile swab was used to create a lawn of cells. Then the MIC strip was applied to the plates and the growth was evaluated after 24–48 h at 37°C. All experiments were repeated at least three times.

Measurement of Membrane Potential

Measurement of membrane potential was done using JC-1 dye as previously described (Panta et al., 2019). Briefly, 1 ml of overnight cultures grown in LB media broth with appropriate antibiotic and reagents were washed with fresh LB and resuspended in 1 ml LB media. 2.5×10^7 cells were inoculated in 25 ml of fresh LB broth in 250 ml flask and grown for about 7 h. Then $\sim 6 \times 10^8$ cells were taken from each culture, washed with permeabilization buffer, PB (10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM glucose) and finally resuspended in PB buffer. Three micromolar of JC-1 dye was added, incubated in the dark at 37°C for 30 min. Cells were washed and resuspended in PB buffer and fluorescence measurements were carried out using a JASCO FP-6300 spectrofluorometer. Membrane potential is determined by the ratio of red (595 nm) to green (530 nm) fluorescence with excitation of 488 nm.

Analysis of Lipid A by TLC

Lipid A analysis was performed as described (Doerrler et al., 2004). Cells were labeled with 5 μCi/ml of ^{32}P in 2 ml of LB broth at an OD_{600} of ∼0.8 for 30 min shaking at 37°C. The cells were collected using a centrifuge and washed with PBS, pH 7.4. To extract the ^{32}P-labeled phospholipids, the cell pellet was resuspended in 1 ml of a single-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (1:2:0.8, v/v). After mixing and incubating for 30 min at room temperature, the insoluble material was removed by centrifugation, and the supernatant containing the ^{32}P-labeled phospholipids was removed. The insoluble residue, which contains the ^{32}P-labeled lipopolysaccharide, was subjected to hydrolysis at 100°C in 0.2 ml 12.5 mM sodium acetate buffer, pH 4.5, in the presence of 1% SDS to cleave the Kdo-lipid A linkage. The released ^{32}P-labeled lipid A species were extracted by the addition of 0.25 ml $CHCl_3$ and 0.5 ml MeOH. Following centrifugation, the supernatant was transferred to new tube to which an additional 0.25 ml $CHCl_3$ and 0.25 ml water was added. The lower phase was washed with fresh upper phase and dried in a speed-vac. Dried material was dissolved in a small volume of chloroform/methanol 4:1 and spotted onto a Silica Gel 60 thin layer chromatography (TLC) plate which was developed in the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v). The plate was dried and exposed to a Phosphor-Imager screen overnight to visualize the ^{32}P-lipid A species.

Quantification of Lipid A Species

A TLC autoradiograph with the resolved lipid A species obtained from Phosphorimager equipped with IQMac software was further analyzed through ImageQuant Tools Version 2.2 for lipid A species quantification. Lipid A species modified with 0, 1, and 2 Ara4N were assigned as "0," "1," and "2," respectively. The intensity of each assigned species was quantified and values for species 0, 1, and 2 were added to get the total amount of lipid A species. Percentage of each species was calculated using the following equations:

$$\% \text{ species } 0 = [0/(0+1+2)] \times 100$$

$$\% \text{ species } 1 = [1/(0+1+2)] \times 100$$

$$\% \text{ species } 2 = [2/(0+1+2)] \times 100$$

Transformation and Complementation Analysis

Transformation of *E. coli* was done using a heat shock method (Froger and Hall, 2007). Transformation of *B. thailandensis* was carried out using conjugation (Garcia, 2017). Briefly, recipient *B. thailandensis* and donor *E. coli* RHO3 strains carrying Tmp^R plasmid(s) to be transferred were grown on LB and LB with 50 μg/ml Tmp and 200 μg/ml DAP, respectively. After 48 h of incubation at 37°C, using a sterile swab, several large colonies of both recipient and donor strains were inoculated by thoroughly spreading them on LB plates supplemented with DAP and incubated at 37°C. Control plates with recipient cells only, and donor cells only were also inoculated in a similar way. After ∼18 h of incubation, a loopful of bacterial cells from conjugation plates and control plates were streaked on LB with 100 μg/ml Tmp plates for selection. DAP was excluded on these plates to select against the donor strain, RHO3. After 48 h of incubation at 37°C, isolated Tmp^R colonies were used for colony PCR using plasmid specific primers (ConfirmpSCrhaB2FW and

ConfirmpSCrhaB2REV) to confirm the introduction of Tmp^R plasmids into *Burkholderia* recipient strains.

For complementation analysis, pSCrhaB2 plasmid was used (Cardona and Valvano, 2005). *BTH_I1512* was PCR amplified using FWBTH_I1512 and REVBTH_I1512 primers. *BTH_I2750* was amplified using FWBTH_I2750 and REVBTH_I2750 primers. These fragments were then ligated into pSCrhaB2 using Nde1 and *Hin*dIII separately giving pSCuppP1 and pSCuppP2. RHO3 strains were then transformed with these plasmids and introduced into E264 and Δ*dbcA*:FRT by conjugation.

Statistical Analyses

Values represent mean ± standard deviation of three independent determinations and statistical significance was calculated by unpaired Student's *t*-test using GraphPad Prism 8.4.3. $^{***}p < 0.001$, $^{**}p < 0.01$. All experiments were repeated at least three times.

RESULTS

Growth Curve for *arn* Conditional Mutants of E264 and Δ*dbcA*

Arn genes in *B. thailandensis* are organized in two transcriptional units as shown in **Figure 1A** (Ortega et al., 2007; Taboada et al., 2012). One transcriptional unit encodes the gene products for synthesis of undecaprenyl-P-Ara4N; BTH_I2193 (41% amino acid identity with *E. coli* K-12 ArnB), BTH_I2192 (48% identity with ArnC), BTH_I2191, BTH_I2190 (28% identity with ArnA), BTH_I2189 (36% identity with ArnD). Another transcriptional unit encodes *arn* transport gene products- BTH_I2196, *BTH_I2195* (23% identity with ArnT), and *BTH_I2194* (27% identity with ArnE/ArnF) (**Figure 1A**). Alignments were performed using Needleman-Wunsch alignment (Altschul et al., 1997, 2005).

In our study, we used the plasmid pSC200 to insert an inducible rhamnose promoter upstream of *arn* synthesis operon (creating strains E264B and Δ*dbcA*B) or upstream of *arn* transport operon (creating strains E264T and Δ*dbcA*T) as shown in **Figure 1A**. The plasmid pSC200 has multiple-cloning site, ori_{R6K}, and mob genes from pGpΩTp and P*rhaB* rhamnose-inducible promoter, *rhaR*, *rhaS*, and the *dhfr* cassette from pSCrhaB2 (Ortega et al., 2007). Ori_{R6k} requires pir protein in trans for replication which is not present in *B. thailandensis*. This allows the plasmid to be integrated into the chromosome at a specific site by homologous recombination. The insertions were confirmed by PCR (**Supplementary Figure S1**) and DNA sequencing.

The strains E264B, Δ*dbcA*B, E264T, and Δ*dbcA*T were grown in LB broth with different concentrations of rhamnose for 24 h and the cell number was measured at different times. None of the strains grew without rhamnose except controls E264; vec and Δ*dbcA*; vec (**Figure 1B**). For other strains, growth was rhamnose dependent, reaching the highest cell numbers at 0.1 and 0.5% rhamnose (**Figure 1B**). These strains were also grown on plates with and without rhamnose (**Figure 1C**) and the results were consistent with growth in liquid media, all suggesting that *arn* genes in *B. thailandensis* are essential, consistent with previous findings (Ortega et al., 2007). We note that E264B, Δ*dbcA*B, E264T, and Δ*dbcA*T struggle to grow in 0.025% LB agar plates whereas they grow better on LB broth as shown in **Figure 1B**.

Overexpression of *arn* Operons Increases the Abundance of Lipid A Doubly Modified With Ara4N

We previously showed using mass spectrometry that Δ*dbcA* had lower amounts of lipid A modified with Ara4N (Panta et al., 2019). Species "2," "1," and "0" marked by arrows in **Figure 2A** represent lipid A with 2 Ara4N, 1 Ara4N, and no Ara4N respectively (**Figure 2A**). Only 27% of the total lipid A from Δ*dbcA* is doubly modified (species 2), compared to 85–90% for the other strains (**Figure 2A**). To investigate whether the overexpression of *arn* operons can increase the abundance of species 2 lipid A in Δ*dbcA*, we analyzed the lipid A profile by ^{32}P labeling and TLC for each strain at different rhamnose concentrations. We found that the level of species 2 increased as rhamnose concentration increased in Δ*dbcA*B, and Δ*dbcA*T strains (**Figure 2B**). At 0.025% and 0.5% rhamnose, species 2 for Δ*dbcA*B increased from 26% of total lipid A to 40%. For Δ*dbcA*T, species 2 increased from 40% to 55% of the total lipid A as rhamnose concentration increased from 0.025% to 0.5%. This trend is consistent with the idea that overexpressing the transport pathways is more efficient in increasing species 2 in Δ*dbcA*.

Overexpression of *arn* Operons Can Partially Complement Colistin Sensitivity

To investigate whether overexpression of *arn* operons can compensate colistin hypersensitivity in Δ*dbcA,* we measured growth on solid plates using a spotting technique with 0.1, 0.5, and 1% rhamnose and different concentrations of colistin (**Figure 3**). After ∼72 h of growth, Δ*dbcA*T and Δ*dbcA*B were able to grow much better than Δ*dbcA* in the presence of colistin. Δ*dbcA*T grew slightly better than Δ*dbcA*B in presence of colistin suggesting that overexpression of the transport operon rescues Δ*dbcA* colistin sensitivity better than overexpression of the biosynthesis operon, consistent with the lipid A profile in **Figure 2B**. It should also be noted that 0.5% rhamnose improved growth of the parent strains E264B and E264T compared to E264; vec in the presence of high concentrations of colistin (**Figure 3**). One possibility is that overexpression of these operons may improve the rate of lipid A modification with Ara4N, leading to better growth in the presence of these high concentrations of colistin. Despite this, it is likely the effects we are seeing in the compared Δ*dbcA* strains are real.

pH Dependence of Colistin Sensitivity, Lipid A Modifications, and Membrane Potential in Δ*dbcA*

Previous reports have shown that cell division defects, antibiotic sensitivity, and temperature sensitivity occur as a result of deletion of two genes belonging to the *E. coli* DedA protein family (*yqjA* and *yghB*) (Thompkins et al., 2008;

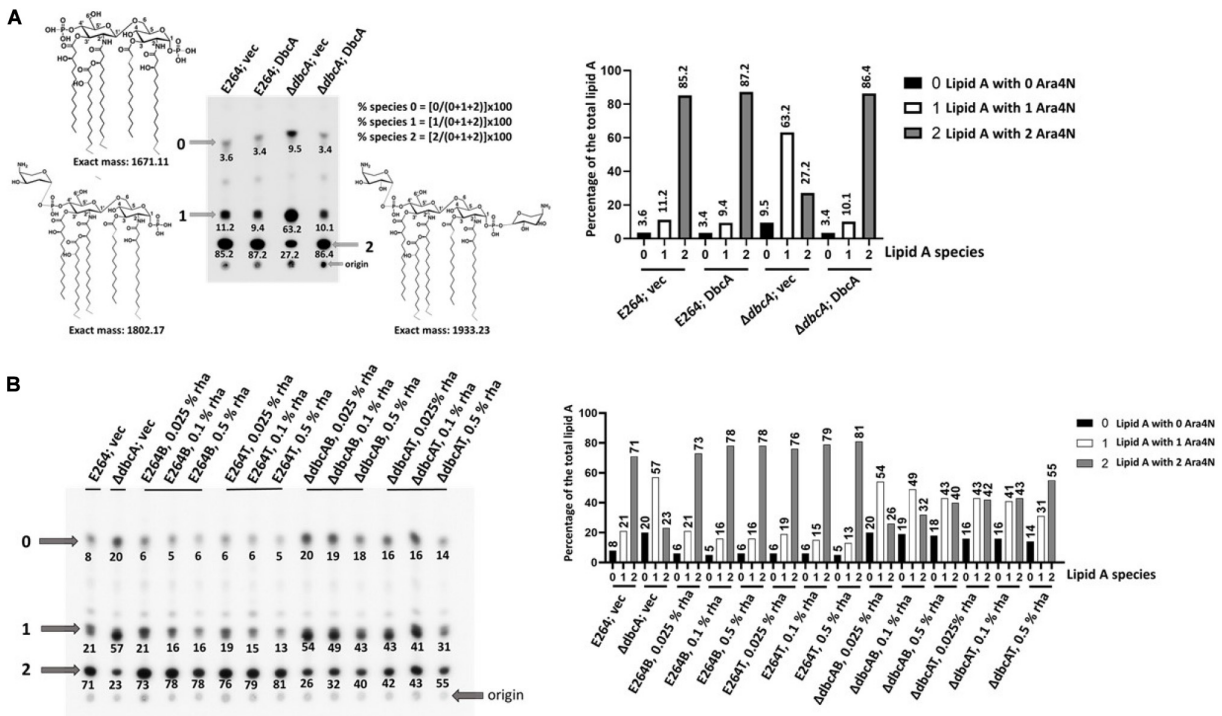

FIGURE 2 | Lipid A analysis by TLC. **(A)** Lower abundance of lipid A modified with two Ara4N (species 2) in Δ*dbcA*. Lipid A was extracted from the indicated strains following labeling with ^{32}P and resolved using thin layer chromatography. Analysis was conducted using a Phosphorimager equipped with IQMac software. Indicated structures were ascertained from comparison with our MS data (Panta et al., 2019). Species "2", "1," and "0" correspond to pentaacylated lipid A modified with two, one, or zero Ara4N, respectively. Numbers below each spot correspond to the percentage of that species of the total signal of species "2", "1," and "0." **(B)** Analysis of species 2 (pentaacylated lipid A modified with two Ara4N) with different concentrations of rhamnose in E264B, E264T, Δ*dbcA*B, and Δ*dbcA*T strains. E264; vec and Δ*dbcA*; vec were included as controls. A bar graph is also included for clarity.

Sikdar and Doerrler, 2010; Kumar and Doerrler, 2014). These phenotypes were rescued by lowering the pH of the media (Sikdar et al., 2013; Kumar and Doerrler, 2014). To test if lowering the pH of the media can rescue colistin sensitivity of Δ*dbcA*, we measured the colistin MIC when grown in media of different pH. Lowering the pH of the media was able to increase the colistin MIC in Δ*dbcA*; vec (**Figure 4A**, upper) from 1.5 at pH 7.5 to 64 at pH 6.5 to more than 1,024 at pH 5.5, all consistent with low pH complementation of different phenotypes of the *E. coli* mutant. In fact, colistin sensitivity of wild type *B. thailandensis* is dependent on the external pH of the media. The colistin MIC of strain E264 dropped to around 32 at pH 8.0 (**Figure 4A**, lower), which suggests that colistin resistance itself is linked to pH homeostasis in *B. thailandensis*. The MIC was also measured using the broth microdilution method (**Supplementary Figure S2**) which is consistent with the colistin E-test strips on solid media (**Figure 4A**).

To investigate whether complementation of colistin sensitivity of Δ*dbcA* at low pH is due to lipid A modification by Ara4N, we looked at the lipid A profile of all 4 strains grown at different pH. To our surprise, the level of species 2 in Δ*dbcA*; vec increased from 18% at pH 7.5 to 30% at pH 7.0 to 51% at pH 5.5 media (**Figure 4B**), consistent with the increase in colistin MIC from pH 7.5 to pH 5.5 in Δ*dbcA*; vec (**Figure 4A**, upper). However, the level of species 2 in E264 remains relatively constant as the pH was altered (**Figure 4B**), suggesting that DbcA helps to maintain lipid A modifications with Ara4N as external pH increases.

We previously proposed that the membrane potential plays an important role in lipid A modification and colistin resistance (Panta et al., 2019). Colistin sensitivity of Δ*dbcA* is rescued by simply growing Δ*dbcA* in acidic pH media (**Figure 4A**). Increased Ara4N modification of lipid A at acidic pH could be due to possible compensation of lower membrane potential at acidic pH media due to increased ΔpH component of the PMF. We measured membrane potential at different pH media and found that lower membrane potential of Δ*dbcA*; vec was corrected at pH 5.5 (**Figure 4C**). This suggests that the restoration of lower membrane potential at pH 5.5 is responsible for increasing the species 2 in Δ*dbcA*; vec (**Figure 4B**) and increasing the colistin MIC in Δ*dbcA*; vec (**Figure 4A**, upper). Another interesting observation was that the membrane potential of wild type E264 at pH 7.5 was significantly lower than that at pH 5.5 (**Figure 4C**).

Products of *arn* Operons Are Essential for Acid Tolerance in *Burkholderia thailandensis*

Growth in mild acid (pH 5.8) has been shown to upregulate Salmonella *arn* genes via crosstalk between PhoP/PhoQ and

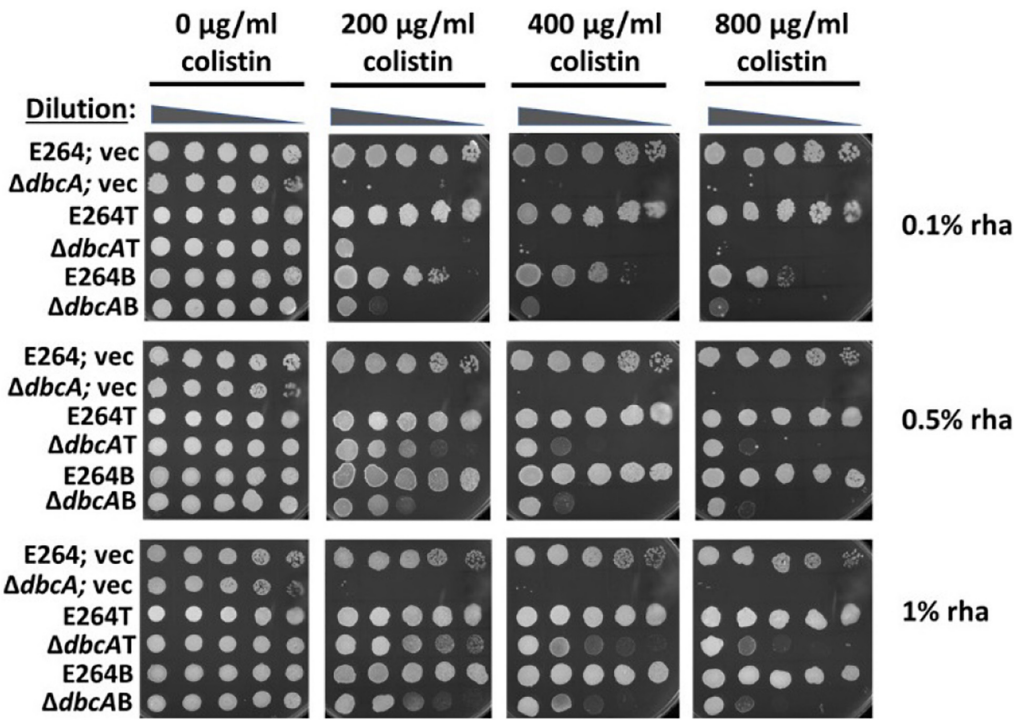

FIGURE 3 | Partial complementation of colistin sensitivity in Δ*dbcAB* and Δ*dbcA*T. All the strains were grown similarly as described in **Figure 1B**. 1:10 dilutions were spotted on LB, 100 Tmp plates with different amount of colistin and rhamnose as indicated. Plates were analyzed after 72 h of incubation at 37°C. Higher concentrations of rhamnose are used for this experiment because the rhamnose promoter is integrated in the chromosome and *arn* genes are transcribed through this promoter as a single copy in compared to multicopy expression plasmids used elsewhere.

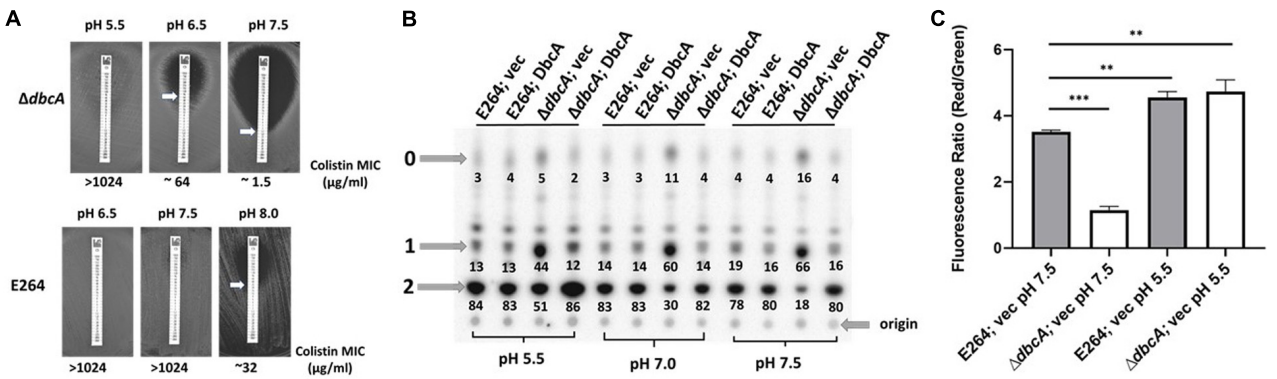

FIGURE 4 | pH dependent colistin sensitivity, lipid A modification and membrane potential in *B. thailandensis*. **(A)** Minimal inhibitory concentration (MIC) was determined for indicated strains using colistin E-test strips on LB, 100 Tmp plates buffered with 100 mM of MES for pH 5.5 and 6.5, and 70 mM BIS-TRIS propane (BTP) buffer for pH 7.5 and 8.0. Approximate MIC is denoted by white arrows. Plates were analyzed after 48 h. **(B)** Analysis of lipid A at different pH media adjusted with either MES or BTP as described above. Lipid A was analyzed as previously described in **Figure 2**. Numbers below each spot correspond to the percentage of that species of the total signal of species "2", "1," and "0." A "percentage of the total lipid A" bar graph for this figure is shown in **Supplementary Figure S3**. **(C)** Assessment of membrane potential (Δψ) of E264; vec and Δ*dbcA*; vec at different pH media using JC-1 dye represented as the red (595 nm)/green (530 nm) ratio. 2.5×10^7 cells from an overnight culture were inoculated in 25 ml LB, 100 Tmp, pH 5.5 (MES adjusted) or pH 7.5 (BTP adjusted). Each experiment was repeated three times. Bars represent mean ± *SD* of three independent determinations and statistical significance was calculated by unpaired Student's *t*-test using GraphPad Prism 8.4.3 ***$p < 0.001$, **$p < 0.01$.

PmrA/PmrB two component systems contributing to polymyxin B resistance (Perez and Groisman, 2007; Chen and Groisman, 2013). It is possible that lipid A modification could be induced in low pH independent of DbcA and compensate colistin sensitivity of Δ*dbcA*. First, we tested whether *arn* operons are essential at low pH. We observed that E264B, Δ*dbcA*B, and Δ*dbcA*T

strains all struggled at pH 5.0 when expression was constrained with 0.05% rhamnose induction (**Figure 5A**). However, at pH 7.0 media plate, all strains grew better (**Figure 5A**), suggesting that *arn* operons are in fact essential for extreme acid tolerance. Second, to examine if complementation of colistin sensitivity of Δ*dbcA* at low pH is through the expression of *arn* operons, we restrained the expression of *arn* operons by using 0.05% rhamnose in growth media and looked at the colistin sensitivity at pH 5.5. While lowering the pH of the media was able to rescue colistin sensitivity of Δ*dbcA*; vec, Δ*dbcA*T, and Δ*dbcA*B still remained sensitive to colistin with 0.05% of rhamnose (**Figure 5B**). E264B also struggles in presence of colistin at pH 5.5 with 0.05% rhamnose, all suggesting that *arn* gene products are essential for acid tolerance. To further support this, we used *Burkholderia cenocepacia ArnT* suppressor mutant strain, MH55 to test if lipid A modification by *ArnT* is essential at acidic pH. *B. cenocepacia* strains K56-2, and MH55 were generous gifts of Dr. Miguel A. Valvano (Queen's University Belfast, United Kingdom). MH55 is a strain lacking Ara4N transferase *ArnT* harboring suppressor mutation in *lptG*, which has been shown to lack Ara4N both in the lipid A and LPS core oligosaccharide (Hamad et al., 2012). MH55 was sensitive to pH 4.5 and pH 5.5 compared to its parent strain K56-2 (**Figure 5C**) consistent with an essential role for Ara4N for adaptation to acidic environments.

DbcA Is Required for Bacitracin Resistance

Previously, we proposed that lower membrane potential observed in Δ*dbcA* might affect the transport activity of undecaprenyl pyrophosphate [C_{55}P(P)] phosphatase UppP/BacA (Panta et al., 2019), which bears similarity to MdfA, a prototypical H^+-coupled multidrug transporter, known to utilize PMF (Fluman et al., 2012). UppA/BacA has been shown to be involved in bacitracin resistance and is the C_{55}P(P) phosphatase in *E. coli* (El Ghachi et al., 2004). To examine whether UppP/BacA plays a role in efficient lipid A modification and colistin resistance, we first tested if Δ*dbcA* is sensitive to bacitracin. Bacitracin is an antibiotic produced by *Bacillus licheniformis* which has been shown to inhibit enzymatic dephosphorylation of C_{55}P(P) in the presence of divalent cations (Stone and Strominger, 1971). To our surprise, Δ*dbcA*; vec is sensitive to bacitracin at high levels (**Figure 6A**) and overexpression of DbcA can also sensitize E264 to bacitracin (**Supplementary Figure S4**). We cloned two homologs of UppP/BacA from *B. thailandensis* (*BTH_I1512* and *BTH_I2750*) and overexpressed them individually in E264 and Δ*dbcA* strains to examine if they could complement bacitracin and colistin sensitivity of Δ*dbcA*. BTH_I1512 displays 45% amino acid identity with *E. coli* UppP/BacA and BTH_I2750 has 36% amino acid identity with *E. coli* UppP/BacA. *BTH_I1512* was able to partially complement bacitracin sensitivity of Δ*dbcA* with 0.001% rhamnose induction, but it was unable to complement colistin sensitivity of Δ*dbcA* (**Figure 6B**). Overexpression of *BTH_I1512* with 0.1% inducer was toxic (**Figure 6B**). Interestingly, BTH_I2750 was unable to complement bacitracin sensitivity but was able to partially complement colistin sensitivity of Δ*dbcA* (**Figures 6B,C**). To determine if this partial complementation of colistin sensitivity of Δ*dbcA* is due to increase in species 2, we analyzed lipid A with BTH_I2750 overexpressed in E264 and Δ*dbcA*. There was no increase in species 2 by BTH_I2750 with either 0.01 or 0.1% rhamnose induction (**Figure 6D**), suggesting that this partial complementation was independent of lipid A modification, and supporting a possible multifactorial mechanism of extreme colistin resistance in *Burkholderia* spp. (Loutet and Valvano, 2011).

DISCUSSION

The increasing threat of multidrug-resistant (MDR) bacterial pathogens with stagnant discovery of new class of effective antibiotics has led to renewed interest in reviving older antibiotics like colistin (polymyxin E), a polycationic cyclic polypeptide belonging to polymyxin family of antibiotics (Talbot et al., 2006). Despite its reported nephrotoxicity and neurotoxicity, colistin still remains the most reliably active agent against many MDR gram-negative bacterial infections (Koch-Weser et al., 1970; Falagas and Kasiakou, 2005; Li et al., 2005). Colistin has been classified as "Reserve Group" of antibiotics to be used as a last-resort antibiotic against MDR infections that cannot be treated by any other conventional antibiotics (Hsia et al., 2019). However, the efficacy of colistin has been challenged by emerging acquired colistin resistance in several human pathogens and the global spread of plasmid acquired colistin resistance (Liu et al., 2016; McGann et al., 2016; Rolain et al., 2016; Elbediwi et al., 2020; Marano et al., 2020). Interestingly, species like *Burkholderia* are intrinsically resistant to colistin and the resistance may be multifactorial (Loutet and Valvano, 2011; Hamad et al., 2012; Malott et al., 2012; El-Halfawy and Valvano, 2014). We have previously shown that DedA protein, DbcA is required for extreme colistin resistance in *B. thailandensis* (Panta et al., 2019). We had proposed a model that the lower membrane potential observed in Δ*dbcA* may cause defects in PMF dependent step(s) in lipid A modification. Here, we demonstrate that overexpression of *arn* transport genes can partially complement the colistin sensitivity of Δ*dbcA* by increasing the abundance of lipid A modified with two Ara4N (species 2). We also show that the lower membrane potential of Δ*dbcA* is corrected at pH 5.5 media compared to pH 7.5 and this correction of membrane potential at pH 5.5 is consistent with the increase of species 2 at pH 5.5, suggesting that the lipid A modification at pH 7.5 by DbcA is dependent on PMF. We show for the first time that DbcA is also required for bacitracin resistance. This is also the first study to show that the lipid A modification, maintenance of membrane potential, and the colistin resistance in *Burkholderia thailandensis* is all linked to the pH of the media.

One of the steps of lipid A modification includes flipping of undecaprenyl-P-Ara4N from the inner leaflet of the cytoplasmic membrane to outer leaflet, carried out by EmrE like transporters ArnEF in *E. coli* (Yan et al., 2007). The small multidrug resistance transporter EmrE uses PMF to efflux a broad range of cationic compounds (Morrison et al., 2015; Robinson et al., 2017;

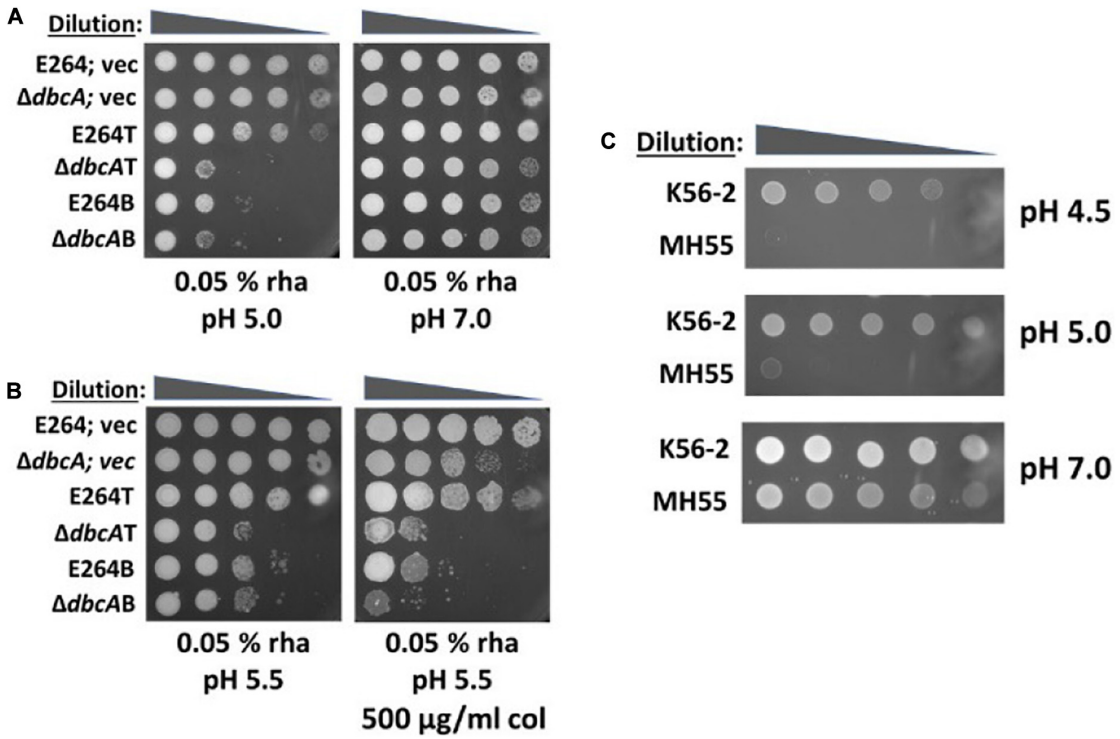

FIGURE 5 | A link between low pH and lipid A modification with Ara4N. **(A)** Lipid A modification with Ara4N is essential for acid tolerance. 1:10 dilutions of indicated strains were spotted on different pH media, LB and 100 Tmp agar plates with indicated concentration of rhamnose. Plates were buffered with either MES or BTP. The plates were analyzed after 72 h of incubation at 37°C. **(B)** Low pH complementation of colistin sensitivity of Δ*dbcA* depends on the expression of *arn* operons. 1:10 dilutions of indicated strains were spotted on LB, 100 Tmp, pH 5.5 with 0.05% of rhamnose and colistin as indicated. The plates were analyzed after 72 h of incubation at 37°C. **(C)** *Burkholderia cenocepacia* ArnT suppressor mutant strain, MH-55 is sensitive to acid stress. 10-fold dilution of 3×10^8 cells from overnight cultures of K-56 and MH-55 strains were spotted on LB plates at different pH media plates adjusted with either MES or BTP.

Hussey et al., 2020). The ArnEF homolog, *BTH_I2194* is present in an operon along with *BTH_I2196* and the ArnT homolog, *BTH_I2195* that we designate as the transport operon (T). The genes involved biosynthesis are present in a nearby operon that we designate as the biosynthesis operon (B). We constructed several conditional mutants (E264B, E264T Δ*dbcA*B, and Δ*dbcA*T) by genomic insertion of an inducible rhamnose promoter in front of either *arn* "B" operon or *arn* "T" operon (**Figure 1A**). This allowed us to control the expression of these operons by the addition of rhamnose in the media.

The death of all the conditional mutants (E264B, E264T Δ*dbcA*B, and Δ*dbcA*T) when grown without rhamnose (**Figure 1B**) is consistent with the essentiality of *arn* genes in *B. thailandensis*, and as reported for *B. cenocepacia* (Ortega et al., 2007; Hamad et al., 2012) and also consistent with unavailability of transposon insertion mutants of *arn* genes (Gallagher et al., 2013). Slight growth of E264T without rhamnose (**Figures 1B,C**) might arise from residual expression of *arn* transport operon since the rhamnose promoter appears to be leaky in several of our experiments (data not shown). Next, we looked at the lipid A profile for all our conditional mutants along with E264 and Δ*dbcA*. Since Ara4N modification is an essential process, we were unable to measure lipid A in the complete absence of rhamnose in either strain. The level of lipid A species 2 increased with increasing amount of rhamnose in Δ*dbcA*B, and Δ*dbcA*T (**Figure 2B**). The amount of species 2 for Δ*dbcA*B was less compared to Δ*dbcA*T even with the same amount of rhamnose induction (**Figure 2B**). It is possible that overexpression of the transport operon might have stepped up the already PMF deficient transport step of lipid A modification in Δ*dbcA*. It should also be noted that species 2 in wild type E264 strains were largely independent of rhamnose concentration, suggesting efficient modification even at very low expression levels. It is also possible that there might be unknown post-transcriptional regulation or energy requiring steps facilitating such an efficient modification of lipid A in E264. We propose that this unknown factor might be PMF, which might be acting as a limiting factor in lipid A modification process.

To examine if this increase in species 2 is enough to complement colistin sensitivity of Δ*dbcA*, we performed a spot assay with varying colistin and rhamnose concentrations. We observed that overexpression of *arn* operons can partially complement colistin sensitivity of Δ*dbcA* (**Figure 3**). It is possible that lower PMF observed in Δ*dbcA* is limiting proper lipid A modification. Inefficient modification of lipid A in Δ*dbcA* is probably one of the effects of perturbed PMF, but there may be additional effects of this mutation that impact colistin resistance. Disturbed PMF in Δ*dbcA*

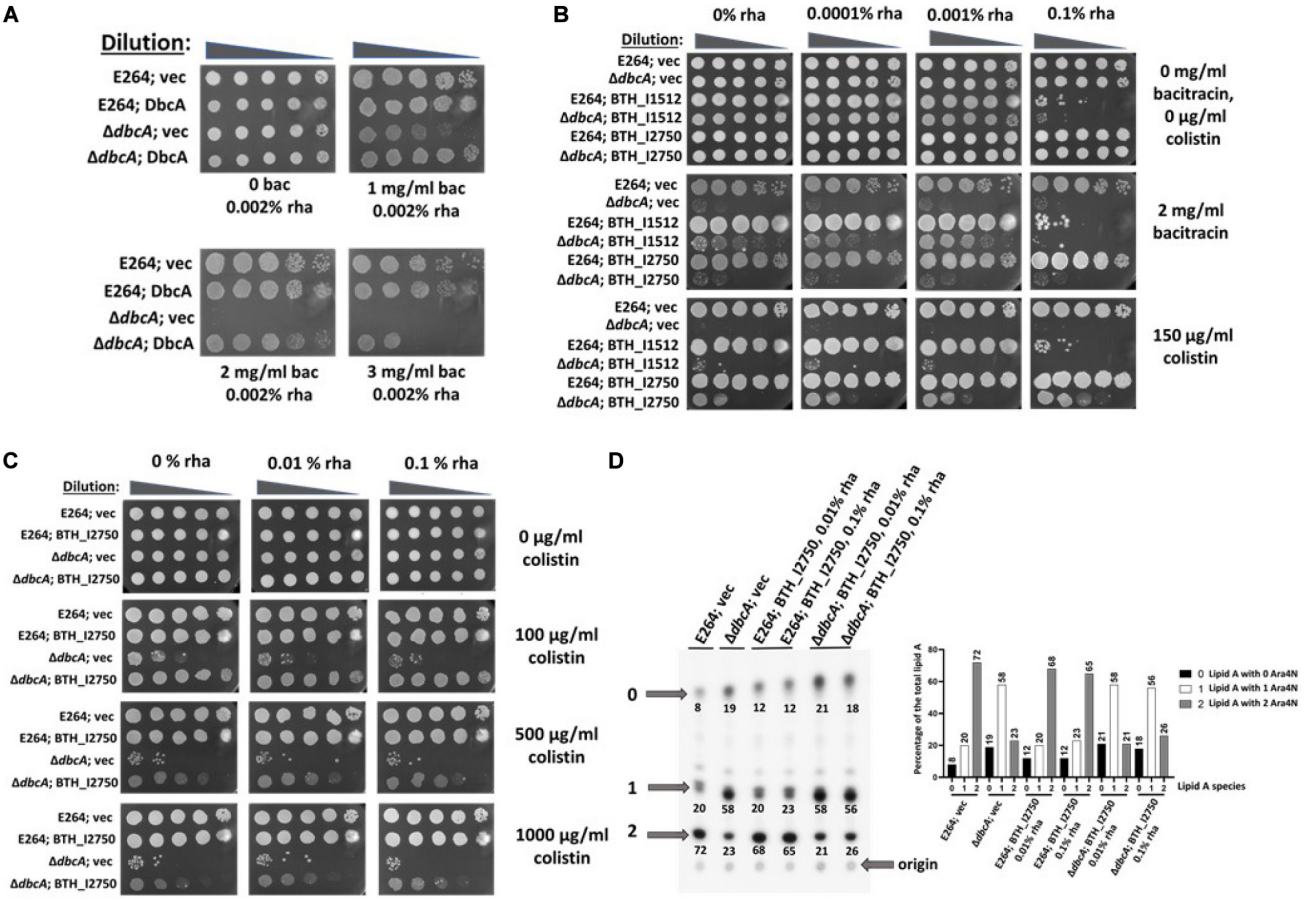

FIGURE 6 | Complementation of bacitracin and colistin sensitivity of Δ*dbcA*; vec by two *B. thailandensis uppP* homologs (*Bth_I1512* and *Bth_I2750*) **(A)** Δ*dbcA* is sensitive to bacitracin. 10-fold dilution of cells from overnight cultures of indicated strains were spotted on LB, 100 Tmp, 0.002% rha and different amount of bacitracin. **(B)** Complementation of bacitracin and colistin sensitivity of Δ*dbcA* by *Bth_I1512* and *Bth_I2750*, respectively. **(C)** Partial complementation of Δ*dbcA* colistin sensitivity by *Bth_I2750*. Here, we used a multicopy expression plasmid that requires a lower concentration of rhamnose for adequate expression, compared to the integrated construct used in **Figure 3**. **(D)** Lipid A analysis by TLC with overexpressed *Bth_I2750* in E264 and Δ*dbcA*. Lipid A was extracted and analyzed for indicated strains. Species 2 was compared for all the strains. A "percentage of the total lipid A" bar graph is also shown on the right-hand side for more clarity.

might also have negative impacts on other PMF dependent efflux pumps, or PMF dependent processes that might be involved in colistin resistance directly or indirectly. Colistin resistance of *B. thailandensis* is defined by a complex synergistic relationship between active efflux and outer membrane diffusion (Krishnamoorthy et al., 2017).

Based on our previous findings in *B. thailandensis* and *E. coli*, we proposed that DedA proteins might be proton dependent transporters (Kumar and Doerrler, 2014, 2015; Kumar et al., 2016; Panta et al., 2019), suggesting that lower membrane potential observed in Δ*dbcA* might be caused by a defect in proton influx. We first examined whether artificially increasing proton influx by simply acidifying the media can compensate colistin sensitivity of Δ*dbcA*. The colistin MIC for Δ*dbcA* increased as the pH of the media decreased (**Figure 4A**, upper). In fact, the colistin resistance in E264 also depends on the pH of the media. Extreme alkaline pH of 8.0 can sensitize E264 to colistin (**Figure 4A**, lower), suggesting that colistin resistance in *B. thailandensis* is linked to pH homeostasis. This is the first study to our knowledge that establishes such a link and emphasizes a need to understand more about alkaline pH homeostasis in *B. thailandensis*. Why does alkaline pH sensitize Δ*dbcA* and E264 to colistin? We analyzed lipid A profiles at different pH. Interestingly, we found that lipid A modification in Δ*dbcA* is dependent on pH of the media. Higher the pH of the media, lower the amount of species 2 in Δ*dbcA* suggesting that lipid A modification with Ara4N is dependent on pH of the media in Δ*dbcA*. (**Figure 4B**). However, the lipid A modification was similar for E264 at all pH's (**Figure 4B**), suggesting that DbcA is responsible in lipid A modification with Ara4N at higher pH's. Lipid A analysis at pH 8 could not be done since the mutant strain underwent growth arrest under these conditions. Surprisingly, we found that lower membrane potential of Δ*dbcA* was corrected at pH 5.5 (**Figure 4C**), suggesting that the lipid A modification at pH 7.5 is PMF dependent. In addition, the membrane potential of E264 turned out to be significantly lower at pH 7.5 than that in pH 5.5 (**Figure 4C**). This suggests that increasing the pH of the media increases the possibility of E264 to

have lower membrane potential and this might be the reason for lower colistin MIC in E264 at pH 8.0. This is further supported by our previous finding that lowering the membrane potential in E264 by CCCP also decreases the colistin MIC in E264 (Panta et al., 2019).

Why Δ*dbcA* has lower membrane potential at pH 7.5 compared to pH 5.5 remains an important question. We have observed that Δ*dbcA* is sensitive to divalent cations (Mg^{++} and Ca^{++}), but not sensitive to monovalent cations (Na^{+} and K^{+}) (**Supplementary Figures S5A,B**). The sensitivity of Δ*dbcA* to divalent cations can also be compensated by acidic pH (**Supplementary Figure S5C**). The presence of additional Mg^{++} and Ca^{++} in MH2 broth also decreased the colistin MIC of Δ*dbcA* (∼6 μg/ml colistin) compared to Mueller Hinton broth without added Mg^{++} and Ca^{++} (MIC ∼128 μg/ml colistin) (**Supplementary Figure S5D**). These observations suggest that DbcA is involved in divalent cations homeostasis, either directly by coupling proton movement to antiport of calcium or magnesium, or indirectly by other yet unidentified mechanisms. We have previously reported that overexpression of *mdfA*, a Na^{+}-K^{+}/H^{+} antiporter of the major facilitator superfamily essential for alkaline tolerance in *E. coli* (Lewinson et al., 2004), can compensate cell division defects and drug sensitivity of an *E. coli* strain lacking two partially redundant DedA family genes *yqjA* and *yghB* (Kumar and Doerrler, 2014). In *E. coli and S. aureus*, alkaline pH was shown to induce calcium influx and cytoplasmic calcium accumulation compared to acidic pH (Naseem et al., 2008; Nava et al., 2020). In light of these studies; it is possible that lower membrane potential of Δ*dbcA* at pH 7.5 is related to inefficient efflux of divalent cations at slightly alkaline pH.

The repression of lipid A modification through two component systems such as PhoPQ (Garcia Vescovi et al., 1996) by Ca^{++} and Mg^{++} could decrease the colistin MIC. Regulation of lipid A modifications in *B. thailandensis* is still largely unknown. The repression of lipid A modification by Ca^{++} and Mg^{++}, however, cannot explain why Δ*dbcA* is sensitive to these divalent cations. Complementation of Ca^{++} and Mg^{++} toxicity in Δ*dbcA* by acidic media also could be due to increased lipid A modification with Ara4N. It is possible that reduced Ara4N modified lipid A in Δ*dbcA* at pH 7.0 increases Ca^{++} or Mg^{++} influx causing toxicity or that lipid A modification with Ara4N protects against divalent cation toxicity in this species.

We also found that the acidic pH complementation of colistin sensitivity of Δ*dbcA* requires expression of *arn* operons (**Figure 5B**). The construction of *arn* conditional mutants allowed us to look at the role of lipid A modification with Ara4N at acidic pH. Here we show that restraining *arn* expression can sensitize *B. thailandensis* to extreme acidic pH (**Figure 5A**). Furthermore, *B. cenocepacia* ArnT suppressor mutant MH-55 is also sensitive to acidic pH (**Figure 5C**) compared to its parental strain K56-2. These observations suggest that the physiological role of lipid A modification by Ara4N might be acid tolerance. Several reports show that the majority of *Burkholderia* species could only be isolated from acidic environments, suggesting a sensitivity to naturally occurring alkaline environments (Curtis et al., 2002; Caballero-Mellado et al., 2004; Kaestli et al., 2009; Stopnisek et al., 2014).

The link between pH sensing and lipid A modification is critical for virulence of many species of bacteria. Maximal Salmonella PhoPQ-dependent gene expression in murine RAW264.7 macrophages is seen following acidification of the phagosome (Alpuche Aranda et al., 1992) and TLR-dependent phagosomal acidification is required for Salmonella's ability to survive phagocytosis (Arpaia et al., 2011). The lipid A profile from *S. typhimurium* infecting RAW264.7 cells showed modifications with aminoarabinose (Ara4N), phosphoethanolamine (PEtN), 2-hydroxymyristate, and palmitate, which resembled with the lipid A profile of *S. typhimurium* grown in acidic and low Mg^{++} minimal media (Gibbons et al., 2005). *Burkholderia cenocepacia* ArnT suppressor mutant strain MH55, that we reported to be acid sensitive, has been shown to cause reduced pathogenicity in both *Arabidopsis thaliana* and *Galleria mellonella* insect larvae without impacting the hosts' ability to recognize pathogens (Khodai-Kalaki et al., 2015). Our study further supports the notion that lipid A modification with Ara4N is not only important for acidic tolerance, but also important for intracellular pathogens to establish infections, survive in acidic compartments of host immune cells, and evade host defenses.

Bacitracin sensitivity of Δ*dbcA* is intriguing, although the sensitivity is only observed at higher concentrations. *E. coli* bacitracin resistance and undecaprenyl pyrophosphate [$C_{55}P(P)$] phosphatase have been shown to be conferred by two different classes of enzymes, either by UppP/BacA (Cain et al., 1993) or by YbjG, lpxT, pgpB, and $BrcC_{EC}$ (all belonging to the phosphatidic acid phosphatase 2 (PAP2) superfamily of enzymes) (Harel et al., 1999; El Ghachi et al., 2005). Undecaprenyl-P has been proposed to flip back to the cytoplasm via UppP/BacA, which displays some amino acid identity to drug efflux pump MdfA, and other secondary transporters known to utilize PMF for their activity (El Ghachi et al., 2018; Workman et al., 2018). It is therefore possible that there is a PMF-dependent step during recycling of the bacterial carrier lipid $C_{55}P(P)$. If so, then Δ*dbcA* mutation might also cause defect in $C_{55}P(P)$ recycling and may result in colistin sensitivity. We found that one of the UppP/BacA homologs of *B. thailandensis*, BTH_I2750, can partially compensate colistin sensitivity of Δ*dbcA*, independent of lipid A modification, supporting the notion that extreme colistin resistance of *B. thailandensis* is multifactorial and more complex than we thought. One of the members of PAP2 superfamily in *H. pylori*, HupA was shown to be involved in UppP activity and polymyxin B resistance independent of lipid A modification (Gasiorowski et al., 2019). Plasmid-mediated colistin resistance determinants *mcr*-1 and *mcr*-3 were found to be linked to PAP2 containing gene *hpap*2 (Anyanwu et al., 2020; Gallardo et al., 2020). More studies need to be conducted to further explore possible role of $C_{55}P(P)$ recycling in colistin resistance in *Burkholderia* spp. Whether DbcA has any direct or indirect link in $C_{55}P(P)$ recycling also needs further investigation. The DedA protein family consists of highly

conserved membrane proteins whose precise function remains a mystery. Here we show that DbcA is involved in PMF-dependent lipid A modification with Ara4N, which appears to be a major factor for maintaining extreme colistin resistance in *Burkholderia thailandensis,* E264.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

WD and PP: conception and design of the study, the acquisition, analysis, and interpretation of the data, and writing of the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.618389/full#supplementary-material

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Colistin Resistance Among Multiple Sequence Types of *Klebsiella pneumoniae* Is Associated With Diverse Resistance Mechanisms: A Report From India

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Background: The resistance to colistin and carbapenems in *Klebsiella pneumoniae* infections have been associated with increased morbidity and mortality worldwide. A retrospective observational study was conducted to determine the prevalence and molecular events contributing to colistin resistance.

Methods: Clinical samples were screened for colistin resistance and underlying mechanisms were studied by PCR-based amplification and sequence analysis of genes of two-component regulatory system (*phoPQ* and *pmrAB*), regulatory transmembrane protein-coding *mgrB*, and mobilized colistin resistance genes (*mcr-1-8*). Gene expression of *pmrC* and *pmrK* was analyzed by qRT-PCR, and the genetic relationship was assessed by MLST. The putative effect of amino-acid substitutions was predicted by a combination of bioinformatics tools.

Results: Of 335 *Klebsiella* spp. screened, 11 (3.2%) were identified as colistin-resistant (MIC range, 8 to >128 μg/ml). *K. pneumoniae* isolates belonged to clonal complex-11 (CC11) with sequence types (STs): 14, 16, 43, 54, 147 and 395, whereby four isolates conferred three novel STs (3986, 3987 and 3988) profiles. Sequence analysis revealed non-synonymous potentially deleterious mutations in *phoP* (T151A), *phoQ* (del87–90, del263–264, L30Q, and A351D), *pmrA* (G53S), *pmrB* (D150V, T157P, L237R, G250C, A252G, R315P, and Q331H), and *mgrB* (C28G) genes. The *mgrB* gene in three strains was disrupted by insertion sequences encoding IS*1*-like and IS*5*/IS*1182* family-like transposase genes. All 11 isolates showed an elevation in the transcription level of *pmrC* gene. Mobilized colistin-resistance (*mcr*) genes were not detected. All but one of the colistin-resistant isolates was also resistant to carbapenems; β-lactamase genes *blaNDM-1-like*, *blaOXA-48-like*, and *blaCTX-M-like* were detected in eight, five, and nine isolates, respectively.

Conclusion: All the studied colistin- and carbapenem-resistant *K. pneumoniae* isolates were genetically distinct, and various mechanisms of colistin resistance were detected, indicating its spontaneous emergence in this bacterial species.

Keywords: colistin resistance, *Klebsiella pneumoniae*, *phoPQ*, *pmrAB*, carbapenem resistance

INTRODUCTION

Klebsiella pneumoniae, a nosocomial pathogen, accounts for one-third of worldwide reported Gram-negative infections (Navon-Venezia et al., 2017). Multidrug-resistant (MDR) *K. pneumoniae* are resistant to extended-spectrum cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones and present a significant challenge to the clinicians. Available data showcase a significant increase in *K. pneumoniae* in India's carbapenem-resistant isolates from 29% in 2008 to 57% in 2016, limiting the treatment of life-threatening infections (Gandra et al., 2016; Dixit et al., 2019). The unavailability of new antimicrobial agents to combat carbapenem-resistant *K. pneumoniae* infections has revived the use of polymyxins (colistin and polymyxin B). However, the indiscriminate use of polymyxins in animals, aquaculture, and agriculture in the last decades has compounded the issue of drug resistance (Nguyen et al., 2016; Zhang et al., 2019).

Colistin-resistant *K. pneumoniae* exhibits a high degree of genetic plasticity where a point mutation and/or genetic disruption in two-component regulatory systems (TCRS), i.e., *pmrAB* and *phoPQ*, are known to confer polymyxin resistance (Cannatelli et al., 2014a; Jayol et al., 2014). Besides, polymyxin resistance is often observed by the inactivation of *mgrB*, a regulatory trans-membrane protein that controls the kinase activity of *phoQ* in *phoPQ* TCRS, by point mutations, indels, or insertion sequences (IS*5-like*, IS*1F*, IS*Kpn13*, IS*Kpn14*, IS*10R*; Cannatelli et al., 2015; Aires et al., 2016). The spread of colistin resistance has accelerated by acquiring plasmid-encoded mobile colistin resistance (*mcr*)- genes. After the first report in China (November 2015), *mcr*-genes were detected in *E. coli*, *K. pneumoniae*, *Shigella sonnei*, *Salmonella enterica*, and many other bacterial strains from colonized and infected humans, food (meat and vegetables), farm and wild animals, and aquatic environments (Liu et al., 2016; Carroll et al., 2019; Zhang et al., 2019). Selective colistin pressure in different sectors has induced constant emergence and evolution of *mcr*-genes, and *mcr-1* to *mcr-10* genes with multiple variants have been identified in colistin-resistant bacteria isolated from several sources (Gharaibeh and Shatnawi, 2019; Wang et al., 2020).

Increasing incidences of colistin resistance among nosocomial *K. pneumoniae* isolates have been reported from Europe, Asia, North America, South America, and Africa (Rojas et al., 2017; Lomonaco et al., 2018; Boszczowski et al., 2019; Zafer et al., 2019). Many isolated case reports and outbreaks of MDR *K. pneumoniae* infections were reported from different parts of India (Goel et al., 2014; Bhaskar et al., 2017; Kaur et al., 2017; Mohanty et al., 2017; Pragasam et al., 2017; Aggarwal et al., 2018). High mortality rate (approximately 69%) in bloodstream infections due to carbapenem- and colistin-resistant *K. pneumoniae* was also noted among Indian patients (Kaur et al., 2017; Manohar et al., 2017; Pragasam et al., 2017; Jajoo et al., 2018; Palani et al., 2020).

The accretion in clinical isolates of colistin- and carbapenem-resistant *K. pneumoniae* warrants further investigation into the epidemiology and underlying molecular mechanisms. Detection of colistin resistance is a challenge in clinical diagnosis, and evolving breakpoints have complicated the problem. Furthermore, horizontal gene transfer and the spread of clones with resistance traits confront the therapeutic control in nosocomial settings (Diene and Rolain, 2014). This study aimed to determine the prevalence of colistin-resistant *K. pneumoniae* in a tertiary care hospital in India; their clonal relationship and the molecular events contributing to colistin resistance.

MATERIALS AND METHODS

Bacterial Strains

Clinical samples collected at Safdarjung Hospital and directed to the Department of microbiology for routine identification from August 2017 to January 2018 were included in the study. *Klebsiella* spp. were identified and screened for colistin resistance following standard broth microdilution method using cation-adjusted Mueller Hinton Broth (HiMedia, India) with EUCAST interpretation (S: ≤2 mg/L, R: >2 mg/L) guidelines (2018). Isolates were identified by a VITEK 2 GN card and confirmed by 16S rRNA gene sequence analysis (Azam et al., 2016). Ethics clearance was obtained from the institutional ethics committee: (i) ICMR-National Institute of Pathology, New Delhi, India (IEC No: NIP-IEC/2/3/17/06) and (ii) VMMC and Safdarjung Hospital, New Delhi, India (IEC No: IEC/VMMC/SJH/Project/1028). The demographic and clinical details of the patients were obtained from electronic medical records available in the hospital intranet.

Antimicrobial Susceptibility Tests

Colistin-resistant isolates were subjected to antibiotic susceptibility testing by Kirby-Bauer disc diffusion method on Mueller Hinton Agar plates (HiMedia, India) against the ampicillin, ampicillin/clavulanic acid, amikacin, azithromycin, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, ertapenem, imipenem, tazobactam/piperacillin, tetracycline, and trimethoprim.

Minimum inhibitory concentrations (MICs) were determined by *E*-test (trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, and imipenem) and broth microdilution (chloramphenicol, ciprofloxacin, colistin, gentamicin, kanamycin, polymyxin B, rifampicin, nalidixic acid, tigecycline, and tetracycline). *E. coli* ATCC 25922 (antibiotic-susceptible), *K. pneumoniae* ATCC 700603 (ESBL-producing), and *E. coli* NCTC 13846 (colistin-resistant) isolates were used as quality control strains. Results were interpreted as per CLSI (2018) guidelines and for tigecycline, EUCAST (2018) guidelines were followed. The susceptibility profile of *K. pneumoniae* isolates was determined using the WHONET (v20.8.21) database software.

Multilocus Sequence Typing

Multilocus sequence typing (MLST) for seven housekeeping genes, *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*, was performed using the Pasteur institute MLST scheme (Diancourt et al., 2005). A total of 3,550 MLST profiles of *K. pneumoniae* available till 2nd March 2019, were extracted from Pasteur institute

MLST database.[1] Sequence types (STs) were determined by comparing the sequence of isolates against *K. pneumoniae* MLST database (Feil et al., 2004). Novel ST Profiles were submitted to the curator for assignment of new sequence type. Clustering and comparative analysis of related STs were performed using default conservative definition of sharing six of the seven loci of eBURST version 3 software.

Molecular Characterization of Genes Contributing to Colistin and β-Lactam Resistance

Lipid A modifying genes, i.e., *phoP*, *phoQ*, *pmrA*, *pmrB*, and *mgrB*, associated with colistin resistance were amplified and sequenced using gene-specific primers designed using the external region of the gene sequence (**Supplementary Table S1**). To determine the presence of plasmid-encoded colistin resistance, gene-specific primers were used to amplify *mcr-1-8* genes. *K. pneumoniae* isolates were also analyzed for the presence of the ESBL gene ($bla_{\text{CTX-M-like}}$) and carbapenemase genes ($bla_{\text{KPC-like}}$, $bla_{\text{NDM-1-like}}$, and $bla_{\text{OXA-48-like}}$), conferring resistance to a broad range of β-lactam antibiotics (primer sequence information used for the amplification of specific genes is given in **Supplementary Table S1**). Genomic DNA from *K. pneumoniae* ATCC 700603 (colistin-susceptible), *E. coli* NCTC 13846 (*mcr-1* positive), *K. pneumoniae* ATCC BAA1705 (bla_{KPC} positive), *K. pneumoniae* ATCC BAA2156 (bla_{NDM} positive), and *E. coli* MRE2 ($bla_{\text{CTX-M}}$ positive, GenBank accession # KM873162) were used as control strains.

Gene Expression Analysis by qRT-PCR

The expression level of *pmrK* (encoding L-Ara4N transferase) and *pmrC* (encoding PEtN transferase) genes were analyzed using gene-specific primers by qRT-PCR in colistin treated and untreated samples. Colistin-resistant isolates and *K. pneumoniae* ATCC 700603 were grown to the mid-log phase in cation-adjusted Mueller Hinton Broth supplemented with and without colistin sulphate (4 μg/ml; Sigma). Bacterial cells were harvested, and total RNA was extracted using RNeasy kit (Qiagen) and treated with DNaseI. cDNA synthesized from 1 μg of total RNA using RevertAid first-strand cDNA synthesis kit (Thermo Scientific) was subjected to Real-time PCR amplification in 25 μl reactions (in triplicates) containing 10 pmoL forward and reverse primers and 1x Fast SYBR green PCR master mix (Applied BioSystems) under the following conditions: 1 cycle of 95°C for 20 s, 40 cycles of 95°C for 3 s, and 60°C for 30 s. After each run, melt curve analysis was performed to ensure single amplicon production, under the conditions 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Relative gene expression levels were calculated using the $2^{\Delta\Delta CT}$ formula, and ≥2 fold change in the expression (relative to wild type) was considered the increase in expression and below ≤0.5 as repression. The *rpsL* gene (encoding ribosomal protein) was used as an internal control. Colistin-susceptible *K. pneumoniae* ATCC 700603 was used for normalization. The student's paired "*t*" test was performed for calculating the significance of differences observed in the expression levels under colistin untreated and treated conditions with the help of GraphPad Prism software version 8.0 (GraphPad software inc. CA, United States).

Analysis of Deleterious Substitution

The amino acid substitutions in the genes (*mgrB*, *phoP*, *phoQ*, *pmrA*, and *pmrB*) associated with the colistin resistance phenotype in clinical isolates were defined by comparing the query sequence against *K. pneumoniae* ATCC 700603. To predict the phenotypic effect of these substitutions on protein structure and function, these were analyzed by three different bioinformatics tools: sorting intolerant from tolerant (SIFT), polymorphism phenotyping (PolyPhen-2), and protein variation effect analyzer (PROVEAN) with default parameters (Ng and Henikoff, 2003; Adzhubei et al., 2010; Sim et al., 2012; Choi and Chan, 2015). The mutations found to be deleterious by at least two of the three analysis software were considered potentially deleterious, while mutations with low confidence intervals were treated as neutral.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of colistin-resistant *K. pneumoniae* isolates under study were deposited at a GenBank nucleotide sequence database under the following accession numbers: *16S rRNA* gene (MH411220, MH410611, MH411072–MH411080), *phoP* gene (MH424384–MH424392, MH450205, MH450206), *phoQ* gene (MH424393–MH424402, MH450207), *pmrA* gene (MH450188–MH450197), and *pmrB* gene (MH450198–MH450204, MH688167–MH688170). Nucleotide sequences of the *mgrB* genes have been deposited under GenBank accession numbers: wild type *mgrB* gene (MH424403–MH424409, MH671333) and mutated/disrupted *mgrB* gene (MK625062–MK625064).

RESULTS

A total of 335 *K. pneumoniae* were isolated from urine (n = 153), blood (n = 20), pus (n = 85), tracheal aspirate (n = 9), wound (n = 7), and other specimens (n = 61) within a period of 6 months (2017–2018) in the hospital. Of the total, 11 (3.2%) *Klebsiella* isolates representing one isolate per patient from the clinical samples viz. urine (5), pus (3), tissue (1), wound swab (1), and blood (1) were identified to be colistin-resistant. All 11 patients had a hospital stay for more than 10 days and encountered invasive devices like intravenous lines, ventilators, or catheters. These patients received colistin during or before their sample collection for microbiology culture analysis. Molecular identification confirmed the isolates as *K. pneumoniae* and MLST analysis revealed the sequence types ST14 (n = 1), ST16 (n = 1), ST43 (n = 1), ST54 (n = 1), ST147 (n = 1), and ST395 (n = 2; **Table 1**). Four of the isolates belonged to three novel ST profiles and were assigned new sequence type numbers as ST3986 (MRK1), ST3987 (MRK5 and MRK6), and ST3988 (MRK10). Comparative and clustering analysis by eBURST v3 of query dataset with a total of 3,550 already reported MLST profiles of *K. pneumoniae* suggested that all 11 isolates of the query

[1]https://bigsdb.pasteur.fr/klebsiella/klebsiella.html

dataset belonged to the most prominent and diverse clonal complex 11 (CC11). The novel sequence types ST3986, ST3987, and ST3988 had evolutionarily related sub-founders as ST16, ST43, ST39, respectively (**Figure 1**). The reference strain *K. pneumoniae* ATCC 700603 used in this study belonged to ST489 and was designated as a singleton by eBURST v3.

Antimicrobial Susceptibility Testing

All 11 tested *K. pneumoniae* isolates were resistant to cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, macrolides, and trimethoprim by disc diffusion method (**Table 1**). Three *K. pneumoniae* isolates (MRK1, MRK9, and MRK10) were resistant to all antibiotics tested by disc diffusion assay except with intermediate resistant phenotype to chloramphenicol (MRK1 and MRK10) and tetracycline (MRK9), as per CLSI standards. MIC-values for colistin and polymyxin B ranged from 8 to >128 μg/ml and 16 to >64 μg/ml, respectively. Unexpectedly, MICs of 5 (45.5%) isolates to colistin were ≥128 μg/ml (**Table 2**). MICs of amoxicillin/clavulanic acid and imipenem were >256 μg/ml and ≥8 μg/ml, respectively, for the 11 tested isolates except for MRK5 (4 μg/ml and 2 μg/ml). High MIC values were noted for kanamycin (≥256 μg/ml; n = 10), gentamycin (≥256 μg/ml; n = 6), co-trimoxazole (>32 μg/ml; n = 9), and tetracycline (>128 μg/ml; n = 5) among colistin-resistant *K. pneumoniae*.

Molecular Determinants for Drug Resistance

Sequence analysis of Lipid A modifying genes of all the 11 isolates of *K. pneumoniae* revealed non-synonymous nucleotide

TABLE 1 | Demographic, clinical, and microbiological features of 11 colistin-resistant *K. pneumoniae* isolates.

Bacterial isolate	Source, year of isolation	Hospital ward	Sequence type	Carbapenemase genes(*blaNDM-1*, *blaOXA-48*)	ESBL gene (*blaCTX-M-like*)	Non-susceptibility to antibiotics by disc diffusion assay	Susceptibility profile[b]	ICU stay, outcome
MRK1	Urine, 2017	-	ST3986[a]	*blaNDM-1*	*blaCTX-M-like*	AMP, AMC, AMK, CIP, CTX, ETP, GEN, IPM, TMP, AZM, TET, TZP	PDR	no, recovered
MRK2	Pus, 2017	Surgical ICU	ST43	*blaOXA-48*	*blaCTX-M-like*	AMP, AMC, AMK, CIP, CTX, ETP, GEN, IPM, TZP	MDR	no, recovered
MRK3	Urine, 2017	Rehabilitation	ST54	*blaNDM-1*	*blaCTX-M-like*	AMP, AMC, AMK, AZM, CIP, CTX, ETP, IPM, GEN, IPM,TMP, TZP	XDR	no, recovered
MRK4	Tissue, 2017	-	ST16	*blaNDM-1*	*blaCTX-M-like*	AMP, AMC, AMK, AZM, CIP, CTX, ETP, GEN, IPM, TMP, TET, TZP	PDR	yes, death
MRK5	Urine, 2017	Orthopedics	ST3987[a]	*blaOXA-48*	-	AMP, AMC, AMK, AZM, CIP, CTX, GEN, TZP	XDR	no, recovered
MRK6	Blood, 2017	Medical ICU	ST3987[a]	*blaNDM-1*, *blaOXA-48*	*blaCTX-M-like*	AMP, AMC, AMK, AZM, CIP, CTX, ETP, GEN, IPM, TMP, TZP	XDR	yes, death
MRK7	Urine, 2017	-	ST395	*blaOXA-48*	*blaCTX-M-like*	AMP, AMC, AZM, CIP, CHL, CTX, ETP, GEN, IPM, TMP, TZP	XDR	no, recovered
MRK8	Wound Swab, 2018	Burns	ST395	*blaNDM-1*	*blaCTX-M-like*	AMP, AMC, AMK, CIP, CTX, ETP, GEN, IPM, TET, TMP, TZP	PDR	yes, death
MRK9	Urine, 2018	Obstetrics and gynecology	ST14	*blaNDM-1*	*blaCTX-M-like*	AMP, AMC, AMK, AZM, CIP, CHL, CTX, ETP, GEN, IPM, GEN, TMP	XDR	no, recovered
MRK10	Pus, 2018	Burns	ST3988[a]	*blaNDM-1*, *blaOXA-48*	-	AMP, AMC, AMK, AZM, CIP, CTX, ETP, GEN, IPM, TMP, TET, TZP	PDR	yes, death
MRK11	Pus, 2018	Medical ICU	ST147	*blaNDM-1*	*blaCTX-M-like*	AMP, AMC, AMK, CIP, CTX, ETP, GEN, IPM, TMP, AZM, TET, TZP	XDR	yes, recovered

Three-letter abbreviation code and amount of antibiotic (μg) in the disc: AMP, ampicillin (10 μg); AMC, ampicillin/clavulanic acid (20/10 μg); AMK, amikacin (30 μg); AZM, azithromycin (15 μg); CTX, cefotaxime (30 μg); CHL, chloramphenicol (30 μg); CIP, ciprofloxacin (5 μg); GEN, gentamicin (10 μg); ETP, ertapenem (10 μg); IPM, imipenem (10 μg); TZP, tazobactam/piparacillin (100/10 μg); TET, tetracycline (30 μg); and TMP, trimethoprim (5 μg).

[a]*Novel sequence type identified in this study. MLST analysis was performed following Pasteur institute MLST database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).*

[b]*Susceptibility profile as determined by WHONET (v20.8.21) database software. MDR: multidrug resistant; PDR- pan-drug resistant, XDR- extensively drug resistant.*

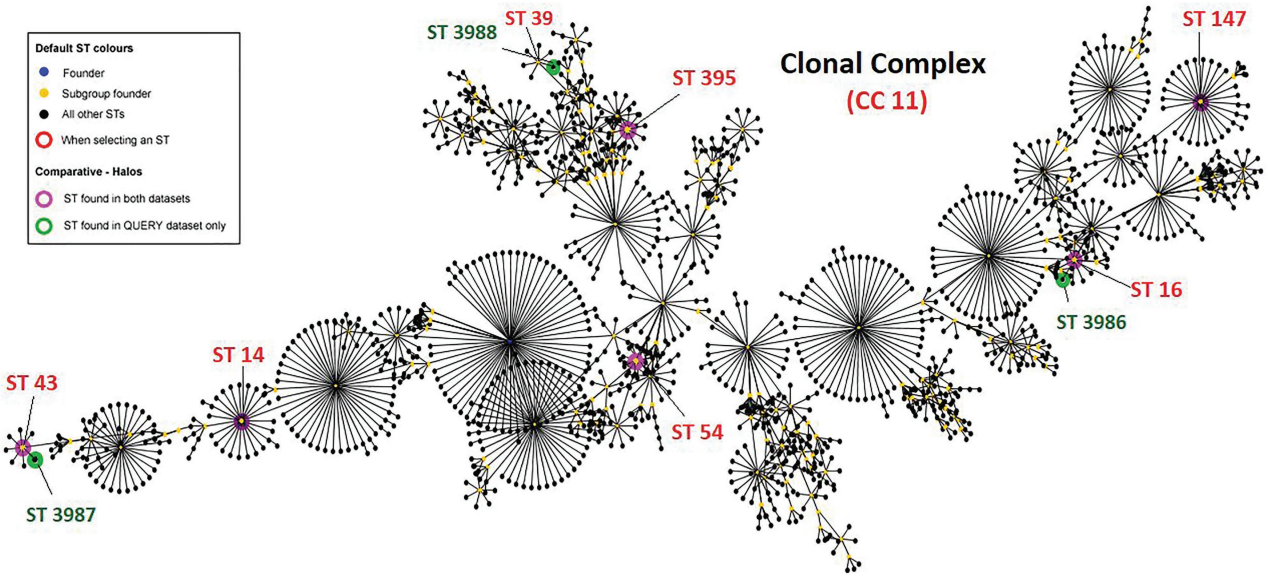

FIGURE 1 | Clonal complex CC11 with 1,562 sequence types. Green halos represents novel sequence types found in this study and submitted to curator of *Klebsiella pneumoniae* MLST database Pasteur institute (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html), pink halos represent isolates with defined sequence types. *K. pneumoniae* ATCC 700603 is ST489 and designated as singleton by eBURST v3 and hence not shown here.

TABLE 2 | Minimum inhibitory concentration (MIC) in μg/ml of different antibiotics tested by *E*-test and broth microdilution method for 11 *K. pneumoniae* isolates.

Antibiotic name	Breakpoints	%R	%I	%S	MIC50	MIC90	MIC range
Amoxicillin/ Clavulanic acid*	S ≤ 8 R ≥ 32	90.9	0	9.1	>256	>256	4 to >256
Imipenem*	S ≤ 1 R ≥ 4	90.9	9.1	0	24	32	2 to >256
Gentamicin	S ≤ 4 R ≥ 16	100	0	0	256	>256	128 to >256
Kanamycin	S ≤ 16 R ≥ 64	100	0	0	256	>256	64 to >256
Rifampin	S ≤ 1 R ≥ 4	100	0	0	128	256	4 to >128
Nalidixic acid	S ≤ 16 R ≥ 32	100	0	0	256	>256	64 to >256
Ciprofloxacin	S ≤ 1 R ≥ 4	100	0	0	8	32	8 to >256
Trimethoprim/ Sulfamethoxazole*	S ≤ 2 R ≥ 4	81.8	0	18.2	64	64	0.064 to >32
Colistin	S ≤ 2 R ≥ 8	100	0	0	64	256	8 to >128
Polymyxin B	S ≤ 2 R ≥ 8	100	0	0	64	128	16 to >64
Chloramphenicol	S ≤ 8 R ≥ 32	63.6	18.2	18.2	32	256	4 to 256
Tetracycline	S ≤ 4 R ≥ 16	54.5	0	45.5	16	256	1 to >128
Tigecycline	S ≤ 1 R ≥ 4	63.6	9.09	27.2	0.5	1.5	0.25 to >256

Susceptibility results were interpreted as per CLSI (2018) guidelines and for tigecycline EUCAST (2018) guidelines were followed. *R, resistant; I, intermediate; S, susceptible.* **E-test was performed to determine the MIC values.*

mutations at two different positions of the *phoP* gene, 18 positions of the *phoQ* gene, nine positions of the *pmrA* gene, 37 positions of the *pmrB* gene, and two positions of the *mgrB* gene. Of these mutations, one in *phoP*, four in *phoQ*, one in *pmrA*, eight in *pmrB*, and one in *mgrB* were found to be potentially deleterious (**Table 3**). The protein sequence of *phoP* genes of all colistin-resistant *K. pneumoniae* isolates showed no difference with the wild type strain (*K. pneumoniae* ATCC 700603) except for MRK9 strain that exhibited T151A substitution. *phoQ* gene was mutated in two strains, i.e., MRK8 (L30Q) and MRK11(A351D), whereas, MRK5 and MRK6 isolates exhibited deletion of four and two amino acids at 87–90 and 267–268 positions, respectively. All other isolates exhibited neutral changes compared to sensitive strain at *phoQ* locus.

The insertional inactivation of the *mgrB* gene was observed in three isolates, i.e., MRK1, MRK3, and MRK4. IS*1* family transposase was observed in *K. pneumoniae* MRK1 strain. In *K. pneumoniae* MRK3, *mgrB* gene was interrupted with a 1,066 bp fragment composed of the IS*5*/IS*1182* family transposase gene, non-coding sequence, and nine base pair inverted repeats (ACCAGGATG). An insertion sequence of 768 base pairs comprising IS*1* family transposase and a non-coding fragment was observed interrupting the *mgrB* gene of *K. pneumoniae* MRK4 strain. A base change T to G at position 82, leading to

TABLE 3 | List of amino acid substitutions or disruptions among *mgrB*, *phoP*, *phoQ*, *pmrA*, and *pmrB* proteins among 11 colistin-resistant *K. pneumoniae* isolates.

Bacterial strain	Colistin MIC (μg/ml)	Amino acid substitutions[#]				
		mgrB	***phoP***	***phoQ***	***pmrA***	***pmrB***
MRK1	128	IS Insertion (IS*1*-like element, position 116–117)	-	-	-	G250C A252G
MRK2	64	-	-	-	-	D150V
MRK3	>128	IS Insertion (IS*5*/IS*1182*-like element, position 94–95)	-	-	-	-
MRK4	>128	IS Insertion (IS*1*-like element, position 116–117)	-	-	-	G250C
MRK5	128	-	-	Deletion (87–90)	-	L237R G250C A252G H267P R315P Q331H
MRK6	64	-	-	Deletion (267–268)	-	A252G
MRK7	32	-	-	-	-	- H267P
MRK8	8	-	-	L30Q	-	-
MRK9	32	C28G	T151A	-	-	T157P
MRK10	16	-	-	-	G53S -	-
MRK11	8	-	-	A351D	-	-

-, no deleterious mutation observed. [#]SIFT, PolyPhen-2, and PROVEAN were used to determine the nature (neutral or deleterious) of amino acid substitutions. Mutations that were found to be potentially deleterious with at least two of the three software's used were taken into account. Mutations showing low confidence intervals were treated as neutral.

an amino acid substitution of C28G being observed in the *mgrB* gene of *K. pneumoniae* MRK9, which was found to be deleterious.

Analysis of *pmrA/pmrB* genes regulating the expression of *arnBCADTEF* operon decorating LPS showed amino acid changes at five positions in *pmrA* gene of four different isolates, none being deleterious except for MRK-10 exhibiting G53S. The *pmrB* gene was mutated in most isolates with a varying number of potentially deleterious mutations ranging from one mutation in MRK2, MRK6 and MRK9 to six deleterious mutations in MRK5 strain. In total, 23 neutral changes and eight potentially deleterious changes were observed in the *pmrB* gene. L30Q substitution of *phoQ* and D150V substitution of *pmrB* were observed to be highly intolerant under PROVEAN, SIFT, and PolyPhen-2 scoring criteria. Plasmid-encoded colistin resistance genes (*mcr-1-8*) did not amplify in any of the tested colistin-resistant *K. pneumoniae* isolates.

Colistin-resistant *K. pneumoniae* isolates resistant to carbapenems showed positive amplification of $bla_{\text{NDM-1-like}}$ and $bla_{\text{OXA-48-like}}$ gene in eight and five isolates, respectively. Furthermore, ESBL encoding $bla_{\text{CTX-M-like}}$ gene was found in six isolates, and one of the isolates, MRK6, showed positive amplification for three β-lactamase ($bla_{\text{NDM-1-like}}$, $bla_{\text{OXA-48-like}}$, and $bla_{\text{CTX-M-like}}$) genes (**Table 1**).

Expression of *pmrK* and *pmrC* Genes

The fold change in the expression level of *pmrC* and *pmrK* genes is given in **Figure 2**. All the colistin-resistant *K. pneumoniae* isolates exhibited increased transcript levels of *pmrC* gene both in the presence or absence of colistin sulphate. The fold change in *pmrC* expression ranged from 2.7 ± 1.29 to 226.37 ± 118.63 when bacteria was subcultured with 4 μg/ml of colistin sulphate and 13.76 ± 4.091 to 404.71 ± 116.25-fold under untreated conditions. MRK4, MRK8, and MRK10 exhibited a comparable level of *pmrC* expression in both treated and untreated conditions (p = >0.05) whereas two isolates (MRK1 and MRK2) showed more than 2-fold increase in expression ($p < 0.05$) upon colistin treatment, and the remaining six isolates exhibited a decrease in *pmrC* expression from 1.5- to 5-fold ($p < 0.05$) upon colistin treatment. The expression level of *pmrK* gene under untreated condition was comparable to colistin-susceptible isolate in seven isolates (MRK1, MRK2, MRK3, MRK5, MRK6, MRK7, and MRK8), high (3.42 ± 1.171 to 21.67 ± 6.87 fold) in three isolates and down in isolate MRK11. Upon colistin treatment, the *pmrK* expression increased by 1.63- to 36.9-fold in seven isolates (MRK1, MRK2, MRK6, MRK7, MRK9, MRK10, and MRK11; $p < 0.05$) and was comparable in three isolates except for MRK4 where it decreased approximately 3-fold.

DISCUSSION

The rise in carbapenem-resistant *K. pneumoniae* isolates has enforced the increased application of polymyxin based therapies (mono and combination), ensuing in the development of colistin- and carbapenem-resistant *K. pneumoniae* strains. Reports of colistin resistance are emerging from different countries (Monaco

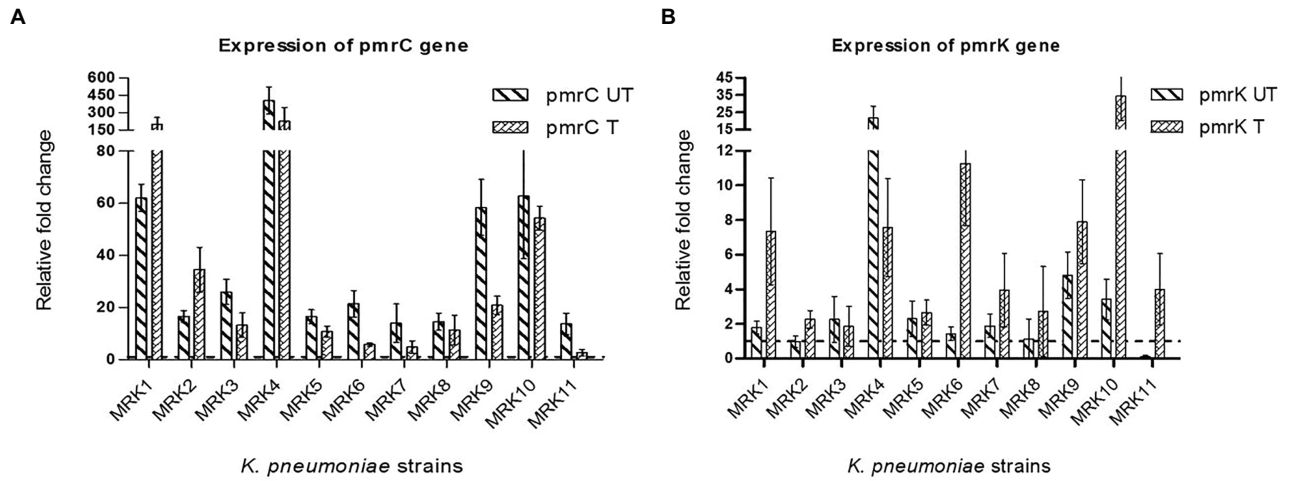

FIGURE 2 | Fold changes ±SD in the expression of *pmrC* and *pmrK* genes among colistin-resistant *K. pneumoniae* isolates (n = 11) with respect to colistin-susceptible *K. pneumoniae* ATCC 700603 isolate are represented here. The *rpsl* gene was used as internal control. All the reactions (both under colistin treated and untreated conditions) were normalized using colistin-susceptible *K. pneumoniae* ATCC 700603 (colistin untreated- wild type). **(A)** relative fold-change of *pmrC* gene expression under colistin treated (*pmrC* T) and untreated conditions (*pmrC* UT); **(B)** relative fold-change of *pmrK* gene expression under colistin treated (*pmrK* T) and untreated conditions (*pmrK* UT). Values given are mean ± SD of three different experiments with qRT-PCR reactions performed in triplicate.

et al., 2014; Rojas et al., 2017; Boszczowski et al., 2019). Our data showed 3.2% (11/335) of *K. pneumoniae* as colistin-resistant with minimal clonal relatedness. All 11 *K. pneumoniae* isolates belonged to same most prominent and diverse clonal complex-11 with sequence types (STs): 14, 16, 43, 54, 147, and 395, whereby four isolates conferred three novel ST (3986, 3987, and 3988) profiles. Although isolates MRK5 and MRK6 belonged to ST3987 and isolates MRK7 and MRK8 belonged to ST395, all four were isolated from patients of different wards of the hospital. The PubMed search for published literature identified limited studies describing the molecular mechanism of colistin resistance among *K. pneumoniae* isolates in India and abroad (**Table 4**). A thorough analysis showed that compared to other global studies, *K. pneumoniae* of very different STs conferred colistin resistance in India. However, Mavroidi et al. (2016) demonstrated colistin resistance due to clonal spread of KPC-producing *K. pneumoniae* belonging to clonal complex CC258 in Greece. Moubareck et al. (2018) showed 59% of colistin resistance in carbapenem-resistant *K. pneumoniae* was associated with locally prevalent ST14 clone in Dubai. High MIC values for colistin and carbapenems in *K. pneumoniae* causing bloodstream infections were observed in different studies from India (Ramesh et al., 2017; Aggarwal et al., 2018). *K. pneumoniae* resistant to both colistin and carbapenems have also been reported to be associated with increased risk of in-hospital mortality from India (Kaur et al., 2017; Aggarwal et al., 2018) and other parts of the world (Giacobbe et al., 2015; Rojas et al., 2017).

In this study, *in vitro* antimicrobial susceptibility profiling and WHONET analysis categorized four *K. pneumoniae* isolate as pan drug-resistant and six as extensively drug-resistant (XDR) as per the definition by Magiorakos et al. (2012). Additionally, these isolates fall to the category of difficult-to-treat Gram-negative infections, where resistance to aminoglycosides emphasizes the difficulty of choosing salvage antibiotics for clinical containment (Kadri et al., 2018). The mortality in the present study was 36.4%, where only 7 out of 11 patients could recover. Three of the four patients deceased were infected with pan-drug resistant (PDR) *K. pneumoniae*. Among the 11 study isolates, positive amplification of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ carbapenemase genes was found in 8 (73%) and 5 (45%) of the isolates, and two isolates were positive for both $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$. Colistin-resistant *K. pneumoniae* with PDR and XDR phenotype co-producing $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ carbapenamases have been reported to cause severe nosocomial infections in several countries (Guducuoglu et al., 2018; Moubareck et al., 2018; Haller et al., 2019).

In *K. pneumoniae*, the positively charged groups 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN) mediate the covalent modifications of lipid A moiety of LPS reducing the net negative charge and subsequently binding affinity of colistin (Tamayo et al., 2005a). Mutations in TCRSs and/or genetic alteration in the *mgrB* gene, the negative regulator of *phoP*/*phoQ* TCRS can cause constitutive expression of the *pmrHFIJKLM* and *pmrCAB* operons transferring the L-Ara4N and PEtN respectively, to lipid A of the cell membrane (Tamayo et al., 2005b). Along with six potentially deleterious mutations in *pmrB* gene, a deletion of 12 nucleotides resulting in the omission of four amino acids in the phospho-transfer domain of *phoQ* gene was observed in *K. pneumoniae* MRK5 strain tolerating 128 μg/ml of colistin. Marina et al. also reported similar deletions in the *phoQ* gene reducing the colistin susceptibility (Marina et al., 2001). MRK7 strain also demonstrated a MIC of 128 μg/ml for colistin having potentially deleterious substitution (H267P) in *pmrB* gene, underlining its significance. L30Q substitution in *phoQ* gene of MRK8 was similar to the earlier report (Cheng et al., 2015). The L30Q

TABLE 4 | Studies performed in India and abroad to understand the molecular mechanisms of colistin resistance among *K. pneumoniae* isolates.

Reference	Sequence Type (ST)[a]	WGS/ targeted sequencing	Mutations observed in colistin-resistant isolates[b]			Transcript expression analysis of LPS modifying genes	*mcr-1 to 10* genes
			phoPQ	***mgrB***	***pmrAB***		
Studies from India							
This study (*n* = 11)	14,16, 43, 54, 147, 395, 3,986, 3,987, 3,988	Targeted sequencing	*phoP* (T151A) *phoQ* (L30Q, A351D), Deletion (87–90, 267–268)	*mgrB* (C28G) Insertion by IS*1*, IS*5*/ IS*1182*-like elements	*pmrA* (Q140L[$], G53S) *pmrB* (G250C, A252G,D150V, L237R, H267P, R315P, Q331H, R256G, T157P[#])	*pmrC*-↑ *pmrK*-↑	negative
Palani et al. (2020) (*n* = 25)	-	Targeted sequencing	-	*mgrB* (C88T) Insertion by IS*Kpn14*, IS*Kpn26*-like elements, complete deletion	-	-	negative
Shankar et al. (2019a) (*n* = 1)	2,957	WGS	*phoP* (G273C) *phoQ* (P424L)	-	*pmrB* (A774T)	-	negative
Shankar et al. (2019b) (*n* = 19)	23, 147, 86, 11, 231, 14, 2096, 2,957	Targeted sequencing	*phoP* (A114R[@], T151A[@], E22K) *phoQ* (L209C[@], W161L, G117D, V370E[@], L172Q[@], W182S[@], V444F[@], P424L, V446W[@])	*mgrB* (C28G[@], M1R[@]) Insertion by IS*903*, IS*Kpn14* and IS*Kpn26* elements	-	-	negative
Mathur et al. (2018) (*n* = 8)	11, 14, 231	WGS	*phoP* (R114A) *phoQ* (D150G)	*mgrB* (V1A, L24H)	*pmrB* (D150H, R256G[$], L344P, T157P[#], A246T)	-	negative
Pragasam et al. (2017) (*n* = 8)	14, 147, 231	WGS	*phoP* (R114A, R128A) *phoQ* (D146G, D150G)	*mgrB* -deletion of A at position 10, premature stop codon	*pmrB* (T157P[#], A245T, R256G[$], L344P)	-	negative
Studies across the world							
Cheong et al. (2019), Korea (*n* = 13)	11,461, 3,217	Targeted sequencing	*phoP* (R198H, K189D) *phoQ* (N152D, L414R)	-	*pmrA* (R203K) *pmrB* (N150D)	-	negative
Zhu et al. (2019), Greece (*n* = 8)	258, 147,	WGS	*phoP* (V53G[$]) *phoQ* (N253P, D438H, T439P).	*mgrB* insertion by IS*Kpn26*-like elements	*pmr*B (T140P[$]) *pmr*C (E307Stop[$])	-	-
Zafer et al. (2019), Egypt (*n* = 22)	11, 101, 147, 16, 37, 383, 785, 1,399	Targeted sequencing	-	*mgrB* (P178Y)	-	-	*mcr-1* positive
Boszczowski et al. (2019), Brazil (*n* = 6)	11, 23, 340, and 437	WGS	*phoQ* (G150D)	*mgrB* (M1V, N25K, V26E, M27G, C28A, D29Y, I45R, P46L, W47F, N42L, K43D, F44P, I45P, P46S)	*pmrA* (T245A, R255G, P345L)	-	negative
Pitt et al. (2018), Brazil (*n* = 19)	147, 258, 11	WGS	*phoP* (A95S, P74L) *phoQ* (N253T, T281M[#], G385C[#], V446G)	*mgrB* (C28STOP[#], Q30STOP[#], D29E[#]) Insertion by IS*Kpn13*, IS*1*, IS*Kpn26*-like elements	*pmrB* (T140P, P158R[#])	-	negative
Lomonaco et al. (2018), Pakistan (*n* = 10)	11, 14, 15, 101	WGS	-	Insertion by IS*Kpn25*, IS*1*, IS*5* elements	*pmrB* (T93P, N110T, T112P, T127P, T128P, L130P, L141P, V151G, T157P[#], L159P, L164P, L213M, A246T, R256G[$])	-	negative

(Continued)

TABLE 4 | Continued

Reference	Sequence Type (ST)[a]	WGS/ targeted sequencing	Mutations observed in colistin-resistant isolates[b]			Transcript expression analysis of LPS modifying genes	*mcr-1 to 10* genes
			phoPQ	***mgrB***	***pmrAB***		
Lu et al. (2018), West China (*n* = 5)	23, 412, 660 and 700	WGS	- *phoQ* (D150G)	-	*pmrB* (P344L)	-	*mcr-1* positive
Cain et al. (2018), UK (*n* = 1)	-	WGS	*phoQ* (K46Q[#])	-	-	-	-
Leung et al. (2017), USA (*n* = 22)	17, 37, 258	WGS	-	*mgrB* (Q30R, Q30stop) Insertion by IS*Kpn26*-like, IS*903B*- like elements	*pmrB* (S85R, T157P[#], H340R) *pmrF* (F280L, K322Q) *pmrJ* (E25A, R29K, I53V, L94I) *pmrK* (I117V, H156Q, D441E)	-	-
Jaidane et al. (2018), Tunisia (*n* = 13)	101, 15, 11, 147, 392	WGS	-	*mgrB* (F28C[#]), insertion by IS*1*-like elements	*pmrA* (A217V) *pmrB* (T246A, R256G[$], T157P[#])	-	negative
Haeili et al. (2017), Iran (*n* = 20)	-	Targeted sequencing	-	*mgrB* (Q30stop[#], C39stop[#]) Insertion by IS5-like and IS*1*-like elements	*pmrB* (A246T, L213M, R256G[$])	*pmrC*-↑ *pmrK*-↑	negative
Halaby et al. (2016), Netherlands (*n* = 13)	43, 1,423	WGS	*phoQ* (A21S[#])	Insertion by IS*3*-like and IS*Kpn14* like elements	-	-	-
Cheng et al. (2015), Taiwan (*n* = 26)	11, 15, 29, 48, 421	Targeted sequencing	*phoP* (V3F, S86L) *phoQ* (L26P[#], A150G, V258F)	*mgrB* (Stop48Y[#])	*pmrB* (R256G[$])	*pmrH*-↑ *pmrK*-↑ *mgrB*-↓	-
Jayol et al. (2015), South Africa (*n* = 1)	-	Targeted sequencing	*phoP* (N191Y[#])	-	-	*phoP*-↑ *phoQ*-↑ *pmrD*-↑ *pmrC*-no change, *pmrA*-no change, *pmrB*-no change, *pmrK*-↑	-

↑, *Increase in transcript expression level;* ↓, *decrease in transcript expression level; -, not detected/analyzed; n, Number of colistin-resistant isolates analyzed for molecular mechanisms of resistance.*

[$]*Mutations observed in colistin-susceptible as well as colistin-resistant isolates.*

[#]*Experimental evidence for their role in imparting colistin resistance.*

[@]*Deleterious role on protein function predicted by bioinformatics tools.*

[a]*Sequence types of colistin-resistant K. pneumoniae.*

[b]*Genes analyzed in the study are included in the table, and associated mutations identified in a respective gene are given in the brackets.*

substitution in the hydrophobic domain (constituting 17–44 amino acid residues) of *phoQ* influences the protein conformation and oligomer stability potentially changing the phosphate transfer and the phosphatase ability (Goldberg et al., 2010). In two of *K. pneumoniae* isolates (MRK1 and MRK4), point mutation A170G was detected in the *pmrA* gene resulting in amino acid change (E57G) potentially deleterious while R256G in *pmrB* of MRK7 predicted not to cause any functional change in the protein function (Pragasam et al., 2017). The novel G250C potentially deleterious substitution in the *pmrB* gene was observed in three of *K. pneumoniae* isolates (MRK1, MRK4, and MRK5) that exhibited high MIC (≥128 μg/ml) for colistin. Several other deleterious substitutions in the *pmrB* (D150V, A252G, L237R, H267P, R315P, and Q331H) genes with their scores in the intolerant and potentially intolerant range are being reported here for the first time. These amino acid substitutions (L237R, A252G, H267P, R315P, Q331H, except D150V) were observed in a single isolate (MRK5) along with a four amino acid deletion in *phoQ* gene, emphasizing the need for further evaluation for their role in colistin resistance. Predicted deleterious substitution *pmrA*-Q140L (found in all 11 test isolates) and *pmrB* R256G (present in MRK7) have been previously reported in colistin-resistant as well as susceptible isolates negating its decisive role in colistin resistance (Pitt et al., 2018). However, the other mutations observed here, L30Q and A351D substitutions in the *phoQ* gene of MRK8 and MRK11 strains, respectively, and G53S in the *pmrA* gene of MRK10 and D150V and H267P in the *pmrB* gene of the MRK2 and MRK7 strain, respectively, may impart resistance in the absence of any other contributing factor.

The *mgrB* gene-mediated inactivation of the *phoP/phoQ* TCRS has been extensively reported to play a prominent role in polymyxin resistance in *K. pneumoniae* (Cannatelli et al., 2014b; Poirel et al., 2017). Three of 11 *K. pneumoniae* isolates MRK1, MRK3 and MRK4 had insertion inactivation of *mgrB* gene disrupting the translation of a functional protein, along with mutations in *pmrA* and *pmrB* genes showing high tolerance to colistin (MIC ≥128 μg/ml). The role of IS elements (IS*5*-like, IS*1F* and IS*Kpn14*, IS*Kpn13*, and IS*10R*) in *mgrB* inactivation that inhibits *phoQ* phosphorylation resulting in increased expression of *pmrHFIJKLM* mRNA and leading to reduced colistin susceptibility in *K. pneumoniae* have been extensively demonstrated (Cannatelli et al., 2014a; Poirel et al., 2017; Zhu et al., 2019). *K. pneumoniae* MRK1 and MRK4 were interrupted by an IS*1*-like transposase elements. The inactivation of *mgrB* protein with a similar IS*1*-like element interrupting at the same nucleotide position (117–118) was observed in *K. pneumoniae* recovered from a dead broiler from Iran (Pishnian et al., 2019). The *mgrB* gene in *K. pneumoniae* MRK3 isolate was disrupted by 969 bp long IS*5*/IS*1182*-like element at a different position. IS*5*/IS*1182* fragment is present in various plasmids isolated from different Gram-negative bacteria (Hala et al., 2019). Cannatelli et al. (2013) and Poirel et al. (2015) have reported insertional inactivation of *mgrB* gene with a similar IS*5*-like insertion sequence at 74–75 nucleotide position; however, the IS*5*-like element reported in MRK3 isolate of this study showed a different interruption of the nucleotide sequence at position 86–87. To the best of our knowledge, this insertional inactivation of *mgrB* gene by the IS*5*/IS*1182* fragment has not been reported before. Isolate MRK7 had M23R substitution having SIFT score of 0 with low confidence, PROVEAN score of −3.121 depicting intolerance and is found to be neutral with PolyPhen-2 hence treated as neutral while MRK9 had C28G substitution also observed by Shankar et al. (2019b), which shows highly intolerant tendency (**Table 4**). The remaining seven isolates had an intact wild type *mgrB* gene. Mutations observed in this study in the *pmrB* (T157P) and *mgrB* (C28Y) gene have been reported in previous studies to be linked with reduced colistin susceptibility in *K. pneumoniae* (Cannatelli et al., 2014a, 2015; Jayol et al., 2014; Lomonaco et al., 2018; Mathur et al., 2018). Isolates harboring IS*Kpn26*-like and other IS elements disrupting *mgrB* also exhibited mutations in *pmrA* and *pmrB* genes (**Table 4**).

To establish a correlation between lipid A modifying operon (*pmrCAB* and *pmrHFIJKLM*) expression pattern and colistin resistance among the test isolates, the expression level of *pmrK* and *pmrC* genes was evaluated. All isolates showed an over-expressed *pmrC* gene in comparison to the *pmrK* gene, implying that PEtN-mediated LPS modification plays a significant role in conferring colistin resistance in tested isolates. Among colistin-resistant *K. pneumoniae* isolates, fold-change increase in expression of the *pmrC* gene was higher in 6/11 (54%) isolates that decreased significantly upon colistin treatment ($p < 0.05$), though overall in both the conditions that level of expression of *pmrC* was higher compared to colistin sensitive isolate. For three isolates, the expression in both colistin treated and untreated conditions did not differ significantly, while the expression of *pmrC* increased significantly upon colistin treatment in two isolates.

The basal expression level of *pmrK* gene in 6/11 (54%) colistin-resistant *K. pneumoniae* isolates was similar to colistin sensitive isolate and showed significantly higher expression in three isolates. The expression of *pmrK* increased significantly upon colistin treatment in 7/11 (63%) isolates, remained unaltered in 3/11 (27%) isolates and repressed in one isolate MRK4. Isolate MRK11 exhibited repressed expression of *pmrK* in untreated condition exhibited >30 fold increase in the expression of *pmrK* gene upon colistin treatment. This isolate harbored deleterious change in *phoQ* gene that upon activation may increase phosphorylation of *phoP* activating transcription of *pmrHFIJKLM* operon resulting in increased *pmrK* expression. A similar increase in the expression of *pmrC* gene as operon regulators has been reported previously (Cheng et al., 2015; Haeili et al., 2017). T157P substitution in *pmrB* (observed in MRK9 isolate) has been shown to contribute to the overexpression of *pmrCAB* and *pmrHFIJKLM* operons with the *pmrC* being highly overexpressed (170-fold) compared to *pmrK* (40-fold; Jayol et al., 2014). The majority of the tested isolates (8/11) harbored *pmrB* gene with several mutations possibly contributing to constitutive expression of *pmrA* gene that may preferentially activate *pmrCAB* promoter compared to *pmrHFIJKLM* resulting in increased expression of the *pmr*C gene in these isolates. Similar to previous reports, no direct correlation between colistin exposure and gene upregulation was observed (Can et al., 2018). A single mechanism may not explain the role of *pmrC* and *pmrK* in mediating colistin resistance in *K. pneumoniae*, as we observed diverse strain-specific mutations that may affect the expression of these operons differentially. Our data indicates discrete emergence of colistin resistance in clinical isolates through strain-specific pathways where multiple mechanisms might be involved in resistance development.

CONCLUSION

All 11 colistin and carbapenem-resistant *K. pneumoniae* isolates under study were distinct (with nine different ST types) and present the emergence of discrete colistin resistance mechanisms. The role of *mgrB* gene as a hot-spot for insertion inactivation and its functional loss associated with colistin resistance was observed in three isolates. Multiple mutations in regulatory genes (*phoP*, *phoQ*, *pmrA*, *pmrB*, and *mgrB*) and their association in the expression pattern of LPS decorating operons raises the colistin MIC values. The 11 test isolates were negative for the mobilized colistin resistance genes *mcr-1-8*, however, the occurrence of other *mcr* gene variants not investigated here could not be excluded as a contributory factor towards increased colistin MIC values. The clinical and public health concerns in a background where the pipeline for new antibiotics is limited, demand implementation of antimicrobial stewardship and infection control measures to prevent the spread of resistant bacteria in the health care settings. The upshot of the exorbitant and lavish use of antibiotics has developed severe concern regarding multidrug-resistant bacteria, especially nosocomial pathogens that necessitates the rational use of colistin as a last-resort antibiotic.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ICMR-National Institute of Pathology, New Delhi, India (IEC No: NIP-IEC/2/3/17/06) and VMMC and Safdarjung Hospital, New Delhi, India (IEC No: IEC/VMMC/SJH/Project/January/2018/1028). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, writing – review and editing: MA, RS, and RG. Data curation: MA, GY, AS, and KU. Formal analysis: MA, GY, AS, KU, and MJ. Funding acquisition: MA and RS. Investigation: RS and RG. Validation: RS, RG, and MJ. Writing – original draft: MA and GY. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.609840/full#supplementary-material

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METHODS
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Evaluation of the Clinical Systems for Polymyxin Susceptibility Testing of Clinical Gram-Negative Bacteria in China

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Objectives: The performance of mainstream commercial antimicrobial susceptibility testing systems on polymyxins has not been well evaluated in China. In this study, three antimicrobial susceptibility testing systems were evaluated for polymyxin B and colistin.

Methods: The MICs of 257 Gram-negative strains collected from clinical cases and livestock were determined and analyzed. Using Broth Microdilution as the gold standard, the performance of VITEK 2® COMPACT, Phoenix™ M50, and Bio-kont AST System were evaluated. Essential agreement (EA), category agreement (CA), very major error (VME), and major error (ME) were calculated for comparison. The results of *mcr-1* positive strains were separately discussed.

Results: The EA, CA, VME, and ME to polymyxin B for Bio-kont were 83.5, 95.6, 13.1, and 0.6%, respectively. The EAs, CAs, VMEs, and MEs to colistin were as follows: Bio-kont, 86.7%/96.5%/7.2%/1.7%; Vitek 2, 64.2%/86.8%/41.0%/0%, and Phoenix M50, 92.9%/92.9%/21.7%/0%. The performance of Bio-kont to polymyxin B and colistin for *Pseudomonas* spp. (EA, CA < 90%, VME > 1.5%, ME = 5.6%/10%) and *Enterobacter* spp. (EA, CA < 90%, VME > 1.5% and ME = 0%), Vitek to colistin for most genera, and Phoenix to colistin for *Enterobacter* spp. (EA, CA < 90%, VME > 1.5%, ME = 0%) were unsatisfactory compared with other genera. The performance of Bio-kont to polymyxins for *Escherichia* spp. and Phoenix to colistin for *Citrobacter* spp., *Escherichia* spp., and *Klebsiella* spp., which all met the CLSI standard, were satisfactory. When the susceptibility of *mcr-1* positive *E. coli* was tested, Bio-kont and Phoenix M50 presented excellent performance with no category errors, while Vitek 2 performed a high VME (25.5%).

Conclusion: With relatively more accurate results for polymyxin B and colistin and lower VME, Bio-kont has an advantage in polymyxin antimicrobial susceptibility testing, especially for *Escherichia* spp., *Klebsiella* spp., *Citrobacter* spp. and *Acinetobacter* spp.

Keywords: antimicrobial susceptibility testing, polymyxin, essential agreement, category agreement, very major error, major error

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INTRODUCTION

Polymyxin B and polymyxin E (colistin) are components of antibiotics produced by fermentation of *Paenibacillus polymyxa*. Despite the high incidence of nephrotoxicity and neurotoxicity associated with these agents, the family of polymyxins includes A, B, C, D, and E (Nation et al., 2014). Polymyxins have gained more attention in clinical practice due to the high susceptibility of multidrug-resistant (MDR) Gram-negative bacteria. Colistin and polymyxin B differ by just one amino acid in the peptide ring (Nation et al., 2014). Furthermore, polymyxin B is administered as an active form, while colistin in the form of an inactive prodrug, colistin methanesulfonate (CMS), which is also known as colistimethate (Tran et al., 2016).

Colistin or polymyxin B was first used in the 1950s for treating infections caused by Gram-negative MDR pathogens. These antibiotics fell out of favor and were replaced by agents with wider therapeutic indexes and less toxicity (Loho and Dharmayanti, 2015). The polymyxin class has recently re-emerged as a last-line agent in the treatment of multi-drug resistant pathogens non-susceptible to fluoroquinolones, aminoglycosides, and beta lactams (Bialvaei and Samadi Kafil, 2015). However, limited experience and lack of reliable consensus guidelines could potentially result in inappropriate use of these last-line antibiotics by clinicians.

As resistance to polymyxins has posed a great challenge in the treatment of infectious diseases, rapid and accurate detection of polymyxin-resistant strains is needed to prevent and control the outbreak of resistant strains. At present, the broth microdilution (BMD) method is the standard reference method for antimicrobial susceptibility testing (AST). However, the whole procedure is cumbersome and the testing requirements are strict, so it is rarely performed manually in clinical practice. At present, VITEK 2® COMPACT (BioMérieux) and Phoenix™ M50 (Becton Dickson Diagnostics) are the most commonly used AST systems in China. However, these systems have unsatisfactory performance when utilized to determine polymyxin susceptibilities. The purpose of this study is to evaluate the performance of three mainstream antimicrobial susceptibility systems in China on colistin and polymyxin B and to select the most accurate one.

MATERIALS AND METHODS

Strains

A total of 257 non-repetitive Gram-negative strains were collected from clinical cases (202 *mcr-1* negative strains) and livestock (55 *mcr-1* positive strains) in China with different polymyxin MICs, including *Escherichia* spp. (n = 136), *Klebsiella* spp. (n = 27), *Citrobacter* spp. (n = 23), *Enterobacter* spp. (n = 25), *Acinetobacter* spp. (n = 23), and *Pseudomonas* spp. (n = 23). All strains were identified by the Vitek MS MALDI-TOF (BioMérieux) system.

Antimicrobial Susceptibility Testing

BMD, the gold-standard reference method for AST, was performed strictly in accordance with the CLSI M7-A10 document (Clinical and Laboratory Standards Institute, 2015). The minimum inhibitory concentrations (MICs) of colistin or polymyxin B were measured only when the growth control was acceptable. *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as quality controls. The performance of three commercial methods, including VITEK 2® COMPACT (BioMérieux) with AST-N335 card (colistin), Phoenix™ M50 (Becton Dickson Diagnostics) with NMIC-413 card (colistin), and Bio-kont AST System (Wenzhou Bio-kont) with polymyxin AST card (colistin and polymyxin B) were evaluated. The AST results of each test strain were considered accurate only when the MIC results of quality control strains were in the QC range.

Data Analysis

MICs were analyzed according to the CLSI M100-E30 document (CLSI, 2020) and EUCAST clinical breakpoints-bacteria (v 10.0) (EUCAST, 2020). To date, the MIC of 2 mg/L has been the CLSI intermediate breakpoints of polymyxin B and colistin for Enterobacteriaceae, Pseudomonas, and Acinetobacter. Given that there is no breakpoint for the susceptible, results that were interpreted as "intermediate" were treated as "susceptible" in order to better calculate ME and VME, which is in accordance with the EUCAST standard. We interpreted the results in this manner: susceptible $\leq$2mg/L, resistance $\geq$4mg/L (Humphries et al., 2019).

Using BMD as the reference method, the following parameters are included in the assessment: Essential agreement (EA) was defined as a percentage of MICs measured by the system within $\pm$ 1 dilution of reference MICs. Category agreement (CA) represents the percentage of results with the same susceptibility categorization as BMD. Very major error (VME) stands for the percentage of false susceptible results compared to BMD. Major error (ME) means the percentage of false resistant results compared to BMD. According to CLSI recommendations, a new system can be acceptable when it meets the standards as follows: CA $\geq$ 90%, EA $\geq$ 90%, VME $\leq$ 1.5%, and ME $\leq$ 3% (CLSI, 2015).

RESULTS

Antimicrobial Susceptibility Test

The MICs of quality control strains were all within the expected reference ranges specified by CLSI M100-S30 (CLSI, 2020). Susceptibilities to polymyxin B and colistin were tested in all 257 strains by BMD. Due to differences in AST cards, both drugs were not always tested by each system. Susceptibilities to polymyxin B were not reported by Vitek 2 and Phoenix M50. Due to loss in the tests, not all strains were reported by each system.

The general agreement between BMD and three AST systems for polymyxin B and colistin is shown in **Figure 1**. The performance of Bio-kont for polymyxin B and colistin both showed acceptable CAs (both 99.4%) and unacceptable EAs (77.8 and 82.2%, respectively) for susceptible strains identified by BMD. For resistant strains, the performances resulted in lower CAs (88.1 and 92.6%, respectively) and improved EAs (89.3 and 93.8%, respectively). The Vitek 2 system performed poorly with only 86.8% of results classified as CA and 62.6% classified as EA

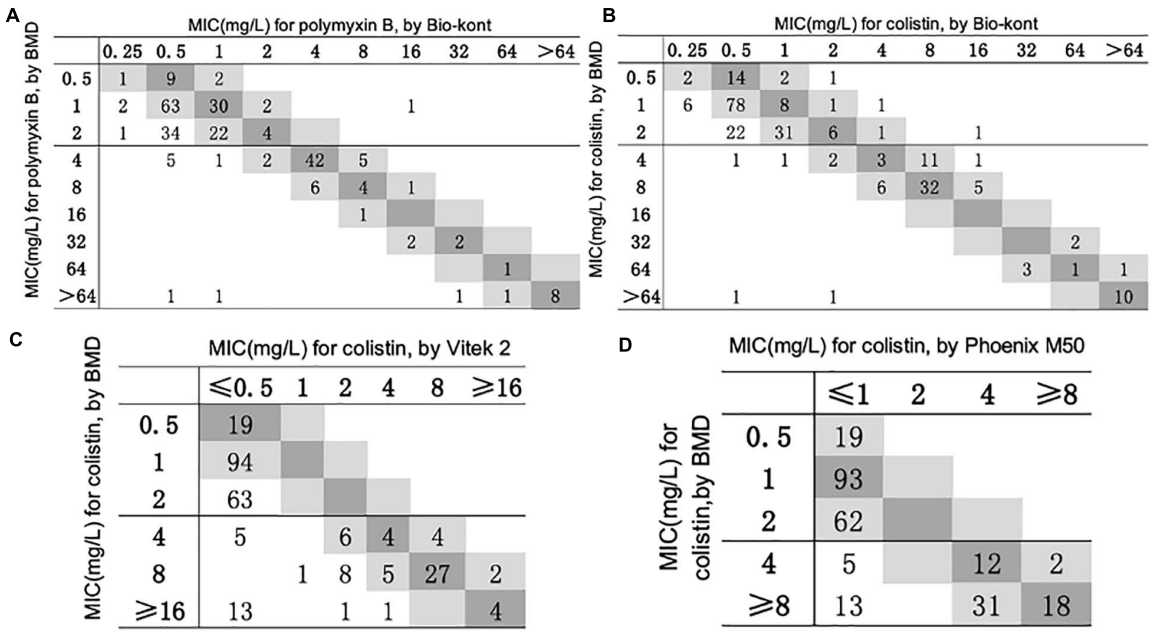

FIGURE 1 | Scatterplot of three AST methods versus reference MIC obtained from BMD. The BMD MICs have been normalized in the scatterplots of Phoenix M50 and Vitek 2. When compared with Vitek 2, the maximum test limit is ≥ 16 mg/L, while compared with Phoenix M50, it is ≥ 8mg/L. **(A)** Scatterplot of Bio-kont versus reference MIC obtained from BMD for polymyxin B. **(B)** Scatterplot of Bio-kont versus reference MIC obtained from BMD for colistin. **(C)** Scatterplot of Vitek 2 versus reference MIC obtained from BMD for colistin. **(D)** Scatterplot of Phoenix M50 versus reference MIC obtained from BMD for colistin.

TABLE 1 | Comparison of performance characteristics to polymyxin B between Bio-kont AST System and BMD method for the seven genera in the 257 strains.

Genera	Method	Total	Polymyxin B					
			S	R	Performance [*n* (%)]			
					EA	CA	VME	ME
Acinetobacter spp.	BMD	23	22	1				
	Bio-kont	22	21	1	14 (53.7%)	22 (100%)	0 (0%)	0 (0%)
Pseudomonas spp.	BMD	23	18	5				
	Bio-kont	23	21	2	18 (78.33%)	18 (78.3%)	4 (80%)	1 (5.6%)
Citrobacter spp.	BMD	23	22	1				
	Bio-kont	23	23	0	17 (73.9%)	22 (95.7%)	1 (4.3%)	0 (0%)
Enterobacter spp.	BMD	25	10	15				
	Bio-kont	25	13	12	19 (76.0%)	22 (88.0%)	3 (12.0%)	0 (0%)
Escherichia spp.	BMD	136	78	58				
	Bio-kont	136	80	56	123 (90.4%)	134 (98.5%)	2 (1.5%)	0 (0%)
Klebsiella spp.	BMD	27	23	4				
	Bio-kont	26	22	4	22 (84.6%)	26 (100%)	0 (0%)	0 (0%)
Total	BMD	257	173	84				
	Bio-kont	255	180	75	213 (83.5%)	244 (95.6%)	10 (13.1%)	1 (0.6%)

The interpretation rules: S (susceptible): MIC ≤ 2mg/L, R (resistance) MIC ≥ 4mg/L.
*EA (Essential agreement) = N_{EA}*100/N.*
*CA (Category agreement) = N_{CA}*100/N.*
*%VME (Very major error) = N_{VME}*100/total isolates resistance by BMD.*
*%ME (Major error) = N_{ME}*100/total isolates susceptible by BMD.*

compared to the reference method. The performance of Phoenix M50 was better especially for susceptible strains with 100% results in both CA and EA. And for resistant strains, CA was 77.8% and EA was 70.4%.

The detailed statistics of three AST systems for 257 strains to polymyxin B and colistin are presented in **Tables 1**, **2**.

As for polymyxin B, the Bio-kont AST System showed a CA ≥ 90% and a ME = 0% for most genera except *Pseudomonas*

TABLE 2 | Comparison of performance characteristics to colistin between each of the three AST systems and BMD method for the seven genera in the 257 strains.

Genera	Method	Total	Results		Performance [*n* (%)]			
			S	R	EA	CA	VME	ME
Acinetobacter spp.	BMD	23	21	2				
	Bio-kont	22	21	1	19 (86.4%)	21 (95.5%)	1 (50%)	0 (0%)
	Vitek	23	23	0	0 (0%)	21 (91.3%)	2 (100%)	0 (0%)
	Phoenix	22	22	0	20 (90.1%)	20 (90.9%)	2 (100%)	0 (0%)
Pseudomonas spp.	BMD	23	20	3				
	Bio-kont	23	20	3	19 (82.6%)	19 (82.6%)	2 (66.7%)	2 (10%)
	Vitek	23	23	0	4 (17.4%)	20 (87.0%)	3 (100%)	0 (0%)
	Phoenix	23	23	0	20 (87.0%)	20 (87.0%)	3 (100%)	0 (0%)
Citrobacter spp.	BMD	23	23	0				
	Bio-kont	23	22	1	15 (65.2%)	22 (95.7%)	0 (0%)	1 (4.3%)
	Vitek	23	23	0	14 (60.9%)	23 (100%)	0 (0%)	0 (0%)
	Phoenix	23	23	0	23 (100%)	23 (100%)	0 (0%)	0 (0%)
Enterobacter spp.	BMD	25	10	15				
	Bio-kont	25	13	12	19 (76.0%)	22 (88.0%)	3 (20.0%)	0 (0%)
	Vitek	25	24	1	8 (32%)	11 (44.0%)	14 (93.3%)	0 (0%)
	Phoenix	25	23	2	12 (48%)	12 (48.0%)	13 (86.7%)	0 (0%)
Escherichia spp.	BMD	136	79	57				
	Bio-kont	136	79	57	129 (94.9%)	136 (100%)	0 (0%)	0 (0%)
	Vitek	136	94	42	122 (89.7%)	121 (89.0%)	15 (26.3%)	0 (0%)
	Phoenix	136	79	57	136 (100%)	136 (100%)	0 (0%)	0 (0%)
Klebsiella spp.	BMD	27	23	4				
	Bio-kont	26	22	4	20 (76.9%)	26 (100%)	0 (0%)	0 (0%)
	Vitek	27	23	4	17 (63.0%)	27 (100%)	0 (0%)	0 (0%)
	Phoenix	26	22	4	26 (100%)	26 (100%)	0 (0%)	0 (0%)
Total	BMD	257	176	81				
	Bio-kont	255	177	78	221 (86.7%)	246 (96.5%)	6 (7.2%)	3 (1.7%)
	Vitek	257	210	47	165 (64.2%)	223 (86.8%)	34 (41.0%)	0 (0%)
	Phoenix	255	192	63	237 (92.9%)	237 (92.9%)	18 (21.7%)	0 (0%)

The interpretation rules: S (susceptible): MIC ≤ 2mg/L, R (resistance) MIC ≥ 4mg/L.
*EA (Essential agreement) = N_{EA}*100/N.*
*CA (Category agreement) = N_{CA}*100/N.*
*%VME (Very major error) = N_{VME}*100/total isolates resistance by BMD.*
*%ME (Major error) = N_{ME}*100/total isolates susceptible by BMD.*

spp. and *Enterobacter* spp. However, immense diversity in EA was observed from 53.7 to 90.4% and high VMEs were showed in *Pseudomonas* spp. (80%), *Citrobacter* spp. (4.3%), and *Enterobacter* spp. (12%). The only species meeting the requirement of an acceptable system (CA = 90.4%, EA = 98.5%, VME = 1.5%, and ME = 0%) was *Escherichia* spp. against polymyxin B. EA for strains of *Acinetobacter* spp. for polymyxin B was merely 53.7%, though with high CA of 100%. The MICs of these strains tested by Bio-kont AST System are generally lower than those of BMD. The total EA rate of Bio-kont for polymyxin B was 83.5%, while the CA rate was 95.6%. Additionally, several VMEs (13.1%) and few ME (0.6%) were observed. CA and ME of Bio-kont met the required standards, and EA and VME did not meet the standards.

As for colistin, compared with BMD, the total EA was 86.7% for Bio-kont, 64.2% for Vitek 2, and 92.9% for Phoenix M50. Among the three AST systems, Vitek 2 showed the worst performance with the lowest EAs for each genus tested in the study, especially in *Acinetobacter* spp., *Pseudomonas* spp., and *Enterobacter* spp. In general, Phoenix M50 presented better EAs, followed by Bio-kont. The total CA was 96.5% for Bio-kont, 86.8% for Vitek 2 and 92.9% for Phoenix M50. Of particular attention, the systems yielded numerous VMEs: 7.2% for Bio-kont, 41.0% for Phoenix M50, and 21.7% for Vitek 2, mainly in *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacter* spp. along with a high VME (26.3%) from Vitek 2 in *Escherichia* spp. Very limited ME rates of 1.7% from Bio-kont and 0% from both Vitek 2 and Phoenix M50 were observed. The respective MEs were all 0% except *Pseudomonas* spp. from Bio-kont (10%) and *Citrobacter* spp. from Bio-kont (4.3%). On the whole, only the EA of Phoenix M50, CAs of Bio-kont and Phoenix M50, and MEs of three systems met the required standards.

To be more specific, although CAs were all >90%, the particularly high VMEs of *Acinetobacter* spp. from three systems were worth exploring. Additionally, the performance

characteristics of *Pseudomonas* spp. to colistin were barely satisfactory with low EAs (Bio-kont 82.6%, Vitek 2 17.4%, Phoenix M50 87.0%), low CAs (Bio-kont both 82.6%, Vitek 2 and Phoenix M50 both 87.0%), and high VMEs (Bio-kont 66.7%, Vitek 2 and Phoenix M50 both 100%) and MEs (Bio-kont 10%, Vitek 2 0%, Phoenix M50 0%). The similar unsatisfactory performance of *Enterobacter* spp. to colistin was presented (low EAs: Bio-kont 76.0%, Vitek 2 32%, Phoenix M50 48%, low CAs: Bio-kont 88.0%, Vitek 2 44.0%, Phoenix M50 48.0%; high VMEs: Bio-kont 20.0%, Vitek 2 93.3%, Phoenix M50 86.7%).

The EAs for *Escherichia* spp. to colistin were highest (Bio-kont 94.9%, Vitek 2 89.7%, Phoenix M50 100%). No VMEs or MEs for *Escherichia* spp. to colistin from Bio-kont and Phoenix M50 were observed to have fully consistent agreement with BMD, which indicated the agreement of Bio-kont and Phoenix M50 with the rules (CA $\geq$ 90%, EA $\geq$ 90%, VME $\leq$ 1.5%, ME $\leq$ 3%).

Except for EAs, the performance characteristics of Vitek 2 and Phoenix M50 were similar in terms of colistin in three genera (for *Acinetobacter* spp.: CA $\geq$ 90%, VME = 100%, and ME = 0%; for *Citrobacter* spp. and *Klebsiella* spp.: CA = 100%, VME = 0%, ME = 0%).

In all, Bio-kont presented the best performance in the tested genus. However, the performance of *Pseudomonas* spp. and *Enterobacter* spp. requires further research and a larger sample size.

Detection of *mcr*-1 Gene and Performance Evaluation on *mcr-1*-Positive/Negative Strains

Among 257 Gram-negative bacteria strains, 55 strains were positive for *mcr*-1 gene and all of them were *E. coli*. As *mcr-1* gene strongly indicates the resistance of colistin and polymyxin B, the susceptibilities of 55 *mcr-1*-positive strains are verified according to BMD. The MIC distribution of 55 *E. coli* strains is as follows. For polymyxin B, the MICs of 45 *mcr-1*-positive strains were 4 mg/L and those of 10 strains were 8 mg/L. For colistin, the MICs of 12 strains were 4 mg/L and those of 43 strains were 8 mg/L. All *mcr-1*-positive strains were low-level resistant to colistin (MIC: 4–8 mg/L) and polymyxin B (MIC: 4–8 mg/L). These strains can be classified as colistin-resistant and polymyxin B-resistant strains according to either CLSI standard document (CLSI, 2020) or EUCAST standard (EUCAST, 2020), which corresponds to the genomic explanation.

Utilizing BMD as standard, the performance of three AST systems for 55 *mcr-1* positive *E. coli* strains and other *mcr-1*-negative strains to polymyxin B and colistin are presented in **Table 3**.

Compared with BMD, Bio-kont system and Phoenix M50 system presented excellent performance with no category errors when the susceptibility of *mcr-1*-positive *E. coli* was tested. It was worth noting that high VME rates (14/55, 25.5%) were observed from Vitek 2 system in *mcr-1*-positive *E. coli* strains. As for *mcr-1*-negative strains, each system showed acceptable rates of CA with several errors. The performance of Bio-kont was better than the other two systems with fewer errors. Noticeably high error rates were presented in both Vitek 2 system and Phoenix M50 system in *mcr-1*-negative strains. In conclusion, influence from *mcr-1* gene to Bio-kont and Phoenix M50 is minor, while that to Vitek 2 maybe need more data for analysis.

DISCUSSION

In this study, we evaluated the performance of three systems: Vitek 2, Phoenix M50, and Bio-kont. Vitek 2 and Phoenix M50

TABLE 3 | Comparison of performance characteristics to colistin and polymyxin B between each of the three AST systems and BMD method for strains of different *mcr-1* genetic conditions.

	mcr-1 gene	Method	Total	S	R	Performance [*n* (%)]			
						EA	CA	VME	ME
Polymyxin B	+	BMD	55	0	55				
		Bio-kont	55	0	55	55 (100%)	55 (100%)	0 (0%)	0 (0%)
Colistin	+	BMD	55	0	55				
		Bio-kont	55	0	55	55 (100%)	55 (100%)	0 (0%)	0 (0%)
		Vitek	55	14	41	47 (85.5%)	41 (74.5%)	14 (25.5%)	0 (0%)
		Phoenix	55	0	55	55 (100%)	55 (100%)	0 (0%)	0 (0%)
Polymyxin B	-	BMD	202	173	29				
		Bio-kont	200	180	20	158 (79.0%)	189 (94.5%)	10 (50%)	1 (0.6%)
Colistin	-	BMD	202	174	28				
		Bio-kont	200	174	23	166 (83%)	191 (95.5%)	6 (21.4%)	3 (1.7%)
		Vitek	202	196	6	118 (58.4%)	182 (90.1%)	20 (71.4%)	0 (0%)
		Phoenix	200	192	8	182 (91%)	182 (91.0%)	18 (64.3%)	0 (0%)

The interpretation rules: S (susceptible): MIC $\leq$ 2mg/L, R (resistance) MIC $\geq$ 4mg/L.
*EA (Essential agreement) = N_{EA}*100/N.*
*CA (Category agreement) = N_{CA}*100/N.*
*%VME (Very major error) = N_{VME}*100/total isolates resistance by BMD.*
*%ME (Major error) = N_{ME}*100/total isolates susceptible by BMD.*

are two of the most commonly used AST systems in China while Bio-kont is a newly developed Chinese system that has obtained a marketing license. A joint EUCAST and CLSI polymyxin breakpoint working group recommended standard BMD as the reference method for the MIC testing of colistin (Carretto et al., 2018; Tsuji et al., 2019). However, diversity in instrument manufacturers, software stability, and even AST panels with different concentration gradients could make a difference in MIC results. With the preclinical PK/PD, clinical PK/TD, and MIC distribution data reviewed, the category of susceptibility was deemed to be inappropriate by CLSI, and an intermediate-only category was established as this category identifies isolates "that approach usually attainable blood and tissue levels and/or for which response rates may be lower than for susceptible isolates" (CLSI, 2020; Satlin et al., 2020). The absence of a susceptible category of the CLSI standard (CLSI, 2020) promoted us to cast light on the analysis referred to EUCAST standard (EUCAST, 2020).

Based on our study, none of the systems in this study met the standards for colistin and polymyxin B AST compared to BMD. Generally, Bio-kont was the most satisfactory system with the highest CA and least errors; it was followed by Phoenix M50, which had higher EA (92.9%), acceptable CA (92.9%), but more errors (21.7%). Vitek 2 showed the worst performance with low EA (64.2%), unacceptable CA (86.8%), and unexpectedly high VME (41.0%). The performances of the Vitek 2 and Phoenix M50 systems for colistin susceptibility test have been estimated before by Vourli et al. with a similar CA as this study (89.7% vs. 86.8% for Vitek 2 and 88.9% vs. 92.9% for Phoenix M50) (Vourli et al., 2017). In a study conducted by Ka Lip Chew et al., a VME rate of 36% for colistin testing by Vitek 2 was demonstrated (Chew et al., 2017). However, research into the Bio-kont system is limited as it is mainly applied in China. The evolution results of this system in this study has proved that this system—with satisfactory CA and an acceptable error rate—has better promotion value.

The problem of considerable errors in the detection of *Pseudomonas* spp. and *Enterobacter* spp. existed in all systems. In terms of *Pseudomonas* spp., the MICs of these error-prone strains were 4 mg/L which were interpreted as ATU (EUCAST) or R—the dividing value (CLSI). It is worth mentioning that the error-prone *Enterobacter* spp. strains tested by Vitek 2 and Phoenix M50 were all highly resistant to colistin (MIC>64 mg/L). False susceptibility in polymyxin B for *Enterobacteriaceae* from Vitek 2 and Phoenix M50 has been reported before, which was suspected to be the result of a smaller inoculum size effect (Doern et al., 2011; Lat et al., 2011; Bobenchik et al., 2015; Zhou et al., 2018). Currently, the CLSI still felt it important to acknowledge that available data suggested limited clinical effectiveness of the polymyxins for the *Enterobacterales*, *P. aeruginosa,* and *Acinetobacter* spp. (Satlin et al., 2020). The results of this study require validation through further research.

The notable errors in *Escherichia* spp. from Vitek 2 were mainly from *mcr-1*-positive strains. In 2017, colistin was formally forbidden from animal feeds in China (China, 2017). Whether poor reliability of colistin susceptibility testing by Vitek 2 for *mcr-1*-positive *E. coli* is an objective existence or an occasional occurrence remains unknown.

Meanwhile, it could be observed that the MICs of colistin in several susceptible isolates tested by the Vitek 2 system were one-to two-fold dilutions lower than those of BMD, indicating that strains classified as S and I by Vitek 2 system should be verified by BMD (Vourli et al., 2017). More studies are needed to further interpret the poor performance of systems and to support the interpretation of AST results.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The Human Research Ethics Committee of Peking Union Medical College Hospital approved this study and waived the need for consent (Ethics Approval Number: S-K771).

AUTHOR CONTRIBUTIONS

YZ wrote the manuscript. PJ, MZ, QY, and YX revised the manuscript. JZ, GZ, WK, SD, and TW performed the experiments. All authors approved the final version of the manuscript.

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WGS Analysis of Clonal and Plasmidic Epidemiology of Colistin-Resistance Mediated by *mcr* Genes in the Poultry Sector in Lebanon

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Poultry and poultry meat are important contributors to the global antimicrobial burden. Unregulated and illegal use of extended-spectrum cephalosporins (ESC) in this sector has long been identified as a major cause of massive spread of ESC-resistant *Escherichia coli*, and colistin usage is considered a main driver of plasmid-mediated *mcr* genes dissemination. In Lebanon, the first *mcr-1*-positive *E. coli* found in poultry dates back to 2015, followed by a few reports of *mcr-1*-positive *E. coli* in poultry, swine, humans, and the environment. On the contrary, a comprehensive picture of the population structure of *mcr-1*-positive *E. coli* and *mcr-1*-bearing plasmids carrying the *mcr-1* gene using whole-genome analysis is largely lacking. This study reports the prevalence of *mcr-1*-positive *E. coli* in poultry originating from 32 farms across three Lebanese governorates and slaughtered in the same place. We report 27/32 (84.4%) *mcr-1* positive farms, leading to a total of 84 non-duplicate *E. coli* collected, of which 62 presented the *mcr-1* gene. Numerous associated resistances were identified, including to ESC through the presence of bla_{CTX-M} or bla_{CMY} genes. The *mcr-1* gene was mostly carried by IncX4 (n = 36) and IncI2 (n = 24) plasmids, which are both known for their efficient transfer capacities. A high genetic diversity was detected, arguing for the lack of contamination during the slaughter process. ST744 and ST1011 were the most widely identified clones, which have been both regularly associated to *mcr-1*-carrying *E. coli* and to the poultry sector. The wide dissemination of colistin-resistance, coupled to resistances to ESC and numerous other molecules, should urge authorities to implement efficient guidelines for the use of antibiotics in the poultry sector in Lebanon.

Keywords: *mcr-1*, poultry, Lebanon, IncX4, IncI2

INTRODUCTION

Since the discovery of the plasmid-mediated colistin-resistance gene *mcr-1* in 2015 (Liu et al., 2016), this gene has been extensively described in numerous animal settings, and notably in the poultry sector worldwide (Apostolakos and Piccirillo, 2018). Besides living animals, the *mcr-1* gene has also been detected in retail meat, suggesting a possible transfer to humans through under-cooked meat or cross-contamination (Nishino et al., 2017; Budel et al., 2020). To date, up to 10 *mcr* gene variants (*mcr-1* to *mcr-10*) have been recognized from different sources, of which *mcr-1* and *mcr-3* are the most widespread (Nang et al., 2019). Moreover, most studies have reported *mcr* genes in association with other resistance genes, including to critically important antimicrobials such as extended-spectrum cephalosporins (ESC) (Grami et al., 2016; Maciuca et al., 2019). In the poultry sector, *mcr-1* gene has mostly been found located on IncX4 and IncI2 plasmids, and to a lesser extent on IncHI2 plasmids (Perreten et al., 2016). The usually high transfer capacity of both IncX4 and IncI2 most probably explains their wide geographical dissemination and their occurrence in a large variety of hosts, both human and animals. Colistin-resistance has often been studied under the prism of plasmid-mediated resistance, so that only a few studies reported the characterization of *mcr*-negative but colistin-resistant isolates and the role of PmrAB and PhoPQ mutations (Quesada et al., 2015).

Regarding animals in Lebanon, *mcr-1*-mediated colistin-resistance was first reported in 2015 in poultry (Dandachi et al., 2018). A single *mcr-1*-positive *Escherichia coli* was recovered from one rectal swab over 982 samples (0.1%) taken in 49 farms for surveillance purposes. In 2017, a follow-up study was performed in this first *mcr*-1-positive farm, which showed that colistin-resistance had widely disseminated since 181/200 chicken and 6/6 workers carried *mcr-1*-producing *E. coli* or *Klebsiella pneumoniae*, while litter and feed were less heavily contaminated (6 and 20%, respectively) (Dandachi et al., 2020). In 2017 as well, 23 over 114 fecal samples from swine were resistant to colistin due to the presence of the *mcr-1* gene, and four of these isolates co-harbored resistances to ESC (Dandachi et al., 2019). Between 2017 and 2018, 88/93 (94.6%) fecal samples collected from the three major poultry farms in Lebanon presented the *mcr-1* gene, among which 35.5% co-harbored a $bla_{\mathrm{CTX-M}}$ gene (Hmede and Kassem, 2018). The presence of the *mcr-1* gene was also detected in water samples, from either irrigation water or the Mediterranean Sea (Hmede et al., 2019; Sourenian et al., 2020), suggesting environmental contamination. Finally, *mcr-1* has also been reported in human clinical isolates in Lebanon that had been collected as early as 2011 (Al-Mir et al., 2019). The plasmidic location of the *mcr-1* gene has, however, rarely been investigated in those studies: it was found located on an IncX4 plasmid in human isolates and in a rainbow trout isolate, and on IncX4 and IncI2 in water isolates (Al-Mir et al., 2019; Hassan et al., 2020; Sourenian et al., 2020). Likewise, data on the bacterial population structure hosting *mcr-1*-mediated colistin-resistance remain largely unknown.

The goal of this study was thus to look for the presence of colistin-resistance in chicken fecal samples collected from 32 chicken farms located in three governorates of Lebanon. Based on both phenotypic and molecular analyses (including next-generation sequencing), we characterized the population structure of colistin-resistant *E. coli*, the genetic support of *mcr*-dependent or *mcr*-independent colistin-resistance, and the plasmid types carrying the *mcr* genes in order to lay the foundations for a better understanding of colistin-resistance spread in poultry, but also in humans and the environment in Lebanon.

MATERIALS AND METHODS

Ethics

This investigation was approved by the Azm Center/Lebanese University ethical committee (document CE-EDST-3-2018), authorized by the Lebanese Ministry of Public Health.

Bacterial Isolation and Identification

Between May and August 2018, poultry samples were collected in one big slaughterhouse in Chekka, North Lebanon. Animals were originated from 32 individual farms hosting from 4500 to 195,000 chicken individuals, and located in seven districts from Akkar, North Lebanon, and Mount Lebanon governorates (**Figure 1**). At slaughterhouse, five different samples from each farm were collected: three caeca and two necks corresponding to five different chicken carcasses were randomly sampled with all precautions needed to avoid inter-sample contamination. Each farm was sampled once, except farm 2 that was sampled twice. All samples were put in plastic bags, conserved at 4°C, and rapidly transported to the Laboratoire Microbiologie Santé et Environnement (LMSE) in Tripoli, Lebanon. Resistant Enterobacterales were isolated on MacConkey agar (Bio-Rad, Hercules, CA, United States) supplemented with colistin (3.5 mg/L) (Sigma–Aldrich, St. Louis, MO, United States). Selective plates were incubated at 37°C for 24 h. One presumptive *E. coli* colony per morphology was arbitrarily selected from each selective plate. Identification of isolates was performed using matrix-assisted laser desorption/ionization time-of-flight MALDI TOF VITEK MS Version 3.0 (bioMérieux, Marcy L'Etoile, France).

Antibiotic Susceptibility Testing and Phenotypic Characterization

Susceptibility testing was performed by the disc diffusion method on Mueller–Hinton agar according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM)[1]. The *E. coli* ATCC 7624 strain was used as quality control. A total of 16 beta-lactam (amoxicillin, piperacillin, ticarcillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, ticarcillin-clavulanic acid, cefalotine, cefuroxime, cefotaxime, ceftiofur, ceftazidime, cefoxitin, cefepime, cefquinome, aztreonam, and ertapenem) and 14 non-beta-lactam (tetracycline, kanamycin, tobramycin, gentamicin, amikacin, apramycin, netilmicin, streptomycin, florfenicol,

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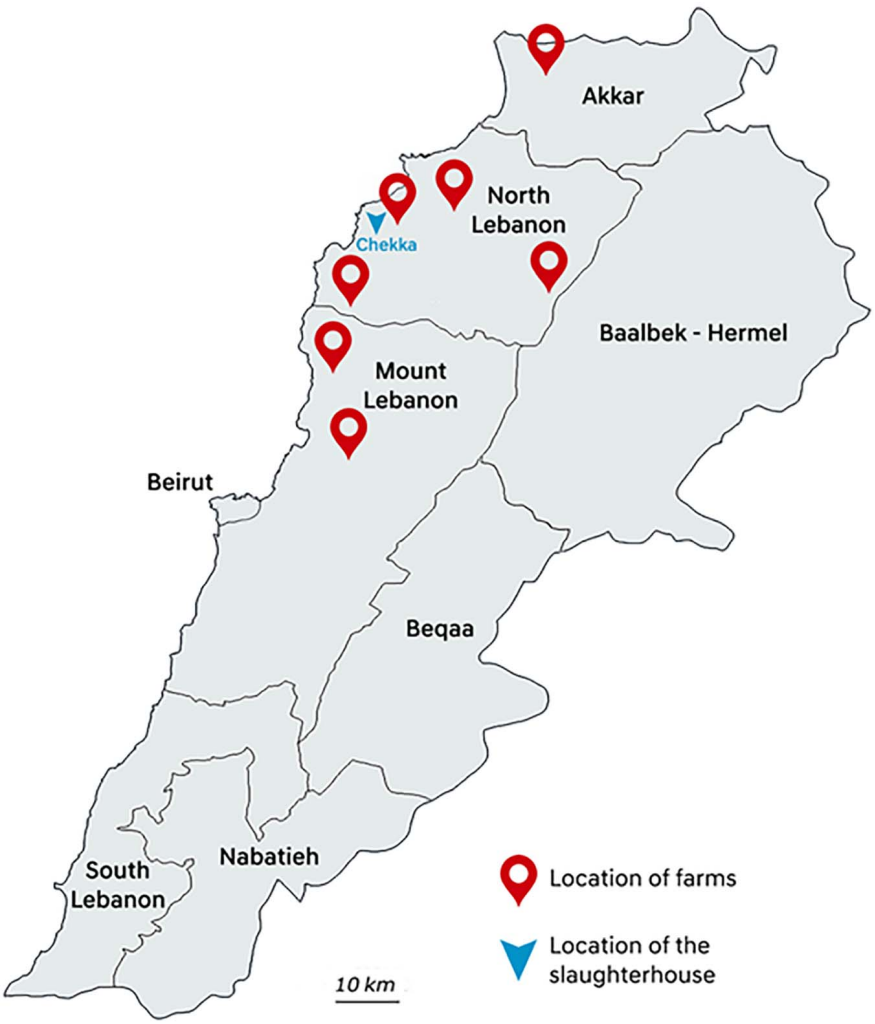

FIGURE 1 | Map of the districts where sampling was performed.

chloramphenicol, sulfonamides, trimethoprim, nalidixic acid, and enrofloxacin) antibiotics of both veterinary and human interest were tested. Minimum inhibitory concentrations (MICs) were determined by broth microdilution for colistin, according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST).

Molecular Typing of the Isolates

The detection of the major *E. coli* phylogenetic groups (A, B1, B2, or D) was performed as described by Doumith et al. (2012). Duplicate isolates collected from the same farm were detected by multiple-locus variable-number tandem-repeat analysis (MLVA) using the multiplex-based PCR described by Caméléna et al. (2019).

Whole-Genome Sequencing (WGS)

DNA was extracted using the NucleoSpin Microbial DNA extraction kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. Library preparation was performed using the Nextera XT technology, and sequencing was performed on a NovaSeq instrument (Illumina, San Diego, CA, United States). After sequencing, reads were quality trimmed and *de novo* assembled using Shovill v1.0.4, and the quality of assemblies was assessed using QUAST v5.0.2. STs were determined using the CGE online tool MLSTFinder v2.0.4[2], while resistance genes and replicon content were inferred using ABRicate v1.0.1[3]. Mutations in *gyrA* and *parC* were searched using PointFinder (see text footnote 2). The PmrA, PmrB, PhoP, and PhoQ amino acid sequences were extracted from the assemblies of all isolates and compared using Clustal Omega with the *E. coli* K12 reference strain (NP_418537.1 for PmrA, NP_418536.1 for PmrB, NP_415648.1 for PhoP, and NP_415647.1 for PhoQ). Virulence factors (VFs) were determined using VirulenceFinder, and serotypes

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

[3]https://github.com/tseemann/abricate

were determined using SeroType Finder (see text footnote 2). Avian pathogenic *E. coli* (APEC) was defined according to Johnson et al. (2008).

Characterization of the *mcr-1*-Carrying Plasmids

The replicon content was determined from the whole-genome sequencing (WGS) data using PlasmidFinder 2.0.1 (see text footnote 2). Plasmids carrying the *mcr-1* gene were assigned *in silico* when the *mcr-1* gene was located on the same contig as the plasmidic marker. When *in silico* data showed no co-occurrence on the same contig, plasmids carrying the *mcr-1* gene were detected using PFGE-S1 gels (6 V/cm for 20 h with an angle of 120° at 14°C with pulse times ranging from 1 to 30 s) followed by Southern blot using adequate probes as previously described (Saidani et al., 2019). Plasmid co-localization was assessed by comparison between the bands corresponding to the resistance gene and those corresponding to the Inc type of the plasmid. When Southern blots did not lead to interpretable results and for all isolates that could not be typed by PFGE (smearing profile), the plasmid of interest was transferred by conjugation in an *E. coli* J53 rifampicin-resistant recipient strain. Conjugation was performed in liquid medium using rifampicin and cefotaxime (5 mg/L) or colistin (2 mg/L) to select transconjugants (TC). Only TC presenting a unique plasmid, as assessed by S1-PFGE, were further characterized by PBRT and Southern blots as described above.

Phylogenetic Analysis

The core genome multi-locus sequence type (cgMLST) was extracted from the WGS data. The pan-genome was determined, and core gene alignments were generated, for each collection, with Roary v. 3.13.0 (Page et al., 2015) using a Protein BLAST identity of 80% and a core definition of 90%. In the first step, all assemblies were annotated *de novo* with Prokka v1.14.6 using default settings (Seemann, 2014). The Prokka annotations were provided to Roary as input. Subsequently, recombination was removed with gubbins v2.4.1 and a maximum likelihood tree was constructed from the core gene alignment produced by Roary using RAxML v.8.2.12 using default parameters. Pairwise single nucleotide polymorphism (SNP) distances were calculated from core genome alignments generated by Roary using snp-dists[4]. The SNP distance matrix is provided as **Supplementary Table S1**. The resulting tree for both analysis/collections was visualized using iTol v.5.5.1[5] (Letunic and Bork, 2019).

Statistical Analyses

Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, United States) using chi-square test in order to search for an association between *mcr-1* gene and ESBL or AmpC genes among colistin-resistant isolates. The tests were two-sided, with a type I error set at $\alpha = 0.05$.

[4]https://github.com/tseemann/snp-dists

[5]http://itol.embl.de/itol.cgi

Accession Number(s)

The whole genome shotgun project was deposited in DDBJ/EMBL/GenBank under the BioProject accession number PRJNA671785.

RESULTS

Detection of Colistin-Resistance

All presumptive colistin-resistant *E. coli* isolated on selective plates was characterized according to their resistance phenotype, phylogroup, and MLVA profiles, and only non-duplicate isolates were kept for further studies. Among the 32 farms tested, 27 presented at least one colistin-resistant isolate (27/32, 84.4%), with MICs ranging from 4 to 64 mg/L. Since several different isolates were retrieved from one farm (up to 12), and also from one animal (up to three), a total of 84 colistin-resistant isolates were collected (**Supplementary Table S1**).

E. coli Characterization and Virulence Patterns

The 84 *E. coli* isolates belonged to phylogroups A ($n = 44$, 52.4%), B1 ($n = 22$, 26.2%), B2 ($n = 1$, 1.2%), and D ($n = 17$, 20.2%). Thirty-one different serogroups were identified, but only O21 ($n = 14$), O102 ($n = 8$), O101 ($n = 6$), O38 ($n = 5$), O1 ($n = 3$), and O109 ($n = 3$) were found in more than two isolates. Twenty-four isolates (24/84, 28.6%) could be considered as APEC according to the definition by Johnson et al. (2008), i.e., concomitantly presenting the *iss*, *iutA*, *hlyF*, *iroN*, and *ompT* virulence genes (**Supplementary Table S1**).

Plasmid-Mediated Colistin-Resistance

Whole-genome sequencing data revealed that no other *mcr* gene than *mcr-1* was present in the *E. coli* genomes. The *mcr-1* gene was detected in 62 *E. coli* isolates (62/84, 73.8%) originating from 21 different farms. Two copies of *mcr-1* were found in one isolate, located on the same contig. The plasmidic location of the *mcr-1* gene was proved in 61/62 of the isolates, mostly by deduction from WGS data when *mcr-1* and Inc genes were located on the same contig (37 isolates) or next to conjugation and Southern blot experiments (14 isolates). For one isolate, the plasmidic or chromosomal location of *mcr-1* was not resolved despite numerous attempts (absence of conjugation and smearing PFGE profile). In all, the *mcr-1* gene was mostly carried by an IncX4 plasmid ($n = 36$), followed by an IncI2 ($n = 24$) and IncHI2 ($n = 1$) plasmid.

Chromosome-Mediated Colistin-Resistance

Amino-acid variations in the PhoP, PhoQ, PmrA, and PmrB were extracted from the WGS data of all *mcr-1*-positive and *mcr-1*-negative isolates (**Supplementary Table S1** and **Table 1**). Modifications were found at one site in PhoP, 11 sites in PhoQ, two sites in PmrA, and six sites in PmrB. Four modifications (H2R and D283G in PmrB, I44L in PhoP, and A482T in PhoQ) were found in ≥25 isolates. Eleven modifications (one in PmrA

TABLE 1 | Amino-acid modifications (PmrA, PmrB, PhoP, and PhoQ) in *mcr-1*-positive and *mcr-1*-negative isolates.

	PmrA		PmrB					PhoP		PhoQ							
	G29S	V129L	H2R	A242V	D283G	V351F	Y358N	A360V	I44L	I175F	V228I	A341T	N346K	T348I	T348N	V373I	V386L	A390T	E464D	L467M	A482T
mcr-1-positive isolates	1	2	61	15	27	15	11	2	21	2	0	1	1	0	2	1	1	1	19	2	30
mcr-1-negative isolates	1	0	21	1	17	2	7	2	4	0	3	0	0	1	0	0	0	0	0	0	0
Total	2	2	82	16	44	17	18	4	25	2	3	1	1	1	2	1	1	1	19	2	30

and 10 in PhoQ) were found only in *mcr-1*-positive isolates (**Table 1**), while two modifications in PhoQ (three isolates with the V228I modification and one isolate with the T348I modification) were exclusively found in *mcr-1*-negative isolates. However, no pattern and not even one specific mutation were found to be associated with all 22 *mcr-1*-negative colistin-resistant isolates.

Associated Resistance Genes

Among the 62 *mcr-1*-positive *E. coli* isolates, 30 presented an associated ESBL gene, including $bla_{\mathrm{CTX-M-65}}$ (n = 15), $bla_{\mathrm{CTX-M-3}}$ (n = 9), $bla_{\mathrm{CTX-M-14}}$ (n = 2), $bla_{\mathrm{CTX-M-15}}$ (n = 2), $bla_{\mathrm{CTX-M-64}}$ (n = 1), and $bla_{\mathrm{SHV-12}}$ (n = 1) (**Figure 2**). No ESBL/*mcr-1* co-localization could be evidenced, neither by conjugation nor by analyzing WGS data. Plasmidic AmpC genes were also detected in 17 isolates, with $bla_{\mathrm{CMY-2}}$ in 16 of them. All but one CTX-M-3-positive *E. coli* isolate also displayed the CMY-2 enzyme. On the contrary, only one CTX-M-65 and nine CMY-2-producing *E. coli* were identified among *mcr-1*-negative colistin-resistant isolates. The presence of the ESBL genotype was 20-fold more common in *mcr-1*-positive than in *mcr-1*-negative colistin-resistant isolates (OR: 19.7, CI: 2.5–155.6, P = 0.0003). On the contrary, no significant difference related to AmpC phenotype was observed between the two aforementioned colistin-resistant groups.

In addition to genes responsible for colistin-resistance, *E. coli* isolates mostly presented resistance genes to aminoglycosides (75/84, 89.3%), tetracyclines (71/84, 84.5%), sulfonamides (66/84, 78.6%), trimethoprim (62/84, 73.8%) and phenicols (53/84, 63.1%). The corresponding genes were mainly *tet*(A), *sul2*, *dfrA12*, and *floR*, while aminoglycoside resistance was largely multifactorial (**Supplementary Table S1**). Quinolone-resistance was observed in 70 isolates, of which 56 were also resistant to fluoroquinolones. Variants of the plasmid-mediated resistance gene *qnrB* (n = 4), as well as the *qnrS1* (n = 14) gene were detected in 14 different isolates. The mutations S83L and D87N in GyrA and S80I in ParC were concomitantly detected in 47/56 fluoroquinolone-resistance isolates (**Supplementary Table S1**). Only one apramycin-resistant isolate was identified, which presented the *aac*(3′)-IV gene, while none of the isolates was resistant to ertapenem and amikacin (**Table 2**).

Population Structure of the *mcr-1*-Positive and *mcr-1*-Negative *E. coli* Isolates

The 84 isolates belonged to 38 different STs (**Figure 2** and **Supplementary Table S1**). Two STs were predominant [ST93 (n = 14) and ST1011 (n = 11, including one SLV)], while two were each found in five different isolates (ST744 and ST1140). The remaining isolates were identified in three or less isolates. Within one ST, colistin-resistance was usually mediated by the same mechanism (either *mcr-1* or chromosomal mutations). However, six STs (ST10, ST48, ST155, ST744, ST1140, and ST1635) encompassed isolates that were resistant to colistin through either plasmid- or chromosome-mediated mechanisms.

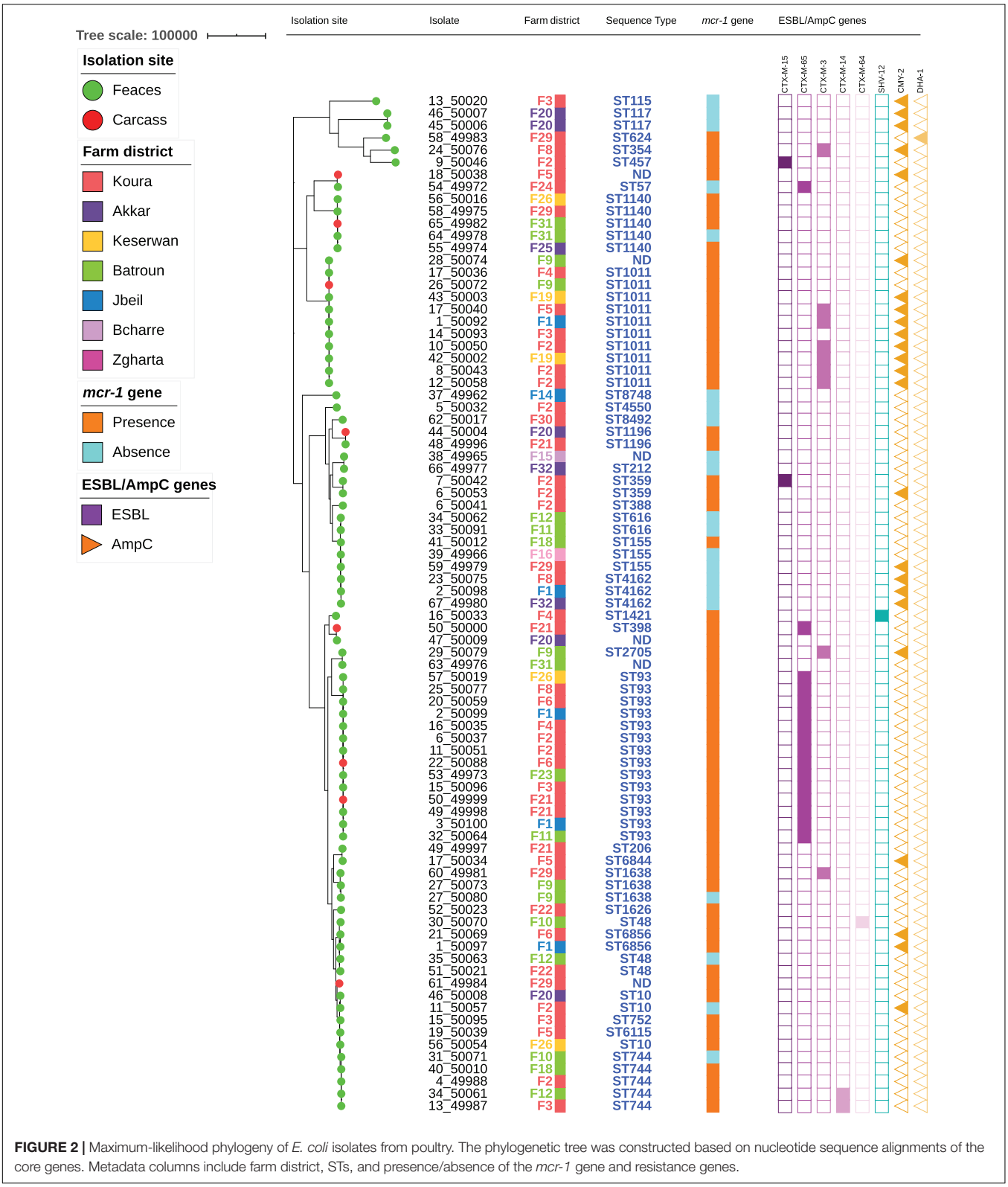

FIGURE 2 | Maximum-likelihood phylogeny of *E. coli* isolates from poultry. The phylogenetic tree was constructed based on nucleotide sequence alignments of the core genes. Metadata columns include farm district, STs, and presence/absence of the *mcr-1* gene and resistance genes.

Among the STs presenting five isolates or more, only ST93 formed a homogeneous group, with isolates differing by 14–72 SNPs. On the other side, ST744, ST1011, and ST1140 were split into two groups, each differing by > 900, > 2700 SNPs, and > 600 SNPs, respectively (**Supplementary Table S2**). The number of SNPs was also detailed for all isolates sharing the same ST. ST359

TABLE 2 | Associated resistance phenotypes.

	mcr-1-positive isolates (*n* = 62)		*mcr-1*-negative isolates (*n* = 22)		Total (*n* = 84)	
	No.	Percentage	No.	Percentage	No.	Percentage
Streptomycin	31	50.0	17	77.3	48.0	57.1
Kanamycin	44	71.0	15	68.2	59.0	70.2
Amikacin	0	0.0	0	0.0	0.0	0.0
Apramycin	1	1.6	0	0.0	1.0	1.2
Gentamicin	26	41.9	9	40.9	35.0	41.7
Tobramycin	24	38.7	9	40.9	33.0	39.3
Netilmicin	11	17.7	6	27.3	17.0	20.2
Chloramphenicol	42	67.7	11	50.0	53.0	63.1
Florfenicol	35	56.5	9	40.9	44.0	52.4
Tetracyclines	51	82.3	17	77.3	68.0	81.0
Sulfonamides	46	74.2	18	81.8	64.0	76.2
Trimethoprim	47	75.8	17	77.3	64.0	76.2
Nalidixic acid	53	85.5	17	77.3	70.0	83.3
Enrofloxacin	47	75.8	9	40.9	56.0	66.7

presented two highly similar isolates (25 SNPs) originating from two different animals from the same farm, while ST616 and ST1196 each comprised two isolates from different farms that only differed by 3 SNPs. On the contrary, ST48 and ST6856 each included two isolates from different farms, respectively, differing by > 5000 and 391 SNPs. ST4162 comprised three isolates from different farms, of which two (farms F1 and F32) were fully identical, while the third one (F8) differed by 27 SNPs. ST155 encompassed three isolates from three different farms: isolates from farms F16 and F29 displayed the *mcr-1* gene and differed by 34 SNPs, while the isolate from farm F18 was *mcr-1*-negative and differed from the two others by > 5000 SNPs. ST1638 comprised three isolates: two were from the same farm F9 and were highly similar (5 SNPs), while the third one from farm F22 clearly diverged (>5000 SNPs). Finally, ST10 included three isolates from three different farms that were not similar, isolates from farm F2 and F20 differing by > 200 SNPs, and further differing by > 6000 SNPs from the isolate from farm F26.

DISCUSSION

This study first reveals a massive spread of colistin-resistance in poultry farms in Lebanon (27/32, 84.4%), with the wide dissemination of the *mcr-1* gene (21/27 positive farms). The poultry sector is the very first one where *mcr-1* was detected in Lebanon (Dandachi et al., 2018), and a recent study in three poultry farms reported high *mcr-1* prevalence in broilers (Hmede and Kassem, 2018). The present work on 32 farms therefore expands our knowledge on the magnitude of *mcr-1* spread in the poultry sector in Lebanon. This high prevalence can most probably be explained by the unregulated use of colistin in this country in the poultry sector, without implementation of antibiotic stewardship programs (Kassem et al., 2019). Even if we have no specific information on colistin use in the farms from which our samples originated, it is known that colistin is easily available without the requirement of a veterinarian's prescription. Around 12 different drug brands that contain colistin are legally available over the counter in agriculture stores in Lebanon and particularly advised for the treatment and prevention of infections in poultry. Consequently, there is a high probability that colistin had been used in several of the tested farms. Additional ESBL/pAmpC genes were detected in 49/84 *E. coli* isolates, and ESBL genes were statistically more associated with *mcr-1*-positive isolates. These 49 ESBL/pAmpC-positive isolates, as well as 28 of the 35 remaining isolates, were multidrug-resistant (resistance to three or more antibiotic families). This suggests that colistin-resistance would be easily co-selected in the poultry gut by the use of most of the veterinary-licensed antibiotics. The bla_{ESBL} genes found in these isolates ($bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-65}}$) do not betray a human origin, where the $bla_{\text{CTX-M-15}}$ gene (which was only detected in two poultry isolates) is clearly dominating.

The mechanism of colistin-resistance in *mcr*-negative isolates was not elucidated here. Numerous mutations were observed compared to the K12 reference strain, most of which were detected in both *mcr*-positive and *mcr*-negative isolates, if not only in *mcr*-positive ones. The rare mutations that have been associated with colistin-resistance (R81S in PmrA; T156K, A159V, G161V in PhoP; E375K in PhoQ) were not detected in our dataset (Jeannot et al., 2017). Likewise, the N346K modification highlighted by Luo et al. (2017) as a possible colistin-resistance-related modification was found here associated to *mcr-1*-positive isolates. We might hypothesize that colistin-resistance-related mutations in PmrAB and PhoPQ may also arise in *mcr-1*-positive isolates and contribute to an increased MIC to colistin, but the highest MICs to colistin (≥16mg/L) observed in this study did not correlate with a specific modification pattern. Moreover, several modifications detected here (S29G in PmrA, H2R in PmrB, and D283G in PhoQ) have also been described in susceptible isolates (Luo et al., 2017). Consequently, further studies are needed to identify other genes that may be associated to colistin-resistance.

The *E. coli* population structure described here appeared very diverse, with 38 different STs detected, as it is mostly the case when samples are originating from different farms. Since all animals were sampled in the same slaughterhouse (even though poultry originated from 32 different farms), we looked for a potential contamination related to the slaughterhouse. Such a massive contamination is very unlikely because collected *E. coli* were genetically diverse, which rather indicates multiple origins. Nevertheless, a one-source contamination cannot be excluded in the cases of ST616 and ST1196 (two isolates each, originating from different farms) and ST93 (recovered in 14 isolates collected from 10 different farms), which only differed by a few SNPs. Clonal spread of *mcr-1*-carrying ST93 clinical isolates has been described in companion animals attending a veterinary hospital in China (Wang et al., 2018), suggesting that this clone may survive in the environment (a clinic or a slaughterhouse) before further dissemination. Interestingly, the *mcr-1* gene has also been detected in ST93

E. coli isolated from a human patient in Uruguay and from one healthy person in Finland (Grondahl-Yli-Hannuksela et al., 2018; Papa-Ezdra et al., 2020).

Besides ST93, and despite the high clonal diversity, two other main STs (ST744 and ST1011) were detected. These STs have already been both concomitantly reported in *mcr-1*-positive isolates of poultry origin in Czechia and in colistin-susceptible poultry isolates from Algeria (Belmahdi et al., 2016; Gelbicova et al., 2019). ST1011 has also been reported in *mcr-1*-positive environmental samples of swine farms in Germany, in pigs in China and Belgium, in poultry in Egypt, and in companion animals in China, as well as in colistin-susceptible poultry meat isolates in Egypt (Elnahriry et al., 2016; El Garch et al., 2017; Guenther et al., 2017; Wang et al., 2018; Ramadan et al., 2020; Shen et al., 2020). Interestingly, *mcr-1*-positive ST1011 *E. coli* isolates have also been recently identified from poultry farm workers in Lebanon (Dandachi et al., 2020). Consequently, the *mcr-1*-positive ST1011 *E. coli* isolate that was identified in 2013 in a Lebanese patient may well have a poultry (or at least an animal) origin (Al-Mir et al., 2019). ST744 has also been reported in *mcr-1*-positive isolates from poultry in Romania and from swine in China, and from *mcr-3*-producing *E. coli* in veal calves in France (Haenni et al., 2018; Maciuca et al., 2019; Shen et al., 2020), but also from *mcr-1*-positive clinical *E. coli* in Portugal (Tacao et al., 2017), suggesting that this clone may be particularly prone to harbor colistin-resistance. Nonetheless, it should be kept in mind that WGS data nowadays strongly challenge any lineage distribution based on MLST only, as also highlighted in some occasions in the present work. Therefore, WGS approaches are required for any further comprehensive pictures of the cross-sectorial distribution of *mcr-1*-positive *E. coli*.

A strength of our work refers to the identification of *mcr-1*-bearing plasmids, which was almost absent from other studies in Lebanon (Al-Mir et al., 2019; Hassan et al., 2020; Sourenian et al., 2020). With the single exception of one isolate where plasmid or chromosomal location was not clarified, all *mcr-1* genes were detected on plasmids, mostly on IncX4 (n = 36) but also on IncI2 (n = 24). IncX4 is the main plasmid spreading *mcr* genes worldwide, and notably *mcr-1* (Matamoros et al., 2017). IncX is a family of small and narrow-range plasmids, and experiments proved that it has a very weak fitness cost and high transfer frequencies at 30°C, allowing its wide spread in environmental settings (Lo et al., 2014). IncI2 plasmids are also spreading efficiently globally, and a recent study proved *in situ* in a mouse model that this plasmid family had a particularly high capacity to transfer DNA in the gut (Neil et al., 2020). This high transfer capacity of both IncX4 and IncI2 plasmids may explain the occurrence of *mcr-1* in such a high number of genetic backgrounds of *E. coli*, and the relative absence of clonal transmission on farm, since animals from the same farm mostly carried different *E. coli* clones.

CONCLUSION

We report a high prevalence and a massive spread of *mcr-1*-positive *E. coli* in poultry farms in Lebanon. Based on WGS analysis, we deciphered that the colistin-resistance gene *mcr-1* has widely disseminated in the poultry sector in diverse genetic backgrounds of *E. coli*, and principally on IncX4 and IncI2 plasmids. Interestingly, no other *mcr* variant than *mcr-1* was found in this sector, while other still unknown non-*mcr* genes most likely also contribute to colistin-resistance. Also, a comprehensive WGS-based picture of the global clonal and plasmidic epidemiology of *mcr-1*-positive *E. coli* in a One Health perspective still lacks in all sectors to further conclude or hypothesize on major sources and routes of transmission in Lebanon. Nonetheless, some situations would warrant further investigations, such as the occurrence of ST1011 in poultry (where it is a major *E. coli* clone), in a human patient and a poultry farm worker in the same country. These results should be used to inform and increase breeders' awareness of the consequences of uncontrolled use of antibiotics in their daily practices. Overall, the wide dissemination of colistin-resistance, coupled to a low-level awareness of antibiotic stewardship in the Lebanese community (Al Omari et al., 2019) and high resistance rates to ESC and numerous other molecules, should urge authorities to implement efficient guidelines for the use of antibiotics in the poultry—and more globally the Agri-food sector in Lebanon.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Azm Center/Lebanese University Ethical Committee (document CE-EDST-3-2018), authorized by the Lebanese Ministry of Public Health.

AUTHOR CONTRIBUTIONS

MH and MO designed the experiments and supervised the sampling campaign. HA-M performed the experiments. AD performed all WGS analyses. MH, HA-M, and J-YM analyzed the data. MH drafted the manuscript. All authors approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.624194/full#supplementary-material

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Colistin-Resistant *mcr-1*-Positive *Escherichia coli* ST131-*H22* Carrying $bla_{CTX-M-15}$ and *qnrB19* in Agricultural Soil

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The pandemic *Escherichia coli* sequence type 131 (ST131) carrying plasmid-mediated colistin resistance *mcr* genes has emerged worldwide causing extraintestinal infections, with lineages belonging to three major clades (A, B, and C). Clade B is the most prevalent in animals, contaminating associated meat products, and can be transmitted zoonotically. However, the $bla_{CTX-M-15}$ gene has only been associated with C2 subclade so far. In this study, we performed a genomic investigation of an *E. coli* (strain S802) isolated from a kale crop in Brazil, which exhibited a multidrug-resistant (MDR) profile to clinically significant antimicrobials (i.e., polymyxin, broad-spectrum cephalosporins, aminoglycosides, and fluoroquinolones). Whole-genome sequencing analysis revealed that the S802 strain belonged to serotype O25:H4, ST131/CC131, phylogenetic group B2, and virotype D5. Furthermore, S802 carried the clade B-associated *fimH22* allele, genes encoding resistance to clinically important antimicrobials, metals, and biocides, and was phylogenetically related to human, avian, and swine ST131-*H22* strains. Additionally, IncHI2-IncQ1, IncF [F2:A-:B1], and ColE1-like plasmids were identified harboring *mcr-1.1*, $bla_{CTX-M-15}$, and *qnrB19*, respectively. The emergence of the *E. coli* ST131-*H22* sublineage carrying *mcr-1.1*, $bla_{CTX-M-15}$, and *qnrB19* in agricultural soil represents a threat to food and environmental safety. Therefore, a One Health approach to genomic surveillance studies is required to effectively detect and limit the spread of antimicrobial-resistant bacteria and their resistance genes.

Keywords: acquired polymyxin resistance, emerging zoonotic *E. coli*, extended-spectrum β-lactamase, food and environmental safety, genomic surveillance, *mcr-1*, multidrug-resistant, One Health

INTRODUCTION

The rapid spread of plasmid-mediated colistin resistance *mcr* genes has gained worldwide attention as a critical public health issue, since colistin is a last resort antimicrobial used to treat severe infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria (Perez et al., 2016; Tsuji et al., 2019).

Currently, epidemiological studies have shown that the spread of colistin-resistant *mcr*-positive bacteria is not a concern restricted to hospitals, but also represents a growing problem involving environmental and food safety. In this regard, various factors such as environmental sources, food-producing animals, international travel, and food trade, have accelerated the worldwide spread of *mcr*-type genes at the human-animal-environment interface (Liu et al., 2016; Hassan and Kassem, 2020; Johura et al., 2020).

In this context, the pandemic *Escherichia coli* sequence type 131 (ST131) carrying *mcr*-type genes has emerged causing extraintestinal infections (Liu et al., 2018; Mamani et al., 2019; Reid et al., 2019; Li et al., 2021). The complex subclonal structure of ST131 elucidated three major clades, each associated with a specific allele of the type 1 fimbrial adhesin gene (*fimH*), namely clade A with *fimH41*, clade B with *fimH22*, and clade C with *fimH30* (Petty et al., 2014; Stoesser et al., 2016).

Most studies have focused on the ST131-*H*30 sublineage, which is one of the leading causes of extraintestinal infections in humans, including C2 subclade associated with the $bla_{\mathrm{CTX-M-15}}$ gene (Dahbi et al., 2014; Matsumura et al., 2015; Mamani et al., 2019). In contrast, the most prevalent animal ST131 strains belong to the ST131-*H*22 sublineage and can be transmitted zoonotically, presenting a public health challenge (Liu et al., 2018; Roer et al., 2019; Saidenberg et al., 2020).

Specifically, contamination of crops by critical priority pathogens is of great concern, since these pathogens can also contaminate vegetables for consumption (Cantas et al., 2013; Araújo et al., 2017; Lopes et al., 2017; Reid et al., 2020; Lopes et al., 2021), increasing the risk of human exposure to antimicrobial-resistant bacteria, including *mcr*-positive strains. Despite this, little is known about the occurrence of bacteria carrying *mcr*-type genes in soils. Therefore, in this study, we performed a genomic investigation of an *mcr-1*-positive *E. coli* strain exhibiting an MDR profile to clinically significant antimicrobials and isolated from agricultural soil in the light of the One Health context that integrates human, animal, and environmental health.

MATERIALS AND METHODS

Soil Sampling and Bacterial Isolation

During a surveillance study conducted between October and December 2019 to monitor the presence of clinically significant MDR Gram-negative bacteria in crops, 15 soil samples with a history of cow manure use were collected at a depth of ∼5 cm from chicory (n = 3), kale (n = 3), mustard (n = 3), parsley (n = 3), and chive (n = 3) crops on a farm in the state of São Paulo (21°00′36.0″ S; 47°27′00.0″ W), Brazil. All samples were stored at 4 °C and processed within 24 h. For bacterial isolation, 1 g of soil was inoculated in Luria-Bertani broth (Oxoid Ltd., United Kingdom) and incubated at 37 °C for 24 h. Subsequently, the cultures were streaked onto MacConkey agar plates (Oxoid Ltd., United Kingdom) supplemented with ceftriaxone (2 μg/ml) or colistin (2 μg/ml). Colonies were picked from the selective plates, subcultured, and streaked to obtain pure cultures. Bacterial identification was initially performed using 16S rRNA gene sequencing (Weisburg et al., 1991).

Antimicrobial Susceptibility Testing and Detection of Resistance Genes

Antimicrobial susceptibility testing was performed by disk diffusion, VITEK 2 (biomérieux, France), and/or agar dilution methods with interpretative criteria from CLSI guidelines [CLSI (Clinical and Laboratory Standards Institute), 2020]. Colistin minimum inhibitory concentration (MIC) was determined by broth microdilution according to EUCAST[1]. Extended-spectrum β-lactamase (ESBL) production was screened by the double-disk synergy test (Jarlier et al., 1988). Additionally, *mcr*-type (*mcr-1* to *mcr-9*) and $bla_{\mathrm{CTX-M}}$-type ($bla_{\mathrm{CTX-M-1}}$, $bla_{\mathrm{CTX-M-2}}$, $bla_{\mathrm{CTX-M-8}}$, and $bla_{\mathrm{CTX-M-9}}$ groups) genes were investigated by PCR (Dallenne et al., 2010; Liu et al., 2016; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017; Yang et al., 2018; Wang et al., 2019).

DNA Isolation and Whole-Genome Sequencing

For whole-genome sequencing (WGS), total DNA was extracted from an overnight culture using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, United States) according to the manufacturer's instructions. Sequencing was performed using the Illumina HiSeq 4000 (2 × 150 bp) platform (Illumina, United States).

Data Processing, Assembly, and Genome Analysis

A quality check of the raw sequencing data was performed using the FastQC v.0.11.9 program[2] and the reads were trimmed with Trimmomatic v.0.39 (Bolger et al., 2014). The quality value used for the base-calling program was Q = 20. In the next step, *de novo* genome assembly was carried out with SPAdes v.3.15.0 (Bankevich et al., 2012) and annotation was performed with Prokka v.1.14.5 (Seemann, 2014). Sequence type, serotype, FimH type, and clonotype were identified using MLST v2.0 (Larsen et al., 2012), SerotypeFinder v.2.0 (Joensen et al., 2015), FimTyper v.1.0 (Roer et al., 2017), and CHTyper v.1.0 (Roer et al., 2018), respectively. Antimicrobial resistance genes were detected using ResFinder v.4.1 (Zankari et al., 2012) and Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) v.4 (Gupta et al., 2014). Metals and biocides resistance genes were analyzed by BacMet v.2.0 (Pal et al., 2014). VirulenceFinder v.2.0 (Joensen et al., 2014) and the Virulence Factor Database (VFDB) v.R5 (Chen et al., 2005) were used to detect virulence genes, whereas virulence phylogroup was determined using the online Clermont typing tool[3].

Phylogenetic Analysis

For phylogenetic analysis, we selected the *E. coli* strain reported in this study and 849 other strains representative of all clades

[1] www.eucast.org

[2] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[3] http://clermontyping.iame-research.center/

(A, B, and C) of *E. coli* ST131. A minimum spanning tree was constructed based on the MSTree v.2 algorithm and the wgMLST scheme in Enterobase[4]. The tree was visualized with iTOL v.5.7 (Letunic and Bork, 2019).

Plasmid Assembly, Annotation, and Typing

Putative plasmid contigs were assembled using plasmidSPAdes v.3.15.0 (Antipov et al., 2016) and subjected to BLASTn analysis followed by gap closure. Annotation was performed by the Rapid Annotations using Subsystems Technology (RAST) server (Aziz et al., 2008) and manually curated with Geneious v.11.1.5 (Biomatters Ltd., Auckland, New Zealand). Plasmid replicon types and multilocus sequence typing were determined using PlasmidFinder v.2.1 and pMLST v.2.0 (Carattoli et al., 2014), respectively.

Conjugation Assays

Conjugation assays were conducted using azide-resistant *E. coli* C600 as recipient strain. Overnight cultures of donor and recipient strains were mixed (ratio 1:1) and incubated for 18 h at 37 °C without shaking as previously described (Furlan et al., 2020a). Transconjugants were selected using MacConkey agar (Oxoid Ltd., United Kingdom) supplemented with sodium azide (200 μg/ml) and ceftriaxone (2 μg/ml), or sodium azide (200 μg/ml) and colistin (2 μg/ml), and confirmed by PCR for the detection of *mcr*- and $bla_{\mathrm{CTX-M}}$-type genes as described above.

RESULTS

MDR *mcr-1*-Positive ESBL-Producing *E. coli* Isolated From Agricultural Soil

In this study, the presence of a *mcr-1*-positive ESBL-producing *E. coli* strain (named S802), identified by 16S rRNA gene sequence analysis and pairwise genome comparison of average nucleotide identity, was confirmed in one soil sample from the kale crop. In addition, the *E. coli* strain S802 displayed an MDR profile, defined as resistant to at least one antimicrobial of three or more different categories (Magiorakos et al., 2012). The MDR profile of *E. coli* S802 included resistance to colistin, penicillin, cephalosporins, aztreonam, aminoglycosides, quinolones, tetracycline, and chloramphenicol. In contrast, the strain displayed an intermediary resistance profile to ampicillin/sulbactam, remaining susceptible to piperacillin/tazobactam, amikacin, and carbapenems (**Table 1**).

TABLE 1 | MICs of antimicrobials for *mcr-1*-positive ESBL-producing *E. coli* strain S802 from agricultural soil.

Antimicrobials	MIC (μg/ml)[a]
Ampicillin	**≥256**
Ampicillin/sulbactam	16/8
Piperacillin/tazobactam	2/4
Ceftazidime	**32**
Ceftriaxone	**≥256**
Cefotaxime	**≥256**
Cefepime	**32**
Aztreonam	**16**
Ertapenem	0,5
Imipenem	1
Meropenem	1
Gentamicin	**64**
Amikacin	2
Ciprofloxacin	**8**
Tetracycline	**≥256**
Chloramphenicol	**32**
Colistin	**4**

[a]*MIC values indicating resistance are shown in bold.*

Identification of the Pandemic *Escherichia coli* ST131 Lineage and Phylogenetic Analysis

WGS revealed that *E. coli* strain S802 belonged to serotype O25:H4 and phylogroup B2, known for including highly virulent extraintestinal lineages. Strain S802 carried *fimH22* allele and was assigned to the clade B pandemic ST131/CC131 lineage (**Figure 1**). In addition, the clonotype CH40-22 was determined.

Phylogenetic relatedness among 850 genomes of globally reported *E. coli* ST131 strains (**Figure 2A**) assigned S802 to a cluster comprising human *E. coli* ST131-*H*22 strains from Spain, Netherlands, Germany, and Belgium; one avian strain from Germany; and one swine strain from Spain. *E. coli* strain S802 was most related to two strains isolated from humans in Spain in 2010 (**Figure 2B**).

Wide Resistome Against Multiple Antimicrobial Categories

In addition to the colistin resistance gene *mcr-1.1*, WGS analysis showed that ESBL production in the S802 strain was associated with the presence of the $bla_{\mathrm{CTX-M-15}}$ gene. Furthermore, a wide resistome was detected encoding other resistance determinants to β-lactams ($bla_{\mathrm{TEM-1A}}$, $bla_{\mathrm{TEM-1B}}$), aminoglycosides [*aac(3)-IIa*, *aadA1*, *aadA2b*, *aph(3′)-Ia*, *aph(3″)-Ib*, *aph(6)-Id*], fluoroquinolones (*qnrB19*), sulphonamides (*sul1*, *sul2*, *sul3*), trimethoprim (*dfrA1*, *dfrA5*), macrolides (*mdfA*), phenicols (*catA1*, *cmlA1*), and tetracyclines (*tetA*), as well as mutations in the quinolone resistance-determining region of *gyrA* (Ser83Leu, Asp87Asn) and *parC* (Ser80Ile) (**Table 2**).

Genes predicted to confer tolerance to metals, including copper (*cueOR*, *cutACEF*, *pcoEABCDRSE*), silver (*silESRCBAP*), copper/silver (*cusSRCFBA*), mercury (*merRTPCADE*), tellurium (*tehAB*, *terY3Y2XY1W*, *terZABCDE*), tellurium/selenium/chromium (*ruvB*), nickel (*nikABCDE*), nickel/cobalt/iron (*rcnABR*), cobalt/magnesium/manganese (*corAB*), and zinc (*zraP*) were also identified.

Regarding biocides resistance, genes encoding efflux pumps, transport modulators, and other proteins associated with resistance to acridines (*acrAEFS*, *tehAB*, *tolC*), chlorhexidine

[4]https://enterobase.warwick.ac.uk/species/index/ecoli

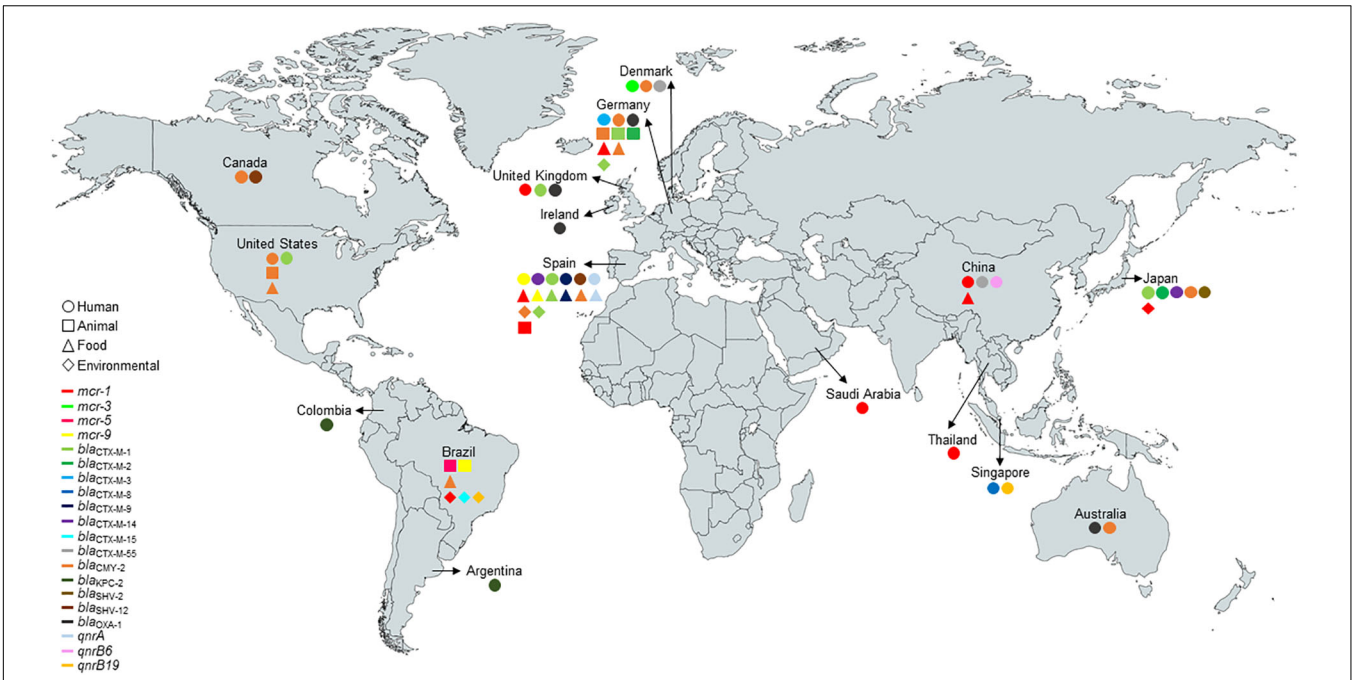

FIGURE 1 | Worldwide distribution and sources of *E. coli* ST131-*H22* carrying *mcr*-type, β-lactamase, and *qnr*-type genes at the human-animal-environment interface. The figure was produced using data retrieved from Enterobase (http://enterobase.warwick.ac.uk/species/index/ecoli) and Pubmed (https://pubmed.ncbi.nlm.nih.gov/) (bla_{TEM}-types were not included in the figure).

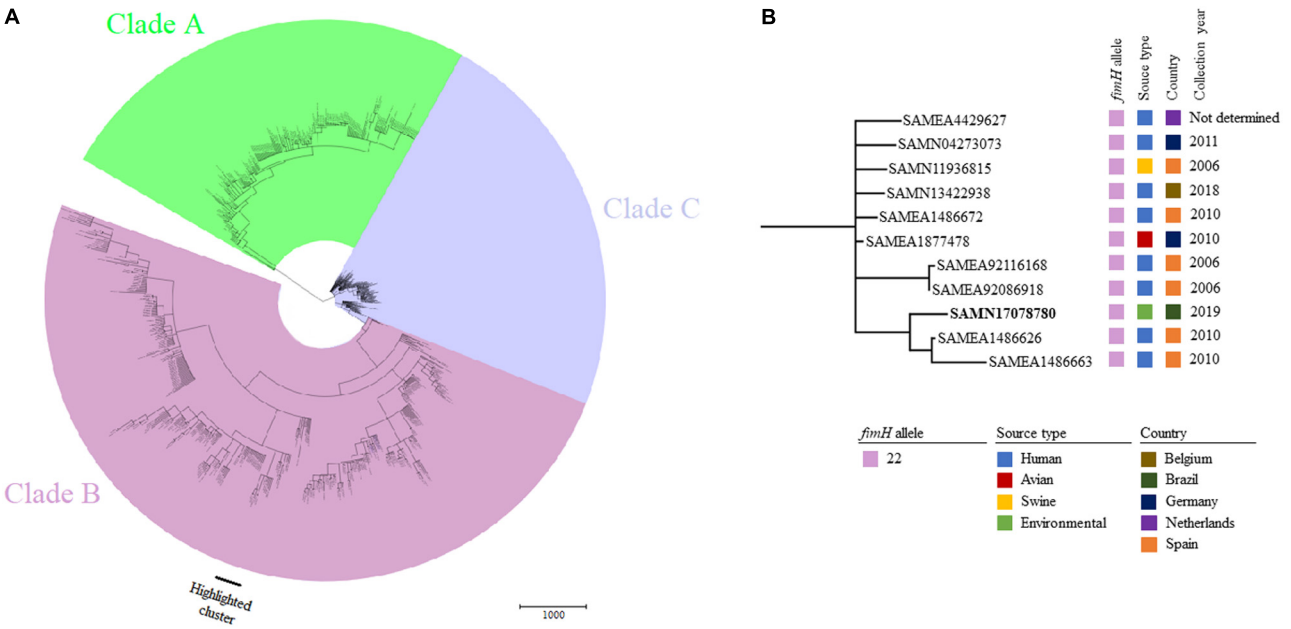

FIGURE 2 | (A) Minimum spanning tree based on wgMLST of 850 worldwide distributed *E. coli* strains belonging to ST131. **(B)** Highlighted cluster containing *E. coli* S802 (BioSample accession indicated in bold) and showing the *fimH* allele, source of origin, country, and collection year of closely related strains.

(*cpxA*), crystal violet (*mdtABCEFGHKNOP*, *tehAB*), ethidium bromide (*acrAEFS*, *sugE*, *tehAB*, *tolC*), hydrochloric acid (*gadCEWX*), hydrogen peroxide (*cpxA*, *fetAB*, *sitABCD*), organic solvents (*marRAB*), quaternary ammonium compounds (*acrAEFS*, *cpxA*, *emrABEKRY*, *mdtABCEFGHKNOP sugE*, *tolC*), sodium deoxycholate (*evgAS*), and sodium dodecyl sulfate (*acrAEFS*, *emrABEKRY*, *mdtABCEFGHKNOP sugE*, *tolC*) were detected (**Table 2**).

TABLE 2 | Genome features, resistome, and virulome of *E. coli* S802.

Feature[a]	Chromosome[b]	pS802-MCR	pS802-CTX-M	pS802-QnrB
Size (bp)	4,963,667 (94 contigs)	285,390	162,792	3,041
GC content (%)	50.47	48.47	52.48	52.09
No. of genes	4,688	315	174	2
Inc group (pMLST)	NA	HI2 (ST4)-Q1	F [F2:A-:B1]	ColE1-like
Resistome				
Polymyxins		*mcr-1.1*		
β-Lactams		bla_{TEM-1A}	$bla_{CTX-M-15}$, bla_{TEM-1B}	
Aminoglycosides		*aac(3)-IIa*, *aadA1*, *aadA2b*, *aph(3″)-Ib*, *aph(6)-Id*	*aph(3′)-Ia*, *aph(3″)-Ib*, *aph(6)-Id*	
Quinolones				*qnrB19*
Sulphonamides		*sul1*, *sul2*, *sul3*	*sul2*	
Trimethoprim		*dfrA1*	*dfrA5*	
Macrolides	*mdfA*			
Phenicols		*catA1*, *cmlA1*		
Tetracyclines		*tetA*	*tetA*	
Metals	*corAB, cueOR*, *cutACEF*, *cusSRCFBA, nikABCDER*, *pcoCD*, *rcnABR*, *ruvB*, *tehAB*, *zraP*	*merRTPCADE*, *pcoEABCDRSE*, *silESRCBAP*, *terY3Y2XY1W*, *terZABCDEF*	*merRTPCADE*	
Biocides	*acrAEFS*, *cpxA, emrABEKRY*, *evgAS*, *fetAB*, *gadCEWX*, *marRAB*, *mdtABCEFGHKNOP sugE*, *tehAB*, *tolC*	*qacEΔ1*	*sitABCD*	
QRDR mutations	*gyrA* (Ser83Leu, Asp87Asn), *parC* (Ser80Ile)			
Virulome	*chuA*, *cnf1*, *fyuA*, *gad*, *hlyCABD*, *hra*, *ibeA, irp1*, *irp2, kpsE*, *kpsM II-K5*, *ompT*, *papIBAHCDJKEFG*, *usp*, *ybtAEPQSTUX*, *yfcV*		*cia*, *cvaABC/cvi*, *etsC*, *hlyF*, *iroBCDEN*, *iss*, *iucABCD*, *iutA*, *mchF*, *ompT*, *sitABCD*, *traT*	

[a] *QRDR, Quinolone resistance-determining region.* [b] *NA, Not applicable.*

Virulome

Virulome analysis of *E. coli* S802 revealed a diversity of virulence factors, including *chuA* (outer membrane hemin receptor), *cia* (colicin Ia), *cnf1* (cytotoxic necrotizing factor), *cvaABC/cvi* [colicin (microcin) V operon], *etsC* (putative type I secretion outer membrane protein), *fyuA* (yersiniabactin receptor), *gad* (glutamate decarboxylase), *hlyCABD* (α-hemolysin operon), *hlyF* (hemolysin F), *hra* (heat-resistant agglutinin), *ibeA* (invasin of brain endothelial cells), *iroBCDEN* (salmochelin operon), *irp1-2*/*ybtAEPQSTUX* (yersiniabactin synthesis), *iss* (increased serum survival lipoprotein), *iucABCD*/*iutA* (aerobactin operon), *kpsE* (capsule polysaccharide export inner-membrane protein), *kpsM II-K5* (polysialic acid transport protein; group II capsule), *mchF* (ABC transporter protein), *ompT* [outer membrane protease (protease 7)], *papIBAHCDJKEFG* (P fimbriae operon), *sitABCD* (iron and manganese transport system), *traT* (complement resistance protein), *usp* (uropathogenic specific protein), and *yfcV* (fimbrial protein) (**Table 2**). In this regard, *E. coli* S802 was assigned to the virotype D5, based on the presence of the *cnf1*, *hlyA*, *ibeA*, *kpsM II-K5*, and *papGIII* genes (Dahbi et al., 2014).

Plasmids and Horizontal Transfer

Three plasmids, named pS802-MCR, pS802-CTX-M, and pS802-QnrB, were harbored by the S802 strain and carried *mcr-1.1*, $bla_{CTX-M-15}$, and *qnrB19*, respectively (**Figure 3**).

The pS802-MCR plasmid was 285,390 bp in length, containing 48.47% GC and 315 coding regions (CDSs), and belonged to the HI2 (ST4) and Q1 incompatibility groups. Besides *mcr-1.1*, this plasmid carried bla_{TEM-1A}, *aac(3)-IIa*, *aadA1*, *aadA2b*, *aph(3″)-Ib*, *aph(6)-Id*, *sul1*, *sul2*, *sul3*, *dfrA1*, *catA1*, *cmlA1*, *tetA*, *merRTPCADE*, *pcoEABCDRSE*, *silESRCBAP*, *terY3Y2XY1W*, *terZABCDEF*, and *qacEΔ1* resistance genes (**Table 2**). Analysis of the genetic context of *mcr-1.1* revealed the presence of IS*Apl1* upstream and *pap2* downstream of the gene. Moreover, pS802-MCR showed a high nucleotide identity with other IncHI2 plasmids of *E. coli* strains isolated from animal, human, and food in European and Asian countries, as well as with plasmids of *Salmonella* Schwarzengrund strains isolated from poultry in Brazil (**Figure 4**).

The pS802-CTX-M plasmid was a 162,792 bp IncF [F2:A-:B1] plasmid, containing 52.48% GC and 174 CDSs. In addition to the bla_{TEM-1B}, *aph(3′)-Ia*, *aph(3″)-Ib*, *aph(6)-Id*, *sul2*, *dfrA5*, *tetA*, *merRTPCADE*, and *sitABCD* resistance genes (**Table 2**), the IS*Ecp1*-$bla_{CTX-M-15}$-Δ*orf477* transposition unit was identified inserted in the transposon Tn*2* truncated by IS*26* in this plasmid. Virulence genes, namely *cia*, *cvaABC/cvi*, *etsC*, *hlyF*, *iroBCDEN*, *iss*, *iucABCD*, *iutA*, *mchF*, *ompT*, *sitABCD*, and *traT*, were also carried on pS802-CTX-M. The pS802-CTX-M plasmid exhibited a high nucleotide identity with IncF plasmids of *E. coli* strains isolated from animal, human, and food in North American, Asian, Australian, and European countries (**Figure 5**).

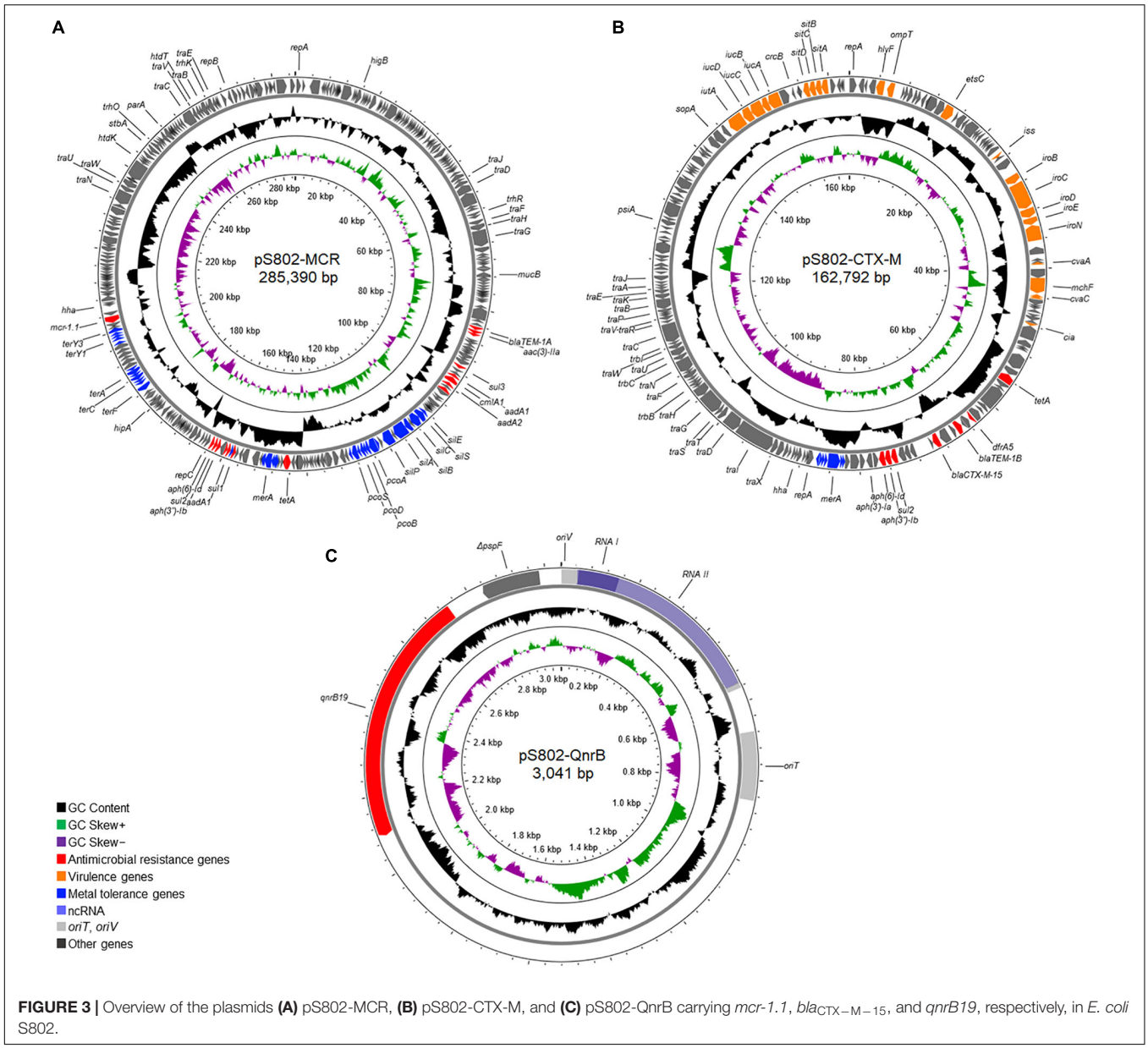

FIGURE 3 | Overview of the plasmids **(A)** pS802-MCR, **(B)** pS802-CTX-M, and **(C)** pS802-QnrB carrying *mcr-1.1*, $bla_{CTX-M-15}$, and *qnrB19*, respectively, in *E. coli* S802.

Additionally, pS802-MCR and pS802-CTX-M harbored genes involved in the replication (*rep* genes), partition/maintenance (*par* genes), conjugation (*tra*, *trb*, *trh* operons), toxin-antitoxin systems (*higB/higA*, *hipB/hipA*, *hok/sok*, *relE/parE*), and inhibition of SOS response (*psiAB*). Conjugation assays confirmed transfer of pS802-MCR and pS802-CTX-M from *E. coli* S802 at frequencies of 4.25×10^{-6} and 5.32×10^{-3} transconjugants/receptor, respectively.

The pS802-QnrB was a small 3,041 bp ColE1-like plasmid, containing 52.09% GC and only the *qnrB19* and Δ*pspF* (truncated transcription activator) genes (**Table 2**). The *qnrB19* gene was located in the conserved genetic context between the sequence encoding the regulatory RNAII and the Xer-mediated recombination site. The pS802-QnrB plasmid was related to other ColE1-like plasmids of Enterobacterales isolated worldwide at the human-animal-environment interface and shared 70% query coverage and ∼99.5% nucleotide identity with plasmids of the same incompatibility group of *E. coli* strains isolated from poultry in Brazil (GenBank accession numbers: KX452393.1 and KX452394.1), similarly to the IncHI2-IncQ1 plasmid in this study.

DISCUSSION

The emergence of clinically relevant bacterial strains in soils is an underestimated public and environmental health problem that requires attention. In this regard, *mcr*-positive *E. coli* lineages from farming soil and agricultural soil have been previously reported in China (Zheng et al., 2017) and Algeria

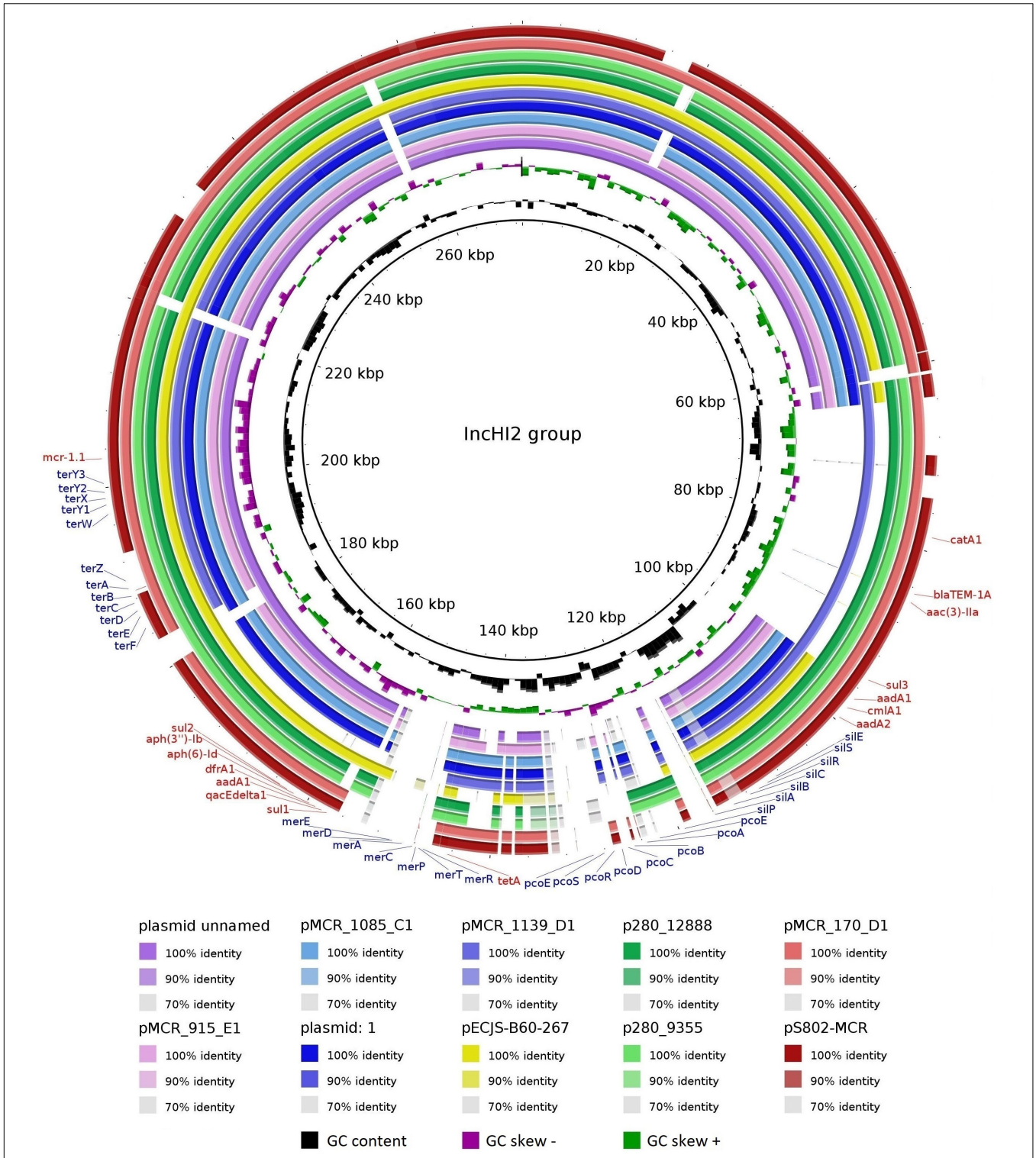

FIGURE 4 | Comparison between IncHI2 plasmids. Plasmids of *E. coli* strains: plasmid unnamed (GenBank: CP015833.1) from a human in the United Kingdom; plasmid: 1 (GenBank: LR882919.1) from a human in the Netherlands; pECJS-B60-267 (GenBank: KX254341.1) from a pig in China; pS802-MCR (GenBank: MW495059.1) from soil in Brazil; pMCR_915_E1 (GenBank: MT929285.1), pMCR_1085_C1 (GenBank: MT929286.1), pMCR_1139_D1 (GenBank: MT929287.1), and pMCR_170_D1 (GenBank: MT929288.1) from turkey meat in the Czech Republic. Plasmids of *Salmonella* Schwarzengrund strains: p280_12888 (GenBank: CP045449.1) and p280_9355 (GenBank: CP045446.1) from poultry in Brazil. Matches with less than 70% identity and no matches appear as blank spaces. Resistance genes to antimicrobials/biocides and metals are indicated in red and blue, respectively.

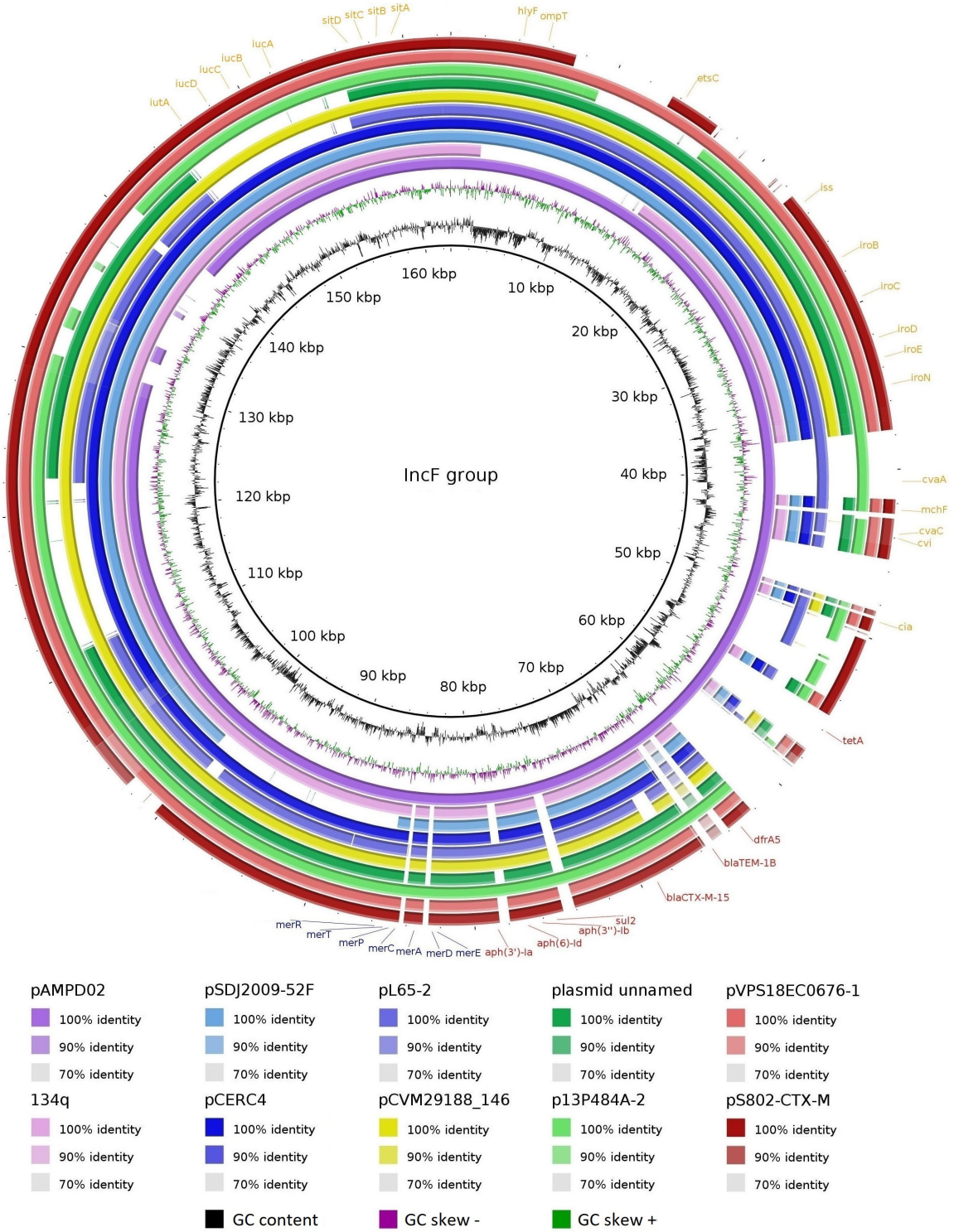

FIGURE 5 | Comparison between IncF plasmids. Plasmids of *E. coli* strains: pAMPD02 (GenBank: CP058310.1) from a giant panda in China; 134q (GenBank: CP023363.1) from a canine in Scotland; pSDJ2009-52F (GenBank: MH195200.1) and pCERC4 (GenBank: KU578032.1) from a human in Australia; plasmid unnamed (GenBank: CP027485.1) from a human in the United States; pL65-2 (GenBank: CP034739.1) from a goose in China; p13P484A-2 (GenBank: CP019282.1) from a pig in China; pVPS18EC0676-1 (GenBank: CP063726.1) from veal in the United States; pS802-CTX-M (GenBank: MW495061.1) from soil in Brazil. Plasmid of *Salmonella* Kentucky: pCVM29188_146 (GenBank: CP001122.1) from poultry in the United States. Matches with less than 70% identity and no matches appear as blank spaces. Resistance genes to antimicrobials and metals are indicated in red and blue, respectively, whereas virulence genes are indicated in orange.

(Touati et al., 2020), respectively. In Brazil, *mcr*-type genes from soil samples have only been detected in total DNA or cultivable microbiota so far (Oliveira et al., 2019; Furlan et al., 2020b; Dos Santos et al., 2020). In this study, we report for the first time the presence of an *mcr-1*-positive *E. coli* isolated from the soil ecosystem in American countries, representing a potential risk of human exposure to antimicrobial-resistant bacteria.

E. coli belonging to the ST131 pandemic high-risk clone has been identified in human, animal, environmental, and food samples (**Figure 1**). In addition, *E. coli* ST131 has been frequently reported carrying clinically significant antimicrobial resistance genes, such as *mcr*-types and/or ESBL genes (Rodrigues et al., 2017; Reid et al., 2019), and associated with extraintestinal diseases, mainly bloodstream and urinary tract infections (Liu et al., 2018; Mamani et al., 2019; Reid et al., 2019).

Whereas *E. coli* ST131-*H*30 is the most prevalent sublineage causing extraintestinal infections in humans (Dahbi et al., 2014; Matsumura et al., 2015; Mamani et al., 2019), ST131-*H*22 predominates in animals, contaminating associated meat products, and can be transmitted zoonotically (Liu et al., 2018; Roer et al., 2019; Saidenberg et al., 2020). Findings from our phylogenetic analysis showed that avian, swine, and human ST131-*H*22 strains were closely related, supporting results from previous studies (Liu et al., 2018; Reid et al., 2019; Roer et al., 2019; Saidenberg et al., 2020), and also included our environmental strain in the same cluster as those strains (**Figure 2**), highlighting their transmission at the human-animal-environment interface.

Notably, IncF [F1:A2:B20] plasmids without $bla_{\mathrm{CTX-M-15}}$, the most clinically relevant ESBL gene worldwide (Bevan et al., 2017), and IncF [F2:A1:B-] plasmids with this gene are the most frequently associated with the C1 and C2 subclades of ST131, respectively (Johnson et al., 2016; Pitout and DeVinney, 2017). In contrast, IncF [F2:A-:B1] without $bla_{\mathrm{CTX-M-15}}$ is commonly detected in clade B (Reid et al., 2019; Flament-Simon et al., 2020). Interestingly, we reported the presence of $bla_{\mathrm{CTX-M-15}}$ in clade B of ST131 in this study (**Figure 1**). Analysis of pS802-CTX-M, an IncF [F2:A-:B1] plasmid, revealed that the IS*Ecp1*-$bla_{\mathrm{CTX-M-15}}$-Δ*orf477* transposition unit was inserted in a truncated Tn*2* transposon, highlighting the role of the insertion sequence IS*Ecp1* for the mobilization of $bla_{\mathrm{CTX-M-15}}$ onto plasmids (Dhanji et al., 2011; Zong et al., 2015).

Additionally, pS802-CTX-M harbored the ColV region, frequently identified in avian pathogenic *E. coli* (APEC) and associated with increased fitness and virulence of these strains (Johnson et al., 2006). The presence of ColV plasmid in *E. coli* strains isolated from humans can indicate evidence of zoonotic transmission (Rodriguez-Siek et al., 2005; Liu et al., 2018). As detected in the present study, ColV plasmids can also carry multiple antimicrobial resistance genes, which is clinically relevant due to the combination of virulence and resistance determinants in a single mobile genetic element (Flament-Simon et al., 2020).

Although the origin of the *E. coli* ST131-*H*22 high-risk sublineage carrying the *mcr-1.1*, $bla_{\mathrm{CTX-M-15}}$, and *qnrB19* genes was not investigated, cow manure used for soil fertilization was the most likely source. In addition, other animal (e.g., wild animal feces), human (e.g., sewage), and environmental (e.g., contaminated irrigation water) sources could be involved in the dissemination of clinically relevant bacterial strains (Beuchat, 2002; Cantas et al., 2013; Araújo et al., 2017).

The range of hosts and sources of the *E. coli* ST131-*H*22 sublineage, including soil detected here, supports a genetic versatility and adaptation mediated by the gene content, which includes genes encoding resistance to antimicrobials, biocides, and heavy metals. In fact, the plasmids pS802-MCR and pS802-CTX-M co-harboring resistance genes to antimicrobials, biocides, and heavy metals were identified (**Figures 3–5**). In this regard, heavy metals could come from sources such as contaminated irrigation water, inorganic fertilizers, and pesticides commonly used in agricultural practices, remaining in the environment for long periods (Gimeno-Garcia et al., 1996; Sipter et al., 2008; Osaili et al., 2016; Bhilwadikar et al., 2019). Consequently, these compounds, as well as biocides, may act as selectors of strains resistant to antimicrobials.

Finally, the presence of MDR pathogens displaying a broad resistome in agricultural soil could lead to contamination of vegetables and, since these foods are usually consumed raw, the risk of human exposure to antimicrobial-resistant bacteria with clinical interest increases (Reid et al., 2020; Lopes et al., 2021). Although ingestion of these bacteria may not immediately have a direct impact on health, colonization by this pathway can contribute to the horizontal gene transfer of antimicrobial resistance to the gut microbiome (Maeusli et al., 2020). Thereafter, a potential threat to human health would be associated with future endogenous infections, mainly in immunosuppressed patients, in whom therapeutic failure could occur.

CONCLUSION

The emergence of zoonosis-associated *E. coli* ST131-*H*22 carrying a broad resistome, including *mcr-1.1*, $bla_{\mathrm{CTX-M-15}}$, and *qnrB19*, in agricultural soil represents a potential risk of human and animal exposure to antimicrobial-resistant bacteria and/or their resistance genes, posing a threat to public and environmental health. Also considering the possible contamination of vegetables for consumption from soil pathogens, appropriate measures, such as the improvement of agricultural practices, in addition to stricter regulations, need to be taken. Therefore, a One Health approach is required to effectively limit the spread of MDR bacteria and prevent their health impacts.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JAENHI000000000.1, https://www.ncbi.nlm.nih.gov/genbank/, MW495059.1, https://www.ncbi.nlm.nih.gov/genbank/, MW495060.1, https://www.ncbi.nlm.nih.gov/genbank/, MW495061.1.

AUTHOR CONTRIBUTIONS

RL, JF, LS, and IG carried out the research. RL and JF performed data curation and formal analysis. RL, JF, and ES conceived and designed the study, and reviewed and edited the manuscript. RL drafted the original manuscript. ES coordinated and acquired funding for the study. All authors read and approved the final manuscript.

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Prevalence and Relatedness of *mcr-1*-Mediated Colistin-Resistant *Escherichia coli* Isolated From Livestock and Farmers in Japan

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Colistin is used to treat infectious diseases in humans and livestock; it has also been used as a feed additive for livestock for approximately 50 years. Since the *mcr-1* plasmid-mediated colistin resistance gene was discovered in China in 2015, it has been detected worldwide, mainly in livestock. In this study, we investigated the prevalence and characteristics of *mcr*-mediated colistin-resistant *Escherichia coli* in livestock and farmers in Japan. We collected fecal samples from 295 healthy livestock (202 cattle and 93 swine) and 62 healthy farmers from 72 livestock farms (58 cattle farms and 14 swine farms) between 2013 and 2015. Twenty-eight *mcr-1*-harboring *E. coli* strains were isolated from 25 livestock (six cattle and 19 swine) and three farmers (two cattle farmers and one swine farmer). The prevalence rates of *mcr-1*-harboring *E. coli* in livestock and farmers were 8.47 and 4.84%, respectively. Of the 28 strains, the resistance genes of three were transferable *via* the *mcr-1*-coding plasmids to *E. coli* J53 at low frequencies (10^{-7}–10^{-8}). Six strains coharbored *mcr-1* with CTX-M β-lactamases (CTX-M-14, CTX-M-27, or CTX-M-156). Of the isolates obtained from livestock and farmers in four farms (farms C, I, N, and P), nine strains had the same genotypical characteristics (sequence types and pulsed-field gel electrophoresis band patterns), plasmid characteristics (incompatibility group and plasmid transferability), and minimum inhibitory concentrations. Thus, the findings suggested that clonal strains could spread among livestock and farmers within farms. To our knowledge, this is the first study to detect clonal relatedness of *mcr-1*-mediated colistin-resistant *E. coli* in livestock and farmers. It is suggested that farmers are at a higher risk of acquiring *mcr-1*-harboring strains, calling for our attention based on the One Health concept.

Keywords: colistin resistance, *mcr-1*, *Escherichia coli*, livestock, farmer, genotype, one health

INTRODUCTION

Colistin is a cationic antimicrobial peptide that damages bacterial cell membranes by targeting the lipid A moiety of lipopolysaccharides (LPSs), which are present in the outer cell membrane of Gram-negative bacteria. Although the clinical usage of colistin induces strong adverse effects (i.e., renal dysfunction and neurotoxicity), it is still an extremely important antibiotic against

infections caused by multidrug-resistant Gram-negative bacteria resistant to carbapenems and fluoroquinolones (Falagas and Kasiakou, 2005). In animals, colistin sulfate has been used as a therapeutic drug and feed additive worldwide. There is a concern that drug-resistant bacteria that have increased due to the use of antibiotics in livestock will not only make it difficult to treat infectious diseases in livestock but also make it difficult to treat infectious diseases in humans acquired through the consumption of livestock products. Therefore, the Food Safety Commission of Japan conducted a risk assessment of colistin sulfate for livestock in 2017 and estimated the risk posed by the drug as "Medium" (Food Safety Commission of Japan, 2017). In response to this estimation, the Japanese government shifted colistin sulfate for livestock as a therapeutic drug to a second-choice drug and withdrew colistin sulfate as a feed additive (Makita et al., 2020).

Colistin resistance is mediated by chromosomes or plasmids. The most common mechanism of chromosomally mediated colistin resistance is mainly through modification of the bacterial outer membrane through alteration of LPSs, by the addition of 4-amino-4-deoxy-l-arabinose and/or phosphoethanolamine, *via* amino acid changes in the PmrA/PmrB and PhoP/PhoQ two-component regulatory systems (Olaitan et al., 2014; Poirel et al., 2017; Aghapour et al., 2019). The other mechanism is the overexpression of efflux-pump systems or overproduction of capsule polysaccharide (Bengoechea and Skurnik, 2000; Campos et al., 2004). Plasmid-mediated colistin resistance is attributable to the transmissible gene *mcr-1*, which is a phosphoethanolamine transferase, leading to a more cationic LPS structure and consequently resistance to polymyxins (Liu et al., 2016). It was first discovered in swine in China in 2015; subsequently, *mcr-1*-harboring *Escherichia coli* has been reported worldwide, mainly in livestock and meat and even in humans (Nang et al., 2019). In addition, there are reports of the chromosomal localization of *mcr-1* in *E. coli* (Zurfluh et al., 2016; Li et al., 2018; Peng et al., 2019).

The spread of antimicrobial resistance genes should be controllable using the One Health concept, which involves the collaborative effort of health science professionals from multiple spheres toward attaining optimal health conditions for people, domestic animals, wildlife, plants, and the environment. The drivers of antimicrobial resistance include antimicrobial use and abuse in humans, animals, and the environment and the spread of resistant bacteria and resistance determinants within and between these spheres and around the world (McEwen and Collignon, 2018). Even in Japan, *mcr-1*-harboring *E. coli* strains have been isolated from livestock, imported and domestic meat, human clinical specimens, and the environment (Kusumoto et al., 2016; Nishino et al., 2017; Ohsaki et al., 2017; Tada et al., 2017; Hayashi et al., 2019). The possibility of crossing of the strains among the spheres is considered. Therefore, the relatedness of *mcr-1*-harboring strains from these different spheres needs to be determined based on the One Health concept.

The clonal relatedness of *mcr-1*-harboring *E. coli* from livestock on farms has been previously reported (Zheng et al., 2019). However, the clonal spread and relatedness of isolates of *mcr*-harboring *E. coli* from other spheres (e.g., among humans, livestock, and environment) remain unclear. To evaluate the relatedness of *mcr-1*-harboring *E. coli* isolated from livestock and farmers, we identified and compared the characteristics [sequence types (STs), pulsed-field gel electrophoresis (PFGE) band patterns, plasmid incompatibility (Inc) groups, plasmid transferability, and minimum inhibitory concentrations (MICs)] of the isolates. In this study, we investigated the prevalence, characteristics, and relatedness of *mcr*-mediated colistin-resistant *E. coli* in livestock (cattle and swine) and farmers in Japan.

MATERIALS AND METHODS

Bacterial Isolates

Between 2013 and 2015, fecal samples from 295 healthy livestock (202 cattle and 93 swine) and 62 healthy livestock farmers (53 cattle farmers and nine swine farmers) were collected from 72 livestock farms (58 cattle farms and 14 swine farms) in the southern part of the Kyushu island, a major production area of cattle farming and swine farming in Japan. The livestock included 80 calves, 101 parent cattle, 21 fattening cattle (1–10 cattle per cattle farm), 26 piglets, 29 parent swine, and 38 fattening swine (3–10 swine per swine farm). Only one *E. coli* isolate was obtained per sample upon selection using Deoxycholate-hydrogen sulfide-lactose agar. The isolates were identified using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS; Vitek MS system; bioMérieux, Co., Ltd.). The studies involving human participants were reviewed and approved by the Ethical Review Committee at the Teikyo University School of Medicine (no.13–118). The participants provided written informed consent to participate in this study.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibilities to β-lactamase and levofloxacin (except for colistin) were determined using the agar dilution method (Clinical and Laboratory Standards Institute, 2020), and quality control was performed using *E. coli* ATCC 25922. The colistin concentration was determined using the broth dilution method. MICs were interpreted according to the breakpoints defined by the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Detection of Antimicrobial Resistance Genes

The presence of *mcr* genes, extended-spectrum beta-lactamase (ESBL)-encoding genes, and carbapenemase-encoding genes was determined using PCR and sequencing as described in previous studies (Dallenne et al., 2010; Poirel et al., 2011; Ye et al., 2016). DNA sequencing was conducted using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, United States) and an Applied Biosystems ABI3730xl analyzer (Thermo Fisher Scientific K.K.). BLAST version 1.12[1] was used to process the sequencing data

[1]https://blast.ncbi.nlm.nih.gov/Blast.cgi

and identify the genes. Sequences were deposited in the GenBank database under accession numbers LC618530 through LC618535 for bla_{CTX-M} genes and LC618536 through LC618563 for *mcr-1* gene.

Plasmid Incompatibility Groups and Conjugation Experiments

Plasmid Inc. groups were identified using PCR-based replicon typing (Carattoli et al., 2005; Johnson et al., 2012). Conjugation experiments were performed using a broth-mating method with *mcr-1*-harboring *E. coli* as the donor and sodium azide-resistant *E. coli* J53 as the recipient, as described previously (Nakano et al., 2004). Transconjugants were selected on Luria-Bertani agar plates containing colistin (4 mg/L) and sodium azide (100 mg/L).

Escherichia coli Genotyping

For multilocus sequence typing (MLST), the protocol reported by Achtman et al. was applied to *E. coli* (Wirth et al., 2006). Seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) were sequenced. DNA sequence variations were analyzed using the MLST database for *E. coli*[2] to determine STs. The similarity of the isolates was compared by PFGE of *Xba*I-digested genomic DNA (*Xba*I-PFGE) with a CHEF-MAPPER System (BioRad Laboratories, Japan), as previously described (Yano et al., 2013). PFGE was performed at 14°C for 20 h at 6 V/cm with a pulse time of 5.3–49.9 s, and the angle was set at 120°C. PFGE patterns were interpreted according to the criteria described by Tenover et al. (1995): "indistinguishable," isolate with only one band different from that of the reference strain; "closely related," isolate with 2–3 bands different from those of the reference strain; and "possibly related," isolate with up to six bands different from those of the reference strain.

RESULTS

Prevalence of *mcr-1*-Harboring *E. coli* Among the Livestock and Farmers

Colistin-resistant *E. coli* strains were isolated from 25 (8.47%) of the 295 livestock. The 25 strains were isolated from six cattle (three calves, two parent cattle, and one fattening cattle) from five farms and 19 swine (15 piglets, one parent swine, and three fattening swine) from 11 farms. All strains harbored *mcr-1*, as confirmed by PCR and sequencing. The prevalence of *mcr-1*-harboring *E. coli* strains was 2.97% (6/202 strains) for cattle and 20.43% (19/93 strains) for swine. *mcr-1*-harboring *E. coli* strains were also isolated from three healthy farmers (two cattle farmers and one swine farmer; 4.84%) among 62 farmers. The prevalence of *mcr-1*-harboring *E. coli* strains was 3.77% (2/53 strains) for cattle farmers and 11.11% (1/9 strains) for swine farmers.

[2]https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef

Antimicrobial Susceptibility and Resistance Genes

The antimicrobial susceptibilities of 28 *mcr-1*-harboring *E. coli* strains are shown in **Table 1**. All the strains were resistant to colistin (4–16 mg/L), and 16 strains were co-resistant to levofloxacin (≥8 mg/L). Carbapenem-resistant strains were not found, and six strains were resistant to cefpodoxime (≥4 mg/L) and coharbored the CTX-M β-lactamase gene. The bla_{CTX-M} genotype belongs to the CTX-M-9 group (three CTX-M-27 strains and one CTX-M-14 strain) and CTX-M-1 group (two CTX-M-156 strains).

Genetic Characteristics of *mcr-1*-Harboring *E. coli*

All *mcr-1*-harboring *E. coli* strains were genotyped using MLST analysis (**Table 1**). Six *mcr-1*-harboring *E. coli* strains from cattle belonged to five different STs (two strains belonged to ST2929 and one strain each to ST69, ST95, ST106, and ST617). Nineteen *mcr-1*-harboring *E. coli* strains from swine belonged to 13 different STs (three to ST10, three to ST744, and three to ST746, followed by 10 STs). The STs of *E. coli* isolated from cattle completely differed from those of *E. coli* isolated from swine. *mcr-1*-harboring *E. coli* strains from three farmers belonged to three different STs (ST10, ST746, and ST2929). All *mcr-1*-harboring *E. coli* strains possessed diverse types of plasmid Inc. Of the 28 strains, 22 possessed F replicon regions, 17 possessed IncFIB, eight possessed IncI1Iγ, six possessed IncX4, four possessed IncHI2, four possessed IncX1, four possessed IncY, three possessed nontypeable, two possessed IncA/C, two possessed IncFIC, two possessed IncHI1, and two possessed IncP. By performing conjugation experiments, transconjugants containing the *mcr-1*-encoding plasmid were obtained at low frequencies (1.0×10^{-7} to 4.2×10^{-8}) from three strains. These three strains coharbored the CTX-M-27 β-lactamase gene with a nontypeable Inc. plasmids and were isolated from farm P.

Clonal Relatedness of *mcr-1*-Harboring *E. coli* Among Livestock and Farmers

Nine strains from four farms were found to have similar CTX-M types, STs, Inc. group, plasmid transferability, and MICs. The strains were as follows: TK3085 and TK3091 from farm C; TK3965 and TK3981 from farm I; TK3473 and TK3491 from farm N; and TK5269, TK5276, and TK5282 from farm P. *Xba*I-PFGE was used to confirm the clonal relatedness of the strains among the farms (**Table 1**). Five PFGE band patterns were obtained for the nine strains. Each PFGE pattern represented only one farm: Pattern A was noted in the farm C strains; pattern B in the farm I strains; pattern C in the farm N strains; and patterns D and D' in the farm P strains. The PFGE D' band patterns differed by three bands from the D band patterns; the strain with the D' band patterns may have been derived from that with the D band patterns. The strains with similar PFGE band patterns were considered to represent clonal strains.

TABLE 1 | Characteristics of *mcr-1*-harboring *Escherichia coli* isolated from livestock and farmers.

Farm	Strains	Source	Resistance gene	Sequence type	PFGE pattern	Plasmid incompatibility group[a]	Conjugation efficiency[b]	MIC (mg/L)[c]			
								CPDX	IPM	LVFX	CL
A	TK3004	piglet	CTX-M-14	744	-	F	No	128	0.125	16	8
B	TK3067	piglet	-	165	-	F, X4	No	0.5	0.125	16	8
C	TK3085	piglet	-	744	A	F, FIB, X1, X4	No	0.5	0.125	32	8
	TK3091	fattening swine	-	744	A	F, FIB, X1, X4	No	1	0.125	32	8
D	TK3105	fattening cattle	-	95	-	F, FIB, FIC, I1Iγ	No	1	0.25	0.125	8
E	TK3121	piglet	-	10	-	F, FIB, HI1, I1Iγ	No	0.5	≤0.06	≤0.06	8
	TK3125	parent swine	-	34	-	F, I1Iγ, Y	No	1	0.25	0.125	8
F	TK3140	piglet	-	398	-	X1, Y	No	1	0.125	4	8
G	TK3149	piglet	-	48	-	F, FIB, HI2	No	0.5	0.125	1	8
H	TK3157	piglet	-	349	-	HI2, P, X1	No	2	0.125	4	8
I	TK3166	piglet 1	-	5229	-	F, FIB	No	1	0.25	32	8
	TK3965	piglet 2	-	746	B	F, FIB, A/C	No	4	0.25	32	8
	TK3981	swine farmer	-	746	B	F, FIB, A/C	No	4	0.125	32	8
J	TK3226	calf	CTX-M-156	617	-	F	No	256	0.125	16	4
K	TK3238	calf	-	69	-	HI1, I1Iγ	No	0.5	0.125	≤0.06	8
L	TK3247	piglet	-	10	-	F, FIB, HI2, I1Iγ	No	0.5	≤0.06	1	8
M	TK3347	parent cattle	-	106	-	F, FIA, FIB	No	0.5	0.125	≤0.06	8
N	TK3469	piglet 1	-	93	-	F, FIB, I1Iγ, X4, Y	No	0.5	0.125	8	8
	TK3479	piglet 2	-	88	-	F, FIB, Y	No	0.5	0.125	0.5	4
	TK3473	piglet 3	-	746	C	F, FIB, X4	No	2	0.125	32	8
	TK3491	piglet 4	-	746	C	F, FIB, X4	No	4	0.25	32	8
	TK3500	piglet 5	-	10	-	F, FIB, HI2, I1Iγ	No	1	≤0.06	8	16
O	TK3562	fattening swine 1	-	Novel[d]	-	F, FIB, FIC	No	1	0.125	≤0.06	4
	TK3573	fattening swine 2	-	410	-	F, FIB, I1Iγ	No	1	0.125	32	8
	TK5269	calf	CTX-M-27	2929	D'	NT	1.0×10^{-7}	>256	0.125	16	8
P	TK5276	parent cattle	CTX-M-27	2929	D	NT	4.2×10^{-8}	256	0.125	8	8
	TK5282	cattle farmer	CTX-M-27	2929	D	NT	1.3×10^{-7}	>256	0.125	8	4
Q	TK5359	cattle farmer	CTX-M-156	10	-	F, P	No	256	0.125	4	8

[a]*NT, nontypeable.*

[b]*No, not transferable.*

[c]*CPDX, cefpodoxime; IPM, imipenem; LVFX, levofloxacin; CL, colistin.*

[d]*Novel type consisting of the following allelic combination: adk 6, fumC 474, gyrB 32, icd 109, mdh 8, purA 1, and recA 2.*

DISCUSSION

mcr-1-harboring *E. coli* has increased worldwide. The prevalence of *mcr-1*-harboring *E. coli* in healthy livestock has been reported to be as follows: 0.6% in Great Britain in 2013–2015 (from swine); 4.45% in Thailand (from swine); 71.43, 68.86, and 87.58% in China in 2015–2016 (from cattle, swine, and chickens, respectively; Duggett et al., 2017; Zhang et al., 2019; Khine et al., 2020); and 0.42% in Japan in 2000–2014 (cattle, swine, and chickens; Kawanishi et al., 2017). Our study revealed a high prevalence of *mcr-1*-harboring *E. coli* in healthy livestock (8.47%); the prevalence in swine (20.43%) was higher than that in cattle (2.97%). Furthermore, we found that the prevalence was 4.84% in farmers in Japan. A previous study showed that the prevalence rates of *mcr-1*-harboring *E. coli* strains were 1.43 and 0.65% in inpatients, wherein it was associated with infection, and healthy volunteers, respectively, in China in 2007–2015 (Wang et al., 2017); our study showed comparatively higher distribution among livestock farmers.

The high prevalence is likely to be associated with colistin use (Tong et al., 2018). Continuous selection pressure might have promoted the dissemination of *mcr-1*, ultimately resulting in its exceedingly high prevalence in livestock. Colistin is no longer used as a feed additive in some countries, such as the United States of America and the EU (European Commission, 2001; European Medicines Agency, 2016). Similarly, the use of colistin as a feed additive in livestock farms was banned from July 2018 in Japan; 52.5% of test farms showed a decrease in the prevalence of *E. coli* with plasmid-mediated colistin resistance 12 months after colistin use was banned in Japan (Makita et al., 2020).

We determined the genetic characteristics of 28 *mcr-1*-harboring *E. coli* strains isolated from livestock and farmers. The genotypes of the 28 strains consisted of 18 different STs; dominant STs were not observed. Major genotypes of ESBL-producing *E. coli* in humans, such as ST131 (Nicolas-Chanoine et al., 2014), were not detected in this study. Seven strains (four strains, ST10; one strain, ST34; one strain, ST48; and one strain, ST617) were found to belong to clonal complex 10 (CC10), including two strains of CTX-M-156 coharboring *E. coli* CC10 (ST10 and ST617), which is considered to have a broad range of hosts, including humans, animals, vegetable, wastewater, and urban streams (Manges et al., 2015; Varela et al., 2015; Reid et al., 2019; Massella et al., 2020). Furthermore, the strains harboring antimicrobial resistance genes were frequently coharboring virulence-associated genes. There is growing concern about the spread of *E. coli* CC10 across the spheres.

Three *mcr-1*-harboring *E. coli* strains (TK5269, TK5276, and TK5282) isolated from a calf, parent cattle, and cattle farmer in farm P had transferable resistance genes to *E. coli*. J53, which is considered as a plasmid-mediated colistin-resistant *E. coli*. We also isolated *mcr-1*-harboring *Klebsiella pneumonia* from the same individual (calf of farm P; data not shown). Herein, we selected only one *E. coli* from one sample, even though there was a possibility that *mcr-1*-harboring *E. coli* strains with different characteristic (STs, Inc. groups, or PFGE band patterns) were present in these samples. There is also a possibility of *mcr-1*-coding gene transfer across different species and strains by horizontal gene transfer, such as conjugation and transformation. A recent study reported that *E. coli* strains harboring chromosomally encoded *mcr-1* were isolated from 3.5% of *mcr-1*-positive *E. coli* in China from 2016 to 2018 (Shen et al., 2020). Therefore, the whole genome of the other 25 strains of *mcr-1*-positive *E. coli* should be analyzed to characterize the genetic construction and determine whether the *mcr-1* gene is encoded on a plasmid or chromosome in the future.

Since resistance genes (e.g., *mcr-1*, ESBL-encoding genes, and carbapenemase-encoding genes) are being detected in humans, animals, and the environment worldwide, it is necessary to take comprehensive measures to prevent the spread based on the One Health concept (Baquero et al., 2019). It is considered that one of the epidemic routes of resistant bacteria is from livestock to humans, but ESBL-harboring strains from livestock and humans show low relatedness (Dorado-García et al., 2018; Mughini-Gras et al., 2019). On the contrary, ESBL-harboring strains from companion animals and humans show high relatedness (Hong et al., 2019). Our study revealed the clonal relatedness of *mcr-1*-harboring *E. coli* isolated from livestock and farmers. It is suggested that farmers are at a higher risk than individuals living outside farms because farmers come into close contact with livestock every day, such as companion animals.

In conclusion, we determined the prevalence of *mcr-1*-harboring *E. coli* in healthy livestock (8.47%) and farmers (4.84%). Some strains with the same genotype characteristics (ST and PFGE band patterns), plasmid characteristics (Inc groups and plasmid transferability), and MICs were isolated from livestock and farmers at the farms. Thus, the findings suggest the spread of *mcr-1*-mediated colistin-resistant *E. coli* among livestock and farmers within the farms studied. To our knowledge, this is the first study to identify the clonal relatedness of *mcr-1*-mediated colistin-resistant *E. coli* in livestock and farmers. Based on the concept of One Health, it is required to identify the association of *mcr-1*-harboring *E. coli* among healthy individuals, hospital patients, animals, wastewater of the livestock, and river water by characterizing the isolates.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GeneBank database repository, accession numbers (LC618530 through LC618563).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Review Committee at the Teikyo University

School of Medicine (no.13–118). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AN and RNa: conceptualization, methodology, investigation, and writing – original draft. RNi: conceptualization and resources. YS, SH, TK-U, and TU: validation and data curation. YO and HY: supervision and project administration. All authors contributed to the article and approved the submitted version.

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Antimicrobial Resistance and Genomic Characterization of Two *mcr-1*-Harboring Foodborne *Salmonella* Isolates Recovered in China, 2016

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The *mcr-1* gene mediating mobile colistin resistance in *Escherichia coli* was first reported in China in 2016 followed by reports among different species worldwide, especially in *E. coli* and *Klebsiella*. However, data on its transmission in *Salmonella* are still lacking. This study analyzed the antimicrobial resistance (AMR) profiles and the *mcr-1* gene presence in 755 foodborne *Salmonella* from 26 provinces of mainland, China in 2016. Genomic features of two *mcr-1*-carrying isolates, genome sequencing, serotypes and further resistance profiles were studied. Among the 755 *Salmonella* tested, 72.6% were found to be resistant to at least one antimicrobial agent and 10% were defined as multi-drug resistant (MDR). *Salmonella* Derby CFSA231 and *Salmonella* Typhimurium CFSA629 were *mcr-1*-harboring isolates. Both expressed an MDR phenotype and included a single circular chromosome and one plasmid. Among the 22 AMR genes identified in *S*. Derby CFSA231, only the *mcr-1* gene was localized on the IncX4 type plasmid pCFSA231 while 20 chromosomal AMR genes, including four plasmid-mediated quinolone resistance (PMQR) genes, were mapped within a 64 kb *Salmonella* genomic island (SGI) like region. *S*. Typhimurium CFSA629 possessed 11 resistance genes including an *mcr-1.19* variant and two ESBL genes. Two IS*26*-flanked composite-like transposons were identified. Additionally, 153 and 152 virulence factors were separately identified in these two isolates with secretion system and fimbrial adherence determinants as the dominant virulence classes. Our study extends our concern on *mcr-1*-carrying *Salmonella* in regards to antimicrobial resistance and virulence factors, and highlight the importance of surveillance to mitigate dissemination of *mcr*-encoding genes among foodborne *Salmonella*.

Keywords: *Salmonella*, colistin, antimicrobial resistance (AMR), *mcr-1*, plasmids, China

INTRODUCTION

Salmonella, one of the top-ranking foodborne pathogens worldwide, is known to cause mild to severe foodborne infections, and has posed a significant public health challenge globally (Jazeela et al., 2020). Antimicrobial compounds are used to treat both human infections and food animal production and evidence suggests that antimicrobial use in food-producing animals contributes to resistance among foodborne *Salmonella* (Crump et al., 2011). This usage also increases the risk of failure when clinical treatment measures are platformed (Bai et al., 2016). On-going surveillance is a necessary step toward monitoring the emergence of multi-drug resistance (MDR) isolates of *Salmonella*.

Colistin (polymyxin E) is considered to be an antimicrobial agent of last-resort for treatment of MDR Gram-negative bacterial infections (Elbediwi et al., 2019). A plasmid-mediated polymyxin resistance mechanism MCR (mobile colistin resistance) and the first *mcr-1* gene was reported in China in 2016 (Liu et al., 2016). The key point of this mechanism is that it encodes a phosphoethanolamine transferase and confers a transferable colistin resistance (Zhao et al., 2017), thus accelerating the therapeutic failure of colistin as a last-resort treatment option for many MDR Gram-negative bacteria (Janssen et al., 2020). Global reports of the identical *mcr-1* gene among several different bacterial species were published shortly thereafter (Sun et al., 2018) along with various publications on related *mcr-1* variants and more divergent *mcr* genes (*mcr-2* ∼ *mcr-10*) (Kluytmans, 2017; Partridge et al., 2018; Osei Sekyere, 2019). According to the proposal for assignment of allele numbers for *mcr* genes and relevant variants (Partridge et al., 2018), *mcr-1.27* and *mcr-10* were designated as the latest *mcr-1* variant and *mcr* gene, respectively. Fifteen bacterial genera have been reported to carry *mcr* genes to date, and with the majority within the Enterobacteriaceae (11/15) family. Enterobacteriaceae members *Escherichia coli*, *Salmonella*, *Klebsiella,* and *Aeromonas* of the Aeromonadaceae family are the most common bacteria from which *mcr* genes have been detected with the highest *mcr* gene prevalence reported in non-pathogenic *E. coli* (Elbediwi et al., 2019). China reported the highest number of *mcr*-positive strains in a recent meta-analysis (Elbediwi et al., 2019), and identified *mcr-1* gene in Guangdong, Shanghai, Zhejiang, Hubei, Jiangsu, Sichuan, Shandong, Anhui, Chongqing, Hong Kong, and Taiwan. Based on literature review in public scientific databases, *mcr*-like genes are reported at lower rates in *Salmonella* when compared to *E. coli*, however there have been increased reported numbers of *mcr*-mediated colistin resistance in *Salmonella* spp. from humans, animals, and foods after 2016 (Li et al., 2016; Sun et al., 2018; Lima et al., 2019; Borowiak et al., 2020; Sia et al., 2020).

In this report, a surveillance of the overall antimicrobial resistance (AMR) features of 755 foodborne *Salmonella* isolates in mainland China in 2016 and an investigation of the genomic characteristics of AMR determinants and virulence factors (VFs) of the two *mcr-1*-habouring isolates is conducted to address this data gap.

MATERIALS AND METHODS

Bacterial Isolates

A total of 755 foodborne *Salmonella* isolates were collected from 26 provinces across mainland China in 2016. *Salmonella* isolates were collected from broad food categories including: special nutritional products (powder infant formula, PIF), raw meat and meat products, aquatic and aquatic products, egg and egg products, soy products, frozen drinks, rice and flour products, nuts and seed products, beverages, cocoa products, and local foods among others.

Antimicrobial Susceptibility Testing (AST) and Screening of the *mcr-1* Gene

All collected *Salmonella* were subjected to AST testing against a panel of compounds (**Table 1**) by broth micro-dilution using the Biofosun® Gram-negative panel which contained 10 classes (16 kinds) of drugs (Fosun Diagnostics, Shanghai, China). Data obtained was interpreted following recommendations described by Clinical and Laboratory Standards Institute guidelines (CLSI, M100-S28). Additionally, CLSI (M31-A3) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, version 2018) documents were consulted when CLSI M100 standards were not available for some antimicrobial compounds. *E. coli* ATCC™25922 was included as a reference strain.

All 755 foodborne *Salmonella* isolates were screened for the presence of *mcr-1* gene by qPCR as described previously (Hu et al., 2019). Isolates that carry *mcr-1* gene were selected for further analysis as described below.

Serotyping and Further AST

Serotypes of *mcr-1* positive isolates were identified by both classical slide agglutination with commercialized antisera (SSI, Denmark) following the Kauffman White Le Minor scheme (WKLM, version 2007, 9th edition), and also by molecular serotyping with xMAP® *Salmonella* Serotyping Assay Kit (SSA, Cat No. AGSSA4502, Luminex, United States) following the manufacturer's protocol. Extended AST tests using additional selected antimicrobial agents relevant to Enterobacteriaceae, including 13 classes (composing 27 compounds, **Table 2**), were carried out on the *mcr-1* positive isolates. Extended Spectrum Beta-Lactamase (ESBL) phenotype testing was also performed according to the protocol and breakpoints described in CLSI (M100-S28). *Klebsiella pneumoniae* ATCC™700603 was included as a suitable positive control.

Plasmid Conjugal Transfer

The transfer ability and frequency of *mcr*-carrying plasmids was investigated by broth mating conjugation experiments with plasmid-free and sodium azide-resistant *E. coli* J53 as the recipient strain. The transconjugants were selected on MacConkey agar plates (Beijing Landbridge, China) supplemented with 100 mg/L sodium azide (Sigma–Aldrich) and 2 mg/L colistin (Sigma–Aldrich). Two different conjugation temperatures (30 and 37°C) were used for the transfer in this study. Transfer frequencies were calculated as the number of

TABLE 1 | Antimicrobial susceptibility of 755 foodborne *Salmonella* isolates against to a panel of antimicrobial agents.

Antimicrobial class	Antimicrobial agent (abbreviation)[a]	Number of resistant isolates	Resistant rate (%)	Number of intermediate isolates	Intermediate rate (%)	Number of susceptible isolates	Susceptible rate (%)
Penicillins	Ampicillin (AMP)	291	38.5	3	0.4	461	61.1
β-Lactam combination agents	Ampicillin/sulbactam (SAM)	263	34.8	32	4.2	460	60.9
Cephalosporins	Cefotaxime (CTX)	89	11.8	4	0.5	662	87.7
	Ceftazidime (CAZ)	45	6.0	11	1.5	699	92.6
	Cephalothin (KF)	103	13.6	47	6.2	605	80.1
	Cefepime (FEP)	40	5.3	7	0.9	708	93.8
Carbapenems	Imipenem (IMP)	0	0.0	0	0.0	755	100.0
	Meropenem (MEM)	0	0.0	0	0.0	755	100.0
Aminoglycosides	Gentamicin (GEN)	83	11.0	0	0.0	672	89.0
Tetracyclines	Tetracycline (TET)	358	47.4	11	1.5	386	51.1
(Fluoro)Quinolones	Nalidixic (NAL)	396	52.5	–	–	359	47.5
	Ciprofloxacin (CIP)	161	21.3	289	38.3	305	40.4
Folate pathway inhibitors	Trimethoprim/ sulfamethoxazole (SXT)	179	23.7	–[e]	–[e]	576	76.3
Phenicols	Chloramphenicol (CHL)	187	24.8	87	11.5	481	63.7
	Florfenicol (FFC)[b,c]	170	22.5	65	8.6	520	68.9
Polymyxin	Polymyxin E (Colistin, CT)[c,d]	61	8.1	–[e]	–[e]	694	91.9

[a]*Interpretation according to the CLSI guidelines M100-S28, 2018, for all drugs except FFC and CT;* [b]*interpretation according to the CLSI guidelines M31-A3, 2008;* [c]*used as a feed additive in animal production;* [d]*interpretation according to EUCAST clinical breakpoints, 2018;* [e]*no break-point data.*

transconjugants obtained per recipient. Transfer of *mcr-1* to transconjugants was confirmed by PCR (Liu et al., 2016). The colistin MIC value of the J53 and transconjugants were tested according to the description above.

DNA Extraction, Whole Genome Sequencing (WGS), Assembly and Annotation

DNA extraction and WGS were carried out for *mcr-1*-carrying isolates to obtain complete genomes. Briefly, a single colony for each isolate was cultured overnight in brain heart infusion (BHI) broth at 37°C. A TIANamp Bacterial DNA extraction kit (DP302, TIANGEN BIOTECH, Beijing, China) was used to extract the genomic DNA from each bacterial culture according to the manufacturer's instructions, followed by a 10-kbp template library preparation step with PacBio® Template Prep Kit. Sequencing was performed commercially using SMRT® Pacific Biosciences RS II platform (Tianjin Biochip Corporation, Tianjin, China) with C4 sequencing chemistry and P6 polymerase within one SMRT®cell.

SMRT® Analysis v2.3.0 was used for demultiplexing, base calling, raw reads quality filtering, and *de novo* assembly according to RS Hierarchical Genome Assembly Process (HGAP) workflow v3.0. Subsequently, Consed software version 28.0 (Gordon and Green, 2013) was used to manually inspect and trim duplicate ends to generate single, complete and closed sequences for each chromosome and plasmid. The genomes assembled from PacBio data were then error corrected by Pilon software (version 1.23) (Walker et al., 2014) with Illumina MiSeq sequencing reads data, of which a library was prepared with a NEBNext® Ultra DNA Library Prep Kit for Illumina (NEB#E7370) followed by sonication fragmentation (350-bp insert), before being loaded on an Illumina HiSeq platform with PE 150 sequencing strategy (Novogene, Beijing, China) with a HiSeq X Ten Reagent Kit v2.5 (Illumina, San Diego, CA, United States). The corrected and assembled contigs were deposited in National Center for Biotechnology Information (NCBI) and automatically annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAP).

Genomic Information Mining

Plasmid replicon types (Inc groups) were identified through the center for genomic epidemiology (CGE) website with PlasmidFinder 2.0 (Carattoli et al., 2014). The predicted serotypes were confirmed and the multi-locus sequence typing (MLST) type were identified using *Salmonella In Silico* Typing Resource (SISTR) (Yoshida et al., 2016). CRISPR *loci* in the genomes were predicted using CRISPRfinder (Grissa et al., 2007). Similarly, prophage sequences were identified using the PHAge Search Tool Enhanced Release (PHASTER) (Arndt et al., 2016).

Assessment of Virulence

Seven extensively used *Salmonella* reference complete genomes LT2 (NC_003197), 14028s (NC_016856), DT104 (NC_022570), CT18 (NC_003198), Ty2 (NC_004631) and two hypervirulent isolates [D23580 (LS997973) and 4/74 (NC_016857)] (Canals et al., 2019), available from GenBank, were used to confirm and compare the presence of *Salmonella* pathogenicity islands (SPIs) and the potential virulence factors (VFs) with two *mcr-1*-carrying isolates in this study by SPIFinder (Roer et al., 2016) and

TABLE 2 | Antimicrobial susceptibility of *Salmonella* isolate CFSA231 and CFSA629 to a further panel of antimicrobial agents and acquired antimicrobial resistance-encoding genes identified in the bacterial genome with online retrieval in Resfinder database.

		CFSA231		CFSA629		CFSA231		CFSA629	
Antimicrobial class	**Antimicrobial agent (abbreviation)**	**MIC (mg/L)**	**R/I/S[a]**	**MIC (mg/L)**	**R/I/S[a]**	**Resistance genes or point mutation**		**Resistance genes or point mutation**	
						Chromosome	**Plasmid**	**Chromosome**	**Plasmid**
β-lactam combination agents	Ampicillin /sulbactam (SAM)	≥32/16	R	≥32/16	R	bla_{OXA-1}		bla_{TEM-1B}	$bla_{CTX-M-14}$
Penicillins	Ampicillin (AMP)	≥32	R	≥32	R				
Cephalosporins	Cefotaxime (CTX)	0.12	S	64	R				
	Cefotaxime + clavulanate (CTX + CLA)	0.06/4	–	0.12/4	–				
	Ceftazidime (CAZ)	0.5	S	2	S				
	Ceftazidime + clavulanate (CAZ + CLA)	0.25/4	–	0.25/4	–				
	Cephalothin (KF)	2	S	≥32	R				
	Cefoxitin (FOX)	2	S	8	S				
	Ceftriaxone (CRO)	0.12	S	≥4	R				
	Cefepime (FEP)	2	S	≥16	R				
Carbapenems	Imipenem (IMP)	0.25	S	0.25	S				
	Meropenem (MEM)	0.03	S	0.03	S				
	Ertapenem (ETP)	0.015	S	0.5	S				
Monobactams	Aztreonam (ATM)	0.06	S	4	S				
Aminoglycosides	Gentamicin (GEN)	≥16	R	8	I	*aac(3)-IV, aac(6′)-Iaa, aac(6′)-Ib-cr, aadA1, aadA2,aaaA2b, aadA8b, aph(3′)-Ia, aph(4)-Ia*		*aac(6′)-Iaa, aph(3′′)-Ib, aph(6)-Id*	*aac(3)-IV, aph(4)-Ia*
	Amikacin(AK)	8	S	1	S				
Tetracyclines	Tetracycline (TET)	≥16	R	≥16	R	*tetA*		*tetB*	
	Tigecycline (TGC)	0.12	S	0.25	S				
(Fluoro)Quinolones	Nalidixic acid (NAL)	≥32	R	≥32	R	ParC T57S; *aac(6′)-Ib-cr, oqxA, oqxB, qnrS2*		GyrA D87Y	
	Ciprofloxacin (CIP)	4	R	0.5	I				
Folate pathway inhibitors	Trimethoprim/ sulfamethoxazole (SXT)	≥8/152	R	0.25/4.75	S	*sul1, sul2, sul3, dfrA12*		*sul2*	
	Trimethoprim(TMP)	≥16	R	0.25	S				
Phenicols	Chloramphenicol (CHL)	≥32	R	8	S	*catB3, cmlA1, floR*			
	Florfenicol (FFC)[b,c]	≥16	R	8	I				
Nitrofurans	Nitrofurantoin (NIT)	32	S	16	S				
Polymyxins	Polymyxin E (Colistin, CT)[c,d]	2	S	4	R		*mcr-1.1*		*mcr-1.19*
	Polymyxin B (PB)[d]	4	I	4	I				
Fosfomycins	Fosfomycin (FOS)	–[e]	–[e]	–[e]	–[e]	*fosA7*			*fosA3*

R, resistant; I, intermediate; S, susceptible; [a]R/I/S according to the CLSI guidelines M100-S28, 2018; [b]R/I/S according to the CLSI guidelines M31-A3, 2008; [c]used as a feed additive in animal production; [d]R/I/S according to EUCAST clinical breakpoints, 2018; [e]no data.

Virulence factor database (VFDB) (Liu et al., 2019). To explore the prevalence of different VFs among various *Salmonella*, a heatmap was made with R and pheatmap, providing for a comparison of the presence or absence of different VFs among the above nine listed genomes and thirteen representative genomes already in the VFDB database with known VFs.

AMR Analysis

Antimicrobial resistance genes were identified through the Center for Genomic Epidemiology (CGE) website with ResFinder 3.0 (Zankari et al., 2012). DNA sequences of each identified AMR gene regions were selected for detailed BLAST analysis. A MUSCLE alignment was performed in Geneious prime software (version 2019.2.3) between *mcr-1.1* (NG_050417.1) and the two *mcr-1* genes sequences in this study. All genes, plasmids and chromosome sequences used in this study were managed and analyzed by Geneious.

A further comparative sequence alignments were performed in Geneious to identify the nucleotide polymorphism and related amino acid substitution sites compared with the original *mcr-1.1* and all variants which could be found on NCBI to date (*mcr-1.2* ∼ *mcr-1.27*). An unrooted rectangular cladogram tree was also generated for all currently known representatives of the MCR protein subgroups (MCR-1 through MCR-10) and related alleles or variants using the Geneious Tree Builder, with UPGMA tree build method and Jukes-Cator genetic distance model in Geneious software, followed by visualization on EvolView (Subramanian et al., 2019). To better understand the genetic environment of the *mcr-1* locus on plasmids of *mcr*-carrying isolates, these sequences were extracted with Geneious, and compared and displayed using Easyfig v2.2.2 (Sullivan et al., 2011).

Genome Data Availability

The genome data of chromosome and plasmid sequences of the two *mcr*-1 gene positive *Salmonella* isolates was submitted to the NCBI nucleotide database under BioProject no. PRJNA498334 with Biosample no. of SAMN10291561 and SAMN10291586, and related accession numbers for chromosome and plasmid sequences were: CP033349, CP033350, CP033351, and CP033352.

RESULTS

AST for 755 Foodborne *Salmonella* Isolates

The percentages related to AMR for all 755 *Salmonella* isolates recovered from various foods are shown in **Table 1**. Among these, 206 isolates (27.3%) were susceptible to all antimicrobial agents and 549 (72.7%) exhibited resistance to at least one compound. The resistance rates were classified into three categories: (1) higher than 34%: NAL, TET, AMP, and SAM; (2) between 11.0 and 24.8%: CHL, SXT, FFC, CIP, KF, CTX and GEN; (3) lower than 10%: CT, CAZ, FEP, IMP, and MEM. Resistance to four cephalosporin-type compounds demonstrated a decreasing trend across the generations of this drug class (KF > CTX/CAZ > FEP). No isolate was resistant to carbapenem-type compounds (IMP and MEM). Sixty five (65/755, 8.6%) isolates were co-resistant to both cefotaxime and ciprofloxacin, two first-line antimicrobial agents used to treat human salmonellosis clinically. One hundred and twenty eight (17.0%), 86 (11.4%), 90 (11.9%), 79 (10.5%), 39 (5.2%), 49 (6.5%), 52 (6.9%), and 26 (3.4%) isolates were resistant to 1, 2, 3, 4, 5, 6, 7, and 8 classes of antimicrobial agents tested, respectively. In total, 134 different AMR profiles were recorded among 549 AMR *Salmonella* isolates. There were 335 isolates (44.4%) were classified as MDR (resistant to three or more classes of antimicrobial agents) and 78 isolates (10.3%) were identified as high level MDR (resistant to seven or eight classes of antimicrobials). Three isolates were co-resistant to 13 different antimicrobial (GEN-AMP-SAM-FEP-SXT-NAL-CHL-TET-CTX-FFC-CAZ-KF-CIP). Nineteen out of 26 provinces returned a resistant rate of higher than 50%, and 9 were recorded to exceed 80% with the highest of 88.6% (31/35, Zhejiang province). Ten of 26 had an MDR rate of no less than 50%, while 4 of them were found to exceed 60% (Inner Mongolia, Jiangsu, Anhui, and Liaoning). The level of MDR rates of Hubei, Shaanxi, and Hunan provinces were higher than 24% (**Figure 1**). The AMR data of 755 isolates were available in **Supplementary data sheet 1**.

Serotyping, Further AST and Plasmid Conjugation for *mcr-1* Gene Positive Isolates

The complete collection was tested for the presence of the *mcr-1* gene by qPCR and two *Salmonella* isolates, CFSA231 and CFSA629, were positive with an *mcr-1* gene detection rate of 0.26% (2/755) among *Salmonella* from mainland China, 2016. The two isolates were recovered from a pork dumpling sample (Huangshi, Hubei) and from an egg sample (Zhongshan, Guangdong), respectively. Based on both serotyping methods mentioned above, CFSA231 and CFSA629 were separately identified as Derby and Typhimurium serotype with the antigen formulas of 1,4,[5],12:f,g:- and 1,4,[5],12:i:1,2. Both isolates demonstrated an MDR phenotype to six or seven classes of antimicrobials but with some notable differences (**Table 2**). For instance, *S.* Derby CFSA231 expressed resistance to gentamicin, ciprofloxacin, folate pathway inhibitors and phenicols, while resistance against cephalosporins and colistin were observed for *S.* Typhimurium CFSA629. Based on the MIC value change for cefotaxime and ceftazidime in combination with clavulanate compared with when tested alone (**Table 2**), *S.* Typhimurium CFSA629 was confirmed as an ESBL positive strain whilst *S.* Derby CFSA231 was negative. *mcr*-harboring plasmids of both isolates could transfer into *E. coli* J53 at frequencies as below: pCFSA231, 1.5×10^{-7} (30°C) and 2.6×10^{-6} (37°C); pCFSA629, 2.1×10^{-4}–2.0×10^{-2} (30°C), and 3.9×10^{-6} (37°C).

Genome Sequence Features, SPIs, Virulence Factors (VFs) and AMR Genes

Both genomes of *S.* Derby CFSA231 and *S.* Typhimurium CFSA629 consisted of a single circular chromosome and a

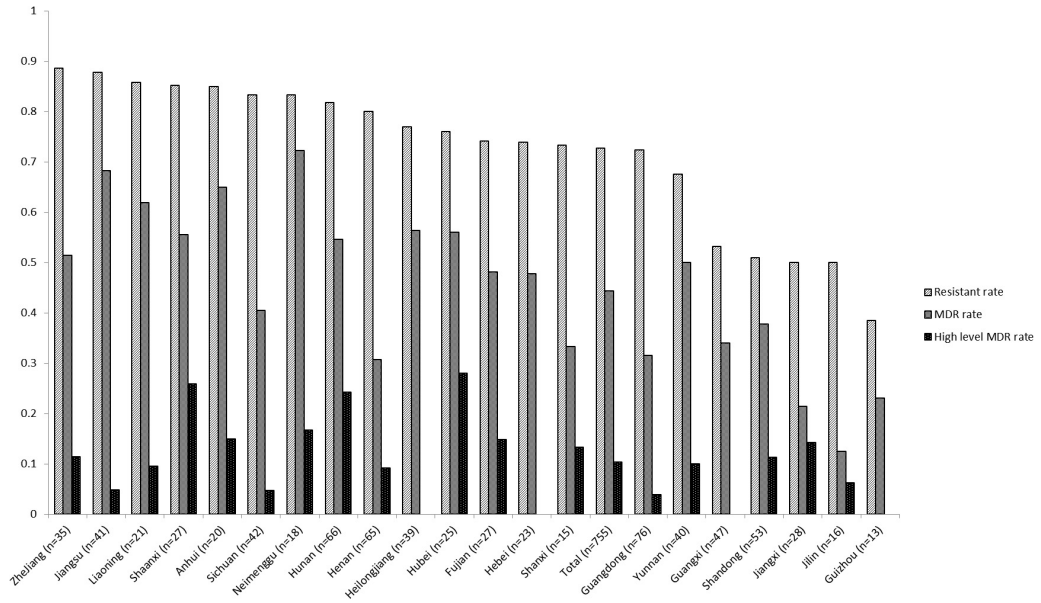

FIGURE 1 | Antimicrobial resistance of foodborne *Salmonella* isolates recovered from different provinces in mainland China, 2016. [Multi-drug resistant (MDR), resistant to three or more classes of antimicrobial agents].

circular plasmid. Details of the genomic features of the bacterial chromosomes and plasmids are shown in **Table 3**. CRISPR features and prophage information is available in **Supplementary Tables 1**, **2**, respectively. Different serotypes showed different SPI genotypes, and SPI details are summarized in **Table 4**, all six *S.* Typhimurium strains contain the same SPI genotype independent of STs; Meanwhile, when compared with two *S.* Typhi (CT18 and Ty2), a small SPI type difference was noted and related to the presence of SPI-6, whilst these could be distinguished from other serotypes in having SPI-7 through SPI-14 and C63PI.

A VF heatmap was generated using R for CFSA231, CFSA629 and 20 additional *Salmonella* reference genomes with known VFs (**Figure 2**). It showed that the bacterial secretion system was the dominant VF class, and the top three VFs included the SPI-1 encoded Type Three Secretion System (T3SS), the SPI-2 encoded T3SS and T3SS effectors denoted by the red and yellow colors in the heatmap (**Figure 2**). The VF pattern of one *Salmonella* isolate appeared to be more closely related to isolates of the same serotype, rather than to the isolates of any other serotypes, such as *S.* Typhi, *S.* Typhimurium, and *S.* Paratyphi A. CFSA629 ST34 was distinguished from those of *S.* Typhimurium ST19 and ST313, with the main difference being the absence of the following VFs: Pef, Mig-5 and Spv. Six typhoidal *Salmonella*, including two *S.* Typhi and four *S.* Paratyphi were clustered into four distinct clades. As listed in **Supplementary Table 3**, the VF number of the 22 *Salmonella* genomes varied from 110 (*S. enterica* subsp. *arizonae* ser.62:z4,z23:– str. RSK2980) to 176 (*S.* Paratyphi C str. RKS4594). The annotated genes belonging to secretion system and fimbrial adherence virulence classes were the top two VFs for CFSA231 and CFSA629.

The resistance genotypes of CFSA231 and CFSA629 identified by Resfinder were shown in **Table 2**. In *S.* Derby CFSA231 the *mcr-1* gene was located on an IncX4-type plasmid pCFSA231 along with a *pap2*-encoding gene distal to this site. We identified a number of similar well conserved plasmids differing by less than 4 nucleotides by BLAST on NCBI, and all of which were distributed in *Enterobacteriaceae,* with a high number of these (>80%) being associated with *E. coli*. Except *mcr-1*, all other 21 AMR-encoding genes identified in the genome of CFSA231, including one ESBL gene ($bla_{\text{OXA-1}}$), were localized to the bacterial chromosome; one point mutation in the *parC* gene, resulting in an amino acid substitution (T57S) was identified. Four ciprofloxacin resistance-encoding genes [*aac(6′)-Ib-cr*, *oqxA*, *oqxB*, *qnrS2*], that are more commonly associated with plasmid-mediated quinolone resistance (PMQR), were mapped on the chromosome. These 21 genes were located within a 64-kbp locus that contained 83 CDS identified which encoded 23 transposases, 20 chromosomal antimicrobial resistant genes mediating resistance to nine drugs in this study, one class 1 integron gene, one recombinase gene and other genes encoding functional and hypothetical proteins (**Figure 3**). A query coverage value of 100% and identity of 100% were recorded with a single nucleotide difference identified between this locus and a similar arrangement reported earlier in a chicken-derived *Salmonella* isolate CD-SL01 recovered from chicken in China, 2015-2016 (NCBI accession number: CP028900.1). Both chromosomal genomes were compared using Geneious and the MAFFT Alignment programme with default settings, and were found to have an identity of 99.947% and 2,565 base/residue differences in total and CD-SL01 was devoid of any plasmids.

In *S.* Typhimurium CFSA629, a mutation in *gyrA* giving rise to a D87Y substitution was found; six and five antimicrobial

TABLE 3 | Genome sequence features for CFSA231 and CFSA629.

Strain or plasmid name	Serotype, MLST type or plasmid replicon type	Number of reads	Mean read length (bp)	Coverage	Size (bp)	G + C content	Number of coding genes, pseudo genes and RNA genes
CFSA231	Derby ST40	65,092	8,680	68.13x	4,834,516	52.1%	4,519; 134; 119
pCFSA231	IncX4				33,309	41.9%	
CFSA629	Typhimurium ST34	54,855	9,083	57.39x	4,999,270	52.1%	4,937; 117; 125
pCFSA629	IncHI2A/IncHI2				210,674	45.2%	

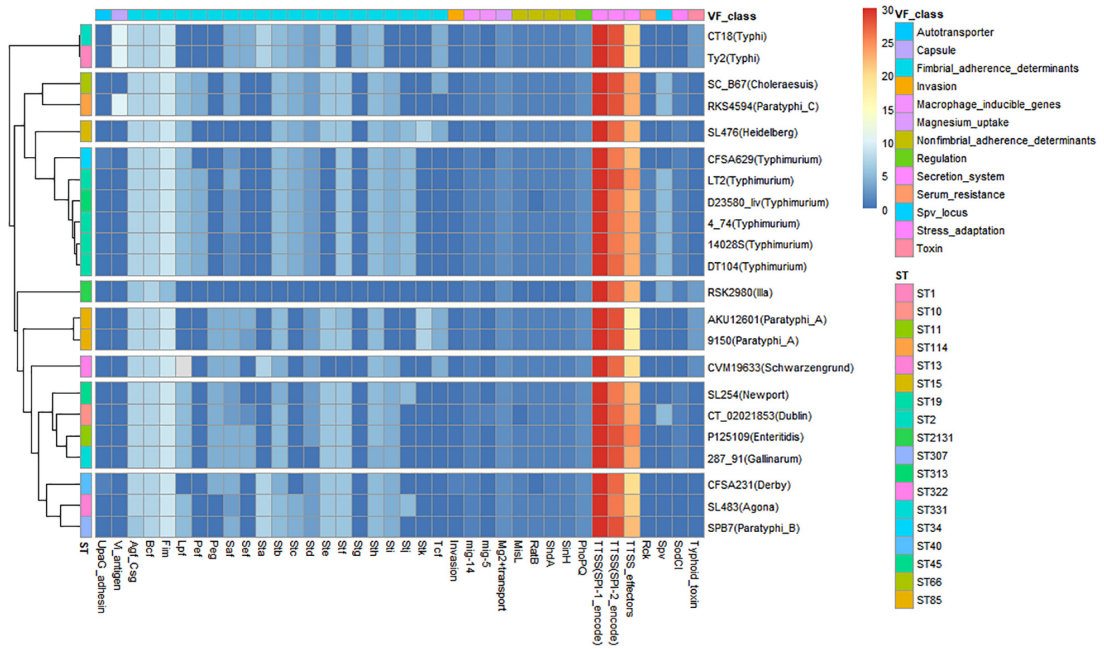

FIGURE 2 | Heatmap showing the relationship of 22 genomes/isolates including *Salmonella* Derby CFSA231 and *Salmonella* Typhimurium CFSA629 and 20 reference genomes based on their differential virulence factor classes. Numbers of virulence factors were represented by different colors, high number were marked with red squares (pixels) and low numbers were represented with blue squares.

resistant genes were located on the chromosome and plasmid, respectively and two different ESBL genes ($bla_{\text{TEM-1B}}$ and $bla_{\text{CTX-M-14}}$) mapped to them each. The *mcr-1* gene was also located on plasmid, an IncHI2 type plasmid pCFSA629, which possessed a single copy of the IS*Apl1* element along with a gene encoding the PAP2 family protein flanking the left and the right side of the *mcr-1* gene. There was two IS*26* composite-like transposonal modules in this isolate which was rich in insertion sequences and transposons: one consisting of IS*26*-*fosA3*-IS*1182*-$bla_{\text{CTX-M-4}}$-IS*26* and another one was IS*26*-[*aac(3)-IV*]-[*aph(4)-Ia*]-IS*6* family-Tn*3*-IS*26*.

Comparison and Relationship Between *mcr* Genotypes and MCR Variants

An *mcr-1* variant was identified and confirmed on pCFSA629 by Geneious (Hu et al., 2021), and was assigned with allele number *mcr-1.19* (MK490674.1, G1,534A) and corresponding protein MCR-1 allele number of MCR-1.19 (QBC35984.1, Val512-to-Ile) in GenBank. Compared with the *mcr-1.1,* no base mutations were observed in the *mcr-1* gene on pCFSA231. The comparative alignment between 26 *mcr-1* variants and *mcr-1.1*, as well as the amino acid differences among their related *mcr-1* coding protein variants was shown in **Table 5**. One single nucleotide difference was observed among most *mcr-1* genotypes (n = 21), and a dinucleotide difference existed in three of these variants. In terms of MCR-1.1 with 541 amino acids, a trinucleotide duplication was found in *mcr-1.11,* resulting in an additional amino acid for an MCR-1 variant of 542 amino acids, while *mcr-1.15* and *mcr-1.26* with 540 amino acids, arising from a single nucleotide polymorphism (SNP) located at initiation codon. It should be noted that three *mcr-1* variants (*mcr-1.10*, *mcr-1.23,* and *mcr-1.24*) had more than 12 nucleotide differences relative to *mcr-1.1,* leading to at least 7 amino acid differences from MCR-1.1. The non-rooted rectangular cladogram showed two distinguished clusters among ten MCR protein sub-groups (**Figure 4**). In the case of the genetic distances within the MCR-1 subgroup, MCR-1.10, MCR-1.23, and MCR-1.24, which exhibited the most amino acid differences relative to MCR-1.1 were also the furthest in

TABLE 4 | Distribution of *Salmonella* pathogenicity island (SPIs) in seven representative genomes of *Salmonella* isolates (Identity threshold: 95%, minimum length: 60%).

Salmonella strain	CFSA231	CFSA629	LT2	14028S	DT104	D23580	4/74	CT18	Ty2
Serotype	Derby	Typhimurium	Typhimurium	Typhimurium	Typhimurium	Typhimurium	Typhimurium	Typhi	Typhi
MLST type	ST40	ST34	ST19	ST19	ST19	ST313	ST19	ST2	ST1
Accession number	CP033350.2	CP033352.2	NC_003197.2	NC_016856.1	NC_022570.1	LS997973.1	NC_016857.1	NC_003198.1	NC_004631.1
SPI-1	–	+	+	+	+	+	+	+	+
SPI-2	+	+	+	+	+	+	+	+	+
SPI-3	+	+	+	+	+	+	+	+	+
SPI-4	+	+	+	+	+	+	+	+	+
SPI-5	–	+	+	+	+	+	+	+	+
SPI-6	–	–	–	–	–	–	–	+	–
SPI-7	–	–	–	–	–	–	–	+	+
SPI-8	–	–	–	–	–	–	–	+	+
SPI-9	–	–	–	–	–	–	–	+	+
SPI-10	–	–	–	–	–	–	–	+	+
SPI-11	–	–	–	–	–	–	–	–	–
SPI-12	–	–	–	–	–	–	–	+	+
SPI-13	–	+	+	+	+	+	+	–	–
SPI-14	–	+	+	+	+	+	+	–	–
C63PI	+	+	+	+	+	+	+	–	–

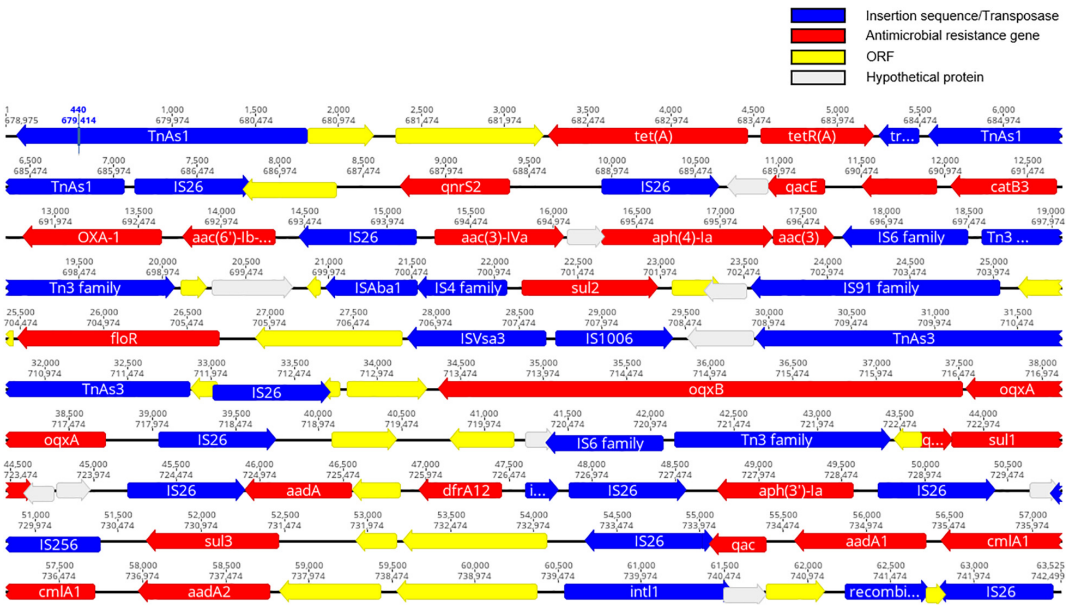

FIGURE 3 | A schematic illustration showing the structural of ∼64kb MDR gene cluster region on chromosome of CFSA231 (created using Geneious software). Antimicrobial resistance (AMR)-encoding genes are indicated in red boxes/arrows. Blue boxes/arrows denote transposon- and integron-associated genes. The individual open reading frame (ORF) are indicated with yellow boxes/arrows. The light gray boxes indicate hypothetical proteins.

genetic distance from MCR-1.1, followed by MCR-1.25, MCR-1.14, MCR-1.3, and MCR-1.15.

Genetic Environment Context of *mcr-1* Gene in pCFSA231 and pCFSA629

The *mcr-1* locus of plasmid pCFSA231 and pCFSA629 were extracted and compared with the relevant *mcr-1* locus on plasmids pHNSHP45-2 (KU341381.1) from *E. coli* and pWW012 (CP022169.1) from *Salmonella* (**Figure 5**). It showed that the gene structures near *mcr-1* on plasmids varied but shared the same regions (genes encoding MCR-1 and PAP2 family proteins), with different presence of insertion sequences (ISs). pCFSA231 had no IS and pCFSA629 obtained only one IS*Apl1;* a tellurium resistance gene cluster was located downstream PAP2 coding gene of pCFSA629 and pHNSHP45-2; pWW012, the *mcr-1*-carrying plasmid from our previous study, consisted of an IS-*mcr-1*-PAP2-IS module which is an IS*Apl1*-flanked composite transposon (Tn*6330*).

TABLE 5 | Nucleotide/amino acid changes of *mcr-1*/MCR-1 alleles/variants compared with *mcr-1.1*/MCR-1.1 (the accession numbers emanated from Reference gene browser with gene family "*mcr-1*," database version: 2020–05-04.1. Nucleotide and amino acid differences were identified by Geneious software).

Allele/variant	RefSeq protein	Refseq nucleotide	GenBank protein	GenBank nucleotide	Nucleotide differences compared with *mcr-1.1*	Amino acid differences compared with MCR-1.1	First discovered host bacteria
mcr-1.1	WP_049589868.1	NG_050417.1	AKF16168.1	KP347127.1	N/A	N/A	*Escherichia coli*
mcr-1.2	WP_065274078.1	NG_051170.1	OBY14952.1	LXQO01000025.1	A8T	Gln3Leu	*Klebsiella pneumoniae*
mcr-1.3	WP_077064885.1	NG_052861.1	ANJ15621.1	KU934208.1	AA111-112GG	Ile38Val	*Escherichia coli*
mcr-1.4	WP_076611062.1	NG_052664.1	APM87143.1	KY041856.1	G1318A	Asp440Asn	*Escherichia coli*
mcr-1.5	WP_076611061.1	NG_052663.1	APM84488.1	KY283125.1	C1354T	His452Tyr	*Escherichia coli*
mcr-1.6	WP_077248208.1	NG_052893.1	AQK48217.1	KY352406.1	G1263A, G1607A	Arg536His	*Salmonella enterica*
mcr-1.7	WP_085562392.1	NG_054678.1	AQQ11622.1	KY488488.1	G643A	Ala215Thr	*Escherichia coli*
mcr-1.8	WP_085562407.1	NG_054697.1	AQY61516.1	KY683842.1	A8G	Gln3Arg	*Escherichia coli*
mcr-1.9[a]	WP_099982800.1	NG_055582.1	ASK38392.2	KY964067.1	T1238C	Val413Ala	*Escherichia coli*
mcr-1.10[b]	WP_096807442.1	NG_055583.1	ASK49940.1	MF176238.1	–	–	*Moraxella* sp.
mcr-1.11[c]	WP_099982815.1	NG_055784.2	ATM29809.1	KY853650.2	GTG19-21dup	Val7dup	*Escherichia coli*
mcr-1.12	WP_104009850.1	NG_056412.1	BBB21811.1	LC337668.1	G9C	Gln3His	*Escherichia coli*
mcr-1.13	WP_109545056.1	NG_057466.1	AVM85874.1	MG384739.1	G465A	Met155Ile	*Escherichia coli*
mcr-1.14	WP_109545052.1	NG_057460.1	ARA74236.1	KX443408.2	AA111-112GG, G591A	Ile38Val, Met197Ile	*Klebsiella pneumoniae*
mcr-1.15[d]	WP_116786830.1	NG_061610.1	AXL06756.1	MG763897.1	AT1-2TA, C836A	Met1del, Thr279Lys	*Klebsiella pneumoniae*
mcr-1.16	WP_136512110.1	NG_064787.1	QBG64271.1	MK568462.1	C952A	Arg318Ser	*Escherichia coli*
mcr-1.17	WP_136512111.1	NG_064788.1	QBG64272.1	MK568463.1	G410C	Ser137Thr	*Escherichia coli*
mcr-1.18	WP_106743337.1	NG_064789.1	–	PGLM01000025.1	T25G	Tyr9Asp	*Escherichia coli*
mcr-1.19	WP_129336087.1	NG_065449.1	QBC35984.1	MK490674.1	G1534A	Val512Ile	*Salmonella enterica*
mcr-1.20	WP_140423329.1	NG_065450.1	SPQ84451.1	LS398440.1	A184C	Met62Leu	*Escherichia coli*
mcr-1.21	WP_140423330.1	NG_065451.1	QCU55424.1	MK965883.1	C1234T	Pro412Ser	*Escherichia coli*
mcr-1.22	WP_148044477.1	NG_065944.1	QDO71694.1	MN017134.1	C1277T	Ser426Phe	*Escherichia coli*
mcr-1.23[e]	WP_160164897.1	NG_067235.1	QHD57408.1	MN873697.1	–	–	*Salmonella enterica*
mcr-1.24[f]	WP_160164898.1	NG_067236.1	QHD64700.1	MN879257.1	–	–	*Escherichia coli*
mcr-1.25	WP_160164899.1	NG_067237.1	QHD64702.1	MN879259.1	G41C, C565G	Ser14Thr, Leu189Val	*Escherichia coli*
mcr-1.26[d]	WP_034169413.1	NG_068217.1	NEU93872.1	JAAGSA010000042.1	T2C	Met1del	*Escherichia coli*
mcr-1.27	WP_163397051.1	NG_068218.1	NEU89143.1	JAAGSB010000042.1	A26G	Tyr9Cys	*Escherichia coli*

[a] *Using start codon that matches other mcr-1 genes rather than the one in the original INSDC entry.*
[b] *Details not listed for 39 nucleotide differences between mcr-1.10 and mcr-1.1 as well as 7 amino acid differences between MCR-1.10 and MCR-1.1.*
[c] *Dup, duplication of nucleotides/amino acids at positions indicated.*
[d] *Del, deletion of amino acids at positions indicated. In comparison of mcr-1.1, the nucleotide at position 1 and 2 (AT) of mcr-1.15 mutate into TA, and also the nucleotide at position 2 (T) of mcr-1.26 mutate into C, resulting in a unavailable translation initiation codon of ATG at 1-3 and a retroposed codon from 4-6(ATG).*
[e] *Details not listed for 12 nucleotide differences between mcr-1.23 and mcr-1.1 as well as 9 amino acid differences between MCR-1.23 and MCR-1.1.*
[f] *Details not listed for 13 nucleotide differences between mcr-1.24 and mcr-1.1 as well as 10 amino acid differences between MCR-1.24 and MCR-1.1.*

DISCUSSION

Salmonella are important foodborne zoonotic pathogens often linked to cases of gastroenteritis and bacteremia, and are one of the leading causes of global bacterial food poisoning worldwide (Threlfall, 2002). The antimicrobial resistance expressed by *Salmonella* is spreading in both developed and developing countries (Parisi et al., 2018). In this study, similar antimicrobial resistance for foodborne *Salmonella* isolates was observed with previous study in China (Hu et al., 2017). The AMR rate recorded against ciprofloxacin (21.3%) indicated a comparatively statistically significant increase when compared with the previous year (Hu et al., 2017). A potential explanation for the high ciprofloxacin intermediate rate in this study (38.3%) may be due to the updated and extended range of criteria in the CLSI guidelines, but this finding partly signal a continuously reduced susceptibility trend among foodborne *Salmonella* to ciprofloxacin in recent years. Gong et al. (2019) found that almost 90% of *S.* Indiana were resistant to both ciprofloxacin and cefotaxime and 8.6% of the tested isolates in this study

demonstrated this feature of resistance, posing a serious threat to public health and ongoing surveillance is clearly necessary in regard to monitoring the emergence of resistance and identify transmission routes.

In this study different serotypes of *Salmonella* possessed different SPI and VF genotypes, and the impact of these differences for a comparison of serotype on virulence potential remains unclear. For example, the hypervirulent invasive non-typhoidal (iNTS) *S.* Typhimurium ST313 str. D23580 could not easily be distinguished from other *S.* Typhimurium of different ST types based on the VFs, since there are several mechanisms that could contribute to its pathogenicity in iNTS (Carden et al., 2015, 2017; Hammarlöf et al., 2018). Even within a serovar, differences in SPI content may also affect virulence potential such as the presence or absence of the *avrA* secreted effector gene in certain *S.* Montevideo lineages (Nguyen et al., 2018). In comparison to many studies of resistance prediction from AMR genes, increased emphasis or the joint on functional transcriptomics, proteomics and genomics techniques are necessary for virulence potential investigation/prediction and pathogenicity modeling.

In this study, two *mcr-1*-carrying foodborne *Salmonella* isolates were recovered among 755 strains (0.26%). According to our latest data related to more than 3,800 foodborne *Salmonella* recovered in mainland China between 2011 and 2019 (data not published), 14 *mcr-1*-harboring isolates were detected corresponding to a positivity rate of 0.4%, which could be regarded as a low prevalence rate. Similar results were reported previously for isolates cultured from clinical, food or food-producing animals (Carnevali et al., 2016; Cui et al., 2017; Lu et al., 2019; Luo et al., 2020). Susceptibility testing of CFSA231 and CFSA629 against colistin, recorded MIC values of 2 mg/L, are consistent with *mcr-1* mediated low-level colistin resistance (2–8 mg/L) (Zhang et al., 2019). Acquisition of this gene could also facilitate further selection of chromosomal mutants in some cases, leading to high-level colistin resistance (HLCR) (Zhang et al., 2019). Thus screening and surveillance for the *mcr-1* in bacteria of importance to human health is critical. Besides their distinct geographic and sample origins, the two *mcr-1*-positive *Salmonella* isolates in this study exhibited different MDR profiles. All 27 compounds tested in the extended AST tests in this study are listed in CLSI and EUCAST and are used in human and veterinary settings. An integrated *One Health* based surveillance system is crucial in tracking these developments, and it should be a more initiative monitoring model which could focus on the antimicrobial resistance dissemination among human beings, animals and environments at the same time (Luo et al., 2020).

The AMR determinants detected in this report were in coherence with the AMR patterns obtained by AST. In the case of *S.* Derby CFSA231 all resistance genes, except for *mcr-1*, were mapped to the chromosome, including four PMQR genes that are more commonly associated with plasmid. The 64 kbp putative SGI-like MDR region in this isolate was largely similar to the *Salmonella* Genomic Island 1 (SGI1) with an ACSSuT phenotype reported earlier (Mulvey et al., 2006), differing in the numbers of mobile genetic elements (MGEs) it contained. BLAST analysis of this locus identified genetically homologous regions in both chromosomal- and plasmid-based sequences in different species including *Salmonella*, *E. coli,* and *K. pneumoniae*, suggesting that this putative SGI1-like region might have already disseminated among the Enterobacteriaceae family, and this will cause a potential of a processed *copy-out-paste-in* transpositional event resulting in dissemination and stabilization of these related resistant genes. The corresponding resistance genes that zoonotic *Salmonella* have acquired are more commonly located on plasmids, in transposons, gene cassettes, or variants of the SGI1 and SGI2 loci (Michael and Schwarz, 2016), thus studies exploring the dissemination of this putative SGI1-like locus may provide further insights into its evolution (Michael and Schwarz, 2016).

The *mcr-1* gene has been found in plasmids with different Inc types such as IncI2, IncHI1, IncHI2, IncP, IncX4, IncFI, and IncFIB (Zhi et al., 2016; Poirel et al., 2017). Based on the epidemiological study (Lu et al., 2019), the *mcr-1*-carrying IncHI2 type plasmid was originally identified in *Salmonella* isolated from diarrhoeal outpatients in Shanghai in 2014 and increasingly detected after the summer of 2015, representing the primary replicon type in 2016. It was reported that foods had played important roles in the expansion of *mcr-1*-carrying IncHI2 plasmids among different members of the Enterobacteriaceae family before 2016, and what is of increasing concern, with usage of antimicrobials other than polymyxins, co-selection of *mcr-1* may happen due to the MDR phenotype and conjugative ability of the IncHI2 plasmids (Zhi et al., 2016). For instance, a *Salmonella* IncHI2 plasmid that predate the era of mass antibiotic usage has been sequenced with no detectable AMR genes (Nguyen et al., 2017), but modern IncHI2 plasmids encoding multiple AMR genes are predominant in MDR *Salmonella* (Chen et al., 2016). Thus, there is a potential for capture of *mcr-1* in MDR *Salmonella* due to co-selection by other antimicrobials other than polymyxin. The prevalence of the *mcr* gene is higher in resistant bacterial isolates cultured from food-producing animals when compared to those cultured from humans. This may be due to the application of antimicrobials in agricultural for production purposes. Although use of colistin in agriculture production has been recently banned in China and Brazil, surveillance must be maintained as increasing clinical usage of polymyxins may also contribute to the risk of dissemination of *mcr* markers in nosocomial settings (Osei Sekyere, 2019).

The co-existence of plasmid-mediated *mcr-1* and carbapenemase-encoding genes has been identified in Enterobacteriaceae and there are increasing reports of this co-occurrence worldwide. Isolates of both clinical and animal origin combining *bla*-encoding genes (such as $bla_{\text{NDM-1}}$, $bla_{\text{NDM-5}}$, $bla_{\text{NDM-9}}$, $bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{VIM-1}}$) with *mcr-1* gene may signal the risk of the emerging of strains expressing pan-drug resistance (PDR) (Yang et al., 2016; Lai et al., 2017; Wang et al., 2018; Le-Vo et al., 2019). It is important to note that the presence of *mcr-1* gene on the chromosome in recent studies (Falgenhauer et al., 2016;

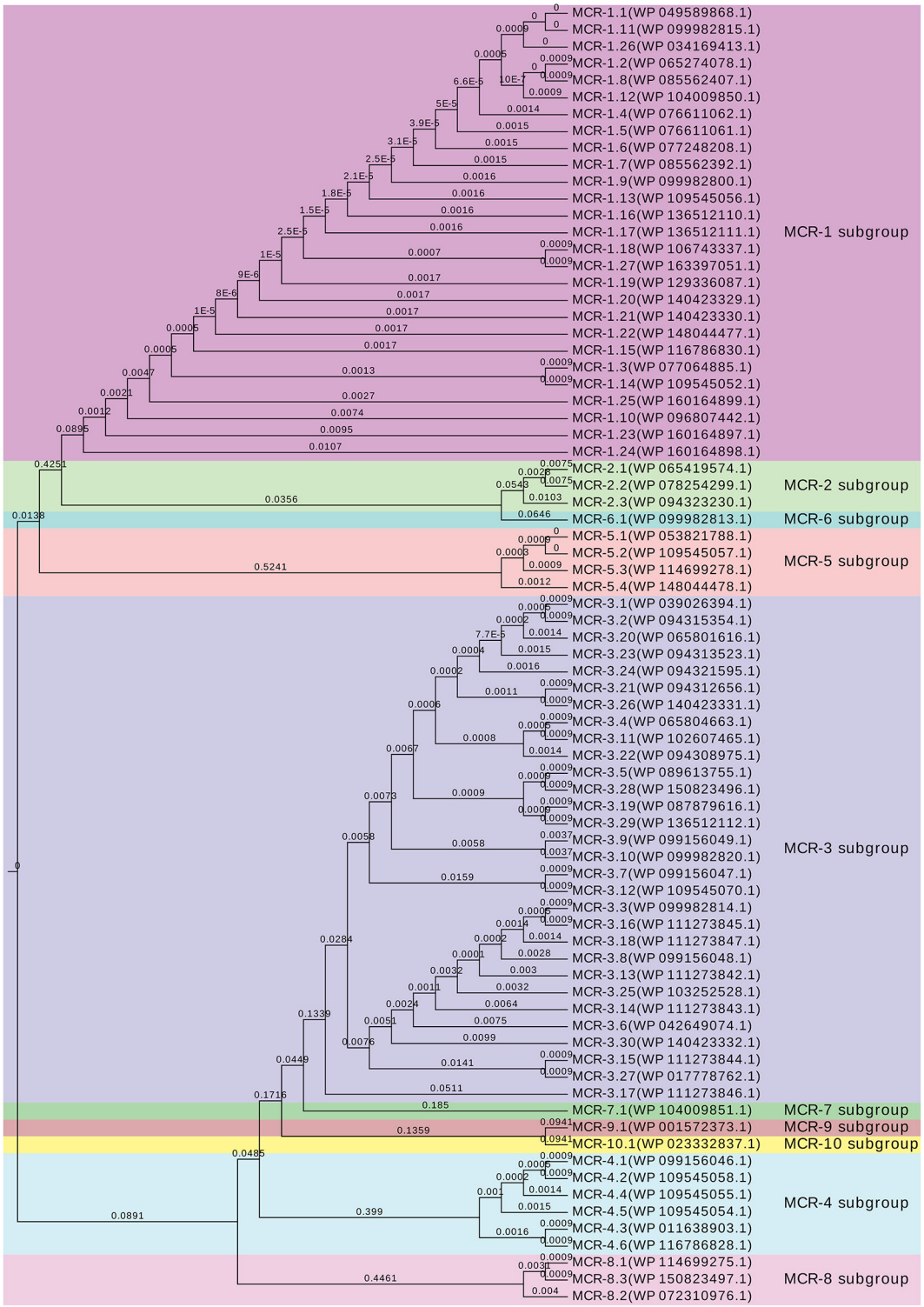

FIGURE 4 | Genetic relationships between MCR protein subgroups and related alleles. UPGMA rectangular cladogram tree of the deduced amino acid sequences of putative phosphoethanolamine transferase belonged to different subgroups was constructed using Geneious prime v2019.0.4 software with Jukes–Cantor genetic distance model, and visualized by EvolView online tool (v2.0, https://www.evolgenius.info/evolview/). NCBI accession numbers are listed following by the MCR alleles and available in GenBank.

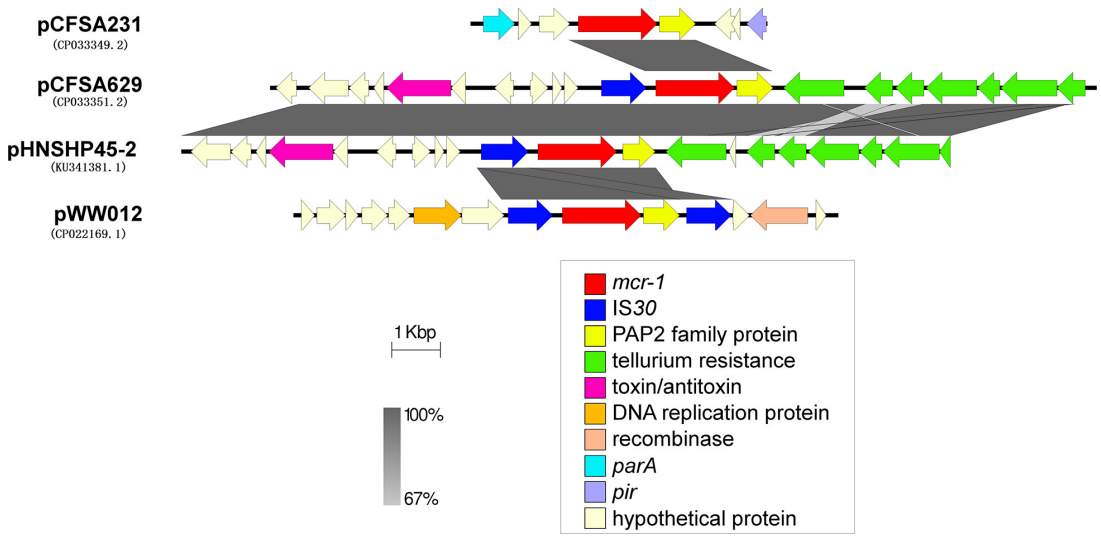

FIGURE 5 | Genetic environments related to *mcr-1* gene in bacterial plasmids. The figure was generated by Easyfig (v2.2.2). Plasmids marked with "pCFSA" were carried by two *mcr-1* positive *Salmonella* isolates in this research, and plasmid pWW012 belonged to a previous research in our lab (accession number: CP022169), while pHNSHP45-2 (accession number: KU341381) belonged to *Escherichia coli* strain SHP45, which is the first isolate reported harboring *mcr-1* gene. Confirmed and putative open reading frames (ORFs) are indicated by block arrows and their orientations with different colors, and arrow size is proportional to the predicted ORF length. *mcr-1* gene is indicated by a red arrow, while genes encoding mobile elements (insertion sequence, IS) are indicated by blue arrows. Regions of homology between the plasmids ranging from 67 to 100% are indicated by the graded shaded regions between sequences.

Zurfluh et al., 2016; Yamaguchi et al., 2020) suggest that the *mcr-1* gene could become more stable through vertical inheritance in *mcr-1*-carrying isolates.

The role of MGEs such as transposons has made an important contribution to the AMR rapid dissemination by horizontal gene transfer under selective pressure imposed by the antimicrobial usage (Snesrud et al., 2018). *mcr-1* gene could be found in various combinations with one or two copies of IS*Apl1* or devoid of the IS element with different replicon types (Li et al., 2017). Snesrud et al. (2018) presented representative sequences of the four general *mcr-1* structures identified to date: (a) composite transposon Tn*6330* that is thought to mediate the initial mobilization of event; (b) a single-ended structure with a distal copy of IS*Apl1*; (c) a structure lacking both copies of IS*Apl1*; (d) and a single-ended structure with a proximal copy of IS*Apl1* only. We can imagine a genetic element with the loss of IS*Apl1* may provide a more stable *mcr-1* state, especially when integrated in the chromosome. In this study, pCFSA231 and pCFSA629 are classified in the structures described in (c) and (b) above, respectively. They did not contain two ISs and the likelihood of losing or moving *mcr-1* gene relatively decreased, however, our results still suggest that plasmids of various replicon types may contribute to the *mcr-1* gene movement and global spread, accelerating the frequency of colistin resistance worldwide.

CONCLUSION

Colistin is an important antibacterial agent used for treating MDR Gram-negative bacteria infections. Intrinsic colistin resistance located on the chromosome was generally thought to be non-transferable until the detection of MCR, a transferable polymyxin resistance reported globally among various bacterial species with comparatively few descriptions in *Salmonella*. In the context of the rapid spreading trend of the clinical *mcr-1*-harboring *Salmonella* and continuous discoveries of novel *mcr* genes and related variants, we report on the AMR profiles of a set of foodborne *Salmonella* cultured from mainland China and describe the complete genomes of two *mcr-1*-positive *Salmonella* isolates, including a *S.* Typhimurium isolate with an *mcr-1*-variant. Improved surveillance is important for understanding the dissemination of *mcr* genes among foodborne *Salmonella* around the world.

DATA AVAILABILITY STATEMENT

The genome data of chromosome and plasmid sequences of the two *mcr-1* gene positive *Salmonella* isolates were submitted to the NCBI nucleotide database under BioProject numbers PRJNA498334 with Biosample No. of SAMN10291561 and SAMN10291586, and related accession numbers for chromosome and plasmid sequences were: CP033349, CP033350, CP033351, and CP033352.

AUTHOR CONTRIBUTIONS

YH performed the literature search. YH, FL, and SF designed the research. YH, SN, WW, XG, YD, CL, and XC performed the experiments and collected the data. YH, SN, WW,

and JX analyzed and interpreted the data and finished the figures and tables. YH and SN wrote the manuscript. FL and SF reviewed and edited the manuscript. All authors read and approved the manuscript. YH, SN, FL, and SF have accessed and verified the underlying 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.2021.636284/full#supplementary-material

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Emergence of IncHI2 Plasmids With *Mobilized Colistin Resistance* (*mcr*)-*9* Gene in ESBL-Producing, Multidrug-Resistant *Salmonella* Typhimurium and Its Monophasic Variant ST34 From Food-Producing Animals in Italy

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A collection of 177 genomes of *Salmonella* Typhimurium and its monophasic variant isolated in 2014–2019 from Italian poultry/livestock (*n* = 165) and foodstuff (*n* = 12), previously screened for antimicrobial susceptibility and assigned to ST34 and single-locus variants, were studied in-depth to check the presence of the novel *mcr-9* gene and to investigate their genetic relatedness by whole genome sequencing (WGS). The study of accessory resistance genes revealed the presence of *mcr*-9.1 in 11 ST34 isolates, displaying elevated colistin minimum inhibitory concentration values up to 2 mg/L and also a multidrug-resistant (MDR) profile toward up to seven antimicrobial classes. Five of them were also extended-spectrum beta-lactamases producers (bla_{SHV-12} type), mediated by the corresponding antimicrobial resistance (AMR) accessory genes. All *mcr*-9-positive isolates harbored IncHI2-ST1 plasmids. From the results of the Mash analysis performed on all 177 genomes, the 11 *mcr-9*-positive isolates fell together in the same subcluster and were all closely related. This subcluster included also two *mcr*-9-negative isolates, and other eight *mcr*-9-negative ST34 isolates were present within the same parental branch. All the 21 isolates within this branch presented an IncHI2/2A plasmid and a similar MDR gene pattern. In three representative *mcr*-9-positive isolates, *mcr-9* was demonstrated to be located on different IncHI2/IncHI2A large-size (∼277–297 kb) plasmids, using a combined Illumina–Oxford Nanopore WGS approach. These plasmids were also compared by BLAST analysis with publicly available IncHI2 plasmid sequences harboring *mcr*-9. In our plasmids, *mcr*-9 was located in a ∼30-kb region lacking different genetic elements of the typical core structure of *mcr*-9 cassettes. In this region were also identified different genes involved in heavy metal metabolism. Our results underline how genomics and WGS-based surveillance are increasingly

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indispensable to achieve better insights into the genetic environment and features of plasmid-mediated AMR, as in the case of such IncHI2 plasmids harboring other MDR genes beside *mcr*-9, that can be transferred horizontally also to other major *Salmonella* serovars spreading along the food chain.

Keywords: *Salmonella* Typhimurium, monophasic variant, *mcr-9*, whole genome sequencing, long-read sequencing, multidrug resistance, ESBL, IncHI2 plasmid

INTRODUCTION

Salmonellosis, among the most important foodborne zoonoses worldwide, is the second most commonly reported gastrointestinal infection in humans after campylobacteriosis in the European Union/European Economic Area, mainly caused by the consumption of contaminated food (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2021). In Europe, among all confirmed salmonellosis cases, the three most commonly reported *Salmonella* serovars in 2019 were *Salmonella* Enteritidis (50.3%), *Salmonella* Typhimurium (11.9%), and monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) (8.2%) (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2021). Sequence types (STs) 19, 34, 313, and 213 represent the most frequently reported STs for *S.* Typhimurium. Starting from the past two decades, the global pandemic *S.* Typhimurium ST34 clone has been increasingly reported, replacing to the "traditional" clone ST19 (Biswas et al., 2019). The efficacy of antimicrobial therapy for salmonellosis may be impaired by the spread of antimicrobial-resistant isolates (Michael and Schwarz, 2016), particularly to last-resort drugs used to treat severe infections in humans.

Colistin is a last-resort antibiotic of the polymyxin family, increasingly used for treating human invasive infections by multidrug-resistant (MDR) or extensively drug-resistant (XDR) Enterobacteriaceae (Poirel et al., 2017). To date, 10 different *mcr* genes have been reported worldwide in Enterobacteriaceae (Wang et al., 2020), with 6 of them (*mcr*-1 to *mcr*-5 and *mcr*-9) identified so far in *Salmonella enterica* (Lima et al., 2019); MDR monophasic *S.* Typhimurium ST34 harboring *mcr* genes (*mcr*-1, *mcr*-3, and *mcr*-5) has also been widely reported worldwide.

mcr-9 is the most recent *mcr* homolog (65 and 63% amino acid identity with *mcr*-3 and *mcr*-7, respectively) so far described in *Salmonella* serovars in different countries in samples from humans (Carroll et al., 2019; Pan et al., 2020; Wang et al., 2021), horses (Elbediwi et al., 2021), livestock (Borowiak et al., 2020; Leite et al., 2020), food (Borowiak et al., 2020; Cha et al., 2020; Tyson et al., 2020; Wang et al., 2021), and environment (Wang et al., 2021).

mcr-9 is an inducible plasmid-borne gene mainly associated with the IncHI2 replicon type (Li et al., 2020), encoding an acquired phosphoethanolamine transferase not conferring by itself clinical resistance to colistin. However, subinhibitory concentrations of colistin could induce its expression, which, in *E. coli*, is mediated by a two-component regulatory system encoded by the *qse*C and *qse*B genes, located downstream of *mcr*-9 (Kieffer et al., 2019).

The aims of this work were to (i) reanalyze by whole genome sequencing (WGS) a historical collection of *S.* Typhimurium and its monophasic variant within ST34 (along with its single-locus variants) to screen for the presence of *mcr-9* and to investigate the relatedness of their genomes and (ii) determine the genetic environment of the *mcr*-9 gene and fully reconstruct the *mcr*-9-harboring plasmids by using a combined Illumina–Oxford Nanopore WGS approach.

MATERIALS AND METHODS

Salmonella Isolates

A collection of 177 genomes of *S.* Typhimurium (n = 39) and its monophasic variant (n = 138) assigned to ST34 and single-locus variants by WGS (**Supplementary Table 1**) was studied in-depth. These isolates were obtained from animal samples (n = 165), collected from Italian poultry (n = 1 broiler chicken, n = 2 laying hens, and n = 14 fattening turkeys) and livestock [n = 106 fattening pigs and n = 42 bovine animals <12 months (veal calves)] and also foodstuff (n = 12), in the frame of antimicrobial resistance (AMR)-monitoring activities (according to Decision 2013/652/EU[1]) conducted from 2014 to 2019 by the National Reference Laboratory for Antimicrobial Resistance (NRL-AR) and previously screened for antimicrobial susceptibility.

Antimicrobial Susceptibility Testing (AST)

AST was performed as minimum inhibitory concentration (MIC) determination by broth microdilution, using the EU consensus 96-well microtiter plates (Trek Diagnostic Systems, Westlake, OH, United States). The results were interpreted according to epidemiological cutoffs (ECOFFs) included in the Annex A of the EU Decision 2013/652/EU, and for sulfamethoxazole the tentative ECOFF of >256 mg/L according to the EURL-AR protocol for antimicrobial susceptibility testing of *Escherichia coli, Salmonella*, and *Campylobacter*[2]. *E. coli* ATCC 25922 was used as quality control strain.

Illumina Short-Read Sequencing and Bioinformatics Analysis

WGS was first performed using an Illumina platform (MiSeq). DNA extraction, library preparation, trimming, and *de*

[1]https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32013D0652&from=it

[2]https://www.eurl-ar.eu/CustomerData/Files/Folders/23-eqas/558_2020-protocol-eurl-ec-salm-camp.pdf

novo assembly of raw reads were performed according to Alba et al. (2020).

Molecular characterization was performed on all the assembled genomes with the ABRicate tool[3] using the Genomic Epidemiology (CGE) databases of ResFinder[4] and PlasmidFinder[5], and the MLST[6] and SeqSero2 (Zhang et al., 2019) tools for the detection of the genetic basis of AMR and plasmid replicon types and to confirm the STs and the serotype *in silico*, respectively.

The pMLST 2.0 online version[7] was also used for pMLST analysis on *mcr*-9-positive isolates and in representative *mcr*-9-negative isolates. The presence of the two-component regulatory system of *mcr*-9 inducible expression encoded by the *qseC* and *qseB* genes (Kieffer et al., 2019), was also searched by BLAST analysis.

In order to determine the genetic relatedness of the whole set of *Salmonella* genomes analyzed, all raw reads from the 177 genomes were also compared and clustered by using the Mash algorithm (Katz et al., 2019).

Oxford Nanopore Technologies (ONT) Long-Read Sequencing and Bioinformatics Analysis

In order to resolve the complete sequence, fully reconstruct the *mcr*-9-harboring plasmids, and precisely identify and locate *mcr*-9-harboring regions, three selected *mcr*-9-positive (IDs: 19063952, 15060500, and 19093665) and one *mcr*-9-negative (ID: 17021625; one of the most closely related to positive ones selected for comparison purposes) isolates were also sequenced using the nanopore-based MinION device (ONT) with the rapid barcoding kit (SQK-RBK004). A hybrid (Illumina–Oxford Nanopore) assembly was performed using the Unicycler pipeline (Branton et al., 2008) with the default parameters.

The assembly obtained was annotated using the RAST Server (Aziz et al., 2008). Additionally, a manual curation for the obtained annotation was performed, especially for the Insertion Sequences (ISs) by using the ISfinder database (Siguier et al., 2006). The presence of virulence genes was also determined on the obtained plasmid sequences using the VF database[8].

The *mcr*-9-carrying plasmids obtained were also compared using BLAST (Altschul et al., 1990) with two previously published complete *mcr*-9-carrying plasmid sequences from Enterobacteriacae: (a) an *mcr*-9/bla_{VIM-1}-carrying plasmid named p3846_IncHI2_mcr (accession number CP052871) with a size of 293,138 bp, isolated from *Enterobacter cloacae* firstly identified in Italy from a human clinical case (Marchetti et al., 2021); (b) an *mcr*-9/bla_{VIM-1}-carrying plasmid named pRH-R27 (accession number LN555650.1) with a size of 299,305 bp from a *Salmonella* infantis isolated from a fattening pig farm in Germany (Falgenhauer et al., 2017).

Graphical representation of the full plasmid comparison, of the *mcr*-9-specific region, and dendrogram was carried out using different tools: BRIG (Alikhan et al., 2011), EasyFig (Sullivan et al., 2011), and iTol (Letunic and Bork, 2021), respectively.

RESULTS

AST

The AMR phenotypes of all Salmonella isolates are reported in **Supplementary Table 1**. As for the 11 *mcr*-9-positive isolates, beside the AMR gene profiles, also the MIC values are reported in detail in **Table 1**.

For isolates reported in **Table 1**, the MIC values of highest priority critically important antimicrobials (HPCIAs) such as colistin, cefotaxime, ceftazidime, and ciprofloxacin were interpreted also according to European Committee on Antimicrobial Susceptibility Testing (EUCAST)[9] clinical breakpoints. In synthesis, out of 11 isolates, 6 showed microbiological and clinical resistance to cefotaxime (FOT) and ceftazidime (TAZ) (MIC range 4–8 mg/L for FOT and 16 mg/L for TAZ). Five showed microbiological resistance (MIC range 0.25–0.5 mg/L), and one also clinical resistance (MIC = 2 mg/L) to ciprofloxacin (CIP). The 11 *mcr*-9-positive isolates displayed elevated colistin MIC values: 4 of them had a MIC of 2 mg/L, on the epidemiological cutoff and clinical breakpoint, and 7 had a MIC of 1 mg/L.

Illumina Short-Read Sequencing and Bioinformatics Analysis

Most of the *S.* Typhimurium and monophasic variant isolates were confirmed as belonging to ST34 (n = 140), while n = 37 to single-locus variants ST19 (n = 29), ST568 (n = 2), and ST376 (n = 6) (**Figure 1** and **Supplementary Table 1**). The study of accessory resistance genes revealed the presence of *mcr*-9.1 in 11 ST34 isolates (1 *S.* Typhimurium and 10 monophasic variant) from the cecal content of seven pigs, three veal calves, and from a pig carcase swab (**Table 1** and **Supplementary Table 1**), but none of them displayed phenotypic resistance to colistin (7 isolates displayed MIC values of 1 mg/L and 4 isolates of 2 mg/L). Details of genomic characteristics analyzed by WGS of the 11 *mcr*-9-positive isolates are reported in **Table 1**. These isolates displayed an MDR gene profile toward up to seven antimicrobial classes (aminoglycosides, beta-lactams, trimethoprim, tetracyclines, sulfamethoxazole (fluoro)quinolones, and phenicols), mediated by AMR accessory genes. In particular, beyond the presence of the *mcr*-9 gene, 9 of the 11 isolates presented the *aac(6′)-Iaa*, *aph(6)-Id*, bla_{TEM-1B} gene pattern associated, respectively, with amikacin/tobramycin, streptomycin, and β-lactam resistance. Eight of them also harbored gentamicin resistance genes as *aac*(3)-IV and/or *aac*(3)-IId and/or *aac*(6′)-IIc combined with *sul*1 and/or *sul*2 (seven isolates), *tet*(B) and/or *tet*(D) (six isolates), *dfrA19* or *dfrA27* (six isolates), the extended-spectrum beta-lactamases (ESBL) gene bla_{SHV-12} (five isolates), *flo*R and/or *catA1* and/or *catA2* (five isolates), *qnrA1* or

[3]https://github.com/tseemann/abricate
[4]https://cge.cbs.dtu.dk/services/ResFinder/
[5]https://cge.cbs.dtu.dk/services/PlasmidFinder/
[6]https://github.com/tseemann/mlst
[7]https://cge.cbs.dtu.dk/services/pMLST/
[8]http://www.mgc.ac.cn/VFs/
[9]https://www.eucast.org/

TABLE 1 | Genotypic and phenotypic characterization of the *mcr*-9 and selected *mcr*-1 carrying isolates analyzed by WGS.

						Antimicrobial resistance profile		Plasmid content	
Isolate ID	**Serotype *in silico***	**Origin**	**Year of isolation**	**ENA accession number**	**ST**	**Horizontally acquired genes**	**Phenotypic AST profile (MIC value mg/L)**	**Plasmid replicons**	**IncHI2 Plasmid MLST (pMLST)**
19015927	I 4,[5],12:i:-	Fattening pigs-cecum	2019	ERS6592624	34	bla_{TEM-1B}, *aph*(3')-Ia, *cat*A2, *aac*(6′)-Iaa, ARR3, *dfr*A27, *aad*A16, *sul*1, *mcr*-9, *aac*(3)-IVa, *aph*(4)-Ia, *flo*R, *tet*D, *aph*(3′′)-*Ib*, *aph*(6)-Id	CHL(256), GEN(32), TMP(64), TET(128), AMP(128), SMX(2048) COL (2)	IncHI2A, IncHI2, IncQ1	ST1
17037369	I 4,[5],12:i:-	Fattening pigs-cecum	2017	ERS6592625	34	*aac(6′)*-Iaa, bla_{SHV}12, *aac*(3)-IId, *sul*2, *aph*(3′′)-Ib, *aph*(6)-Id, *mcr*-9, bla_{TEM-1B}, *cat*A1, *qnr*S1	CHL(256), GEN(64), TET(64), AMP(128), CIP(0,5), FOT(8), TAZ(16), SMX(2048) COL (1)	IncHI2, IncHI2A	ST1
15049009	I 4,[5],12:i:-	Fattening pigs-cecum	2015	ERS2030266	34	*aac*(6′)IIc, *tet*B, *dfr*A19, *flo*R, *tet*D, *mcr*-9, *sul*1, *aph*(6)-Id, *aac*(6′)-Iaa, *aad*A2	CHL(256), GEN(64), TMP(64), TET(128), AMP(128), SMX(2048) COL (1)	IncHI2, IncHI2A	ST1
15060498	I 4,[5],12:i:-	veal calves production animals-cecum	2015	ERS2030252	34	bla_{TEM-1B}, *aac*(6′)-Iaa, *flo*R, *tet*B, *sul*2, *tet*D, *mcr*-9, *qnr*A1, *dfr*A19, *aph*(6)-Id, *aad*A2, *sul*1	CHL(128), TMP(64), TET(64), AMP(128), CIP(0,25) SMX(2048) COL (1)	IncHI2, IncHI2A	ST1
15060500°	I 4,[5],12:i:-	veal calves production animals-cecum	2015	ERZ2110487	34	bla_{TEM-1B}[§], *aac*(6′)-Iaa, *tet*B, *flo*R[§], *sul*2[§], *tet*D[§], *mcr*-9[§], *aph*(6)-Id[§]	CHL(128), TET(128), AMP(128), SMX(2048), COL (1)	IncHI2, IncHI2A	ST1
15083030	Typhimurium	Fattening pigs-cecum	2015	ERS2030200		bla_{TEM-1B}, *aac*(6′)-Iaa, bla_{SHV}12, *tet*B, *mcr*-9, *cat*A1	CHL(256), TET(128), AMP(128)FOT(4), TAZ(16) COL (1)	IncHI2, IncHI2A	ST1
19041082	I 4,[5],12:i:-	Fattening pigs-cecum	2019	ERS6592626	34	bla_{TEM-1B}, *mcr*-9, *aac*(3)-IVa, *aph*(4)-Ia, *tet*B, *aac*(6′)-IIc, *qnr*B2, bla_{SHV}12, *flo*R, *aph*(3′)-Ia, *sul*2, *tet*C, *aac*(6′)Iaa, *dfr*A19, *cat*A2, *aph*(6)-Id, *aad*A2, *sul*1	CHL(256), GEN(64), TMP(64), TET(128), AMP(128), CIP(0,5), FOT(8),TAZ(16), SMX(2048) COL (2)	IncHI2, IncHI2A, IncI1-I(Gamma)	ST1
19093665°	I 4,[5],12:i:-	Fattening pigs-cecum	2019	ERZ2110531	34	*aac*(6′)-Iaa, bla_{TEM-1B}[§], *aac*(3)-IVa, *aph*(4)-Ia, *aac*(6′)-IIc[§], *sul*1[§], bla_{SHV}12[§], *dfr*A19[§], *flo*R, *tet*D[§], *mcr*-9[§], *aph*(6)-Id[§]	CHL(256), GEN(64), TMP(64), TET(128), AMP(128), FOT(8), TAZ(16), SMX(2048) COL (1)	IncHI2, IncHI2A, IncI1-I(Gamma)	ST1
19057303	I 4,[5],12:i:-	veal calves production animals-cecum	2019	ERS6592627	34	*mcr*-9, *qnr*A1, *sul*1, *aph*(6)-Id, *aph*(3′′)-Ib, bla_{TEM-1B}, *aad*A2, *sul*2, *aac*(6′)-Iaa, *tet*B, *aac*(6′)-IIc, *flo*R, *dfr*A19, *tet*D	CHL(256), GEN(64), TMP(64), TET(128), AMP(128), CIP(0,5), SMX(2048) COL (2)	IncHI2, IncHI2A	ST1
15045799	I 4,[5],12:i:-	Fattening pigs-cecum	2015	ERS2030275	34	*aac*(6′)-Iaa, *tet*B, *aac*(6′)-IIc, *dfr*A19, bla_{SHV}12, *tet*D, *mcr*-9, *qnr*A1, *sul*1, bla_{TEM-1B}, *aph*(6)-Id, *aad*A2	GEN(64), TMP(64), TET(128), AMP(128), CIP(0,5), FOT(4), TAZ(16), SMX(2048) COL (1)	IncHI2, IncHI2A	ST1
19063952°	I 4,[5],12:i:-	Carcase swab from pig	2019	ERZ2110542	34	*aph*(6)-Id[§], *aad*A2[§], *aac*(6′)-Iaa, *tet*B, *aac*(6′)-IIc[§], *dfr*A19[§], *sul*2[§], bla_{SHV}12[§], *tet*D[§], *mcr*-9[§], *sul*1[§], bla_{TEM-1B}[§]	GEN(64), TMP(64), TET(128), AMP(128), FOT(8), TAZ(16), SMX(2048) COL (2)	IncHI2, IncHI2A	ST1
17021625*	I 4,[5],12:i:-	Fattening pigs-cecum	2017	ERZ2110516	34	*aac*(6′)-Iaa, *tet*A[§], *mcr*-1.1, *flo*R[§], *sul*1[§], *dfr*A1[§], *tet*M[§], *aph*(3′)-Ia[§], *sul*2[§], *sul*3[§], *qnr*B19, bla_{TEM-1B}[§], *aph*(6)-Id[§]	CHL(256), NAL(64), TMP(64), COL(8), TET(128), AMP(128), CIP(2), TGC(2), SMX(2048) COL (8)	Col(pHAD28), IncHI2, IncHI2A	ST4

*AST, Antimicrobial susceptibility test results associated with AMR genetic background; ENA, European Nucleotide Archive, ST = sequence type following the scheme of Enterobase (https://enterobase.warwick.ac.uk/); AMR, antimicrobial resistance; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; FOT, cefotaxime; GEN, gentamicin; SMX, sulfamethoxazole; TAZ, ceftazidime; TET, tetracycline; TMP, trimethoprim; TGC, tigecycline; *mcr-9-negative isolate, long reads sequenced, °mcr-9-positive isolate, long reads sequenced, § AMR gene located on IncHI2 plasmid revealed by the hybrid (Illumina–Oxford Nanopore) approach.*

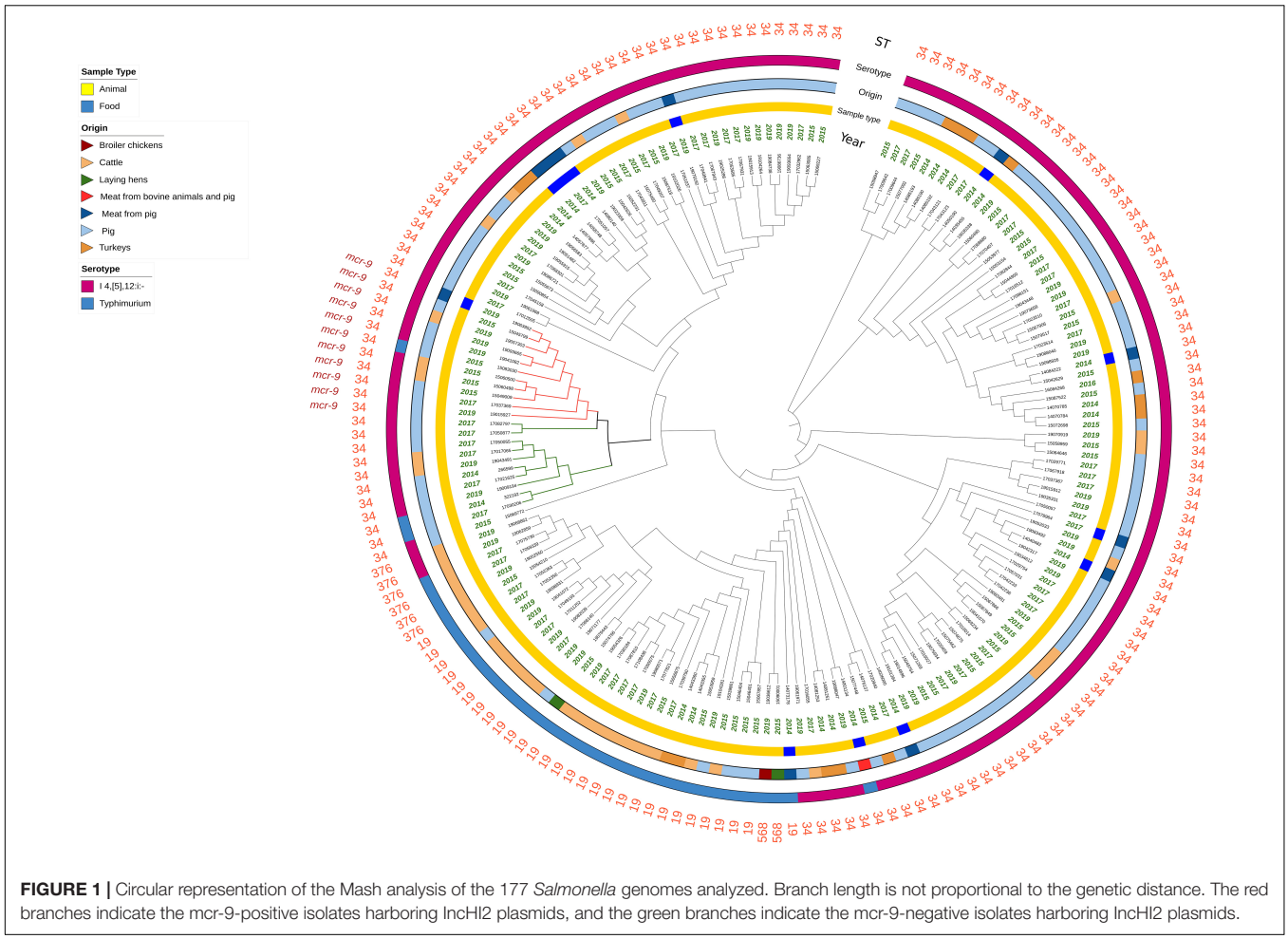

FIGURE 1 | Circular representation of the Mash analysis of the 177 *Salmonella* genomes analyzed. Branch length is not proportional to the genetic distance. The red branches indicate the mcr-9-positive isolates harboring IncHI2 plasmids, and the green branches indicate the mcr-9-negative isolates harboring IncHI2 plasmids.

qnrB2 or *qnrS1* (four isolates) genes, encoding, respectively, sulfamethoxazole, tetracycline, trimethoprim, all beta-lactams, phenicol, and fluoroquinolone resistance. The abovementioned AMR gene profiles were confirmed by the corresponding AMR phenotypes (**Table 1**). No *mcr*-9-positive isolates presented known chromosomal point mutations associated with AMR phenotypes, and all tested negative for the presence of *qseC* and *qseB* regulatory genes. All *mcr*-9-positive isolates harbored an IncHI2/2A, two an IncI1 and one an IncQ replicon type. Moreover, the pMLST analysis revealed that all of them harbored an IncHI2-ST1 plasmid, while one representative *mcr*-9-negative isolate harbored an IncHI2-ST4 replicon (**Table 1**).

The results of the Mash analysis are represented in **Figure 1**. In the Mash tree, three main clusters were identified, with two of them consisting of 7 and 29 ST34 monophasic variant isolates, and the third one, being the most complex, grouped all the remaining isolates in several subclusters. No clear clustering based on year of sampling, sample type, or origin of the isolates was observed. The 11 *mcr*-9-positive isolates fell together in one of the subclusters within the most complex cluster and were all closely related (marked in red, **Figure 1**). This subcluster included also two *mcr*-9-negative isolates (17050877 and 17082797 marked in green, **Figure 1**). Additionally, other eight *mcr*-9-negative ST34 isolates were present within the same parental branch (marked in green, **Figure 1**).

Only the 21 isolates within this branch (both the 11 *mcr*-9 positive and the 10 negative ones) presented an IncHI2/2A plasmid (**Figure 2A** and **Supplementary Figure 1A**) and a similar MDR gene pattern, with three of them being also positive for *mcr*-1.1, associated with colistin resistance (**Figure 2B** and **Supplementary Figure 1B**).

ONT Long-Read Sequencing and Bioinformatics Analysis

Three representative *mcr*-9-positive and one *mcr*-9-negative isolates (**Table 1** and **Supplementary Table 1**) were selected to be sequenced with ONT. From the hybrid (Illumina–Oxford Nanopore) assembly approach, the three plasmids containing *mcr-9* were resolved. *mcr-9* was demonstrated to be located on three different IncHI2/IncHI2A plasmids, named pMOL952, pMOL665, and pMOL500, with sizes of 297, 486, 291, 132, and 277,503 bp, respectively. The *mcr-9*-negative isolate also contained an IncHI2/IncHI2A plasmid, named pMOL625, with a size of 283, 630 bp.

Annotation of plasmid sequences identified four main genetic regions in the four IncHI2/IncHI2A plasmids. The plasmid

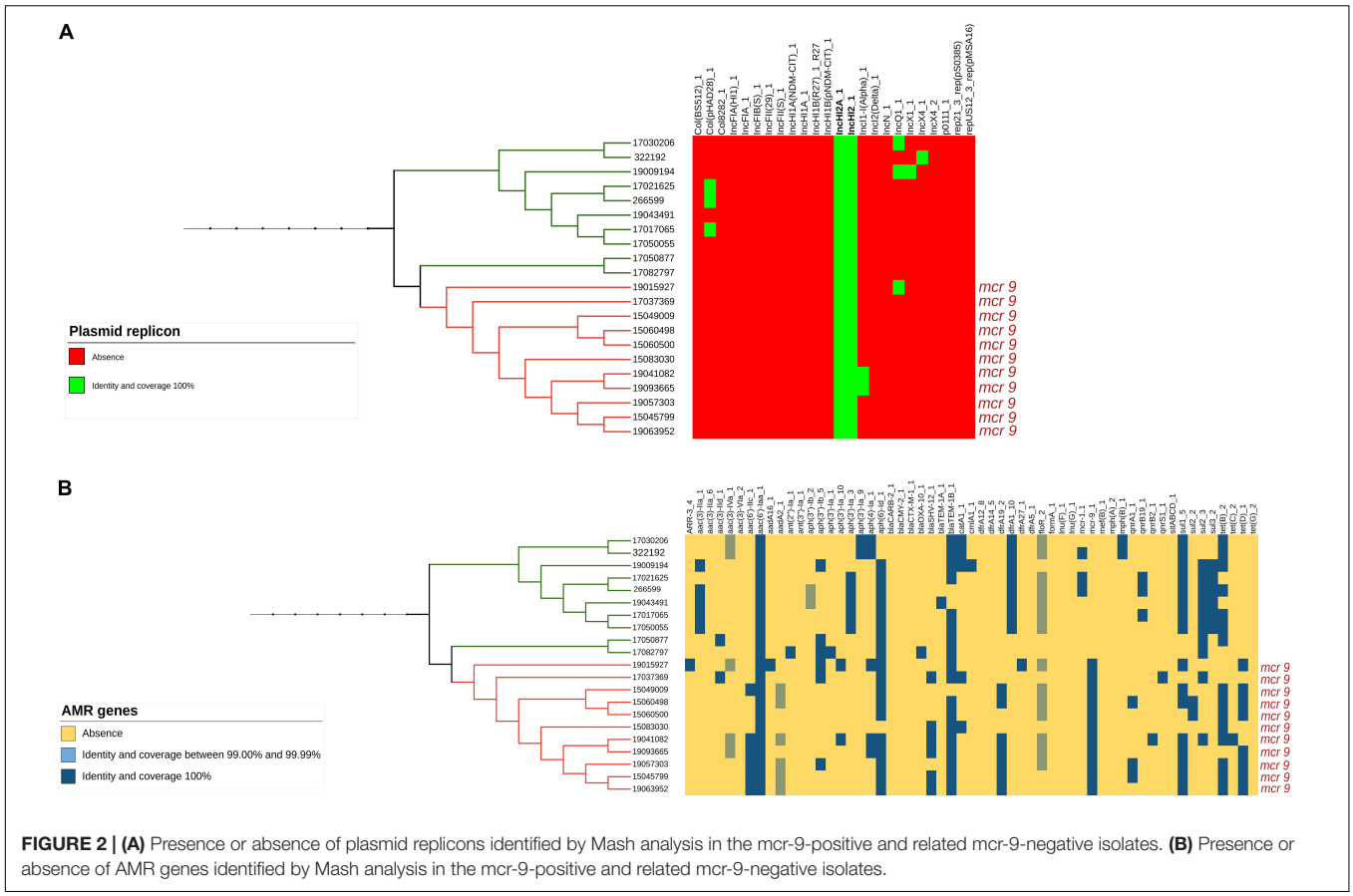

FIGURE 2 | (A) Presence or absence of plasmid replicons identified by Mash analysis in the mcr-9-positive and related mcr-9-negative isolates. **(B)** Presence or absence of AMR genes identified by Mash analysis in the mcr-9-positive and related mcr-9-negative isolates.

backbone (located in the region 1..40.000 nucleotides (nt); ∼13% of the total plasmid size) included a replication region containing the *rep*A gene encoding a replication initiation protein, a stability region, and the conjugative and transfer genes (**Figure 3**).

Comparison of our three *mcr*-9-carrying plasmids revealed that with a coverage range of 94–98%, they shared an identity of 99.9%, while the *mcr*-9-negative pMOL625 presented a lower coverage range (71–75%) and an identity of 98.7% with the abovementioned plasmids. Besides *mcr*-9, all the four IncHI2 plasmids harbored the majority of the AMR genes detected encoding resistance to all beta-lactams, aminoglycosides, trimethoprim, tetracyclines, sulfamethoxazole, and phenicols (**Table 1**). In particular, pMOL-952 and pMOL-665 shared a variable region (240,020..250,837 nt; **Figure 3**) of a class 1 integron carrying several AMR genes and *qacEΔ1* (quaternary ammonium compound resistance gene). The studied plasmids also harbored genes involved in the metabolism of heavy metals as arsenic, nickel, and mercury. In this regard, a mercury-resistance (mer) operon (101,550..105,526 nt; **Figure 3**) was shared by the *mcr*-9-positive pMOL-952, pMOL-665, and pMOL-500 plasmids and was absent in the *mcr*-9-negative pMOL625.

No virulence genes were detected in the four IncHI2 plasmids.

In these plasmids, the *mcr*-9 gene was located on an almost conserved region in the three *mcr*-9-harboring plasmids, of approximately 24,548 bp. *mcr*-9 was located adjacent to a cupin fold metalloprotein gene (*wbu*C) with the insertion sequences IS903 upstream and the IS26 downstream of both genes. Additionally, located upstream of this structure were genes associated with heavy metal metabolism (**Figures 3**, **4**). No other AMR genes have been detected in this region.

The results obtained with BLAST analysis with the two previously published IncHI2 *mcr*-9-carrying plasmids revealed that our three IncHI2 *mcr*-9-carrying plasmids were closely related (88% coverage and 99.9% identity) to the IncHI2 ∼293 kb plasmid (p3846) described in Italy by Marchetti et al. (2021) in *E. cloacae*. As for the *mcr*-9/bla_{VIM-1}-carrying IncHI2 plasmid (pRH-R27, size ∼299 kb) described in Germany in *S.* Infantis from a fattening pig farm, it shared with our three *mcr*-9-carrying plasmids a coverage range of 80–84% and an identity of 99.9%.

The specific region where *mcr*-9 was located in pMOL952, pMOL665, and pMOL500 was almost identical to the corresponding region of p3846 and pRH-R27 with an identity of 99% including the *wbu*C gene. The structural analysis revealed that pMOL952, pMOL665, and pMOL500 presented a mobile element (IS903) that was not identified in p3846 or pRH-R27 (**Figures 3**, **4**).

The complete sequences of the four resolved plasmids were submitted to the European Nucleotide Archive[10] under

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

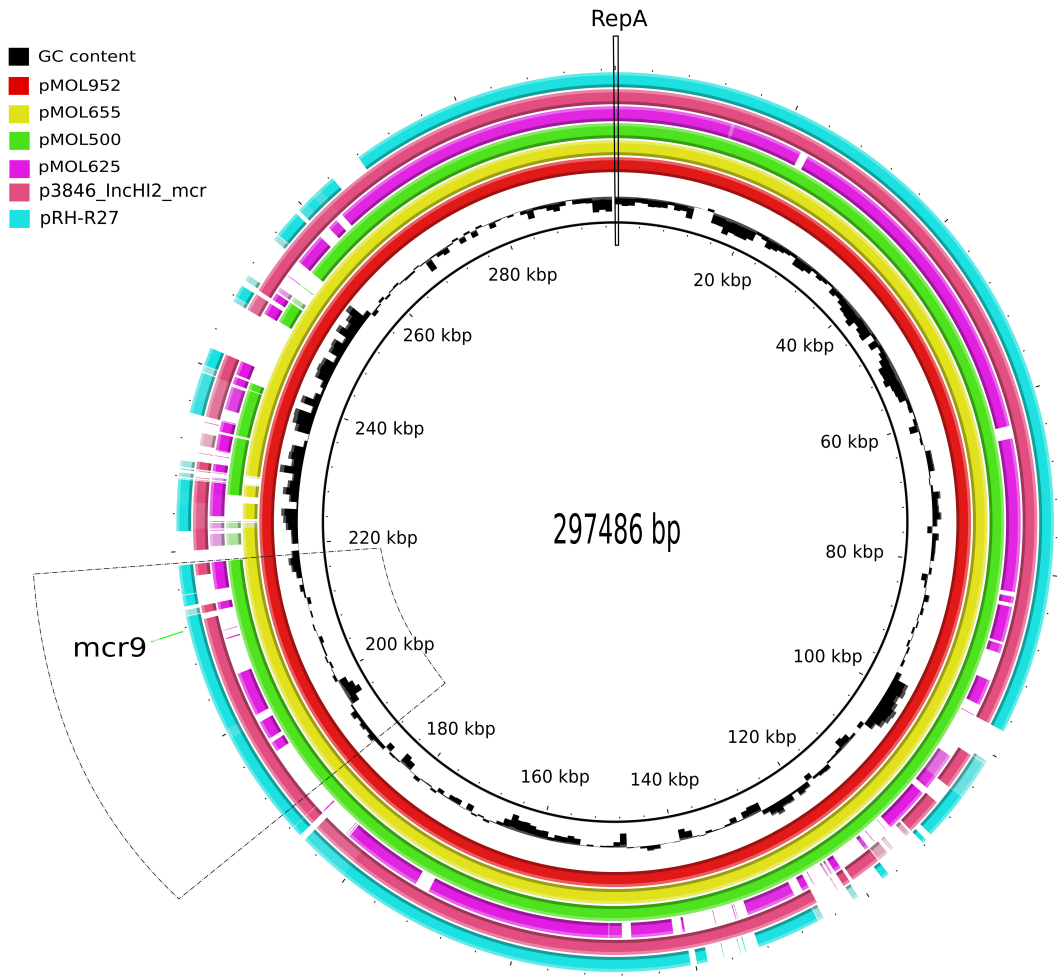

FIGURE 3 | Graphical comparison of the complete sequence of pMOL-952 (red; ERZ2110542), pMOL-665 (yellow; ERZ2110531), pMOL-500 (green; ERZ2110487), pMOL625 (fuchsia; ERZ2110516), p3846_IncHI2 (old pink; CP052871), and pRH-R27 (blue marine; LN555650.1). The repA gene is indicated with a continuous line. The region including mcr-9 is indicated with a dotted line.

the project accession number PRJEB44718 (ERZ2110487, ERZ2110531, ERZ2110542, and ERZ2110516).

DISCUSSION

In this study, we report for the first time in Italy the presence and genomic features of 11 MDR, *mcr*-9-positive *S.* Typhimurium, and single-locus variant isolates from food-producing animals (fattening pigs and veal calves) belonging to the emerging ST34 clone, with some of them also displaying resistance to highest priority critically important antimicrobials (HPCIAs), as third- and fourth- generation cephalosporins. Indeed, plasmid-borne *mcr* genes pose a significant threat to public health at international level, not only because of the colistin-resistant phenotype they can induce but also because they can be transferred horizontally to foodborne pathogens in combination with other resistance genes, with the potential to be transmitted to humans and impair the treatment options.

From the Mash analysis, we identified 21 ST34 isolates within the same parental branch (both the 11 *mcr*-9 positive and the 10 negative ones) harboring large-size (∼277–297 kb) IncHI2/2A plasmids and a similar MDR gene pattern (**Figure 2** and **Supplementary Figure 1**). Interestingly, only these isolates shared the presence of these plasmid types and presented a greater abundance of AMR genes compared to the rest of the *Salmonella* genomes analyzed. Because of the intrinsic characteristic of the Mash approach, the existence of a large-size IncHI2/2A plasmid harboring several similar AMR genes could have determined the high genetic relatedness of these isolates represented on the Mash tree.

Similarly to several previous findings (Carroll et al., 2019; Börjesson et al., 2020; Kananizadeh et al., 2020; Tyson et al., 2020), in our study, the presence of *mcr*-9 was not associated with clinical or microbiological resistance to colistin, and the *qse*C and *qse*B genes were absent in all the *mcr*-9-positive isolates detected. Indeed, the *mcr*-9 expression has been shown to be induced by the presence of colistin when this gene is located upstream of

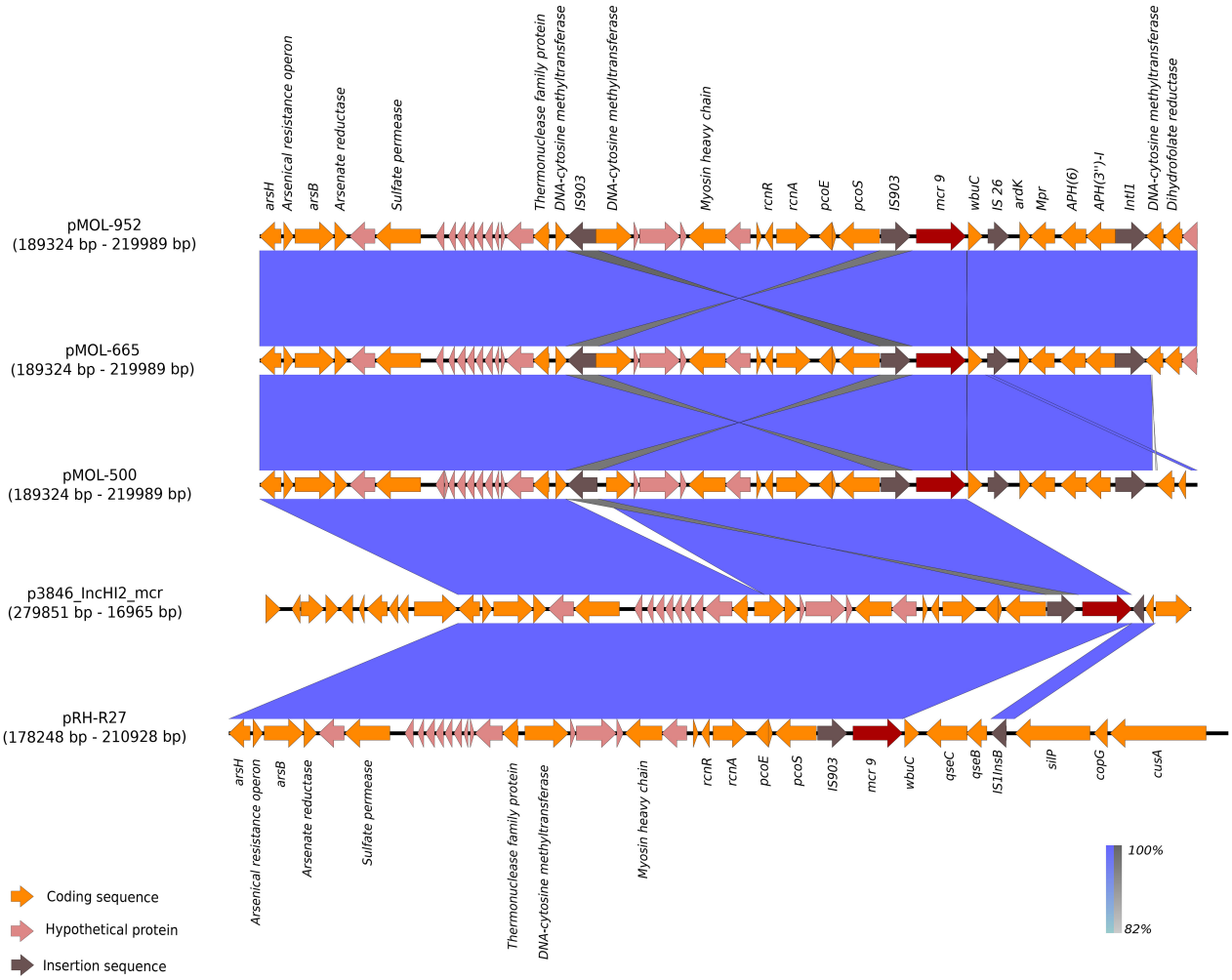

FIGURE 4 | BLAST graphical output of the region where mcr-9 is located in pMOL-952 (ERZ2110542), pMOL-665 (ERZ2110531), pMOL-500 (ERZ2110487), p3846_IncHI2 (CP052871), and pRH-R27 (LN555650.1). Red arrows: mcr-9 gene, orange arrows: Genes encoding functional proteins, pink arrows: Genes encoding hypothetical proteins, brown arrows: Mobile genetic element.

the two-component regulatory system *qse*BC. Therefore, *mcr*-9 may silently spread and remain undetected, unless induced by subinhibitory concentrations of colistin (Kieffer et al., 2019). However, the effectiveness of this regulatory system may be dependent on the genetic context (e.g., *mcr*-9 could be located on a plasmid or integrated into the chromosome) or may differ in relation to different strain backgrounds (e.g., serotype-dependent colistin susceptibility observed in *Salmonella* isolates) (Tyson et al., 2020), or other still unknown mechanisms could be involved. Additionally, the *mcr* gene family may undergo further genetic microevolution (and possibly to changes in the susceptibility pattern to colistin) due to selection pressure in the food-producing animal industry.

In our three selected isolates, *mcr*-9 was demonstrated to be located on IncHI2/IncHI2A plasmids by the hybrid (Illumina–Oxford Nanopore) approach, together with other AMR genes conferring resistance to different antimicrobial classes, including the bla_{SHV-12} ESBL gene type detected in the two plasmids of pig origin, and different genes involved in heavy metals metabolism. Similarly, previous findings detected IncHI2- or IncHI2A-type plasmids in *mcr*-9 positive Enterobacteriaceae, often together with other resistance determinants (including HPCIAs) and to heavy metals mainly from human patients (Carroll et al., 2019; Chavda et al., 2019; Kieffer et al., 2019; Bitar et al., 2020; Faccone et al., 2020; Kananizadeh et al., 2020; Lin et al., 2020; Osei Sekyere et al., 2020; Soliman et al., 2020; Tsui et al., 2020; Umeda et al., 2020; Elbediwi et al., 2021; Marchetti et al., 2021; Sun et al., 2021), and also from animal (Börjesson et al., 2020; Yuan et al., 2019; Borowiak et al., 2020; Haenni et al., 2020; Leite et al., 2020), food (Borowiak et al., 2020; Sadek et al., 2020; Tyson et al., 2020), and environmental (Kamathewatta et al., 2020) sources. Few reports also described *mcr*-9 integrated into the chromosome of *Citrobacter* (Ribeiro et al., 2021) and *Salmonella* (Pan et al., 2020; Tyson et al., 2020) isolates, with a genetic context similar to the structure observed in *mcr*-9-harboring plasmid sequences, suggesting a possible *mcr*-9

transfer as a gene cassette between plasmids and chromosomes (Pan et al., 2020).

However, only very few complete IncHI2 plasmid sequences carrying *mcr*-9 from food-producing animals and related foodstuff are available in public repositories for comparison, so far. In this regard, we fully reconstructed two *mcr*-9-positive IncHI2 plasmids from the monophasic variant of *S.* Typhimurium isolated from pig sources and one from the cecal content of a veal calf. To the best of the authors' knowledge, this represents the first report of MDR, *mcr*-9-positive Enterobacteriaceae detected in bovine animals.

All 11 IncHI2 plasmids harboring *mcr*-9 were assigned to ST1 by pMLST analysis. IncHI2-ST1 plasmids have been the most frequently reported IncHI2 ST, representing a major vehicle in mediating *mcr*-9 and AMR gene dissemination also in Enterobacteriaceae isolates from clinical settings (Li et al., 2020). Interestingly, the *mcr*-9-negative IncHI2 plasmid we sequenced with ONT was assigned to another ST, ST4.

IncHI2 plasmids are known carriers of resistance determinants not only to antibiotics but also to heavy metals such as silver, mercury, arsenic, copper, tellurium, and others with the potential to coselect for the concomitant presence of *mcr*-9 (Tyson et al., 2020).

Moreover, pigs are considered one of the most significant vectors for the monophasic variant of *S.* Typhimurium ST34, and pork meat represents one of the main infection sources for humans (Biswas et al., 2019). As copper and zinc supplementation are commonly used in the swine industry (or in the case of therapeutical use of ZnO also to control postweaning diarrhea), heavy metals could accumulate in the environment, leading to a selection of ST34 isolates resistant to heavy metals and to IncHI2 plasmids frequently associated with various metal tolerance genes (Biswas et al., 2019).

IncHI2 plasmids have been reported to be diverse in terms of the overall genetic structure, while *mcr*-9 has been reported to be consistently located in the sil-cop region (Li et al., 2020). As previously described (Li et al., 2020), in this region, the core structure of all reported *mcr*-9 cassettes was rcnR-rcnA-pcoE-pcoS-IS903-mcr-9-wbuC, with the genetic content immediately upstream of *mcr*-9 mostly conserved. Differently, the gene content located downstream of *mcr*-9 was reported to be genetically diverse (silver resistance determinants and *qse*B-C regulators absent in most plasmids), and transposon elements were not identified.

In our plasmids, *mcr*-9 was located in a plasmid region (∼30 kb) lacking different genetic elements of the core structure, compared to the other plasmid sequences subjected to BLAST analysis (**Figure 4**). In particular, *mcr*-9 was flanked by the *wpu*C gene and the IS903 element with and additional IS element (IS26) located downstream *wpu*C, suggesting the potential ability to mobilize this gene. Heavy metal resistance genes were also identified (as arsenic and nickel) in the same region, but accordingly to previous findings, silver resistance determinants and *qse*B-C regulators were absent.

In conclusion, the spread of MDR *S.* Typhimurium, including monophasic ST34, has widely challenged the treatment options to control foodborne infections (Biswas et al., 2019). This can be of even more concern, especially when there is concomitant evidence of MDR genes against HPCIAs, such as colistin, extended-spectrum cephalosporins, and (fluoro)quinolones. Therefore, genomics and WGS-based surveillance are increasingly indispensable to achieve better insights also into the genetic environment and features of plasmid-mediated AMR and the relationships with bacterial pathogenic hosts, as in the case of such IncHI2 plasmids harboring *mcr*-9, that can be transferred horizontally also to major *Salmonella* serovars spreading along the food chain.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AF, VC, PA, and ED conceived and designed the experiments. FF, PDM, MI, IM, AG, and ED performed the experiments. ED, PA, VC, AF, EC, and VD analyzed the data. VC, PA, ED, and AF wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.705230/full#supplementary-material

Supplementary Figure 1 | (A) Presence or absence of plasmid replicons in the 177 *Salmonella* genomes identified by Mash analysis. **(B)** Presence or absence of AMR genes in the 177 *Salmonella* genomes identified by Mash analysis.

Supplementary Table 1 | Metadata, phenotypic AMR patterns, and STs in 177 *S.* Typhimurium and monophasic variant isolates.

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Prevalence and Characteristic of Swine-Origin *mcr-1*-Positive *Escherichia coli* in Northeastern China

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The emergence of the plasmid-mediated colistin resistance gene *mcr-1* is threatening the last-line role of colistin in human medicine. With *mcr-1*-positive *Escherichia coli* (*E. coli*) isolated from food animal being frequently reported in China, the prevalence of *mcr-1* in food animal has attracted public attention. In the present study, a total of 105 colistin-resistant *E. coli* strains were isolated from 200 fecal samples collected from six swine farms in northeastern China. *mcr*-PCR revealed that the prevalence of *mcr-1* in colistin-resistant *E. coli* was 53.33% (56/105). *mcr-1*-positive *E. coli* showed extensive antimicrobial resistance profiles with the presence of additional resistance genes, increased expression of multidrug efflux pump-associated genes, and increased biofilm formation ability. MLST differentiated all the *mcr-1*-positive *E. coli* into 25 sequence types (STs) and five unknown ST, and the most common ST was ST10 ($n = 11$). By phylogenetic group classification, the distribution of all *mcr-1*-positive *E. coli* belonging to groups A, B1, B2, and D was 46.43, 35.71, 5.36, and 5.36%, respectively. Conjugation experiment demonstrated that most of the *mcr-1* were transferable at frequencies of 2.68×10^{-6}–3.73×10^{-3} among 30 representative *mcr-1*-positive *E. coli*. The plasmid replicon types IncI2 ($n = 9$), IncX4 ($n = 5$), IncHI2 ($n = 3$), IncN ($n = 3$), and IncP ($n = 1$) were detected in the transconjugants. The results of growth assay, competition experiment, and plasmid stability testing showed that acquisition of *mcr-1*-harboring plasmids could reduce the fitness of bacterial hosts, but *mcr-1* remained stable in the recipient strain. Due to the potential possibility of these *mcr-1*-positive *E. coli* being transmitted to humans through the food chain or through horizontal transmission, therefore, it is necessary to continuously monitor the prevalence and dissemination of *mcr-1* in food animal, particularly in swine.

Keywords: colistin resistance, *mcr-1* gene, swine, *Escherichia coli*, prevalence, characteristics

INTRODUCTION

The discovery and use of antibiotics in human medicine was regarded as one of the vast medical advancements over the past decades, and antibiotics also play an important role in food-animal agriculture (Worthington and Melander, 2013). The increasing amount of animal protein for human consumption accelerates the development of modern animal production. However, the widespread use of antibiotics in livestock has posed a significant public health threat, which can potentially increase selection pressure on antibiotic-resistant bacteria (ARB) and further promote the dissemination of ARB in livestock (You and Silbergeld, 2014). Moreover, the animal-origin ARB can be transmitted to humans through the environment and food chain as well as through direct contact (Graham et al., 2009).

Escherichia coli is one of the major pathogens in the swine industry, which is associated with gastrointestinal diseases and systemic infections, including diarrhea, edema disease, septicemia, polyserositis, mastitis, and urinary tract infections (Fairbrother et al., 2005). These diseases can lead to morbidity, mortality, and delayed growth, which are responsible for considerable economic losses and restrict the development of the swine industry. To maintain health and productivity, antibiotics are widely administered to treat *E. coli* infections in farms to swine *via* oral, either in feed or in water (Fairbrother et al., 2005). Among a variety of antibiotics used in swine farms, polypeptides and aminoglycosides are most frequently administrated (Sabine et al., 2017).

Colistin is a kind of cationic polypeptides and a member of the polymyxin family, including polymyxins A, B, C, D, and E. Only polymyxin B and polymyxin E (colistin) are currently used clinically. Due to the broad-spectrum activity against a wide range of Gram-negative bacteria (GNB), colistin is widely used in pig production to control intestinal infections caused by *Enterobacteriaceae* (Landman et al., 2008). The routine use of colistin in human medicine was abandoned in the 1970s due to its major side effects, including nephrotoxicity and neurotoxicity (Landman et al., 2008). However, with the emergence of multidrug-resistant Gram-negative bacteria (MDR-GNB) and the paucity of novel classes of antibiotics entering the clinic, colistin has been reintroduced to human clinical use as a last-line treatment option for severe infections caused by MDR-GNB (Falagas and Kasiakou, 2005). The rapid rise and dissemination of MDR-GNB led to the increased amounts of colistin used in humans and animals with the inevitable risk of accelerating the emergence of colistin resistance (Kempf et al., 2013).

Colistin resistance was commonly thought to be chromosomally mediated, until a novel plasmid-mediated colistin resistance gene *mcr-1* was characterized in *E. coli* isolated from animals and humans in China at the end of 2015 (Liu et al., 2016). Because of the rapid horizontal spread of colistin resistance by plasmids, the discovery of *mcr-1* has attracted public attention among physicians and veterinarians. To date, the cases of bacteria harboring *mcr-1* gene have been found in 47 different countries across six continents (Asia, Europe, Africa, North America, South America, and Oceania) from humans, animals, and environmental samples (Shi et al., 2020). Due to the high prevalence of *mcr-1*-positive *E. coli* originating from food animal than from humans, food animal production, particularly pig production, has been singled out as the major cause of *mcr-1* amplification and spread (Rhouma et al., 2016).

In this study, we aimed to investigate the prevalence and characteristics of *mcr-1* in swine farms in northeastern China by determining (1) the carriage rate of *mcr-1* in colistin-resistant *E. coli* isolated from swine fecal samples; (2) the antimicrobial resistance profiles of *mcr-1*-positive *E. coli* isolates; (3) the presence of additional resistance genes, the relative expression levels of multidrug efflux pump-associated genes, and biofilm formation ability in *mcr-1*-positive *E. coli* isolates; (4) the genetic relationship of the *mcr-1*-positive *E. coli* isolates by multilocus sequence typing (MLST) and phylogenetic group; and (5) the transferability, conjugation frequency, fitness cost, and plasmid stability of *mcr-1*.

MATERIALS AND METHODS

Sample Collection and Bacterial Strain Identification

Between July 2016 and June 2017, a total of 200 fecal swabs were collected from six swine farms in northeastern China, including Heilongjiang (Harbin), Jilin (Changchun), and Liaoning (Shenyang). In each province, two geographically distinct swine farms were selected; 40 fecal swabs were randomly collected from 40 different pigs in each farm in Harbin, and 30 fecal swabs were randomly collected from 30 different pigs in each farm in Changchun and Shenyang. Fecal swabs were collected by placing a wet cotton swab at the animal anus of 2∼5 cm with minor rotation. The samples brought to the laboratory were immediately streaked out on MacConkey agar and incubated at 37°C for 18 h. The putative *E. coli* isolates on MacConkey agar (bright pink with a dimple) per sample were transferred to eosin methylene blue agar for further purification and were incubated at 37°C for 18 h. Randomly selected colonies with typical *E. coli* morphology were selected from each sample for PCR detection of *16S rRNA* gene and for sequencing (Seurinck et al., 2003). All confirmed *E. coli* isolates were stored at −80°C for further studies.

Colistin Resistance Screening and Confirmation of *mcr-1*-Positive Strains

To isolate colistin-resistant *E. coli*, all the strains were screened on the MacConkey agar containing 2 μg/ml of colistin. The DNA templates of all colistin-resistant isolates were extracted using the DNA extraction kit (TIANGEN, Beijing, China) following the instructions of the manufacturer. The presence of *mcr-1* in colistin-resistant *E. coli* was determined by PCR amplification and followed by Sanger sequencing as described previously (Liu et al., 2016).

Antimicrobial Susceptibility Testing

The susceptibility of all *mcr-1*-positive strains to 26 antibiotics, namely meropenem, ertapenem, imipenem,

ampicillin, ampicillin–sulbactam, amoxicillin/clavulanic acid, cefuroxime, ceftazidime, cefepime, ceftriaxone, cefoxitin, aztreonam, gentamicin, amikacin, kanamycin, streptomycin, ciprofloxacin, levofloxacin, tetracycline, doxycycline, tigecycline, chloramphenicol, florfenicol, fosfomycin, sulfisoxazole, and nitrofurantoin, was determined by the standard disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI); the interpretation of the susceptibility result was according to CLSI (document M100), 2018, except those for florfenicol and sulfisoxazole which were interpreted according to the CLSI VET01-A4, and tigecycline was interpreted in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2017. The *E. coli* ATCC 25922 was used as a quality control strain.

Detection of Additional Antimicrobial Resistance Genes

The presence of carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{OXA-48}, and bla_{IMP}) (Doyle et al., 2012), extended spectrum-β-lactamase (ESBL) genes (bla_{CTX-M}) and non-ESBL genes (bla_{TEM}, bla_{SHV}, bla_{OXA-1}) (Dallenne et al., 2010), pAmpC genes (bla_{CMY}, bla_{FOX}, and bla_{DHA}) (Dallenne et al., 2010), tetracycline resistance genes [*tet*(A), *tet*(B), *tet*(C), and *tet*(M)] (Ng et al., 2001), aminoglycoside resistance genes [*rmtA*, *rmtB*, *rmtC*, *rmtD*, *armA*, *nmpA*, and *aac(3)-IV*] (Yeganeh Sefidan et al., 2019), fluoroquinolone resistance genes [*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *oqxAB*, *qepA*, and *aac(6′)-Ib-cr*] (Ciesielczuk et al., 2013), streptomycin/spectinomycin resistance genes (*strA*, *strB*, and *aadA*) (Srinivasan et al., 2007), fosfomycin resistance genes (*fosA* and *fosA3*) (Lee et al., 2012), florfenicol resistance gene (*floR*) (Li et al., 2015), and sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) (Hammerum et al., 2006) was examined by PCR. The positive products were validated with Sanger sequencing, then all the obtained sequences were compared using Blast with those published in the NCBI database[1].

Phylogenetic Groups and Multilocus Sequence Typing Analysis

The genetic relatedness of *mcr-1*-positive strains was investigated by MLST as previously described for *E. coli* (Tartof et al., 2005). Furthermore, a two-step multiplex PCR was performed to determine the phylogenetic group, and the primers used (*chuA*, *yiaA*, and *TspE4.C2*) and details were the same as previously described (Clermont et al., 2000). Phylogenetic trees for all sequence types (STs) were constructed using the neighbor-joining method with MEGA software (Kumar et al., 2018). Annotation for each isolate and tree embellishment were visualized using Itol[2].

[1]http:/www.ncbi.nlm.nih.gov/blast

[2]https://itol.embl.de/

Detection of the Relative Expression Levels of Genes Encoding Efflux Pumps, Porins, and Regulators by Quantitative Real-Time PCR

Eleven representative strains were chosen from all *mcr-1*-positive *E. coli* for the detection of the relative expression levels of genes encoding efflux pumps (*acrA*, *mdfA*, *ydhE*, *acrE*, *tolC*, *mdtE,* and *mdtF*), regulators (*marA*, *soxS*, *fisF*, *dsrA*, and *evgA*), and porin protein-encoding genes (*ompC* and *ompF*). Total RNA of *mcr-1*-positive strains and a reference strain *E. coli* ATCC 25922 was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, United States), and cDNA was synthesized with 5 × All-In-One MasterMix (ABM, Richmond, Canada) following the instructions of the manufacturer. The *mdh* gene was used as the housekeeping gene. Quantitative real-time PCR (BioEasy SYBR Green High ROX Master Mix, Bioer, Hangzhou, China) was performed according to the methods described by Vinué et al. (2015). The relative expression levels of the tested genes were calculated using the $2^{-\Delta\Delta CT}$ method as described by Huang W. et al. (2020).

Detection of Biofilm Formation Ability

All *mcr-1*-positive isolates were inoculated into 15 ml tubes containing 5 ml Luria–Bertani (LB) broth and then cultured overnight in a shaking incubator at 37°C. The biofilm formation assay of these isolates was then conducted in 96-well polystyrene flat-bottom microtiter plates as described previously (Teh et al., 2010). To quantify the biofilm formation ability, the absorbance values of the solution were measured at 590 nm using an automated Multiskan FC reader (Thermo Fisher Scientific). The experiment was repeated independently three times.

Conjugation Experiment and Plasmid Replicon-Type Analysis

The transferability of *mcr-1* was tested by conjugation experiment with *mcr-1*-positive *E. coli* as donors and rifampicin-resistant *E. coli* EC600 as recipient strains. The MacConkey agar plates containing rifampicin (256 μg/ml) and colistin (2 μg/ml) were used to select *mcr-1*-positive transconjugants. PCR analysis and DNA sequencing were carried out to confirm that transconjugants were derivatives of the recipient strain *E. coli* EC600. The transfer frequency of *mcr-1* was determined as described in a previous study (Liu et al., 2016). The replicon types of the transconjugants were determined according to previous studies (Carattoli et al., 2005; Johnson et al., 2012).

Growth Assay and *in vitro* Competition Experiment

To assess the fitness impact of *mcr-1* carriage on the host, growth assay and *in vitro* competition experiment were carried out. Growth curves for the recipient (EC600) and *mcr-1*-positive *E. coli* transconjugants were performed in 96-well flat-bottom plates (Corning Inc., Corning, NY, United States) as described previously (Long et al., 2019).

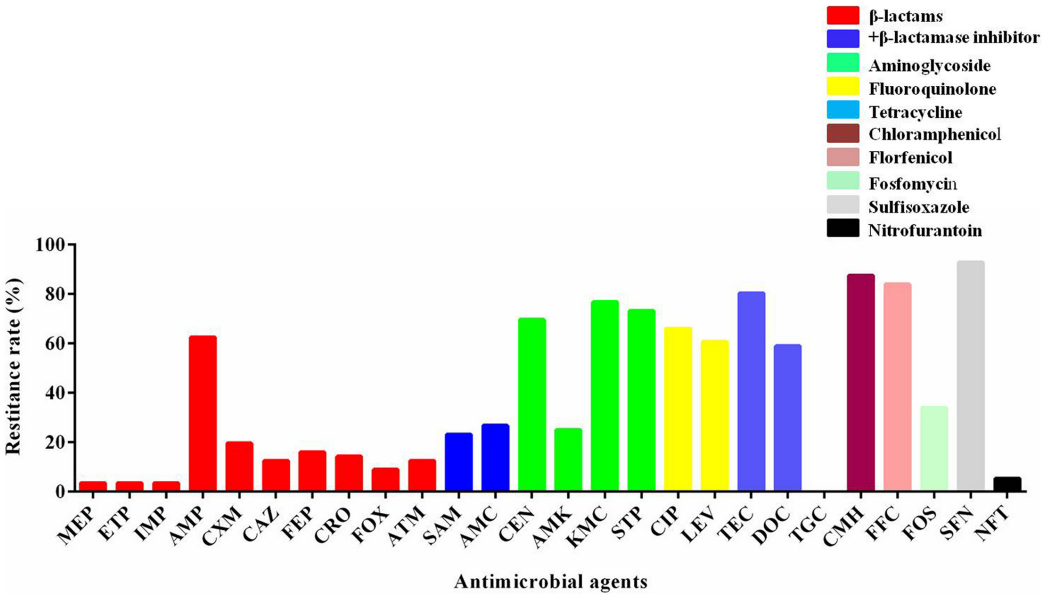

FIGURE 1 | The resistant rate of *mcr-1*-positive *E. coli* to other antibiotics. MEP, meropenem; ETP, ertapenem; IMP, imipenem; AMP, ampicillin; CXM, cefuroxime; CAZ, ceftazidime; FEP, cefepime; CRO, ceftriaxone; FOX, cefoxitin; ATM, aztreonam; SAM, ampicillin–sulbactam; AMC, amoxicillin–clavulanic acid; GEN, gentamicin; AMK, amikacin; KMC, kanamycin; STP, streptomycin; CIP, ciprofloxacin; LEV, levofloxacin; TEC, tetracycline; DOC, doxycycline; TGC, tigecycline; CMH, chloramphenicol; FFC, florfenicol; FOS, fosfomycin; SFN, sulfisoxazole; NFT, nitrofurantoin.

In vitro competition experiments were conducted using *mcr-1*-positive *E. coli* transconjugants competing with EC600. Twenty-four-hour competition experiments were performed as described previously (He et al., 2017). Growth assay and *in vitro* competition experiment were performed in triplicate.

Plasmid Stability Testing

To estimate the stability of the plasmid harboring *mcr-1*, plasmid stability experiments were performed using *mcr-1*-positive *E. coli* transconjugants as described previously (Sota et al., 2010).

RESULTS

Prevalence of *mcr-1* in Colistin-Resistant *E. coli*

A total of 176 *E. coli* strains were isolated from 200 fecal samples collected from six swine farms located in northeastern China, and the *E. coli* isolates showed high resistance rate to colistin (59.66%, 105/176). Colistin-resistant *E. coli* colonies were identified in 66.20% (47/71), 54.90% (28/51), and 55.56% (30/54) *E. coli* strains isolated from swine farms in Heilongjiang, Jilin, and Liaoning, respectively. *mcr*-PCR and sequencing revealed that 56 *E. coli* were positive for *mcr-1*, the carriage rate was extremely high (53.33%, 56/105), and the prevalence of *mcr-1* in colistin-resistant *E. coli* isolated from swine farms in Heilongjiang, Jilin, and Liaoning was 46.81% (22/47), 53.57% (15/28), and 63.33% (19/30), respectively.

Antimicrobial Susceptibility of *mcr-1*-Positive *E. coli*

The susceptibility of 56 *mcr-1*-positive *E. coli* isolates to other antimicrobials was determined. The percentages of resistance rate are presented in **Figure 1**. There were a high rate of resistance (60–100%) to gentamicin, kanamycin, streptomycin, ciprofloxacin, levofloxacin, tetracycline, chloramphenicol, florfenicol, doxycycline, and sulfisoxazole; a moderate rate of resistance (20–60%) to ampicillin, ampicillin–sulbactam, amoxicillin–clavulanic acid, amikacin, and fosfomycin; and a low rate of resistance (<20%) to meropenem, ertapenem, imipenem, cefuroxime, ceftazidime, cefepime, ceftriaxone, cefoxitin, aztreonam, and nitrofurantoin. There were no strains that were resistant to tigecycline. As shown in **Table 1**, most of the *mcr-1*-positive *E. coli* were multidrug resistant.

Presence of Additional Resistance Genes in *mcr-1*-Positive *E. coli*

Molecular features revealed that most *mcr-1*-positive *E. coli* carried additional resistance genes, as shown in **Figure 2**. Overall, bla_{TEM} (n = 56, 100%) was the most common non-ESBL gene in our study, followed by bla_{SHV-1} and bla_{OXA-1} that were identified in three (5.36%) and five (8.93%) isolates, respectively. In addition, the ESBL gene bla_{CTX-M} was detected in eight (14.86%) *mcr-1*-positive *E. coli* isolates. The detected pAmpC genes were bla_{CMY} (n = 10, 17.86%), bla_{FOX-5} (n = 5, 8.93%), and bla_{DHA-1} (n = 2, 3.57%). The carbapenemase genes (bla_{KPC}, bla_{OXA}, and bla_{IMP}) were not detected, and only bla_{NDM-5} was detected in two (3.57%) isolates. Among aminoglycoside resistance genes, only *rmtA* [7, 12.50%) and *aac(3)-IV* (25,

TABLE 1 | Characteristics and antimicrobial resistance profiles of *mcr-1*-positive *E. coli.*

Strains	ST	Phylogroup	Antimicrobial resistance
HLJ173	1,421	B1	ATM/GEN/KMC/STP/CIP/LEV/TEC/CMH/SFN
LN58	410	A	AMP/CAZ/FEP/CRO/FOX/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
LN122	1,463	B1	AMP/SAM/AMC/CIP/LEV/TEC/SFN
LN191	20	B1	GEN/KMC/STP/CIP/LEV/SFN
LN252	20	A	AMP/SAM/AMC/GEN/KMC/CIP/LEV/DOC/CMH/FFC/SFN/AMK
JL124	5,229	B1	AMP/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN/AMK
HLJ226	New ST1	B1	GEN/KMC/STP/CIP/LEV/CMH/FFC/SFN/FOS
LN72	1,0580	A	TEC/CMH/FFC/SFN/FOS
LN176	93	A	AMP/ATM/SAM/AMC/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
JL226	New ST4	B1	AMP/SAM/AMC/GEN/KMC/STP/CIP/TEC/DOC/CMH/FFC/SFN/NFT/AMK
JL125	48	A	GEN/KMC/STP/DOC/CMH/FFC/SFN/FOS
HLJ8	10	Unknown	AMP/CXM/CAZ/FEP/CRO/GEN/KMC/CIP/TEC/DOC/CMH/FFC/SFN/AMK
JL252	9,159	Unknown	GEN/KMC/STP/CMH/FFC/SFN/FOS
LN74	10	A	AMC/SFN
HLJ212	10	A	AMP/TEC/DOC/CMH/FFC/SFN/FOS
HLJ84	New ST5	B1	AMP/GEN/KMC/STP/CIP/LEV/TEC/CMH/FFC/SFN/AMK
LN20	617	A	GEN/KMC/STP/TEC/DOC/CMH/FFC/SFN
LN203	2,935	B1	KMC/STP/CIP/SFN
JL114	3,944	A	AMP/CAZ/FEP/CRO/LEV/TEC/DOC/CMH/FFC/SFN/FOS
HLJ464	3,944	A	AMP/FEP/LEV/DOC/FOS
LN221	3,944	B1	SAM/FOS
LN220	398	B1	AMP/SAM/AMC/KMC/STP/CIP/LEV/DOC/CMH/FFC/SFN
JL7	3,014	B2	AMP/SAM/AMC/CMH/FFC/SFN/FOS
JL127	1,421	B1	SAM/AMC/GEN/KMC/STP/CIP/LEV/DOC/SFN/AMK
HLJ174	3,856	A	CIP/LEV/TEC/DOC/SFN
HLJ456	New ST3	B1	TEC/DOC/CMH/FFC/SFN
HLJ438	New ST2	B1	AMP/CAZ/FEP/CRO/FOX/GEN/KMC/STP/TEC/CMH/FFC/FOS
HLJ56	4,379	B1	GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
LN106	156	B2	AMP/CXM/SAM/AMCGEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN/AMK
LN19	1,589	A	GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
HLJ63	410	A	AMP/CXM/CAZ/FEP/CRO/FOX/TEC/DOC/CMH/FFC/SFN/FOS
LN251	20	B1	CIP/LEV/TEC/DOC/CMH/FFC/SFN
JL128	5,229	B1	ATM/AMC/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
JL47	5,229	B1	AMP/GEN/KMC/STP/CIP/LEV/TEC/SFN/AMK/FOS
HLJ70	898	B1	CXM/ATM/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
HLJ43	898	A	AMP/SAM/AMC/GEN/KMC/STP/CIP/LEV/TEC/CMH/FFC/SFN/FOS
JL43	224	D	CXM/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN/NFT
LN66	131	D	GEN/KMC/STP/TEC/CMH/FFC/SFN
LN186	93	B1	AMP/CXM/ATM/AMC/GEN/KMC/STP/CIP/LEV/TEC/CMH/FFC/SFN/AMK/FOS
JL55	48	A	AMP/SAM/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
JL63	48	A	AMP/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
HLJ79	48	A	AMP/GEN/KMC/STP/TEC/CMH/FFC/SFN/AMK/FOS
HLJ194	772	B2	AMP/ATM/GEN/KMC/STP/TEC/DOC/CMH/FFC/FOS
LN190	772	A	AMP/CXM/TEC/DOC/CMH/FFC/SFN
HLJ188	772	Unknown	CXM/GEN/KMC/STP/TEC/DOC/CMH/FFC/SFN/AMK
LN59	617	B1	AMP/CXM/CAZ/FEP/CRO/GEN/KMC/STP/CIP/LEV/TEC/CMH/FFC/SFN/AMK
JL176	165	A	AMP/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC
HLJ187	6,730	Unknown	AMP/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
HLJ336	10	A	AMP/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
JL9	10	A	AMP/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN
JL33	10	A	AMP/CXM/FEP/CRO/FOX/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/NFT/AMK/FOS
HLJ230	10	D	MEP/ETP/IMP/AMP/CXM/CAZ/FEP/CRO/FOX/GEN/KMC/STP/TEC/CMH/FFC/FOS/SFN
HLJ222	10	A	AMP/SAM/GEN/KMC/STP/CIP/LEV/TEC/DOC/CMH/FFC/SFN/FOS

(Continued)

TABLE 1 | Continued

Strains	ST	Phylogroup	Antimicrobial resistance
LN182	10	A	AMP/SAM/AMC/GEN/KMC/STP/CIP/LEV/TEC/CMH/FFC/SFN/FOS
LN215	10	A	AMP/ATM/GEN/KMC/STP/CIP/LEV/TEC/CMH/FFC/SFN
JL79	10	D	AMP/SAM/AMC/KMC/STP/TEC/CMH/FFC/FOS/SFN

44.64%] were detected. As for fluoroquinolone resistance genes, there were 10 (17.86%), 7 (12.50%), 31 (55.36%), 2 (3.57%), and 9 (16.07%) isolates harboring *qnrD*, *qnrS*, *oqxAB*, *qepA*, and *aac(6′)-Ib-cr*, respectively, but there were no strains harboring *qnrA*, *qnrB*, and *qnr*C. The number of isolates harboring *tet*(A), *tet*(B), and *tet*(M) was 27 (48.21%), 20 (35.71%), and 31 (55.36%), respectively. The plasmid-encoded *floR* gene that conferred chloramphenicol resistance was detected in 38 (67.86%) *mcr-1*-positive strains. The isolates positive for sulfonamide resistance genes comprised 33 (58.93%) strains harboring *sul1*, followed by 24 (42.86%) and 18 (32.14%) strains harboring *sul2* and *sul3*, respectively. The *strA* and *strB* were closely associated with streptomycin resistance, which were detected in 34 (60.71%) and 39 (69.64%) isolates. The *fosA* (n = 9, 16.07%) and *fosA3* (n = 17, 30.36%) were prevalent in fosfomycin-resistant isolates.

Molecular Genotyping of *mcr-1*-Positive *E. coli*

The genotyping results of *mcr-1*-positive *E. coli* are summarized in **Table 2**. The *mcr-1*-positive isolates were distributed into phylogroups A (n = 26), B1 (n = 20), B2 (n = 3), and D (n = 3), and the phylogroup was undefined for four isolates. MLST differentiated the 56 *mcr-1*-positive *E. coli* into 25 STs and five unknown ST (untypable). As shown in **Figure 3**, the most common ST was ST10 (n = 11), followed by ST48 (n = 4), ST20 (n = 3), ST3944 (n = 3), ST772 (n = 3), ST5229 (n = 3), ST617 (n = 2), ST410 (n = 2), ST93 (n = 2), ST898 (n = 2), and ST1421 (n = 2), and then by single ST isolates, including ST165, ST10580, ST3856, ST1589, ST398, ST1463, ST4379, ST2935, ST156, ST3014, ST131, ST224, ST6730, and ST9159. Moreover, ST10, ST48, and ST617 are different by one or two alleles and they correspond to clonal complex CC10. As shown in **Figure 2**, phylogenetic analysis of all *mcr-1*-positive *E. coli* underlined the evidence for the horizontal transfer of *mcr-1*.

Relative Expression Levels of Genes Encoding Efflux Pumps, Porins, and Regulators in *mcr-1*-Positive *E. coli*

According to the results of MLST, 11 STs were predominant among *mcr-1*-positive *E. coli*. One representative strain of *E. coli* was chosen from each ST for subsequent detection. As shown in **Figure 4**, compared with the *E. coli* ATCC 25922, the relative expression levels of *acrA*, *mdtE*, *mdtF*, *marA*, *soxS*, *fisF*, *ompF*, and *ompC* were increased in all tested *mcr-1*-positive *E. coli*. The expression of *mdfA*, *ydhE*, *acrE*, *tolC*, and *dsrA* was increased in four, seven, four, five, and four strains, respectively, and the expression of *evgA* was reduced in all tested *mcr-1*-positive *E. coli*. The results indicated that upregulation of the expression of efflux pump-related genes could be used to explain the multidrug resistance of *mcr-1*-positive *E. coli*.

Biofilm Formation Ability of *mcr-1*-Positive *E. coli*

As shown in **Figure 5**, among the 56 *mcr-1*-positive *E. coli* strains, 28 (50.00%) strains showed significantly increased ability of biofilm formation compared with the *E. coli* ATCC 25922 ($p < 0.05$ or $p < 0.01$), and two (3.57%) strains showed significantly decreased ability of biofilm formation ($p < 0.05$). However, the remaining strains (26/56, 46.43%) showed no significant changes in their ability of biofilm formation.

Transferability of *mcr-1* and Plasmid Replicon Types in *mcr-1*-Positive Transconjugants

The transferability of *mcr-1* and conjugation frequencies are exhibited in **Table 3**. Among 30 representative *mcr-1*-positive *E. coli*, majority of the strains (n = 26) were capable of transferring *mcr-1* to the recipient rifampicin-resistant *E. coli* EC600. The conjugation frequencies of the isolates lay between 2.68×10^{-6} and 3.73×10^{-3}. The detected plasmid replicon types in the transconjugants included IncI2 (n = 8), IncX4 (n = 5), IncHI2 (n = 3), IncN (n = 3), and IncP (n = 1).

The combinations of IncN/IncX4 (n = 2), IncP/IncHI2 (n = 2), and IncI2/IncX4/IncHI2 (n = 1) were detected, indicating some transconjugants harbored several replicon types.

Fitness Cost and Plasmid Stability

As shown in the growth curves of **Figure 6A**, compared with the recipient (EC600), the growth rates at growth phase and cell densities at stationary phase were decreased slightly in *mcr-1*-positive *E. coli* transconjugants. The results of the *in vitro* competition experiment (**Figure 6B**) showed that the relative fitness values of all selected *mcr-1*-positive *E. coli* transconjugants were below 1. These results revealed that the acquisition of *mcr-1*-bearing plasmid could place an energy burden on the bacterial host and incur fitness cost. A total of five *mcr-1*-positive *E. coli* transconjugants were randomly selected and were passaged daily for 10 days in the absence of antibiotic selection. The results (**Figure 6C**) showed that *mcr-1* could be detected in transconjugants after a series of passages, suggesting that the plasmid harboring *mcr-1* remains stable in the hosts.

DISCUSSION

In the 1960s, several countries permitted the use of colistin in food animal production (Rhouma et al., 2016). However,

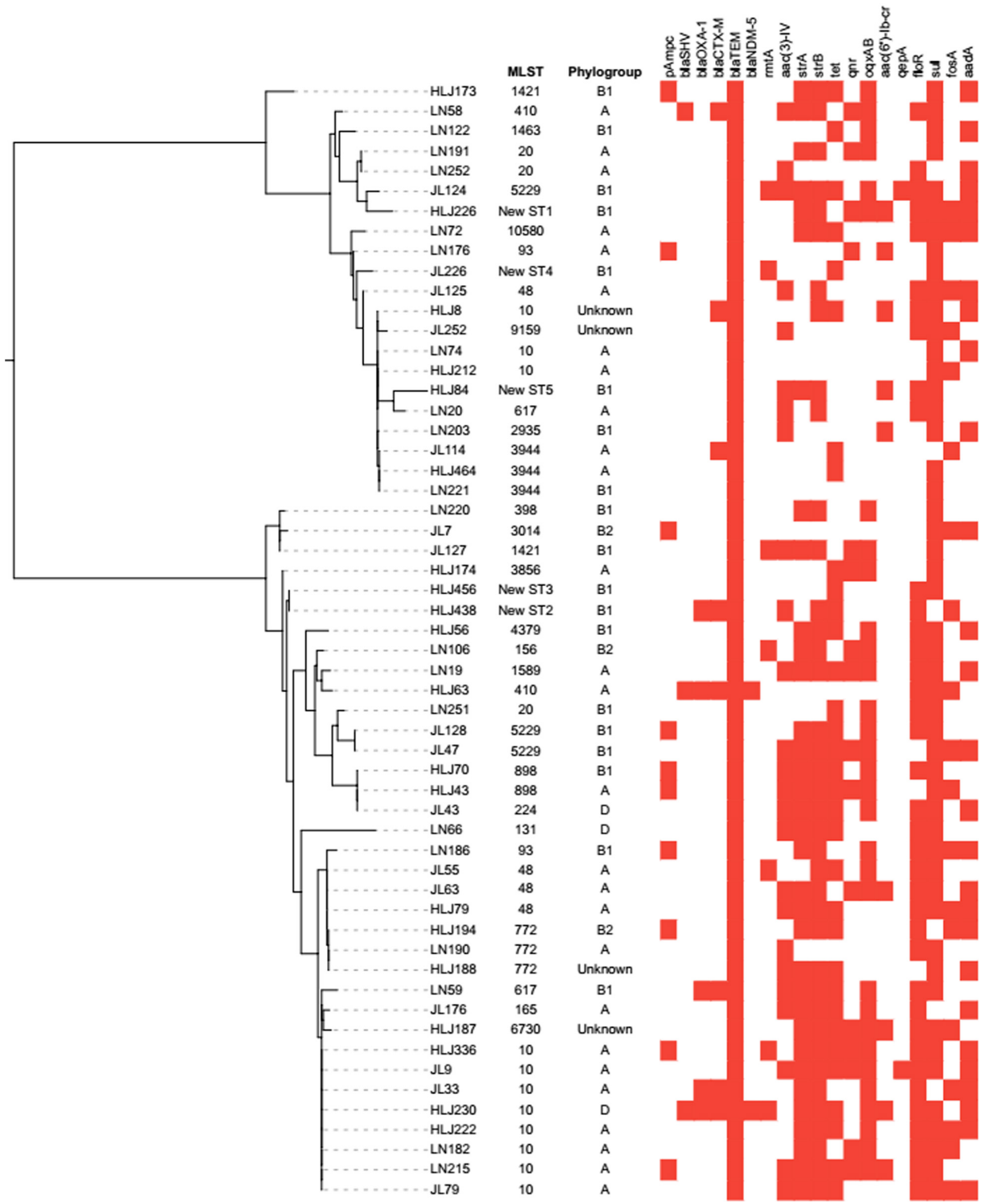

FIGURE 2 | Analysis of phylogroup, antimicrobial resistance genes, and relationships among 56 *mcr-1*-positive *E. coli* isolates from swine farms in northeastern China. Relationships among 56 *mcr-1*-positive *E. coli* isolates are indicated using an unrooted tree based on the alignments of concatenated MLST allelic sequences using the neighbor-joining method. For comparison of resistance genes among the 56 *mcr-1*-positive *E. coli* isolates, the red squares represent positivity for antimicrobial resistance genes.

TABLE 2 | Genotyping of *mcr-1*-positive *E. coli*.

Phylogroup (number of strains)	Clonal complex (number of strains)	Sequence type (number of strains)
A (26)	CC10 (14)	ST10 (9), ST48 (4), ST617 (1)
	CC20 (1)	ST20 (1)
	CC23 (2)	ST410 (2)
	CC165 (1)	ST165 (1)
	CC168 (1)	ST93 (1)
	Other CC (7)	ST3944 (2), ST10580 (1), ST3856 (1), ST898 (1), ST772 (1), ST1589 (1)
B1 (20)	CC10 (1)	ST617 (1)
	CC20 (2)	ST20 (2)
	CC101 (3)	ST5229 (3)
	CC168 (1)	ST93 (1)
	CC398 (1)	ST398 (1)
	Other CC (12)	NS1 (1), ST898 (1), NS2 (1), ST1421 (2), NS3 (1), ST3944 (1), ST1463 (1), ST4379 (1), NS4 (1), ST2935 (1), NS5 (1)
B2 (3)	CC156 (1)	ST156 (1)
	Other CC (2)	ST772 (1), ST3014 (1)
D (3)	CC10 (1)	ST10 (1)
	CC131 (1)	ST131 (1)
	Other CC (1)	ST224 (1)
Unknown (4)	CC10 (1)	ST10 (1)
	Other CC (3)	ST6730 (1), ST9159 (1), ST772 (1)

the regular use of colistin in food animal is recognized as one of the major contributors to the emergence of colistin-resistant *Enterobacteriaceae* in humans (Maamar et al., 2018). The discovery of a novel stable plasmid-mediated gene *mcr-1* in *E. coli* contributed to our understanding of potential colistin resistance transmission between animals and humans (Liu et al., 2016). Moreover, livestock and poultry have been described as the major reservoir for colistin resistance (Rhouma et al., 2016). A survey has been performed to investigate the prevalence of colistin resistance in *E. coli* isolated from farms in different geographic areas of China during 2013–2014, which revealed that colistin resistance rates in *E. coli* from pigs, chickens, and cattle were 26.5, 14.0, and 0.9%, respectively (Zhang et al., 2019). The results demonstrated that colistin resistance was extremely serious in food animals, particularly in pigs.

In this study, *E. coli* strains isolated from swine farms in northeastern China showed significantly higher frequency of colistin resistance (52.5%). This result supports a previous finding that colistin resistance in *E. coli* occurred widely in pigs (54.25%) in intensive breeding farms of Jiangsu Province from 2015 to 2016 (Zhang et al., 2019). The high frequency of colistin resistance in the *E. coli* isolates recovered from food production animals could be explained by the increasing amount of colistin administrated in animal husbandry in the past few years, especially in swine (Zhang et al., 2019). It has been reported that colistin was used in massive quantities in the swine industry for the treatment of gastrointestinal disease worldwide, including France, Belgium, Spain, Austria, Germany, and China (Rhouma et al., 2016). Moreover, the amount of colistin used in agriculture was 11,942 tons per year by the end of 2015 in China, which was predominant all over the world (Liu et al., 2016).

The rapid horizontal spread of *mcr-1* by plasmids is one of the major reasons for the increasing prevalence of colistin resistance. Several studies have reported that many countries and regions found the presence of GNB carrying *mcr-1* in humans, animals, and the environment (Fernandes et al., 2016; Hadjadj et al., 2017). In this study, 56 (53.33%) *E. coli* strains were positive for *mcr-1* among 105 colistin-resistant *E. coli* isolated from swine farms. Similar to our result, a surveillance of colistin resistance performed in Jiangsu Province revealed that the *mcr-1* prevalence was 68.86% in pigs (Zhang et al., 2019). A previous study showed a high *mcr-1*-positive rate (79.2%) in swine-origin *E. coli* isolated from nine provinces in China. Further testing showed that most *mcr-1*-positive bacteria were identified as *E. coli*, demonstrating that *E. coli* was the predominant bacterial host of the *mcr-1* gene (Zhang et al., 2018). With the purpose of promoting growth, colistin had been widely used as a feed additive in farms for many years in China before 2017. The excessive use of colistin potentially increases the selection pressure which can promote the spread of *mcr-1*, finally leading to an exceedingly high prevalence of *mcr-1* in food animals (Tong et al., 2018). Fortunately, the Chinese government has banned the use of colistin as food additive for growth promotion in farms since April 1, 2017.

It has been reported that plasmids harboring *mcr-1* usually carry other resistance genes, encoded for aminoglycosides, quinolones, etc. (Rozwandowicz et al., 2018). Furthermore, the resistance genes can be horizontally transferred *via* plasmids, which is recognized as one of the major reasons for the extensive resistance profiles of the *mcr-1*-positive bacteria (Fan et al., 2020). In the present study, *mcr-1*-positive *E. coli* isolates displayed high resistance rates to antibiotics that are commonly used in veterinary medicine, including florfenicol, doxycycline, ciprofloxacin, chloramphenicol, streptomycin, gentamicin, kanamycin, and ampicillin. They showed low rates of resistance to some important antibiotics in human medicine, such as tigecycline, nitrofurantoin, ertapenem, meropenem, and imipenem. The usage of different antibiotics may lead to various resistance profiles, and antibiotics commonly used in food animals can form selection pressure on bacteria to become resistant. The antimicrobial resistance profiles of *mcr-1*-positive *E. coli* in this study were similar to the large-scale investigation performed in China (Huang et al., 2017).

The emergence of a superbug resistant to all last-line antibiotics (carbopenems, colistin, and tigecycline) was rare in swine farms, and a similar result was also obtained in a previous study about *E. coli* of food-animal origin in China (Tong et al., 2018). However, co-carriage of *mcr-1* and bla_{NDM-5} was detected in this study which has been found in *Enterobacteriaceae* isolated from animals and humans (Du et al., 2016; Paveenkittiporn et al., 2020). Notably, the one isolate harboring *mcr-1* and bla_{NDM-5} belongs to phylogroup D, indicating the possibility of two isolates being pathogenic *E. coli* responsible for extraintestinal infection

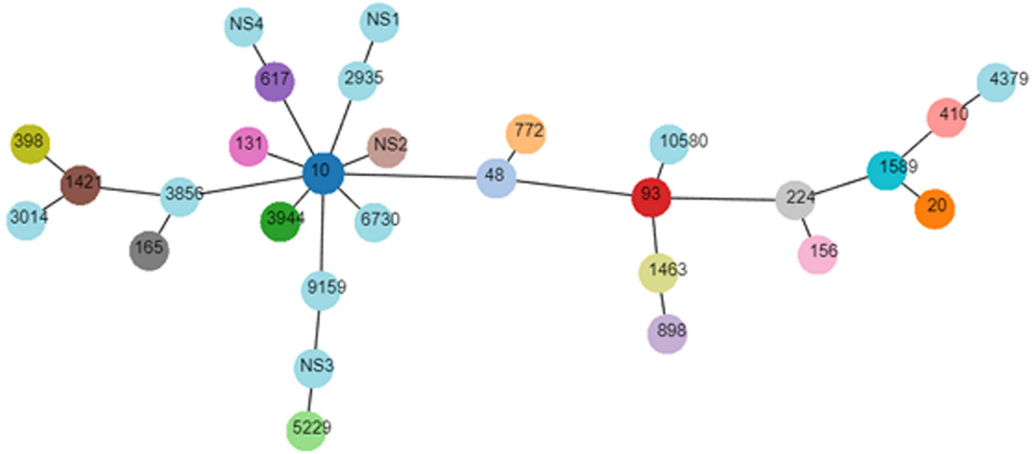

FIGURE 3 | Minimal spanning tree of *mcr-1*-positive *E. coli.* Each circle corresponds to one ST and the size of each circle indicated the number of isolates in this ST type.

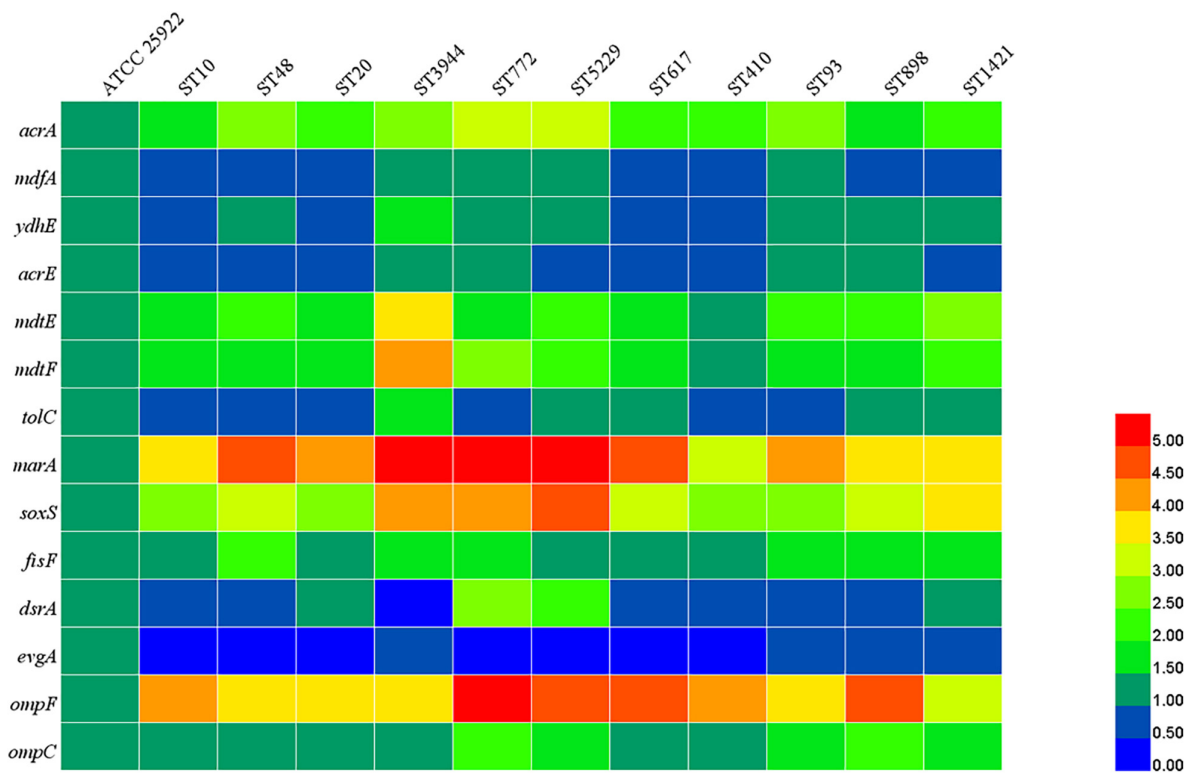

FIGURE 4 | Relative expression levels of efflux pumps, porins, and regulators. The different colors indicate the different expression levels.

(Khanawapee et al., 2020). The extensive resistance profiles of *mcr-1*-positive *E. coli* could be explained by the high frequencies of the presence of other resistance genes, including bla_{TEM}, bla_{CTX-M}, *aac3-IV*, *tet*(A), *tet*(M), *floR*, *sul1*, *sul2*, and *oqxAB*. Multidrug efflux pump in bacteria is a ubiquitous mechanism leading to cross-resistance with several antimicrobial agents and can increase the resistance level by interacting synergistically with other resistance mechanisms (Baron and Rolain, 2018). It has been demonstrated that β-lactams, fluoroquinolones, tetracycline, and chloramphenicol could be the substrates of efflux pumps. In the present study, the relative expression levels of some genes associated with multidrug efflux pumps were increased in *mcr-1*-positive *E. coli*. When the same plasmid carries *mcr-1* and various resistance genes, the frequent use of other antibiotics, such as aminoglycosides, tetracyclines, or sulfonamides, also can promote the selection of colistin resistance

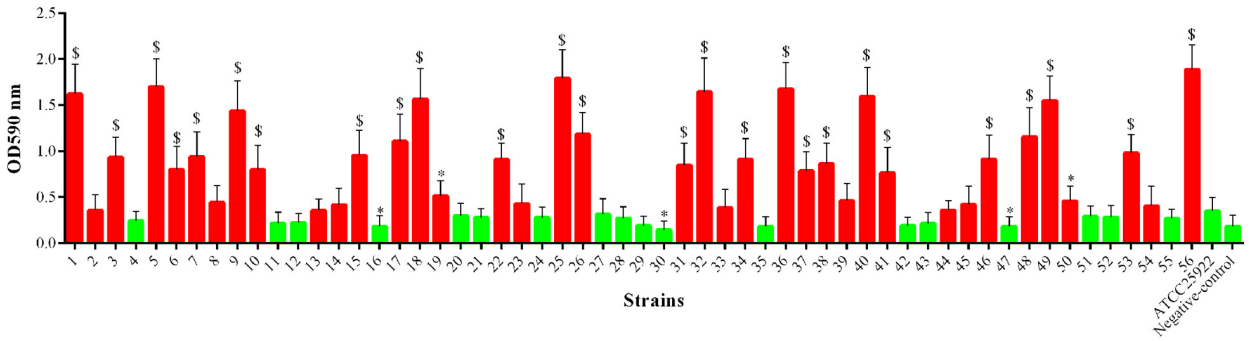

FIGURE 5 | Biofilm formation ability of *mcr-1*-positive *E. coli*; the red indicates increased biofilm formation ability, and green indicates decreased biofilm formation ability compared with *E. coli* ATCC 25922. $^{*}p < 0.05$; $^{\$}p < 0.01$.

TABLE 3 | MLST, transferability, conjugation efficiencies, and plasmid replicon types of 30 *mcr-1*-positive *E. coli*.

Strains	Phylogroup	*mcr-1*	Sequence type	Transferability	Conjugation efficiency	Plasmid replicon types	MIC* (μg/ml)
HLJ8	Unknown	+	10	+	1.85×10^{-4}	IncHI2	8
HLJ63	A	+	410	+	3.73×10^{-3}	IncP/IncHI2	4
HLJ70	B1	+	898	+	1.62×10^{-4}	IncN	4
HLJ173	B1	+	1,421	+	1.97×10^{-4}	IncN/IncX4	2
HLJ194	B2	+	772	−	–	–	–
HLJ187	Unknown	+	6,730	+	2.85×10^{-3}	IncX4	8
HLJ79	A	+	48	+	3.15×10^{-4}	IncI2	8
HLJ56	B1	+	4,379	+	5.36×10^{-4}	IncHI2	4
HLJ174	A	+	3,856	+	2.64×10^{-4}	IncI2	4
HLJ464	A	+	3,944	+	3.18×10^{-4}	IncX4	4
JL124	B1	+	5,229	−	–	–	–
HLJ226	B1	+	NewST 1	+	5.14×10^{-6}	IncN/IncX4	2
JL176	A	+	165	+	3.24×10^{-4}	IncN	4
JL252	Unknown	+	9,159	+	2.76×10^{-4}	IncI2	4
JL7	B2	+	3,014	+	1.96×10^{-3}	IncHI2	4
JL43	D	+	224	−	–	–	–
HLJ438	B1	+	NewST 2	+	3.62×10^{-4}	IncX4	2
HLJ456	B1	+	NewST 3	+	2.84×10^{-4}	IncI2	2
JL226	B1	+	NewST 4	+	2.26×10^{-5}	IncP	4
HLJ84	B1	+	NewST 5	+	4.81×10^{-4}	IncI2	4
LN20	A	+	617	+	3.67×10^{-4}	IncP/IncHI2	4
LN186	B1	+	93	+	2.53×10^{-4}	IncI2	8
LN203	B1	+	2,935	+	2.68×10^{-6}	IncN	2
LN66	D	+	131	−	–	–	–
LN72	A	+	10,580	+	5.64×10^{-5}	IncI2	8
LN106	B2	+	156	+	2.37×10^{-4}	IncX4	4
LN122	B1	+	1,463	+	2.98×10^{-4}	IncI2	4
LN252	A	+	20	+	2.75×10^{-4}	IncI2/IncX4/IncHI2	4
LN220	B1	+	398	+	2.12×10^{-4}	IncP	4
LN19	A	+	1,589	+	9.05×10^{-5}	IncX4	4

**The MIC of colistin against mcr-1-positive transconjugants.*

(Sabine et al., 2017). Therefore, we cannot ignore the effect of the high prevalence of *mcr-1* in swine-origin *E. coli*, increasing the number of multidrug-resistant bacteria.

Biofilm formation is commonly relied on regarding the cooperation of different bacterial strains and species for a common goal. Biofilm shows as bacteria form dense surface-associated communities, which could allow them to prosper and protect each other; bacteria within a biofilm showed enhanced tolerance to harsh environmental conditions and increased antibiotic resistance (Rabin et al., 2015). It has been suspected

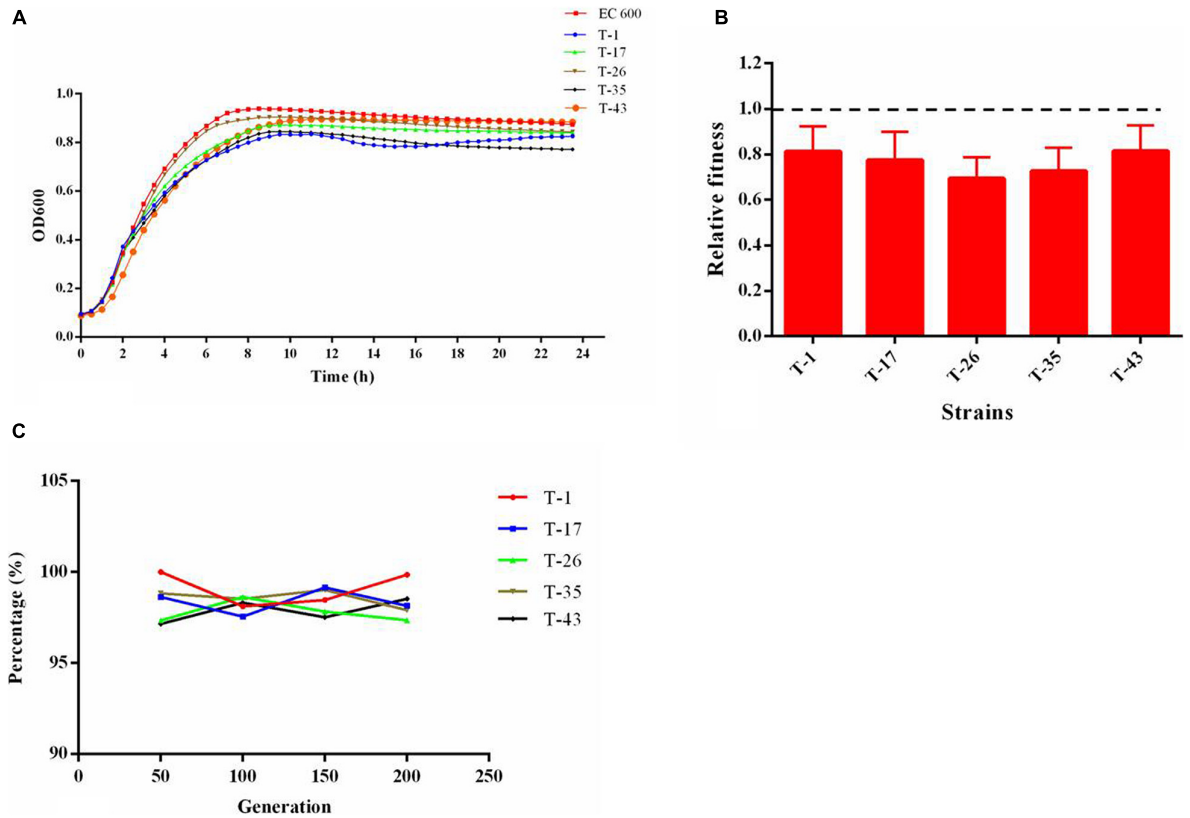

FIGURE 6 | (A) Growth kinetics of transconjugants harboring *mcr-1*; **(B)** relative fitness of transconjugants harboring *mcr-1*, a relative fitness of 1 indicates that the transcoujugants undergo no fitness cost; **(C)** stability of plasmid harboring *mcr-1* in transconjugants.

that biofilm could play a significant role in the persistence of bacterial infections in both clinical and food industries (Bridier et al., 2015). Unfortunately, most of the *mcr-1*-positive *E. coli* isolated from swine in this study were biofilm producers. The result suggested that biofilm formation is one of the strategies used by these bacteria against antibiotics and environmental stress. The prevalence of biofilm in swine-origin *mcr-1*-positive *E. coli* maybe associated with the excessive use of antibiotics in swine farms. A similar idea has been reported that the improper use of antibiotics may select for and further accumulate bacteria with a strong or moderate biofilm formation ability (Ma et al., 2020).

Many studies have demonstrated that the mobile genetic element of *mcr-1* could promote colistin resistance dissemination between animals and humans and result in the high prevalence of *mcr-1* worldwide (Liu et al., 2016; Wang et al., 2018). In this study, the transferability and the dissemination risk of *mcr-1* were assessed among 30 representative strains. The results were in line with previous findings which showed that majority of the reported *mcr* encoded by plasmids were transferable (Wang et al., 2018). Among the reported *mcr-1*, majority of them were mediated by plasmids, but there were some studies that reported the emergence of *mcr-1* on chromosome, or the plasmids harboring *mcr-1* were inconjugative, which could lead to failure of horizontal transfer (Lu et al., 2019).

The plasmid replicon types IncI2 (n = 9), IncX4 (n = 5), IncHI2 (n = 3), IncN (n = 3), and IncP (n = 1) were detected in the transconjugants. Among the already reported plasmids harboring the *mcr-1* gene, they belong to different replicon types, including IncI2, IncHI1, IncHI2, IncFIB, IncFII, IncP, IncX4, and IncY (Huang H. et al., 2020). With the use of colistin in clinical settings, the type of plasmids carrying *mcr-1* became more diverse which was reported by a survey performed in China to investigate the carriage of *mcr-1* among hospital patients, suggesting that colistin administration could promote the dissemination of diverse resistance plasmids among *E. coli* isolates (Huang H. et al., 2020). Moreover, the combinations of IncN/IncX4 (n = 2), IncP/IncHI2 (n = 2), and IncI2/IncX4/IncHI2 (n = 1) were detected, indicating that some transconjugants harbored several replicon types. This could be explained by the co-transfer of *mcr-1* and other resistance genes. The results of growth assay analysis and *in vitro* competition experiment showed that the acquisition of *mcr-1*-harboring plasmids could reduce the fitness of the bacterial host, but plasmid stability testing revealed that *mcr-1*-harboring plasmids remained stable in the recipient strain, which was consistent with a previous study (He et al., 2017). These results indicated that bacterial fitness cost could not cause plasmid loss.

The genetic relationship of the *mcr-1*-positive *E. coli* isolates was analyzed by MLST, which revealed that the most common

ST was ST10, followed by ST48, ST20, ST3944, ST772, ST5229, ST617, ST410, ST93, ST898, and ST1421, and then by single ST isolates. More importantly, three predominant STs (ST10, ST48, and ST617) identified in the current study are different by one or two alleles and they correspond to clonal complex CC10. This result supported the previous finding that the most prevalent ST was ST10 in an investigation of *mcr*-positive *E. coli* isolated from diseased food animals in Europe (Garch et al., 2016). As we all know, ST10 is described as one of the predominant *E. coli* lineages, which is widespread among humans and animals, especially in livestock animals (Manges et al., 2015). By phylogenetic group classification, a total of 46 (82.14%) *mcr-1*-positive *E. coli* belong to groups A and B1 in this study, indicating that most of the swine-origin *mcr-1*-positive *E. coli* were non-pathogenic or commensal strains, consistent with a previous study (Khanawapee et al., 2020).

CONCLUSION

The findings of this study demonstrated the high prevalence of *mcr-1* in swine farms in northeastern China. *mcr-1*-positive *E. coli* showed extensive antimicrobial resistance profiles with the presence of additional resistance genes, increased expression of efflux pump-associated genes, and increased biofilm formation ability. The high diversity of clones and the results of the conjugation experiment underlined the evidence for the horizontal transfer of *mcr-1*. The *mcr-1*-harboring plasmids could reduce the fitness of bacterial hosts but remained stable in the recipient strain. Due to the last-line role of colistin in the treatment option against infection caused by MDR GNB, and livestock production has been described as one of the greatest reservoirs of *mcr-1*, careful monitoring of the spread of *mcr-1* gene in food animals is urgently needed, particularly in swine.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XZ, YY, and PC conceived and designed the experiments. PC, SC, HL, XL, JS, FL, and MI collected the samples and performed the experiments. PC and MI analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Concurrent Resistance to Carbapenem and Colistin Among *Enterobacteriaceae* Recovered From Human and Animal Sources in Nigeria Is Associated With Multiple Genetic Mechanisms

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Resistance to last resort drugs such as carbapenem and colistin is a serious global health threat. This study investigated carbapenem and colistin resistance in 583 non-duplicate *Enterobacteriaceae* isolates utilizing phenotypic methods and whole genome sequencing (WGS). Of the 583 isolates recovered from humans, animals and the environment in Nigeria, 18.9% (110/583) were resistant to at least one carbapenem (meropenem, ertapenem, and imipenem) and 9.1% (53/583) exhibited concurrent carbapenem-colistin resistance. The minimum inhibitory concentrations of carbapenem and colistin were 2–32 μg/mL and 8 to >64 μg/mL, respectively. No carbapenem resistant isolates produced carbapenemase nor harbored any known carbapenemase producing genes. WGS supported that concurrent carbapenem-colistin resistance was mediated by novel and previously described alterations in chromosomal efflux regulatory genes, particularly *mgrB* (M1V), *ompC* (M1_V24del), *ompK37* (I70M, I128M), *ramR* (M1V), and *marR* (M1V). In addition, alterations/mutations were detected in the *etpA, arnT, ccrB, pmrB* in colistin resistant bacteria and *ompK36* in carbapenem resistant bacteria. The bacterial isolates were distributed into 37 sequence types and

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characterized by the presence of internationally recognized high-risk clones. The results indicate that humans and animals in Nigeria may serve as reservoirs and vehicles for the global spread of the isolates. Further studies on antimicrobial resistance in African countries are warranted.

Keywords: concurrent carbapenem-colistin resistance, *Enterobacteriaceae*, high-risk clones, Nigeria, Africa, whole genome sequencing

INTRODUCTION

Antimicrobial resistance, particularly in Gram-negative bacteria, challenges the ability to treat common infections and is one of the greatest threats to global public health systems (Breijyeh et al., 2020). Resistance is more worrisome in resource-limited countries such as in sub-Saharan Africa where infections are common and last-resort antimicrobial agents are scarce and/or unaffordable (Tompkins et al., 2021).

Carbapenems and colistin play a significant role as "last resort" antibiotics in the treatment of infections caused by an extended spectrum of β-lactamase producing *Enterobacteriaceae* and multidrug-resistant *Enterobacteriaceae* (including carbapenem-resistant isolates), respectively. Categorized as the highest priority critically important drugs, the emergence of resistance to them, both individually and concurrently, is a serious source of healthcare concern (World Health Organization [WHO], 2018). Globally, resistance to colistin and carbapenem has been increasingly reported in isolates from animals and humans. The concurrent resistance of *Enterobacteriaceae* to carbapenems and colistin has been reported with increasing frequency in some parts of the world (Du et al., 2016; Yao et al., 2016; Lomonaco et al., 2018). Concurrent resistance determinants are usually located on conjugative plasmids and can thereby be co-transferred, setting the stage for pandrug resistance (Long et al., 2019).

Carbapenem resistant *Enterobacteriaceae* (CRE) comprise both carbapenemase producing (CP-CRE) and non-carbapenemase producing CRE (non-CP-CRE) strains. While CPE produce carbapenemases to hydrolyze carbapenem, non-CP-CRE have β-lactamase (ESBLs and AmpC enzymes) activity combined with structural mutations of the outer membrane protein and drug efflux pumps (Logan and Weinstein, 2017).

Colistin resistance in *Enterobacteriaceae* can be due to structural modifications of the bacterial lipopolysaccharide, such as the addition of 4-amino-4-deoxy-l-arabinose (L-Ara4N) or phosphoethanolamine (pEtN) (intrinsic resistance). Acquired resistance may result from chromosomal mutations in genes encoding the *PhoPQ* and *PmrAB* a two-component regulatory system, the *mgrB*, a negative regulator of *PhoPQ*, or the plasmid-borne mobile colistin resistance genes (*mcr*-1 to *mcr*-10) encoding a group of pEtN transferases (Aires et al., 2016; Zafer et al., 2019; Wang C. et al., 2020).

We recently reported the occurrence of *mcr*-mediated colistin resistance among 99 colistin resistant isolates; *Escherichia coli* (67/99), *Klebsiella pneumoniae* (30/99), *Citrobacter werkmanii* (1/99), and *Alcaligenes faecalis* (1/99) from humans and animals in Nigeria (Ngbede et al., 2020). Studies have also reported a high prevalence, up to 52%, of carbapenem resistance mediated by carbapenemase producing genes in samples from humans in Nigeria (Ogbolu and Webber, 2014; Jesumirhewe et al., 2017; Olowo-okere et al., 2019; Otokunefor et al., 2019; Ogbolu et al., 2020; Olalekan et al., 2020; Olowo-Okere et al., 2020; Shettima et al., 2020). These findings suggest that resistance to the last resort drugs carbapenem and colistin is a significant problem in Nigeria. Despite these increasing reports on colistin and carbapenem resistant bacteria emanating from sub Saharan Africa, the occurrence of co-resistance to both drugs and detailed insight into the molecular mechanism associated with this resistance phenotype has not been a major focus of such studies. Similarly, majority of these studies focus mainly on PCR detection of the most commonly and previously reported mechanisms including those mediated by bla_{KPC}, bla_{NDM}, bla_{IMP} genes for carbapenem and the *mcr*- gene for colistin and rarely followed by WGS analyses of the isolates which is usually targeted at strain typing. This is in spite of the increasing non-detection of these genes in some of the isolates resistant to these antibiotics. Understanding the mechanisms of resistance is crucial to countering the mounting burden of infections caused by multidrug resistant bacteria, and whole-genome sequencing (WGS) has been shown to play a significant role in the rapid and accurate detection and characterization of known and emerging resistance determinants. Rarely, studies from sub Saharan Africa utilized whole genome sequencing to understand the detailed underlying genetic mechanism for carbapenem-colistin co-resistance in *Enterobacteriaceae* to the best of our knowledge. Such information is critical in formulating strategies for the clinical management and control of infections caused by multidrug-resistant *Enterobacteriaceae*. This study aimed to investigate the prevalence and genetic mechanisms underlying colistin-carbapenem co-resistance among *E. coli* and *Klebsiella* species (two important nosocomial pathogens) recovered from animals, humans, and the environment in Nigeria.

MATERIALS AND METHODS

Bacteria Isolates

This study utilized a total of 583 non-duplicate *Enterobacteriaceae* acquired between 2016 and 2019. Isolates comprised 487 *E. coli,* 87 *Klebsiella* species, and nine *Citrobacter* species and were recovered from human clinical sample: stool (n = 60) and urine (n = 35), human hospital environment (n = 15), rectal swabs of camels (n = 40), cattle (n = 36), dogs (n = 42), pigs (n = 65), and cloacal swabs of poultry (n = 250) and clinical samples (liver) of poultry (n = 40) were used in this study

(Ngbede et al., 2020). Procedures for sample collection, isolation, and identification have been previously reported (Ngbede et al., 2020). Ethical approval for the collection of samples from humans was provided by the Health Research and Ethics Committee of the facilities (FMH/FMC/MED.108/VOL.I/X and BSUTH/MKD/HREC/2013B/2018/0027).

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentrations (MICs) of carbapenem (meropenem, ertapenem, and imipenem) were determined on the 583 isolates using the broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2020). Briefly, colonies of each isolate from an overnight growth on tryptone soya agar were suspended in 5 mL normal saline to make an inoculum the equivalent of a 0.5 MacFarland standard. The turbidity of the inoculum was measured using a densitometer (BioScan). A 20 μL volume of the inoculum was dispensed into 2 mL of Mueller–Hinton broth containing different concentrations of the respective carbapenems and incubated at 35°C for 24 h. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 served as controls.

Phenotypic Assay for Carbapenemase Production

The Carbapenem inactivation method (CIM) was used to screen the isolates for carbapenemase production as described (van der Zwaluw et al., 2015). Briefly, a loopful (≈10 μL) of the isolate was suspended in 400 μL of double distilled water and followed by a 10 μg meropenem disk (Oxoid, United Kingdom) which was immersed in the suspension and incubated for 2 h at 35°C. The disk was removed from the suspension with an inoculation loop and placed on a Mueller–Hinton agar plate inoculated with a 0.5 McFarland standard *E. coli* strain ATCC 29522 (a susceptible indicator strain) using a sterile cotton swab; it was subsequently incubated at 35°C for 24 h. A positive results was provided when isolates with carbapenemase production inactivates meropenem in the disk, allowing uninhibited growth of the susceptible indicator strain. A negative result occurred when meropenem disks were incubated in suspensions of isolates without carbapenemases, yielding a clear inhibition zone of the susceptible indicator strain (van der Zwaluw et al., 2015).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility profiles of the isolates were determined by the disk diffusion method (Clinical and Laboratory Standards Institute, 2020) using 10 antimicrobial agents sourced from Oxoid, United Kingdom: Amoxycillin (10 μg), amoxicillin/clavulanic acid (30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), chloramphenicol (10 μg), gentamicin (10 μg), doxycycline (30 μg), enrofloxacin (5 μg), and sulfamethoxazole-trimethoprim (25 μg). The results were interpreted based on the guidelines of the CLSI (Clinical and Laboratory Standards Institute, 2020).

DNA Extraction and Whole Genome Sequencing

Fifty out of the 53 isolates co-resistant to colistin and carbapenem, were randomly selected and subjected to WGS. Only 50 isolates were randomly selected for the WGS due to its associated cost and the selection of isolates represented the different species and susceptibility patterns encountered among the 53 co-resistant (carbapenem-colistin) isolates recovered from humans, animals and the environment. Genomic DNA (gDNA) was extracted from overnight cultures of the isolates using the Wizard Genomic DNA Purification Kit (Promega, United States) following the manufacturer's recommendations. Isolates were sequenced using next-generation sequencing on an Illumina MiSeq platform (OE Biotech, Shanghai, China) using the V2 paired-end chemistry (2 × 250 bp).

Analyses of WGS Data

Quality of the sequencing was assessed using QUAST v5.1[1] (Gurevich et al., 2013) before *de novo* assembly using the SeqMan Pro v.11.2.1 (DNASTAR, United States) followed by annotation using PROKKA v1.14.5. Species identity of the isolates was further confirmed with the WGS data using the KmerFinder v3.2[2] (Hasman et al., 2014; Larsen et al., 2014; Clausen et al., 2018). Genetic relatedness of isolates was analyzed by phylogroup (*E. coli* only), multilocus sequence types (MLST) and core genome MLST (cgMLST). MLST for each isolate was predicted using the pubMLST[3] while the assignment of core genome MLST and phylogroups for the *E. coli* isolates were carried out using the cgMLSTFinder v1.1[4] (Zhou et al., 2020) and the online Clermont tool v20.03[5] (Beghain et al., 2018; Clermont et al., 2019).

In silico serotyping for the *E. coli* and *Klebsiella* isolates was performed using the SeroFinder 2.0[6] (Joensen et al., 2015) and Kaptive[7] (Wick et al., 2018).

The presence of acquired antimicrobial resistance genes were investigated using the ResFinder v4.0[8] (Bortolaia et al., 2020) and CARD RGI 5.2.0[9] (Alcock et al., 2020). Plasmids were identified using PlasmidFinder 2.1[10] (Carattoli et al., 2014) and MGEFinder v1.0.3[11] (Johansson et al., 2021). Virulence genes were identified using the VirulenceFinder 2.0 (Lui et al., 2019).

Detection of Mutations in Genes Related to Colistin and Carbapenem Resistance

Mutations in the genes previously identified as responsible for resistance to colistin (*mgrB, prmAB, phoPQ, arnT, ccrB*) and carbapenem (*ompC, ompF, ompK/35/36/37*, *marR, acrR,*

[1]http://cab.cc.spbu.ru/quast/
[2]https://cge.cbs.dtu.dk/services/KmerFinder/
[3]https://pubmlst.org/bigsdb
[4]https://cge.cbs.dtu.dk/services/cgMLST
[5]http://clermontyping.iame-research.center/index.php
[6]https://cge.cbs.dtu.dk/services/SerotypeFinder/
[7]http://kaptive.holtlab.net/
[8]https://cge.cbs.dtu.dk/services/ResFinder/
[9]https://card.mcmaster.ca/analyze/rgi
[10]https://cge.cbs.dtu.dk/services/PlasmidFinder/
[11]https://cge.cbs.dtu.dk/services/MobileElementFinder/

ramR) were investigated by alignment with wild type reference genomes of *E. coli* strain K-12 substrain MG1655 (NC_000913.3) and *K. pneumonia* subspecies *pneumoniae* MGH 78578 (NC_009648.1). PROVEAN v1.1.3[12] was employed to predict the possible role/effect of observed amino acid substitutions (mutation/alteration) on protein functions i.e., colistin or carbapenem resistance (Choi and Chan, 2015). The standard PROVEAN cutoff score of ≤-2.5 and >-2.5 was used to categorize the mutation with deleterious and/or neutral effects on protein function, respectively (Choi et al., 2012; Choi and Chan, 2015).

Transfer Experiments

The conjugative transferability of colistin and carbapenem resistance determinants was assessed using the solid mating conjugation assay with sodium azide-resistant *E. coli* J53 as the recipient (Ojo et al., 2016). Overnight cultures of the donor and recipient were mixed in a 1:4 ratio in tryptone soya broth and centrifuged at 14,000 × *g* for 1 min. The pelleted cells were resuspended in 15 μL of 0.85% NaCl, spotted onto MHA, incubated at 37°C for 20–24 h. Bacteria growing on the MHA were resuspended in 1 mL of 0.85% NaCl and a 100 μL serially diluted aliquot. Each dilution was placed on Brain Heart Infusion (BHI) agar supplemented with sodium azide (150 μL/mL) + colistin (2 μg/mL), meropenem (2 μg/mL) + sodium azide (150 μL/mL), colistin (2 μg/mL) + meropenem (2 μg/mL) + sodium azide (150 μL/mL) for the selection of transconjugant. The conjugation, or transfer, the frequency was measured based on the ratio of the observed transconjugant CFU (T) divided by the recipient CFU (T/R).

RESULTS

Prevalence of Carbapenem Resistance and Concurrent Carbapenem-Colistin Resistance

Of the 583 isolates tested, 110 (18.9%) comprising 53 *E. coli* and 57 *Klebsiella* species were resistant to at least one of three carbapenems (meropenem, 18.7%; 109/583; ertapenem, 16.8%; 98/583; imipenem, 18.2%; 106/583) and originated from pigs (*n* = 14), poultry (*n* = 39), humans (*n* = 33), environment (*n* = 3), cattle (*n* = 10), camel (*n* = 1), and dogs (*n* = 10). Based on colistin resistance findings determined in a former study (Ngbede et al., 2020) and this study, 53 isolates (9.1%; 53/583) were considered resistant to both colistin and at least one of the carbapenems. Of the 53 isolates expressing concurrent carbapenem-colistin resistance, 50 were randomly selected for further investigation: 23 human isolates (16 *K. pneumoniae*, 2 *K. quasipneumoniae*, and 5 *E. coli*), one hospital environmental isolate (*K. quasipneumoniae*), and 26 animal isolates (1 *K. pneumoniae,* 24 *E. coli*, and 1 *C. werkmanii*). The MIC of carbapenems ranged between 2 and 32 μg/mL (**Supplementary Table 1**).

[12]http://provean.jcvi.org/seq_submit.php

The resistance profile is shown in **Table 1** for the *Citrobacter* and *E. coli* isolates and **Table 2** for the *Klebsiella* isolates. The 50 carbapenem/colistin resistant strains were negative for carbapenemase production, with 98% of the isolates resistant to the β-lactam antibiotics. This particularly included amoxicillin and amoxicillin/clavulanic acid as all the isolates except one *E. coli,* were resistant to amoxycillin. Similarly, 48, 66, and 78% of the isolates were resistant to aminoglycosides, sulphamethaxazole/trimethoprim, and fluoroquinolones, respectively (**Tables 1**, **2**).

Whole Genome Sequence Analysis

Quality assessment of the genome reads revealed the *Citrobacter* isolate had a total genome length of 5.0 MB distributed over 60 contigs with an N_{50} and average GC content of 48,746 bp and 52, respectively. The total genome length of the *E. coli* isolates ranged from 3.7 to 4.2 MB distributed over 50–1465 contigs with an N_{50} length of 2,642–296,122 bp and an average GC content (mol%) of 50 while the *Klebsiella* isolates had an average GC content (mol%) of 50, total genome and N_{50} length of 5.0–5.6 MB and 126,035–462,504 bp, respectively distributed over 24–195 contigs. The WGS data further confirmed the identity of the 50 isolates as *C. werkmanii* (*n* = 1), *E. coli* (*n* = 29), *K. pneumoniae* (*n* = 17), *K. quasipneumoniae* (*n* = 3).

Carbapenem Resistance Mechanisms

The WGS data confirmed that none of the isolates harbored any known carbapenemase genes. However, we found novel deletions, including the deletion of 24 amino acids from Met1 to Val24 (M1_V24del) in the *ompC* gene of all the *E. coli* isolates (29/29) (**Table 3**). Previously known substitutions in the *ompC* gene mediating carbapenem resistance were also detected among *E. coli* isolates (D192G/K in 17/29 isolates; N47D in 5/29 isolates) but, no mutations associated with carbapenem resistance were detected in the *ompF* gene. Among the *Klebsiella* isolates, we detected a HYTH insertion between amino acid Met233 and Thr234 (M233_T234insHYTH) (10/20) insertion in the *ompK37*, substitution A217S (15/20) and N218H (6/20) in *ompK36*, and substitution I70M (20/20), I128M (20/20), N230G (10/20) and T261A (1/20) in *ompK37* (**Table 4**).

Multiple substitutions were detected in the global regulator proteins *marR* and *ramR*, but not in the regulators *AcrR, MarA, RamA*, and *SoxR*. Only the M1V substitution in *E. coli marR* and *K. pneumoniae ramR* was associated with carbapenem resistance (**Tables 3**, **4**).

Colistin Resistance Mechanisms

Sixteen (one *C. werkmanii*, eight *E. coli*, and seven *K. pneumoniae* isolates) of the 50 carbapenem-colistin resistant isolates harbored the plasmid mediated colistin resistant genes: *mcr*-1 (*n* = 9), *mcr*-1 and +*mcr*-5 (*n* = 1), *mcr*-8 (*n* = 4), *mcr*-1, and +*mcr*-8 (*n* = 2) (**Tables 3**, **4**). No point mutations associated with colistin resistance were detected in the *pmrABC* and *phoPQ* genes. Alterations in the *mgrB* were detected in all the isolates viz *E. coli* (29/29), *Klebsiella* (20/20), and *Citrobacter* (1/1) (**Tables 3**, **4**).

TABLE 1 | Phenotypic and genotypic resistance profile of *Enterobacteriaceae* of animal origin expressing concurrent carbapenem-colistin resistance from Nigeria.

ID	Phenotypic resistance profile	β-lactamases	Aminoglycosides	Fluoroquinolones	Sulphamethazoxle-Trimethoprim	Tetracycline	Phenicol
B14	AMC AMX CEF DOX GEN ENR STX	CTX-M-15, TEM-1B	*aph(3′′)-Ib, aph(6)-Id, aac(3)-IId*	*qnrS1, qepA1, gyrA**	*sul2, dfrA14, dfrA17*	*tetA, tetB*	–
B22	AMC AMX CEF ENR STX	CTX-M-15	*aph(3′′)-Ib, aph(6)-Id*	*qnrS1*	*sul2, dfrA14*		–
C40	AMC AMX CEF ENR	CTX-M-15		*qnrS1*		*tetA*	–
E41	AMC AMX CEF STX	CTX-M-15, TEM-1B	*aph(3′′)-Ib, aph(6)-Id*	*qnrS1*	*sul2, dfrA14*	*tetA*	–
L3	AMC AMX CEF DOX GEN STX	CTX-M-55	*aac(3)-IId*	*qnrS1*	*sul2, dfrA14*	*tetA*	
L18	AMC AMX CHL CEF DOX GEN ENR FLOR SXT	TEM-1B	*armA, aadA1, aadA2b, aac(3)-IIa, aph(3′)-Ia, aph(6)-Ic,*	*acrR*, qnrB1, oqxA, oqxB, qnrB17*	*sul1, sul3, dfrB4,*	*ramR*, tetD, tetM*	*cmlA1*
L6	AMC AMX CIP CEF DOX ENR STX	CTX-M-15, OXA-1	*aac(6′)-Ib-cr, aadA5*	*gyrA**	*sul1, dfrA17*	*tetA*	*CatB3*
L13	AMC AMX CIP CEF DOX ENR STX	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*	*gyrA**	*sul1, dfrA17*	*tetA*	*CatB3*
L15	AMC AMX CEF ENR	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*	*gyrA**	*sul1, dfrA17*	*tetA*	*CatB3*
L16	AMC AMX CEF COL DOX ENR STX	CTX-M-15, TEM-1B	*aph(3′′)-Ib, aph(6)-Id*	*qnrS1*	*sul2, dfrA14*	*tetA*	
L17	AMC AMX CHL CIP CEF DOX GEN ENR STX	CTX-M-15	*aadA2, aadA1*	*gyrA**	*sul2, dfrA12*	*tetA*	*cmlA1*
L20	AMC AMX CHL CIP CEF DOX GEN ENR FLOR	CTX-M-65	*aac(3)-IId*	*qnrS13*		*tetA*	*floR*
L22	AMC AMX CHL CIP CEF DOX GEN ENR FLOR STX	-	*aac(3)-IIa, aph(3′)-Ia, aph(3′′)-Ib, aadA1*	*gyrA**	*sul3, dfrA1*	*tetA*	*floR*
L23	AMC AMX CHL CEF DOX GEN ENR	TEM-1B	*aac(3)-Via*	*gyrA*, qnrS1*	*sul1, sul3, dfrA14*	*tetA*	
L25	AMX AMC CIP CEF ENR STX	TEM-1B	*aac(3)-Via*	*gyrA*, qnrS1*	*sul1, sul3, dfrA14*	*tetA*	
L26	AMC AMX CHL CEF DOX ENR FLOR STX	TEM-1B	*aph(3′)-Ia, aph(3′′)-Ib, aadA1*	*gyrA*, qnrS1, parC**	*sul3, dfrA1*	*tetA*	*floR*
L27	AMC AMX CHL CEF COL GEN ENR FLOR STX	TEM-1B	*aac(3)-IIa, aadA1, aph(3′′)-Ib*	*gyrA**	*sul3, dfrA1*	*tetA*	*floR*
L28	AMC AMX CIP CEF DOX GEN ENR	TEM-1A	*aac(3)-IId, aac(3)-Via*	*gyrA*, qnrS1*	*sul1, sul3, dfrA14*	*tetA*	–
L29	AMC AMX CEF DOX GEN ENR STX	TEM-1B	*aac(3)-Via*	*gyrA*, qnrS1*	*sul1, sul3, dfrA14*	*tetA*	–
L31	AMC AMX CEF DOX GEN	CTX-M-55	*aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id*	*gyrA*, parC**	*sul2*	*tetB*	–
L36	AMC AMX CHL CIP CEF DOX GEN ENR STX	TEM-1B	*aac(3)-IId, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id*		*sul2, dfrA14*	*tetA*	*floR*
L38	AMC AMX CHL CEF DOX GEN ENR FOX STX	CMY-98, TEM-1	*aph(3′)-Ia, aph(6)-Id, aadA2b*	*qnrB19, qnrB34*	*sul1, dfrA12*	*tetB, tetM*	*cmlA1*
L39	AMC AMX CIP CEF DOX ENR STX	TEM-1B	*aadA5*	*qnrS1*	*sul2, dfrA17*	*tetA*	–
L40	AMC AMX CHL CIP CEF DOX GEN ENR FLOR STX	TEM-1B	*aac(3)-IId, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id*	*qnrS1*	*sul2, dfrA14*		*floR*
L41	AMC AMX CEF DOX GEN ENR		*aac(3)-IIa, aph(6)-Id*	*qnrS13, gyrA*, parC**,	*sul2*	*tetA*	–
L43	AMC AMX CIP CEF GEN ENR	TEM-1B	–	*qnrS1*	–	–	–

**Mutations on the respective genes.*

AMC, amoxycillin; AMX, amoxycillin-clavulanic acid; CHL, chloramphenicol; CEF, ceftriaxone; CIP, ciprofloxacin; DOX, doxycycline; FLOR, florfernicol; GEN, gentamicin; ENR, enrofloxacin; FOX, cefoxitin; STX, sulfamethazaxole-trimethoprim.

TABLE 2 | Phenotypic and genotypic resistance profile of *Enterobacteriaceae* of human origin expressing concurrent carbapenem-colistin resistance from Nigeria.

ID	Phenotypic resistance profile	β-lactamases	Aminoglycosides	Fluoroquinolones	Sulphamethazoxle-Trimethoprim	Tetracycline	Phenicol
H2	AMC AMX CEF GEN	SHV-11	–	*acrR*, oqxA, oqxB*	–	–	–
H34	AMC AMX CHL CIP CEF DOX ENR FOR STX	CTX-M-15, TEM-1B	*aac(6′)-Ib-cr, aac(6′)-Ib3*	*gyrA*, parC*, parE**	*sul1, dfrA1*	*tetB*	*catA1, catB3*
H35	AMC AMX CHL CIP CEF	CTX-M-15	*aadA1*	*qepA4, gyrA*, parE**	*sul1, sul2, dfrA12*	*tetB*	*catA1, cmlA1*
H36	AMC AMX CEF DOX STX	SHV-1	–	*acrR*, oqxA, oqxB*	–	–	–
H41	CEF COL DOX	–	–	–	–	–	
H6	AMC AMX CHL CIP CEF GEN STX	TEM-1B, SHV-11	*aac(3)-IId, aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB, qnrS1*	*sul2, dfrA14,*	*tetA, tetD*	*catA2*
H50	AMC AMX CEF DOX STX	OKP-B-8	–	*acrR*, oqxA, oqxB*	–	–	–
H4	AMC AMX CHL CIP CEF DOX ENR FOX STX	SHV-1	–	*acrR*, oqxA, oqxB*	–	–	–
H5	AMC AMX CEF DOX ENR STX	TEM-1B, DHA-1, SHV-1	*aph(3′′)-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB, qnrB4*	*sul1, sul2, dfrA1, dfrA14,*		
H7	AMC AMX CEF DOX ENR	SHV-11	*aadA1*	*acrR**	*sul2, dfrA5*		
H23	AMC AMX CIP CEF DOX GEN ENR SXT	CTX-M-15, OXA-1, TEM-1B, SHV-28	*aac(3)-IIa, aac(6′)-Ib-cr, aph(3′′)-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB, qnrB1*	*sul2, dfrA14*	*tetA*	
H25	AMC AMX CIP CEF DOX GEN ENR STX	CTX-M-15, OXA-1, TEM-1B, SHV-28	*aac(3)-IIa, aac(6′)-Ib-cr*	*acrR*, oqxA, oqxB, qnrB1*	*sul2, dfrA14,*	*tetA*	*catB3*
H26	AMC AMX CIP CEF GEN ENR STX	CTX-M-15, OXA-1, TEM-1B, SHV-28	*aac(3)-IIa, aac(6′)-Ib-cr*	*acrR*, oqxA, oqxB, qnrB1*	*sul2, dfrA14,*	*tetA*	*catB3*
H29	AMC AMX CEF FLOR	–	–	–	*sul2, dfrA15*		–
H30	AMC AMX CEF FLOR MER	OKP-B-7	–	*acrR*, oqxA, oqxB*	–	–	–
H31	AMC AMX CEF DOX ENR FLOR STX	SHV-1	–	*acrR*, oqxA, oqxB*	–	–	–
H39	AMC AMX CEF DOX	TEM-1C, SHV-1	*aadA1*	*oqxA, oqxB*	*sul1, dfrA1*	*tetA*	
H40	AMC AMX CEF		*aph(3′)-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB*			
H45	AMC AMX CHL CIP CEF DOX GEN ENR FLOR STX	CTX-M-15, TEM-1B, SHV-11	*aac(3)-IId, aadA2*	*acrR*, oqxA, oqxB*	*sul1, sul2 dfrA1*	*tetD*	*catA2*
H46	AMC AMX CHL CIP CEF DOX GEN ENR FLOR STX	CTX-M-15, TEM-1B, SHV-11	*aac(3)-IId, aadA2, ant(2′′)-Ia, aph(3′)-Ia, aph(3′)-Ib*	*oqxA, oqxB*	*sul1, sul2 dfrA10, dfrA12*	*tetD, tetJ*	
H47	AMC AMX CHL CIP CEF GEN STX	CTX-M-15, TEM-1B, SHV-11	*aac(3)-IId, aadA2, aph(3′)-Ib*	*acrR*, oqxA, oqxB*	*sul1, sul2 dfrA12*	*tetD*	*catA2*
H48	AMC AMX CEF ERY FOX STX	TEM-1B, SHV-11	–	*acrR*, oqxA, oqxB*	*sul2 dfrA26*	–	–
H49	AMC AMX CEF	TEM-1B, DHA-17	*aph(3′′)-Ib, aph(6)-Id, aadA1*	*sul1, sul2*	*dfrA1, dfrA5*	*tetA*	*catA2*
H22	AMC AMX CEF DOX ENR STX	OKP-B-5	*aph(3′′)-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB*			

**Mutations on the respective genes.*

AMC, amoxycillin; AMX, amoxycillin-clavulanic acid; CHL, chloramphenicol; CEF, ceftriaxone; CIP, ciprofloxacin; DOX, doxycycline; FLOR, florfernicol; GEN, gentamicin; ENR, enrofloxacin; FOX, cefoxitin; STX, sulfamethazaxole-trimethoprim.

Novel substitutions were found in some the *E. coli* isolates, including V336M and T411I in the *etpA* and A278T, T157A, L485F, and W100M in the *arnT* (**Table 3**). The majority of the *Klebsiella* isolates harbored the N195S amino acid substitution (18/20), and some harbored the N141I/H (5/20) and W140L/S (5/20) substitutions in the *ccrB,* which were previously reported to mediate colistin resistance (Cheng et al., 2016). Finally, novel mutations as predicted to mediate colistin resistance, G164S in *arnT,* and the M1_L56del deletion were detected in one *Klebsiella* isolate (**Tables 3**, **4**).

TABLE 3 | Characteristics and resistance mechanisms associated with concurrent carbapenem-colistin among *Enterobacteriaceae* isolated from animals in Nigeria.

Sample source	ID no	Species	*mcr*- genes	Mutations and genetic alterations mediating								
				mgrB	*ccrB*	*etpA*	*arnT*	*ompC*	*marR*	*ramR*	*ompK36*	*ompK37*
Cattle Rectal swab	B14	*E. coli*	–	M1V	–	–	A278T	M1_V24del, G29S, D39N, G40A, L41K, D46S, Q54T, Y74F	M1V	–	–	–
	B22	*E. coli*	–	M1V	–	–	–	M1_V24del	M1V	–	–	–
Camel rectal swab	C40	*E. coli*	–	M1V	–	V336M		M1_V24del, D192G	M1V	–	–	–
Pig rectal swab	E41	*E. coli*	–	M1V	–	–	–	M1_V24del, D192G	M1V	–	–	–
	L6	*E. coli*	–	M1V	–	–	–	M1_V24del, D192G	M1V	–	–	–
	L13	*E. coli*	–	M1V	–	–	–	M1_V24del, D192G	M1V	–	–	–
	L15	*E. coli*	–	M1V	–	–	–	M1_V24del, D192G	M1V	–	–	–
	L16	*E. coli*	–	M1V	–	–	–	M1_V24del, D192G, N228_T229insGSYTSNGV	M1V	–	–	–
	L17	*E. coli*	–	M1V	–	–	–	M1_V24del, D192G	M1V	–	–	–
	L20	*E. coli*	–	M1V	–	T411A	T157A	M1_V24del	M1V	–	–	–
Poultry cloacal swab	L3	*E. coli*	–	M1V	–	–	–	M1_V24del	M1V	–	–	–
	L18	*K. pneumonia*	–	M1V	M1_L56del, W140S, N141H, N195S	–	–	–	–	M1V	A217S, N218H	I70M, I128M
	L22	*E. coli*		M1V	–	–	–	M1_V24del, D192G	M1V	–	–	–
	L23	*E. coli*	*mcr-1*	M1V	–	–	–	M1_V24del, N47D, D192K	M1V	–	–	–
	L25	*E. coli*		M1V	–	–	–	M1_V24del, N47D, D192K	M1V	–	–	–
	L26	*E. coli*	*mcr-1*	M1V	–	–	L485F	M1_V24del	M1V	–	–	–
	L27	*E. coli*	*mcr-1*	M1V	–	–	–	M1_V24del, D192G, N228_T229insGSYTSNGV	M1V	–	–	–
	L28	*E. coli*		M1V	–	–	–	M1_V24del, D192G	M1V	–	–	–
	L29	*E. coli*	*mcr-1, mcr-5*	M1V	–	–	–	M1_V24del, N47D, D192K	M1V	–	–	–
	L31	*E. coli*		M1V	–	–	–	M1_V24del	M1V	–	–	–
Liver from sick birds	L36	*E. coli*	*mcr-1*	M1V	–	–	–	M1_V24del	M1V	–	–	–
	L38	*Citrobacter werkmanii*	*mcr-1*	M1V	–	–	–	–	M1V	–	–	–
	L39	*E. coli*	*mcr-1*	M1V	–	–	–	M1_V24del, N47D	M1V	–	–	–
	L40	*E. coli*	*mcr-1*	M1V	–	–	–	M1_V24del, A230_A231insYYISNGVAR	M1V	–	–	–
	L41	*E. coli*	–	M1V	–	–	W100M	M1_V24del, K173T, N176_T186del, G190E, D192G, D225W	M1V	–	–	–
	L43	*E. coli*	–	M1V	–	–	–	M1_V24del, N47D, D192G	M1V	–	–	–

M1V: Depicts a substitution of the amino acid methionine (M) by valine (V) at position 1; M1_V24del: depicts a loss/deletion of the first 24 amino acids, Methionine (M) at position 1 through Valine (V) at position 24; N228_T229insGSYTSNGV: depicts an insertion of 8 amino acids GSYTSNGV between position 228 and 229.

TABLE 4 | Characteristics and resistance mechanisms associated with concurrent carbapenem-colistin among *Enterobacteriaceae* isolated from human sources in Nigeria.

Sample source	ID no	Species	*mcr* gene	Mutations and genetic alterations mediating							
				mgrB	***ccrB***	***arnT***	***ompK36***	***ompK37***	***ompC***	***ramR***	***marR***
Human clinical sample (stool)	H2	*K. pneumonia*	*mcr-1, mcr-8*	M1V	N195S	–	A217S, N218H	I70M, I128M	–	M1V	–
	H6	*K. pneumonia*	*mcr-8*	M1V	N195S	–	A217S	I70M, I128M, N230G, M_233T234insHYTH	–	M1V	–
	H34	*E. coli*	–	M1V	–	–	–	–	M1_V24del	–	M1V
	H35	*E. coli*	–	M1V	–	–	–	–	M1_V24del, D192G	–	M1V
	H36	*K. pneumonia*	–	M1V	M1_A52del, W140L, N141I	–	A217S	I70M, I128M	–	M1V	–
	H41	*E. coli*	–	M1V	–	–	–	–	M1_V24del, R267L	–	M1V
	H50	*K. quasipneumoniae*	–	M1V	M1_A59del, W140S, N141H, N195S	–	A217S, N218H	I70M, I128M	–	M1V	–
Human clinical sample (urine)	H4	*K. pneumonia*	–	M1V	N195S	–	–	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H5	*K. pneumonia*	–	M1V	N195S	–	–	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H7	*K. pneumonia*	*mcr-8*	M1V	N195S	G164S	–	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H23	*K. pneumonia*	–	M1V	N195S	–	–	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H25	*K. pneumonia*	–	M1V	N195S	–	–	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H26	*K. pneumonia*	–	M1V	N195S	–	–	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H29	*E. coli*	–	M1V	–	–	–	–	M1_V24del	–	M1V
	H30	*K. quasipneumoniae*	–	M1V	M1_A59del, W140S, N141H, N195S	–	A217S, N218H	I70M, I128M	–	M1V	–
	H31	*K. pneumonia*	*mcr-1, mcr-8*	M1V	N195S	–	A217S, N218H	I70M, I128M	–	M1V	–
	H39	*K. pneumonia*	*mcr-8*	M1V	N195S	–	–	I70M, I128M, T261A	–	M1V	–
	H40	*K. pneumonia*		M1V	M1_A59del, W140L, N141H	–	A217S, N218H	I70M, I128M	–	M1V	–
	H45	*K. pneumonia*	*mcr-1*	M1V	N195S	–	A217S	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H46	*K. pneumonia*	–	M1V	N195S	–	A217S	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H47	*K. pneumonia*	–	M1V	N195S	–	A217S	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H48	*K. pneumonia*	–	M1V	N195S	–	A217S	I70M, I128M, N230G, M233_T234insHYTH	–	M1V	–
	H49	*E. coli*	–	M1V		–	–	I70M, I128M, N230G, M233_T234insHYTH	M1_V24del, D192G, A231_Y232insNGYGER	–	M1V
Environon-mental sample (sink)	H22	*K. quasipneumoniae*	–	M1V	N195S	–	A217S	I70M, I128M, N230G, M233_T234insHYTH	–	–	–

M1V: Depicts a substitution of the amino acid methionine (M) by valine (V) at position 1; M1_A52del: depicts a loss/deletion of the first 52 amino acids i.e., Methionine (M) at position 1 through Alanine (A) at position 52; M233_T234insHYTH: depicts an insertion of 4 amino acids HYTH between position 233 and 234.

Resistome Other Than Colistin and Carbapenem Resistance of the Isolates

The 50 sequenced isolates harbored a plethora of resistant genes including β-lactamases (bla_{CTX-M}, bla_{SHV}, bla_{OXA-1}, bla_{TEM-1}, bla_{CMY}, bla_{DHA}), aminoglycosides [*aac(3)-IIa*, *aac(6′)-Ib3, aac(3)-VIa, aph(3′)-Ia*, *aph(3″)-Ib*, *aph(6)-Ic, aph(6)-Id*, *armA*, *aadA1, aadA2b, aadA5, aac(6′)-Ib-cr*], fluoroquinolones [*qepA1, qepA4, oqxAB, qnrS1, qnrS13, qnrB4, qnrB17, qnrB19, qnrB34*, *aac(6′)-Ib-cr*], sulfonamides (*sul1*, *sul2, sul3*), trimethoprim (*dfrA1, dfrA10, dfrA12, dfrA14, dfrA15, dfrA17 dfrB4*), phenicols (*catA2, catB3, cmlA1, floR*), tetracyclines [*tet(A), tet(B), tet(D), tet(J), tet(M)*] as well as mutations in *acrR, gyrA*, and *parC* for fluoroquinolones and *ramR* for tetracyclines particularly tigecycline (**Tables 1**, **2**).

Genes bla_{TEM} (15/30) and bla_{CTX-M} (14/30) were found to be the predominant beta-lactamases among the *E. coli* isolates (**Table 1**) while bla_{SHV} was frequently observed among the *Klebsiella* isolates (15/20) (**Table 2**). Some isolates (19/50) harbored more than one *bla* in different combinations. The single *Citrobacter* isolate in the study harbored the only AmpC type *bla* detected i.e., CMY-98 genotype (**Table 1**). Fluoroquinolone resistance was mostly mediated by plasmid mediated quinolone resistance *qnr* (16/29) and chromosomal mutations (S83L, D87N) of the *gyrA* (15/29) in the *E. coli.* Amongst the *Klebsiella* isolates, quinolone resistance was mostly due to a mutation of the *acrR* gene (18/20). Seven isolates harbored the *aac(6)-Ib-cr* gene which confers resistance to both aminoglycosides and fluoroquinolones.

Gene *tet(A)* (20/29) was the most common tetracycline resistance gene carried by the *E. coli* isolates while *tet*(A) (5/20) and *tet(D)* (n = 5/20) were the most common among the *Klebsiella* species. The *tet(M)* commonly restricted to Gram-positive bacteria was detected in one *E. coli* and a *K. pneumoniae* isolated from a cloacal swab from poultry. Additionally, one of the *Klebsiella* isolates (L18) harbored the *ramR* mutation (A19V) responsible for tigecycline resistance.

Genetic Diversity

Multilocus sequence and phylogroup typing showed that the isolates were polyclonal and genetically diverse (**Supplementary Tables 2**, **3**). The *E. coli* isolates clustered within six of the eight known phylogroups: A, B1, B2, C, D, E with phylogroup B1 been the predominantly occurring (12/29) based on the Clermont typing profile. MLST assigned the isolates into 23 sequence types (STs) (and one1 unknown ST) with four of the STs occurring more than once: ST191 (n = 3), ST224 (n = 2), ST410 (n = 3), and ST2485 (n = 3) (**Supplementary Table 2**). cgMLST assigned them into 24 different cgSTs due to genetic differences between the two ST224 strains which were identified as cgST72904 (isolate L17) and cgST38401 (isolate L22) (**Supplementary Table 3**).

In silico serotyping using the WGS data assigned the isolates into 23 serotypes, O128ab/ac:H20 (n = 2). Although the "O"-group antigen of eight isolates were unknown, the isolates belonged to the H9 (n = 3), H21 (n = 1), H23 (n = 1), H30 (n = 1), H37 (n = 1), and H45 (n = 1).

Similarly, the *Klebsiella* isolates were distributed into 14 different multilocus sequence types (STs) with three STs occurring more than once: ST45 (n = 2), ST307 (n = 3), and ST340 (n = 3). We were however unable to infer the ST for one of the *K. quasipneumoniae* isolate (**Supplementary Table 3**). *In silico* serotyping of the *Klebsiell*a isolates assigned them into 15 different serotypes with the most commonly occurring serotype being KL102:O2v2 (n = 4).

Plasmidome and Virolome

Thirty different plasmid replicon types were detected via analyses of the WGS data using PlasmidFinder (**Supplementary Tables 2**, **3**). The majority of the isolates harbored at least two plasmid replicon types with the most abundant being the IncFIB (AP001918) in *E. coli* (n = 17) and IncFIB(K) in *Klebsiella* (n = 16). Other dominant replicon types were IncFII and IncFIA in *E. coli* (n = 9 each), IncFII(K) and IncR in *Klebsiella* (n = 11 each). No plasmids were detected in three *E. coli* and two *Klebsiella* isolates.

Although, the *mcr*-1 was located in a unique contig that had the same sequence as the IncX4 plasmid backbone, no other resistance genes were found on the IncX4 plasmid in this study. Other resistance genes harbored on plasmids include *sul2* and *aph(3″)-Ib* on *IncQ1*, *blaTEM-1B* on *IncX1*, *blaTEM-1C* on *IncFII(K).*

A total of 42 different virulence genes were detected across the 50 isolates (**Supplementary Tables 2**, **3**). Carriage of extra-intestinal pathogenic *E. coli* (ExPEC) virulence associated genes (VAGs): *cva, cvi*, *hylF*, *iroN*, *iss*, *iutA*, *ompT*, *sitA*, *traT* was widespread among the *E. coli* isolates (**Supplementary Table 2**). Pathotype specific VAGs including *astA* (EAEC), *afaD, hlyA, ihA, fyuA* (UPEC), *vat* (APEC), and *papC* (APEC/UPEC) were also detected (**Supplementary Table 2**).

Conjugation Assay

The conjugation assay confirmed transfer of the colistin resistant determinant from two isolate (H2 and L40) to the recipient *E. coli* J53 with conjugation frequencies of 3.4×10^{-1} cfu/recipient cfu and 4.6×10^{-1} cfu/recipient cfu, respectively. None of the isolates transferred the carbapenem resistant determinant. The colistin MIC for the two transconjugants was 4 μg/mL compared with 0.5 μg/mL for the *E. coli* J53. Furthermore, the transconjugants expressed two resistant phenotypes (L40: AMC-AMX-CIP-CEF-DOX-GEN; H2: AMC-AMX-CEF-GEN).

DISCUSSION

The last two decades have witnessed a significant rise in infections caused by multidrug resistant *Enterobacteriaceae* and resulted in an increase in the use of carbapenems and colistin as last resort drugs (Peyclit et al., 2019). Concurrent resistance to last resort drugs represents a serious health concern globally (Chaudhary, 2016; Serwecinska, 2020) and our study has provided evidence of high levels of concurrent resistance to colistin and carbapenem in *Enterobacteriaceae* in Nigeria with a prevalence of 9.1%. This is of major concern as Nigeria is a low-income country with minimal

antimicrobial surveillance. This also indicates the emergence and establishment of potentially pandrug resistant strains, and creates limitations in the ability to control common infections.

While transferable mobile genetic elements such as *mcr*- and carbapenemase genes have generated much interest and are the target of most studies, our results show that concurrent carbapenem–colistin resistance in the *E. coli* and *K. pneumoniae* isolates is also linked to previously reported and novel mechanisms. These mechanisms include chromosomal mutations/disruptions affecting regulatory and non-regulatory genes controlling efflux-reflux pumps and membrane permeability. Such mutations/disruptions may play a greater role in resistance to carbapenem and colistin resistance than previously suspected.

Surprisingly, we found no evidence for carbapenemase producing genes in both our phenotypic and genomic investigations (WGS). This is in contrast to some studies on resistance in the region that reported carbapenemase genes such as bla_{NDM} and $bla_{OXA-181}$ are widespread (Jesumirhewe et al., 2017; Olalekan et al., 2020; Olowo-Okere et al., 2020; Shettima et al., 2020).

Consistent with previous reports on their role in colistin/carbapenem resistance, we detected the plasmid mediated colistin resistance genes; *mcr*-1, *mcr*-5, and *mcr*-8, and alterations/mutations in the *mgrB, ccrB,* that mediates colistin resistance as well as alterations/mutations in the *OmpC, OmpK36, OmpK37* which mediates carbapenem resistance (Olaitan et al., 2014; Poirel et al., 2015; Cheng et al., 2016). We also identified multiple potential novel mutations/alteration associated with colistin resistance: *arnT* (W100M, T157A, G164S, A278T, L485F), *etpA* (V336M, T411H), *pmrB* (G164S, R256G), *ccrB* (M1_L56del, M1_KA52del, M1_A78del, M1_M59del) and carbapenem resistance: *ompC* (M1_V24del, K173T, N228_T229insGSYTSNGV, A231_Y232insNGYGER, A230_A231insYYISNGVAR), ompK37 (K27Q, D28Q, G29V, N30G, K31S, D33T, M1_Y25del, M233_T234insHYTH). Colistin resistance is mediated by LPS modification, which is encoded by the *pmrHFIJKLM* operon and the *pmrC* locus, and regulated by PhoPQ and PmrAB. This modification decreases the negative charge of the outer membrane, reducing its interaction with colistin (Trimble et al., 2016). Alterations in the genes of the two component regulatory systems (2CRS) particularly *mgrB, phoP/phoQ, pmrA, pmrB, pmrC*, and *crrABC* mediate colistin resistance (Wright et al., 2015; Cheng et al., 2016). Genetic alteration in the *mgrB* gene, which was common among the isolates, results in a disruption of the negative feedback loop of the PhoP/PhoQ, overexpression of the PhoP-regulated genes leading to the up-regulation of the *pmrHFIJKLM* operon and an abnormally high levels of lipid A modification, and ultimately a low susceptibility to colistin. Similarly, *crrB* mutations consistent with those detected in this study were shown to mediate reduced colistin susceptibility (Cheng et al., 2016) via induction of CrrC expression, which induces an elevated expression of the *pmrHFIJKLM* operon and *pmrC* via the PmrAB two-component system, loss of regulation of the *crrAB* gene that encodes a glycosyltransferase-like protein, which in turn leads to modification of lipid A and increased autophosphorylation of *ccrB* which leads to colistin resistance (Wright et al., 2015; Cheng et al., 2016; Aghapour et al., 2019). Porins are outer membrane proteins associated with the modulation of cellular permeability and antibiotic resistance. OmpF and *OmpA* in *E. coli* and OmpK35/36/37 in *Klebsiella* play a major role in antibiotic transport into the bacteria cell. Alterations in the gene encoding these proteins have been reported to infer with protein configuration and thus entry of antibiotics, particularly carbapenems (Fernández and Hancock, 2012). We hypothesized that the observed disruptions in these gene as observed among our isolates and identified as deleterious by PROVEAN may be responsible for the high carbapenem MIC and resistance in this study.

While the significance of these mutations and their impact on the MIC and resistance to carbapenem and colistin will require expression level analyses including transcriptomics and complementation assays, we hypothesize that the observed concurrent carbapenem–colistin resistance resulted from a combination of the chromosomal mutations/alterations in the *mgrB* (M1V), *ompC* (M1_V24del), *ompK37* (I70M, I128M) and the regulatory efflux pump genes (*marR* (M1V), and *ramR* (M1V). Other studies have reported that mutations in the efflux system, particularly the global regulator *marR* and *ramR* as found in our study, β-lactamase production, and porin deficiency could play major roles in carbapenem resistance (Girlich et al., 2009; Findlay et al., 2012; Shin et al., 2012; Adler et al., 2013; Tsai et al., 2013; Chetri et al., 2019, 2020). An important finding in this study that we also wish to highlight, is the high probability for an isolate that is colistin resistant to also be carbapenem resistant, and vice versa.

Reports emanating particularly from Asia demonstrate a significant reduction in the rate of colistin resistant isolates due to the effect of the ban on colistin use in the wake of the detection of the plasmid mediated *mcr-1* gene (Walsh and Wu, 2016; EMA/AMEG, 2021; Usui et al., 2021; Wang Y. et al., 2020). Similarly, countries such as the United States, Canada, and United Kingdom which have never approved colistin usage in animal production have continually reported lower rates of colistin resistant strains (EMA/AMEG, 2021). However, this is not the situation in Nigeria and other African countries where there is a rise in the numbers of colistin resistant strains, as demonstrated in this study. No such ban or regulation on colistin use exist in Nigeria where its use is currently widespread and is a common active ingredient in most antibiotic combinations/preparation used in livestock production for the purpose of prophylaxis and therapy. Although carbapenem is rarely used in food animals in Nigeria, the inappropriate use of colistin has been shown to provide selective pressure for the emergence of colistin and multidrug (including carbapenem) resistant strains (Napier et al., 2013). We, therefore, hypothesize that the high rates of co-resistance observed in our study may be connected to the widespread use of colistin, particularly in livestock production in Nigeria.

Majority of the isolates expressed multidrug resistance profiles, including high resistance to amoxicillin-clavulanic acid (98%), fluoroquinolones (78%), and gentamicin (48%) which are widely used in the treatment of infections in Nigerian hospitals.

The high resistance rates we recorded are in accordance with data in other reports on human and animal isolates from Nigeria and Sub-Saharan Africa (Ojo et al., 2016; Chah et al., 2018; Aworh et al., 2019; Olalekan et al., 2020; Shettima et al., 2020). Most of our isolates also carried the $bla_{CTX-M-15}$ which is consistent with the increasing reports of this genotype from animal and human sources in Nigeria (Chah et al., 2018; Okpara et al., 2018; Olowo-Okere et al., 2020). There is thus growing evidence that this genotype is expanding rapidly and might become the dominant mechanism mediating resistance to the β-lactams.

The significant clonal diversity i.e., polyclonality observed amongst our isolates is consistent with previous reports, particularly those of carbapenem and colistin resistant isolates in Nigeria where the population structure is diverse (Ngbede et al., 2020; Olalekan et al., 2020). Some of the animal and human isolates in our study had similar STs with previously reported "high-risk" clones including *K. pneumoniae* ST11, ST17, ST45, ST340 (human isolates) and *E. coli* ST58, ST744, ST410 (animal isolates). These high risk STs are known for their global dissemination, ease of transmission between different hosts, ability to cause disease and acquire genetic determinants such as virulence factors, epidemic plasmids and antibiotic resistance that provide them with a competitive advantage over other bacterial clones (Lee et al., 2016; Roer et al., 2018; Feng et al., 2019; Nadimpalli et al., 2019; Patiño-Navarrete et al., 2020). The *E. coli* ST58, ST744, ST410 virulence profile categorized them to the UPEC, EAEC and DAEC pathotypes. Similarly, the ST11 and ST340 detected in our human isolates are closely related to ST258, all belonging to clonal complex CC258 which has been associated with outbreaks, pandemics and mass dissemination of KPC *K. pneumoniae* (Netikul and Kiratisin, 2015; Sui et al., 2018; Cienfuegos-Gallet et al., 2019; Fu et al., 2019; Zhao et al., 2019).

CONCLUSION

In this study, we report evidence for the occurrence of multidrug resistant *Enterobacteriaceae* with concurrent carbapenem-colistin resistance in 9.1% of the isolates. The genetic mechanism underlying this concurrent resistance phenotype was majorly novel and previously known chromosomal alterations (deletion, insertions, and substitutions). The plasmid-mediated colistin resistance gene *mcr-* in combination with these chromosomal alterations accounted for colistin resistance in few of the isolates. Some of the *E. coli* and *Klebsiella* isolates expressing concurrent carbapenem-colistin resistance in this study belonged to the internationally recognized "high-risk" clones. The combination of diverse drug resistance genes and sequence types highlight the considerable genome plasticity and polyclonality that characterize the population structure of both clinical and non-clinical colistin and CRE in Nigeria. The polyclonality might create considerable problems during outbreak tracing and source attribution.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research and Ethics Committee of the facilities (FMH/FMC/MED.108/VOL.I/X and BSUTH/MKD/HREC/2013B/2018/0027). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

EN and CW: conceptualization and funding acquisition. EN, FA, AP, and CW: methodology. EN, AMA, SD, AAA, CA, PA, LM, NM, and MA: sampling/investigation. EN, FA, AP, AK, YY, and PK: data curation. EN, MA, and CW: supervision. EN and FA: writing—original draft preparation. EN, YY, PK, OL, PB, and CW writing—review and editing. All authors read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Identification of the Plasmid-Mediated Colistin Resistance Gene *mcr*-1 in *Escherichia coli* Isolates From Migratory Birds in Guangdong, China

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We determined the prevalence and transmission characteristics of *mcr-1*-positive *Escherichia coli* (MCRPEC) isolates from migratory birds *Anser indicus* in Guangdong, China. We identified 22 MCRPEC from 303 *A. indicus* fecal samples (7.3%) in Guangzhou, Zhaoqing, and Futian. The *mcr-1* gene coexisted with 24 other types of antibiotic resistance genes (ARG), and 11 ARGs were highly prevalent at levels >50%. The MCRPEC displayed a diversity of sequence types (ST), and 19 distinct STs were identified with ST10, ST1146, and ST1147 as the most prevalent. In addition, these MCRPEC from birds were closely related phylogenetically to those from other sources in China. Whole-genome sequencing analysis demonstrated that *mcr*-1 was located on IncX4 ($n = 9$, 40.9%), IncI2 ($n = 5$, 22.7%) and IncP ($n = 1$, 4.5%) plasmids and the latter shared an identical plasmid backbone with other sources. These results highlight the significance of migratory birds in the transmission of antibiotic resistance and provide powerful evidence that migratory birds are potential transmitters of antibiotic resistance.

Keywords: migratory birds, *mcr-1*, *Escherichia coli*, transmission, antibiotic resistance

INTRODUCTION

Colistin is a polymyxin antibiotic that has been used in veterinary clinics for many years. It is now applied as a last-resort drug for treatment of carbapenem-resistant Enterobacterial infections (Mendes et al., 2018). The emergence of the plasmid-mediated mobile colistin resistance gene *mcr-1* in China and its rapid global spread now poses a serious threat to public health (Liu et al., 2016; Wang et al., 2018). Significantly, these *mcr-1* plasmids often harbor other antibiotic resistance genes (ARG) that encode carbapenemase and extended-spectrum β-lactamase (ESBL) resistance (Haenni et al., 2016; Yao et al., 2016). Therefore, the global distribution and spread of *mcr-1* gene is concerning.

The World Health Organization (WHO) has developed a global strategy to address antimicrobial resistance based on the "One Health" approach that is based on the close links between human, animal, and environmental health (Dafale et al., 2020). Wildlife can act as ARG reservoirs and transmission vectors between microorganisms and the environment (Allen et al., 2010; Arnold et al., 2016). Therefore, a contaminated environment also plays a key role in the spread of antibiotic resistance since this is where bacteria from different sources are able to exchange ARGs (Aeksiri et al., 2019). In particular, birds play a large part in ARG dissemination due to their environmental exposure through ingested food or polluted water (Jarma et al., 2021). This is especially true for migratory birds that can act as ARG reservoirs and spread antibiotic resistance through migration (Vergara et al., 2017; Elsohaby et al., 2021). For instance, the *mcr*-1 gene has been isolated from European herring gulls (Ruzauskas and Vaskeviciute, 2016), from Spanish and Portuguese gulls (Ahlstrom et al., 2021) as well as in Thailand and China in migratory birds (Aeksiri et al., 2019; Lake, 2020).

Since colistin is considered the last-resort antibiotic used to treat multidrug-resistant bacteria (Li et al., 2020), the emergence of the *mcr-1* gene in migratory birds is especially worrisome. Monitoring the level of antibiotic resistance carried in migratory birds is a necessary step to prevent ARG spread. This study investigated the prevalence and genomic structures of *mcr-1* producing *Escherichia coli* (MCRPEC) isolated from migratory birds *Anser indicus* in Guangdong, China.

MATERIALS AND METHODS

Ethics Statement

The Institutional Review Board of South China Agricultural University (SCAU-IRB) approved the Samples and bacteria protocols. All *A. indicus* feces were sampled under authorization from Animal Research Committees of South China Agricultural University (SCAU-IACUC).

Sampling Information

A total of 303 feces samples from *A. indicus* were collected in 2017 in Guangzhou, Zhaoqing, and Futian in Guangdong province. Briefly, all samples were placed to 1.0 ml of LB Broth and incubated for 16–18 h at 37°C followed by inoculation on MacConkey agar plates containing 2.0 mg/L colistin that were incubated for 16 h. Two or three different forms red colonies were selected for identification using the MALDI-TOF 80 MS Axima system (Shimadzu-Biotech Corp., Kyoto, Japan) and 16S rRNA sequencing. The colistin-resistant isolates were screened for the presence of the *mcr*-1 gene by PCR as previously described (Yang et al., 2019a).

Antimicrobial Susceptibility Testing

The antimicrobial sensitivity of 22 MCRPE for 15 antibiotics was examined by measuring the minimal inhibitory concentration (MIC) using the agar dilution method and interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI M100-S28). Susceptibility to colistin was performed by broth micro-dilution as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 92 Version 6.0; EUCAST, 2016). Quality control of the procedure was conducted by using the susceptible *E. coli* ATCC 25922.

Whole-Genome Sequencing and Bioinformatics Analysis

Total genomic DNA was extracted from 22 MCRPEC isolates using a Genomic DNA Purification Kit (Tiangen, Beijing, China) as per the manufacturer's instructions. WGS was performed with the Illumina Hiseq 2500 System (Novogene Guangzhou, China) using the paired-end 2×150-bp sequencing protocol. Raw sequence reads were trimmed using Trim Galore, and the genomes were *de novo*-assembled into contigs using SPAdes (3.9.0) predefined kmers set. All genome assemblies of 22 *E. coli* isolates were deposited in GenBank and are registered with BioProject number PRJNA748548.

Bioinformatics Analysis

The CGE platform[1] was used for analyses of multilocus sequence typing (MLST-2.0), acquired resistance genes (ResFinder 4.1, all antibiotic resistance databases were selected with a cut-off value of 95% identity and 80% minimum coverage) and plasmid incompatibility groups (PlasmidFinder-2.1 version, using Enterobacteriaceae database with parameters of minimum 95% identity and 85% query coverage). Sequence comparisons between *mcr*-1 carrying plasmids were performed using Easyfig and Brig (Alikhan et al., 2011). Phylogenetic trees for MCRPEC isolates in this study were constructed using homologous strains from the NCBI database using CSI Phylogeny (v1.4), and *E. coli* (U4) was used as the reference genome (Kaas et al., 2014). The corresponding characteristics of each isolate visualized using FigTree v1.4.4 and the online tool iTOL.[2] Single polynucleotide pairs (SNP) analysis was visualized as a heatmap of the SNP count matrix using BacWGSTdb 2.0 (Feng et al., 2021). The sequences of *mcr*-1 plasmids were constructed by *de novo* assembly using Genious (10.0.7). Circular comparisons among *mcr*-1-related IncX4 and IncI2 plasmids from this study and NCBI database were performed using BLAST Ring Image Generator (BRIG v0.9555).

RESULTS

Bacterial Isolation and Antibiotic Susceptibility Testing

We recovered 22 (7.3%) MCRPE from 303 *A. indicus* fecal samples in Guangdong Province, China, that included 10% from Futian (12/120), 5.7% from Zhaoqing (2/35) and 5.4% from

[1]http://www.genomicepidemiology.org/
[2]https://itol.embl.de/

Guangzhou (8/148). The MIC values for colistin for these isolates were all ≥4 μg/ml. These isolates also possessed high levels of resistance to other common antibiotics including sulfamethoxazole/trimethoprim (95.4%), fosfomycin (81.8%), florfenicol (81.8%), tetracycline (77.2%), ciprofloxacin (34.1%), and olaquindox (34.1%). Smaller numbers of isolates displayed resistance to nalidixic acid (27.3%), cefotaxime (22.7%), streptomycin (18.1%), cefoxitin (13.6%), gentamicin (4.5%), and amikacin (4.5%). Notably, all strains remained sensitive to meropenem and tigecycline (**Table 1**; **Supplementary Figure S2**).

Whole-Genome Sequencing Analysis

We sequenced 22 of MCRPE isolates using the Illumina HiSeq platform, and the average genome size was 4.9 Mbp. Interestingly, we identified 19 distinct STs that included ST10 (2/22), ST1146 (2/22), and ST1147 (2/22) and the remaining were single STs that included ST198, ST2351, ST4014, ST58, ST5878, ST155, ST746, ST6725, ST101, ST2280, ST46, ST5229, ST9401, ST162, and ST1258. The strain FT109-1 was a member of a previously unreported new ST (**Figure 1**).

We reconstructed a phylogenetic tree using the 22 MCRPE isolates along with 10 MCRPE isolates from the NCBI database. All isolates were classified into four clades lineage except FT109-1 (see above). The major lineage IV included 10 (31%) isolates, and ST10 members accounted for 40% of this lineage. In addition, we also found close phylogenetic relationships between our 22 MCRPE from *A. indicus* and other sources in China that included humans, pigs, chickens, and environmental samples.

TABLE 1 | Bacterial information and antimicrobial resistance profiles.

Isolate	Plasmid type	Resistance phenotype
FT104-1	IncI2	CS-FOS-OLA-FFC-TET-S/T
FT105-1	IncI2	CS-FOS-OLA-GEN-AMK-FOX-FFC-TET-S/T
FT109-1	IncP	CS-FOS-STR-OLA-NAL-CIP-CTX-FFC-TET-S/T
FT11	Untypable	CS-FOS-OLA-CIP-FFC-TET-S/T
FT130-1	IncX4	CS-FOS-FFC-TET-S/T
FT130-2	Untypable	CS-STR-OLA-CIP-FFC-TET-S/T
FT13-1	IncX4	CS-FOS-STR-OLA-CIP-FFC-TET-S/T
FT18-1	IncX4	CS-FOS-OLA-CIP-FFC-S/T
FT30-1	IncX4	CS-FOS-FFC-TET
FT7-2	IncX4	CS-FOS-OLA-CIP-FFC-S/T
FT95-1	Untypable	CS-NAL-CIP-CTX-FFC-TET-S/T
FT99	IncI2	CS-FOS-OLA-CIP-FFC-TET-S/T
U23	IncI2	CS-FOS-TET-S/T
U34-1	IncX4	CS-FOS-STR-S/T
U39-1	IncX4	CS-FOS-NAL-CIP-CTX-FFC-TET-S/T
U4	IncI2	CS-FOS-TET-S/T
U40	Untypable	CS-STR-BAL-CIP-CTX-FOX-FFC-TET-S/T
U48-1	IncX4	CS-FOS-FFC-TET-S/T
U48-2	Untypable	CS-FOS-OLA-FFC-TET-S/T
U7	Untypable	CS-S/T
ZQ28	IncX4	CS-FOS-OLA-NAL-CTX-FFC-TET-S/T
ZQS2-1	Untypable	CS-FOS-FOX-FFC-TET-S/T

CS, colistin; FOS, Fosfomycin; STR, streptomycin; OLA, olaquindox; NAL, nalidixic acid; GEN, gentamicin; AMK, amikacin; FOX, cefoxitin; CTX, cefotaxime; FFC, florfenicol; CIP, ciprofloxacin; TET, tetracycline; S/T, trimethoprim/sulfamethoxazole.

To further assess the relationships between these isolates, we performed a SNP analysis that indicated most of our MCRPE isolates had significant SNP differences. Notably, in two cases, there were two isolates possessing a collection of different genomic characteristics that were recovered from the same sample (FT130 and U48; **Table 1**). For instance, both ST10 (U48-1) and ST9401 (U48-2) were recovered from sample 18FS1 and shared 42,580 SNPs (**Supplementary Figure S1**).

Additionally, our MCRPE group represented 24 ARG types that conferred resistance to 14 classes of antibiotics including colistin, fosfomycin, streptomycin, nalidixic acid, ciprofloxacin, florfenicol, cefotaxime, cefoxitin, tetracyclines, gentamicin, amikacin, olaquindox, and sulfamethoxazole/trimethoprim. Among these, 11 ARGs were highly prevalent with detection rates >50% and included *aad*, *aph*, *cmlA*, *dfrA*, *floR*, *mdf*, *mcr-1*, *qnrs*, *oqx*, *sul*, and *tet* (**Table 1; Figure 1**).

mcr-1-Associated Plasmid Types

Whole-genome sequencing analysis indicated that 12 different Inc types were present on plasmids in the 22 MCRPEC isolates (**Figure 1**). In 15/22 isolates, we could identify the replicon sequence type of *mcr*-1-harboring plasmids and included IncX4 ($n = 9$, 40.9%), IncI2 ($n = 5$, 22.7%) and IncP ($n = 1$, 4.5%). Alignment of *mcr*-1-carrying IncX4 and IncI2 plasmids demonstrated that all *mcr*-1-carrying plasmids shared identical plasmid backbones. More importantly, the backbones of these mcr-1-linked plasmids from *A. indicus* were highly similar to those recovered from other sources including diverse animals, humans, and poultry. Plasmids used for comparison include the following: human plasmids: pMCR_WCHEC1604 (KY829117), pHNSHP45 (KP347127.1), pGZ49260 (MG210937.1) and animals: pNFGDF93 (MF978388.1), pLWY24J (MN689940.1), pNFGDF49 (MF978387.1), and pHNSHP49 (MF774188.1). Interestingly, the insertion element *ISApl1* was not present in our *mcr-1*-associated IncI2 plasmids and *IS26* was ether partially or totally absent from the mcr-1-carrying IncX4 plasmids (**Figure 2**).

Additionally, we also found the presence of broad-host-range IncP plasmids that acted as *mcr*-1 vectors in this study. The *mcr*-1-carrying IncP plasmids shared conserved backbones with the plasmids pMCR_1622 and pZR12 taken from *E.coli* human and pig isolates, respectively (**Figure 3**). *Mcr-1-PAP2* and *ISApl1-mcr-1-PAP2* were inserted within the same locus in pMCR_1622 and pZR12 and suggested that this locus is a hot spot for the insertion of *ISApl1* (see above).

DISCUSSION

The plasmid-mediated colistin resistance gene *mcr-1* was originally identified in *E. coli* and has since been found in many countries on all continents (Liu et al., 2016; Matamoros et al., 2017). The speed and extent of the spread of the *mcr-1* gene are worrying especially since migratory birds carry foreign ARGs and act as a major source of bacterial antibiotic resistance in local environments (Lake, 2020). Long-distance migratory birds are considered key agents in the spread of antibiotic

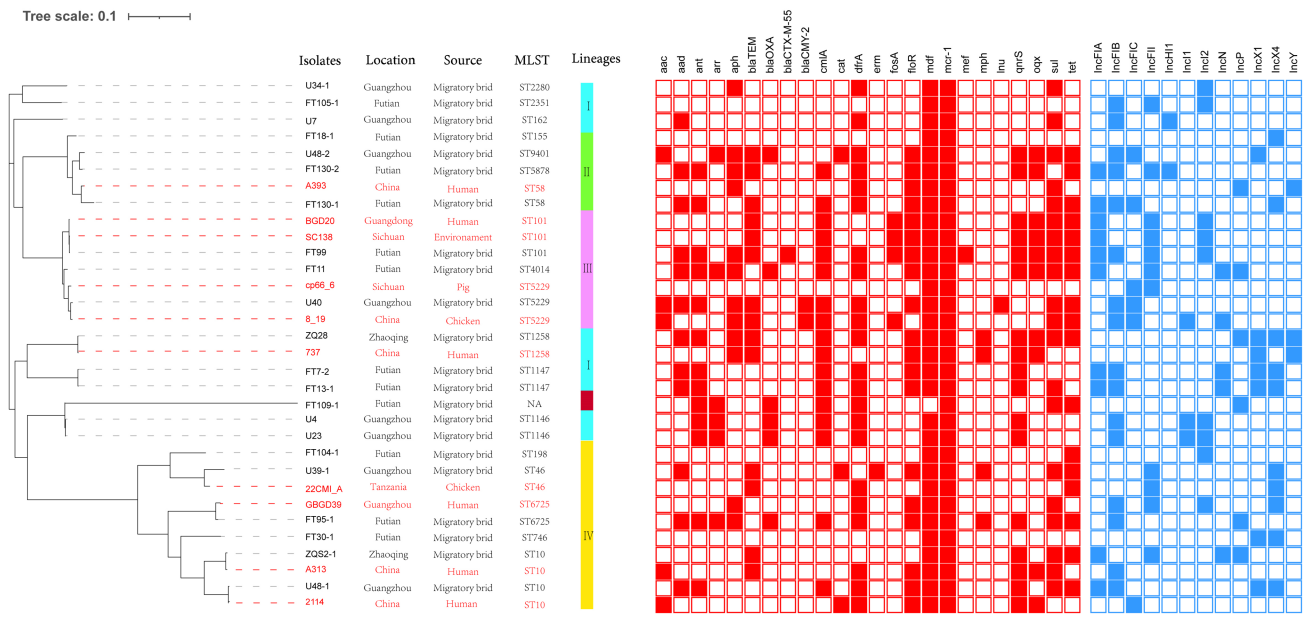

FIGURE 1 | Analysis of MCRPE isolates used in this study. **Left Panel:** Isolates from migratory birds in Guangdong, China (black font) and from the NCBI database (red font). **Right Panel:** Genetic relationships among 32 *mcr-1* positive *Escherichia coli* (MCRPEC) isolates using a maximum likelihood tree. Red-filled squares indicate possession of the indicated antibiotic resistance genes (ARG) and blue-filled squares indicated plasmid Inc type.

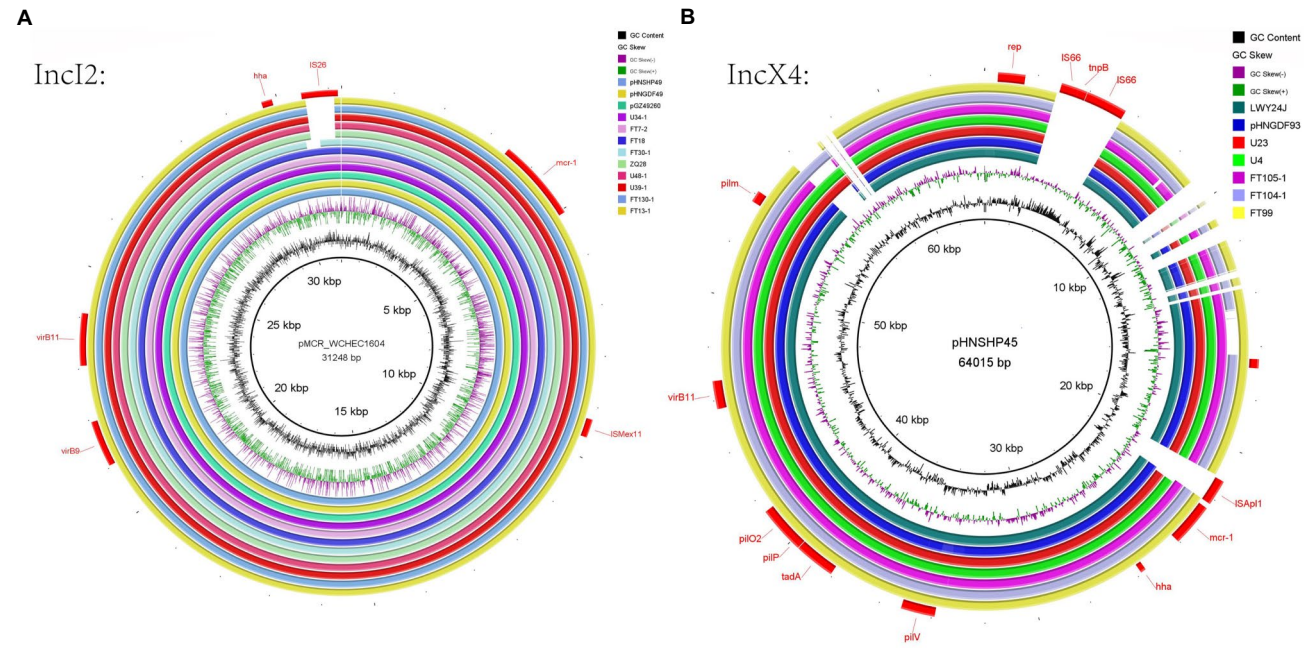

FIGURE 2 | IncI2 plasmid analysis for 21 MCRPEC isolates from migratory birds **(A)**. Alignments of IncI2 plasmids from this study with reference plasmid pHNSHP45. **(B)** Alignment of IncX4 plasmids with reference plasmid pMCR_WCHEC1604. The sources of these plasmids were migratory birds (FT7-2, FT18, FT13-1, FT30-1, FT99, FT104-1, FT105-1, FT130-1, U4, U23, U34-1, U39-1, U48-1, and ZQ28), human plasmids (pMCR_WCHEC1604, pHNSHP45, and pGZ49260) and animals (pNFGDF93, pLWY24J, pNFGDF49, and pHNSHP49).

resistance (Franklin et al., 2020). For antibiotic-resistant bacteria, aquatic systems are major traffic routes between wildlife and humans (Taylor et al., 2011). The resistance genes acquired by birds can be re-introduced in the environment, possibly into different water systems (e.g., by migrating ducks) and might re-infect humans directly *via* contact with contaminated

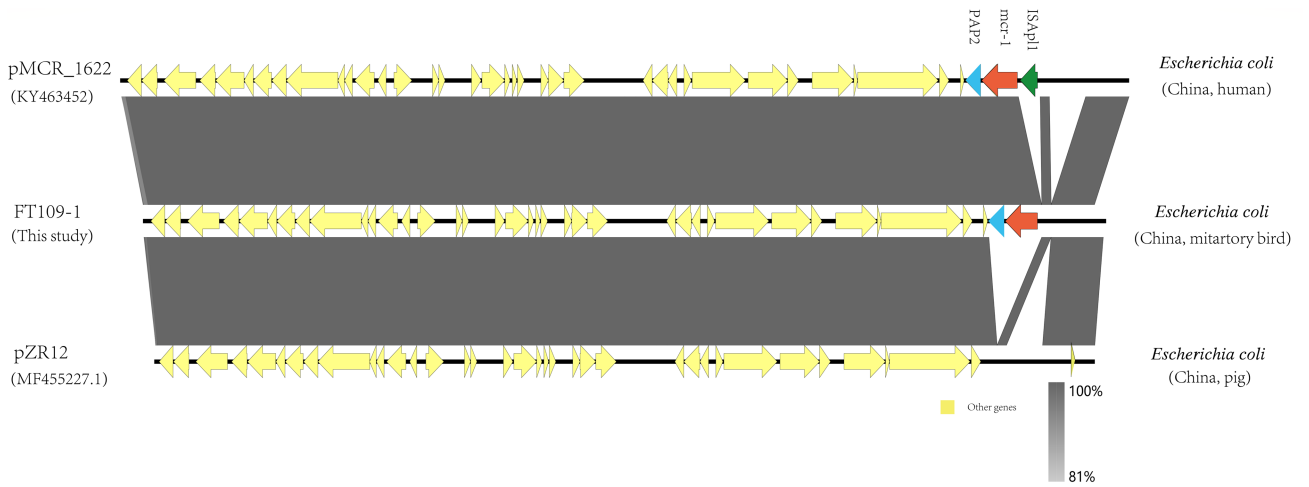

FIGURE 3 | Genomic organization between pFT109-1 and the indicated plasmids obtained from GenBank.

water or indirectly by the introduction of these genes into the food chain (Marcelino et al., 2019), and long-distance migration of migratory birds may accelerate this spread, which poses a great threat to human safety (Chen et al., 2017; Hadjadj et al., 2017; Monte et al., 2017; Mohsin et al., 2019). Therefore, it is very necessary to monitor the flora of migratory birds. In our study, we isolated 22 MCRPEC isolates in fecal samples from *A. indicus* in Guangdong, China. This is the first report of the presence of MCRPEC from migratory birds in Guangdong, and our study provides strong evidence that migratory birds are potential transmitters of the colistin resistance gene *mcr-1*.

Whole-genome sequencing analysis of these *mcr-1* positive *E. coli* strains identified 19 different STs and was consistent with previous reports documenting the ST diversity of MCRPEC (Wang et al., 2018). However, ST10, ST1146, and ST1147 were more prevalent in our isolates and ST10 is frequently found in clinical (Lin et al., 2020), animal (García-Meniño et al., 2018), and environmental samples (Flament-Simon et al., 2020). In addition, we found that the genomes of two MCRPE isolates from the same sample differed by the phylogenetic analysis. This scenario is worrying and indicated that a significant diversity was present in our population of MCRPE isolates from migratory birds. More notably, the emergence and spread of *mcr-1* gene in humans, animals, and the environment have been reported globally. In our study, the close phylogenetic relationships for 22 of the MCRPEC isolates from *A. indicus* and other sources in China indicated the existence of clonal transmission of *mcr-1* in migratory bird populations, and the latter are likely responsible for the widespread occurrence of *mcr-1*. This indicated that migratory birds played an important role in the transmission of the *mcr*-1 gene.

In addition to *mcr-1*, we also identified numerous ARGs in MCRPEC isolates such as the plasmid-mediated quinolone resistance gene *qnrS1*, the tetracycline resistance gene *tet,* and ESBL-dependent $bla_{\text{CTX-M-55}}$ genes. Many previous studies have reported high levels of ARGs in the intestinal flora of migratory birds (Jarma et al., 2021) and thereby indicated that antibiotic resistance can easily be disseminated *via* migration (Elsohaby et al., 2021). According to a survey, *mcr-1* resistance gene is widespread among different birds, accounting for 50% of the total samples (Cao et al., 2020). Notably, silent transmission of *mcr-1* gene was observed, and MCRPE may be difficult to detect whether the *mcr-1* gene is only tested for in colistin-resistant isolates (Moura et al., 2016). As the reservoir of resistance genes, the resistance level of the strains carried by migratory birds should continue to be concerned.

Whole-genome sequencing analysis of our isolates further revealed the presence of different *mcr-1* harboring plasmids, and the IncX4 and IncI2 types were the most prevalent. The IncX4 and IncI2 type plasmids have a high transferability, and this creates favorable conditions for horizontal transmission of *mcr-1* (Yang et al., 2019b). IncX4-type plasmids represent promiscuous plasmids contributing to the intercontinental spread of the *mcr-1* gene (Fernandes et al., 2016), which obtained from different bacterial species, belonging to different STs, isolated in different clinical contexts, including wildlife, and found on different continents are highly similar in the plasmid backbone sequences. The ubiquity and transferability of IncX4 plasmids carrying *mcr-1* sheds light on the role of this incompatibility group in the global spread of colistin resistance (Sellera et al., 2017). In our study, nine IncX4 plasmids and five IncI2 plasmids carrying *mcr-1* in our study shared the same plasmid skeletons as isolates acquired from humans, animals, and the environment as reported elsewhere (**Figure 3**). This indicated that these plasmid types have been diffusing between humans, animals, and the environment (Matamoros et al., 2017), IncX4 and IncI2 plasmids facilitated the transmission of the *mcr*-1 gene. The additional presence of an *mcr-1-pap2* cassette (Yang et al., 2017) can also function as an independent mobile genetic that could facilitate ARG acquisition and mobilization between bacteria by a mechanism known as "copy-paste and paste" (Partridge et al., 2018). For instance, one or two copies of *ISApl1* that flank the *mcr*-1-*pap*2 cassette can actively capture and mobilize *mcr*-1 genes.

Together our data provide evidence for the horizontal transfer of *mcr-1* due to migratory bird long-distance migrations. *ISApl1* is a common mobile genetic element found in *mcr*-1 plasmids

(Snesrud et al., 2016). Interestingly, IS*Apl1* and other mobile elements were not present in the *mcr-1*-related IncX4 and IncI2 plasmid backgrounds in our study isolates. The reason for this was most likely that IS*Apl1* was lost during subsequent recombination steps following the initial mobilization of *mcr*-1 (Snesrud et al., 2016). In addition, in this study, *mcr-1* was also carried by the broad-host-range IncP type plasmid and shared conserved backbones with *E. coli* plasmids pMCR_1622 and pZR12 from human and pig samples, respectively. Moreover, IncP plasmids have been reported to mediate the transmission of *mcr-1* across various hosts and has the potential to become a dominant *mcr*-1 carrier (Zhao et al., 2017; Fish, 2018). The emergence of a large group of host plasmids carrying *mcr-1* in long-haul migratory birds is of great concern for public health and further suggests that the drug-resistant flora in migratory birds should be monitored.

CONCLUSION

In conclusion, we identified 22 *mcr-1*-positive *E. coli* isolates from migratory birds *A. indicus* in Guangdong, China. Notably, this is the first study to report the development of diversity in the population of MCRPE isolates from migratory birds in Guangdong. Phylogenetic analysis proved that the development of diversity in the population of MCRPE isolates from migratory birds. WGS analysis further determined that *mcr-1* coexisted with other ARGs and also demonstrated diversity in the plasmid population, and the latter provides important epidemiological information for the global dissemination of the *mcr-1* gene. These plasmids can serve as vectors for rapid spread of colistin resistance among different hosts over long distances due to bird migration. The antibiotic-resistant flora in migratory birds should therefore be under constant surveillance.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA748548.

AUTHOR CONTRIBUTIONS

YZ and X-PLi: sampling. YZ and R-YS: data analysis. YZ: conceptualization, methodology, and writing-first draft. XK and JL: investigation and writing – review and editing. YY, JS, X-PLa, and Y-HL: conceptualization, writing – review and editing, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.755233/full#supplementary-material

Supplementary Figure S1 | Single polynucleotide pair (SNP)-heatmap analysis of MCRPE isolates used in this study using SNP count matrices.

Supplementary Figure S2 | The antimicrobial sensitivity of 22 MCRPE for 15 antibiotics, yellow indicates that the strain is not sensitive to the drug (CS, colistin; FOS, Fosfomycin; STR, streptomycin; OLA, olaquindox; NAL, nalidixic acid; GEN, gentamicin; AMK, amikacin; FOX, cefoxitin; CTX, cefotaxime; FFC, florfenicol; CIP, ciprofloxacin; TET, tetracycline; MEM, meropenemS/T; TIG, tigecycline; trimethoprim/sulfamethoxazole).

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Colistin Resistance in Monophasic Isolates of *Salmonella enterica* ST34 Collected From Meat-Derived Products in Spain, With or Without CMY-2 Co-production

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Colistin is a last-resort antibiotic in fighting severe infections caused by multidrug resistant Gram negative pathogens in hospitals. Zoonotic bacteria acquire colistin resistance in animal reservoirs and mediate its spread along the food chain. This is the case of non-typhoid serovars of *Salmonella enterica*. Colistin-resistant *S. enterica* in foods represents a threat to human health. Here, we assessed the prevalence of colistin-resistance in food-borne isolates of *S. enterica* (2014–2019; Asturias, Spain), and established the genetic basis and transferability of this resistance. Five out of 231 isolates tested (2.2%) were resistant to colistin. Four of them, belonging to the European monophasic ST34 clone of *S*. Typhimurium, were characterized in the present study. They were collected from pork or pork and beef meat-derived products, either in 2015 (three isolates) or 2019 (one isolate). Molecular typing with XbaI-PFGE and plasmid profiling revealed distinct patterns for each isolate, even though two of the 2015 isolates derived from the same sample. The MICs of colistin ranged from 8 to 16 mg/L. All isolates carried the *mcr-1.1* gene located on conjugative plasmids of the incompatibility groups IncX4 (2015 isolates) or IncHI2 (2019 isolate). Apart from colistin resistance, the four isolates carried chromosomal genes conferring resistance to ampicillin, streptomycin, sulfonamides and tetracycline [bla_{TEM-1}, *strA-strB*, *sul2*, and *tet*(B)] and heavy metals, including copper and silver (*silESRCFBAGP* and *pcoGE1ABCDRSE2*), arsenic (*arsRSD2A2BCA1D1*) ± mercury (*merEDACPTR*), which are characteristically associated with the European ST34 monophasic clone. The 2019 isolate was also resistant to other antibiotics, comprising third generation cephalosporins and cephamycins. The latter phenotype was conferred by the bla_{CMY-2} gene located on

an IncI1-I(α)-ST2 plasmid. Results in the present study identified meat-derived products as a reservoir of a highly successful clone harboring transferable plasmids which confer resistance to colistin and other clinically important antibiotics. An important reduction in the number of food-borne *S. enterica* detected during the period of the study, together with the low frequency of colistin resistance, underlines the success of One Health initiatives, such as those implemented at the UE, to control zoonotic bacteria along the food chain and to halt the spread of antimicrobial resistance.

Keywords: colistin resistance, *mcr-1*, *bla*$_{CMY-2}$, IncX4, IncH12, IncI2, food – borne pathogens, European ST34 monophasic clone

INTRODUCTION

Non-typhoidal serovars of *Salmonella enterica* subsp. *enterica* are one of the leading causes of bacterial food-borne infections in humans and animals worldwide (Majowicz et al., 2010). Up to date, more than 1,500 non-typhoidal serovars of this subsp. have been recognized (Grimont and Weill, 2007; Issenhuth-Jeanjean et al., 2014), with *S.* Typhimurium being one of the most frequently detected. Since around 2005, a monophasic variant of this serovar with the antigenic formula 4,[5],12:i:- and sequence type (ST) 34 has emerged in Europe, originally in pigs and later in other domestic animals (EFSA, 2010; Hauser et al., 2010; Hopkins et al., 2010; Sun et al., 2020). Subsequently, this "European monophasic clone" has experienced a global expansion, being also responsible for multiple outbreaks and sporadic cases of human salmonellosis in America, Asia, and Australia (Soyer et al., 2009; Mulvey et al., 2011, 2013; Arai et al., 2018; Elnekave et al., 2018; Sun et al., 2020).

The European clone harbors two chromosomal genetic elements, namely the RR region and SGI-4, both involved in resistance. Isolates of this clone are typically resistant to ampicillin, streptomycin, sulfonamides and tetracycline (tetra-resistance pattern ASSuT) with the responsible genes, *bla*$_{TEM-1}$, *strA-strB*, *sul2*, and *tet*(B), located on RR that also contains a mercury resistance locus. This region consists of one or two resistance modules flanked and interspersed with IS*26* elements (Lucarelli et al., 2012; Boland et al., 2015; García et al., 2016). In most cases, the tetra-R region is located upstream of *iroB*, replacing a number of chromosomal ORFs including the *fljAB* genes responsible for expression of the second flagellar phase. In addition to the tetra-R region, the European clone contains genomic island SGI-4, formerly known as SGI-3, an integrative conjugative element (ICE), which includes a copper homeostasis and silver resistance island (CHASRI), and an arsenic resistance locus (Petrovska et al., 2016; Arai et al., 2018; Branchu et al., 2019; Mourao et al., 2020). It has been proposed that the use of heavy metals as growth promoters in pork production, particularly after the ban of antibiotics with such an aim in European Union (EU), could have contributed to the epidemiological success of the clone (Mastrorilli et al., 2018; Clark et al., 2020).

Apart from chromosomal resistance to traditional antibiotics and heavy metals, the European monophasic clone is actively evolving mainly through acquisition of plasmids which can confer resistance to additional antimicrobial agents, including those categorized by the World Health Organization (WHO) as critically important with highest priority in human medicine (WHO, 2019). To this category belongs colistin (polymyxin E), a last-resort antibiotic used to treat life-threatening infections caused by multidrug-resistant, carbapenemase-producing Gram-negative bacteria in hospitals (Kaye et al., 2016). In consequence, the emergence and rapid spread of plasmid-borne colistin resistance, encoded by *mcr* genes, is a cause of serious concern in human medicine, and is actively monitored as part of programs implemented by both national and international organizations (Hugas and Beloeil, 2014). Zoonotic bacteria, like *S. enterica*, which have acquired plasmid-mediated colistin resistance, can be transmitted to humans across the food chain. Once in humans, apart from causing disease, they may act as a vehicle for the spread of colistin resistance to other *Enterobacterales*, including important nosocomial pathogens, such as *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter* spp., and to members of the indigenous microbiota.

In the present study, experimentally obtained information was combined with whole genome sequence analysis for in-depth characterization of four food-borne monophasic isolates of *S.* Typhimurium, selected on the basis of colistin resistance, and detected in the frame of the Spanish contribution to the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria (Commission implementing decision 2013/652/EU; European Commission, 2013). One of them was also resistant to many other antibiotics, comprising third generation cephalosporins and cephamycins, aggravating the challenge to food-safety and public health.

MATERIALS AND METHODS

Bacterial Isolates, Antimicrobial Susceptibility Testing and Screening of *mcr* and *bla*$_{CMY-2}$ Genes

All *S. enterica* isolates (n = 231) detected in retail food samples by the Laboratory of Public Health (LSP) of the Principality of Asturias, Spain, along the 2014 to 2019 period, were tested for colistin susceptibility. MICs with Sensititre were determined for isolates recovered during 2014, 2015, 2016, and 2019, using the EUVSEC panel (for susceptibility testing of *Salmonella* and *Escherichia coli* as part of surveillance programs; Thermo Scientific). The panel consisted of ampicillin, cefotaxime, ceftazidime, meropenem, gentamicin, tetracycline,

tigecycline, chloramphenicol, azithromycin, sulfamethoxazole, trimethoprim, nalidixic acid, ciprofloxacin, and colistin. For the 2017 and 2018 isolates, screening of colistin resistance was performed on SuperPolymyxin medium (Nordmann et al., 2016). Colistin MICs of resistant isolates were accurately determined by broth microdilution, according to current EUCAST recommendations[1]. The presence of *mcr-1* to *mcr-5* genes was screened by PCR using previously reported primers and conditions (Liu et al., 2016; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017). Disk diffusion assays were done for all monophasic *S.* Typhimurium isolates (n = 84) detected during the period of study. The following commercially available disks (Thermo Scientific), with the amount in μg shown in parenthesis, were used: ampicillin (10), amoxicillin-clavulanic acid (30), cefepime (30), cefotaxime (30), cefoxitin (30), ertapenem (10), chloramphenicol (30), amikacin (30), gentamicin (10), kanamycin (30), streptomycin (10), tobramycin (10), azithromycin (15), nalidixic acid (30), ciprofloxacin (5), sulfonamides (300), tetracycline (30), trimethoprim (5), fosfomycin (300), and nitrofurantoin (300). Results were interpreted according to EUCAST (2016) or CLSI (2019). For a single colistin-resistant isolate that was also resistant to both cefotaxime and cefoxitin, the presence of the $bla_{\mathrm{CMY-2}}$ gene was tested by PCR, using the primers reported by Pérez-Pérez and Hanson (2002).

To place the colistin-resistant isolates within context, information regarding year of recovery, sample of origin, serotype, as well as phage type and antimicrobial resistance properties, when known, is shown in **Supplementary Table 1** for the 231 food-borne isolates. They were recovered from retail food of different origins, including fresh or processed meat from beef, pig, wild boar and chicken, eggs and derived products, seafood, and also bovine and pig carcasses at slaughterhouses.

Experimental Typing of the Isolates and Plasmid Analysis

The serotype of all isolates, and also the phage type of those recovered before 2017, were determined by the "Agencia Española de Consumo, Seguridad Alimentaria y Nutrición" (AECOSAN). The phage type of the 2019 isolate is unknown, because since 2017 reference laboratories in Spain have discontinued typing of *Salmonella* with this technique. Typing by Pulsed-Field Gel Electrophoresis (PFGE) was done with the XbaI endonuclease (Thermo Fisher Scientific), following the PulseNet protocol for *S. enterica*[2]. XbaI-digested DNA of *S.* Braenderup H9812 was used as size standard (Hunter et al., 2005), and a dendrogram of similarity was constructed with Bionumerics v.6.6 (Applied Maths N.V., Sint-Martens-Latem, Belgium), using the unweighted pair group method with arithmetic means (UPGMA). For plasmid analysis, a modified alkaline lysis method and the S1-PFGE technique were applied (Kado and Liu, 1981; Barton et al., 1995). Plasmids of the *E. coli* strains V517 (Sanchez et al., 1986) and 39R861 (Threlfall et al., 1986) were included as size standards for undigested DNA. Conjugation experiments were performed in Luria-Bertani (LB) liquid medium, using each of colistin-resistant isolates as donors and *E. coli* K-12 J53 (resistant to rifampicin) as the recipient. Cultures of donor and recipient strains were incubated overnight at 37°C. Then 100 μl of the donor culture and 200 μl of the recipient culture were added to 1 ml LB. The mixtures were incubated overnight without shaking either at 37°C (LSP 237/15, LSP 295/15, and LSP 298/15) or at 28 and 37°C (LSP 38/19). Transconjugants were selected on eosin–methylene blue agar (Oxoid, Madrid, Spain), containing rifampicin (50 mg/L) plus either colistin (3.5 mg/L; for all isolates) or cefotaxime (8 mg/L; for LSP 38/19). Eight to twelve transconjugants per mating experiment were tested for antimicrobial susceptibility, plasmid content, PBRT of the IncX4, IncHI2, IncI1 and IncFIB/IncFIC replicons (Carattoli et al., 2005), and PCR detection of the *mcr-1.1* and $bla_{\mathrm{CMY-2}}$ genes. The frequencies of plasmid transfer were calculated as the number of transconjugants per donor cell, with values corresponding to the average of three independent experiments.

Whole Genome Sequencing of the Colistin Resistant Isolates and Bioinformatic Analysis

WGS of colistin resistant isolates was performed with Illumina. Total DNA was extracted from overnight cultures grown in LB broth using the GenElute™ Bacterial Genomic DNA Kit (Sigma) according to the manufacturer's instructions. Paired-end reads of 150 nt were generated from ca. 500–400 bp fragment libraries at the CIBIR (Centro de Investigación Biomédica, La Rioja, Spain; 2015 isolates) or at Eurofins Genomics (Ebersberg, Germany; 2019 isolate), using HiSeq 2500 and NovaSeq 6000 S2 PE150 XP platforms, respectively. Genome reconstruction was achieved with PLACNETw[3]. This tool facilitates full genome analysis, including *de novo* assembly of all the genome with VelvetOptimizer, followed by the separation of contigs belonging to the chromosome or contigs belonging to Mobile Genetic Elements (MGEs), such as plasmids. At the same time, and in a friendly, easy, and graphical way, PLACNETw allows the exploration of close references to each assembled contig, in order to determine its genetic nature (chromosome, plasmid, ICE, IME, etc.). Besides, this tool can ease MGE typing, both by the analysis of replication initiation proteins and relaxases, which are key elements in plasmid replication and mobilization. Finally, an *in silico* incompatibility group classification is performed (Lanza et al., 2014; Vielva et al., 2017).

Information regarding the quality of the assemblies, as provided by VelvetOptimiser, is compiled in **Supplementary Table 2**. The genomes were deposited in GenBank, under the accession numbers indicated below, and annotated by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP[4]). Bioinformatic analysis was performed both with PLACNET and with the MLST, ResFinder, PlasmidFinder and pMLST tools of the Center for Genomic Epidemiology[5] (Camacho et al., 2009; Larsen et al., 2012; Carattoli et al., 2014; Bortolaia et al.,

[1]www.eucast.org

[2]https://pulsenetinternational.org/protocols/pfge/

[3]https://castillo.dicom.unican.es/upload/

[4]https://www.ncbi.nlm.nih.gov/genome/annotation_prok/

[5]https://cge.cbs.dtu.dk/services/

2020; Zankari et al., 2020). SGI-4, the tetra-R and *fljAB-hin* regions, as well as relevant plasmids or plasmid contigs were manually refined by means of BLASTn, CLONE Manager (CloneSuite9), and MyDbFinder (CGE, DTU) using customized databases comprising open reading frames from strains SLK-521 (SGI-4; accession number MN730128), 105/7/03 and 07-2006 (tetra-R regions; accession numbers HQ331538 and KR856283, respectively), and LT2 (*fljAB-hin* region; accession number NC_003197), with STM ORFs following the *S.* Typhimurium LT2 nomenclature. Reconstruction of the IncX4 plasmids from contigs identified with PLACNET, and of the tetra-R regions (guided by the location and orientation of the multiple IS*26* elements carried by them), was achieved by PCR amplifications of the intervening DNA, followed by Sanger sequencing of the obtained amplicons (performed at STAB VIDA, Caparica, Portugal). Once established, the organization of the tetra-R regions, as well as the genetic environments of the *mcr-1.1* and bla_{CMY-2} genes, were represented with EasyFig[6].

Nucleotide Sequence Accession Numbers

The genome sequences of the colistin resistant isolates under study were deposited at the GenBank database with the following accession numbers: JACXKV000000000, JACXKU000000000, JACXKT000000000, and JAGMWH000000000, for LSP 237/15, LSP 295/15, LSP 298/15, and LSP 38/19, respectively. The plasmid sequences of pIncX4_LSP 237/15, pIncX4_LSP 295/15, pIncX4_LSP 298/15, pIncHI2_contig 309_LSP 38/19, and pIncI1-I(α)_contig 9_LSP 38/19 were also deposited in GenBank under accession numbers OK642377, OK642378, OK642379, OK642375, and OK642376.

RESULTS

General Characteristics of the Isolates, Typing and Plasmid Analysis

During the 2014 to 2019 period, 231 isolates of *S. enterica* were recovered from food samples in Asturias. Five of them (2.2%) were resistant to colistin and four of these (LSP 237/15, LSP 295/15 and LSP 298/15 and LSP 38/19), pertaining to the monophasic European clone of *S.* Typhimurium were thoroughly characterized in the present study (**Table 1**). They represent 4.8% of the total number of isolates belonging to this clone (84; 36.4% of the total *S. enterica*) detected in food samples during the period of study. The remaining isolate, LSP 136/15, was identified as *S. enterica* serovar Kedougou and carried the *mcr-4.3* gene on a ColE10 plasmid (Rebelo et al., 2018).

The selected isolates were recovered from retail meat products either in 2015 (LSP 237/15, LSP 295/15, and LSP 298/15) or 2019 (LSP 38/19). Remarkably, although LSP 295/15 and LSP 298/15 derived from the same sample (pork and beef minced meat), the four isolates were different (see below), and could be then assigned to distinct strains. As shown in **Table 1**, the antigenic formula of the colistin resistant isolates was 4,12:i:- (LSP 237/15) or 4,5,12:i:- (LSP 295/15, LSP 298/15, and LSP 38/19). The three isolates from 2015 belonged to phage types DT104B, DT138 and RDNC, while the phage type of the 2019 isolate was not determined.

Draft genomes of the isolates consisted in 79 to 218 total contigs (23 to 88 larger than 1 kb), with assembly sizes ranging from 4.94 to 5.42 Mb (**Supplementary Table 2**). MLST typing performed "*in silico*" assigned the isolates to ST34, as expected for the European monophasic clone. Experimental typing with XbaI-PFGE revealed a distinct pattern for each isolate, though profiles of the two isolates obtained from the same sample (LSP 295/15 and LSP 298/15) were more closely related (**Figure 1A**). Plasmids were found in all isolates, in numbers ranging from one (LSP 295/15) up to six (LSP 38/19). They belonged to different incompatibility groups and each isolate, even those derived from the same sample, had a different plasmid profile (**Figures 1B,C**; **Table 1**).

Resistance Properties and Genetic Basis of Antimicrobial Drug Resistance

As shown in **Table 1**, MICs of colistin for the isolates ranged from 8 to 16 mg/L, and the *mcr-1.1* gene was identified in all of them. The gene was carried by IncX4/MOBP3 plasmids of 33.3 kb in the 2015 isolates, and by a large IncHI2/MOBH plasmid of 242 kb in the 2019 isolate (**Figures 1B,C**; **Table 1**). Chromosomal mutations in the *pmrA/B* regulatory genes, which can also mediate colistin resistance by affecting the expression of enzymes involved in modification of the lipopolysaccharide, were not observed. Apart from colistin resistance, the isolates shared the tetra-resistance pattern (ampicillin, streptomycin, sulfonamides and tetracycline), characteristically associated with the monophasic ST34 clone and conferred by bla_{TEM-1}, *strA-strB*, *sul2*, and *tet*(B) genes of chromosomal location. Moreover, LSP 38/19 was also resistant to cefotaxime and cefoxitin. This phenotype is consistent with production of an AmpC β-lactamase, and the presence of bla_{CMY-2} was indeed demonstrated. The gene, which encodes the CMY-2 AmpC β-lactamase, resided on a IncI1-I(α)/*mckB*/MOBP plasmid, assigned to ST2 by pMLST. The 2019 isolate was additionally resistant to many other antibiotics, including chloramphenicol, aminoglycosides (gentamicin, kanamycin, and tobramycin), and trimethoprim, with the responsible genes located on the large IncHI2 [*aadA1*, *aadA2*, *aac(3″)-IId*, *cmlA1*, *floR*, *dfrA12*, and *tetM*] and IncFIB-IncFIC/*traI*/MOBH [*aadA1*, *cmlA1*, *sul3*, *tet*(A), and *merRTPC*] plasmids, or carried by a small ColE1 plasmid [*aph(3′)-Ia*]. The actual location of two additional aminoglycoside resistance genes, *aph(4)-1a* and *aph(3)-IV*, which were found together, flanked by two copies of IS26, could not be established.

Genetic Environment and Transferability of the *mcr-1.1* and bla_{CMY-2} Genes

The *mcr-1.1* gene was located on IncX4 plasmids in the 2015 isolates, and carried by a large IncHI2 plasmid in the 2019 isolate (**Table 1**). The genetic context of *mcr-1.1* in the 2015

[6]https://mjsull.github.io/Easyfig/

TABLE 1 | Origin and properties of food-borne colistin-resistant isolates of the monophasic ST34 variant of *Salmonella enterica* serovar Typhimurium.

Isolate[a]	Origin	Antigenic formula	Phage type[b]	Colistin MIC (mg/L)	Resistance phenotype (capitalized)[c] Antibiotic resistance genes (italicized)[c]	Plasmid (size in bp)[d]
LSP 237/15	Pork and beef meat processed for raw consumption	4,12:i:-	PT 104B	16	COL, AMP, STR, SUL, TET *mcr-1.1*, bla_{TEM-1}, [*strA*, *strB*], *sul2*, *tet*(B)	IncI2(δ) (61,356) **IncX4** (33,336)* ColE1-like (5,055)* ColE1-like (3,351)*
LSP 295/15	Pork and beef minced meat	4,5,12:i:-	PT 138	8	COL, AMP, STR, SUL, TET *mcr-1.1*, bla_{TEM-1}, [*strA*, *strB*], *sul2*, *tet*(B)	**IncX4** (33,304)*
LSP 298/15	Pork and beef minced meat	4,5,12:i:-	RDNC	8	COL, AMP, STR, SUL, TET *mcr-1.1*, bla_{TEM-1}, [*strA*, *strB*], *sul2*, *tet*(B)	IncI1-I(α)-ST_{unk} (88,771) **IncX4** (33,304)*
LSP 38/19	Fresh pork sausage	4,5,12:i:-	ND	8	COL, AMP, [CTX, FOX], CHL, [STR, GEN, TOB, KAN], SUL, TET, TMP *mcr-1.1, bla_{TEM-1}, bla_{CMY-2}*, [*cmlA1, floR*], [*strA*, *strB*, *aadA1, aadA2*, *aac(3)-IV, aph(3')-Ia, aph(4)-Ia*], *sul2*, *sul3,* [*tet*(A), *tet*(B), *tetM*], *dfrA12*	**IncHI2** (242,363) **IncI1-I(α)-ST2** (105,151) **IncFIB, IncFIC-F46:A-:B20** (84,823) **ColE1** (5,699)* ColE1 (4,664)* UNK (3,751)*

[a]*LSP, "Laboratorio de Salud Pública, Principado de Asturias," Spain. The names of the isolates include a serial number followed by the last two digits of the year of isolation. LSP 295/15 and LSP 298/15 originated from the same sample.*
[b]*PT, phage type; RDNC, reacted but did not conform; ND, not determined.*
[c]*COL, colistin; AMP, ampicillin; CTX, cefotaxime; FOX, cefoxitin; CHL, chloramphenicol; STR, streptomycin; GEN, gentamicin; TOB, tobramicin; KAN, kanamycin; SUL, sulfonamides; TET, tetracycline; TMP, trimethoprim. Resistance to hygromycin, encoded by the aph(4)-1a gene, was not experimentally tested. Antibiotics pertaining to the same family, as well as genes conferring resistance to the same family of antibiotics are enclosed in square brackets.*
[d]*Inc, plasmid incompatibility group; UNK, unknown. IncX4 (mcr-1.1), IncHI2 (mcr-1.1 and other resistance genes) and IncI1-I(α) (bla_{CMY-2}), as well as two other resistance plasmids, IncFIB, IncFIC, and ColE1, are highlighted in bold (see text for details). Circularized plasmids are marked with an asterisk.*

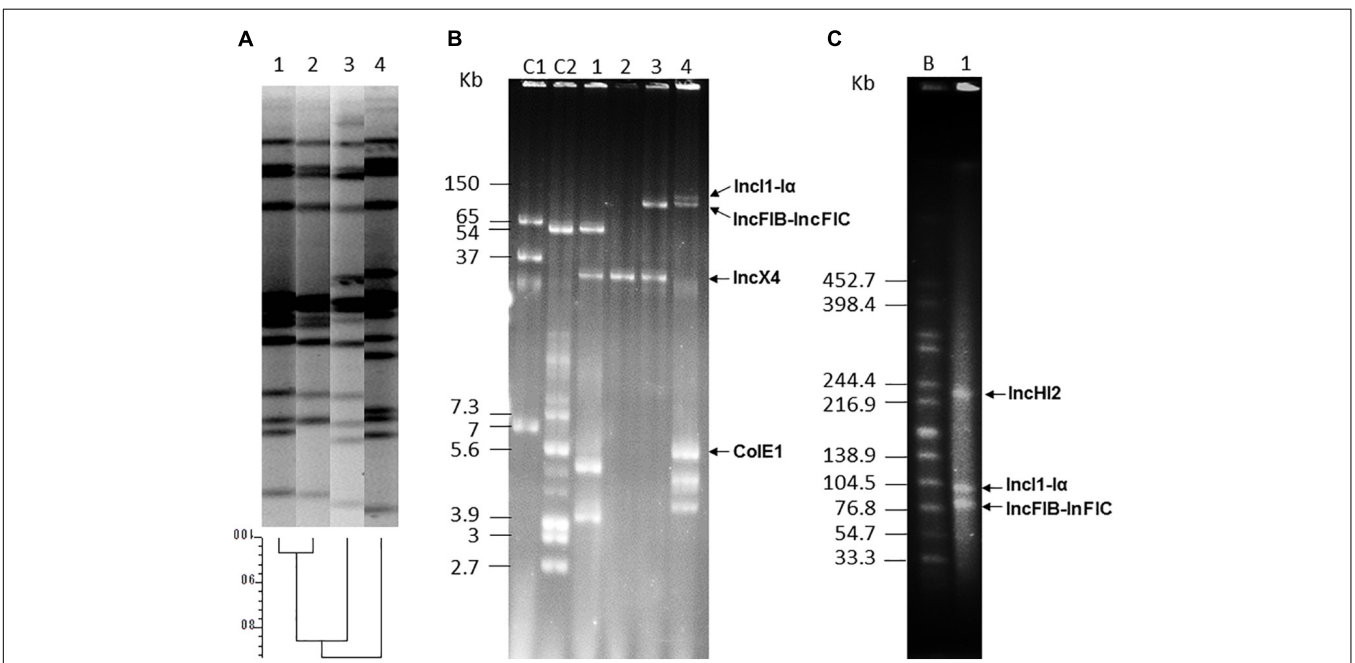

FIGURE 1 | Typing of food-borne isolates belonging to the European monophasic clone, and plasmid analysis. **(A)** XbaI-PFGE and dendrogram showing the similarity between the generated profiles. Lane 1, LSP 295/15; lane 2, LSP 298/15; lane 3, LSP 38/19; lane 4, LSP 237/15. **(B)** Plasmid profiles generated with the Kado-Liu method. Lanes C1 and C2, plasmids obtained from *Escherichia coli* 39R861 (NCTC 50192) and V517 (NCTC 50193) used as size standards for undigested DNA; lane 1, LSP 237/15; lane 2, LSP 295/15; lane 3, LSP 298/15; lane 4, LSP 38/19. **(C)** S1-PFGE. Lane B, XbaI-digested DNA of *Salmonella enterica* serovar Braenderup H9812 used as size standard; lane 1, LSP 38/19. Resistance plasmids are indicated in **(B,C)**.

isolates is shown in **Figure 2A**. As previously reported for other IncX4 plasmids, *mcr-1.1* was found adjacent to a putative *orf*, designated *pap2* since it encodes a transmembrane protein of the PAP2 superfamily. The IS*Apl1* insertion sequence, proposed to be involved in the initial mobilization of *mcr-1*, likely by means of the Tn*6330* composite transposon (see below), is not detected (Li et al., 2017). However, a single copy of IS*26*, flanked by 8 bp target site duplications (CTGTGTGA), is located further

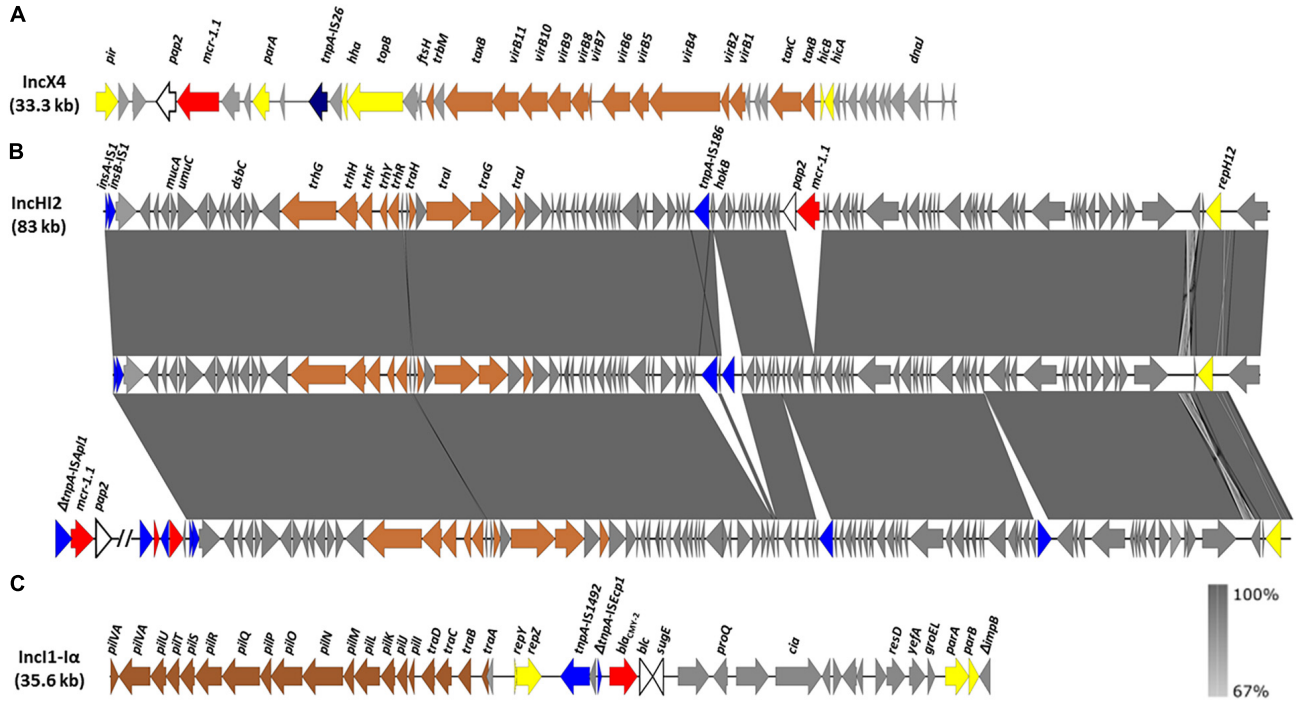

FIGURE 2 | Genetic environment of the *mcr-1.1* and bla_{CMY-2} genes detected in food-borne isolates of the European monophasic clone. **(A)** Genetic context of *mcr-1.1* carried by IncX4 plasmids in the LSP 237/15, LSP 295/15, and LSP 298/15 isolates. **(B)** Genetic organization of a ca. 83 kb contig carrying the *mcr-1.1* gene in the IncHI2 plasmid of LSP 38/19. The alignment of this contig with homologous regions in two related plasmids either lacking *mcr-1.1* (pKUSR18; accession number KM396298) or having the gene outside this region (pSE08-00436-1; accession number NZ_CP020493) was created with EasyFig BLASTn. The gray shading between the regions reflects nucleotide sequence similarity ranging from 67 to 100%, according to the scale shown at the lower right part of the figure. **(C)** Genetic environment of the bla_{CMY-2} gene in a 35.6 kb contig of the IncI1-I(α) plasmid of LSP 38/19. The open reading frames (ORFs) are represented by arrows pointing to the direction of transcription and having different colors according to their function: red, resistance; yellow, plasmid replication, stability and maintenance; brown, conjugation; blue, DNA mobility, with the *tnpA* gene of IS*26* shown in dark blue; gray, other roles; white, *pap2*, adjacent to *mcr-1.1* in the IncX4 and IncHI2 plasmids, and *bcl-sugE*, adjacent to bla_{CMY-2} in the IncI1-I(α) plasmid. The scale is different for **(A–C)**.

downstream. The IncX4 plasmids of LSP 295/15 and LSP 298/15 were identical to each other, and differed from the IncX4 plasmid of LSP 237/15 by a single SNP and a 32 bp insertion present in the latter one but not in the former two. The IncX4 plasmids of these isolates were very closely related (more than 99% identity and coverage) to previously sequenced IncX4 plasmids carrying either *mcr-1.1* (such as pE15004 from *E. coli*; accession number KX772777), or *mcr-1.2* (like pMCR1.2-IT from *K. pneumoniae*; accession number KX236309).

The *mcr-1.1* gene of the 2019 isolate was located on a large contig of 83 kb (**Figure 2B**), which belongs to the IncHI2/MOBH plasmid. As in the 2015 isolates, the gene was adjacent to *pap2* and devoided of IS*AplI*. The insertion site of *mcr-1.1-pap2*, between two *orfs* encoding hypothetical proteins, was previously found only in unnamed plasmid1 from *Salmonella* isolate S15BD05371 which, according to SeqSero and MLST, corresponds to the monophasic ST34 variant of *S.* Typhimurium (accession number GCF_014857405.1). Other IncH12 plasmids contain regions closely related to the 83 kb contig of the LSP 38/19 plasmid, in which the *mcr-1.1* gene is either absent (i.e., pKUSR18; accession number KM396298) or placed outside the corresponding region (i.e., pSE08-00436-1; accession number NZ_CP020493) (**Figure 2B**). On the other hand, the bla_{CMY-2} gene of LSP 38/19 resided on an IncI1-I(α)-ST2 plasmid of 105 kb (**Table 1**). More precisely, bla_{CMY-2} was located on a contig of 35.6 kb, which harbored the IS*Sbo1*-like-ΔIS*Ecp1*-bla_{CMY-2}-*blc-sugE* structure, inserted downstream of the *repZ* gene (**Figure 2C**). This contig was more closely related to regions of IncI1-I(α)-ST2 plasmids found in *E. coli* isolates from a human patient (pC-6; accession number KT186369) and a dog (pR7AC; accession number KF434766).

The IncX4, IncHI2 and IncI1-I(α) plasmids harboring the *mcr-1.1* or bla_{CMY-2} genes in the LSP isolates could be transferred into *E. coli* through conjugation/mobilization experiments (**Table 2**). The frequency of conjugation of the IncX4 found alone in the LSP 295/15 isolate was of 2.6×10^{-6} transconjugants/donor cell. Similar frequencies were obtained for the IncX4 plasmids of LSP 237/15 and LSP 298/15, which each co-resided with a different IncI1 plasmid (**Table 1**), both cryptic. Taking into account that the IncX4 plasmids of the three LSP isolates were nearly identical (see above), the IncX4 plasmids of LSP 237/15 and LSP 298/15 are expected to be conjugative and, in fact, they were found alone in ca. 10% of the transconjugants analyzed. However, mobilization cannot be excluded as most transconjugants carried the IncX4 and IncI plasmids. In the case of LSP 38/19, conjugation

TABLE 2 | Conjugation/mobilization frequencies of IncX4, IncHI2, and IncI1-I(α) plasmids of food-borne colistin resistant isolates of the monophasic ST34 variant of *Salmonella enterica* serovar Typhimurium.

Donor strain / plasmid(s)	Selection for:	Selection with[a]:	Transfer frequency[b] (standard error)	
			28°C	37°C
LSP 237/15 / IncX4; IncI2(δ)[c]	IncX4	RIF + COL	nd	**1.5×10^{-6}** (0.98)
LSP 295/15 / IncX4	IncX4	RIF + COL	nd	**2.6×10^{-6}** (0.85)
LSP 298/15 / IncX4; IncI1-I(α)c	IncX4	RIF + COL	nd	**0.6×10^{-6}** (0.42)
LSP 38/19 / IncHI2; IncI1-I(α)	IncH12	RIF + COL	**2.6×10^{-5}** (1.2)	**1.6×10^{-5}** (1.2)
LSP 38/19 / IncHI2; IncI1-I(α)	IncI1-I(α)	RIF + CTX	$<$**1.9×10^{-8}**	**3.6×10^{-5}** (1.8)

[a] *RIF, rifampicin; COL, colistin; CTX, cefotaxime.*
[b] *Conjugation frequencies (shown in bold) are reported as the number of transconjugants per donor cell, and result from the average of three independent experiments, nd, not determined.*
[c] *Criptic plasmids for which selection is not available.*

experiments were carried out both at 28 and 37°C, since it has been reported that optimal transfer of IncHI2 plasmid occurs between 22 and 30°C (García-Fernández and Carattoli, 2010). Selection was performed either with cefotaxime or colistin, used as markers of the IncI1-I(α) and IncHI2 plasmids, respectively. At 37°C, selection with cefotaxime yielded a transfer frequency of 3.6×10^{-5} transconjugants/donor cell, while at 28°C transconjugants were not obtained (frequency $<1.9 \times 10^{-8}$). These results indicate that the IncI1-I(α) of LSP 38/19 can be efficiently transferred at 37°C but not at 28°C. All transconjugants analyzed from the 37°C mating were resistant to cefotaxime and colistin, PCR-positive for bla_{CMY-2} and *mcr-1.1*, and carried the IncHI2, IncI1-I(α) and IncFIB-IncFIC plasmids. In contrast, when selection was performed with colistin, the conjugation frequencies were of 2.6×10^{-5} and 1.6×10^{-5} at both 28 and 37°C. Transconjugants resulting from matings at the two temperatures were resistant to colistin but susceptible to cefotaxime, PCR positive for *mcr-1.1* but not for bla_{CMY-2}, and carried the IncHI2 and IncFIB-IncFIC plasmids. These results are consistent with efficient transfer of the IncHI2 plasmid at 28°C, either by conjugation and/or mobilization, and also at 37°C, although in the latter case it has to be mobilized by the accompanying IncFIB-IncFIC plasmid.

Chromosomal Regions Involved in Antibiotic Resistance and Heavy Metal Tolerance Genetic Bases of the Monophasic Phenotype

As shown in **Figure 3**, the tetra-R regions of LSP 295/15 and LSP 298/15 were identical to each other and consisted of a contiguous module which also coincided with that of strain 07-2006, previously reported by García et al. (2016). However, the flanking DNA at the left and right borders was different (ΔSTM2756 and a large inverted segment encompassing from ΔSTM2816 to *iroB* and flanked by oppositely oriented copies of IS*26*, in the case of LSP 295/15 and LSP 298/15, and STM2759 and *iroB* in strain 07-2006). In contrast, the tetra-R regions of LSP 237/15 and LSP 38/19 comprised two modules embedded between ΔSTM2753 and *iroB*. The modules of LSP 237/15 resemble those of 105/7/03 (Lucarelli et al., 2012), except for the *mer* locus which is present in the latter but not in the former. In both cases, ΔSTM2759 to ΔSTM2753, inverted with respect to their orientation in the chromosome of *S.* Typhimurium LT2, are located between the two modules. The organization of LSP 38/19 is similar to that of LSP 237/15, except that the first module and adjacent ORFs (ΔSTM2759 to ΔSTM2753) are inverted in 38/19, and additional ORFs (ΔSTM2759 to ΔSTM2761, preceded by an IS*26* element), are found between the two modules. In all strains except 105/7/03, evolution of the tetra-R region was accompanied by deletions of a number of chromosomal ORFs that always included the *fljAB* genes, as well as the entire (LSP 295/15, LSP 298/15, LSP 38/19, and 07-2006) or part (LSP 237/15) of the *hin* gene, linking these regions with the monophasic phenotype.

In contrast to the diversity of the tetra-R regions, SGI-4 was identical in the analyzed isolates, and identical or nearly identical to the genomic islands carried by many other ST34 monophasic strains, including SLK-521 used for comparison (Mourao et al., 2020). It comprised the *silESRCFBAGP*, *pcoGE1ABCDRSE2*, and *arsRSD2A2BCA1D1* gene clusters, accountable for copper, silver and arsenic tolerance. The insertion site of the ICE element, between STM4320 and *yjdC* (both encoding putative transcriptional regulators), also coincides with previous information.

DISCUSSION

During the 2014 to 2019 six year period, 231 isolates of *S. enterica* were recovered from food samples in Asturias, a Northern Spanish region. Interestingly, the number of isolates decreased considerably along time, from nearly 96 in 2014 down to 4–10 since 2017, a favorable trend that probably correlates with implementation in Spain of the integrated EU legislation aimed to monitor and control *Salmonella* along the food chain (European Commission, 2005; Hugas and Beloeil, 2014). Although with some delay, this trend roughly coincides with that reported in the EU for human salmonellosis, which has been stable over the past 5 years after a long period of decline (EFSA and ECDC, 2021). In Asturias, 36.4% of the isolates recovered from foods belonged to the European monophasic clone of *S.* Typhimurium, with sequence type ST34. The incidence of this clone increased considerably along the last years, and four out of five colistin-resistant isolates detected during the period of study belonged to this clone. The ST34 isolates characterized in the present study carried *mcr-1.1* on transferable IncX4 or IncHI2 plasmids. These plasmid types are amongst the most prevalent vehicles leading to the worldwide spread of *mcr-1* in different bacteria, including the European monophasic clone (Nang et al., 2019).

There is strong evidence supporting that the *mcr-1* gene was originally mobilized from the chromosome of a species of the

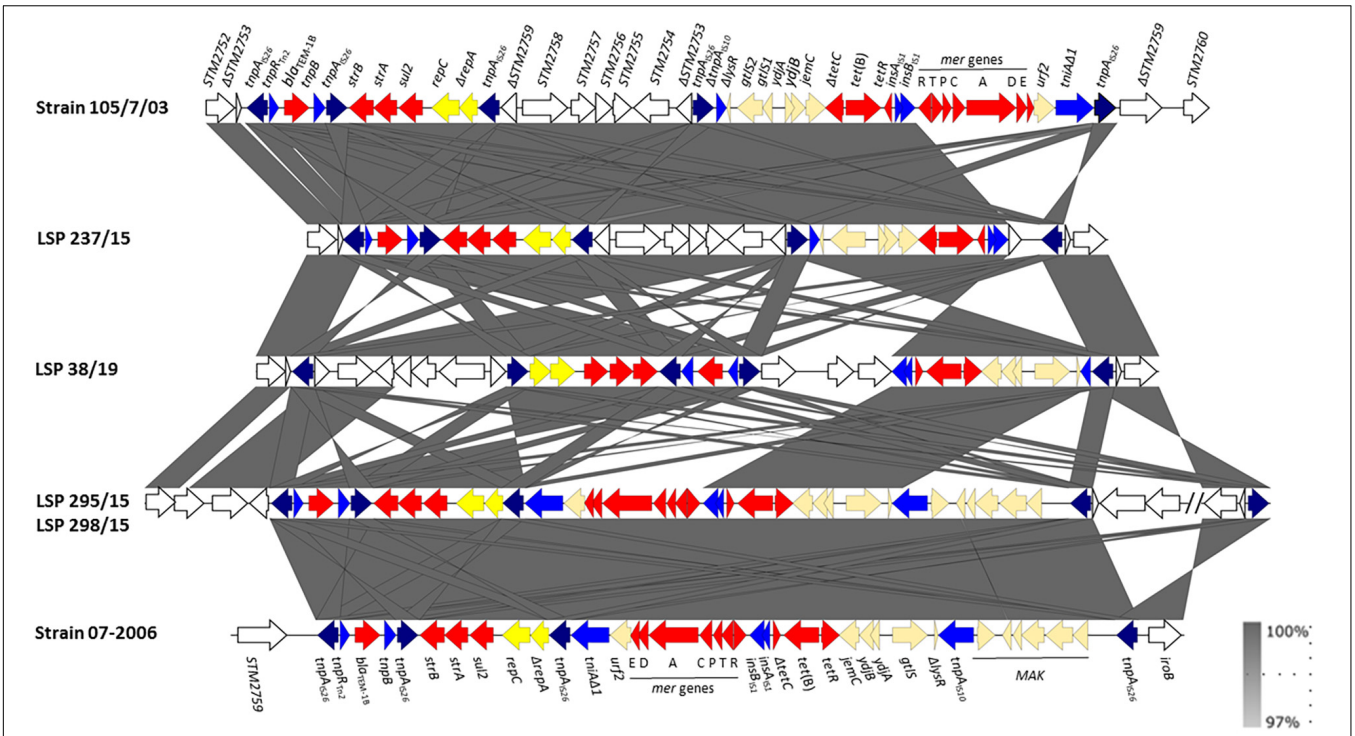

FIGURE 3 | Genetic organization of the tetra-resistance regions found in food-borne isolates of the European monophasic clone. The alignment was created with EasyFig BLASTn including the tetra-resistance regions of strains 105/7/03 (accession number HQ331538, shown above) and 07-2006 (accession number KR856283; shown below) for comparison. The open reading frames (ORFs) are represented by arrows pointing to the direction of transcription and having different colors according to their origin or roles: white, chromosomal ORFs of the *S.* Typhimurium LT2 chromosome (named according to accession number NC_003197); red, resistance; blue, DNA mobility, with the *tnpA* gene of IS*26* shown in dark blue; yellow, plasmid replication genes; pale orange, other roles. The gray shading between the structures connects regions of nucleotide sequence similarity ranging from 97 to 100%, according to the scale shown at the lower right part of the figure.

genus *Moraxella* by an IS*Apl1*-flanked composite transposon termed Tn*6330* (Poirel et al., 2017; Snesrud et al., 2018). However, most of the detected *mcr-1* structures lack either one (single-ended derivatives) or more frequently both copies of IS*Apl1* (Snesrud et al., 2016, 2018). This is regarded as a mechanism of stabilization of the *mcr-1* gene, associated with the loss of the transposition capacity. Neither of the two copies of IS*Apl1* were found surrounding the *mcr-1.1* genes of the isolates in the present study, although a remnant of the downstream IS*Apl1* inverted right repeat (IRR) of 27 bp, which encompasses the last 26 bp of the *pap2* gene, was identified in the IncX4 plasmids of the 2015 isolates, and a shorter version with only 21 bp, overlaps the *pap2* gene in the IncHI2 plasmid. In single-ended variants, a unique upstream copy of IS*Apl1* in conjunction with the IRR of the ancestral downstream IS*Apl1* may still be able to mobilize the *mcr-1* gene. In contrast, variants entirely devoided of IS*Apl1*, like those reported in the present study, have made their way toward stabilization of the resistance gene and maintenance of plasmid integrity (Snesrud et al., 2018). Interestingly, a copy of IS*26* is present in the IncX4 plasmid, close to the *mcr-1.1* gene. In contrast to IS*Apl1*, IS*26*, once gained by a plasmid or the chromosome, is able to proliferate, actively contributing to the generation of complex resistance regions containing multiple copies of the element (Harmer and Hall, 2014; He et al., 2015; Vazquez et al., 2021). It would be worth to follow the activity of IS*26* within IncX4 plasmids carrying *mcr-1.1*. Indeed, IS*26* appears to have played a critical role in the generation of multiple variants of the tetra-R region of the European monophasic clone. It has been proposed that a copy of IS*26*, inserted at exactly the same position adjacent to *iroB*, could have been responsible for the monophasic phenotype of this clone, by causing different deletions affecting the *fljAB* operon. The originally acquired copy of IS*26* could have then acted as a target or recognition site for incorporation of one or more antibiotic resistance modules linked to additional IS*26* elements (García et al., 2016). Such events were associated to further deletions and/or inversions of the chromosomal DNA upstream of *iroB*, yielding multiple variants of the tetra-R region, like those reported in the present study.

Interestingly, although the four isolates characterized in the present study derived from three food samples, each was assigned to a distinct strain, according to differences in phage type, XbaI-PFGE profiles, variations affecting both the tetra-R regions and the deletions responsible of the monophasic phenotype, and plasmid content. The phage type was only determined for the 2015 isolates that were assigned to DT104B, DT138 and to RDNC. Although DT193 and DT120 are the main types associated with the ST34 clone, several others types,

including DT104B and DT138, have been reported (EFSA, 2010; Hopkins et al., 2010; Gallati et al., 2013; Petrovska et al., 2016; Campos et al., 2019). With regard to PFGE and plasmid profiles, the two isolates obtained from the same sample, though different, were more closely related to each other than to those recovered from different samples. Interestingly, a remarkable level of short-term microevolution has accumulated during clonal expansion of monophasic ST34 (Petrovska et al., 2016; Tassinari et al., 2020; Cadel-Six et al., 2021). This led to a large amount of genomic variation, such as that observed for the isolates analyzed in the present study. Of particular interest is the evolution of the clone through acquisition of transmissible IncX4, IncHI2 and IncI1 plasmids, which can play an important role in the spread of the *mcr-1* and bla_{CMY-2} genes through the food chain. CMY-2 is one of the plasmid-determined AmpC-type β-lactamases, enzymes which confer resistance to a wide range of β-lactams, including oxyimino cephalosporins and cephamycins (Philippon et al., 2002). Acquisition of this gene by *S. enterica* serotypes is a matter of concern since these compounds are drugs of choice for the treatment of severe infections caused by this and other bacteria (Gilbert et al., 2021). Furthermore, this species could act as reservoir and vehicle for transmission of *mcr-1.1* and bla_{CMY-2} genes to other clinical important bacterial species throughout the food chain.

In summary, the study identifies meat-derived products as a reservoir of a highly successful clone harboring "epidemic" plasmids which confer resistance to colistin and other clinically important antibiotics. One Health initiatives, as those implemented by the EU, are vital for the containment of these isolates which are potentially dangerous to human health. The observed reduction in the number of food-borne *S. enterica*, together with the low frequency of resistance to the last resort colistin, underlines the success of such measures at a regional level. It would be interesting to investigate whether similar isolates to those reported herein are also circulating in hospitals of the region.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JACXKV000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXKU000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXKT000000000; https://www.ncbi.nlm.nih.gov/genbank/, JAGMWH000000000; https://www.ncbi.nlm.nih.gov/genbank/, OK642377; https://www.ncbi.nlm.nih.gov/genbank/, OK642378; https://www.ncbi.nlm.nih.gov/genbank/, OK642379; https://www.ncbi.nlm.nih.gov/genbank/, OK642375; https://www.ncbi.nlm.nih.gov/genbank/, OK642376.

AUTHOR CONTRIBUTIONS

JF, VL, RR, and MR conceptualized and designed the study. XV, VG, and MB carried out the experiments. XV, MB, MT, RR, and MR did data curation. XV, VG, RR, and MR prepared tables and figures. XV, MT, VL, RR, and MR performed WGS analyses. XV, RR, and MR drafted the manuscript. All authors contributed to analysis and interpretation of the results, reviewed the manuscript, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.735364/full#supplementary-material

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