

Prevalence and molecular characteristics of *mcr-1* gene in *Salmonella typhimurium* in a tertiary hospital of Zhejiang Province

Jun Lu^{1,*}

Jingjing Quan^{2,3,*}

Dongdong Zhao²

Yanfei Wang^{2,3}

Yunsong Yu^{2,3}

Jin Zhu¹

¹Department of Laboratory, Quzhou People's Hospital, Quzhou, Zhejiang, China; ²Department of Infectious Diseases, College of Medicine, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou, Zhejiang, China; ³Key Laboratory of Microbial Technology and Bioinformatics of Zhejiang Province, Hangzhou, Zhejiang, China

*These authors contributed equally to this work

Objectives: *mcr-1* gene has been widely reported in the world. This study aimed to analyze the prevalence and molecular characteristics of *mcr-1* gene in *Salmonella typhimurium* from Quzhou People's Hospital.

Materials and methods: A total of 62 *S. typhimurium* isolates were isolated and preserved in our laboratory from 2007 to 2016. PCR was used to screen plasmid-mediated colistin resistance gene, *mcr-1*. For *mcr-1*-positive isolates, susceptibilities to colistin and other antibiotics were assessed using broth microdilution or agar dilution methods. The genetic location of *mcr-1* was determined by analysis of pulsed-field gel electrophoresis profiles of S1-digested genomic DNA and subsequent Southern blot hybridization. The multi-locus sequence type and other drug resistance genes found in the *mcr-1*-positive isolates were analyzed by performing whole genome sequencing. Genetic environment of *mcr-1* gene was analyzed by RAST and Easyfig.

Results: A total of three *S. typhimurium* isolates were identified to be *mcr-1* positive, with the prevalence rate of 4.8% (3/62). The minimum inhibitory concentration values of colistin for all these isolates were 8 µg/mL. The three *mcr-1*-positive isolates carried *mcr-1* gene on two different types of plasmids having the sizes of ~54.7–78.2 kb and 310.1 kb, respectively. All the three isolates belonged to ST34 and carried various resistant genes.

Conclusion: Colistin-resistant, *mcr-1*-positive *S. typhimurium* isolates belonging to ST34 have been isolated from Quzhou People's Hospital. Surveillance needs to be strengthened to identify colistin resistance and prevent the spread of drug-resistant bacteria in the hospital.

Keywords: *Salmonella typhimurium*, colistin, *mcr-1*

Introduction

Polymyxins, including polymyxin B and E (colistins), are generally regarded as the last-resort therapy for infections caused by multidrug resistant (MDR) Gram-negative bacilli, especially carbapenem-resistant Enterobacteriaceae.^{1,2} In recent years, polymyxin resistance has gained wide attention in the field of global medical research due to the increasing reports of polymyxin-resistant bacteria. In late 2015, Chinese researchers reported the first plasmid-mediated colistin resistance gene, *mcr-1*,³ and since then, it has been reported in other continents as well, including Asia, Europe, America, Africa, and Australia,⁴ as well as in several provinces (Zhejiang, Sichuan, Beijing, Guangdong, etc) in China.^{5–9} The discovery of *mcr-1* gene makes it possible for the horizontal transmission of polymyxin resistance in Enterobacteriaceae, which may in turn lead to a rapid increase in polymyxin resistance. So far, *mcr-1* gene has been found only in Enterobacteriaceae, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp.,

Correspondence: Jin Zhu
Department of Laboratory, Quzhou People's Hospital, 2 Zhongludi, Quzhou 324000, Zhejiang, China
Email qzhosp@163.com

Enterobacter spp., *Kluyvera ascorbata*, *Raoultella ornithinolytica*, *Citrobacter braakii*, etc.^{4,6,10,11}

Studies focusing on the *mcr-1* gene-carrying *E. coli* and *K. pneumoniae* strains are common, while the studies on prevalence and molecular characteristics of the *mcr-1* gene in *Salmonella* spp. are still lacking. *Salmonella typhimurium* is an important zoonotic pathogen that usually causes foodborne diseases, and humans, especially infants, are highly susceptible to this infection.¹² Furthermore, the occurrence of MDR isolates of *S. typhimurium* have become common now and have had great impact on the effectiveness of current strategies to prevent and control foodborne diseases.¹³ This study aims to analyze the prevalence and molecular characteristics of *mcr-1* gene in *S. typhimurium* strains isolated from Quzhou People's Hospital and provide a basis for the prevention and control of polymyxin resistance in the hospital strains of *S. typhimurium*.

Materials and methods

Bacterial isolates

A total of 62 *S. typhimurium* isolates were collected from Quzhou People's Hospital from 2007 to 2016. All the strains were isolated from patients who visited the hospital during the period. Isolates were identified using the automated Vitek 2 system (BioMérieux, Marcy l'Etoile, France).

Screening for *mcr-1* gene

All the isolates were screened for the presence of *mcr-1* gene by PCR with primers *mcr-1-F* (5'-GCTCGGTCAGTC-CGTTT-3') and *mcr-1-R* (5'-GAATGCGGTGCG-GTCTTT-3'), and positive amplicons were subsequently sequenced. All the sequences were further analyzed on the BLAST server (<http://blast.ncbi.nlm.nih.gov/>).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of colistin and other ten antimicrobial agents (including levofloxacin, ciprofloxacin, cefotaxime, cefepime, cefoperazone/sulbactam, imipenem, meropenem, amikacin, tigecycline, and aztreonam) were determined for the *mcr-1*-positive isolates by the broth microdilution method, while the MICs of fosfomycin and sulfamethoxazole/trimethoprim were evaluated by the agar dilution method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). The results were interpreted in accordance with the CLSI and European Committee on Antimicrobial Susceptibility Testing (for colistin, tigecycline, and fosfomycin) breakpoints (http://www.eucast.org/clinical_breakpoints).

Southern blot analysis

To determine the plasmid location of the *mcr-1* gene, genomic DNA digested with S1 nuclease (TaKaRa, Tokyo, Japan) was electrophoresed on a CHEF-mapper XA pulsed-field gel electrophoresis system (Bio-Rad Laboratories Inc., Hercules, CA, USA) for 22 hours at 14°C with run conditions of 6 V/cm and pulse times from 2.16 seconds to 63.8 seconds. The DNA fragments were transferred to a positively charged nylon membrane (EMD Millipore, Billerica, MA, USA) and then hybridized with a digoxigenin-labeled *mcr-1*-specific probe. The fragments were then detected using a NBT/BCIP color detection kit (Hoffman-La Roche Ltd., Basel, Switzerland).

Whole genome sequencing (WGS)

The total genomes of the *mcr-1*-positive isolates were extracted and then sequenced on an Illumina-Hiseq™ 2000 sequencing system (Illumina Inc, San Diego, CA, USA) using a paired-end 2×100 bp protocol. Sequence reads were assembled using CLC Genomics Workbench software package (CLC Bio 8.0, Aarhus, Denmark). The multi-locus sequence type (MLST) and other resistant genes were analyzed on the website of Center of Genomics Epidemiology (<http://www.genomicepidemiology.org/>). Gene prediction and annotation were performed using RAST (<http://rast.nmpdr.org/>). Sequence comparison was performed using BLAST (<http://blast.ncbi.nlm.nih.gov/>), and physical maps were generated by using Easyfig.¹⁴

Ethics approval

The clinical isolates were part of the routine hospital laboratory procedure. The Ethics Committee of the Quzhou People's Hospital approved this study as it mainly focused on bacteria, and not the patients.

Results

Epidemiology data and characteristics of *mcr-1*-positive isolates

A total of three *mcr-1*-positive isolates of *S. typhimurium* were identified (16–541, 16–573, and 16–623), with the prevalence rate of 4.8% (3/62). All the three strains were isolated from feces of patients with infectious diarrhea diseases in 2016. Two patients were infants and the other was 15 years old (male =1, female =2). No underlying diseases were found in all the three patients, and all of them were eventually cured (Table 1).

Table 1 Characteristics of *mcr-1*-positive isolates

Isolates	Age	Sex	Living place	Underlying disease	Outcome	Department	Samples	Isolated date
16-541	15 years	Female	Village	None	Cured	Gastroenterology department	Feces	8/27/2016
16-573	8 months	Female	City	None	Cured	Pediatric department	Feces	9/20/2016
16-623	10 months	Male	Village	None	Cured	Pediatric department	Feces	11/2/2016

Antimicrobial susceptibility of *mcr-1*-positive isolates

All the three *mcr-1*-positive *S. typhimurium* strains were resistant to colistin, with the MIC of 8 µg/mL. Moreover, they were all resistant to the third/fourth-generation cephalosporins (cefotaxime and cefepime) and sulfamethoxazole/trimethoprim, while imipenem, meropenem, amikacin, tigecycline, and fosfomycin showed great activity against these *mcr-1*-positive isolates (Table 2).

mcr-1 gene locations

S1-nuclease digestion and Southern blot analysis indicated that all three *mcr-1* genes were located on plasmids. *mcr-1* genes of isolates 16-541 and 16-623 were located on the same-size plasmids of ~310.1 kb, while the one of isolate 16-573 was located on a ~60 kb plasmid (Figure 1).

WGS analysis

The whole genomes of the three *S. typhimurium* strains were sequenced and analyzed.

Other mechanisms associated with antimicrobial resistance were analyzed by using ResFinder. In addition to *mcr-1*, all the three isolates carried a variety of resistance genes to different antibiotics, including aminoglycosides, β-lactams, fluoroquinolones, sulfonamides, tetracyclines, and trimethoprim. In addition, isolate 16-573 carried *fosA3* gene, which induced fosfomycin resistance, while isolates 16-541 and 16-623 carried *ARR-3* gene, which induced rifampicin resistance (Table 3).

Genetic environment of *mcr-1* gene

One of the three *mcr-1*-carrying plasmids (16-573) was greatly similar to pEc20COE13 (accession number: KY012274), which belonged to plasmid type IncI2 (Figure 2A). Although the *mcr-1* gene was found to be inserted at different locations of the plasmid, the *mcr-1* genes located on the contigs of isolates 16-541 and 16-623 were partly similar to the *mcr-1*-positive plasmid p803-DB-*mcr* (accession number: KY012274), which belonged to plasmid type

Table 2 MICs of *mcr-1*-positive *Salmonella typhimurium* isolates to colistin and other antibiotics (µg/mL)

Antibiotics	16-541	16-573	16-623
Colistin	8 (R)	8 (R)	8 (R)
Levofloxacin	4 (I)	0.25 (S)	4 (I)
Ciprofloxacin	8 (R)	0.125 (S)	8 (R)
Cefotaxime	>256 (R)	>256 (R)	>256 (R)
Cefepime	128 (R)	64 (R)	128 (R)
Cefoperazone/sulbactam	32 (I)	16 (S)	32 (I)
Imipenem	0.5 (S)	0.5 (S)	1 (S)
Meropenem	0.0625 (S)	0.0625 (S)	0.0625 (S)
Amikacin	8 (S)	4 (S)	16 (S)
Tigecycline	1 (S)	0.5 (S)	1 (S)
Fosfomycin	0.25 (S)	64 (R)	0.25 (S)
Sulfamethoxazole/trimethoprim	>32 (R)	>32 (R)	>32 (R)
Aztreonam	8 (I)	4 (S)	16 (R)

Abbreviations: MIC, minimum inhibitory concentration; I, intermediate; R, resistant; S, susceptible.

IncHI2 (Figure 2B). No IS*Apl1* elements were identified at the upstream or downstream of *mcr-1* gene.

Discussion

Since the first report of *mcr-1* gene, it has been widely reported all over the world, and can be isolated from animals, food, environment, and humans (patients and healthy people). Apart from the common strains like *E. coli* and *K. pneumoniae*, *mcr-1* gene has also been reported from *Enterobacter aerogenes* and *Enterobacter cloacae*.¹⁵ *Salmonella* spp., especially *S. typhimurium*, is one of the main hosts of *mcr-1* gene. Although many countries, such as Britain, Italy, the Netherlands, and Portugal, have reported *mcr-1*-carrying *S. typhimurium* isolates, most of them were of animal origin.¹⁶⁻¹⁹

This study analyzed the prevalence and molecular characteristics of *mcr-1* gene in *S. typhimurium* isolates from 2007 to 2016 retrospectively, which were preserved in the laboratory of Quzhou People's Hospital. The results showed that the detection rate of *mcr-1* gene was 4.8%, which was slightly higher than that reported previously (0.06%–1.55%).^{16,20} It is observed that *mcr-1*-positive *S. typhimurium* were

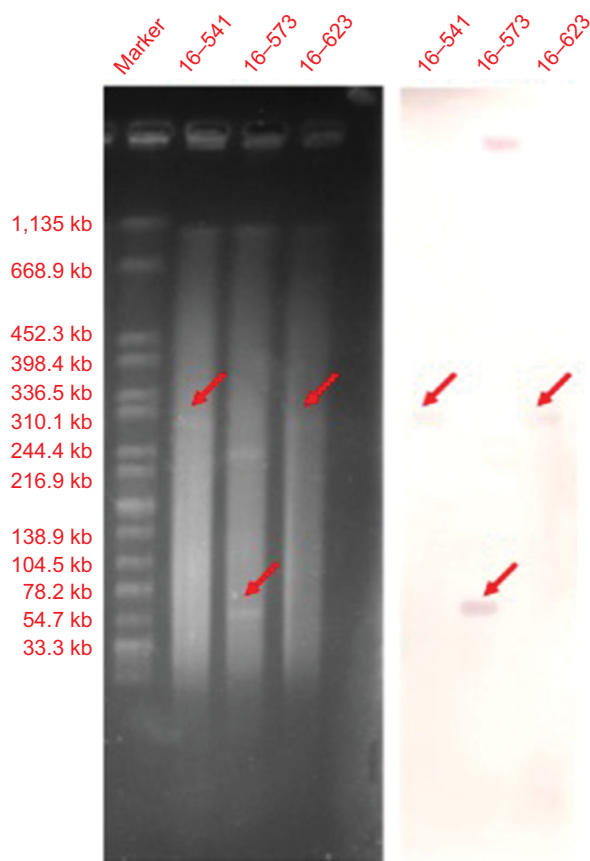


Figure 1 SI-digested plasmid DNA and Southern blot hybridization with *mcr-1* gene of *Salmonella typhimurium* isolates

Notes: The red arrows indicate positive signals via southern blot hybridization with the *mcr-1* probes.

not detected until 2016. Furthermore, all the *mcr-1* genes were located on plasmids. One of the three *mcr-1*-carrying plasmids belonged to plasmid type IncI2, and the other two belonged to IncHI2, which was the same as the recently reported *mcr-1*-horbored plasmid of ST34 *S. typhimurium* isolated from pigs in China.²¹ All these results suggest that *mcr-1* gene may play an important role in polymyxin resistance of clinically isolated *S. typhimurium* strains and lead to the rapid increase of polymyxin resistance in these bacteria.

S. typhimurium is a common clinical pathogenic serotype of *Salmonella* spp. In recent years, there has been a rapid increase in the incidence of antimicrobial resistance among the *Salmonella* spp., which can be attributed to the irrational use of antibiotics in clinics as well as the randomly discharged stools of poultry which can contain the residues of antibiotics.²² According to the previously reported study, *mcr-1*-positive isolates usually remain susceptible to many other antibiotics.²³ In this study, *mcr-1*-positive strains were resistant to colistin as well as to third/fourth-generation cephalosporins and sulfamethoxazole/trimethoprim. WGS analysis showed that these isolates were all CTX-M-14-type extended-spectrum

Table 3 Other resistant genes of *mcr-1*-positive *Salmonella typhimurium* isolates

Isolates/ resistant genes	Aminoglycoside	β -Lactam	Fluoroquinolone	Fosfomycin	Rifampicin	Sulfonamide	Tetracyclines	Trimethoprim
16-541	<i>aph(3'')-Ia/Ib</i> , <i>aadA1/A2</i> , <i>aac(6'')Ib-cr</i> , <i>aph(6)-Ia</i> , <i>aph(4)-Ia</i> , <i>aac(3)-Iva</i>	<i>bla_{TEM-1B'}</i> , <i>bla_{CTX-M-14'}</i> <i>bla_{OXA-1}</i>	<i>oqxA</i> , <i>oqxB</i> , <i>qnrS2</i> , <i>aac(6'')Ib-cr</i>	None	ARR-3	<i>sul1</i> , <i>sul2</i> , <i>sul3</i>	<i>tet(A)</i> , <i>tet(B)</i>	<i>dfrA1/2</i>
16-573	<i>aph(3'')-Ia</i> , <i>aadA1/A2</i> , <i>aph(4)-Ia</i> , <i>aac(3)-Iva</i>	<i>bla_{CTX-M-14}</i>	<i>oqxA</i> , <i>oqxB</i>	<i>fosA3</i>	None	<i>sul1</i> , <i>sul2</i> , <i>sul3</i>	<i>tet(B)</i>	<i>dfrA1/2</i>
16-623	<i>aph(3'')-Ia</i> , <i>aadA1/A2</i> , <i>aac(6'')Ib-cr</i> , <i>aph(4)-Ia</i> , <i>aac(3)-Iva</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Ia</i>	<i>bla_{TEM-1B'}</i> , <i>bla_{CTX-M-14'}</i> <i>bla_{OXA-1}</i>	<i>oqxA</i> , <i>oqxB</i> , <i>qnrS2</i> , <i>aac(6'')Ib-cr</i>	None	ARR-3	<i>sul1</i> , <i>sul2</i> , <i>sul3</i>	<i>tet(B)</i>	<i>dfrA1/2</i>

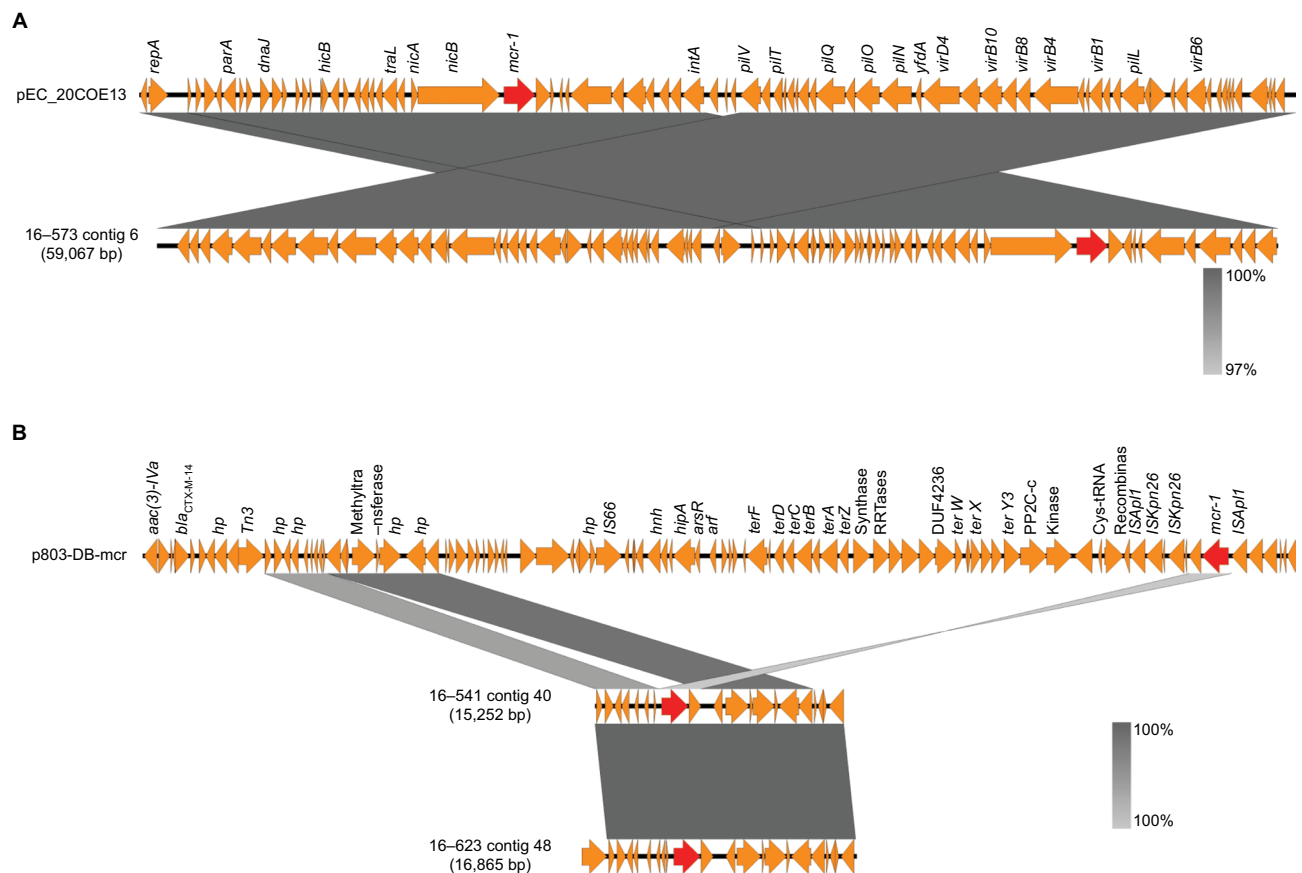


Figure 2 Genetic structures of two representative types of plasmids carrying *mcr-1* gene.

Notes: (A) Comparison of *mcr-1*-located contig of isolate 16-573 and pEC_20COE13. (B) Comparison of *mcr-1*-located contigs of isolates 16-541 and 16-623 and p803-DB-mcr. The red arrows represent the *mcr-1* gene.

β -lactamase producing strains and carried sulfonamide and trimethoprim resistance genes, which were in accordance to the resistant phenotype. The MIC of fosfomycin of isolate 16-573 was 64 $\mu\text{g/mL}$, which was far higher than the MIC of other two isolates (0.25 $\mu\text{g/mL}$). It was due to the co-expression of *fosA3* in isolate 16-573. In addition, although the isolate also carried quinolone-resistance genes *oqxA* and *oqxB*, it was susceptible to both levofloxacin and ciprofloxacin, which might be due to the non-expression of the two genes.

In China, the most common ST of *S. typhimurium*, especially MDR *S. typhimurium*, is ST34,¹³ which is also prevalent in Europe.²⁴ In this study, all the three *S. typhimurium* isolates belonged to ST34. At the same time, most of the *S. typhimurium* strains carrying *mcr-1* gene were isolated from animals and belonged to ST34,^{21,25} which suggested that ST34 was closely related to antimicrobial resistance and the spread of this clone would pose a great threat to the prevention and control of clinical *S. typhimurium* infections.

Conclusion

In conclusion, colistin-resistant *mcr-1*-positive *S. typhimurium* strains have emerged in Quzhou People's Hospital and belong to the most common sequence type ST34, which is associated with MDR *S. typhimurium*. It is necessary to strengthen the surveillance of polymyxin resistance rate in our hospital to prevent the spread of resistant bacteria.

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Disclosure

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

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