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ORIGINAL RESEARCH

Coproduction Of MCR-9 And NDM-1 By Colistin-Resistant *Enterobacter hormaechei* Isolated From Bloodstream Infection

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Yi Yuan[1,*]
Ying Li[2,*]
Guangxi Wang[3]
Chengwen Li[2]
Li Xiang[3]
Junping She[3]
Yan Yang[3]
Fangcai Zhong[1]
Luhua Zhang[3]

[1]Department of Clinical Laboratory, The First People's Hospital of Neijiang, Sichuan, People's Republic of China; [2]Department of Immunology, School of Basic Medical Sciences, Southwest Medical University, Luzhou, Sichuan, People's Republic of China; [3]Department of Pathogenic Biology, School of Basic Medical Sciences, Southwest Medical University, Luzhou, Sichuan, People's Republic of China

*These authors contributed equally to this work

Background: Colistin acts as the last line of defense against severe infections caused by carbapenem-resistant *Enterobacteriaceae*. Infections caused by extensively drug-resistant isolates coproducing MCR and carbapenemases have posed a serious public health concern.
Purpose: In this study, we reported the first clinical colistin and carbapenem-resistant *Enterobacter hormaechei* isolate SCNJ07 coharboring $bla_{\text{NDM-1}}$ and *mcr-9* from a patient with bloodstream infection in China.
Methods: Bacterial antimicrobial susceptibility testing was performed using the broth microdilution method. Conjugation assay was carried out to investigate the transferability of *mcr-9* and $bla_{\text{NDM-1}}$. Whole-genome sequencing of strain SCNJ07 was performed using an Illumina HiSeq system and the genetic characteristics of the *mcr-9*- and $bla_{\text{NDM-1}}$-harboring plasmids were analyzed.
Results: Conjugation assays revealed that both $bla_{\text{NDM-1}}$ and *mcr-9* genes could successfully transfer their resistance phenotype to *Escherichia coli* strain J53. Whole genome sequencing showed that SCNJ07 possessed an FIB36:FIIY4 type self-transmissible plasmid bearing $bla_{\text{NDM-1}}$, which possessed high similarity to previously reported pRJF866 in China. *mcr-9* was located on a ~28-kb self-transmissible plasmid pMCR-SCNJ07 with both IncHI2 and IncR replicons. Two copies of intact IS903 that bracketed a ~8-kb region containing the *mcr-9* gene were identified in pMCR-SCNJ07. BLASTn analysis revealed that a number of *mcr-9*-positive plasmids have been around for a while among *Enterobacteriaceae* worldwide.
Conclusion: This study reveals the likelihood of a wide dissemination of this newly identified colistin resistance gene *mcr-9* among *Enterobacteriaceae*. Further surveillance is urgently needed to understand the prevalence and dissemination of *mcr-9*, thereby facilitating establishment of measures to control its spread.
Keywords: colistin, *mcr-9*, CRE, IS*903*

Introduction

Carbapenem-resistant *Enterobacteriaceae* (CRE)-related infections are global public health issues.[1] The New Delhi metallo-β-lactamase (NDM) is one of the most common carbapenemases worldwide.[2] Polymyxins are among the last-line therapeutic options to treat serious infections caused by CRE.[3] However, concerns were raised regarding the increasing prevalence of the plasmid-borne colistin resistance gene, *mcr-1*, which has been detected from the environment, food, animals and humans around the world since its first discovery in China in late 2015.[4–6] More worrisomely, cases of infection caused by extensively drug-resistant isolates that

Correspondence: Luhua Zhang
Department of Pathogenic Biology, School of Basic Medical Sciences, Southwest Medical University, Xiang Lin Road, Longmatan District, Luzhou City 646000, Sichuan Province, People's Republic of China
Tel/Fax +86 830 3160073
Email zhluhua@swmu.edu.cn

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coproduce MCR-1 and NDM have been reported by groups from different regions.[7–13]

Following a recent study which identified an additional *mcr* homolog, *mcr-9*, in a *Salmonella enterica* serotype Typhimurium isolate,[14] we isolated an extensively drug-resistant *Enterobacter hormaechei* strain, which coproduces NDM-1 and MCR-9, from a patient with bloodstream infection. The genetic characteristics of the bla_{NDM-1} and *mcr-9*-harboring plasmids were analyzed. In addition, comparative genetic analysis of *mcr-9* in pMCR-SCNJ07 and closely related plasmids were also carried out. To the best of our knowledge, this is the first report describing a clinical colistin and carbapenem-resistant *E. hormaechei* isolate coharboring bla_{NDM-1} and *mcr-9* in China.

Materials And Methods

Bacterial Isolation And Identification

The *E. hormaechei* strain SCNJ07 was recovered from the blood sample of a 50-year-old male patient with obstructive jaundice in a hospital in Sichuan, China, in September 2018. It was initially identified as *Enterobacter cloacae* using the Vitek-2 compact system (bioMérieux, Marcy-l'Étoile, France) and by species identification established by sequencing of the 16S rRNA gene amplified with the universal primers 27F and 1492R.[15] The presence of the acquired carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{GES}, bla_{IMP}, bla_{OXA-48}, and bla_{VIM}) and *mcr* genes (*mcr-1* to *mcr-8*) in SCNJ07 was determined by PCR amplification as described previously.[16–21] The detection of *mcr-9* was amplified using the primers MCR9-F (5′- CTTTCCATAACAGCGAGACA C-3′) and MCR9-R (5′- GTATCCTTCCTGCCATCCTC-3′).

Antimicrobial Susceptibility Testing

In vitro susceptibility tests of ceftazidime, ceftriaxone, cefepime, cefazolin, cefotetan, imipenem, aztreonam, ciprofloxacin, gentamycin, tobramycin, levofloxacin, amikacin, piperacillin-tazobactam and trimethoprim-sulfamethoxazole were performed using Vitek-2 system. The minimum inhibitory concentrations (MICs) of meropenem, colistin, polymyxin B, doxycycline, fosfomycin and tigecycline against the strain were determined using the microdilution broth method following recommendations of the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2019). Breakpoints of colistin and tigecycline were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/); otherwise, we applied those defined by the CLSI.

Conjugation Assay

Conjugation experiments were carried out using broth-based methods with *Escherichia coli* J53 (azide-resistant) as the recipient strain. Transconjugants were selected on LB agar plates containing 150 μg/mL sodium azide plus 1 μg/mL meropenem or 4 μg/mL colistin. The presence of bla_{NDM-1} or *mcr-9* in transconjugants was confirmed by PCR and Sanger sequencing.

Genome Sequencing And Analysis

Total genomic DNA of *E. coli* isolates was extracted using Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. Purified DNA was subjected to whole genomic sequencing on the Illumina HiSeq 2000 system with the 150-bp paired-end approach and 150× coverage. Reads were trimmed using Trimmomatic[22] and were then assembled using the SOAP *de novo* program.[23] Annotation was carried out using Prokka.[24] The species identification was performed by average nucleotide identity (ANI) analysis with JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/#analyse). Antimicrobial resistance genes were identified using ResFinder v3.1 of the Center for genomic epidemiology (http://genomicepidemiology.org/).

Plasmid Sequencing And Analysis

Plasmid DNA from transconjugants containing bla_{NDM-1} or *mcr-9* was extracted and sequenced using the Illumina HiSeq system. After filtering J53 chromosomal DNA data and assembling the remaining reads, the plasmid carrying bla_{NDM-1} or *mcr-9* was completely circularized using PCR and Sanger sequencing to fill in gaps between contigs. The plasmid replicon type and MLST were determined using the PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and pMLST tools (https://cge.cbs.dtu.dk/services/pMLST/). The annotations of the plasmid sequences were conducted using the RAST tools and edited manually.[25] Sequence alignment of *mcr-9*-carrying plasmids was performed using BLAST and visualized with Easyfig v 2.2.3.[26] Alignments with highly homologous complete plasmid sequences of pMCR-SCNJ07 available in NCBI were performed by using the BRIG tool.[27]

Nucleotide Sequence Accession Numbers

Draft genome sequence of the strain SCNJ07 has been deposited into GenBank under the accession no. VBSC00000 000. The complete sequences of pNDM-SCNJ07 and pMCR-SCNJ07 have been deposited into GenBank under accession no. MK933278 and MK933279, respectively.

Ethics Statement

The clinical isolate *E. hormaechei* SCNJ07 was generated as part of the routine hospital laboratory procedure. The Ethics Committee of the Southwest Medical University exempted this study from review because the present study only focused on bacteria and patient intervention was not required.

Results And Discussion

Antimicrobial Susceptibility Of The Strain *E. hormaechei* SCNJ07

Susceptibility testing by Vitek-2 system showed that the strain SCNJ07 was resistant to all tested drugs, including ceftazidime (MIC, ≥64 μg/mL), ceftriaxone (MIC, ≥64 μg/mL), cefepime (MIC, ≥64 μg/mL), cefazolin (MIC, ≥64 μg/mL), cefotetan (MIC, ≥64 μg/mL), imipenem (MIC, ≥16 μg/mL), aztreonam (MIC, ≥64 μg/mL), ciprofloxacin (MIC, ≥4 μg/mL), gentamycin (MIC, ≥16 μg/mL), tobramycin (MIC, ≥16 μg/mL), levofloxacin (MIC, ≥8 μg/mL), amikacin (MIC, ≥64 μg/mL), piperacillin-tazobactam (MIC, ≥128 μg/mL) and trimethoprim-sulfamethoxazole (MIC, ≥320 μg/mL). Additional broth microdilution testing[28] showed that it also exhibited resistance to meropenem (MIC, 256 μg/mL), doxycycline (MIC, 128 μg/mL), colistin (MIC, 16 μg/mL) and polymyxin B (MIC, 8 μg/mL) but was susceptible to fosfomycin (MIC, 64 μg/mL) and tigecycline (MIC, 2 μg/mL). PCR and sequencing analysis showed that the strain SCNJ07 harbored both a $bla_{\text{NDM-1}}$ carbapenemase gene and the recently identified plasmid-borne colistin resistance gene *mcr-9*.

Location And Transferability Of *mcr-9* And $bla_{\text{NDM-1}}$

Conjugation assays showed that $bla_{\text{NDM-1}}$ and *mcr-9* were successfully transferred to *E. coli* J53 at the frequency of ~10^{-2} and 10^{-4} (transconjugant/recipient), respectively. Compared with the MICs for the recipient strain J53, transformants containing $bla_{\text{NDM-1}}$ showed a 128-fold increase for meropenem (from 0.5 to 64 μg/mL) and those harboring *mcr-9* 16-fold for colistin (from 0.5 to 8 μg/mL). These findings revealed that $bla_{\text{NDM-1}}$ and *mcr-9* were functional and carried by two self-transmissible plasmids.

Genome Characteristics Of The Strain SCNJ07

Draft genome sequence of SCNJ07 was assembled into 120 contigs (112 were >1000 bp in length), which comprises 5,321,397 bp, with a 54.77% GC content. Species identification based on ANI analysis confirmed that the strain SCNJ07 actually belongs to *E. hormaechei*, as it only had an 86.32% identity (75.45% query coverage) to *E. cloacae subsp. cloacae* ATCC 13,047, but a 98.41% identity (80.30% coverage) to *E. hormaechei subsp. steigerwaltii* strain DSM 16691, obviously above the 95–96% cut-off for defining a bacterial species.[29]

Resistance Profile Of The Strain SCNJ07

Analysis of the whole genome sequence of SCNJ07 allowed us to more confidently conclude that $bla_{\text{NDM-1}}$ and *mcr-9* co-existed in this strain. Besides, in consistence with its multidrug resistance phenotype, SCNJ07 also had multiple genes mediating resistance to β-lactams ($bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{SHV-12}}$ and $bla_{\text{ACT-7}}$), aminoglycosides (*aac(6')-IIa, aadA16, aac(6')-Ib3* and *rmtC*), fluoroquinolones (*qnrS1* and *aac(6')-Ib-cr*), macrolide (*mph*(A)), rifampicin (*ARR-3*), tetracycline (*tet(D)*), sulfonamide (*sul1*) and trimethoprim (*dfrA16*). *E. hormaechei*, a common nosocomial pathogen of clinical significance, was reported in several outbreaks of sepsis in neonatal intensive care units in the USA and in Brazil,[30] while it was only sporadically reported in China.[31] The identification of *E. hormaechei* SCNJ07 coproducing NDM-1 and MCR-9 in this study highlights the need to enhance the epidemiologic surveillance of this novel colistin resistance gene in CRE.

Analysis Of The $bla_{\text{NDM-1}}$-Harbouring Plasmid pNDM-SCNJ07

Plasmid analysis revealed that the $bla_{\text{NDM-1}}$ was carried by an FIB36:FIIY4 replication type plasmid, designated pNDM-SCNJ07, which was 110,786 bp in length and had an average GC content of 54.84%. pNDM-SCNJ07 is almost identical (99% identity and 99.98% coverage) to the plasmid pRJF866 (GenBank accession no. KF732966) from *K. pneumoniae* from Shanghai, China, in 2015.[32] Besides, similar FIB36:FIIY4-type plasmids carrying $bla_{\text{NDM-1}}$ have been widely found among *Enterobacteriaceae*, including pKOX_NDM1 (Accession no. JQ314407) from *Klebsiella michiganensis* from Taiwan,[33] pNDM_20ES (Accession no. MF042356) from *E. cloacae* and pNDM_4TM (Accession no. MF042352) from *Serratia marcescens* from Romania.[34] On these plasmids, the $bla_{\text{NDM-1}}$ gene was embedded in the same genetic environment and its flanking miniature inverted-repeat transposable elements (MITEs, positions

70834 to 71089 and 77035 to 77290 of pNDM-SCNJ07) were suggested to be responsible for the mobilization of bla_{NDM-1} onto the FIB36:FIIY4 plasmids.[33,35]

Genetic Characteristics Of The Plasmid-borne *mcr-9*

Complete sequence of the *mcr-9*-carrying plasmid, designated pMCR-SCNJ07, was circularized by PCR mapping using pT5282-mphA (Accession no. KY270852) as the template. pMCR-SCNJ07 was 285,587 bp in size with an average G+C content of 47.31%. The plasmid contained 327 predicted ORFs, and two replicons, IncHI2 (ST1) and IncR, and carried a number of additional resistance genes, including bla_{SHV-12}, *aadA16, aac(6')-IIa, mph(A), sul1* and *tet(D)*. Sequence alignment showed that pMCR-SCNJ07 displayed 97% query coverage and 99.99% identity with the reference plasmid pT5282-mphA from an *E. cloacae* isolated in 2012 from a teaching hospital in Chongqing, China.[36] By BLAST, pMCR-SCNJ07 was also similar to several previously sequenced IncHI2 plasmids (Figure 1), including pMRVIM0813 (86% query coverage and 99.97% identity, accession no. KP975077), pCTXM9_020038 (85% query coverage and 99.97% identity, accession no. CP031724), pC45-VIM4 (85% query coverage and 99.96% identity, accession no. LT991958) and pSE15-SA01028 (83% query coverage and 99.99% identity, accession no. CP026661), from a clinical *E. cloacae* isolate from USA in 2015, an *E. hormaechei* from China in 2016, an *E. cloacae* from France in 2018 and a *Salmonella enterica subsp. Enterica* isolate from Germany in 2015, respectively. It is noteworthy that all these plasmids were *mcr-9*-positive, highlighting an earlier presence of *mcr-9* among *Enterobacteriaceae* around the world than previously known and raising the likelihood of ongoing undetected transmission. Close surveillance is urgently needed to determine *mcr-9* prevalence and effective actions are required to control its further dissemination.

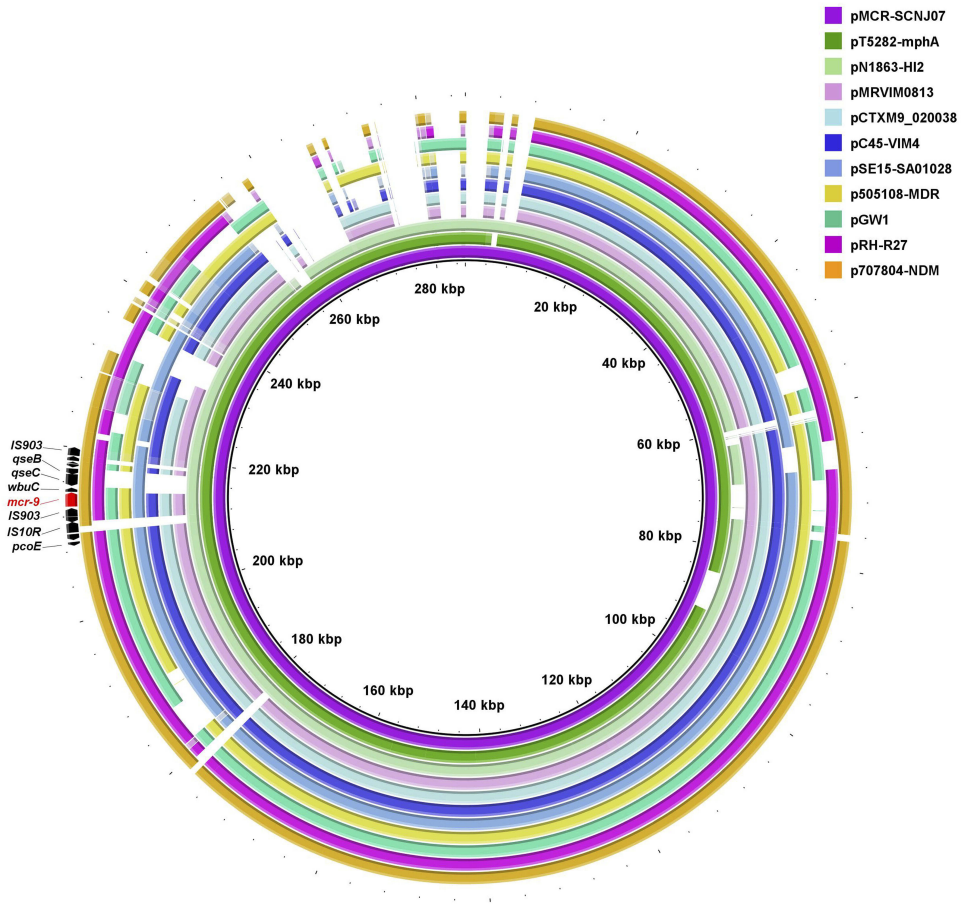

Figure 1 Circular comparison of *mcr-9*-carrying IncHI2 plasmids. The complete sequence of pMCR-SCNJ07 was used as the reference. The arrows indicate deduced ORFs and their orientations. The *mcr-9* gene is indicated in red. The circular maps were generated using BRIG[27] and plasmids were included in the following order (inner to outer circles): pMCR-SCNJ07 (this study, accession no. MK933279), pT5282-mphA (KY270852), pN1863-HI2 (MF344583), pMRVIM0813 (KP975077), pCTXM9_020038 (CP031724), pC45-VIM4 (LT991958), pSE15-SA01028 (CP026661), p505108-MDR (KY978628), pGW1 (CP028975), pRH-R27 (LN555650) and p707804-NDM (MH909331).

The genetic environment of *mcr-9* has not been described before. On pMCR-SCNJ07, *mcr-9* was located in a ~8kb region bracketed by two copies of intact IS903, an insertion sequence originating from *E. coli*. In this region, *wbuC* (encoding a cupin fold metalloprotein) was located downstream of *mcr-9*, followed by a two-component system encoding genes *qseC* and *qseB*, a truncated insertion sequence ΔIS1R, and a remnant of Δ*silR* (encoding a transcriptional regulatory protein) (Figure 2A). The two copies of intact IS903 could form a composite transposon or a circular intermediate, which has the potential to mobilize the *mcr-9* gene. However, no circular intermediate was detected despite repeated attempts via inverse PCR in our work. Whether *mcr-9* could transfer through the formation of a circular intermediate requires further investigation. Of note, similar to the scenario in all known *mcr-1* cassettes, in which one or both ISApl1 sequences are consistently absent in the *mcr-1*-bearing region,[37] the downstream IS903 was absent on some *mcr-9*-carrying plasmids (Figure 2A).

A pairwise comparison of the *sil–cop* region of IncHI2 plasmids R478 and pRH-R27 enabled us to learn that *mcr-9* was likely to be transferred by the IS1R to IS903 region initially, and that insertion of this *mcr-9*-carrying region between *sil* and *cop* resulted in truncation of *cop* resistance gene clusters into Δ*copS–copE1* (Figure 2B).[38] BLASTn

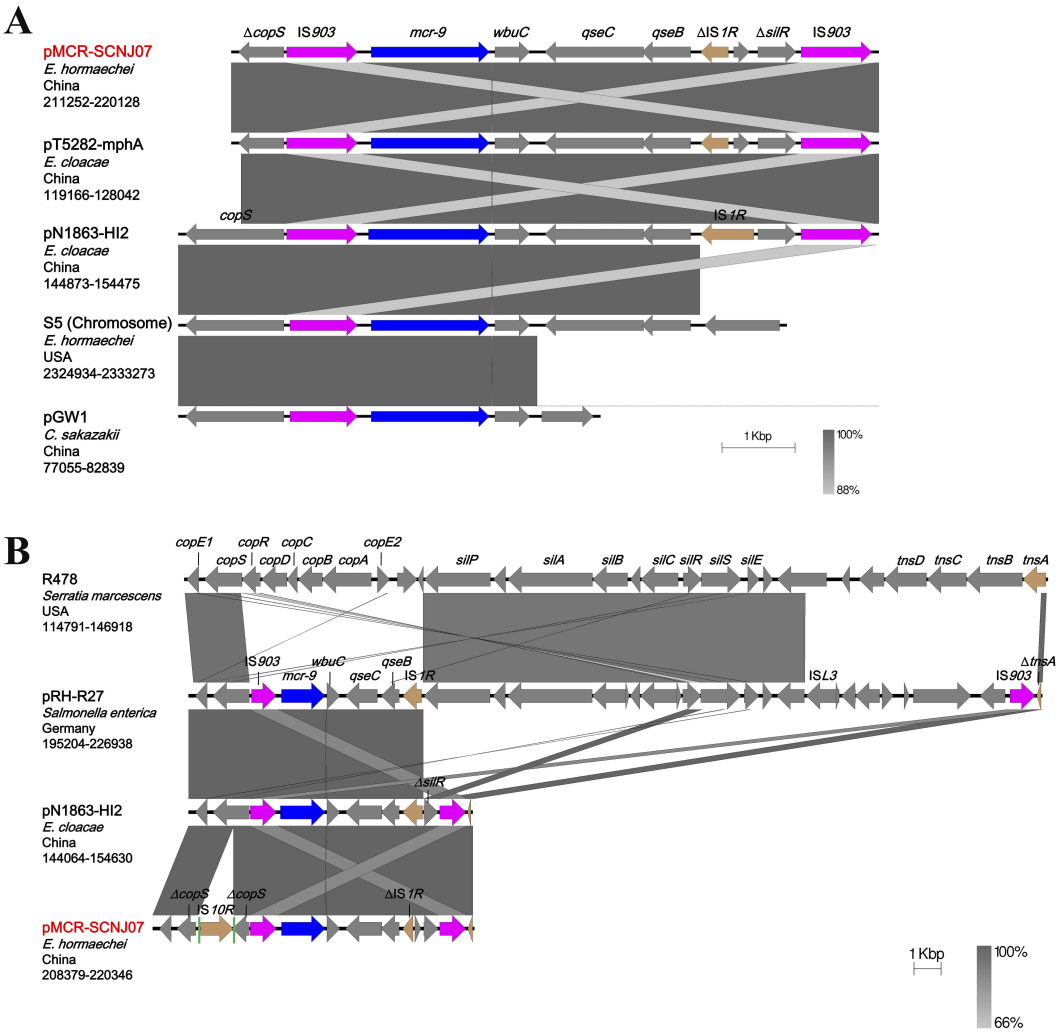

Figure 2 Colinear analyses for genetic environment of *mcr-9*. (**A**) Comparison of the *mcr-9*-containing regions from different plasmid reservoirs and the chromosome of *E. hormaechei* strain S5. (**B**) Comparison of the *mcr-9*-neighboring genetic contexts on IncHI2-type plasmids. The corresponding region on non-*mcr-9*-carrying plasmid R478 (top) is shown for comparison. Arrows indicate the positions and directions of the genes and Δ indicates the truncated gene. *mcr-9* genes are indicated in dark blue and IS903 are highlighted in purple. Gray shades denote shared regions with a high degree of homology. Vertical green bars represent the direct repeats of IS10R. The accession numbers were: *E. hormaechei* strain S5 (Accession no. CP031571), pT5282-mphA (KY270852), pN1863-HI2 (MF344583), pGW1 (CP028975), R478 (NC_005211), pRH-R27 (LN555650), pN1863-HI2 (MF344583), and pMCR-SCNJ07 (this study, MK933279). The construction of sequence comparison was performed using BLAST (http://blast.ncbi.nlm.nih.gov) and Easyfig version 2.2.3.[26]

analysis suggested that the *mcr-9*-carrying region of pMCR-SCNJ07 could be derived from pRH-R27 as a result of multiple genetic events (Figure 2B). No 9-bp target site duplication repeats, which are characteristic of the insertion of IS903, could be identified in the sequence adjacent to both IS903 elements. The detail of the evolutionary route of the *mcr-9*-carrying region, therefore, remains unclear.

To address the possible origin of *mcr-9*, a BLAST search against the NCBI's non-redundant protein sequence database (nr) was conducted by using the amino acid sequence of *mcr-9* as a probe. The result identified hits aligned to phosphoethanolamine transferases from multiple genera of *Enterobacteriaceae* (100% query coverage and 94–100% identity), followed by that from *Buttiauxella brennerae* (WP_064558897, 100% query coverage and 86.83% identity). It should be noted that of the flanking genes of *mcr-9* within the ~8kb region, none but the *wbuC* gene showed homologous to that from *Buttiauxella*, with 98% query coverage and 85.9% identity between their amino acid sequences. These findings suggested that the disseminated *mcr-9* gene in *Enterobacteriaceae* might have originated from *Buttiauxella* species and that the *wbuC* gene had been likely comobilized with *mcr-9* from its original genetic context. Additional studies are needed to fully characterize the origin of *mcr-9*.

Conclusion

In summary, we here report the discovery of a clinical *E. hormaechei* strain carrying both bla_{NDM-1} and *mcr-9*. Despite few reports characterizing *mcr-9*-harboring plasmids, this newly identified mobile colistin resistance gene is likely to have already been widely disseminated. Yet more worryingly, two copies of IS903 encompassing the *mcr-9* gene have the potential to mobilize *mcr-9* by hijacking more plasmids as the vehicle to disseminate this gene. Therefore, screening for the *mcr-9* gene should be urgently included in the surveillance of colistin-resistant *Enterobacteriaceae* from humans, animals, and the environment.

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Author Contributions

LZ designed the experiments. YY, GW, and CL performed the experiments. LX, JS and YY analyzed the data. YL and LZ analyzed the data and wrote the manuscript. FZ edited the original draft. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, 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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