ORIGINAL RESEARCH

Characterization of a novel bla_{NDM-5}-harboring IncFII plasmid and an *mcr-1*-bearing IncI2 plasmid in a single *Escherichia coli* ST167 clinical isolate

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Background and purpose: The spread of the plasmid-mediated, colistin-resistance gene *mcr-1* into New Delhi metallo-β-lactamase (NDM)-producing bacterial isolates can cause untreatable infections. In this study, we conducted a molecular characterization of a novel, conjugative, bla_{NDM-5}-positive IncFII plasmid (pNDM-EC16-50) together with an *mcr-1*-bearing IncI2 plasmid in a single *Escherichia coli* ST167 clinical isolate EC16-50.

Methods and results: EC16-50, which belongs to the *E. coli* strain ST167 and phylogroup A, was identified to co-produce NDM-5 and MCR-1. S1-PFGE and Southern blotting showed that bla_{NDM-5} and *mcr-1* genes were located on ~95 kb and ~65 kb plasmids, respectively. A conjugation assay revealed that both bla_{NDM-5}- and *mcr-1*-bearing plasmids were self-transmissible. Comparative plasmid analysis suggested that bla_{NDM-5}-harboring F2:A-:B-plasmid might have evolved from the well-reported NDM-carrying pMC-NDM-like plasmid via recombination with a locally emerged pSJ_94 plasmid, whereas the *mcr-1*-carrying IncI2 plasmid was similar to previously reported *mcr-1*-bearing plasmids in China.

Conclusion and impact: This study represents the first report of the NDM-5 carrying Inc-FII- but not IncX3-type plasmid in an MCR-1-producing *E. coli* isolate. More striking was the dissemination of *mcr-1* in a successful epidemic NDM-5-producing *E. coli* clone ST167, which could facilitate the spread of colistin resistance in carbapenemase-producing *E. coli* isolates.

Keywords: *Escherichia coli*, ST167, MCR-1, IncFII plasmid, NDM-5

Introduction

Colistin is considered to be the last resort for the treatment of serious infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE). However, the emergence and dissemination of the plasmid-mediated, colistin-resistance gene *mcr-1* have seriously compromised current treatment options for CRE infections.[1] The spread of the *mcr-1* gene into CRE, such as New Delhi metallo-β-lactamase (NDM)-producing isolates, is of great clinical concern as this results in the emergence of true pan-drug-resistant pathogens. NDM-5, which differs from NDM-1 by two amino-acid substitutions (Val88Leu and Met154Leu) and possesses increased resistance to carbapenems and extended-spectrum cephalosporins, has become a predominant variant expressed by NDM-producing *Escherichia coli* since they were first reported in 2014 in China.[2,3] To date, several cases of infection caused by NDM-5 and MCR-1 co-producing *E. coli* have been reported in the United States and China.[4–10] In these bacterial isolates, the *mcr-1* gene was carried by plasmids of different replicon types, including IncX4, HI2, and I2, whereas the bla_{NDM-5} gene in each isolate was located on IncX3 plasmids. Nearly all of the published studies

from China revealed that the bla_{NDM-5} gene was carried by IncX3-type plasmids in *E. coli* isolates, and *E. coli* ST167 was regarded as an epidemic clone involved in the transmission of the bla_{NDM-5} gene.[11–14] More recently, two NDM-5-carrying IncF plasmids were identified in *E. coli* ST167 strains from China (pNDM5_020007, CP025626) and Italy (pNDM-5-IT, MG649062),[15,16] but there have been no reports of NDM-5-encoding IncF plasmids in MCR-1-producing *E. coli*.

In this study, we conducted a molecular characterization of a novel NDM-5-carrying F2:A-:B-plasmid and an IncI2 plasmid co-harboring *mcr-1* and $bla_{CTX-M-55}$ in a single *E. coli* ST167 clinical isolate. Our results revealed new molecular features of *E. coli* that co-produce NDM-5 and MCR-1.

Materials and methods
Bacterial isolates and antimicrobial susceptibility testing
A male patient without a history of foreign travel was admitted to the department of hepatobiliary surgery in a teaching hospital of Zhengzhou University in July 2016. He was diagnosed with bile-duct stones complicated by cholangitis. During the surgery, a bile sample was collected from this patient for microbiological analysis. A multidrug-resistant *E. coli* isolate named EC16-50 was recovered from this bile culture; the isolate was identified using the VITEK®2 system (bioMérieux, Marcy-l'Étoile, France) and further confirmed by 16S-rRNA sequencing. Antimicrobial susceptibility testing was conducted using several different assays: the broth microdilution method for ampicillin, piperacillin-tazobactam, cefazolin, ceftazidime, aztreonam, imipenem, meropenem, ciprofloxacin, gentamicin, amikacin, chloroamphenicol, doxycycline, tigecycline, and colistin; the agar dilution method for fosfomycin; and the Etest method (AB bioMérieux, Sweden) for trimethoprim/sulfamethoxazole. The results were analyzed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[17] Breakpoints for colistin and tigecycline were interpreted according to European Committee on Antimicrobial Susceptibility testing (EUCAST) guidelines (http://www.eucast.org/clinical_breakpoints/). *E. coli* ATCC 25922 was used as a quality control strain.

Detection of resistance determinants and bacterial genotyping
The modified Hodge test and the imipenem-EDTA double-disk synergy test were performed according to CLSI guidelines to detect carbapenemase activity.[17] PCR and nucleotide sequencing were employed to screen for the presence of carbapenemase-encoding genes ($bla_{OXA-48-like}$, bla_{IMP}, bla_{VIM},

bla_{KPC}, and bla_{NDM}) and the *mcr-1* gene as described previously.[1,18] Multi-locus sequence typing and phylogenetic grouping were performed according to published protocols (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/) and a previously described method, respectively.[19]

S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE) and Southern blot analysis
To evaluate whether the bla_{NDM} and *mcr-1* genes were present in plasmids, genomic DNA of *E. coli* EC16-50 was used for S1-PFGE and Southern blotting with bla_{NDM}- and *mcr-1*-specific probes as previously described.[18] The *Salmonella enterica* serotype Braenderup H9812 was used as the size marker.

Conjugation assay
A conjugation assay was conducted as previously described using *E. coli* J53 (sodium-azide-resistant strain) as the recipient. Transconjugants were selected on Mueller-Hinton (MH) agar supplemented with sodium azide (100 µg/mL) and imipenem (1 µg/mL) or colistin (2 µg/mL).[18] PCR sequencing and antimicrobial susceptibility testing of the transconjugants were subsequently performed to confirm whether the plasmid was successfully transferred to the recipient.

Plasmid analysis
For plasmid analysis, each plasmid was transferred to *E. coli* DH5α by electroporation. Transformant colonies were selected on Luria-Bertani agar plates containing imipenem (1 µg/mL) or colistin (2 µg/mL) and were examined for the presence of the bla_{NDM} and *mcr-1* genes by PCR and sequencing. Plasmid DNA was extracted from a confirmed transformant using a QIAGEN Midi Kit (QIAGEN, Hilden, Germany) and subjected to complete plasmid sequencing using an Illumina MiSeq platform (Shanghai Majorbio Company, Shanghai, China). Sequencing reads were assembled de novo using SOAPdenovo v.2.04 (http://soap.genomics.org.cn/). The gaps between different contigs were closed by PCR and sequencing. Prediction and annotation of open reading frames (ORFs) were done with Glimmer 3.02 (http://cbcb.umd.edu/software/glimmer/) and Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nucleotide sequence accession numbers
The complete nucleotide sequences of plasmids pEC16-50-MCR and pNDM-EC16-50 have been deposited in GenBank under accession nos. MG515249 and MG515250, respectively.

Ethics statement

The bile sample and clinical isolate of *E. coli* EC16-50 were generated as part of routine hospital laboratory procedures. The patient's next of kin provided written, informed consent for details that were included in the manuscript.

Results and discussion

Isolate characteristics

The antimicrobial susceptibility testing results showed that strain EC16-50 was resistant to all of the β-lactams including carbapenems, fluoroquinolones, gentamicin, amikacin, doxycycline, and colistin, but susceptible to fosfomycin, tigecycline, and trimethoprim/sulfamethoxazole (Table 1). The positive result for the imipenem-EDTA double-disk synergy test revealed that EC16-50 was a metallo-β-lactamase producer. Finally, PCR and sequencing showed that EC16-50 co-harbored both bla_{NDM-5} and *mcr-1* genes.

Strain EC16-50 was assigned to the *E. coli* ST167 strain and phylogroup A. To date, *E. coli* ST167 is classified as an internationally disseminated clonal lineage associated with the global spread of CTX-M-15 extended-spectrum-β-lactamase and NDM metallo-β-lactamases in humans and other animals.[20,21] Recently, reports from different parts of China indicated that *E. coli* ST167 exhibited close linkages with the bla_{NDM-5} gene,[13,14] and dissemination of *mcr-1* within the successful epidemic clone may facilitate further spread of colistin resistance in carbapenemase-producing *E. coli* isolates. This led to the emergence of extensively drug-resistant pathogens. To prevent such pathogens from developing further, the spread of *E. coli* ST167 that co-produces MCR-1 and NDM-5 should be closely monitored in China.

Plasmid location

S1-PFGE followed by Southern blotting was able to identify that the bla_{NDM-5} and *mcr-1* genes in EC16-50 were on ~95 kb and ~65 kb plasmids, respectively (Figures S1 and S2). Then we performed conjugative assays to confirm their transferability, and both *mcr-1*- and bla_{NDM-5}-carrying plasmids in EC16-50 were successfully transferred into *E. coli* J53.

Characterization of the bla_{NDM-5}-carrying plasmid

We found that the bla_{NDM-5}-carrying plasmid, named pNDM-EC16-50, was 95,724 base pairs (bp) in size. It consisted of an

Table 1 Antibiotic susceptibilities of *E. coli* EC16-50, its transformants *E. coli* T-MCR and *E. coli* T-NDM, its conjugants *E. coli* J53-MCR and *E. coli* J53-NDM, and their recipient strains *E. coli* J53 and DH5α

Antimicrobial category	Antimicrobial agent	MIC (µg/mL)[a]						
		EC16-50[a]	T-MCR[b]	T-NDM[b]	J53-MCR[c]	J53-NDM[c]	J53	DH5α
Penicillins	Ampicillin	>512	>512	>512	>512	>512	4	<2
Antipseudomonal penicillins + β-lactamase inhibitors	Piperacillin/tazobactam	>512	>512	>512	>512	>512	4	<4
Non-extended-spectrum cephalosporins	Cefazolin	>512	>512	>512	>512	>512	2	<4
Extended-spectrum cephalosporins	Ceftazidime	>512	32	256	32	512	<0.25	<0.06
Carbapenems	Imipenem	128	<0.25	32	<0.25	64	<0.25	<0.06
	Meropenem	128	<0.25	32	0.25	32	4	<0.06
Monobactams	Aztreonam	512	32	1	64	<1	0.25	<0.06
Fluoroquinolones	Ciprofloxacin	64	<0.25	<0.25	<0.25	<0.25	<0.25	<0.06
Aminoglycosides	Gentamicin	>512	2	64	<1	64	<0.25	1
	Amikacin	>512	2	512	1	>512	4	1
Folate pathway inhibitors	Trimethoprim/ sulfamethoxazole	2.5	2.5	5	2.5	2.5	<0.15	<0.15
Phenicols	Chloramphenicol	128	4	2	256	4	4	1
Phosphonic acids	Fosfomycin	<1	<1	<1	<1	<1	<1	<1
Tetracyclines	Doxycycline	32	1	1	16	2	1	1
Glycylcyclines	Tigecycline	0.5	0.25	0.5	0.5	0.25	0.25	0.25
Polymyxins	Colistin	8	2	<0.25	4	1	1	1

Notes: [a]MICs were determined by broth dilution as proposed by CLSI and interpreted using its guidelines. [a]*E. coli* EC16-50 is positive for *mcr-1* and NDM-5. [b]T-MCR and T-NDM are transformants of *E. coli* EC16-50. [c]J53-MCR and J53-NDM are conjugants of *E. coli* EC16-50.
Abbreviations: MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute.

average guanine and cytosine (GC) content of 51.2% and 84 predicted ORFs. The replication region of pNDM-EC16-50 comprised *repA2*, *repA1*, and *repA4* and was completely identical to other F2:A-:B-plasmids, such as pMC-NDM (HG003695) and pGUE-NDM (JQ364967). BLASTn results for pNDM-EC16-50 against the NCBI non-redundant database showed that the plasmid shared 99% identity with several IncFII-type plasmids carrying bla_{NDM} (including pMC-NDM, pGUE-NDM, pEC1188-NDM16 [MH213345], pLZ135-NDM [MF353156], and pCC1409 [KT725789]), with query coverages ranging from 70% to 81%. The bla_{NDM-5}-containing multidrug resistance mosaic region (MRR) in pNDM-EC16-50 was almost identical to that of pMC-NDM, except for 1) two point mutations in bla_{NDM-1} leading to the occurrence of the bla_{NDM-5} allele, 2) a 160-bp sequence insertion between the IS*26* and ΔIS*Aba125* elements, and 3) an intact IS*CR1* element without IS*26* inserted. The extensive backbone region upstream of the conjugative transfer operon *tra-trb* in pNDM-EC16-50 showed new features compared to typical bla_{NDM}-harboring IncFII plasmids, such as pGUE-NDM (bla_{NDM-1}) and pMC-NDM (bla_{NDM-1}) reported in Europe

and pCC1409 (bla_{NDM-5}) reported in Korea (Figure 1B).[22–24] A 13,592 bp fragment in pNDM-EC16-50's backbone region included scaffold genes responsible for plasmid partition (*parM*) and stability (*stbB*) and a group II intron that was virtually identical (100% query coverage, 99.6% identity by BLASTn analysis in NCBI) to the plasmid pGUE-NDM/pMC-NDM (the group II intron is missing from pMC-NDM). As shown in Figure 1A, Region 1 (*ydaAB-ydbA-ycdA-ssb-parB-psiAB-mok-hok-yubOP-X-polypeptide*), which is present in both pGUE-NDM and pMC-NDM, was replaced by a 16,182 bp pSJ_94 (CP011064)-derived accessory module (100% query coverage and 100% nucleotide identity; designated as Region 2) composed of mobile genetic elements and a number of functional genes, such as those encoding putative alpha-galactosidase, lactose permease, and sucrose-6-phosphate hydrolase (Figure 1A). In addition, we observed that the entire *tra-trb* region, excluding *traX* gene in pNDM-EC16-50, was more similar to its counterpart on pSJ_94, except for 1) a 50-nucleotide difference in the *traI* gene, 2) a 1-nucleotide difference in the *traT* and *traP* genes, and 3) the number of CAACAGCCA tandem repeats in the *traD* gene
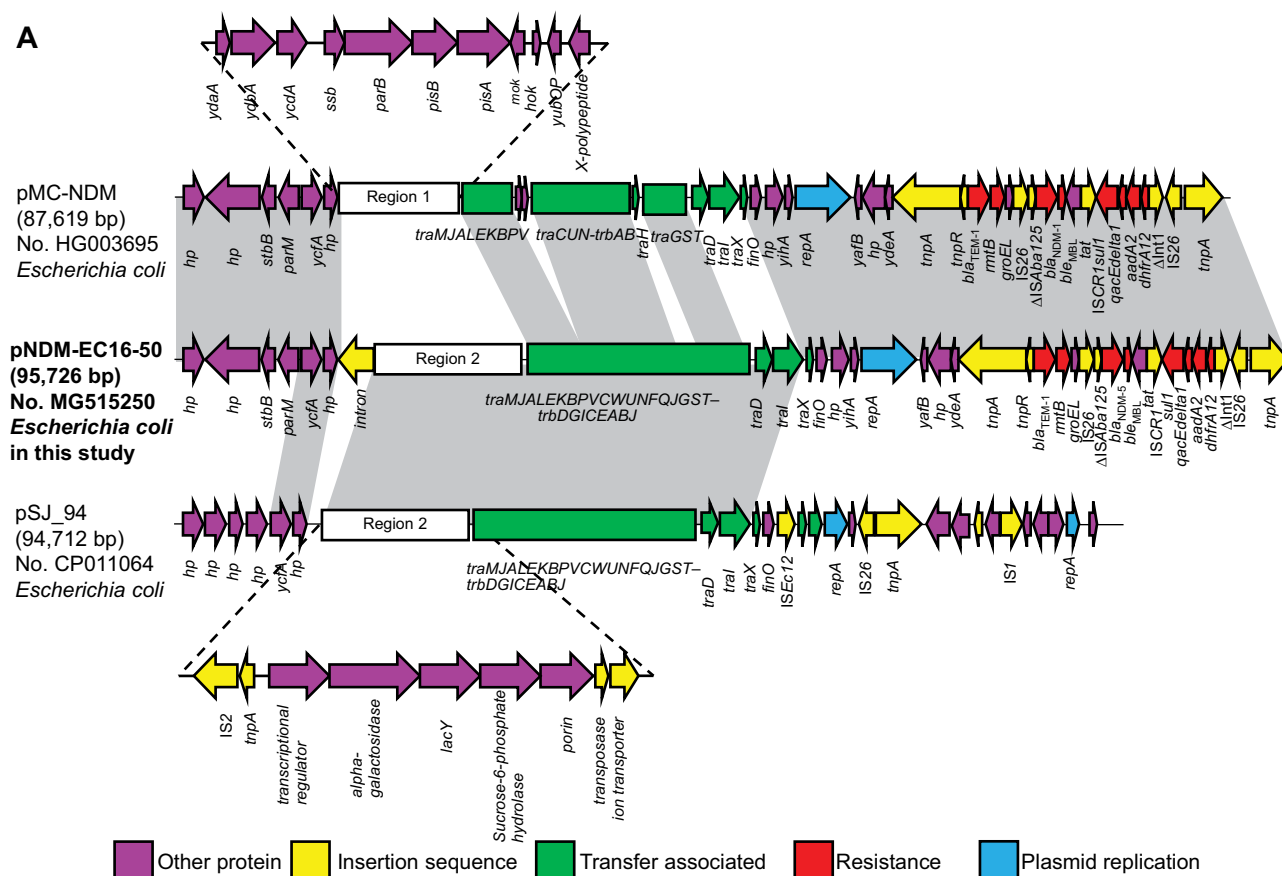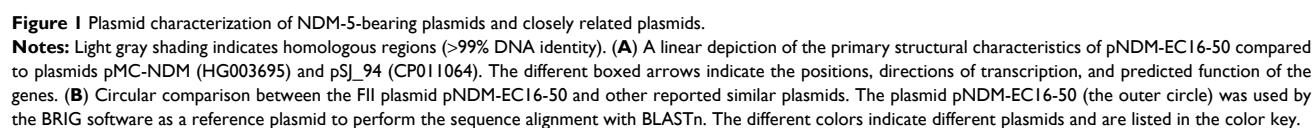

Figure 1 (*Continued*)

B

Figure 1 Plasmid characterization of NDM-5-bearing plasmids and closely related plasmids.
Notes: Light gray shading indicates homologous regions (>99% DNA identity). (**A**) A linear depiction of the primary structural characteristics of pNDM-EC16-50 compared to plasmids pMC-NDM (HG003695) and pSJ_94 (CP011064). The different boxed arrows indicate the positions, directions of transcription, and predicted function of the genes. (**B**) Circular comparison between the FII plasmid pNDM-EC16-50 and other reported similar plasmids. The plasmid pNDM-EC16-50 (the outer circle) was used by the BRIG software as a reference plasmid to perform the sequence alignment with BLASTn. The different colors indicate different plasmids and are listed in the color key.

(61 and 11 repeats in pNDM-EC16-50 and pSJ_94, respectively). Thus, the overall structure of pNDM-EC16-50 can be divided into two distinct modules (Figure 1A): an ~47.9 kb pMC-NDM-derived module containing the IncFII replication region, partial backbone region, and the entire MRR and an ~47.8 kb pSJ_94-like module encompassing multiple functional genes and the *tra-trb* gene clusters. These findings suggested that pNDM-EC16-50 might have evolved from a recombination of the bla_{NDM}-carrying F2:A-:B- type pMC-NDM-like plasmid and the pSJ_94-like plasmid belonging to

F36:A4:A20:B- type. Moreover, we speculated that pNDM-EC16-50 may have been created by Region 2 of a pSJ_94-like plasmid displacing Region 1 on a pMC-NDM-like plasmid.

Previous studies from China revealed that the IncX3-type pNDM-MGR194-like plasmid contributed to the dissemination of the bla_{NDM-5} gene among *Enterobacteriaceae*, and bla_{NDM-5} gene is mostly carried on this type of plasmid in *E. coli* ST167.[12,13] Recently, two novel bla_{NDM-5}-positive IncF plasmids harboring two or more IncF replicons belonging to F36:A4:B- (pNDM5_020007, China) and F36:F31:A4:B1

(pNDM-5-IT, Italy) plasmid types were identified in *E. coli* ST167 strains.[15,16] In addition, three bla_{NDM-5}-carrying IncFII-type plasmids, whose sequences were submitted to the GenBank database in 2017, were detected in clinical *E. coli* (pGZ3_NDM5, CP017981 and pLZ135-NDM, MF353156) isolates and the *S. enterica* serovar Typhimurium strain (pST41-NDM, CP019444) in China.[8,25] These observations, together with our findings, suggested that conjugative IncFII-type plasmids might have emerged as a potential vehicle (other than the IncX3-type plasmid) to mediate the spread of bla_{NDM-5} in China. In this study, the transfer fre-

quency of pNDM-EC16-50 determined by conjugation assay (2.5×10^{-7} transconjugants per donor cell) was much lower than that of the representative bla_{NDM}-bearing IncFII plasmids pGUE-NDM (2.5×10^{-4} transconjugants per donor cell) and pMC-NDM (2×10^{-3} transconjugants per donor cell)[22,23] and bla_{NDM-1}-bearing IncX3 plasmids (10^{-1} to 10^{-5} transconjugants per donor cell).[26] In addition, Shin et al reported a prominent instability profile of the bla_{NDM-5}-bearing IncFII plasmid in a *Klebsiella pneumoniae* isolate.[27] In light of such evidence, we speculated that the low transfer frequency and instability in the host of the IncFII-type plasmid compared to the
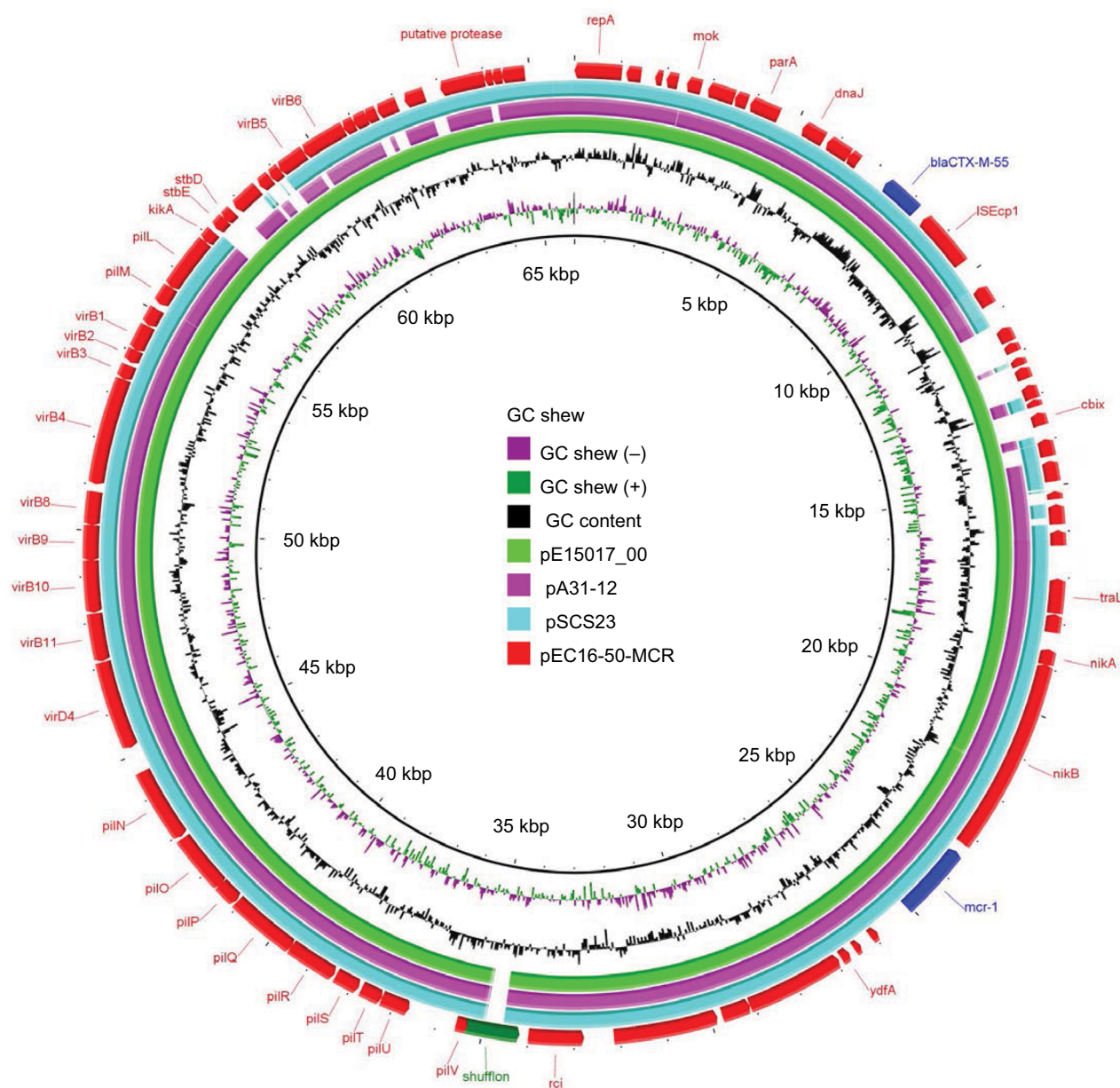
Figure 2 Circular comparison between the *mcr-1*-bearing plasmid pEC16-50-MCR and other reported similar plasmids.
Notes: The plasmid pEC16-50-MCR (the outer circle) was used by the BRIG software as a reference plasmid to perform sequence alignments with BLASTn. The different colors indicate different plasmids and are listed in the color key.

IncX3-type plasmid may be responsible for the scarce reporting of bla_{NDM-5}-bearing IncFII plasmids in NDM-producing *E. coli* isolates. Clearly, these elusive plasmids need closer investigation.

Characterization of the plasmid bearing mcr-1

We found that the *mcr-1*-carrying plasmid in EC16-50, named pEC16-50-MCR, was 65,978 bp in size and belonged to the IncI2 incompatibility group. It contained 80 ORFs and had an average GC content of 42.6%. We further revealed that pEC16-50-MCR consisted of 1) a typical IncI2 plasmid backbone region that is responsible for plasmid replication, maintenance, and transfer, 2) a 3,090 bp IS*Ecp1-bla*$_{CTX-M-55}$ transposition unit, and 3) a 2,471 bp *mcr-1* cassette composed of the *mcr-1* gene and a PAP2-like ORF (Figure 2). Similar IncI2 plasmids co-harboring *mcr-1* and *bla*$_{CTX-M-55}$ genes have been described, such as pA31-12 (KX034083) and pSCS23 (KU934209) in *E. coli* and *S. enterica* isolates, respectively.[28,29] It is noteworthy that an IS*Apl1* mobile element was present upstream of the *mcr-1* cassette in both pA31-12 and pSCS23, whereas it was absent in pEC16-50-MCR. Snesrud et al hypothesized that the loss of IS*Apl1* during the course of evolution increased the stability of *mcr-1* in plasmid vectors due to IS*Apl1*'s pivotal role in the mobilization of *mcr-1*.[30] Therefore, the *mcr-1* cassette lacking IS*Apl1* and co-occurring with *bla*$_{CTX-M}$ genes on pEC16-50-MCR-like conjugative plasmids may facilitate widespread dissemination of *mcr-1*. A BLASTn comparison revealed that the sequence of pEC16-50-MCR was very similar to that of the plasmid pE15017_00 (KX772778), with 99% query coverage and 99% nucleotide identity. We found that the major difference between pEC16-50-MCR and pE15017_00 lay in the shufflon region, a DNA segment that consists of multiple DNA inversions and is involved in site-specific recombination in IncI2-type plasmids.[31] Both segments C and BD of IncI2 shufflons were found to be present in the shufflon region of pEC16-50-MCR, while segment C was found to be missing from pE15017_00, indicating that rearrangement events had taken place in the shufflon region of pE15017_00 mediated by the recombinase (Figure 2).

Conclusion

In summary, we characterized a novel, conjugative, bla_{NDM-5}-carrying IncFII plasmid that coexists with a self-transmissible IncI2 plasmid harboring both *mcr-1* and *bla*$_{CTX-M-55}$ genes in a successful epidemic *E. coli* clone ST167. Unlike IncX3-type plasmids, which are common vehicles for dissemination of bla_{NDM-5}, the IncF plasmid is currently only sporadically reported to carry bla_{NDM-5} in *E. coli* from China. Therefore, the role of this type of plasmid in the dissemination of bla_{NDM-5} in *E. coli* needs further investigation. In addition, the acquisition of *mcr-1*-bearing plasmids in widespread NDM-5-producing *E. coli* ST167 clones could contribute to the spread of polymyxin resistance within CRE strains. Overall, our study highlights the urgent need for enhanced surveillance of the spread of *E. coli* ST167 strains co-producing MCR-1 and NDM-5.

Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work. LX, SQ, and WX designed and supervised the study. PW conducted the primary and confirmatory diagnostics for carbapenemase gene presence, species identification, and bacterial genotyping. PW and JC prepared the samples for Illumina MiSeq, performed the data analyses, and conducted most of the molecular experiments. SQ wrote the manuscript. All of the authors reviewed and approved the final manuscript.

Disclosure

The authors report no conflicts of interest in this work.
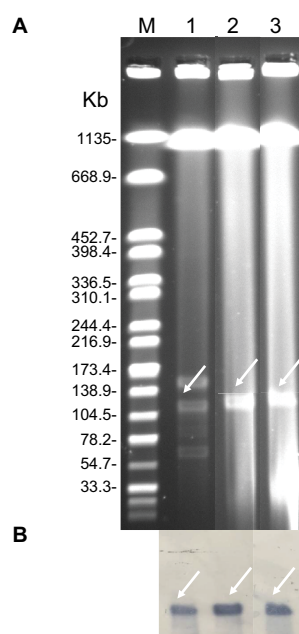
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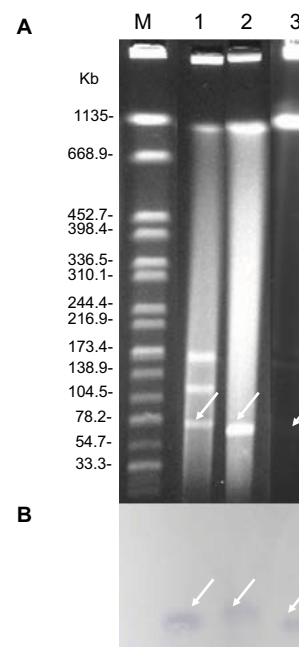
Supplementary materials

Figure S1 Identification of NDM-5-carrying plasmids.
Notes: (**A**) Plasmid profiles revealed by S1 nuclease pulsed-field gel electrophoresis.
(**B**) Southern blotting with bla_{NDM-5}-specific probe. Lane M, reference standard strain H9812 restricted with *Xba*I; Lane 1, 2, and 3 were *E. coli* EC16-50, its transformant T-NDM, and its conjugant J53-NDM, respectively.

Figure S2 Identification of *mcr-1*-carrying plasmids.
Notes: (**A**) Plasmid profiles revealed by S1 nuclease pulsed-field gel electrophoresis.
(**B**) Southern blotting with *mcr-1*-specific probe. Lane M, reference standard strain H9812 restricted with *Xba*I; Lane 1, 2, and 3 were *E. coli* EC16-50, its transformant T-MCR, and its conjugant J53-MCR, respectively.