

Detection and characterization of a clinical *Escherichia coli* ST3204 strain coproducing NDM-16 and MCR-1

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Objectives: A plasmid-mediated colistin resistance gene, *mcr-1*, has been reported worldwide and has caused concern regarding a major therapeutic challenge. Alarmingly, *mcr-1* has spread into clinical carbapenem-resistant Enterobacteriaceae isolates, resulting in extensively drug-resistant and even pan drug-resistant isolates that can cause untreatable infections. In this study, we report isolation of an extensively drug-resistant *Escherichia coli* strain EC1188 that coproduces NDM-16 and MCR-1 from a urine sample taken from a patient with craniocerebral injury.

Materials and methods: *E. coli* strain EC1188 was identified and subjected to genotyping, susceptibility testing and conjugation experiments. The genetic locations of *bla*_{NDM-16} and *mcr-1* were established with southern blot hybridization. The complete genome sequence of this strain was obtained and the genetic characteristics of the *mcr-1*- and *bla*_{NDM-16}-harboring plasmids were analyzed. In addition, comparative genetic analyses of *mcr-1* and *bla*_{NDM-16} with closely related plasmids were also carried out.

Results: Whole-genome sequencing revealed that strain EC1188 possess various resistance genes and virulence genes. S1-pulsed-field gel electrophoresis and southern blot suggested that the *bla*_{NDM-16} and *mcr-1* genes were located on an ~65 kb plasmid and an ~80 kb plasmid, respectively. Moreover, the two genes could successfully transfer their resistance phenotype to *E. coli* strain C600. Sequence analysis showed that these two plasmids possessed high sequence similarity to previously reported *bla*_{NDM-5}-harboring and *mcr-1*-harboring plasmids in China.

Conclusion: To the best of our knowledge, this is the first report to isolate an *E. coli* strain that coproduces NDM-16 and MCR-1. In addition, we characterized the *bla*_{NDM-16}-harboring plasmid for the first time. Our study further emphasizes that the co-occurrence of the two prevalent transferrable resistance plasmids in a single isolate is highly significant because infections caused by MCR-1-producing carbapenem-resistant Enterobacteriaceae isolates are increasing each year. It is imperative to perform active surveillance to prevent further dissemination of MCR-1-producing CRE isolates.

Keywords: *E. coli*, *mcr-1*, NDM-16, CRE, colistin resistance, extensively drug-resistant bacteria

Introduction

Polymyxins are regarded as last-line therapeutic options to treat the growing number of infections caused by multidrug-resistant Gram-negative bacteria, especially carbapenem-resistant Enterobacteriaceae (CRE).¹ Meanwhile, since the initial discovery of the plasmid-mediated colistin resistance gene, *mcr-1*, this gene has been reported in Enterobacteriaceae isolates from the environment, food, animals and humans worldwide.² Even more worrisome, *mcr-1* has spread into clinical CRE isolates, resulting in extensively drug-resistant isolates that can cause untreatable infections.

Our research group and others have reported on a number of clinical isolates that coproduce MCR-1 and carbapenemase. Among these clinical MCR-1-producing CRE isolates, the NDM-type carbapenemases are highly represented, including NDM-1^{3–5} and NDM-5.^{6–11} Other carbapenemases have also been identified, such as KPC-2,^{12,13} VIM-1¹⁴ and OXA-48.^{15,16} Such strains have the potential to seriously compromise current treatment options for CRE infections. Moreover, these resistance genes are located on different plasmids, allowing their further spread. It is clear that MCR-1-producing CRE isolates result in so-called “superbug” isolates, accelerating the entry into a “post-antibiotic” era.¹⁷

In the present study, we report the isolation of an extensively drug-resistant *Escherichia coli* strain that coproduces NDM-16 and MCR-1 from a urine sample taken from a patient with a craniocerebral injury. The genetic characteristics of the *mcr-1*- and *bla*_{NDM-16}-harboring plasmids were analyzed. In addition, comparative genetic analyses of *mcr-1* and *bla*_{NDM-16} with closely related plasmids were also carried out. To the best of our knowledge, this is the first report describing the isolation of an isolate *E. coli* strain coproducing NDM-16 and MCR-1.

Materials and methods

Patient and isolate data

A carbapenem-resistant strain of *E. coli* EC1188 was isolated from a 40-year-old female patient who was admitted to a hospital for a craniocerebral injury caused by a traffic accident in Hangzhou, Zhejiang, China in September 2017. The strain was cultured from urine during hospitalization of the patient and was preliminarily identified by the VITEK 2 system (Sysmex-bioMérieux, Marcy l’Etoile, France) and further confirmed by 16S rRNA sequencing. Multilocus sequence typing (MLST) of resistance genes and the Inc-type plasmid of the strain were performed using the MLST 1.8 server, ResFinder 3.0, Virulence Finder 1.5 and Plasmid Finder 1.3, which are available at the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org/>). Antibiotic susceptibilities were determined using the broth microdilution method and the results were interpreted according to the Clinical and Laboratory Standard Institute guidelines,¹⁸ except for tigecycline and colistin, which were interpreted according to the European Committee on Antimicrobial Susceptibility Testing breakpoints for *Enterobacteriaceae* (http://www.eucast.org/clinical_breakpoints). *E. coli* ATCC 25922 was used as a quality control strain of antibiotic susceptibility test.

S1-pulsed-field gel electrophoresis (PFGE) and southern blot analysis

To ascertain the plasmids location of the *bla*_{NDM-16} and *mcr-1* genes, genomic DNA digested with S1-nuclease (TaKaRa, Osaka, Japan) was electrophoresed on a CHEF-mapper XA PFGE 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 to 63.8 seconds. The DNA fragments were transferred to a positive-charged nylon membrane (EMD Millipore, Billerica, MA, USA) and then hybridized with a digoxigenin-labeled *bla*_{NDM-16}- and *mcr-1*-specific probe. An NBT/BCIP color detection kit (Hoffman-La Roche Ltd., Basel, Switzerland) was then used to detect the fragments. The *Salmonella enterica* serotype Braenderup H9812 was used as the size marker.

Conjugation experiments

A filter-mating experiment was carried out between the isolate and rifampicin-resistant *E. coli* C600 as the recipient strain.⁸ Transconjugants were selected for Mueller–Hinton (MH) agar plates containing 500 mg/L rifampicin and 100 mg/L ampicillin or 500 mg/L rifampicin and 2 mg/L colistin. Polymerase chain reaction sequencing and antimicrobial susceptibility testing of the transconjugants were subsequently performed to confirm whether the plasmid was successfully transferred to the recipient.

Genomic DNA extraction and analysis

Total genomic DNA extraction and analysis was performed as previously described.¹² Briefly, the genomic DNA of *E. coli* strain EC1188 was extracted using a QIAamp DNA MiniKit (Qiagen, Valencia, CA, USA) following the manufacturer’s recommendations. Sequencing was performed via a single molecule real-time technique using a PacBio RS II platform and the resulting sequences were assembled de novo using the hierarchical genome assembly process with the default settings of the single molecule real-time Analysis v2.3.0 software package. Next, the Rapid Annotation using Subsystems Technology annotation website server (<http://rast.nmpdr.org/rast/cgi>) was used to annotate the genomes. A comparison of pEC1188-NDM16 and pEC1188-MCR and their related plasmids was performed with EasyFig 2.2.2.¹⁹

Plasmid stability

Plasmid stability was determined according to a previous study.²⁰ Briefly, the isolate was individually streaked out on the MH agar, incubated at 37°C for 24 hours and then

transferred to a fresh MH agar plate. After repeating this procedure for 10 days, 12 individual colonies were randomly selected. Subsequently, the *mcr-1* and *bla*_{NDM-16} genes were screened by polymerase chain reaction and sequenced.

Nucleotide sequence accession numbers

The complete nucleotide sequences of the plasmids pEC1188-NDM16 and pEC1188-MCR reported in the present study have been deposited in the GenBank nucleotide database under accession numbers MH213345 and MH213346, respectively.

Ethical approval

The clinical isolate *E. coli* EC1188 was part of the routine hospital laboratory procedure.

The Ethics Committee of the Zhejiang Provincial People's Hospital exempted this study from review because the present study only focused on bacteria.

Results and discussion

Isolate characteristics

The antimicrobial susceptibility testing results showed that strain EC1188 was resistant to the majority of the antimicrobial agents tested (Table 1) and was classified as an extensively drug-resistant bacterium according to the recently proposed international classification scheme.²¹ Acquired resistance to antimicrobial agents was further identified, including beta-lactams (*bla*_{TET-1B}, *bla*_{CTX-199} and *bla*_{NDM-16}), aminoglycoside (*aadA5*, *rmtB* and *aadA2*), colistin (*mcr-1*), macrolide (*mph(A)*), sulfonamide (*sulI*), tetracycline (*tetA*) and trimethoprim (*dfrA12* and *dfrA17*). MLST analysis showed the isolate belonged to ST3204.

To date, some clinical isolates coproducing MCR-1 and various carbapenemases have been identified since an MCR-1 and NDM-5 coproducing isolate was first reported from a duck sample.²² However, strains coproducing NDM-16 and MCR-1

have not been reported. At present, *E. coli* is the primary host among strains coproducing MCR-1 and carbapenemase (Table 2). According to the MLST results (Table 2), these isolates have various sequence types, indicating that these isolates are not clonally related to each other, even though some of the strains were collected from the same hospital.

In addition, the type one fimbriae genes *fimABCDEFGHI*, which encode virulence determinants, were identified by the Rapid Annotation using Subsystems Technology annotation. Furthermore, the type IV fimbrial genes *pilABC* were also identified. Other virulence genes for increased serum survival (*iss*), glutamate decarboxylase (*gad*), enterobactin siderophore receptor protein (*iron*), colicin M (*cma*) and several putative virulence factors were also identified in the genome of this strain. The identified virulence determinants may have contributed to the infection or colonization of the strain.

Plasmid location

S1-PFGE followed by southern blotting showed that the *mcr-1* and *bla*_{NDM-16} genes were located on an ~65 kb IncHI2 plasmid and an ~80 kb IncF plasmid, respectively (Figure 1). Filter mating experiments were then performed to confirm their transferability and the two genes could successfully transfer their resistance phenotype to *E. coli* strain C600 (Table 1). In addition, after ten rounds of subculture on MH agar without the addition of antibiotics, randomly selected colonies were confirmed to carry *mcr-1* and *bla*_{NDM-16} genes and plasmids identical to their parental isolate in size, indicating that the plasmids harboring the *bla*_{NDM-16} or *mcr-1* genes may be stably maintained in the hosts.

Genetic context of plasmids bearing *mcr-1*

The whole genome sequence of strain EC1188 was obtained to better characterize the *mcr-1*- and *bla*_{NDM-16}-positive plasmids. The *mcr-1* gene was located in a plasmid named pEC118-MCR. Sequence analysis showed that the plasmid

Table 1 Antibiotic susceptibility of *Escherichia coli* strain EC1188 and its transconjugants

Isolates	MICs (mg/L)									
	FEP	IMP	NIT	CAZ	AMK	CIP	ATM	TGC	MNO	COL
EC1188	64	8	64	>128	>128	8	128	2	16	8
JH-NDM16	32	16	32	>128	0.5	0.5	0.125	0.125	1	<0.25
JH-MCR1	32	0.5	8	>128	0.5	0.5	64	0.125	1	4
<i>E. coli</i> C600	0.125	0.5	8	0.25	0.5	0.125	0.25	0.125	1	<0.25
ATCC 25922 ^a	0.125	0.5	<8	0.125	0.5	0.125	0.125	0.125	0.25	<0.25

Notes: Drug susceptibility was determined with broth microdilution method. All susceptibility tests were repeated at least three times according to the CLSI method. The results of colistin and tigecycline susceptibility were interpreted according to EUCAST breakpoints. ^aQuality control strain of antibiotic susceptibility test.

Abbreviations: AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CLSI, Clinical and Laboratory Standard Institute; COL, colistin; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FEP, cefepime; IMP, imipenem; MIC, minimum inhibitory concentration; MNO, minocycline; NIT, nitrofurantoin; TGC, tigecycline.

Table 2 Detailed information of strains coproducing MCR-I and carbapenemases isolated from clinical patients

Carbapenemase resistance gene	Isolate name	Year of isolation	Country	Sex	Host strain	MLST	Sample	Incompatibility and size of mcr-I	Incompatibility and size of carbapenemase	References
NDM-I	EC1002	–	China	Male	<i>Escherichia coli</i>	ST2373	Blood	IncI2, ~63 kb	IncA/C2, ~112 kb	4
NDM-I	EC2474	–	China	Male	<i>E. coli</i>	ST131	Blood	IncHI2, ~224 kb	IncFII, ~76 kb	4
NDM-I	GB090	2016	China	Female	<i>E. coli</i>	ST744	Feces	IncFI, –	–	5
NDM-I	BB1290	2015	Venezuela	Male	<i>E. coli</i>	ST19	Feces	IncHI2, ~60 kb	–	3
NDM-5	MCR1_NJ	2014	US	Male	<i>E. coli</i>	ST405	Urine	IncX4, ~33 kb	IncX3, ~40 kb	6
NDM-5	07HAE27	2014	China	Female	<i>E. coli</i>	ST167	Blood	IncHI2, ~60 kb	–	8
NDM-5	CDA6	2015	China	Male	<i>E. coli</i>	ST167	Urine	IncX4, ~35 kb	IncX3, –	9
NDM-5	BJ10	2015	China	Female	<i>E. coli</i>	ST156	Ascites	IncI2, ~65 kb	IncX3, –	9
NDM-5	SZ03 ^a	2015	China	–	<i>Klebsiella pneumoniae</i>	ST25	Surgery wound	IncX4, ~33 kb	IncX3, ~46 kb	10
NDM-5	SZ04 ^a	2015	China	–	<i>K. pneumoniae</i>	ST25	Peritoneal fluid	IncX4, ~33 kb	IncX3, ~46 kb	10
NDM-5	GZ1 ^a	2015	China	Female	<i>E. coli</i>	ST10	Abdominal drainage	–	–	11
NDM-5	GZ2 ^a	2015	China	Female	<i>E. coli</i>	ST10	Urine	IncI2, ~60 kb	IncX3, ~102 kb	11
NDM-5	E159	2016	China	Male	<i>E. coli</i>	ST206	Urine	IncHI2, ~240 kb	IncX3, ~46 kb	7
NDM-16	EC1188	2017	China	Female	<i>E. coli</i>	ST3104	Urine	IncI2, ~65 kb	IncFII, ~100 kb	This study
KPC-2	H17	2015	China	Male	<i>E. coli</i>	ST354	Urine	IncHI2, ~60 kb	IncR, ~104.5 kb	12
KPC-2	NRZ14408	2014	Germany	–	<i>E. coli</i>	ST362	Foot wound	IncI2, –	–	13
VIM-I	KRI	2015	Switzerland	Male	<i>E. coli</i>	CC23	Urine	–	–	14
OXA-48	GN775	2011	Canada	–	<i>E. coli</i>	–	Drainage liquid	IncHI2, –	–	15
OXA-48	DC2562	2010	China	Female	<i>E. coli</i>	ST44	Urine	IncII, ~62 kb	IncII, ~62 kb	16
OXA-48	DC90	2012	China	Male	<i>E. coli</i>	ST2	Pus	IncP, ~33.3 kb	IncFIB, ~104.5 kb	16

Notes: ^aIsolated from the same patient. “–” indicates data not applicable.

Abbreviation: MLST, multilocus sequence typing.

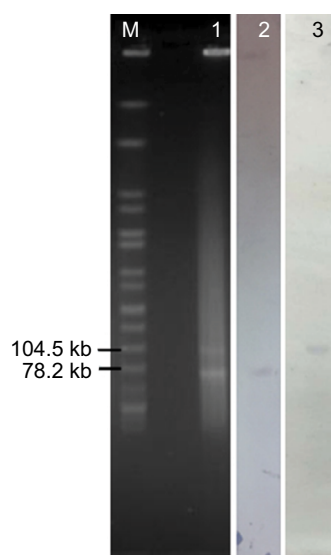


Figure 1 S1-digested plasmid DNA and Southern blot hybridization of the *E. coli* EC1188 isolate.

Notes: Bands with arrows pointing to them showed positive signals via Southern blot hybridization with the *bla*_{NDM-16} and *mcr-I* probes. M indicates *Salmonella* serotype Braenderup strain H9812 molecular marker; 1 indicates *E. coli* EC1188; 2 indicates *mcr-I* positive signal; and 3 indicates *bla*_{NDM-16} positive signal.

was 65,806 bp in length (Figure 2), belonged to IncI2 incompatibility group and had a G+C content of 42.5%.

Sequence analysis showed that the genes coding for plasmid transfer functions, such as *tra* and *pil*, and the genes responsible for plasmid stability functions, such as *mok* and *parA*, were identified. BLASTn search results showed that pEC1188-MCR exhibited 91% query coverage and 99% identity to the originally described *mcr-I*-encoding plasmid pHNSHP45.²³ The pEC118-MCR plasmid sequence was nearly identical to the plasmid pHNSHP45, primarily differing by the following: 1) the lack of a 2,704 bp insertion of IS683 downstream of the *repA* gene, which is consistent with another plasmid (pH17-1) carrying the *mcr-I* gene but lacking the inserted sequence;¹² 2) an IS*AplI* transposable element was not observed in this plasmid, but an insertion sequence ISE*cpI* was present (Figure 2) and 3) an extended-spectrum β-lactamase gene *bla*_{CTX-M-199} was identified in this plasmid. Note that the insertion sequence IS*AplI* gene was not found upstream of *mcr-I*, but the *pap2* gene encoding the PAP2-family protein

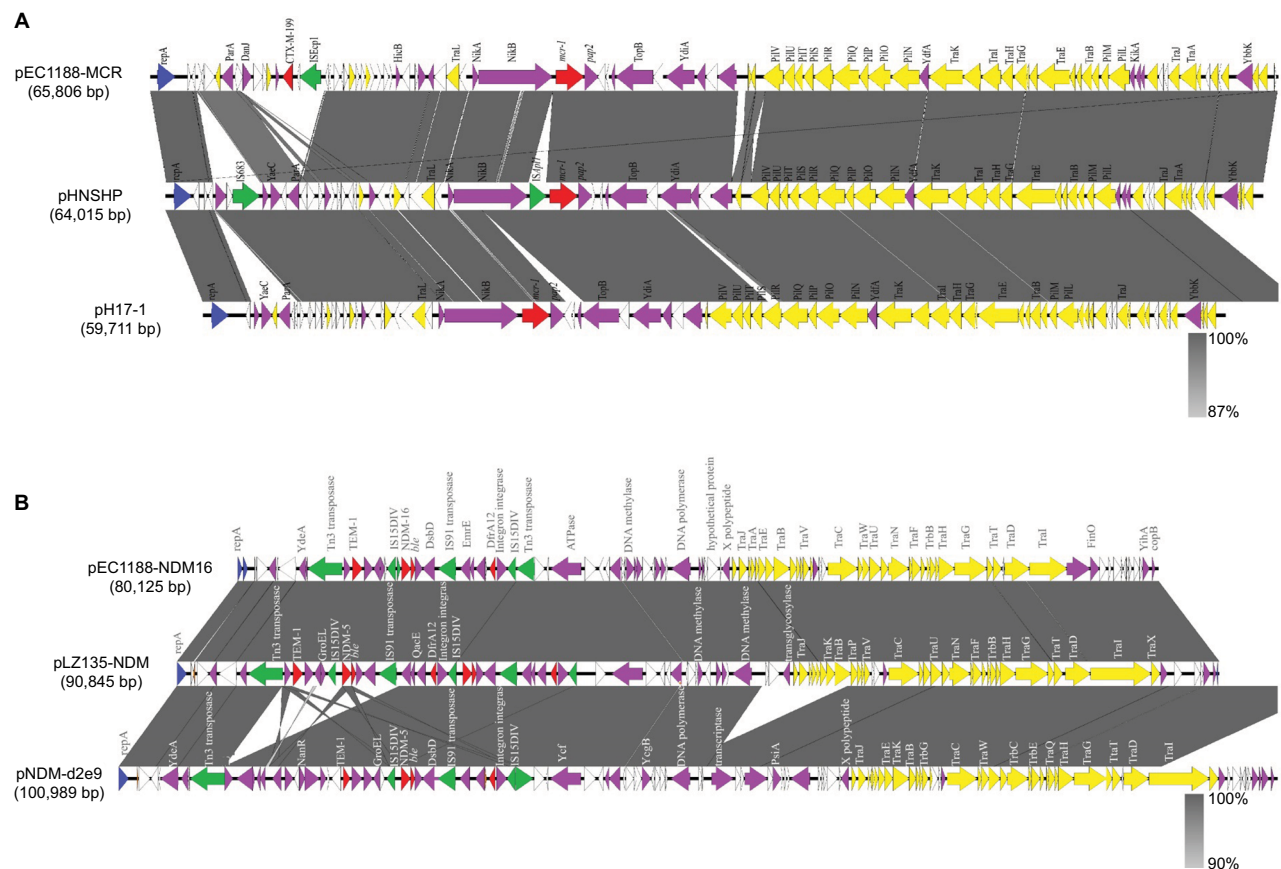


Figure 2 Linear plasmid characterization of NDM-16 and MCR-1-bearing plasmids with closely related plasmids.

Notes: The gray regions between plasmids indicate nucleotide identity (87–100%) by BLASTn. Arrows indicate predicted ORFs. **(A)** Primary structural characteristics of pEC1188-MCR compared to plasmids pHNSHP (KP347127) and pH17-1 (CP021194). **(B)** Primary structural characteristics of pEC1188-NDM16 compared to plasmids pLZ135-NDM (MF353156) and pNDM-d2e9 (CP026201). Colored arrows represent open reading frames, with red, purple, yellow, green and white representing antibiotic resistance gene, replication, recombination and repair genes, plasmid stability genes, mobile elements and plasmid transfer related genes and unknown function genes, respectively.

was identified downstream of the *mcr-1* gene. The insertion sequence of *ISAp11* is not always associated with *mcr-1* and it is absent in some *mcr-1*-positive plasmids, implying a relic for frequent conjugation of *ISAp11-mcr-1-pap2*.²⁴

So far, *mcr-1* has been reported to be carried in different plasmids incompatibility such as IncI2, IncX4, IncHI2 and IncP.²⁴ The IncI2-type plasmid was the first reported *mcr-1*-harboring plasmid. In addition, in our previous study, of 21 *mcr-1*-positive isolates recovered from bloodstream infections in China, 15 isolates contained *mcr-1* on the IncI2-type plasmid.⁸ Furthermore, incompatibility of strains coproducing MCR-1 and carbapenemases isolated from humans being was analyzed, indicating that IncI2-type plasmid was also prevalent (Table 2). Overall, IncI2-type plasmid played an important role in the dissemination of *mcr-1* in *E. coli*, even in strains coproducing MCR-1 and carbapenemases.

Genetic characteristics of plasmids bearing *bla*_{NDM-16}

The *bla*_{NDM-16} gene is located in a plasmid named pEC1188-NDM16. Sequence analysis showed that the plasmid was

80,215 bp in length (Figure 2), belonged to the IncF incompatibility group and had a G+C content of 51.8%. The plasmid was further analyzed in the nr/nt database and showed an overall 99% nucleotide identity and 100% query coverage to the *bla*_{NDM-5}-positive plasmids pLZ135-NDM (GenBank Accession Number MF353156) and pNDM-d2e9 (GenBank Accession Number CP026201), as shown in Figure 2. Interestingly, analysis of the typical segment flanking *bla*_{NDM-16} on pEC1188-NDM16 revealed that the *bla*_{NDM-16} gene was likely to be transferred by two flanking *IS15DIV* elements, which share the same flanking sequence (*IS15DIV-bla*_{NDM-16-ble-dsbD-IS91-dfrA12-IS15DIV) with the two *bla*_{NDM-5} gene-bearing IncX3 plasmids.}

In addition, NDM-5 is the primary type of carbapenemase among MCR-1-producing CRE isolates (Table 2). Our recent study also found that *bla*_{NDM-5} was able to coexist in the MCR-1-producing strain isolated from bloodstream infections.⁸ Furthermore, NDM-16, which differs from NDM-5, has aspartate to valine amino acid substitution at position 233. Note that dissemination of the *bla*_{NDM-5} gene via a plasmid among non-clonal *E. coli* isolates has been observed in our

hospital.²⁵ Although no genetic association was observed among MCR-1-producing CRE isolates, the widespread dissemination of *bla*_{NDM-5} in recent years in Enterobacteriaceae highlights the need for active surveillance to prevent further dissemination of MCR-1-producing isolates.

Conclusion

In summary, to the best of our knowledge, this is the first report of a clinical *E. coli* strain coproducing MCR-1 and NDM-16. In addition, this study also characterizes the *bla*_{NDM-16}-harboring plasmid for the first time. Our study further underscores that the co-occurrence of the two prevalent transferrable resistance plasmids in a single isolate raises extensive concern, because infections caused by MCR-1-producing CRE isolates increase each year. It is imperative to perform active surveillance to prevent further dissemination of MCR-1-producing CRE isolates.

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Author contributions

YY and YZ conceived and designed the experiments; XL, PZ, and XM performed the experiments; DZ, JJ, and JQ analyzed the data; XL and XM wrote the manuscript. All authors read and approved the final manuscript. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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