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 blaNDM-1 and blaSHV-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 blaNDM-1 and blaSHV-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 19896 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
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. The *Enterobacter cloacae* strain was identified by the heat shock protein 60-encoding (*hsp60*) gene. The sequence of which was obtained through amplification and sequencing using the primers of Hsp60-F (5ʹ-GGT AGA AGA AGG CGT GGT TGC-3ʹ) and Hsp60-R (5ʹ-ATG CAT TCG GTG GTG ATC ATC AG-3ʹ) as previously described. 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.

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). The following agents were tested: Ampicillin, Ampicillin/sulbactam, Piperacillin, Piperacillin/tazobactam, Cefazolin, Cefuroxime, Cefotetan, Cefazidime, 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*~NDM-1~ was performed using the Probe-NDM (5ʹ-GGC GGA ATG GCT CAT CAC GA-3ʹ) and Probe-NDM-R (5ʹ-CGC AAC ACA GCC TGA CTT TC-3ʹ) as previously described. Conjugation experiment was carried out by broth and filter mating using strain EcHK001 and *E. coli* strain J53 Az 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*~NDM-1~ and *bla*~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 MiION 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). The *bla*~NDM~ carried plasmid was designated as pNDM-EcHK001 here and annotated by RAST. PlasmidFinder 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 ftsA*) of *E. cloacae* on the MLST web server. 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). The average nucleotide identities (ANIs) between strain EcHK001 and other isolates were calculated by JSpecies WS web service.

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*~NDM-1~ carrying strain EcHK001 was further classified into the *E. hormaechei*. 


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/taizobactam | ≥128/R  | ≤4/S| ≥128/R                    |
| Cefazolin      | ≥64/R   | ≤4/S| 64/I                      |
| Cefuroxime     | ≥64/R   | ≤1/S| ≥64/R                     |
| Cefotetan      | ≥64/R   | ≤4/S| 64/I                      |
| Ceftriaxime    | ≥64/R   | ≤1/S| ≥64/R                     |
| Cefepime       | ≥64/R   | ≤1/S| 8/I                       |
| Aztreonam      | ≥64/R   | ≤1/S| 8/I                       |
| 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.

Echko01 exhibited resistance to most tested antimicrobial agents, but was still susceptible to amikacin, ciprofloxacin and levofloxacin (Table 1). S1-PFGE showed that Echko01 contained three plasmids which sizes were ~33 kb, ~54kb and ~130 kb (Figure 1). Electrophoresis and southern blotting revealed that the blaNDM-1 gene was located on the ~54kb plasmid. This plasmid, designated as pNDM-Echko01, was successfully transferred via conjugation at a frequency of 2.94×10^-2. Susceptibility testing showed the transconjugants acquired resistance to most tested β-lactams including ampicillin, ampicillin/sulbactam, pipericillin, cefazolin, cefuroxime, cefazidime, ceftriaxone, aztreonam, imipenem and meropenem. In addition to blaNDM-1, multiple resistance genes including blasIV-12, blaCTX-M-14, blasHA-1, blaACT-17, blaTEM-1, qnrB4, brp, sul2, sul1, strA, strB, dfrA14 and acs(6)-IIc were identified through genome sequencing and analysis. Genome analysis indicated the blasHA-1, blasCTX-M-14 and blaACT-17 genes were located on the chromosome, while other bla genes were on plasmids. The plasmid pNDM-Echko01 had a length of 54,035 bp and carried both the blasNDM-1 and blasIV-12 genes. The blaTEM-1 gene was located on a 124,544 bp plasmid which had an 11,452-bp resistance region containing Δmpa, mpR, blaTEM-1, ISy3a3, strB, strA, sul2, IS321, ΔmpA and IS26, same as the region from plasmid p34998-210.894kb (GenBank accession no. CP012169.1). Of note, synonymous mutations in the blaTEM-1 (T396G) and blaACT-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 blaTEM-1 and blaACT-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 Echko01 was assigned a new sequence type 1000. Whole-genome phylogenetic analysis revealed Echko01 formed a clade with strains isolated in USA and China (Figure 2). Sequence alignments revealed the ANI values between Echko01 and three isolates from Chengdu, China ranged from 99.22% to 99.23%, while the ANI between Echko01 and the strain UCI_5 from Irvine, California, USA was 99.30%. However, the ANI between Echko01 and the other Chinese isolates ranged from 86.5 to 87.2. These results indicated that Echko01 had a close relationship with overseas strains and may have originated from an earlier ancestor than domestic strains.

Characterization of Plasmid pNDM-Echko01

Plasmid pNDM-Echko01 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, IS3000, the transposon Tn125 encoding blaNDM-1, a mobile element containing blasIV-12 and ISL3.

The genome sequence of pNDM-Echko01 shared >99% similarity with plasmid pNDM-HN380 (GenBank accession no. JX104760) in K. pneumoniae, pNDM-HF727 (GenBank accession no. KF976405) in E. cloacae, 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 blaNDM-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 blaNDM-1. The black arrows indicate the plasmid pNDM-EcHK001.
pEC55-NDM4 (GenBank accession no. KX470734) and pZHDC33 (GenBank accession no. KX094555) also shared >99% identity with pNDM-EcHK001 but containing different blaNDM alleles (Figure 4). The blaNDM-4 (T354G) and blaNDM-13 genes (C531T) differ from blaNDM-1 and blaNDM-4 by only a single nucleotide, respectively, suggesting a close relationship among these three plasmids. On these above plasmids, the blaNDM-1 gene was located in a Tn125 variant while the blaSHV-12 gene in a composite transposon. The transposon Tn125 served as a mobile element for the transfer of blaNDM-1 and comprised ΔISSAbal25, blaNDM-1 (locus tag, CS291_RS00145), the bleomycin resistance gene ble (locus tag, CS291_RS00140), ΔtrpF (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 ΔISSAbal25 is disrupted by IS5 while the downstream copy is absent. The ΔISSAbal25 and IS5 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 blaSHV-12-carrying transposon is sequentially organized as ΔygbJ (locus tag, CS291_RS00085), ygbI (locus tag, CS291_RS00080) and blaSHV-12 (locus tag, CS291_RS00075), which were located within two opposite

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**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.
IS26 elements. And each IS26 was surrounded by 14 bp IRs.
In comparison with pNDM-EcHK001, the inversion of the
blaSHV-12–carrying transposon happened in plasmids
pKP04NDM (GenBank accession no. KU314941)\textsuperscript{26} and
p112298-NDM (GenBank accession no. KP987216).\textsuperscript{27} In
addition, p112298-NDM had a 546-bp-deletion in the IS26
gene. Plasmid pIncX-SHV\textsuperscript{28} had a similar backbone as
pNDM-EcHK001 but with the deletion of Tn125 and a
different blaSHV allele, while pSCE516-2 (GenBank acces-
sion no. JN247852) had only the NDM-1-carrying Tn125
with an inversion of IS5 and a shorter ΔISAb125 region
with only 65 bp remaining, suggesting the composite trans-
poson 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 blaNDM-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 blaNDM Among Enterobacterales

| Plasmid       | Host         | Location          | Bla Gene                  | Coverage | Similarity | GenBank Accession | Reference |
|--------------|--------------|-------------------|---------------------------|----------|------------|-------------------|-----------|
| pNDM-HF727   | E. cloacae   | Haifeng, China    | blaNDM-1, blaSHV-12       | 100%     | 99.99%     | KF976405          | 25        |
| pNDM-HN380   | K. pneumonia | Hunan, China      | blaNDM-1, blaSHV-12       | 100%     | 99.99%     | JX104760          | 25        |
| pYE315203    | C. freundii  | Zhejiang, China   | blaNDM-1, blaSHV-12       | 100%     | 99.99%     | JX254913          | Unpublished |
| pKPN5047     | K. pneumonia | Beijing, China    | blaNDM-1, blaSHV-12       | 100%     | 99.99%     | KC311431          | Unpublished |
| pEC55-NDM4   | E. coli      | Henan, China      | blaNDM-1, blaSHV-12       | 100%     | 99.99%     | KX470734          | Unpublished |
| pZHD33       | K. pneumonia | Zhejiang, China   | blaNDM-1, blaSHV-2        | 100%     | 99.99%     | KX094555          | Unpublished |
| pKPN4NDM     | E. coli      | Hong Kong, China  | blaNDM-1, blaTEM          | 100%     | 99.99%     | KU314941          | 26        |
| p112298-NDM  | C. freundii  | Guangzhou, China  | blaNDM-1                   | 99.99%   | 99.99%     | KP987216          | 27        |
| pIncX-SHV    | K. pneumonia | Rome, Italy       | blaNDM-1                   | 80.28%   | 99.99%     | JN247852          | 28        |
| pSCE516-2    | E. coli      | Chengdu, China    | blaNDM-1                   | 85.6%    | 99.99%     | KX023261          | Unpublished |
We provided a detailed description of a multidrug-resistant *E. hormaechei* strain EcHK001, which had an IncX plasmid pNDM-EcHK001 co-harboring *bla*<sub>NDM-1</sub> and *bla*<sub>SHV-12</sub>. The IncX plasmids have been repeatedly reported in *Enterobacterales*, carrying different resistance genes. The plasmid pNDM-EcHK001 had a relatively high conjugation frequency compared with other IncX3 plasmids, which may facilitate rapid dissemination of the *bla*<sub>NDM-1</sub> gene. The pNDM-EcHK001-like plasmids, which share the same backbone and have both *bla*<sub>NDM-1</sub> and *bla*<sub>SHV-12</sub>, have a broad host range and the ability of transfer among *Enterobacterales*. However, it is noteworthy that there were no previous reports of such plasmids in *E. hormaechei*. The co-occurrence of *bla*<sub>NDM-1</sub> 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*<sub>NDM-1</sub> and *bla*<sub>SHV-12</sub> 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 *Enterobacterales*. More attention should be devoted to the surveillance of the rapid dissemination of the NDM-1 gene.
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