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ORIGINAL RESEARCH

# Characterization of blaNDM-1- and blaSHV-12-Positive IncX3 Plasmid in an *Enterobacter Hormaechei* New Sequence Type 1000 from China

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**Purpose:** Carbapenem-resistant *Enterobacter cloacae* complex has been reported worldwide and becomes a new challenge for clinical management. The present study was to characterize the IncX3 plasmid encoding $bla_{NDM-1}$ and $bla_{SHV-12}$ gene in *E. hormaechei* sequence.

**Materials and Methods:** EcHK001 was recovered from the sputum sample of a patient. Species identification and antimicrobial susceptibility testing were performed using the VITEK 2 system, while further classification was carried out by hsp60 typing. The presence of NDM-1 was detected by PCR and sequencing. Conjugation experiments and southern blotting were carried out to determine the transferability of the NDM-1-carrying plasmid. Whole-genome sequencing and analysis were conducted to better understand the molecular characteristics of the multi-drug resistant isolate.

**Results:** Strain EcHK001 was classified as *E. hormaechei* of new sequence type 1000. Multiple drug-resistant genes were detected. The $bla_{NDM-1}$ and $bla_{SHV-12}$ genes were located on a self-transferable IncX3 plasmid. Synonymous mutations were identified in the genes encoding TEM-1 and ACT-17. Phylogenetic analysis indicated that EcHK001 clustered into a different clade from domestic strains.

**Conclusion:** The rapid spread of the recurrent IncX3 plasmid highlights the need for continuous surveillance of the NDM-1 dissemination. The presence of mutations in existing carbapenem-resistant genes may generate potential new variants and raise serious challenges for clinical treatment.

**Keywords:** multidrug-resistant, NDM-1, SHV-12, enterobacter hormaechei

## Introduction

New Delhi metallo-β-lactamase-1 (NDM-1) is an enzyme with the ability to hydrolyze most β-lactams. Since first detected in 2009 from a *Klebsiella pneumoniae* strain,[1] 21 NDM variants have been reported from a variety of *Enterobacterales* species.[2] The widespread of NDM is mainly due to diverse self-transferable plasmids. The co-existence of NDM and other resistance genes in a single plasmid is increasingly detected and confers higher resistance to carbapenems, raising great challenges for clinical management.[3,4]

As important opportunistic pathogens, *Enterobacter cloacae* complex is responsible for a variety of human infections in the respiratory tract, wound and urine.[5] Among the members of *the Enterobacter cloacae* complex, *E. hormaechei* was first reported in 1989[6] and has been increasingly detected worldwide with the acquisition of different beta-lactamase genes.[7,8] In this study, we report a carbapenem-resistant *E. hormaechei*

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strain with an IncX3 plasmid coproducing NDM-1 and SHV-12. Susceptibility test, conjugation experiment and whole-genome sequencing were performed to investigate the molecular characteristics of the multiple drug-resistant strain.

# Materials and Methods

## Bacterial Strain Identification

The NDM-1-producing strain EcHK001 was isolated from the sputum sample of an 83-year-old male patient through routine surveillance in Haikou, China, in 2015. The *E. cloacae* strain was identified by the VITEK2 compact system (bioMérieux, France) and 16S rRNA gene sequencing.[9] The *Enterobacter cloacae* complex can be further classified by the heat shock protein 60-encoding (*hsp60*) gene,[10,11] the sequence of which was obtained through amplification and sequencing using the primers of Hsp60-F (5′-GGT AGA AGA AGG CGT GGT TGC-3′) and Hsp60-R (5′-ATG CAT TCG GTG GTG ATC ATC AG-3′) as previously described.[12] Phylogenetic analysis of the *hsp60* sequences from GenBank was performed to confirm the identification of *E. hormaechei*.

The presence of NDM, SHV, TEM, and ACT genes was detected by PCR and sequencing as previously described.[13–16]

## Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of *E. hormaechei* strain EcHK001 and the transconjugants were determined using the VITEK2 system with AST-GN09 card (bioMérieux) and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).[17] The following agents were tested: Ampicillin, Ampicillin/sulbactam, Piperacillin, Piperacillin/tazobactam, Cefazolin, Cefuroxime, Cefotetan, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nitrofurantoin, and Sulfamethoxazole/trimethoprim.

## Southern Blotting and Conjugation Experiments

DNA fragments of strain EcHK001 were prepared in agarose plugs by electrophoresis and digested with S1 endonuclease (Takara, Dalian, China). Subsequent fragments were further separated by PFGE through a CHEF-DR III system (Bio-Rad, Hercules, USA). A sheet of nylon membrane (Roche) was used to transfer the plasmid DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane. Southern blotting specific to $bla_{\text{NDM-1}}$ was performed using the Probe-NDM-F (5′-GGC GGA ATG GCT CAT CAC GA-3′) and Probe-NDM-R (5′-CGC AAC ACA GCC TGA CTT TC-3′) as previously described.[18]

Conjugation experiment was carried out by broth and filter mating using strain EcHK001 and *E. coli* strain J53 $Az^r$ as the donor and recipient, respectively. The mixture was then incubated at 30°C for 18 hrs. Transconjugants were selected on MacConkey agar plates containing meropenem (4 μg/mL) plus sodium azide (100 μg/mL) at 37°C for 48 hrs. PCR amplification and sequencing were further performed to determine the presence of the $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-12}}$ genes in the transconjugants.

## Whole Genome Sequencing and Phylogenetic Analysis

Genomic DNA was extracted from the bacterial culture of *E. hormaechei* strain EcHK001 using the QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA). Whole-genome sequencing was performed using Illumina HiSeq 2500 sequencer at Novogene Company (Beijing, China) with 109-fold coverage and the MinION sequencer with the nanopore library prepared using the Rapid Sequencing Kit SQK-RAD004 (Oxford Nanopore Technologies, UK). Hybrid assembly of Illumina and Nanopore sequencing reads was carried out with Unicycler (v0.4.7).[19] The $bla_{\text{NDM}}$-carried plasmid was designated as pNDM-EcHK001 here and annotated by RAST.[20] PlasmidFinder[21] was employed to determine the replicon type.

Strain typing was carried out by using the genomic sequence to query the seven house-keeping genes (*pyrG, gyrB, rplB, leuS, dnaA, rpoB* and *fusA*) of *E. cloacae* on the MLST web server.[22] All 7 strains of *E. hormaechei* from China and other 11 sequences of *E. hormaechei* were obtained from NCBI for phylogenetic tree construction using kSNP (v3.0).[23] The average nucleotide identities (ANIs) between strain EcHK001 and other isolates were calculated by JSpeciesWS web service.[24]

## Nucleotide Sequence Accession Numbers

Genomic and plasmid sequences of strain EcHK001 have been deposited into GenBank under accession NZ_PEIL01000000 and NZ_CM008823, respectively.

# Results

## Microbiological Features of Strain EcHK001

Based on the *hsp60* gene, the $bla_{\text{NDM-1}}$ carrying strain EcHK001 was further classified into the *E. hormaechei*.

**Table 1** Antibiotic Susceptibilities of Strain EcHK001 and the *E. coli* J53 Transconjugants

| Antimicrobial | MIC (ug/mL)/Susceptibility Result | | |
|---|---|---|---|
| | EcHK001 | J53 | J53 (the Transconjugant) |
| Ampicillin | ≥32/R | 8/S | ≥32/R |
| Ampicillin/sulbactam | ≥32/R | 4/S | ≥32/R |
| Piperacillin | ≥128/R | ≤4/S | ≥128/R |
| Piperacillin/tazobactam | ≥128/R | ≤4/S | 64/I |
| Cefazolin | ≥64/R | ≤4/S | ≥64/R |
| Cefuroxime | ≥64/R | ≤1/S | ≥64/R |
| Cefotetan | ≥64/R | ≤4/S | 32/I |
| Ceftazidime | ≥64/R | ≤1/S | ≥64/R |
| Ceftriaxone | ≥64/R | ≤1/S | ≥64/R |
| Cefepime | ≥64/R | ≤1/S | 8/I |
| Aztreonam | ≥64/R | ≤1/S | 32/R |
| Imipenem | ≥16/R | ≤0.05/S | ≥16/R |
| Meropenem | ≥16/R | ≤1/S | 8/R |
| Amikacin | 4/S | ≤2/S | ≤2/S |
| Gentamicin | ≥16/R | ≤1/S | ≤1/S |
| Tobramycin | ≥16/R | ≤1/S | ≤1/S |
| Ciprofloxacin | 1/S | ≤0.25/S | ≤0.25/S |
| Levofloxacin | 1/S | ≤0.25/S | ≤0.25/S |
| Nitrofurantoin | 128/R | ≤16/S | ≤16/S |
| Sulfamethoxazole-trimethoprim | ≥320/R | ≤20/S | ≤20/S |

**Abbreviations:** S, susceptible; R, resistant; I, intermediate.

EcHK001 exhibited resistance to most tested antimicrobial agents, but was still susceptible to amikacin, ciprofloxacin and levofloxacin (Table 1). S1-PFGE showed that EcHK001 contained three plasmids which sizes were ~33 kb, ~54kb and ~130 kb (Figure 1). Electrophoresis and southern blotting revealed that the $bla_{NDM-1}$ gene was located on the ~54kb plasmid. This plasmid, designated as pNDM-EcHK001, was successfully transferred via conjugation at a frequency of $2.94\times10^{-2}$. Susceptibility testing showed the transconjugants acquired resistance to most tested β-lactams including ampicillin, ampicillin/sulbactam, piperacillin, cefazolin, cefuroxime, ceftazidime, ceftriaxone, aztreonam, imipenem and meropenem. In addition to $bla_{NDM-1}$, multiple resistance genes including $bla_{SHV-12}$, $bla_{CTX-M-14}$, $bla_{DHA-1}$, $bla_{ACT-17}$, $bla_{TEM-1}$, *qnrB4, brp, sul2, sul1, strA, strB, dfrA14* and *aac(6')-IIc* were identified through genome sequencing and analysis. Genome analysis indicated the $bla_{DHA-1}$, $bla_{CTX-M-14}$ and $bla_{ACT-17}$ genes were located on the chromosome, while other *bla* genes were on plasmids. The plasmid pNDM-EcHK001 had a length of 54,035 bp and carried both the $bla_{NDM-1}$ and $bla_{SHV-12}$ genes. The $bla_{TEM-1}$ gene was located on a 124,544 bp plasmid which had an 11,452-bp resistance region containing Δ*tnpA, tnpR,* $bla_{TEM-1}$, IS*Vsa3, strB, strA, sul2*, IS*4321*, Δ*tnpA* and IS*26*, same as the region from plasmid p34998-210.894kb (GenBank accession no. CP012169.1).[10] Of note, synonymous mutations in the $bla_{TEM-1}$ (T396G) and $bla_{ACT-17}$ (C96A, T276G, T675C, T690C, A708G, G777A and G807A) genes were detected and verified by PCR, indicating the potential to generate new variants of these β-lactamase genes. The variants of $bla_{TEM-1}$ and $bla_{ACT-17}$ were deposited in GenBank under accession number MG818166 and MG865974.

## Strain Typing and Phylogenetic Analysis

In silico MLST analysis identified a new allele of the housekeeping genes *pyrG* designated as *pyrG*-281, and strain EcHK001 was assigned a new sequence type 1000. Whole-genome phylogenetic analysis revealed EcHK001 formed a clade with strains isolated in USA and China (Figure 2). Sequence alignments revealed the ANI values between EcHK001 and three isolates from Chengdu, China ranged from 99.22% to 99.23%, while the ANI between EcHK001 and the strain UCI_5 from Irvine, California, USA was 99.30%. However, the ANI between EcHK001 and the other Chinese isolates ranged from 86.5 to 87.2. These results indicated that EcHK001 had a close relationship with overseas strains and may have originated from an earlier ancestor than domestic strains.

## Characterization of Plasmid pNDM-EcHK001

Plasmid pNDM-EcHK001 had a length of 54,035 bp and 52 ORFs (Figure 3). The plasmid, assigned to replicon type IncX3, consists of a 39.6 kb backbone and a 19.2 kb multiple drug resistance (MDR) region. The backbone carries genes responsible for plasmid replication (*repB*), conjugal transfer (*taxA, taxB, taxC* and *pilx1-pilx11*, etc.) and plasmid stability (*parA, parB*, etc.). The MDR region is composed of Tn3, IS*3000*, the transposon Tn125 encoding $bla_{NDM-1}$, a mobile element containing $bla_{SHV-12}$ and IS*L3*.

The genome sequence of pNDM-EcHK001 shared >99% similarity with plasmid pNDM-HN380 (GenBank accession no. JX104760) in *K. pneumoniae*, pNDM-HF727 (GenBank accession no. KF976405) in *E. cloacae*,[25] pYE315203 (GenBank accession no. JX254913) in *Citrobacter freundii* and pKPN5047 (GenBank accession no. KC311431) (Table 2), which are differed by only 14 SNPs in the IS5 element upstream of the $bla_{NDM-1}$ gene. Plasmids

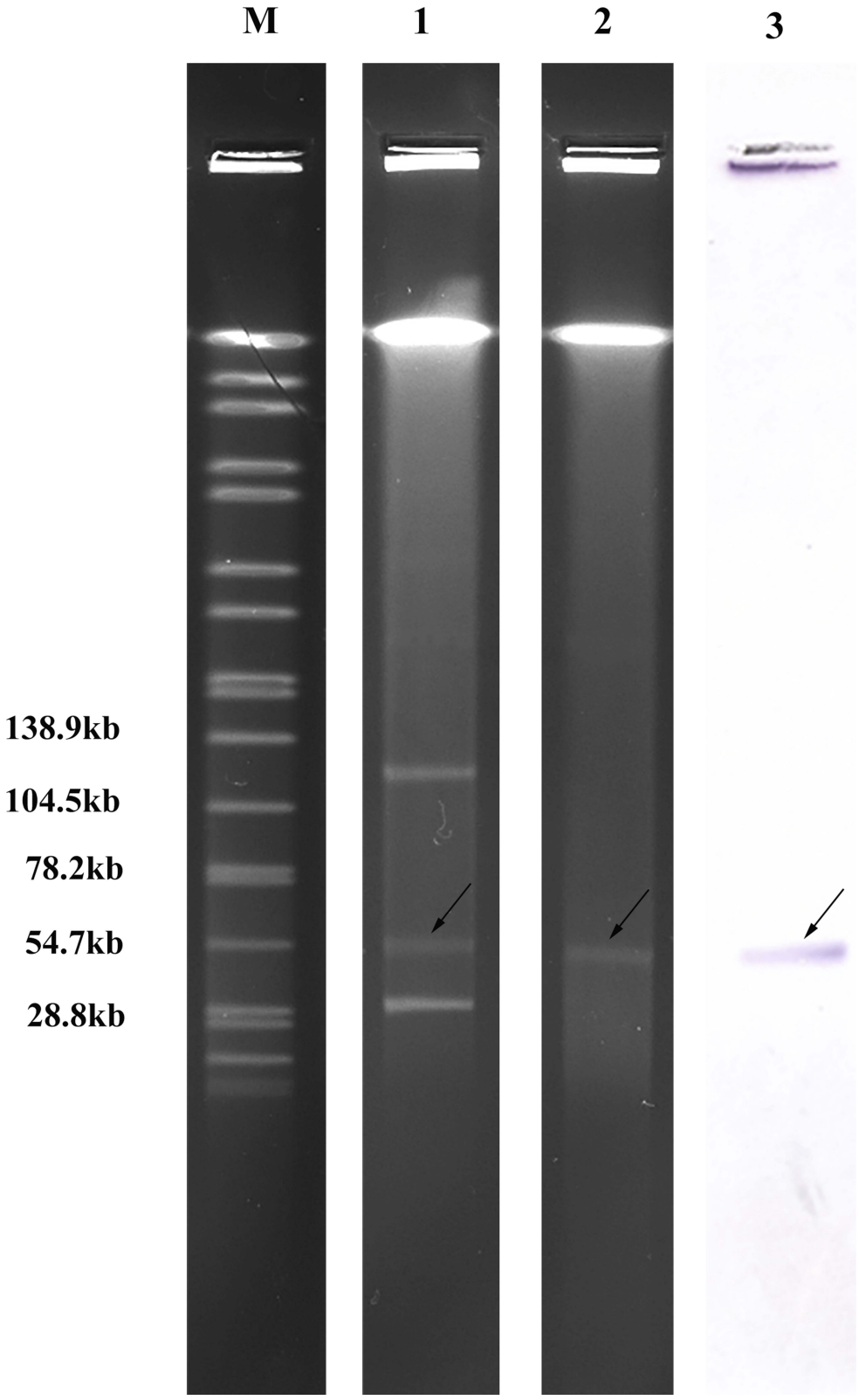


**Figure 1** S1-PFGE and Southern blotting. Lane M, marker (*Salmonella* H9812); Lane 1, *E. hormaechei* EcHK001; Lane 2, the transconjugants; Lane 3, Southern blotting of EcHK001 with the probes specific to $bla_{NDM-1}$. The black arrows indicate the plasmid pNDM-EcHK001.

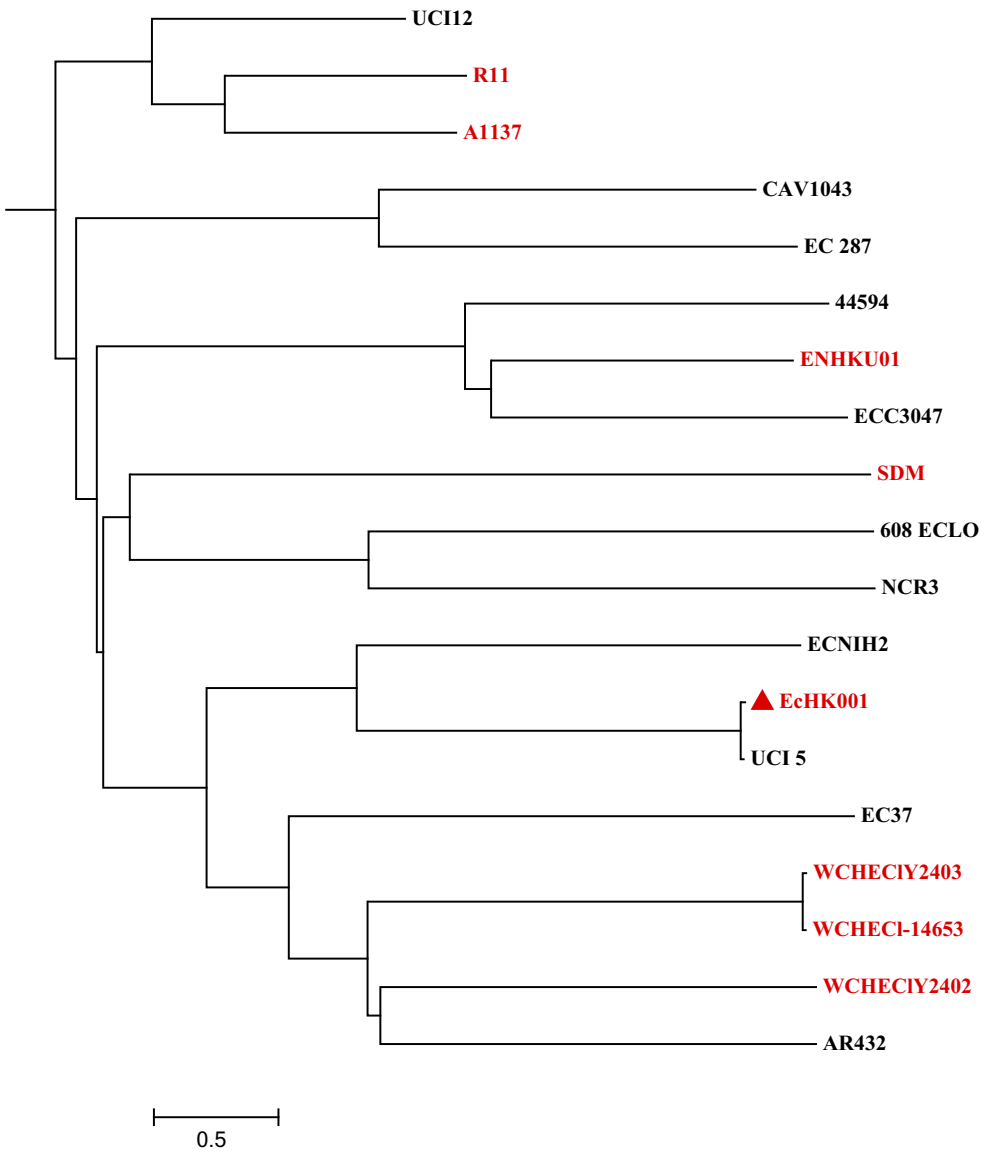


**Figure 2** Phylogenetic tree of *E. hormaechei* strain EcHK001 with other 19 available *E. cloacae* complex genomes from GenBank. Strain EcHK001 is marked with a solid triangle. Isolates from China are indicated with red shading.

pEC55-NDM4 (GenBank accession no. KX470734) and pZHDC33 (GenBank accession no. KX094555) also shared >99% identity with pNDM-EcHK001 but containing different $bla_{NDM}$ alleles (Figure 4). The $bla_{NDM-4}$ (T354G) and $bla_{NDM-13}$ genes (C531T) differ from $bla_{NDM-1}$ and $bla_{NDM-4}$ by only a single nucleotide, respectively, suggesting a close relationship among these three plasmids. On these above plasmids, the $bla_{NDM-1}$ gene was located in a *Tn125* variant while the $bla_{SHV-12}$ gene in a composite transposon. The transposon *Tn125* served as a mobile element for the transfer of $bla_{NDM-1}$ and comprised ΔIS*Aba125*, $bla_{NDM-1}$ (locus tag, CS291_RS00145), the bleomycin resistance gene *ble* (locus tag, CS291_RS00140), Δ*trpF* (locus tag, CS291_RS00135), *tat* (locus tag, CS291_RS00130), *dct* (locus tag, CS291_RS00125), *groES* (locus tag, CS291_RS00120), *groEL* (locus tag, CS291_RS00115) and *insE* (locus tag, CS291_RS00115) as previously described.[25] Compared with the prototype *Tn125*, the upstream ΔIS*Aba125* is disrupted by IS*5* while the downstream copy is absent. The ΔIS*Aba125* and IS*5* were each surrounded by two inverted repeats (IRs) (15 bp and 12 bp), respectively. Two direct repeats (DRs) (21 bp and 11 bp) were located at the end of *groEL* and *insE*.

The $bla_{SHV-12}$-carrying transposon is sequentially organized as Δ*ygbJ* (locus tag, CS291_RS00085), *ygbI* (locus tag, CS291_RS00080) and $bla_{SHV-12}$ (locus tag, CS291_RS00075), which were located within two opposite

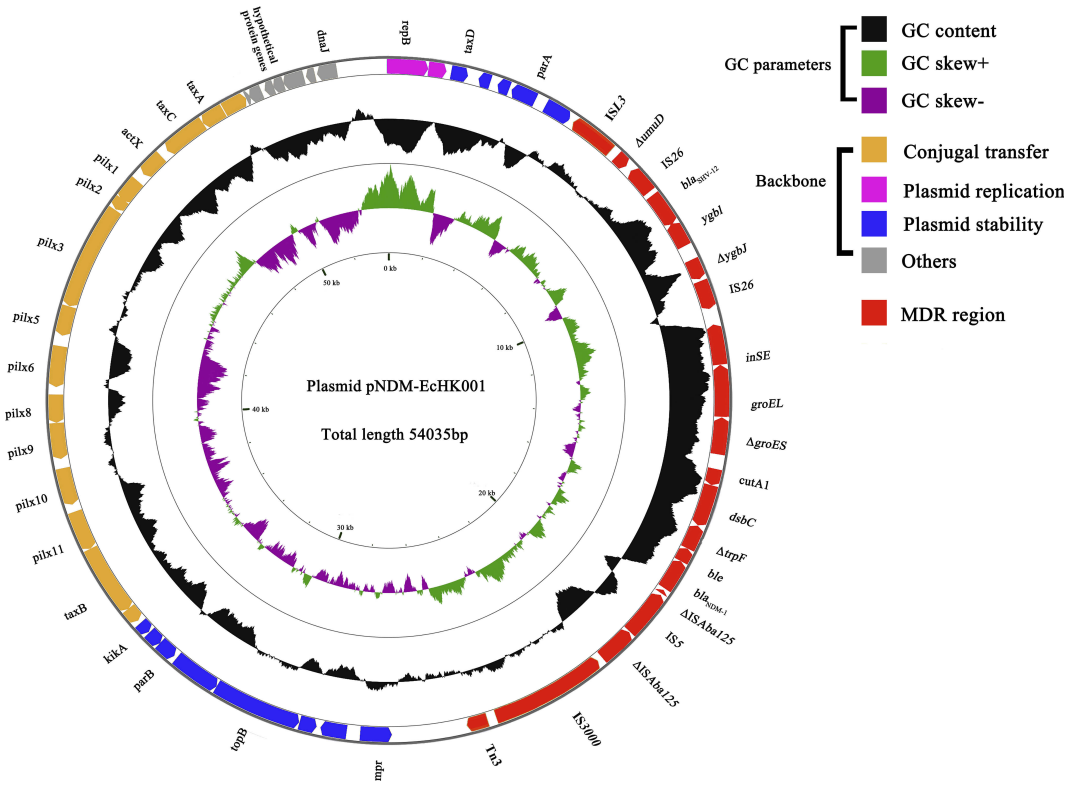


**Figure 3** The genetic map of plasmid pNDM-EcHK001. GC content, GC skew+ and GC skew- are, respectively, indicated in black, green and purple. The MDR regions are indicated in red. The modules of conjugal transfer, plasmid replication and plasmid stability are, respectively, indicated in orange, pink and blue. The other elements are indicated in grey.

IS*26* elements. And each IS*26* was surrounded by 14 bp IRs. In comparison with pNDM-EcHK001, the inversion of the $bla_{SHV-12}$–carrying transposon happened in plasmids pKP04NDM (GenBank accession no. KU314941)[26] and p112298-NDM (GenBank accession no. KP987216).[27] In addition, p112298-NDM had a 546-bp-deletion in the IS*26* gene. Plasmid pIncX-SHV[28] had a similar backbone as pNDM-EcHK001 but with the deletion of *Tn125* and a different $bla_{SHV}$ allele, while pSCE516-2 (GenBank accession no. JN247852) had only the NDM-1-carrying *Tn125* with an inversion of IS*5* and a shorter ΔIS*Aba125* region with only 65 bp remaining, suggesting the composite transposon may serve as the major vehicle for SHV-12 (GenBank accession no. KX023261) (Figure 4). The information of all the above plasmids is shown in Table 2.

## Discussion

Previous studies have shown that the $bla_{NDM-1}$-positive isolates in diverse species of *Enterobacterales* often exhibited high resistance to beta-lactam antibiotics and posed

**Table 2** pNDM-EcHK001-Like Plasmids Harboring $bla_{NDM}$ Among *Enterobacterales*

| Plasmid | Host | Location | *Bla* Gene | Coverage | Similarity | GenBank Accession | Reference |
|---|---|---|---|---|---|---|---|
| pNDM-HF727 | *E. cloacae* | Haifeng, China | $bla_{NDM-1}$, $bla_{SHV-12}$ | 100% | 99.99% | KF976405 | 25 |
| pNDM-HN380 | *K. pneumoniae* | Hunan, China | $bla_{NDM-1}$, $bla_{SHV-12}$ | 100% | 99.99% | JX104760 | 25 |
| pYE315203 | *C. freundii* | Zhejiang, China | $bla_{NDM-1}$, $bla_{SHV-12}$ | 100% | 99.99% | JX254913 | Unpublished |
| pKPN5047 | *K. pneumoniae* | Beijing, China | $bla_{NDM-4}$, $bla_{SHV-12}$ | 100% | 99.99% | KC311431 | Unpublished |
| pEC55-NDM4 | *E. coli* | Henan, China | $bla_{NDM-4}$, $bla_{SHV-12}$ | 100% | 99.99% | KX470734 | Unpublished |
| pZHDC33 | *E. coli* | Zhejiang, China | $bla_{NDM-13}$, $bla_{TEM}$ | 100% | 99.99% | KX094555 | Unpublished |
| pKP04NDM | *K. pneumoniae* | Hong Kong, China | $bla_{NDM-1}$ | 100% | 99.99% | KU314941 | 26 |
| p112298-NDM | *C. freundii* | Guangzhou, China | $bla_{NDM-1}$, $bla_{SHV-2}$ | 98.99% | 99.99% | KP987216 | 27 |
| pIncX-SHV | *K. pneumoniae* | Rome, Italy | $bla_{SHV-11}$ | 80.28% | 99.99% | JN247852 | 28 |
| pSCE516-2 | *E. coli* | Chengdu, China | $bla_{NDM-1}$ | 85.6% | 99.99% | KX023261 | Unpublished |

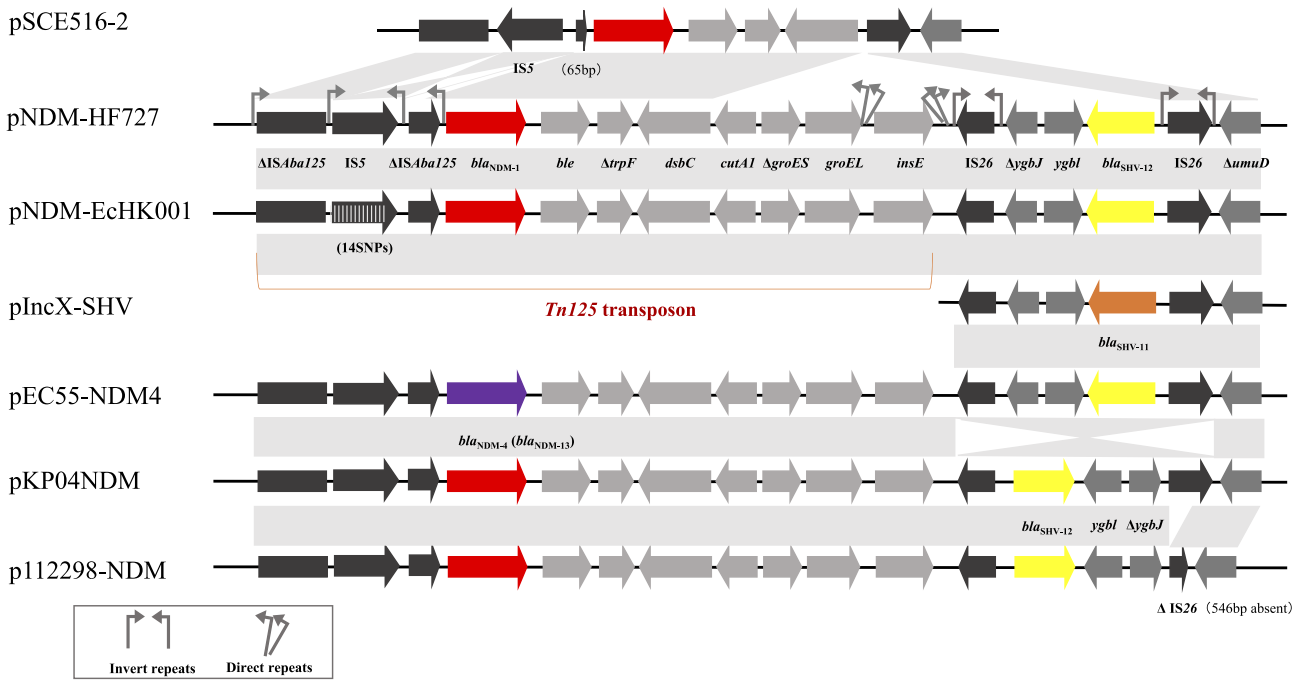


**Figure 4** The genetic structure features of pNDM-EcHK001-like plasmids. The *bla*$_{NDM-1}$ *and bla*$_{SHV-12}$ gene are indicated in red and yellow. The *bla*$_{NDM}$ variants and the *bla*$_{SHV-11}$ gene are indicated in purple and dark orange. The insertion sequences ΔIS*Aba125*, IS*5* and IS*26* are indicated in black. Other genes in Δ*Tn125* and *bla*$_{SHV-12}$-carrying transposon are indicated in light gray and dark gray, respectively.

a significant threat to public health.[3,29] We provided a detailed description of a multidrug-resistant *E. hormaechei* strain EcHK001, which had an IncX plasmid pNDM-EcHK001 co-harboring *bla*$_{NDM-1}$ and *bla*$_{SHV-12}$. The IncX plasmids have been repeatedly reported in *Enterobacterales*, carrying different resistance genes.[30] The plasmid pNDM-EcHK001 had a relatively high conjugation frequency compared with other IncX3 plasmids,[31] which may facilitate rapid dissemination of the *bla*$_{NDM-1}$ gene.

The pNDM-EcHK001-like plasmids, which share the same backbone and have both *bla*$_{NDM-1}$ and *bla*$_{SHV-12}$, have a broad host range and the ability of transfer among *Enterobacterales*.[32] However, it is noteworthy that there were no previous reports of such plasmids in *E. hormaechei*. The co-occurrence of *bla*$_{NDM-1}$ and other beta-lactamase genes mediated resistance to broad-spectrum antibiotics such as carbapenems and cephalosporins, raising challenges in rapidly tailoring clinical therapy.

These plasmids were first detected and repeatedly reported from different geographical locations mainly in China. The isolates carrying these plasmids were all recovered from samples of human patients, which had no history of foreign travel or epidemiological relationship. These evidences indicated a natural reservoir of these plasmids may exist in China. The slight variations among the pNDM-EcHK001-like plasmids suggesting they may be descended from a common ancestor. Whole-genome phylogenetic analysis showed that EcHK001 had a close relationship with foreign isolates. More data are needed to further understand the mechanisms underlying the dissemination of these pNDM-EcHK001-like plasmids.

## Conclusions

In summary, our study characterized a novel *E. hormaechei* ST1000 strain EcHK001, which had a different origination with domestic isolates through phylogenetic analysis. The strain carried multiple *bla* genes and conferred increased resistance to carbapenems. The coexistence of the *bla*$_{NDM-1}$ and *bla*$_{SHV-12}$ genes is repeatedly reported on the pNDM-EcHK001-like plasmids, which had a high transferability and may serve as a common vehicle for rapid dissemination of carbapenemase-encoding genes. Our findings further highlight the spread of NDM-1 in *EnterobacteralesEnterobacteriaceae*. More attention should be devoted to the surveillance of the rapid dissemination of the NDM-1 gene.

## Ethics Approval

The authors state that all experimental protocols were approved by the institutional ethics committees of Academy of Military Medical Sciences.

## Data Sharing Statement

Genomic and plasmid sequences of strain EcHK001 were deposited in GenBank under accession NZ_PEIL01000000 and NZ_CM008823. The variants of blaTEM-1 and blaACT-17 were deposited in GenBank under accession number MG818166 and MG865974.

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## Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; 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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