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ORIGINAL RESEARCH

Co-Occurrence of NDM-9 and MCR-1 in a Human Gut Colonized *Escherichia coli* ST1011

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Background: The emergence of the plasmid-borne colistin-resistant gene (*mcr-1*) poses a great threat to human health. What is worse, the recent observations of the coexistence of *mcr-1* with carbapenemase encoding genes in some bacteria caused even more concern. Yet, there is a lack of observations of such strains in the human gut.

Methods: The isolation of *E. coli* L889 was performed on selective medium plates. Antibiotic susceptibilities were determined by an agar dilution and a broth microdilution method. Multi-locus sequence typing (MLST) and acquired resistance genes were also characterized. Transferability of $bla_{\text{NDM-9}}$/*mcr-1*-carrying plasmids was determined by conjugation, replicon typing and S1-Pulsed-field gel electrophoresis (S1-PFGE), and Southern blotting. The sequences of these plasmids were analyzed by using whole-genome sequencing with Illumina Novaseq and Nanopore platforms.

Results: *E. coli* L889 was identified as ST1101 concomitantly carrying $bla_{\text{NDM-9}}$ and *mcr-1* from a stool sample. Antimicrobial susceptibility tests showed that it was resistant to various antimicrobial agents and only susceptible to tigecycline. Notably, $bla_{\text{NDM-9}}$ was located on a ~114-kb untypable plasmid, while *mcr-1* was located on a ~63-kb IncI2 plasmid.

Conclusion: Our research, to our knowledge, first reported an ST1101 *E. coli* strain with an untypeable $bla_{\text{NDM-9}}$-harbouring plasmid and an IncI2 *mcr-1*-carrying plasmid. The colonized *E. coli* strains potentially contribute to the dissemination and transfer of $bla_{\text{NDM-9}}$ and *mcr-1* to clinical isolates, which is a considerable threat to public health and should be closely monitored.

Keywords: $bla_{\text{NDM-9}}$, *mcr-1*, gut, *Escherichia coli*, ST1101

Background

The global dissemination of colistin resistance, due to transferable *mcr*-genes, threatens public and animal health as there are limited therapeutic options. Since the plasmid-mediated colistin resistance gene *mcr-1* was first reported in *Escherichia coli* isolates in China, several reports confirmed that *mcr-1* has spread in several Enterobacteriaceae species on different continents and from various samples.[1–7] Of great clinical concern are the inevitable co-occurrence of *mcr*-genes and carbapenem-resistance genes among Enterobacteriaceae and the widespread resistance genes in the environment, which eventually aggravate the selection process in the occurrence of true pan-drug resistance.[1,8,9]

The first NDM-9-producing *K. pneumoniae* strain was isolated in 2014 in China, which showed that the $bla_{\text{NDM-9}}$ gene encodes a protein with one amino acid substitution (E152K) compared with NDM-1.[10] Subsequently, the NDM-9 variant was sporadically reported in Asian and European countries.[11–13] It is worthy to note

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that NDM-9 presented more significant enzyme activity than NDM-1 on all tested β-lactams except monobactams, slightly higher hydrolytic activity for cefotaxime, cefoxitin, imipenem, and meropenem, and higher affinity for imipenem and meropenem.[11]

Previously we conducted a prospective, observational cohort study involving inpatients to screen carbapenemase-producing Enterobacteriaceae (CPE) from stool samples.[14] In this work, we report the isolation of co-producing of MCR-1 and NDM-9 in a human gut colonized *E. coli* L889 from that follow-up investigation. We also described the antimicrobial susceptibility profile and plasmid characteristics of this isolate.

Materials and Methods

Bacterial Isolation and Susceptibility Testing

Previously, we sampled 811 nonduplicate stool samples from 443 inpatients and screened for carbapenemase-producing Enterobacteriaceae isolates.[14] The *E. coli* L889 strain was isolated from a fecal sample of a 21-years old male patient admitted with abdominal pain. Bacterial identification was confirmed by matrix-assist laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker, Bremen, Germany). The carbapenemase encoding gene (bla_{NDM}) was identified using PCR (Primer: Forward-ATGGAATTGCCCAATATTATGCAC, Reverse-TCAGCGCAGCTTGTCGGC), and DNA sequencing was performed on the PCR positive isolates using Sanger sequencing. Antimicrobial susceptibility testing (AST) was conducted by agar dilution and broth microdilution, using *Escherichia coli* (ATCC 25922) as the control. AST results were interpreted following the Clinical and Laboratory Standards Institute (CLSI) 2020 standards.[15]

Whole-Genome Sequencing and in silico Analysis

Whole-genome sequencing (WGS) of L889 was performed using Illumina NovaSeq 6000 (Illumina, USA) and Nanopore (Oxford Nanotechnology, UK) platforms in Novogene (Beijing, China). The hybrid assembly of Illumina and Nanopore reads was performed using Unicycler 20 (v0.4.7). PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and Resfinder[16] were used to determine the plasmid replicon type and acquired resistance genes. Genotyping was performed to query the seven via the multi-locus sequence typing MLST web service (https://cge.cbs.dtu.dk/services/MLST/).

Plasmid Analysis and Conjugation Assays

The number and size of the plasmid of the strains were characterized by S1-PFGE. The location of bla_{NDM-9} and *mcr-1* genes was confirmed by Southern blotting and hybridization with a digoxigenin-labeled bla_{NDM-9} and *mcr-1* probe using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). Conjugation transfer experiments were conducted to explore the transferability of plasmids with rifampicin-resistant *E. coli* 600 as the recipient strain as recipients, as described previously.[17] After that, using Mueller-Hinton agar (OXOID, Hampshire, United Kingdom) plates that contained both 200 mg/L rifampicin (Meilunbio, Dalian, China) and 2 mg/L meropenem to select bla_{NDM-9} carrying transconjugants, and 200 mg/L rifampicin with 2 mg/L colistin to select *mcr-1* carrying transconjugants, respectively. The final identification of transconjugants, including MALDI-TOF/MS identification, resistance genes detection, and AST, to confirm whether the experiments succeed.

Results and Discussion

E. coli L889 was isolated from a 21-years old patient on November 2017. Antimicrobial susceptibility testing by showed that L889 was resistant to various types of antimicrobial agents, including amoxicillin/clavulanate (MIC = 32 μg/mL), piperacillin/tazobactam (MIC > 128 μg/mL), cefotaxime (MIC > 128 μg/mL), ceftazidime (MIC > 128 μg/mL), cefpirome (MIC > 128 μg/mL), cefepime (MIC = 128 μg/mL), meropenem (MIC = 4 μg/mL), ertapenem (MIC = 16 μg/mL), imipenem (MIC = 8 μg/mL), aztreonam (MIC = 128 μg/mL), amikacin (MIC > 128 μg/mL), gentamicin (MIC ≥ 128 μg/mL), levofloxacin (MIC = 32 μg/mL), ciprofloxacin (MIC > 64 μg/mL), trimethoprim/sulfamethoxazole (MIC > 8 μg/mL), tetracycline (MIC = 64 μg/mL), fosfomycin (MIC > 512 μg/mL), and colistin (MIC = 16 μg/mL). It only susceptible to tigecycline with the MIC value of 0.125 μg/mL.

Analyzing the genome sequence of L889 by MLST 2.0 showed that it belonged to ST-1011. It is worthy to note that NDM-producing *E. coli* ST-1011 is prevalent in duck farms in southeast coastal China.[18] A previous study conducted in Lebanon found that ST1011 was one of the most widely identified clones associated with *mcr-1*-carrying *E. coli* and to the poultry sector. Our work further highlights the co-occurrence of NDM-9 and MCR-1 in *E. coli* ST1011 from

human gut colonized isolate. The dissemination of *E. coli* ST1011 from the poultry sector to the human sector indicated that we should closely monitor this clone among clinical settings.

A total of 26 acquired resistance genes were predicted from the genome sequence of L889 by ResFinder. These ARGs enabled L889 to exhibit resistance to different types of antimicrobial agents, including beta-lactams ($bla_{\text{CTX-M-123}}$, $bla_{\text{NDM-9}}$, $bla_{\text{CT-M-164}}$, $bla_{\text{TEM-1B}}$, and $bla_{\text{OXA-1}}$), fosfomycin (*fosA3*), colistin (*mcr-1*), sulphonamide (*sul* and *sul2*), phenicol (*floR*), aminoglycosides (*aadA2, aadA22, aac(3)-Iid, aac6'-Ib-cr, aph3''-Ib, aph6-Id, armA*, and *aph3'-Ia*), macrolide (*mdfA* and *mph(A)*), quinolone (*oqxA* and *oqxB*), tetracycline (*tet(A)*), chloramphenicol (*catB4*), rifampin (ARR-3), and trimethoprim (*dfrA12* and *dfrA17*). To a further extent, the co-occurrence of *mcr*-1 and bla_{NDM} genes in *E. coli* has been occasionally detected in human and animal sectors.[6,19–22]

As far as we know, L889 is the first reported gut-originated *E. coli* strain that harbored both *mcr-1* and $bla_{\text{NDM-9}}$ genes. S1-PFGE revealed that L889 carried four plasmids (Figure 1). Further investigation by Plasmidfinder and Blastn confirmed that *mcr-1* carried by a 63 kp plasmid (pL889-MCR1), and $bla_{\text{NDM-9}}$ encoded by a 114 bp plasmid (pL889-NDM9). Plasmids were sequenced on Illumina and Nanopore platforms. Two complete plasmids were assembled by the sequencing reads from both two platforms. The complete sequences of these two plasmids were deposited in The National Center for Biotechnology Information with the accession numbers of MZ062604 (pL889-MCR1) and MZ062605 (pL889-NDM9).

Plasmid pL889-NDM9 is a 114,985 bp circular untypeable plasmid. Nucleotide sequence alignment revealed that the backbone of pL889-NDM9 exhibited high similarity to plasmid pHNTH02-1 (MG196294), which was previously recovered from *E. coli* THSJ02 from retail chicken meat in

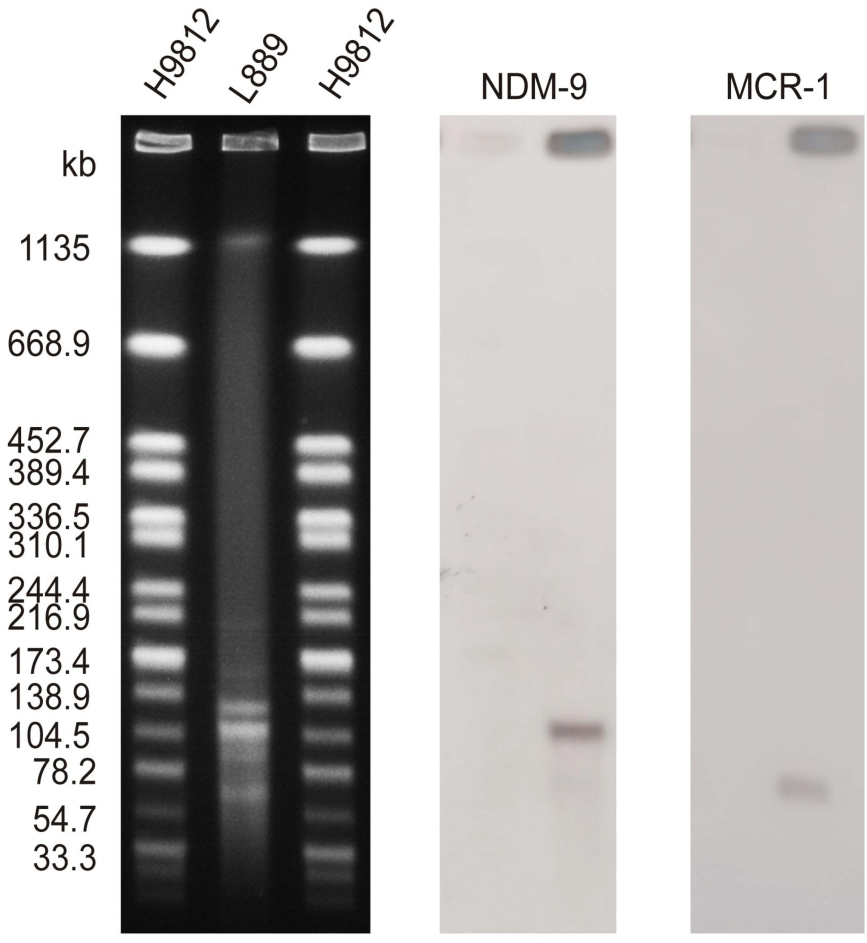

Figure 1 S1-PFGE pattern for *E. coli* L889 and Southern blot analysis indicating the $bla_{\text{NDM-9}}$-and *mcr-1*-carrying plasmids. Lane marker, *Salmonella* serotype Braenderup strain H9812 as a reference size standard; L889, PFGE result of S1-digested plasmid DNA of strain *E. coli* L889; NDM-9, Southern blotting of L889 with the probes specific to the $bla_{\text{NDM-9}}$; MCR-1, Southern blotting of L889 with the probes specific to the *mcr-1*.

Guangzhou, China,[23] possesses 99% coverage and 99.62% identity (Figure 2A). pL889-NDM9 also showed highly homologous to pEC013 (MG545909), pNDM-T2 (MN335919) and pHNSD138-1 (MG271839), which were all found in *E. coli* isolated from chicken samples (83–90% coverage and 99.14–99.66% identity). Furthermore, genetic environment characterization revealed that bla_{NDM-9} was located in an IS*CR1* complex class 1 integron with two copies of IS*26*, with a conserved structure of IS*26*-ΔIS*Aba125*-bla_{NDM-9}-ble_{MBL}-*trpF*-*tat*

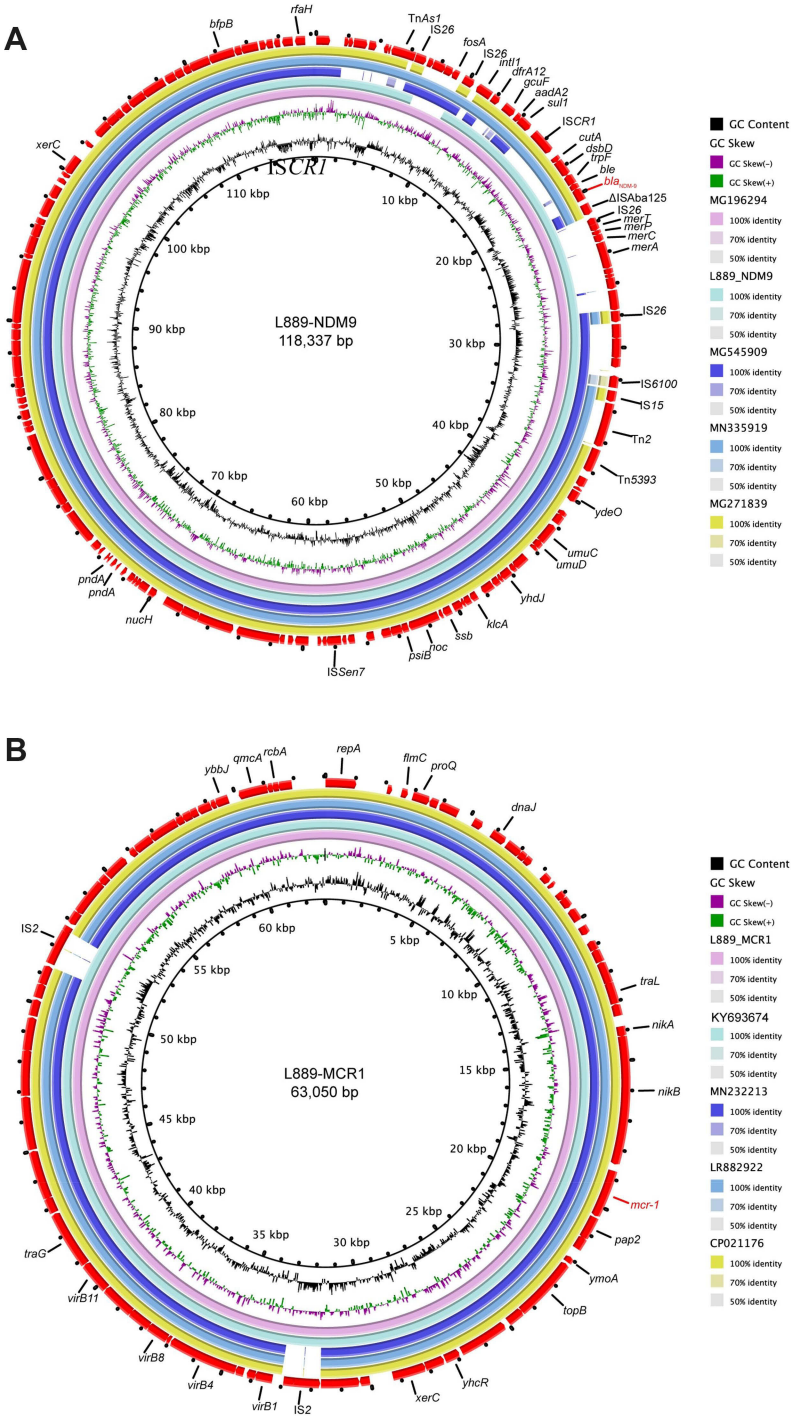

Figure 2 Comparative analysis of plasmids pL889-NDM9 and pL889-MCR1 detected in *E. coli* L889. (**A**) Comparison of bla_{NDM-9} coding region of plasmid pL889-NDM9 with plasmid pHNTH02-1 (MG196294), pEC013 (MG545909), pNDM-T2 (MN335919), and pHNSD138-1 (MG271839). (**B**) Comparison of *mcr-1*-carrying plasmid pL889-MCR1 with plasmids pHNTH02-1 (KY693674), pHLJ179-34 (MN232213), and p5CRE51-MCR-1 (CP021176). The circular map was generated with the BLAST Ring Image Generator (http://brig.sourceforge.net/).

Table 1 Minimum Inhibitory Concentrations (MICs) of Tested Antibiotics for the bla_{NDM-9}- and *Mcr-1*- Positive *Escherichia coli* ST1101 Strain and Transconjugants

Agents	*E. coli* L889	Transconjugant (NDM-9)	Transconjugant (MCR-1)
Amoxicillin/clavulanate	32 (R)	32 (R)	64 (R)
Piperacillin/tazobactam	>128 (R)	>128 (R)	>128 (R)
Cefotaxime	>128 (R)	>128 (R)	>128 (R)
Ceftazidime	>128 (R)	>128 (R)	>128 (R)
Cefpirome	>128 (R)	64 (R)	>128 (R)
Cefepime	128 (R)	64 (R)	128 (R)
Meropenem	4 (R)	2 (I)	2 (I)
Imipenem	8 (R)	8 (R)	4 (R)
Ertapenem	16 (R)	8 (R)	2 (S)
Aztreonam	128 (R)	0.5 (S)	128 (R)
Gentamicin	>128 (R)	1 (S)	>128 (R)
Amikacin	>128 (R)	4 (S)	>128 (R)
Levofloxacin	32 (R)	0.5 (S)	64 (R)
Ciprofloxacin	>64 (R)	0.5 (S)	>64 (R)
Trimethoprim/sulfamethoxazole	>8 (R)	<0.125 (S)	>8 (R)
Tetracycline	64 (R)	0.5 (S)	64 (R)
Fosfomycin	>512 (R)	1 (S)	>512 (R)
Nitrofurantoin	64 (I)	4 (S)	16 (S)
Tigecycline	0.125 (S)	0.25 (S)	0.125 (S)
Colistin	16 (R)	2 (S)	16 (R)

-ΔcatA-IS*CR1-qacEΔ1-sul1-aad2-gcuF-dfrA12-intI1*-IS *26*, which is consistent with the plasmid pHNTH02-1. Our results further supported that this conserved structure may be associated with the transfer and spread of NDM-9-carrying plasmids.[23] Moreover, an antimicrobial resistance gene conferring resistance to fosfomycin, *fosA3*, is located upstream of NDM-9 region. To date, the emergence of bla_{NDM-9} has been reported in *E. coli*,[24,25] *Klebsiella pneumoniae*,[10] *Klebsiella variicola*,[11] and *Cronobacter sakazakii*.[26] Our discovery of this plasmid supplemented previous studies and further highlighted the dissemination of bla_{NDM-9} gene-carrying plasmids in human gut colonized isolates.

Plasmid pL889-MCR1 is a 63,050 bp circular plasmid belonging to IncI2, which was associated with the global dissemination of MCR-1-producing *E. coli* from animal and human sectors.[27–31] Nucleotide sequence alignment indicated that it aligned very well to plasmids pHNTH02-1 (KY693674), pHLJ179-34 (MN232213), and p5CRE51-MCR-1 (CP021176) (Figure 2B). Of note, p5CRE51-MCR-1 carrying isolate was recovered from a urinary tract infection, which represents the first case reported an *E. coli* strain co-producing MCR-1 and NDM-9.[32] Annotation of the plasmid sequence revealed a typical structure surrounding the *mcr-1* gene (*nikA-nikB-mcr -1-pap2*) in pL889-MCR1. Interestingly, this conserved structure was popularly identified in clinical and animal isolates.[28,33]

Conjugation analysis confirmed that both bla_{NDM-9} and *mcr-1* genes were transferable to the recipient cells (Table 1). These data are consistent with previous investigations that MCR-1- or NDM-9-positive Enterobacteriaceae exhibit in vitro antibiotic resistance against most antimicrobial agents.[23–26,32] It is worthy to note that tigecycline showing well in vitro activity against these bacteria carrying MCR-1 and NDM-9 in the current case and previous investigations,[24,32] a further large-scale study to evaluate the activity of tigecycline s is warranted.[32]

Collectively, the present case reported the complete sequences of an IncI2 type *mcr-1* carrying plasmid and an untypeable type bla_{NDM-9} carrying plasmid in an *E. coli* isolated from a stool sample. Furthermore, our data also clearly demonstrated that colonized *E. coli* strains potentially contribute to the dissemination and transfer of bla_{NDM-9} and *mcr-1* to clinical isolates. To lower the risk of disseminating this multidrug-resistant strain in stool samples, closely monitoring is needed in the future.

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Disclosure

The authors report no conflicts of interest in this work.

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