Identification and Characterization of the First *Escherichia coli* Strain Carrying NDM-1 Gene in China

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Abstract

New Delhi metallo-β-lactamase-1 (NDM-1), an acquired class B carbapenemase, is a significant clinical threat due to its extended hydrolysis of β-lactams including carbapenems. In this study, we identified the first confirmed clinical isolate of *Escherichia coli* BJ01 harboring *bla*NDM-1 in China. The isolate is highly resistant to all tested antimicrobials except polymyxin. *bla*NDM-1, *bla*CTX-M-57, and *bla*TEM-1 were identified in the isolate. *bla*NDM-1 was transferable to *E. coli* EC600 and DH5α in both plasmid conjugation experiments and plasmid transformation tests. BJ01 was identified as a new sequence type, ST224, by multilocus sequence typing. Analysis of genetic environment shows complex transposon-like structures surrounding the *bla*NDM-1 gene. Genetic analysis revealed that the region flanking *bla*NDM-1 was very similar to previously identified NDM-positive *Acinetobacter* spp. isolated in China. The findings of this study raise attention to the emergence and spread of NDM-1-carrying *Enterobacteriaceae* in China.

Introduction

Antimicrobial resistance is a growing global challenge to human health[1]. The emerging New Delhi metallo-β-lactamase (NDM-1), an acquired class B carbapenemase that was first clinically detected in a patient at a hospital in New Delhi, India, has brought up worldwide public attention again[2–4]. It confers resistance to a broad-spectrum of β-lactams, including carbapenems, which are the mainstream treatment for antibiotic-resistant bacterial infections[5]. Although current reports indicate that NDM-1 does not hydrolyze monobactams, most of NDM-1-carrying strains also express enzymes that could hydrolyze monobactams, making NDM-1-producers very difficult to control[6]. The rapid dissemination of NDM-1-producing gram-negative species also contributes to this major concern of public health. It has been reported in 50 countries across five continents, in the last 2 years[2,4].

NDM-1 is detected mainly in *Escherichia coli* and *Klebsiella pneumonia* but occasionally in *Klebsiella oxytoca*, *Citrobacter freundii*, *Morganella morganii*, *Providencia spp.*, *Enterobacter cloacae*, *Proteus spp.*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, and *Pseudomonas aeruginosa* [3,7,8]. To our knowledge, NDM-1-producing strains have been seen only in *Acinetobacter* spp. [9–12] and *Enterococcus faecium* [13] in China. There is no evidence of the emergence of NDM-1-producing *Enterobacteriaceae* in China at this point, although it was found in the stool samples of a 1-year-old infant and his mother in Hong Kong who once traveled to and were hospitalized in Hunan Province, China[14]. In the present study, we report the first confirmed case of NDM-1-producing *E. coli* infection in Beijing, China.

Materials and Methods

Patient Information

A 75-year-old Chinese patient with diabetes, who came from the Anhui province in southern China, was admitted for diabetes-related foot complications comprising a swollen peak of the left foot and ulceration of the fourth and fifth toes on September, 2011. The NDM-1-producing *E. coli*, BJ01, strain was isolated from the ulcer secretion. Since the strain was found to be resistant to almost all tested antibiotics, a combination of intravenous levofloxacin and etimicin was given empirically. Amputation of the fifth metatarsal bones and an incisional drainage operation were performed. The wound started healing nicely. The patient was discharged from the hospital one month after admission. Neither the patient nor the patient’s family members had a history of traveling to the Indian subcontinent or other countries. The patient in this manuscript has given written informed consent, as outlined in the PLOS consent form to publication of their case details.

Bacterial Isolate and Phenotypic Screening for MBL

The clinical isolate BJ01 of the metallo-β-lactamase (MBL)-producing *E. coli* was derived from the ulcer secretion of the
patient collected on the admission day. The strain with same phenotype was still positive on day 5 after the operation. The identification and drug susceptibility of the bacteria were tested by using Vitek 2 Compact (bioMerieux, Marcy l’Etoile, France). Minimal inhibitory concentrations (MICs) of 18 antibiotics and combinations of antimicrobials were determined by broth microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[13]. Most of antimicrobial agents were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), except for imipenem (Merck Sharp & Dohme, Whitehouse Station, USA), meropenem (Dainippon Sumitomo Pharma, Chuo-ku, Japan) and polymyxin (Sigma, St. Louis, USA). E.coli strain ATCC25922 was used as a quality control for MIC determination. MBL production was tested by comparing the specific primers for the CTX-M group 1 (CTX-M-1grpF, 5'-CGCAACACAGGCTGACTTTC-3' and CTX-M-1grpR, 5'-GGTCGCCAGTTTCCATTTGC-3') were those recommended by the Chinese Center for Disease Control and Prevention. For the specific detection of the MBL, including IMP, VIM, SPM, and NDM, the regions flanking the blaNDM-1 gene were sequenced by primer walking strategy, starting from each end of the blaNDM-1 transformant. The plasmid containing blaNDM-1 gene was used as a template for primer walking. The first set of primers was designed targeting each end of the blaNDM-1 sequence (5'-GGTGCCAGTTTCCATTTGC-3' and 5'-TGCCGACACCTGAGCACC-3'). The amplification products were sequenced. Then new primers complementary to the known sequence were designed and synthesized for sequencing the unknown DNA sequence next to it. Totally, 13 pairs of primers were synthesized. Sequence files were assembled and aligned by using ContigExpress software.

Results

Antimicrobial Susceptibility of E. coli BJ01 Strain

E. coli BJ01 isolated from the patient displayed high resistance to all tested antibiotics, including ampicillin, ampicillin-sulbactam, cefazolin, aztreonam, ceftaxone, cepafeline, imipenem, meropenem, ceftazidime, piperacillin-tazobactam, cefotetan, tobramycin, gentamicin, ciprofloxacin, levofloxacin, amikacin and trimethoprim-sulfamethoxazole, except polymyxin, which showed bacteriostatic activity to BJ01 (MIC 1 µg/ml) (Table 1, Fig. 1). On HMA, amoxicillin-clavulanic acid showed no synergy with cephalosporins except for aztreonam since metalloenzymes do not hydrolyze aztreonam (Fig. 1a). On HMA containing EDTA, metalloenzyme activity was inhibited, the diameters of the inhibition zones for MEM (meropenem) and IPM (imipenem) were both 18 mm greater than on HMA without EDTA, and synergistic function was observed between amoxicillin-clavulanic acid and ceftazidime, ceftaxone, aztreonam and cepafeline (Fig. 1b). Above findings are suggestive of ESBL (extended-spectrum β-lactamase) production in E. coli BJ01[20].

Transfer of Antibiotic Resistance

In the conjugation experiments, two resistant phenotypes of E. coli EC600 transconjugants (EC600ESBL and EC600NDM₁) were found by using replica plating methods, presenting with different drug resistant patterns (Table 1). The IMP (imipenem) and MEM (meropenem) sensitivity of EC600ESBL and the synergy between AMC (amoxicillin-clavulanic acid) and FEP (cephapline), CAZ (ceftaxidime), CRO (ceftazidime), ATM (aztreonam) on the strain suggested ESBL production (Fig. 1c). EC600NDM₁ grew on both plates containing rifampin (Sigma, St. Louis, USA, 100 µg/mL) plus ceftazidime (F. Hoffmann-La Roche Ltd., Basel, Swiss, 4 µg/mL) for 24 h at 37℃. The bacterial colonies were then transferred to a plate containing rifampin (Sigma, St. Louis, USA, 100 µg/mL) plus imipenem (Merck Sharp & Dohme, Whitehouse Station, USA, 4 µg/mL) for 24 h at 35℃ by replica plating, which maintains the original colony pattern.

Plasmid DNA was extracted using a Qiagen Mini Kit (Qiagen, Hilden, Germany) and was transformed into E. coli DH5α competent cells (TAKARA, Shanghai, China). Transformants were selected on MHA plates containing IPM (4 µg/mL).

The identification and susceptibility of the transconjugant and transformant were confirmed via using the VITEK 2 system. PCR analysis was used to determine the presence of carbapenemase genes.

Multilocus Sequence Typing (MLST) and Sequencing of Genetic Environment

MLST with seven housekeeping genes (adh, fumC, gnyB, iod, mdh, purA, and recA) was performed according to the protocol described on the E. coli MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli/). The regions flanking the blaNDM-1 gene were sequenced by primer walking strategy, starting from each end of the blaNDM-1 gene in the EC600ESBL transformant. The plasmid containing blaNDM-1 gene was used as a template for primer walking. The first set of primers was designed targeting each end of the blaNDM-1 sequence (5'-GGTGCCAGTTTCCATTTGC-3' and 5'-TGCCGACACCTGAGCACC-3'). The amplification products were sequenced. Then new primers complementary to the known sequence were designed and synthesized for sequencing the unknown DNA sequence next to it. Totally, 13 pairs of primers were synthesized. Sequence files were assembled and aligned by using ContigExpress software.

Polymerase Chain Reaction (PCR) Amplification for the Detection of MBL and other β-lactamase genes

The primers used (NDM-F1, 5'-GGCCGGAATGGCTCATCACGA-3'; NDM-R1, 5'-CGGAACACAGGCTGACTTTC-3') were those recommended by the Chinese Center for Disease Control and Prevention. For the specific detection of bladNDM-1, primers NDM-F2 (5'-ATGGATGTCCCAATATTAGTC-3') and NDM-R2 (5'-TCAGCCGACGTGTCGCCAT-3') were used to amplify the entire bladNDM-1 gene. At that time, we performed molecular testing for other MBL, including blablaIMP, blablaVIM, blablaSPM, and bladNDM-1[17,18]. PCR was also undertaken for other β-lactamase genes, including bladTEM, bladSHV, bladCTX-M, and bladOXA-1[19]. bladCTX-M was further identified using the specific primers for the CTX-M group 1 (CTX-M-1grpF, 5'-CCGAAATAAGGAATCCCAT-3' and CTX-M-1grpR, 5'-GGCCGTCTAAGGCGATAAAC-3'), bladTEM, bladSHV, bladCTX-M, and bladOXA-1[19]. bladCTX-M was further identified using the specific primers for the CTX-M group 1 (CTX-M-1grpF, 5'-CCGAAATAAGGAATCCCAT-3' and CTX-M-1grpR, 5'-GGCCGTCTAAGGCGATAAAC-3'), bladTEM, bladSHV, bladCTX-M, and bladOXA-1[19].
Figure 1. Phenotypic detection of carbapenemases and extended-spectrum β-lactamase (ESBL) in *E. coli* *BJ01*. **a.** Antibacterial susceptibility testing for *BJ01* on the Mueller-Hinton agar (MHA). **b.** Antimicrobial susceptibility testing for *BJ01* on MHA impregnated with 2 mL of ethylenediaminetetraacetic acid (EDTA) 5 mM. **c.** Antimicrobial susceptibility testing for the *EC600* transconjugant (*EC600ESBL*). **d.** Antimicrobial susceptibility testing for the *EC600* transconjugant (*EC600NDM-1*). *EC600NDM-1* was resistant to imipenem, meropenem, and cephalosporins except for ATM and showed no synergy between AMC and cephalosporins. **e.** Antimicrobial susceptibility testing for the *DH5α* transformant (*DH5αNDM-1*). The phenotype was the same as that of *EC600NDM-1*. IMP, imipenem; MEM, meropenem; CRO, ceftriaxone; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; ATM, aztreonam; FEP, cefepime.
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Table 1. Antibiotic susceptibility profiles of different strains (MIC (μg/mL)).

| MIC (μg/mL) | *BJ01* | *EC600* | *EC600ESBL* | *EC600NDM-1* | *DH5α* | *DH5αNDM-1* |
|-------------|--------|---------|-------------|--------------|--------|--------------|
| **Ampicillin** | >256 | 2 | >256 | >256 | 2 | >256 |
| **Ampicillin-sulbactam** | >256 | 2 | >256 | >256 | 2 | >256 |
| **Cefazolin** | >256 | 2 | >256 | >256 | 1 | >256 |
| **Aztreonam** | 256 | 0.25 | 256 | 0.50 | 0.50 | 0.50 |
| **Ceftriaxone** | >256 | 0.25 | 256 | 256 | 0.25 | 256 |
| **Cefepime** | 256 | ≤0.125 | 128 | 128 | ≤0.125 | 128 |
| **Imipenem** | 32 | ≤0.125 | ≤0.125 | 32 | ≤0.125 | 32 |
| **Meropenem** | 32 | ≤0.125 | ≤0.125 | 16 | ≤0.125 | 32 |
| **Ceftazidime** | >256 | 0.25 | 16 | >256 | 0.25 | >256 |
| **Piperacillin-tazobactam** | 256 | 1 | 1 | 128 | 1 | 128 |
| **Cefotetan** | 256 | 0.5 | 0.5 | 128 | 1 | 128 |
| **Tobramycin** | 256 | 0.25 | 128 | 0.25 | 0.25 | 0.25 |
| **Gentamicin** | 256 | 0.25 | 128 | 0.25 | 0.25 | 0.25 |
| **Ciprofloxacin** | 32 | 0.25 | 1 | 0.25 | 0.25 | 0.25 |
| **Levofoxacin** | 16 | 1 | 1 | 0.5 | 0.25 | 0.25 |
| **Amikacin** | >256 | 0.25 | 256 | 0.50 | 0.50 | 0.50 |
| **Trimethoprim-sulfamethoxazole** | >32/608 | 0.5/9.5 | 0.5/9.5 | 0.5/9.5 | 0.5/9.5 | 0.5/9.5 |
| **Polymyxin** | 1 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |

MIC, minimum inhibitory concentration; ESBL, extended-spectrum β-lactamase; NDM, New Delhi metallo-β-lactamase-1.
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MHA containing rifampin plus imipenem and MHA containing rifampin plus ceftriaxone. While, EC600ESBL grew only on MHA containing rifampin plus ceftriaxone. This indicates that the carbapenemase gene and ESBL gene may be on different plasmids and therefore transferred independently. EC600ESBL had the same resistance phenotype as BJ01 on MHA containing EDTA because the carbapenemase in BJ01 was inhibited on MHA impregnated with EDTA. (Fig. 1c). The EC600NDM-1 transconjugant was resistant to carbapenems and cephalosporins but sensitive to aminoglycosides, fluoroquinolones, and aztreonam, which indicated that plasmid harboring blaNDM-1 did not carry other ESBLs, aminoglycoside-resistance, or quinolone-resistance genes (Fig. 1d). The prepared plasmid was transformed into E. coli DH5α. Antimicrobial susceptibility profiles of the DH5αNDM-1 transformant exhibited antibiotic sensitivities similar to the EC600NDM-1 isolate (Fig. 1e; Table 1).

Detection of Drug Resistance Genes

By PCR and sequencing, we detected the presence of MBL genes in BJ01 strain. The blaNDM-1, blaTEM-1 and blaCTX-M-57 genes detected (Fig. 2), but not any other MBL genes (blaVIM, blaIMP, blaGIM, and blaSIM) or β-lactamase genes (blaatm, and blaOXA-1). The presence of blaNDM-1 gene was further confirmed by Southern blot (Fig. 3). CTX-M-β-lactamases can be divided into five groups based on their amino acid sequence identities. Group I includes CTX-M-1, -28, -29, etc. CTX-M-57 is a group I CTX-M and shared 99% amino acid identity with CTX-M-15[21,22], which is one of the most common types of ESBL found in bacterial isolates[23]. The sequences of the blaNDM-1, blaTEM, and blaCTX-M-57 genes were analyzed and deposited in GenBank under accession numbers HQ603057, JX036279, and JX036278. Only blaTEM-1 and blaCTX-M-57 were detected in the EC600ESBL transconjugant and only blaNDM-1 was detected in the EC600NDM-1 transconjugant and DH5αNDM-1 transformant, which indicated that blaTEM-1 and blaCTX-M-57 were located on a different plasmid from blaNDM-1 (Fig. 2).

MLST genotype analysis

MLST revealed that E. coli BJ01 belonged to a new sequence type 224 (ST224).

**Figure 2.** Polymerase chain reaction-amplified CTX-M-57, TEM-1, and NDM-1 genes in BJ01 (1), EC600ESBL (2), EC600NDM-1 (3), and DH5αNDM-1 (4). CTX-M-57 and TEM-1 were detected in BJ01 and EC600ESBL; blaNDM-1 was detected in BJ01, EC600NDM-1, and DH5αNDM-1.

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**Figure 3.** Southern blot hybridization on blaNDM-1 gene of BJ01. The band marked with the white arrow indicated positive signals by Southern blot hybridization with the specific NDM-1 probe.

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Genetic Environment of the blaNDM-1 gene

A 17,924-bp fragment was obtained from plasmid DNA of strain E. coli BJ01, and sequenced by primer walking. The sequence has been deposited in GenBank with accession No. JX296013. Sequencing of the blaNDM-1 upstream region identified the presence of an IS4ba125 insertion sequence. Further sequence analysis revealed that the region flanking the IS4ba125 was the IS3000 gene. The tufE gene flanked the 3’ end of blaNDM-1, followed by dhaC, truncated cutl gene disrupted by IS26, wmuD and a transposase gene. (Fig. 4)

**Discussion**

In contrast to most other countries where NDM has been mainly identified in Enterobacteriaceae, in China to date NDM has only been reported in Acinetobacter[9,11,12,24] and Enterococcus faecium[13]. However, other types of MBLs, such as IMP, and other classes of acquired carbapenemases, such a KPC, have been reported in Enterobacteriaceae in China[25–29], but isolates of NDM-1-producing E.coli have not previously been identified in China.

NDM-1-harboring strains could be highly multidrug resistant[3]. Previous reports on NDM-1-producing Acinetobacter spp. have demonstrated that strains resistant to all available antimicrobials except colistin were common in China[10,30]. Our results showed resistant to almost all tested antimicrobials of the identified NDM-1-producing E.coli isolate. The patient had a good general condition and the infection was localized. Ultimately, the patient survived the infection. But this case should have raised public concerns again over increasing incidence of highly multidrug resistant NDM-1-harboring strains in China.

Travelers contribute significantly to the global movement of microbes and resistance genes [31]. Although nosocomial trans-
mission of NDM-1 has occurred in many countries[26,32,33], medical tourism plays a significant role in the spread of NDM-1[34], and traveling to the Indian subcontinent is a significant risk factor of infection with an NDM-1-producing bacterium[35]. Patients in China who were found to have blaNDM-1-carrying bacteria had no history of traveling to the Indian subcontinent or another country[9,11,13,24]. In this study, the patient carrying the blaNDM-1-positive E. coli strain had no foreign travel history.

The identified R701 strain demonstrated phenotypes of both ESBL and NDM-1 (Table 1). This is consistent to previous reports on NDM-1-producers. For example, a study from China indicates that all 4 identified NDM-1-carrying A. baumanii isolates expressed both genes[30]. Data from the SMART study shows that blaNDM-1-positive enterobacteriaceae isolates often carry additional β-lactamase genes. Among 53 blaNDM-1-carrying strains, blCTX-M-15 is detected in 30[36]. However, our data also suggested that ESBL and NDM-1 genes may be carried by distinct plasmids and could be transferred separately (Fig. 1). This has to be further confirmed by sequence analysis.

In our study, MLST analysis revealed that R701 belonged to sequence type 224 (ST224), which was different from the NDM-1-producing ST101 E. coli isolated from Australia [37], Germany [38], Canada [39], the UK [40], and New Zealand [41]. This is the first reported case of infection due to a NDM-1-producing E. coli, which is highly drug-resistant and comes with a new MLST genotype in E. coli. The patient carrying this strain does not have a travel history. The detection of the NDM-1-positive E. coli isolate in our study indicates immediate importance of strengthening surveillance to prevent the nosocomial infection and dissemination of NDM-1 in China.

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