Epidemiological Characteristics of bla_{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 bla_{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 bla_{NDM-1} using PCR. For those bla_{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 bla_{NDM-1}. The antibiotic susceptibilities were assessed by determining minimum inhibitory concentration (MIC) of them using two-fold 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 bla_{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 bla_{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 nonsusceptible 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 bla_{NDM-1} genes were located on plasmids and one on chromosome.

Conclusions: Compared with the previous reports, the numbers and species of the bla_{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 bla_{NDM-1} among gram-negative clinical isolates as well.

Keywords: New Delhi metallo-β-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-β-lactamase 1 (NDM-1), a new type of carbapenemase, can hydrolyze almost all antimicrobials except colistin, tigecycline and sometimes aztreonam, and was thus referred to “superbug” by media. This article will focus on the problem of carbapenem resistance mediated by NDM-1.

NDM-1, a new type of Ambler class B metallo-β-lactamases (MBLs), encoded by bla_{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, bla_{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 bla_{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 bla_{NDM-1} gene was mostly on different large plasmids and partly on chromosome [24]. Those plasmids carrying bla_{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 bla_{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.
from July 1, 2011 to June 30, 2012. 338 Enterobacteriaceae and 395 ABCs which were nonsusceptible to carbapenem were selected from 2170 Enterobacteriaceae and 600 ABCs clinical isolates. Standard strains for antimicrobial susceptibility were E. coli ATCC25922, E. coli ATCC35218 and Pseudomonas aeruginosa ATCC27853. Salmonella serotype 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:TG CAT AAA ACG CCT CTG; R:GAA ACT GTC GCA CCT CAT. The reaction mixtures were 20 µl 2X Tap 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 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 bla<sub>NDM-1</sub>-positive organisms were affirmed for bacterial genus by the sequence analysis of the 16S rDNA, using the universal primers of 27F-AGAGTTTGATCCTGGCTCAG and 1492R-GGCTACCTTGTTACGACTT [25]. The thermal cycling conditions were: 5 min at 94°C, 30 cycles of amplification consisting of 60 s at 95°C, 60 s at 45°C, and 90 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.

**Antimicrobial susceptibility**

Susceptibility testing for bla<sub>NDM-1</sub>-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 family Enterobacteriaceae were as follows: susceptible (S), ≤ 0.5 µg/ml; resistant(R), ≥ 4 µg/ml; for ertapenem were as follows: S, ≤ 0.5 µg/ml; R, ≥ 2 µg/ml. Likewise, the breakpoints of imipenem and meropenem for A.baumannii were: S, ≤ 4 µg/ml; R, ≥ 16 µg/ml. Both of the two species, the breakpoints of meropenem were used for panipenem.

**PFGE**

Bacterial DNA was prepared in agarose blocks and digested with restrict enzyme XbaI (four K. pneumonia and Salmonella serotype Braenderup strain H9812) and Apal (two 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 Enterobacteriaceae while 24 h and a switch time from 5 to 20s in A. calcoaceticus. The gel was stained with ethidium bromide to make the PFGE banding patterns visible.

**S1-PFGE and southern hybridization**

Refer to the literature published early [28], bacterial DNA was prepared in agarose blocks and digested with S1 nuclease, and then separated by PFGE as above with conditions of 14 h at 6 V/cm and 14°C, with a pulse angle of 120° and a switch time from 1 to 10 s. The gel was stained with ethidium bromide to make the bands visual. After that, the DNA fragments were transferred to nylon membranes (GE, China), hybridized with digoxigenin-labelled bla<sub>NDM-1</sub>-specific probes and detected using an NBT/BCIP colour detection kit (Roche, Switzerland).

**Plasmid analysis and southern hybridization**

Plasmids were extracted according to Molecular Cloning; a laboratory manual then digested with 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 visible. The plasmid fragments were then transferred to nylon membranes hybridized with digoxigenin-labelled bla<sub>NDM-1</sub>-specific probes and detected using an NBT/BCIP colour detection kit as above.

**Results**

The identification of bla<sub>NDM-1</sub>-positive bacteria

All PCR detection for bla<sub>NDM-1</sub> results was positive. The sequencing

<table>
<thead>
<tr>
<th>Strains</th>
<th>PRL</th>
<th>TSP</th>
<th>CTX</th>
<th>CRO</th>
<th>CAZ</th>
<th>CIP</th>
<th>SCA</th>
<th>FEP</th>
<th>ETP</th>
<th>AMK</th>
<th>GEN</th>
<th>AMK</th>
<th>TCY</th>
<th>CIP</th>
<th>TGC</th>
<th>LVP</th>
<th>NIT</th>
<th>POL</th>
<th>POS</th>
</tr>
</thead>
<tbody>
<tr>
<td>M186</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>32</td>
<td>512</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>32</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.031</td>
<td>0.031</td>
<td>0.128</td>
</tr>
<tr>
<td>M187</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>64</td>
<td>512</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.031</td>
<td>0.031</td>
</tr>
<tr>
<td>M194</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>64</td>
<td>512</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.031</td>
<td>0.062</td>
<td>0.128</td>
</tr>
<tr>
<td>U091</td>
<td>512</td>
<td>256</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>32</td>
<td>64</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>0.25</td>
<td>256</td>
<td>64</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>32</td>
</tr>
<tr>
<td>Q297</td>
<td>512</td>
<td>256</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>64</td>
<td>256</td>
<td>4</td>
<td>8</td>
<td>64</td>
<td>32</td>
<td>128</td>
<td>2</td>
<td>128</td>
<td>64</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Q442</td>
<td>512</td>
<td>256</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>64</td>
<td>256</td>
<td>4</td>
<td>8</td>
<td>64</td>
<td>128</td>
<td>0.5</td>
<td>128</td>
<td>16</td>
<td>0.5</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>G113</td>
<td>256</td>
<td>256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>—</td>
<td>128</td>
<td>&gt;256</td>
<td>—</td>
<td>128</td>
<td>128</td>
<td>—</td>
<td>—</td>
<td>256</td>
<td>8</td>
<td>0.125</td>
<td>—</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>X231</td>
<td>256</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>&gt;256</td>
<td>—</td>
<td>128</td>
<td>128</td>
<td>256</td>
<td>—</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0.062</td>
<td>0.1</td>
<td>0.062</td>
<td>0.062</td>
</tr>
</tbody>
</table>


**Table 1:** The MICs of bla<sub>NDM-1</sub>-positive bacteria.

---

**References**

results of the amplicons showed all were 100% identity with *K. pneumoniae* strain 05-505 (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* 

The MICs of *bla* 

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* 

Except U091 on chromosome and X122 not succeed, the other seven strains all displayed the *bla* 

**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* 

**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* 

**Figure 2:** Results of S1-PFGE(left) and Southern blot hybridization(right). M:Low Range PFGE Marker.

(\[a\] )                             (\[b\])
Our study reported nine bla<sub>NDM-1</sub>-producing strains in all. Except the U091 strain the bla<sub>NDM-1</sub> gene was on chromosome, all other eight Southern blot hybridization results showed that bla<sub>NDM-1</sub> 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


