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Characteristics of the plasmid-mediated colistin-resistance gene *mcr-1* in *Escherichia coli* isolated from a veterinary hospital in Shanghai

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The mobile colistin-resistance (*mcr-1*) gene is primarily detected in *Enterobacteriaceae* species, such as *Escherichia coli* and *Salmonella enterica*, and represents a significant public health threat. Herein, we investigated the prevalence and characteristics of *mcr-1*-positive *E. coli* (MCRPEC) in hospitalized companion animals in a pet hospital in Shanghai, China, from May 2021 to July 2021. Seventy-nine non-duplicate samples were collected from the feces ($n=52$) and wounds ($n=20$) of cats and dogs and the surrounding hospital environment ($n=7$). Seven MCRPEC strains, identified using screening assays and polymerase chain reaction, exhibited multidrug-resistant phenotypes in broth-microdilution and agar-dilution assays. Based in whole-genome sequencing and bioinformatics analyses, all seven isolates were determined to belong to sequence type (ST) 117. Moreover, the IncI2 plasmid was prevalent in these MCRPEC isolates, and the genetic environment of the seven *E. coli* strains was highly similar to that of *E. coli* SZ02 isolated from human blood. The isolates also harbored the β -lactamase gene *bla*_{CTX-M-65}, and florfenicol resistance gene *floR*, among other resistance genes. Given that horizontal transfer occurred in all seven strains, *E. coli* plasmid transferability may accelerate the emergence of multidrug-resistant bacteria and may be transmitted from companion animals to humans. Therefore, the surveillance of MCRPEC isolates among companion animals should be strengthened.

KEYWORDS

pet hospital, colistin, *mcr-1*, *Escherichia coli*, multidrug-resistant bacteria

Introduction

Antibiotics have remained a key means of treating bacterial infections in humans and animals; however, bacterial resistance reduces the effectiveness of treatment, posing a serious threat to public health (Sun et al., 2020). In particular, the continued spread of multidrug-resistant (MDR) bacteria has caused high morbidity and mortality rates worldwide. In fact, an estimated 10 million people will die as a result of drug-resistant infections by 2050 (O'Neill, 2016). Antimicrobial resistance (AMR) and antibiotic-resistance genes (ARG) pose multi-faceted challenges and are among the greatest threats to human health in the 21st century (Torres et al., 2021). Meanwhile, colistin was once reintroduced as the last resort against MDR and carbapenem-resistant infections (Shen et al., 2019) and is, thus, included in the World Health Organization's list of antibiotics of critical importance in human medicine (World Health Organization, 2019). Bacterial resistance to colistin is thought to be acquired by chromosomal point mutations (Carroll et al., 2019). Moreover, colistin-resistance mechanisms are related to modulation of the bacterial cell surface, including variations in LPS structure, or due to shedding of the capsular polysaccharides that bind, or trap, colistin (Olaitan et al., 2014). However, the first plasmid-mediated colistin-resistance gene, mobile colistin-resistance 1 (*mcr-1*), was first reported in China in 2015 (Liu et al., 2016), thereby breaking the "last line of defense," and increasing the threat of ARGs to public health.

Several *mcr-1* variants (*mcr-1* to *mcr-10*) have been identified (Hussein et al., 2021) with plasmid-mediated colistin-resistance genes having been reported in 47 countries as widely distributed in humans, animals, water, food, and the environment (Shen et al., 2020). *Escherichia coli* is the most prevalent species harboring *mcr*-positive isolates, followed by *Salmonella enterica* and *Klebsiella pneumoniae*. The *mcr-1* gene is widely distributed in three major plasmid types, namely, IncI2, IncHI2, and IncX4 (Nang et al., 2019). A previous study revealed that the tandem configuration of ISAp11-*mcr-1* with different components enables a more diverse genetic context for *mcr-1* (Yu et al., 2017). Meanwhile, comparative analyses of pMCR_1410 and pHNSHP45 revealed that conjugative plasmid transfer genes may be involved in interspecies plasmid transfer (Zhao and Zong, 2016), suggesting the potential spread of *mcr-1* to a more diverse bacterial pool.

Drug-resistant bacteria can spread from animals to humans through direct contact, the food chain, and the environment (World Health Organization, 2014). In recent years, *mcr-1* and its variants have primarily been detected in livestock such as pigs and

cattle (Wang et al., 2019, 2021; Cheng et al., 2021; Liu et al., 2022), and increasingly in companion animals, in China (Wang et al., 2018; Zhang et al., 2019, 2021; Lei et al., 2021). In fact, *Mcr-1* has been found in 8.7% of *Enterobacteriaceae* isolates from companion animals in Beijing, and *mcr-1*-positive *E. coli* (MCRPEC) transmission between companion animals *via* close contact has been observed in a pet shop in Guangzhou (Zhang et al., 2016; Lei et al., 2017). Importantly, colistin is routinely used for the treatment of bacterial infections in both humans and companion animals, while MCRPEC strain transmission occurs readily *via* close contact. Thus, to assess the potential risk of transmitting resistant bacteria to humans, it is necessary to accurately determine whether companion animals host bacteria carrying the *mcr-1* gene. Therefore, in the current study, we investigate the prevalence and characteristics of MCRPEC isolates from companion animals at a typical pet hospital in Shanghai, China.

Materials and methods

Sample collection and bacterial identification

As a first-tier city in China, Shanghai has a large population of pet owners; therefore, it was selected as a representative city for this study, and a typical pet hospital in the city was selected. With the consent of the pet owners, 79 non-duplicate samples (environment: $n=7$, fecal: $n=52$, wounds: $n=20$) were collected from 22 cats and 9 dogs and the general clinic environment between May 2021 and July 2021. To identify colistin-resistant gram-negative strains, the samples were enriched in Mueller-Hinton (MH) broth (Landbridge, Beijing, China) at 37°C for 24 h, without shaking; thereafter, they were inoculated on MacConkey agar (Landbridge, Beijing, China) supplemented with 2 mg/L colistin (Meilun Biotechnology Co. Ltd., Dalian) and 30 mg/L vancomycin (Meilun Biotechnology Co. Ltd. Dalian) and cultured at 37°C for an additional 24 h. Single, pure colonies were subsequently selected and inoculated into 500 μ l of fresh Luria-Bertani broth containing 2 mg/L colistin (Hu et al., 2022). The presence of *mcr* genes was confirmed using polymerase chain reaction (PCR) with primers that amplify *mcr-1* to *mcr-10* (Mentasti et al., 2021). PCR with 16S rDNA primers was used to identify the species, as described previously (Srivastava et al., 2008). The *mcr*-positive strains were stored at -80°C until further use.

Antimicrobial susceptibility testing for MCRPEC

Broth-microdilution and agar-dilution assays were performed to determine the minimum inhibitory concentrations (MICs) of colistin and 10 antibiotics (cefotaxime, ceftiofur, gentamicin, amikacin, ciprofloxacin, meropenem, florfenicol, tetracycline,

Abbreviation: AMK, amikacin; AMR, antimicrobial resistance; CEF, ceftiofur; CIP, ciprofloxacin; CL, colistin; CTX, cefotaxime; ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; FFC, florfenicol; GEN, gentamicin; MCRPEC, *mcr-1*-positive *E. coli*; MDR, multidrug-resistant; MEM, meropenem; MH, Mueller-Hinton; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; PTZ, piperacillin-tazobactam; ST, sequence type; TCY, tetracycline; TGC, tigecycline.

tigecycline, and piperacillin-tazobactam Meilun Biotechnology Co. Ltd., Dalian) as recommended by the Clinical and Laboratory Standards Institute (CLSI)¹ and the European Committee on antimicrobial susceptibility testing (EUCAST).² *Escherichia coli* ATCC25922 was used as the quality-control strain. According to a previous report, bacteria resistant to three or more antibiotics were considered to be MDR strains (Zhao et al., 2021).

Plasmid conjugation assays

The transferability of the colistin-resistance gene *mcr-1* was determined using filter mating with sodium azide-resistant *E. coli* J53 as the recipient strain and the MCRPEC isolates as the donor. During the logarithmic growth phase, the recipient and donor strains were mixed in a volume ratio of 1:3. A sterilized 0.45 µm microporous filter membrane was applied to MH medium, and the mixture was spread onto the membrane. After culturing at 37°C for 12 h, transconjugants were selected on MacConkey agar containing 4 mg/L colistin and 200 mg/L sodium azide and confirmed using enterobacterial repetitive intergenic consensus (ERIC)-PCR and PCR analysis of *mcr-1* (Liu et al., 2020; Shafiq et al., 2022).

Whole-genome sequencing and bioinformatics analysis

DNA was extracted from all *mcr-1*-positive isolates using the TIANamp Bacteria DNA kit (Tiangen Biotech Co. Ltd., Beijing, China), according to the manufacturer's instructions. Whole-genome sequencing was performed by Annoroad Gene Technology (Beijing, China) on the NovaSeq 6,000 S4 sequencing platform with NovaSeq 6,000 S4 Reagent kit V1.5. Bacterial genome assembly was performed using the SPAdes software (version 3.11) (Bankevich et al., 2012), and the draft sequence was annotated using PATRIC (version 3.6.9)³ (Antonopoulos et al., 2019). The virulence factors and AMR genes of the strains were analyzed using the ResFinder and VirulenceFinder tools of the Center for Genomic Epidemiology.⁴ The relationship with MCRPEC was evaluated using core-genome alignments and phylogenetic trees, constructed using Parsnp with the neighbor-joining method, and visualized using the online tool iTOL 6.5.7⁵ (Hu et al., 2022). The genetic environment of *mcr-1* was investigated using the Easyfig 2.2.215 tool (Sullivan et al., 2011). The comparative genomic map was generated using the BRIG 0.9516 tool (Alikhan et al., 2011).

1 <https://clsi.org>

2 <https://eucastr.org>

3 <https://patricbrc.org/>

4 www.genomicsequencing.org

5 <https://itol.embl.de>

Results

MCRPEC isolation and genus identification

Colistin-resistant bacteria were detected in 56 of the 79 samples. Seven MCRPEC isolates were identified using PCR with multiple primers (dogs: $n=2$, cats: $n=5$; Table 1).

Antimicrobial susceptibility profiles

The resistance profiles of the seven MCRPEC isolates to various antimicrobials are shown in Table 1. All seven isolates exhibited multidrug resistance, including resistance to colistin, cefotaxime, and florfenicol. The MIC of florfenicol was 256 mg/L. Three isolates (L25cr-2, L30cr-1, and L36cr-1) were also resistant to gentamicin. All seven isolates were susceptible to amikacin, ciprofloxacin, tigecycline, piperacillin-tazobactam, and ceftiofur.

Plasmid conjugation assay

Conjugation assay results revealed that the *mcr-1*-carrying plasmid from all seven isolates was successfully transferred to recipient *E. coli* J53 cells. After three repeated conjugation experiments, the transfer success rate reached 100% (Supplementary Table S1).

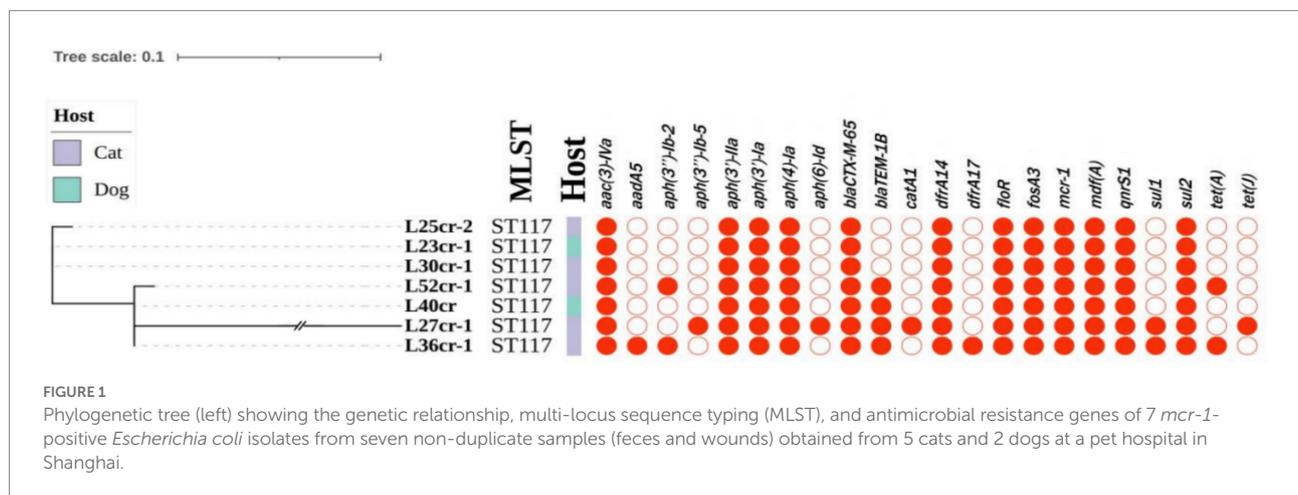
Genomic epidemiology

Multi-locus sequence typing assigned all seven isolates to the ST117 sequence type (ST; Figure 1). The genetic environment of the seven *E. coli* strains was highly similar to that of *E. coli* SZ02 isolated from human blood (GenBank accession number: KU761326.1; Figure 2). All strains were identified to have the same plasmid combination and the same serotype. The six *mcr-1*-positive plasmids were identified as IncI2 plasmids. However, the plasmids in L25cr-2 were difficult to characterize owing to their short sequences (Supplementary Table S1). All MCRPEC isolates harbored the common structure of *nikA-nikB-mcr-1-PAP2* (Figure 3) that is reportedly readily transferred horizontally to different plasmids (Shen et al., 2019, 2020). Mobile genomic elements (*ISKpn8*, *ISKpn19*, *ISKpn24*, *ISEc1*, *ISEc38*, *ISEc39*, *ISEc48*, *ISEc59*, *IS30*, *IS102*, *IS421*, *IS629*, *IS911*, *IS1006*, *ISSf110*, *MITEEc1*, *cn_5813_IS911*, and *cn_3566_ISEc1*) co-existed in the seven MCRPEC isolates (Supplementary Table S1). However, no *ISApI1* insertion sequences were observed upstream or downstream of *mcr-1*. Plasmid conjugative transfer genes (*traA*, *traD*, *traJ*, *traG*, *traE*, *traH*, *traL*, *pilL*, *pilU*, *pilR*, *pilQ*, *pilN*, *pilM*, *pilP*, *pilS*, *pilT*, and *pilV*) and those associated with the type IV secretion system (*virB1*, *virB2*, *virB8*, *virB10*, *virB11*, and *virD4*)

TABLE 1 Antimicrobial susceptibility profiles of 7 *mcr-1*-positive *Escherichia coli* (MCRPEC) isolates from companion animals at a pet hospital in Shanghai, China.

Isolate	Species	Source	Animal procedure	Minimum inhibitory concentration (mg/L)										
				MEM	CL	AMK	CIP	GEN	CTX	TGC	PTZ	FFC	CEF	TCY
L23cr-1	<i>E. coli</i>	Dog feces	Blood test	0.007	8	1	1	4	8	≤ 0.015	1	256	8	0.5
L25cr-2	<i>E. coli</i>	Cat feces	Sterilization operation	0.007	8	1	1	8	16	0.25	1	256	8	≤ 0.25
L27cr-1	<i>E. coli</i>	Cat feces	Soft tissue contusion	0.007	8	1	1	4	8	0.25	1	256	8	≤ 0.25
L30cr-1	<i>E. coli</i>	Cat feces	Sterilization operation	≤ 0.004	16	1	1	8	8	0.5	1	256	8	0.5
L36cr-1	<i>E. coli</i>	Cat feces	Medical checkup	0.007	16	1	1	8	8	0.25	1	256	8	≤ 0.25
L40cr	<i>E. coli</i>	Dog wound	Abdominal surgery	0.007	8	1	1	4	8	0.25	2	256	8	≤ 0.25
L52cr-1	<i>E. coli</i>	Cat feces	Sterilization operation	0.007	8	1	1	4	8	0.25	1	256	8	≤ 0.25

Minimum inhibitory concentrations indicating resistant isolates are shown in bold text. The drug resistance breaking points of MEM, TCY, and FFC were according to Clinical and Laboratory Standards Institute standard. The drug resistance breaking points of CL, AMK, CIP, GEN, CTX, TGC, CEF, and PTZ were according to the European Committee on antimicrobial susceptibility testing standard. AMK, amikacin; CEF, ceftiofur; CIP, ciprofloxacin; CL, colistin; CTX, cefotaxime; FFC, florfenicol; GEN, gentamicin; MEM, meropenem; PTZ, piperacillin-tazobactam; TCY, tetracycline; TGC, tigecycline.



were located in the *mcr*-bearing plasmids, facilitating *mcr-1* transfer to other bacteria.

Distribution of AMR and virulence-associated genes among *mcr-1* isolates

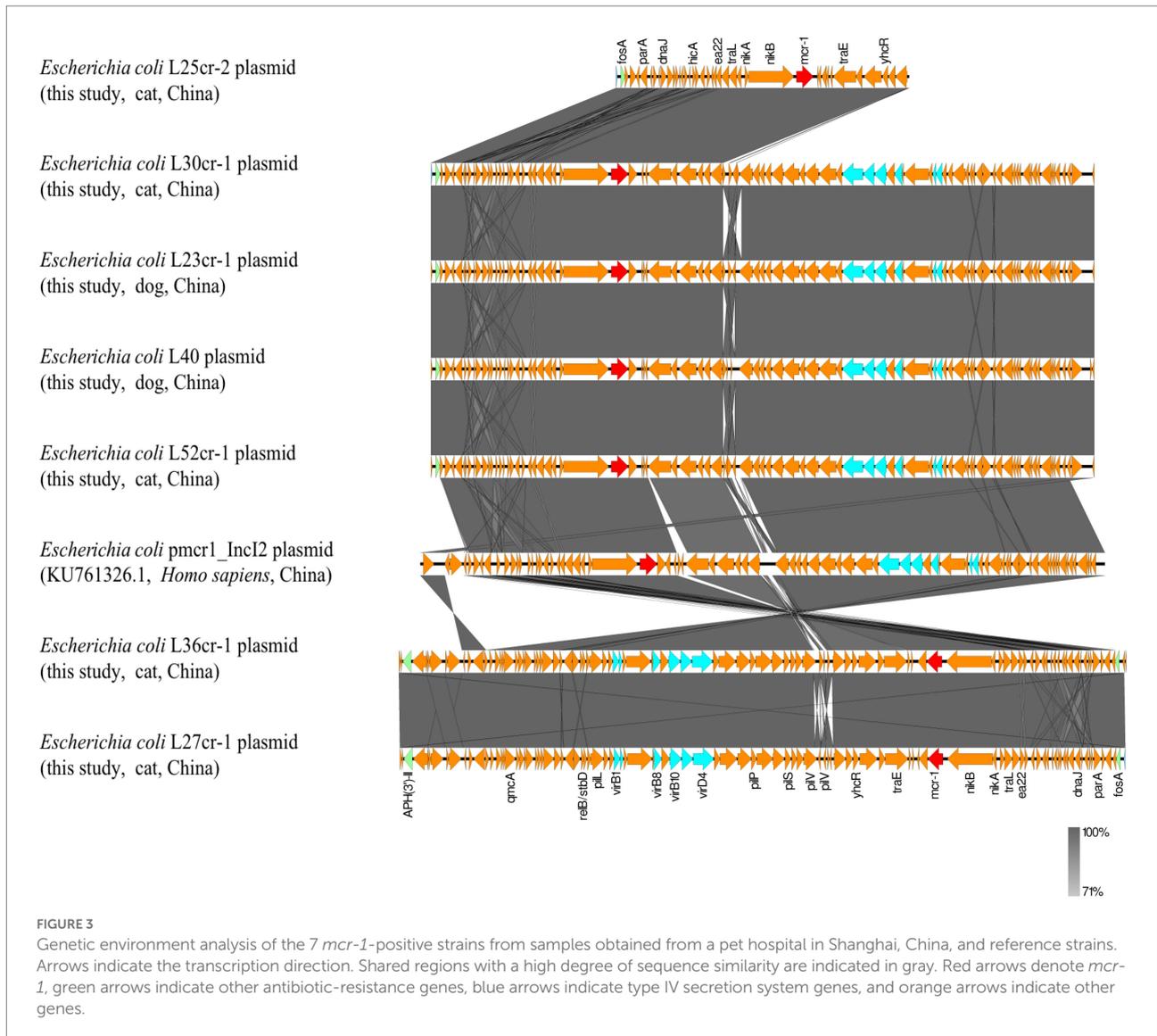
Whole-genome sequencing analysis revealed that all MCRPEC strains carried additional resistance genes (Figure 1). In addition to *mcr-1*, aminoglycoside resistance genes [*aac(3)-IVa*, *aph(3')-IIa*, *aph(3')-Ia*, and *aph(4)-Ia*], a trimethoprim resistance gene (*dfrA14*), fosfomycin resistance gene (*fosA*), tigecycline resistance genes (*tet(A)* and *tet(J)*), and sulfonamide resistance genes (*sul1* and *sul2*) were present in the seven isolates. All strains contained the β-lactamase gene (*bla_{CTX-M-65}*) and the florfenicol resistance gene (*floR*) (Figure 1).

The seven isolates were screened for virulence factor genes using the VirulenceFinder tool (version 2.0); the associated gene profiles are presented in Supplementary Figure S1. Many virulence genes, namely fimbrial-related genes (*papB/C/D/E/F/G/H/I/J/K*

and *yfcV*), heme absorption-related genes (*chuT/U/V/W/Y*), a secretion system gene (*espL1*), and vacuolating autotransporter toxin gene (*vat*), as well as those encoding the siderophore receptor (*fyuA*), aerobactin synthetases (*iucA/B/C/D* and *iutA*), common pili (*ecpA/B/C/D/E/R*), and type I fimbriae (*fimA/B/C/D/E/F/G/H/I*) were present in all seven MCRPEC strains. However, tetracycline resistance genes [*tet(A)* and *tet(J)*], and *vat* were only isolated in the five cat samples.

Discussion

Reports on the emergence of AMR, particularly colistin-resistance, are increasing worldwide (Shafiq et al., 2019). Meanwhile, the close contact between humans and companion animals may lead to bacterial horizontal transmission. However, the presence of ARGs in companion animals was not previously investigated in-depth (Chen et al., 2019). Nevertheless, *mcr-1* has previously been detected in companion animals in China, with an average detection rate of 6.9% (Zhang et al., 2016, 2021; Lei et al., 2017, 2021; Wang et al., 2018). *E. coli* is a common pathogen



Importantly, all antibiotics tested, herein, excluding florfenicol, are also commonly administered to humans, which provides high selection pressure for MDR bacteria. However, in this study, although the L36cr-1 isolate harbored several resistance genes, the host animal (a cat) was only 1 month old and had not yet received any antibiotics. Hence, this animal may have become infected with this isolate *via* nosocomial or community contact, as the host animal lived in a city and moved around frequently.

All seven MCRPEC isolates had lost the *ISApI1* sequence on both sides of *mcr-1*, resulting in a structure lacking *ISApI1*, which may further reduce chromosomal integration, thereby increasing the stability of *mcr-1* in plasmid vectors and facilitating its widespread dissemination (Du et al., 2020; Mohsin et al., 2021). This finding, combined with the plasmid conjugation assay results, suggests that other mobile genetic elements (such as the type IV secretion system, *pil*, and *ter*) and *mcr-1* may have played an important role in its horizontal transmission in the seven strains. In fact, all seven strains

harbored a type IV secretion system gene. Plasmid-mediated conjugated antibiotic-resistance and virulence gene transfer between different bacterial genera requires the type IV secretion system, wherein type IV pili enhance conjugative plasmid transfer (Christie, 2016; Kohler et al., 2018). Hence, the type IV secretion system can lead to an explosive emergence of multidrug resistance among populations of clinically significant pathogens (Cascales and Christie, 2003). Therefore, the surveillance of bacteria containing type IV secretion systems must be emboldened.

In a previous study (Li et al., 2016), we found a similar segment (*nika-nikB-mcr-1-pap2*) among plasmids of strains from both humans and animals, further supporting the hypothesis that *mcr-1* can readily spread between humans and companion animals. Furthermore, all plasmids had the same ST, indicating that *mcr-1* was likely derived from a single source, and suggesting the occurrence of *E. coli* clonal and vertical transmission in this area. The coexistence of horizontal and vertical transmission in

these seven MCRPEC strains may increase the risk of *mcr-1* transmission, thereby posing a threat to human health.

This study had certain limitations, our experiment was limited to detecting the prevalence of *mcr-1* in a small area. Nevertheless, these results highlight the need for urgent implementation of a wide-range surveillance plan in other regions of China. Furthermore, other important emerging resistance genes in companion animals should be monitored to better quantify their risk to human health.

In summary, the observed spread of *mcr-1* in companion animals could be attributable to the horizontal and vertical transmission of plasmids. We demonstrated that MCRPEC from companion animals represents a potential risk to human health by highlighting the possible transmission of *mcr-1*. This gene confers colistin-resistance to *E. coli*, thereby limiting clinical treatment options for humans. To prevent the spread of *mcr-1* from having a negative impact, minimizing the opportunities for *mcr-1*-harboring strains to infect and proliferate in humans is necessary. Therefore, surveillance programs should be implemented globally to monitor the prevalence of MCRPEC in companion animals, standards for rational antibiotic use in companion animals must be established, and pet owners must be encouraged to adhere strictly to veterinary-directed antibiotic regimens.

Data availability statement

The genome assemblies of the seven MCRPEC strains were deposited in GenBank under the BioProject accession number PRJNA812731.

Author contributions

XC and JL: conceptualization and methodology. HL, WC, and JZ: investigation. HL, RZ, and SF: data curation and formal

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analysis. HL: writing – original draft. XC, HL, ZS, JY, YW, and GW: writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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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.2022.1002827/full#supplementary-material>

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