

Co-Occurrence of *mcr-9* and *bla*_{NDM-1} in *Enterobacter cloacae* Isolated from a Patient with Bloodstream Infection

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Background: Bloodstream infection (BSI) caused by carbapenem-resistant Enterobacteriaceae are potentially life-threatening related to poorer outcomes. Colistin is considered one of the last-resort treatments against human infections caused by multidrug-resistant (MDR) Gram-negative bacteria. Therefore, emergence of strains from the blood that co-harboring *mcr* and carbapenem resistance genes were considered as a serious problem.

Purpose: In this study, two *mcr-9*-harboring MDR *Enterobacter cloacae* isolates BSI034 and BSI072 recovered from BSI patients were identified, one of which co-harbored *mcr-9* and *bla*_{NDM-1}. The genetic characteristics of the MDR plasmid needed to be clarified.

Methods: S1-PFGE and Southern blotting were conducted to determine the location of *mcr-9*. Whole-genome sequencing was performed to obtain the complete genome and plasmid sequences. The resistome and virulence genes of the strains, accompanied by the genetic characteristics of *mcr-9*- and *bla*_{NDM-1}-harboring plasmids, were analyzed.

Results: Whole-genome sequencing showed that BSI034 harbored *mcr-9*-carrying IncHI2-type pBSI034-MCR9 and *bla*_{NDM-1}-carrying IncX3-type pBSI034-NDM1. The 278,517 bp pBSI034-MCR9 carried *mcr-9* along with the other 19 resistance genes. *mcr-9* was flanked by IS903B (1057 bp) and IS26 (820 bp) in the same orientation. In addition to resistance genes, strain BSI034 also carried a chromosome-located *Yersinia* high-pathogenicity island, which harbored genes of yersiniabactin biosynthesis operon *ybtSXQPAUTE*, *irp1/2*, and *fyuA*.

Conclusion: We described the complete genome and *mcr-9/bla*_{NDM-1}-co-harboring plasmid of *E. cloacae* from a BSI patient. Notable differences were observed within mosaic modules between pBSI034-MCR9 and other *mcr-9*-harboring plasmids due to extensive recombination via horizontal gene transfer.

Keywords: MDR, *mcr-9*, colistin, virulence genes

Introduction

Colistin is considered one of the last-resort treatments against human infections caused by multidrug-resistant Gram-negative bacteria.¹ The first plasmid-mediated colistin resistance gene *mcr-1* was identified on an IncI2 plasmid from *Escherichia coli* and *Klebsiella pneumoniae* in China.² While *mcr-1* remains the predominant plasmid-mediated colistin resistance gene, *mcr-2* to *mcr-8*^{3,4} have been identified in various species from humans and animals. Recently, *mcr-9* was identified from a colistin-susceptible *Salmonella enterica* serotype Typhimurium isolate recovered from a human patient in the USA in 2010. Induced expression of *mcr-9* in *E. coli* conferred MIC of colistin at 2.5 mg/L.⁵ *mcr-9* shared 65% and 63% amino acid

identity with the most closely related MCR-3 and MCR-7 enzymes, respectively, and between 33% and 45% with the other MCR-like enzymes.⁶ *mcr-9* has also been identified among ESBL-producing Enterobacteriaceae isolates from horses in Sweden, including *Enterobacter cloacae*, *E. coli*, *Klebsiella oxytoca* and *Citrobacter freundii*.⁷

Carbapenem-resistant Enterobacteriaceae (CRE), which is a member of the ESKAPE group of pathogens, is an emerging pathogen and a leading cause of nosocomial infections⁸ associating with great difficulty of clinical treatment.⁹ Bloodstream infection (BSI) caused by CRE are potentially life-threatening related to poorer outcome and longer hospital stays.¹⁰ To better understand the characteristic of *mcr-9*-harboring plasmid, the *mcr-9*-positive isolates from BSI patients in China were identified.

Materials and Methods

Bacterial Strains

We collected 188 Enterobacteriaceae from the blood of BSI patients in Guangdong province, China. The isolation of those strains was part of the routine hospital laboratory procedure. Preliminary species identification was achieved by MALDI-TOF MS (BrukerDaltonik GmbH, Bremen, Germany) and 16s rRNA sequencing, and the identification of *E. cloacae* species was confirmed by WGS. The *mcr-9* gene was detected by using WGS data in two *E. cloacae* isolates BSI034 and BSI072 from two patients.

Antimicrobial Susceptibility Testing

MICs were determined for colistin, tigecycline, cefotaxime, ceftazidime, cefepime, gentamicin, amikacin, imipenem, ertapenem, meropenem, ciprofloxacin, fosfomycin, trimethoprim-sulfamethoxazole, piperacillin-tazobactam, aztreonam for both *mcr-9*-carrying isolates, using the agar dilution method except for colistin, for which used the broth dilution method was used, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. The *E. coli* ATCC25922 was the quality control strain used for the MIC measurement. The results interpreted according to CLSI instructions, while colistin and tigecycline resistance was defined according to EUCAST clinical breakpoints.

S1-PFGE and Southern Blotting

The plasmid and/or chromosomal locations of *mcr-9* were determined by S1-PFGE, followed by Southern blotting hybridizations.¹¹ Southern blotting hybridizations of

plasmid DNA were performed with a digoxigenin-labeled *mcr-9* probe, according to the manufacturer's instructions (Roche Diagnostics, Germany).

Whole-Genome Sequencing and Analysis

The five *E. cloacae* isolates were subjected to genomic DNA extraction using the Qiagen Blood & Tissue kit (Qiagen, Hilden, Germany) and whole-genome sequencing. DNA libraries were constructed with 350-bp paired-end fragments and sequenced using an Illumina HiSeq 2000 platform. The sequencing reads were assembled into contigs using SPAdes version 3.10.¹² The transferrable antimicrobial resistance genes and virulence genes were identified using ABRicate version 0.5 (<https://github.com/tseemann/abricate>). In silico multilocus sequence typing (MLST) was performed by MLST 1.8 (<https://cge.cbs.dtu.dk/services/MLST/>). Considering strain BSI034 co-harboring *mcr-9* and *bla*_{NDM-1} and other MDR features, we further used the long-read MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) to sequencing this strain. De novo hybrid assembly both of short Illumina reads and long MinION reads was performed using Unicycler v.0.4.3.¹³ Complete circular contigs were then corrected using Pilon v.1.22 with Illumina reads. The complete nucleotide sequence of pBSI034-MCR9 and pBSI034-NDM1 reported in this study has been submitted to the NCBI database and assigned accession numbers MN937241 and MN937240.

Results and Discussion

Characteristics of Patients Infected by Both Isolates

The ST114 strain BSI034 was isolated from a 35-year-old male patient in September 2013, who was suffering the renal calculus and urinary tract infection. After receiving the percutaneous nephrolithotomy, the man developed fever and bloodstream infection. Then, he was treated with meropenem and discharge from the hospital alive. While the ST190 strain BSI072 was recovered from a 3-year-old boy in June 2018, who was suffering a Burkitt lymphoma and acute kidney failure in a pediatric ICU. After receiving chemotherapy, the boy developed myelosuppression and bloodstream infection, which the source was considered as catheter-associated. Before infection, the patient was treated with piperacillin-tazobactam and imipenem. He then received the therapy of meropenem, cefoperazone sulbactam, levofloxacin, and tigecycline subsequently after infection. The boy eventually developed septic shock and died four days later.

Antimicrobial Susceptibility and Location of *mcr-9*

MICs of 15 antimicrobial agents for strain BSI034 and BSI072 were determined (Table S1). Except for colistin, strain BSI034 was resistant to all the tested antimicrobial agents. While strain BSI072 was susceptible to colistin, tige-cycline, and amikacin (Table S1). Both BSI034 and BSI072 did not show resistance to colistin, with 0.25 µg/mL and 0.5 µg/mL, respectively. This further support that *mcr-9* only causes colistin resistance under induction expression.⁶

S1-PFGE and Southern blotting hybridization revealed that *mcr-9* located on plasmids in size of ~270 Kb and ~320 Kb in BSI034 and BSI072, respectively (Figure 1).

Resistome and Virulence Genes in *E. cloacae* Isolates

It showed that BSI034 co-harbored *mcr-9* and *bla*_{NDM-1} genes, and the other 24 antimicrobial resistance genes mediating resistance to aminoglycosides (*aac*(6')-IIc, *aadA5*, *aph*(3'')-Ib, *aph*(3')-Ia, *aph*(6)-Id, *aph*(3')-Ia, *armA*), β-lactams (*bla*_{ACT-16},

*bla*_{DHA-1}, *bla*_{OXA-1}, *bla*_{SHV-12}), chloramphenicol (*catA2*, *catB3*), fosfomycin (*fosA*), macrolide (*msr*(E), *mph*(E), *ere*(A)), quinolones (*aac*(6')-Ib-cr, *oqxB*, *oqxA*, *qnrB4*), sulphona-mides (*sulI*), and trimethoprim (*dfpA1*, *dfpA19*). By contrast, BSI072 harbored *mcr-9* along with other 18 resistance genes mediating resistance to aminoglycosides (*ant*(2'')-Ia, *aadA16*), β-lactams (*bla*_{ACT-7}, *bla*_{CTX-M-65}, *bla*_{OXA-10}), chloramphenicol (*catA1*, *catB8*), fosfomycin (*fosA*), quinolones (*oqxB*, *oqxA*, *aac*(6')-Ib-cr, *qnrB6*), rifampicin (*ARR-3*), sulphonamides (*sulI*), tetracycline (*tet*(A), *tet*(G)), and trimethoprim (*dfpA27*).

In addition to resistance genes, both strains carried many virulence genes. A total of 35 different virulence genes were identified among these *mcr-9* positive isolates. Both isolates carried virulence genes including *acrB*, *fepA*, *fepC*, *fepD*, *fepG*, *ybdA*, *entE*, *entA*, *entB*, *ompA*, *gnd*, *galF*, *rscB*, *vipA/tssB*. *acrB*, part of the AcrAB-TolC tripartite system, belonging to the RND family, have been proved to be associated with increasing antibiotic resistance and virulence in *E. cloacae*.¹⁴ Besides the virulence genes above, BSI034 also carried a chromosome-located *Yersinia* high-pathogenicity island (HPI) which harbored yersiniabactin biosynthesis operon *ybtSXQPAUTE*, *irp1/2*, and *fyuA*. This HPI was inserted at the 3'-end of a tRNA gene (tRNA-Asn) and has no other mobile elements.

Genetic Characteristics of the Plasmid-Borne *mcr-9*

The *mcr-9*-carrying pBSI034-MCR9, which has IncHI2 and IncHI2A replicons, was found to encode 297 open reading frames (ORFs) (Figure 2). In pBSI034-MCR9, *mcr-9* was flanked by two insertion sequences (IS) in the same orientation. With 132 bp upstream of *mcr-9*, an IS903B (1057 bp, IS6 family) was identified. This element included the intact IRL and IRR sequences but lacked the 8 bp direct repeat (DR) sequence surrounding it. Besides, downstream of *mcr-9* with 655 bp interval is an IS26 (820 bp, IS6 family). Similar to the conclusion in another study, IS903 and IS903-like have been found upstream of *mcr-9*, and IS1R, IS26-like, IS15DII have been found downstream of *mcr-9*.^{6,15} The consistent adjacency of *mcr-9* to IS903-like element suggests the acquisition of *mcr-9* by an IS903-dependent mechanism. The major differences of the genetic context of *mcr-9* were various IS elements and ORFs downstream of *mcr-9*, thus forming diverse *mcr-9* gene contexts. Aside from *mcr-9*, pBSI034-MCR9 harbored other 22 resistance genes including four copies of *sulI* to form a large MDR plasmid, including those involved in resistance to aminoglycoside, β-lactams, chloramphenicol, macrolide, quinolones, sulphonamides, and trimethoprim. Those

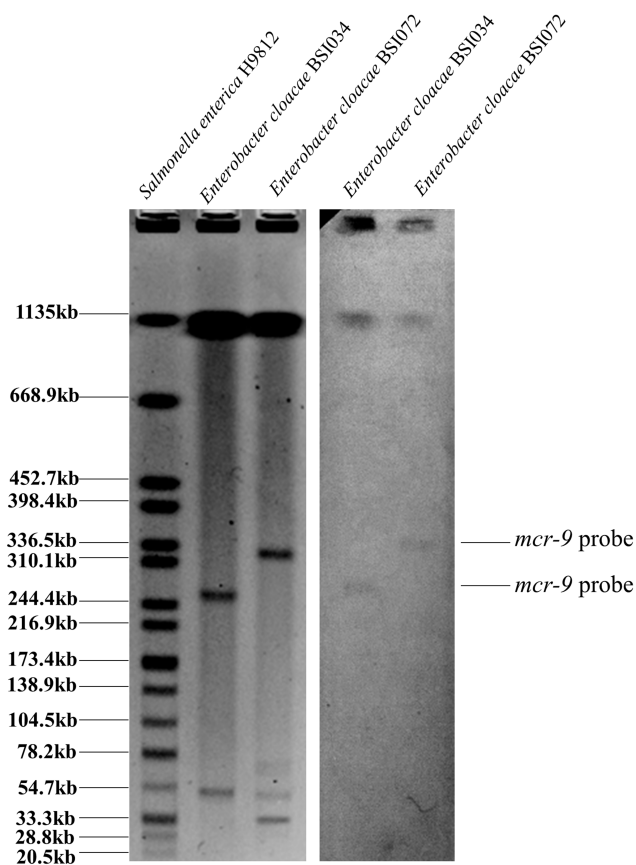


Figure 1 S1-PFGE and Southern blotting hybridization with the *mcr-9* probe. The left image represents the S1-PFGE map of *E. cloacae* strains; the right image is the result of Southern blotting hybridization using *mcr-9* probe.

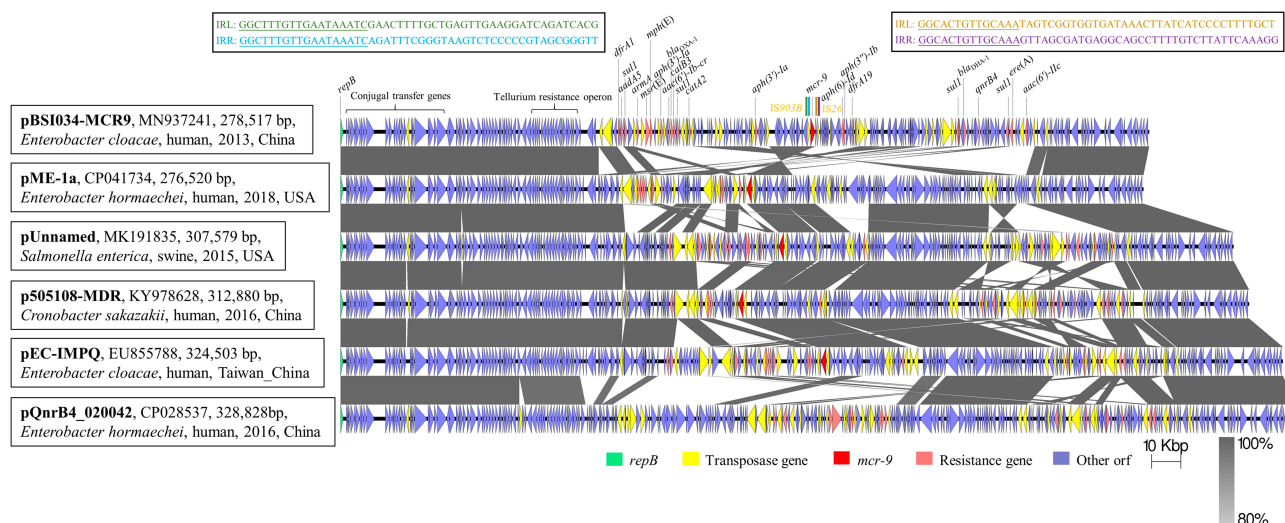


Figure 2 Schematic presentation of major structural features of pBSI034-MCR9 in comparison with five reference plasmids. Areas shaded in gray indicate homologous regions of $\geq 80\%$ nucleotide sequence identity in the plasmid scaffold regions. ORFs are portrayed by arrows to indicate the direction of transcription and colored based on their predicted gene functions. The figure was drawn to scale.

resistance genes were surrounded by various IS elements or transposons. A BLAST comparison of pBSI034-MCR9 screened five most close plasmids (Figure 2). These included a newly identified *mcr-9* and *bla*_{VIM-4}-co-harboring plasmid pME-1a (CP041734) in an *E. hormaechei* isolate from a pediatric patient in the USA;¹⁶ pUnnamed (MK191835) from *Salmonella enterica* subsp. *enterica* serovar Braenderup strain from swine in the USA;¹⁷ p505108-MDR (KY978628) in *Cronobacter sakazakii* isolated from a patient in China;¹⁸ pEC-IMPQ (EU855788) in a *bla*_{IMP-8}-carrying *E. cloacae* from a patient in Taiwan;¹⁹ non-*mcr-9*-carrying plasmid pQnrB4_020042 (CP028537) in a clinical *E. hormaechei* in China. They all carried a backbone related to a conjugational transfer locus (~30 Kb), a tellurium resistance operon (*ter* locus, 16785 bp) and *parAB* and *parMR* for partition. Additionally, pUnnamed, p505108-MDR, and pEC-IMPQ carried a mercuric resistance operon (*merEDACPTR*, 3976 bp). However, the accessory modules exhibited some similarities but also notable differences across those plasmids, which are likely to contain several MDR modules to form large MDR plasmids due to extensive recombination mediated by multiple mobile elements.

Analysis of the Plasmid Carrying *bla*_{NDM-1}

*bla*_{NDM-1} was located on the IncX3 plasmid pBSI034-NDM1. The *bla*_{NDM-1} gene accompanied by *ble*_{MBL} (bleomycin resistance gene) located downstream of an *IS*_{Aba125} element (1092 bp). This *IS*_{Aba125} was disrupted by the insertion of *IS*₅ (1195 bp) at the 917 bp position site. Furthermore, an *IS*₃₀₀₀ (3236 bp) and a truncated Tn2

(580 bp) element were found upstream of the *IS*_{Aba125} element. Indeed, *bla*_{NDM-1} has always been found in association with an upstream insertion sequence *IS*_{Aba125} which provides the -35 promoter sequence.²⁰ pBSI034-NDM1 also harbored *bla*_{SHV-12}, which was bounded by two *IS*₂₆ (820 bp) elements. pBSI034-NDM1 is organized very similarly (100% query coverage, 99% identity) to that of plasmids pNDM1_020135 (*K. pneumoniae*, CP037965), p112298-NDM (*Citrobacter freundii*, KP987216), and pNDM-HF727 (*E. cloacae*, KF976405) (Figure 3). The main difference between them is pBSI034-NDM1 carries an additional segment that contains an *IS*₅ (1195 bp).

Conclusion

BSI caused by carbapenem-resistant and colistin-resistant Enterobacteriaceae differs from most other multidrug-resistant bacterial pathogens in that there is no reliable treatment. In summary, we reported the complete sequence of plasmid co-harboring the *mcr-9* and other resistance genes in NDM-1-producing *E. cloacae*. The linkage is a matter of concern since it could herald the possibility of a co-spread of the two genes, both involved in resistance to last-resort agents. Screening for the *mcr-9* gene in other species in animals, the environment, and humans is necessary to understand its dissemination throughout the world.

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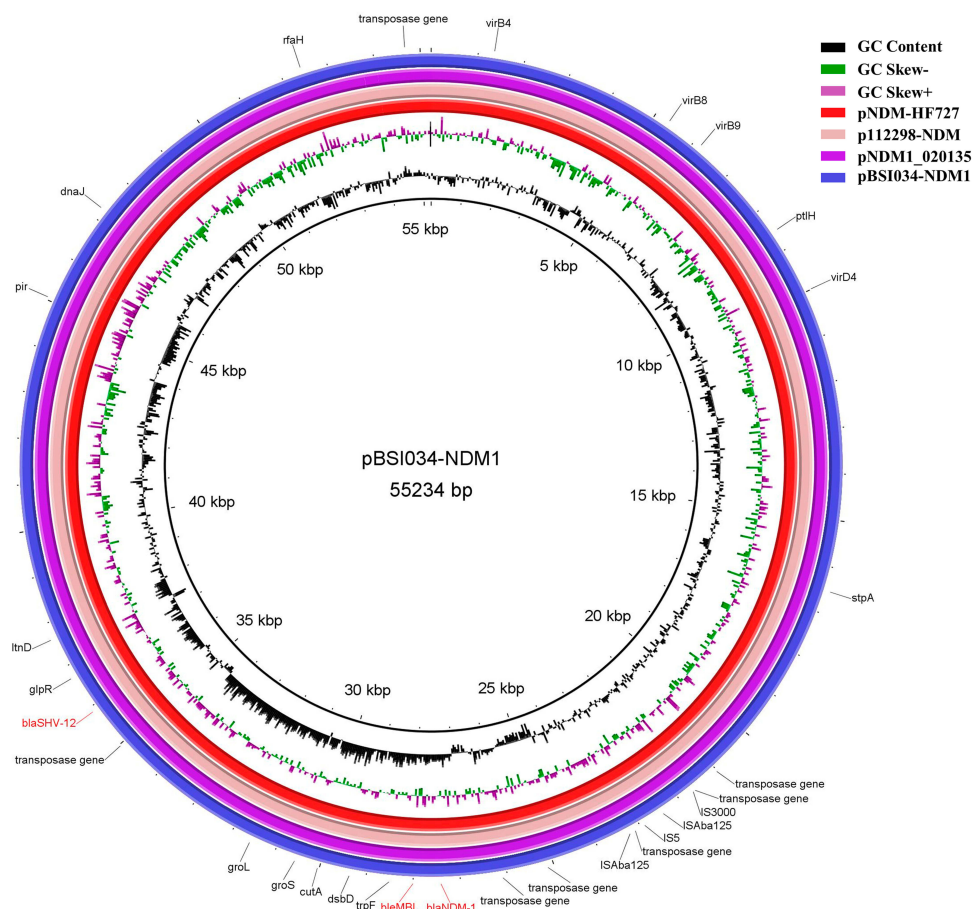


Figure 3 Circular genetic map of pBSI034-NDM1 and three reference plasmids. pNDM-HF727 (accession no. KF976405), p112298-NDM (accession no. KP987216), pNDM1_020135 (accession no. CP037965).

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Disclosure

The authors report no conflicts of interest in this work.

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