Infection and Drug Resistance

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ORIGINAL RESEARCH

Prevalence and molecular characteristics of *mcr-1* colistin resistance in *Escherichia coli*: isolates of clinical infection from a Chinese University Hospital

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Background: Colistin has been considered as one of the most effective treatments in clinical infections, especially multidrug-resistant (MDR) bacteria-infected patients. The *mcr-1* gene leads to polymyxin resistance in China. The present study investigated the prevalence of *mcr-1* in a Chinese teaching hospital, and the molecular phenotypes of the positive strains were analyzed.
Methods: A total of 1,112 *Escherichia coli* strains were collected from a Chinese University Hospital from January 2015 to January 2016. The *mcr-1* gene was detected by PCR. All positive specimens were subjected to susceptibility testing, clinical analysis, phylogenetic analysis, DNA Southern blot hybridization, and gene sequencing.
Results: Six (0.6%) strains of *mcr-1*-positive *E. coli* were susceptible to imipenem, meropenem, and tigecycline, except for one that presented moderate levels of tigecycline resistance. The six isolates were resistant to cefotaxime and cefepime and divided into six types of sequences. These positive strains carried a total of three plasmids: approximately 33, 61, and >92 kb plasmids. All patients were eventually cured using different types of antibiotics and discharged.
Conclusion: The current study showed that the *mcr-1* gene was responsible for the majority of colistin resistance in clinical isolates of *E. coli*. The gene can be transferred into plasmids containing other drug resistance genes by plasmid–DNA conjugation, which might cause severe consequences in drug-resistant strains. Thus, the widespread popularity of *mcr-1* gene should be prevented.

Keywords: *mcr-1*, plasmid, colistin, clinical infections

Background

The rapid increase in infections caused by antibiotic-resistant Gram-negative bacteria, such as carbapenem-resistant Enterobacteriaceae, has raised great concern worldwide. Colistin was used as an alternative therapy to treat infections caused by multidrug-resistant (MDR) Gram-negative bacteria.[1] Colistin was one of the few options for such type of bacterial infections. As colistin is currently used for treating human or animal infections, an increasing number of colistin and drug resistance mechanisms are reported.[2,3] Nevertheless, the prevalence of the colistin-resistant issue has gained worldwide attention. *Mcr-1* genes were first reported as mediated by plasmid and can be spread between different bacteria through plasmids. Previously, the primary mechanism underlying colistin resistance stated that the bacterial chromosome coding of the binary regulatory system pmr AB and pho PQ and the associated regulatory gene *mgr B* mutations led to altered lipid A modification, thereby reducing the affinity of bacteria for polymyxin.[4] In 2015, some investigators identified a novel mechanism mediating the low level of resistance to colistin.[5] These resistant strains carry a novel gene *mcr-1* encoding phosphoethanolamine transferase that

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reduces the affinity of colistin to lipopolysaccharide, which was identified on an IncI2 plasmid and pHNSHP45, isolated from an *Escherichia coli* isolate from a pig in China; thus, these bacteria are not deemed sensitive to colistin.[6,7] Currently, the *mcr-1* gene has been detected in approximately 40 countries. The distribution of these plasmids among carbapenem-resistant organisms might produce "super bacteria."

Herein, we aimed to determine the prevalence of *mcr-1* gene in a Chinese-teaching hospital and understand its molecular characteristics. Furthermore, we assessed the prognostic impact of this gene on clinical patients and the drug resistance of patients infected with *mcr-1*-positive strains. Hence, monitoring the resistance of colistin, delaying the spread of bacterial resistance, and providing effective advice for clinical treatment are essential.

Methods

Clinical bacterial isolates

The clinical isolates were collected from the Microbiology Laboratory of Anhui Medical University Union Hospital (Heifei, Anhui, China) from January 2015 to 2016, which is the largest tertiary hospital in Anhui Province, Central China. The inclusion criteria were distinct for the diagnosis of bacterial infections. All the isolates were identified using the Gram-negative bacteria identification card of the Vitek system (BioMèrieux, Missouri, France).

Extract template DNA

Several monoclonal bacterial colonies were suspended in 100 μL of sterile distilled water and heated at 100°C for 10 minutes and centrifuged at 10,000× *g* for 10 minutes. The resulting supernatant contained the bacterial genomic DNA.

mcr-1 screening

The primers for the PCR amplification of *mcr-1* gene were as follows: mcr-1-forward (5′-GCTCGGTCAGTCCGTTTG-3′) and mcr-1-reverse (5′-GAATGCGGTGCGGTCTTT-3′). A total of 35 cycles were conducted as follows: 94°C predenaturation for 5 minutes, 94°C denaturation for 1 minute, 55°C annealed 30 seconds, and 72°C extension for 1 minute. The PCR products were analyzed by agarose gel electrophoresis (Figure 1), and the positive products were sequenced.

Molecular typing

Pulsed-field gel electrophoresis (PFGE) was used for the molecular typing of six *mcr-1*-positive strains. The bacteria were embedded in SeaKem Gold Agarose (Lonza, Rockland, MD, USA), and the genome was subjected to restriction

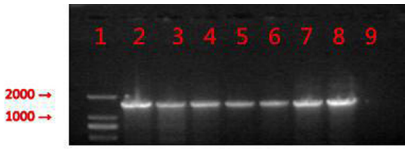

Figure 1 PCR products from six *mcr-1*-positive isolates.
Notes: 1: marker, DL2000; 2: positive control; 3: E4857; 4: E6512; 5: E0964; 6: E2069; 7: E9497; 8: E1825; and 9: negative control.

cleavage using *XbaI*. Subsequently, the DNA fragments were electrophoresed by the CHEF-mapper XA PFGE system (Bio-Rad Laboratories Inc., Hercules, CA, USA) for 22 hours at 14°C, 6 V/cm, and from 5 to 35 seconds pulses. The PFGE patterns were compared using BioNumerics Version 7.6. Furthermore, whole genome sequencing was used for determining the molecular typing and all the resistance genes.

mcr-1-resistant gene detection and analysis

The *mcr-1* DNA was digested using the S1 nuclease (Takara, Otsu, Japan), and then, the genomic DNA resolved by PFGE system for 18 hours at 14°C with 2.16–63.8 seconds pulses. The DNA fragments were transferred to nylon membrane (EMD Millipore, Billerica, MA, USA) that was hybridized with digoxigenin-labeled *mcr-1*-specific probes in the hybridization oven at 40°C overnight. The NBT/BCIP color detection kit (Hoffman-La Roche Ltd., Basel, Switzerland) was used for staining the nylon membrane after hybridization.

We used filter mating to investigate whether the *mcr-1*-resistant gene can be transferred. *E. coli* EC600 with rifampicin resistance was employed as the recipient strain; all the *mcr-1*-positive isolates were tested, ratio of the donor and recipient strains was 1:1, and the agar with colistin (4 mg/L) and rifampicin (700 mg/L) was utilized for selection. The transconjugants were demonstrated as *mcr-1* positive by PCR and further validated by PFGE. Then, the plasmids from *mcr-1*-positive transconjugants were extracted. Next, a strain of *Klebsiella pneumoniae* was preserved at the Department of Microbiology of our hospital. The growth did not produce mucus on the plate and resistance to imipenem; *K. pneumoniae* served as the recipient strain. The recipient bacteria were mixed with the extracted plasmid in a ratio of 10:1, and the electrotransformation mixture was set at a voltage of 2.1 kV. The electrotransformed mixture was incubated in the broth for 3 hours and allowed to grow overnight on plates containing polymyxin (2 mg/L).

Antimicrobial susceptibility testing (AST)

All mcr-1-positive specimens were tested for AST. The antimicrobial agents tested included meropenem, imipenem,

tigecycline, colistin, aztreonam, amikacin, levofloxacin, cefoperazone-sulbactam, cefotaxime, cefepime, and trimethoprim/sulfamethoxazole. The Clinical and Laboratory Standards Institute (CLSI) was used to explain the results of AST.[8] *E*-test was used for supplementing the susceptibility testing, and the results were interpreted based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints.[9] *E. coli* 25922 was used as a standard quality control strain.

Gene sequence analysis

Positive strains were analyzed by whole-genome sequencing.

Clinical data collection

The clinical data including specimen type, age, sex, infection type, and clinical outcomes were collected from patients. These patients exhibited a clear infection during hospitalization. Also, they were treated with antibiotics during the hospital stay. The cases of these patients were analyzed retrospectively.

Results

In this study, we collected 1,112 nonduplicate clinical infection isolates from patients. Of these isolates, six (0.6%) strains carried the *mcr-1* gene, which was similar to that reported previously.[10–13] These *mcr-1*-positive isolates were collected from five different departments. The age of the patients ranged from 30 to 70 years, except for one of the patients, who was only 3 months old. The baseline data of the patients with *mcr-1*-positive *E. coli* infection are shown in Table 1. In addition, AST was estimated in all positive isolates and the MIC of colistin ranged from 4 to 16 mg/L, indicating a low level of resistance to polymyxin. Of the six *mcr-1*-positive isolates, a majority were susceptible to tigecycline, amikacin, cefoperazone sulbactam, meropenem, and imipenem, while only one was susceptible to levofloxacin, aztreonam, and trimethoprim/sulfamethoxazole, and all isolates were resistant to polymyxin, cefotaxime, and cefepime. The characteristics of *mcr-1*-positive isolates are shown in Tables 1–3.

Table 1 Clinical information of six patients with *mcr-1*-positive specimens

Isolates	Department	Specimen	Gender	Age	Underlying diseases	Length of hospital stay (days)	Treatments used	Outcomes
E6512	Nephrology	Urine	Female	30 years	Diabetes, urinary tract infection	6	Cefoxitin, levofloxacin	Cured
E1825	Urinary surgery	Urine	Female	36 years	Pyelolithotomy, percutaneous nephrostomy	20	Cefoperazone sulbactam, imipenem, meropenem	Cured
E2069	Pediatric department	Sputum	Male	5 months	Severe pneumonia, mechanical ventilation	15	Ceftriaxone, erythromycin, imipenem	Cured
E9497	Burns surgery	Pus	Male	48 years	Hypertension, history of spinal surgery	40	Cefoperazone, sulbactam, vancomycin, imipenem	Cured
E0964	Urinary surgery	Urine	Female	62 years	Hypertension, urinary tract infection	9	Ceftriaxone, imipenem, rifamycin	Cured
E4857	Dermatology	Pus	Male	70 years	Hypertension, skin and soft tissue infections	24	Mezlocillin sulbactam, imipenem, gentamicin	Cured

Table 2 Characteristics of colistin-resistant and mcr-1-positive isolates

Isolates	Number of mcr plasmid	Location of mcr-1	Antimicrobials isolate was susceptible to	ST	MIC of colistin (mg/L)
E1825	1	Plasmid, 61 kb	Meropenem, imipenem, amikacin	ST 457	4
E2069	1	Plasmid, 33 kb	Tigecycline, levofloxacin, amikacin, meropenem	ST 160	4
E0964	2	Plasmid, 33 and 61 kb	Imipenem, Trimethoprim/sulfamethoxazole, cefoperazone sulbactam	ST 6706	8
E4857	2	Plasmid, 61 and >92 kb	Meropenem, imipenem, tigecycline, cefoperazone sulbactam, aztreonam	ST 2847	8
E6512	1	Plasmid, 61 kb	Meropenem, imipenem, tigecycline, cefoperazone sulbactam, amikacin	ST 156	8
E9497	1	Plasmid, 33 kb	Meropenem, imipenem, tigecycline, cefoperazone sulbactam, amikacin	ST 354	16

Note: In the first column, the first number represents the isolate of *E. coli* and the latter numbers represent the bacteria number.
Abbreviations: *E. coli*, *Escherichia coli*; ST, sequence type.

Table 3 Drug sensitivity test value of *mcr-1*-positive strain

Isolates	CTX	FEP	TGC	MEM	IPM	LEV	AK	CO	ATM	TS	CPS
E1825	>256	>256	4	0.125	0.25	256	2	4	>265	>32	48
E2069	>256	>256	0.5	<0.03125	0.25	<0.25	4	4	32	0.047	32
E0964	>256	>256	1	<0.03125	0.125	64	>256	8	32	>32	16
E4857	128	128	0.5	<0.03125	0.125	32	>256	8	6	>32	16
E6512	>256	>256	0.5	0.0625	0.125	4	16	8	64	>32	32
E9497	>256	>256	1	0.0625	0.125	32	8	16	48	>32	6

Note: The data in the table represent the MIC value of each drug.
Abbreviations: AK, amikacin; ATM, aztreonam; CO, colistin; CPS, cefoperazone sulbactam; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; LEV, levofloxacin; MEM, meropenem; TGC, tigecycline; TS, trimethoprim/sulfamethoxazole.

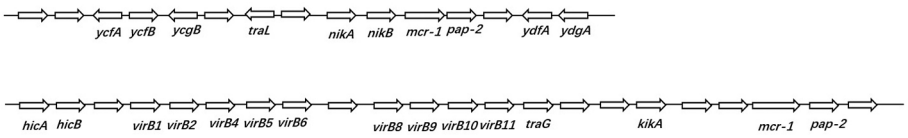

Figure 2 Two representative*mcr-1* gene environments.

Mcr-1 was usually found to be present in the bacterial genome with other resistance genes, such as *ESBL*.[23] Nevertheless, all the six positive isolates were susceptible to carbapenems and meropenem. Moreover, the whole genome sequencing did not show any coexistence of carbapenem-resistant genes with *mcr-1*. We also found that the ESBL genes, including *blaTEM-1B*, *blaCTX-M14*, *blaCTX-M132*, *blaCTX-M55*, and *blaCTX-M123* were contained in the genomes of these six strains that can lead to resistance to antibiotics, such as cephalosporins and penicillins. Notably, these strains contain other resistance genes: *fosA3*, *fosA*, *aph*(*4*)-*Ia*, *sul2*, *oqxA*, *oqxB*, and *aadA2*. A large number of studies have found that the transfer of the *mcr-1* gene was usually associated with the movable element *ISApl1-mcr-1*. Our sequencing analysis did not reveal significant differences around the *mcr-1* gene in the six *mcr-1*-positive strains, and two genetic environments were detected (Figure 2). We did not find the *IsApl1* insert upstream to the *mcr-1* gene, rather the *pap-2* gene was found downstream to the *mcr-1* gene. Thus, we speculated that the *ISApl1* gene may be recombined, resulting in only partial sequences remaining upstream of *mcr-1*.

XbaI-PFGE (Figure 3) showed that these *mcr-1*-positive strains were divided into six genotypes that indicated non-clonal transmission. The six *mcr-1*-positive strains were grouped into six distinct sequence types (STs) that were highly heterogeneous and a variety of plasmid type bacteria that carry *mcr-1*.

In the current filter mating study, two strains were successfully transferred to EC600 simultaneously, while the remaining four strains were tested two times, suggesting

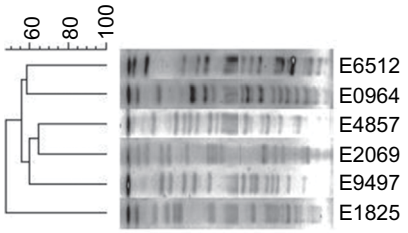

Figure 3 Cluster analysis of six strains of bacteria by *XbaI* digestion.

that all the *mcr-1* genes were harbored in the plasmids. After 1 week of continuous subculture, the transconjugants can still detect the mcr-1 gene after PCR verification, which proves that the plasmid carrying mcr-1 can be stable in the transconjugants. In the plasmid conjugation test, the transconjugants did not grow on the plates, which might be attributed to the low efficiency of electrotransformation. This phenomenon might be related to the bacterial characteristics of *K. pneumoniae*.

The results of the S1 nuclease digestion (Figure 4) and Southern blot analysis (Figure 5) showed that the *mcr-1* genes were localized on the following three different types of plasmids: approximately 33, 61, and >92 kb.

Discussion

The current study showed that the *mcr-1* gene could be detected in various *Enterobacteriaceae* bacteria, including *E. coli*, *K. pneumoniae*, *Salmonella*, and *Enterobacter cloacae*, and reported in several countries.[14–18] Some studies demonstrated that the *mcr-1* gene could be transmitted through the food chain model, leading to its widespread occurrence in poultry farming, and sources of water contaminated with

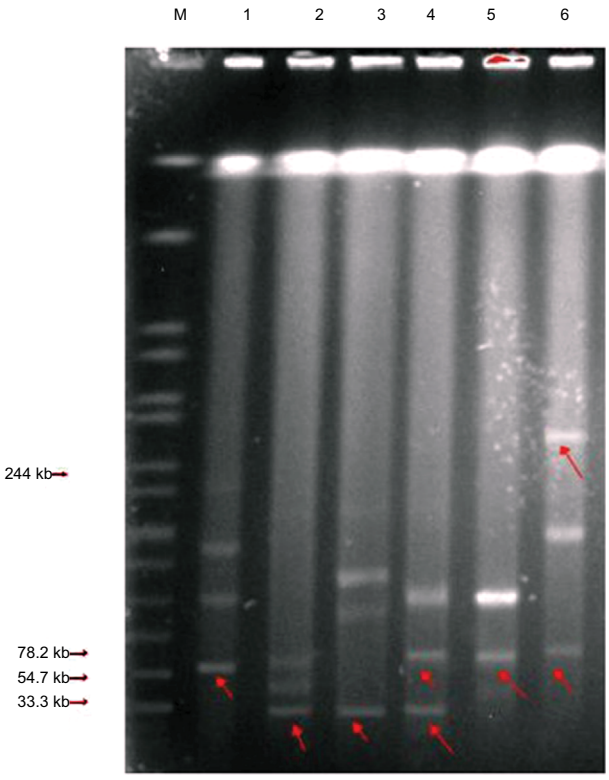

Figure 4 S1-digested plasmid DNA.
Notes: M: *Salmonella* H9812 (20.5–1135 kb); 1: E1825; 2: E9497; 3: E2069; 4: E0964; 5: E6512; and 6: E4857. The arrow in the figure represents the position of the *mcr-1* plasmid.

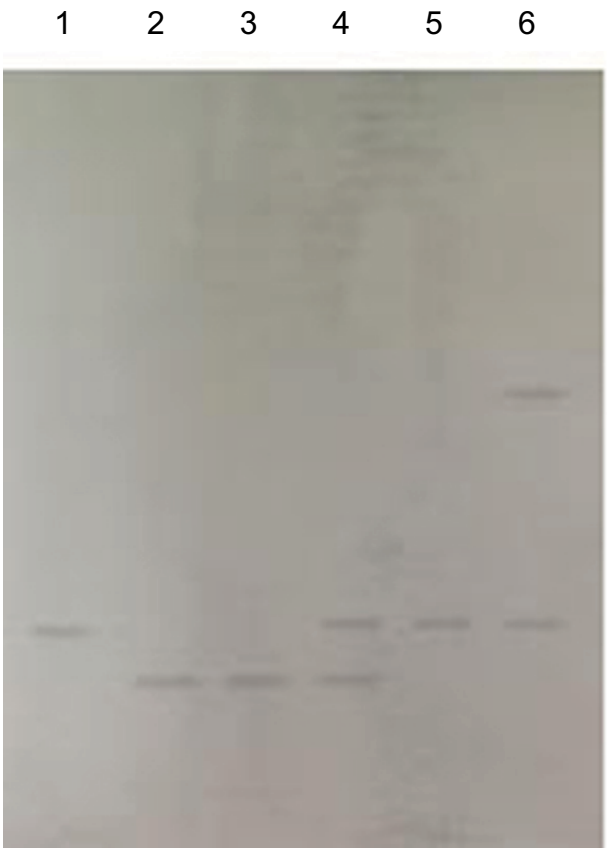

Figure 5 Southern blot hybridization of *E. coli* isolates with *mcr-1*.
Notes: 1: E1825; 2: E9497; 3: E2069; 4: E0964; 5: E6512; and 6: E485.
Abbreviation: *E. coli*, *Escherichia coli*.

mcr-1 can also be considered as another route of transmission beyond the chain.[5,12,19–21] The following two cases of *mcr-1*-positive *E. coli* were identified in the population: infected bacteria and fixed-value bacteria; the use of antibiotics and the patient's immunosuppression are the putative causes of the epidemic.[24] Currently, the detection of human *Enterobacter mcr-1* has been reported in various countries at a rate of 1–2%, which is significantly lower than that of the animal sources.[5,12] Usually, the *mcr-1* gene coexists with other drug-resistant genes, which is in agreement with the current study. Other drug-resistant genes copromote *mcr-1* that might indicate a selective role for mcr-1 and promote its epidemic spread; however, the underlying mechanism is not yet clarified. Based on the relevant research results, the lack of appropriate pressure selection did not prompt the occurrence of *mcr-1* in the human body for a prolonged period.[18]

More than 10 plasmids have been found to carry the *mcr-1* gene, including *IncX4*, *Incl2*, *IncP*, *IncX3-IncX4*, *IncFII*, *Incl2-IncFIB*, *IncFI*, *IncX1-IncX2*, *IncHI1*, and *IncHI2*. The most prevalent plasmids are IncHI2, Inc2, and Inc2, followed by IncX4 and other plasmids. The genome of these plasmids carries the *mcr-1* gene, as well as, other drug-resistant genes, leading to the cotransmission of multiple drug-resistant genes. The insertion sequence ISApl1 is often detected upstream or downstream of the *mcr-1* gene, and this mobile element causes the spread of *mcr-1* gene. Some studies have shown that the *mcr-1* gene can be inserted into other plasmids by its upstream and downstream looping in ISApl1.[22]

Nevertheless, the current study showed that the prevalence of colistin resistance was extremely low in *E. coli* based on the clinical infections in the hospital. This result was similar to that of the previous study.[5,10–13] However, we need to further expand the number of strains and the sample collection areas. The diversity of PFGE types and STs showed that all of these *mcr-1*-positive isolates were from sporadic cases, and this low prevalence might be the background of the presence of the *mcr-1* gene in microorganisms; however, additional data are essential for the substantiation of these findings. Hitherto, according to the results of Chinese studies, the *mcr-1* gene has a high prevalence rate in animals and a low prevalence rate in hospital patients.[10,25,26] Furthermore, in China, due to the large quantities of polymyxin used in agriculture and animal

husbandry, the spread of *mcr-1* gene between bacteria cannot be ignored. Although no *mcr-1*-positive *K. pneumoniae* was detected in the collected isolates in this study, a low level of *mcr-1* prevalence was detected in *K. pneumoniae*.[5,12,14–18] Considering that less clinical data are available for *mcr-1*, we aimed to establish an animal infection model to understand the correlation between the new resistance genes and other resistance genes and the threat to human infection.

mcr-1 can be transmitted between the natural environment, human body, and different strains through different transfer elements. Therefore, we can control the spread of mcr-1 by several effective measures. mcr-1 spreads in the hospital environment in the absence of colistin. Thus, patients with mcr-1 infection in the hospital, ward environment, medical equipment, as well as, doctors should be monitored promptly to administer antibiotics effectively in order to prevent the prevalence of *mcr-1* in the hospital.

The major concern for the clinicians is the *mcr-1* transfer into carbapenemase producers in a hospital setting, which could lead to the production of pandrug-resistant (PDR) strain.[27,28] Thus, effective measures are imperative to monitor the prevalence of multislime resistance, thereby preventing the further spread of bacterial resistance.

Conclusion

The current study showed that the *mcr-1* gene was responsible for the majority of colistin resistance in clinical isolates of *E. coli*. The gene can be transferred into plasmids containing other drug resistance genes by plasmid-DNA conjugation, which might cause severe consequences in drug-resistant strains. Thus, the widespread popularity of *mcr-1* gene should be prevented.

Ethical approval

All patients provided written informed consent, and the Ethics approval was obtained from the medical ethics committee of the First Affiliated Hospital of Anhui Medical University with the following reference number: Quick-PJ 2018-05-14.

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Author contributions

Prof Jilu Shen is responsible for the experimental design and is the corresponding author. Other authors are responsible for the experimental procedure and clinical data collection. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

None of the authors have any personal or financial involvement with the organizations that have financial interest in its content. The authors report no conflicts of interest in this work.

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