Epidemiology and Molecular Characteristics of mcr-9 in *Citrobacter* spp. from Healthy Individuals and Patients in China

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**ABSTRACT** With the globally prevailing carbapenemase-producing (CP) *Citrobacter* spp., polymyxin antibiotics have been reconsidered as one of the last-resort treatment options. Our study was conducted to investigate the prevalence of mcr-9 in *Citrobacter* species. From October to November 2021, 650 fecal samples and 215 *Citrobacter* isolates were collected from healthy individuals and infected patients, respectively. Isolates were screened for the presence of the mcr-9 gene by the PCR method. mcr-9-carrying strains were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Due to the susceptibility to colistin, *Citrobacter* spp. isolates were first induced to increase the expression of mcr-9 on China blue agar plates containing colistin and were then subjected to conjugation experiments. Whole-genome sequencing was performed on the Illumina NovaSeq PE150 system. The prevalence of mcr-9 in the *Citrobacter* genus from healthy guts and infected patients was 0.62% and 1.86%, respectively. In all mcr-9-positive strains, MICs of polymyxin B were observed at ≤2 μg/mL, displaying a nonresistant phenotype. As for conjugation experiments, only one isolate successfully transferred the mcr-9 gene to *Escherichia coli* C600. Whole-genome sequencing showed that eight mcr-9-positive *Citrobacter* isolates carried mcr-9 and genes encoding resistance to beta-lactam antibiotics, including *blaCTX-M*, *blaKPC*, *blaTEM*, and *blaSHV*. We also discovered that mcr-9 could be located on the pKPC-CAV1321 plasmid. Our study investigated the prevalence of mcr-9 in *Citrobacter* spp. in both healthy individuals and infected patients and described the carriage of mcr-9 on the pKPC-CAV1321 plasmid for the first time.

**IMPORTANCE** The emergence of mcr homologues posed a serious threat to the therapeutic efficiency of polymyxin antibiotics. *Citrobacter freundii* is generally regarded as an opportunistic pathogen associated with a variety of nosocomial infections. In this study, we investigated the prevalence of mcr-9 in *Citrobacter* spp. isolates from healthy individuals and infected patients and highlighted the importance of the rational use of antibiotics. In addition, this epidemiological investigation is the first to describe the carriage of mcr-9 on plasmid pKPC-CAV1321 and confirms the horizontal transfer of this plasmid. Our research may shed new light on further studies of mcr-9 dissemination in humans.

**KEYWORDS** mcr-9, *Citrobacter* spp., colistin, plasmid, *Citrobacter freundii*
of a variety of opportunistic infections involving urinary tract, respiratory tract, and wound infections (1–3). Recently, the escalating increase in multidrug-resistant (MDR) strains, particularly carbapenemase-producing (CP) C. freundii, poses a serious threat to public health on an international scale (4, 5). Due to the lack of effective antibiotics, polymyxin (colistin and polymyxin B), a neglected antibiotic, returned to the spotlight as one of the last resorts against serious infections caused by MDR strains (6).

In 2016, mcr-1, the first mobile colistin resistance gene was first reported in humans and food animals (7). It not only presented an enormous challenge to the therapeutic efficiency of colistin but also caused a global panic over antibiotic barrenness (8). To date, 10 plasmid-borne mcr homologues (mcr-1 to mcr-10) have been detected in multidinous genera of Enterobacterales from animals, humans, and the environment (9, 10). Among them, mcr-9 is the second most widely spread gene, following mcr-1, and has been identified in 40 countries across six continents (11).

The human gut is considered as a reservoir of resistance genes and acts an important role in horizontal gene transfer. According to the reports from Wang et al., the prevalence of mcr-1-positive Escherichia coli in healthy individuals was as high as 14.3% in 2016. Since China banned colistin as an animal feed additive in 2017, the prevalence of mcr-1 has displayed a marked decline ($P < 0.0001$) (12).

Considering the importance of mcr genes, we carried out the mcr-9 screening in fecal samples and clinical isolates from healthy individuals and patients, respectively, to investigate its prevalence and gain an insight into the microbiological features of mcr-9-carrying Citrobacter spp. from both healthy individuals and patients.

**RESULTS**

**Prevalence of mcr-9-carrying Citrobacter spp.** A total of eight mcr-9-positive strains were initially identified as C. freundii by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF), but two of strains were further confirmed to be Citrobacter portucalensis using average nucleotide identity (ANI). Three C. freundii strains and one C. portucalensis strain were from healthy individuals, and the others were from infected patients (Table 1). Of the 215 Citrobacter spp. isolated from the infection samples, 4 (1.86%) were confirmed to carry mcr-9, which was higher than the prevalence of mcr-9-carrying Citrobacter spp. from healthy guts (0.62%, 4/650), but no significant difference was observed via Chi-square test ($P = 0.214,$ $>0.05$).

**Antimicrobial susceptibility testing and conjugation experiments.** According to CLSI breakpoints, all Citrobacter spp. isolates verified to carry mcr-9 are nonresistant to polymyxin B (Table 2), and the MICs of these strains were $<2 \mu g/mL$. None of the 56 healthy human intestinal isolates were mcr-9-positive.

**TABLE 2** Antimicrobial susceptibility testing results of eight mcr-9-positive Citrobacter spp. ($\mu g/mL$)

| Group                      | Strain | IPM | MEM | ETP | CMZ | CAZ | CTX | TGP | SCF | CAV | FEP | PB | TGC | CIP | AK | ATM |
|----------------------------|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|----|-----|
| Clinical isolates          | ZY-5   | $\leq 1$ | $\leq 1$ | $\leq 2$ | 128 | $>128$ | 16  | 16/4| $\leq 8/4$ | $\leq 0.5/4$ | $\leq 4$ | $\leq 4$ | 4   | 16  | 8   | 128 |
|                           | wm52   | $\leq 1$ | $\leq 1$ | $\leq 2$ | 32  | 64  | 128 | $\leq 8/4$ | $\leq 0.5/4$ | $\leq 4$ | $\leq 1$ | 0.5 | $\leq 1$ | 8   | 64  |
|                           | 21435  | $\leq 1$ | $\leq 1$ | $\leq 2$ | 16  | $\leq 2$ | $\leq 4$ | $\leq 8/4$ | $\leq 0.5/4$ | $\leq 4$ | $\leq 0.5$ | 1   | $>32$ | $\leq 4$ | $\leq 4$ |
|                           | F1-34  | $\leq 1$ | $\leq 1$ | $\leq 2$ | 32  | $>128$ | 32  | 64/4| $\leq 16/8$ | $\leq 0.5/4$ | $\leq 4$ | $\leq 0.5$ | 1   | $\leq 1$ | $\leq 4$ |
| Healthy human intestinal isolates | 56    | $\leq 1$ | $\leq 1$ | $\leq 2$ | 64  | $\leq 2$ | 8   | $\leq 8/4$ | $\leq 8/4$ | $\leq 0.5/4$ | $\leq 4$ | 1   | 0.5 | 2   | $\leq 4$ |
|                           | 82     | $\leq 1$ | $\leq 1$ | $\leq 2$ | 16  | $\leq 2$ | $\leq 4$ | $\leq 8/4$ | $\leq 8/4$ | $\leq 0.5/4$ | $\leq 4$ | 0.5 | $\leq 1$ | 8   | $\leq 4$ |
|                           | 146    | $\leq 1$ | $\leq 1$ | $\leq 2$ | 32  | $\leq 2$ | $\leq 4$ | $\leq 8/4$ | $\leq 8/4$ | $\leq 0.5/4$ | $\leq 4$ | 1   | 0.5 | $\leq 1$ | $\leq 4$ |
|                           | 156    | $\leq 1$ | $\leq 1$ | $\leq 2$ | 32  | $\leq 2$ | 16  | $\leq 8/4$ | $\leq 8/4$ | $\leq 0.5/4$ | $\leq 4$ | $\leq 0.5$ | $\leq 0.25$ | $\leq 1$ | $\leq 4$ |

$^{a}$IPM, imipenem; MEM, meropenem; ETP, ertapenem; CMZ, cefmetazole; CAZ, cefazidime; CTX, cefotaxime; TGP, piperacillin/tazobactam; SCF, cefoperazone/sulbactam; CAV, ceftazidime/avibactam; FEP, ceftepime; PB, polymyxin B; TGC, tigecycline; CIP, ciprofloxacin; AK, amikacin; ATM, aztreonam.
isolates were resistant to carbapenem antibiotics, including imipenem, meropenem, and ertapenem. The conjugation experiments were carried out for eight mcr-9-positive *Citrobacter* spp. isolates, while only one isolate, number 156, recovered from healthy individuals, successfully delivered the mcr-9 to *E. coli* EC600.

**Genetic analysis of mcr-9-positive *Citrobacter* spp.** The genetic characteristics of the eight mcr-9-positive isolates are presented in Table 3. Compared with isolates from healthy people, *Citrobacter* spp. from infected patients presented more abundant resistance genes and plasmid types. All of the eight strains were found to carry mcr-9 and genes encoding resistance to beta-lactam antibiotics, including *bla*<sub>CMY</sub>, *bla*<sub>DHA</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>TEM</sub>. In addition, aminoglycoside (aac) and sulfonamide (sul1) resistance genes were detected in all four *Citrobacter* spp. strains from patients.

Furthermore, mcr-9-positive *Citrobacter* spp. isolated from sites of infection, except for one isolate, carried IncHI2 and IncHI2A replicons, among which IncHI2 is the predominant replicon type reported to carry mcr-9 (13). The exception was isolate F1-34, which contained the pKPC-CAV1321 type plasmid rather than IncHI2 or IncHI2A. Surprisingly, the majority of *Citrobacter* spp. isolated from healthy guts appeared to carry the pKPC-CAV1321 plasmid but not IncHI2 or IncHI2A. In addition, no plasmid was observed in isolate number 56.

**DISCUSSION**

In this study, we describe the prevalence of mcr-9 in *Citrobacter* spp. from both healthy individuals and infected patients. Several scattered mcr-9-carrying *C. freundii* isolates were reported in animal and patient samples in previous studies (14–16), but information regarding clinical epidemiology and mechanisms of resistance to colistin in *Citrobacter* genera is lacking. According NCBI databases ([https://www.ncbi.nlm.nih.gov/pathogens/isolates/#AMR_genotypes:mcr-9](https://www.ncbi.nlm.nih.gov/pathogens/isolates/#AMR_genotypes:mcr-9)), isolates of *C. freundii* carrying mcr-9 have been detected in 12 countries across 4 continents as of March 2022, including two isolates in China. However, no reports of the mcr-9 gene located in *C. portucalensis* were reported. *C. portucalensis* is a novel species of the genus *Citrobacter* that was closely related to *C. freundii* and first isolated from an aquatic sample in Portugal in 2017 (17).

It is of great significance to detect *Citrobacter* isolates carrying mcr-9 in both healthy people and infected patients. In recent years, MDR *Citrobacter* spp. attracted increasing attention, since infections caused by these bacteria were always life-threatening and difficult to treat. What’s more, *C. freundii* is a commensal of the intestinal tract of humans and animals and plays an important role in carrying and transferring various resistance genes.

Although the majority of previously reported mcr-9-carrying isolates, including eight *Citrobacter* spp. isolates in this study, were not resistant to colistin (18), it has been proved that the expression of mcr-9 could be inducible by subinhibitory concentrations of colistin in the presence of qseB and qseC genes, the MIC levels were therefore increased (19). This suggests that the clinical use of colistin may induce resistance to colistin in mcr-9-positive isolates and accelerate the dissemination of mcr-9 among potential
pathogens. Subsequently, the emergence of colistin-resistant strains will further limit the clinical antibiotic options, resulting in more serious global resistance. It is suggested that rational use of antibiotics is crucial to reduce microbial resistance.

IncHII plasmids were the predominant replicon type carrying mcr-9 (13) and could increase the dissemination of mcr-9 in carbapenem-resistant Enterobacteriaceae (CRE) (20). Surprisingly, our data suggested that mcr-9 can also be located on the type of pKPC-CAV1321 plasmid. To the best of our knowledge, our study is the first report of such a gene on plasmid pKPC-CAV1321. As described above, only isolate 156 successfully transmitted the mcr-9 gene to E. coli EC600 via conjugation experiment, and the conjugant was also tested by whole-genome sequencing. The WGS analysis of the conjugant indicated that the mcr-9 gene of isolate 156 was indeed located on the pKPC-CAV1321 plasmid. Our finding suggested that mcr-9 can be transferred into other microbial pathogens along with this plasmid, thereby accelerating the spread of mcr-9.

In addition, analysis of the plasmids showed that mcr-9 spread in Citrobacter spp. may be related to the antibiotic environment where the isolates were grown. For clinical isolates, transmission of mcr-9 is largely by means of IncHII and IncHIIA among multidrug-resistant pathogens, as these superplasmids may carry a large number of resistance genes required for survival. However, spread of mcr-9 in Citrobacter spp. among healthy person possibly was connected with plasmid pKPC-CAV1321.

Our study investigates the prevalence of mcr-9 in the genus Citrobacter from both healthy individuals and patients and reports the carriage of mcr-9 on plasmid pKPC-CAV1321 for the first time.

MATERIALS AND METHODS

Sample collection. From October to November 2021, a total of 650 fecal samples were collected from healthy individuals. Fecal samples were collected from subjects who underwent routine physical examinations within 3 days, excluding those with gastroenteritis or chronic diseases. A total of 215 Citrobacter isolates were collected from infected patients. Infected patients were those who had been diagnosed by a doctor with a bacterial infection in at least one body system or region, including sputum, secretions, urine, blood, feces, and tissues.

After being enriched in LB broth at 37°C for 6 to 8 h, all fecal samples were screened for the mcr-9 gene by PCR using previously published primers (21), and the clinical isolates obtained from infected patients were directly screened for the mcr-9 gene using the PCR method. PCR-positive samples were further purified and subjected to verification of mcr-9 by Sanger sequencing. Next, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF; Bruker, Germany) was performed to confirm the identification of mcr-9-carrying isolates.

Antimicrobial susceptibility testing. The broth microdilution method was used to examine the sensitivity of mcr-9-positive isolates to common antibiotics, including imipenem, meropenem, ertapenem, cefmetazole, cefazidime, cefotaxime, piperacillin/tazobactam, cephalazin, aztreonam, ciprofloxacin, amikacin, and gentamicin. The MICs of most antimicrobial agents, with the exception of tigecycline, were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (22). Results of tigecycline were judged with reference to the breakpoints of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (https://www.eucast.org/). Based on the MIC interpretation criteria of the CLSI guidelines, isolates with a MIC of ≤2 μg/mL were classified as polymyxin B intermediate and those with a MIC of ≥4 μg/mL as polymyxin B resistant.

Conjugation experiments. Considering that mcr-9-positive isolates showed a nonresistant phenotype but could be induced in the colistin-containing plate (19), we induced the expression of mcr-9 by subculturing successive generations onto a China blue agar plate containing colistin to increase the MIC levels. Conjugation experiments were carried out between mcr-9-carrying Citrobacter spp. isolates, the MICs of which were induced to 2 μg/mL, and rifampin-resistant Escherichia coli C600. The donor was a Citrobacter spp. with EC600 as a recipient. Conjugants were selected on Mueller-Hinton (MH) agar plates supplemented with 600 μg/mL rifampicin and 1.5 μg/mL colistin.

Whole-genome sequencing. Genomic DNA was separated from mcr-9-positive isolates with the PureLink genomic DNA minikit (Invitrogen, USA), following the instructions provided, and sequenced on the Illumina NovaSeq PE150 system by Novogene, China. The short-read data were assembled using SPAdes v3.15.4 (http://cab.spbu.ru/software/spades/). Identification of antibiotic resistance genes and plasmid replicon typing were conducted using the Center for Genomic Epidemiology website (http://www.genomicepidemiology.org/).

Data availability. The data sets presented in this study can be found under BioProject number in online repositories. The names of the repository/repositories and accession numbers can be found below: PRJNA827125.
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