Epidemiological Characteristics of \( \text{bla}^{\text{NDM-1}} \) in Enterobacteriaceae and Acinetobacter calcoaceticus – Acinetobacter baumannii Complex in China from 2011 to 2012

Weimei Ou and Yuan Lv*

The Institute of Clinical Pharmacology, Peking University First Hospital, China

Abstract

**Objectives:** The study aimed to investigate the prevalence and the epidemiological characteristics of \( \text{bla}^{\text{NDM-1}} \) in Enterobacteriaceae and Acinetobacter calcoaceticus–Acinetobacter baumannii complex (ABC) in China from July 2011 to June 2012.

**Methods:** All organisms studied were screened for the presence of \( \text{bla}^{\text{NDM-1}} \) using PCR. For those \( \text{bla}^{\text{NDM-1}} \)-positive strains, 16S rRNA along with API strips were performed to validate the bacterial genus and species. The ABCs were reconfirmed by PCR detection of \( \text{bla}_{\text{NDM-1}} \). The antibiotic susceptibilities were assessed by determining minimum inhibitory concentration (MIC) of them using two-folder agar dilution test recommended by the Clinical and Laboratory Standards Institute (CLSI). Molecular typing was performed using pulsed-field gel electrophoresis (PFGE). An S1 nuclease PFGE (S1-PFGE) and Southern blot hybridization were conducted to ascertain the gene location of \( \text{bla}_{\text{NDM-1}} \).

**Results:** Among 2170 the family Enterobacteriaceae and 600 ABCs, seven Enterobacteriaceae strains and two A. calcoaceticus isolates from five different provinces carried the \( \text{bla}_{\text{NDM-1}} \) gene. The seven Enterobacteriaceae strains were four Klebsiella pneumoniae, one Enterobacter cloacae, one Enterobacter aerogenes and one Citrobacter freundii, respectively. All of them showed non-susceptible to any agent of imipenem, meropenem, panipenem and ertapenem. Two A. calcoaceticus were both resistant to imipenem and meropenem. Three K. pneumoniae showed the same PFGE profiles. Eight \( \text{bla}_{\text{NDM-1}} \) genes were located on plasmids and one on chromosome.

**Conclusions:** Compared with the previous reports, the numbers and species of the \( \text{bla}^{\text{NDM-1}} \) in Enterobacteriaceae have been significantly increased in China and most of them can disseminate which should be drawn great attention. Consecutive surveillance should be implemented and focused on the dissemination of \( \text{bla}^{\text{NDM-1}} \) among gram-negative clinical isolates as well.

Keywords: New Delhi metallo-\( \beta \)-lactamase 1 (NDM-1); Enterobacteriaceae; Acinetobacter baumannii; Epidemiology

Introduction

Carbapenems are of choice antibiotics to many infections, especially those triggered by multi-drug resistant gram-negative bacteria. Therefore, carbapenemase in clinical gram-negative organisms which can hydrolyze carbapenems are an important threat to public health. What is more worse, New Delhi metallo-\( \beta \)-lactamase 1(NDM-1), a new type of Ambler class \( B \) metallo-\( \beta \)-lactamases (MBLs), encoded by \( \text{bla}^{\text{NDM-1}} \) was first reported in K. pneumoniae and *Escherichia coli* derived from a Swedish patient of Indian origin who was admitted to hospital in New Delhi, India in 2009 [1]. Since then, \( \text{bla}^{\text{NDM-1}} \)-positive bacteria have disseminated worldwide, including almost all seven continents except the Antarctica [2]. Indian subcontinent and China were the major reservoirs, Balkan states like Serbia, Montenegro and Bosnia–Herzegovina may be considered as a ‘secondary’ reservoir area while the Middle East (Morocco, Algeria, Libya, Egypt, Iraq, Kuwait, Oman, Lebanon and Afghanistan), southeast Asia (South Korea, Indonesia, Vietnam and Thailand) and parts of Europe (France, Italy) may be additional reservoir areas. The \( \text{bla}^{\text{NDM-1}} \) gene was identified in K. pneumoniae, *E. coli*, Klebsiella oxytoca, Enterobacter cloacae, Enterobacter aerogenes, Proteus spp., Citrobacter freundii, Morganella morganii, Providencia spp., Acinetobacter spp. and Raoultella ornithinolytica [3-23]. The \( \text{bla}^{\text{NDM-1}} \) gene was mostly on different large plasmids and partly on chromosome [24]. Those plasmids carrying \( \text{bla}^{\text{NDM-1}} \) were mostly transferable and coexisted with many other resistant determinants [9,11,17], making treatment of NDM-1-producing bacteria a further complication.

This study retrospectively survey the nationwide epidemiology of \( \text{bla}^{\text{NDM-1}} \) in Enterobacteriaceae and ABCs strains derived from 18 tertiary hospitals presenting different provinces in China from July 1, 2011 to June 30, 2012.

Materials and Methods

**Bacterial strains**

The species of the family Enterobacteriaceae and ABCs were collected from 18 tertiary hospitals in different provinces in China

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*Corresponding author: Yuan Lv, The Institute of Clinical Pharmacology, Peking University First Hospital, China, Tel: +86-139-1033-2958; E-mail: lyzx5857@163.com

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from July 1, 2011 to June 30, 2012. 338 \textit{Enterobacteriaceae} and 395 ABCs which were nonsusceptible to carbapenem were selected from 2170 \textit{Enterobacteriaceae} and 600 ABCs clinical isolates. Standard strains for antimicrobial susceptibility were \textit{E. coli} ATCC25922, \textit{E. coli} ATCC35218 and \textit{Pseudomonas aeruginosa} ATCC27853. \textit{Salmonella} serotype \textit{Braenderup} strain H9812 was used as the marker for PFGE.

### PCR amplification

The DNA extraction was performed from fresh culture using boiling techniques. The primers used in this study were based on primers published by the Chinese Center For Disease Control and Prevention (CDC). F:TCG CAT AAA ACG CCT CTG; R:GAA ACT GTC GCA CCT CAT. The reaction mixtures were 20 μl 2x Taq PCR MIX (TaKaRa, Dalian, China) 10 μl; 20 μM each primer 1 μl; DNA sample 2 μl and ddH2O 6 μl. Amplification was carried out under the following thermal cycling conditions: 5 min at 94°C; 30 cycles of amplification consisting of 15 s at 94°C, 30 s at 51°C, and 30 s at 72°C; and 10 min at 72°C for the final extension. The amplicon were analyzed by electrophoresis in a 1.5% agarose gel and were sequenced.

### Species confirmation

The \textit{bla}_{NDM-1}-positive organisms were affirmed for bacterial genus by the sequence analysis of the 16S rRNA, using the universal primers of 27F-AGAGTTTGTATCCTGCTCAG and 1492R-GGCTACCCTTGGTATCAGCT [25]. The primers used in this study were based on primers previously reported as F:TAA TGC TTT TTA TAC GCA ATT GCA CTT CAT CTT GG [26].

### Antimicrobial susceptibility

Susceptibility testing for \textit{bla}_{NDM-1}-positive isolates was performed by determining MICs by two-folder agar dilution test on Mueller-Hinton agar plates at 37°C. The results were interpreted according to the CLSI2013 M100-S23 guidelines [27]. The breakpoints of imipenem and meropenem for \textit{Enterobacteriaceae} were as follows: susceptible (S), ≤ 0.5 μg/ml; resistant (R), > 2 μg/ml. Likewise, the breakpoints of imipenem and meropenem for \textit{A. baumannii} were: S, ≤ 4 μg/ml; R, > 16 μg/ml. Both of the two species, the breakpoints of meropenem were used for panipenem.

### S1-PFGE and southern hybridization

Refer to the literature published early [28], bacterial DNA was prepared in agarose blocks and digested with restrict enzyme XbaI (four \textit{K. pneumonia} and \textit{Salmonella} serotype \textit{Braenderup} strain H9812) and Apal (two \textit{A. calcoaceticus}). The DNA fragments were separated by use of a CHEF-Mapper XA PFGE system (Bio-Rad, USA) at 6 V/cm and 14°C, with a pulse angle of 120°, for 23 h and a switch time from 4 to 40 s in \textit{Enterobacteriaceae} while 24 h and a switch time from 5 to 20 s in \textit{A. calcoaceticus}. The gel was stained with ethidium bromide to make the PFGE banding patterns visual.

### Plasmid analysis and southern hybridization

Plasmids were extracted according to Molecular Cloning: a laboratory manual then digested with \textit{EcoRI} and agarose gel electrophoresis at 90V 45 min after prepared in agarose holes. The gel was stained with ethidium bromide to make the plasmid profiles visual. The plasmid fragments were then transferred to nylon membranes (GE, China), hybridized with digoxigenin-labelled \textit{bla}_{NDM-1}-specific probes and detected using an NBT/BCIP colour detection kit (Roche, Switzerland).

### Results

The identification of \textit{bla}_{NDM-1}-positive bacteria

All PCR detection for \textit{bla}_{NDM-1} results was positive. The sequencing

### Table 1: The MICs of \textit{bla}_{NDM-1}-positive bacteria.

<table>
<thead>
<tr>
<th>Strains</th>
<th>PRL</th>
<th>TZIP</th>
<th>CTX</th>
<th>CRO</th>
<th>CAZ</th>
<th>CFP</th>
<th>SCF</th>
<th>FEP</th>
<th>GEN</th>
<th>AMK</th>
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<th>TGC</th>
<th>CIP</th>
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<td>512</td>
<td>512</td>
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<td>512</td>
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<td>256</td>
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<td>&gt;256</td>
<td>—</td>
<td>128</td>
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<td>512</td>
<td>512</td>
<td>256</td>
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<td>—</td>
<td>128</td>
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<td>—</td>
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results of the amplicons showed all were 100% identity with *K. pneumoniae* strain 05-506 (Genbank accession number: FN396876). 16S rRNA sequencing and biochemical API strips revealed that four were *K. pneumoniae* (M186, M187, M194, U091), two were ABCs (G113, X231), one was *Enterobacter cloacae* (Q297), one *Enterobacter aerogenes* (Q442) and one *Citrobacter freundii* (X122), respectively. The *bla*\textsubscript{OXA-51-like} detection of the two ABCs was negative, indicating that both G113 and X231 were *A. calcoaceticus*.

The MICs of *bla*\textsubscript{NDM-1}-positive bacteria

All nine strains showed highly resistant to broad spectrum penicillin, cephalosporins, β-lactamase inhibitor combinations, most carbapenem and nitrofurantoin, but showed variable susceptibilities to aminoglycosides and tetracyclines. The good news was that most strains show susceptible to fluoroquinolones and tigycycline (Table 1).

PFGE

The three *K. pneumonia* from the same provinces (M186, M187, M194) had the same PFGE profiles while the two *A. calcoaceticus* showed different profiles (Figure 1).

Plasmid analysis of *bla*\textsubscript{NDM-1}-positive bacteria

Except U091 on chromosome and X122 not succeed, the other seven strains all displayed the *bla*\textsubscript{NDM-1} gene were on plasmid, with size ranging from ~23 to ~96 kb (Figure 2).

Plasmid analysis and southern hybridization

Since S1-PFGE results weren’t good and repeated results weren’t stable, we conducted plasmid extraction and Southern blot to make sure whether the *bla*\textsubscript{NDM-1} was on plasmid or not. U091 didn’t have plasmid, which could further explain the above S1-PFGE & Southern blot result that why its *bla*\textsubscript{NDM-1} gene was on chromosome. Other eight strains all had plasmids. The sizes of plasmids were consistent with S1-PFGE results above. Southern blot hybridation showed the *bla*\textsubscript{NDM-1} gene were all on plasmids in the eight strains that haboured plasmids (Figure 3).

Discussion

In the past few decades, an alarming increase in the prevalence of antimicrobial resistant pathogens of serious community- and hospital-acquired infections has been shown worldwide. The increase in carbapenem resistance in Gram-negative bacteria has become a major concern. Bacteria producing NDM-1 had ever caused global panic because they can hydrolyze almost all antimicrobial agents except few, which were referred to “Superbug” by media. To further complicate matters, the *bla*\textsubscript{NDM-1} gene encoding NDM-1 has disseminated rapidly over distantly related geographical areas around the world [8,12,18,23]. In terms of human hosts, there are three major routes to acquire an NDM-1 producing organism: nosocomial, personal travel and...
community acquisition. The bla<sub>NDM</sub>-<sup>1</sup>-carrying bacteria have been reported as gut colonizers in human with or without clinical symptoms, they can survive in the local environment as well, which may result in human acquiring the bla<sub>NDM</sub>-<sup>1</sup>-positive bacteria unconsciously. Hence, the bacteria possessing the bla<sub>NDM</sub>-<sup>1</sup> gene constitute tremendous health threat to human.

There are three main resistant mechanisms against β-lactam antibiotic: 1. the production of β-lactamases which cleave the amide bond of the β-lactam ring; 2. the possession of an altered or acquired penicillin binding protein with low affinity for β-lactams; 3. over expression of efflux pump mechanism [29]. The most common mechanism of resistance in carbapenem is the production of carbapenemases (one of β-lactamases), including enzymes of Ambler classes A, D and B (MBLs). NDM-1 is one of MBLs that mediates carbapenem-resistant. In this study, we confirmed those bla<sub>NDM</sub>-<sup>1</sup>-bearing strains were resistant if not intermediate to carbapenem.

China has reported bla<sub>NDM</sub>-<sup>1</sup>-producing bacteria since 2010 by Chinese CDC. Since then, many researches have been on this issue. Chen et al. [13] reported four <i>A. baumannii</i> at the mainland of China, Ho et al. [17] reported one <i>E. coli</i> in Hong Kong, Wu et al. [20] reported <i>K. pneumoniae</i> in Tai Wan. By now, a number of bla<sub>NDM</sub>-<sup>1</sup>-positive bacteria have been reported. So far, the species China has discovered have <i>E. coli</i>, <i>K. pneumoniae</i>, <i>K. oxytoca</i>, <i>K. ozaenae</i>, <i>E. cloacae</i>, <i>E. aerogen</i>, <i>C. freundii</i>, <i>Salmonella enteritidis</i>, <i>Morganella morganii</i>, <i>Providencia spp.</i>, <i>Alcaligenes faecalis</i>, <i>Kocuria varians</i>, <i>Moraxella group</i>, <i>Comamonas testosteroni</i>, <i>Stenotrophomonas maltophilia</i>, <i>Staphylococcus capitis</i>, <i>Methylobacterium species</i>, <i>Raoiella ornithinolytica</i>, <i>Acinetobacter spp.</i> and <i>E. faecium</i>. Though there are several molecular level researches on the genetic context of bla<sub>NDM</sub>-<sup>1</sup>, there are limited studies about epidemiology of bla<sub>NDM</sub>-<sup>1</sup>-containing isolates [6,10,13-15,17]. Compared with previously reports [10,13], this study demonstrates that the numbers and species of bla<sub>NDM</sub>-<sup>1</sup> in family Enterobacteriaceae have been significantly increased in China, including <i>Klebsiella pneumoniae</i>, <i>Enterobacter cloacae</i>, <i>Enterobacter aerogen</i> and <i>Citrobacter freundii</i>. This situation should draw intensive attention since Enterobacteriaceae are the main cause of nosocomial infection. It will be undoubtedly much troublesome once they acquire bla<sub>NDM</sub>-<sup>1</sup>.

Our study reported nine bla<sub>NDM</sub>-<sup>1</sup>-producing strains in all. Except the U091 strain the bla<sub>NDM</sub>-<sup>1</sup> gene was on chromosome, other eight Southern blot hybridization results showed that bla<sub>NDM</sub>-<sup>1</sup> were all on plasmid, which may result in horizontal transmission rapidly. Further studies are being done to elucidate the transmissibility and the background of resistance determinants.

References


