Emergence of NDM-1-producing Acinetobacter baumannii in China

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Objectives: To investigate the prevalence of blaNDM-1 in Gram-negative bacilli in China.

Methods: A total of 11,298 clinical Gram-negative bacilli, covering Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii and Pseudomonas aeruginosa, were collected for PCR-based surveillance of blaNDM-1 from 57 hospitals representing 18 provinces in China. For blaNDM-1-positive isolates, antibiotic susceptibilities were assessed and molecular typing was performed using PFGE. The genetic location of blaNDM-1 was determined by analysis of PFGE profiles of S1 nuclease-digested genomic DNA and Southern blot hybridization. Plasmid transfer to E. coli recipients was investigated using filter mating and electroporation.

Results: Four A. baumannii isolates with blaNDM-1 were identified in four different provinces in China: no positive isolates were detected among E. coli, K. pneumoniae and P. aeruginosa. These blaNDM-1-positive A. baumannii were resistant to all carbapenems and cephalosporins, and three remained susceptible to fluoroquinolones, aminoglycosides and colistin. The four NDM-1-producing A. baumannii were clonally diverse and carried blaNDM-1 on different plasmids. Plasmids carrying blaNDM-1 were successfully transferred from three of the four isolates to E. coli recipients, although the transconjugants and transformants were prone to lose the transferred plasmids after passage in the absence of ampicillin selection.

Conclusions: We describe the emergence of A. baumannii producing NDM-1 in China. Systemic surveillance network should be established for monitoring these resistant bacteria.

Keywords: carbapenem resistance, metallo-β-lactamases, plasmids

Introduction

The increase in carbapenem resistance in Gram-negative bacteria has become a major concern worldwide. The most common mechanism of resistance is the production of carbapenemases, including enzymes of Ambler classes A, D and B (metallo-β-lactamases (MBLs)), with the corresponding genes often being associated with mobile genetic elements.1,2 KPC-type class A carbapenemase, which is commonly plasmid-encoded in Klebsiella pneumoniae, is widespread in China, Israel, Greece, South America and the USA.3,4 The production of MBLs, mainly of the VIM and IMP types, has mostly been associated with Pseudomonas aeruginosa, Acinetobacter spp. and, more recently, Enterobacteriaceae.5,6

New Delhi MBL 1 (NDM-1), a new type of MBL, was first reported in K. pneumoniae and Escherichia coli recovered from a Swedish patient who was admitted to hospital in New Delhi, India.7 In recent months, the emergence and dissemination of NDM-1-producing isolates have been reported in several countries, including the USA, Canada, Sweden, the UK, Austria, Belgium, France, the Netherlands, Germany, Japan, Africa, Oman and Australia.8 The blaNDM-1 gene was identified in K. pneumoniae, E. coli, Enterobacter cloacae, Proteus spp., Citrobacter freundii, Klebsiella oxytoca, Morganella morganii, Providencia spp. and Acinetobacter baumannii.8–10 NDM-producing bacteria are commonly resistant to almost all groups of antibiotics, including fluoroquinolones, aminoglycosides and β-lactams (especially carbapenems), but susceptible to colistin and sometimes tigecycline. The blaNDM-1 gene has been detected on different large plasmids, which were readily transferable among bacteria,8,11 making NDM-1-producing bacteria a serious clinical and public health threat.

In the present study, we report on the detection of four blaNDM-1-positive A. baumannii during large-scale PCR-based surveillance for NDM-1 in China.

Materials and methods

Bacterial strains and species identification

A nationwide survey of multidrug resistance in Gram-negative bacteria was undertaken in China from January 2009 to September 2010.
In this study, sequential clinical isolates (up to a maximum of 50 per hospital) were collected from 57 hospitals representing 18 provinces. Each hospital undertook surveillance for three consecutive months. All isolates were identified using Vitek GN+ cards (bioMérieux, France). A. baumannii were confirmed by PCR detection of blaOXA-51-like and sequence analysis of the 16S–23S rRNA gene spacer region.12

Molecular detection of resistance genes

Isolates were screened for the presence of blaNDM-1 by PCR with primers NDM-F-38 (5′-GCCGGGAATGGCTCAGACG-3′) and NDM-R-344 (5′-CGCAACACAGCTCGACTTT-3′); presumptive positive results obtained during screening were validated by PCR using the primers NDM-up168 (5′-GAATGTCTGGCAGACACACTT-3′) and NDM-dw647 (5′-TGGGCCTTGCTGTCCTTGAT-3′). PCR experiments were performed according to standard conditions with an annealing temperature of 58°C. PCR screening and sequencing primers were designed using GenBank accession numbers FN396876. PCR screening was performed for additional resistance genes and insertion elements of the NDM-1-positive isolates, including blaOXA-51-like, blaOXA-23-like, ISAb1-blaOXA-51-like, ISAb1-blaOXA-23-like, blaOXA-58-like, blaOXP-like, blaVIM-like, blaNDM-1, blaTEM, blaVEB, blaSHV, blaPER, blaCTX-M and armA.13-15

Antimicrobial susceptibility testing

MICs of a range of antibiotics for the four NDM-1-positive isolates were determined by Etest (AB bioMérieux, Solna, Sweden) and results of susceptibility testing were interpreted according to CLSI guidelines.16 The breakpoint for Enterobacteriaceae of the European Committee on Antimicrobial Susceptibility Testing was used for tigecycline (http://www.srga.org/eucastwt/MICTAB/MICtigecycline.htm).

PFGE

Genomic DNA was prepared in agarose blocks and digested with the restriction enzyme Apal. The DNA fragments were separated by use of a CHEF-Mapper XA PFGE system (Bio-Rad, USA) for 22 h at 6 V/cm and 14°C, with a pulse angle of 120° and pulse times from 5 to 20 s. PFGE banding patterns were analysed visually.17

Plasmid analysis and Southern blot

Genomic DNA was digested with S1 nuclease and separated by PFGE as above, but with a switch time from 2.16 to 63.8 s for 20 h runtime. Then, the DNA fragments were transferred to nylon membranes (Millipore, USA), hybridized with digoxigenin-labelled blaNDM-1-specific probes and detected using an NBT/BCIP colour detection kit (Roche Applied Sciences).

Conjugation and transformation

Filter mating was performed with each of the four NDM-1-positive isolates of A. baumannii using E. coli J53 (azide resistant) as the recipient strain, with selection based on growth on agar in the presence of ampicillin (50 mg/L) and azide (200 mg/L). Transconjugants were confirmed as blaNDM-1-positive by PCR analysis.

Plasmid DNA was extracted with the Qiagen Midi Kit (Qiagen, Germany). Transformation of plasmids was carried out using electroporation and E. coli DH5α cells (TAKARA, China). Transformants were selected on agar plates containing ampicillin (50 mg/L) and confirmed blaNDM-1 positive by PCR analysis.

Results

Identification of NDM-positive isolates

A total of 11,298 clinical isolates of Gram-negative bacilli, including 2109 A. baumannii, 2910 P. aeruginosa, 3439 E. coli and 2840 K. pneumoniae, were collected and screened for the presence of blaNDM-1. Of these, four isolates (designated ABCA207, ABC3229, ABC4289 and ABWA7), all identified as A. baumannii, were positive. These PCR screening results were validated by sequencing and the sequence of the blaNDM-1 genes showed 100% identity with previously reported genes. No blaNDM-1 positive isolates were detected among the E. coli, K. pneumoniae and P. aeruginosa isolates.

These four NDM-positive A. baumannii were isolated from Xinjiang province, Jilin province, Shaanxi province and Hubei province, respectively. Specimens included sputum (two patients), blood (one patient) and secretion (one patient) from three male patients and one female patient. Three of these patients were neonates (Table 1).

Antimicrobial susceptibility testing

All these NDM-1-producing A. baumannii were resistant to all carbapenems, cephalosporins and β-lactamase inhibitor combinations tested. A. baumannii ABC3229 was resistant to gentamicin, amikacin, ciprofloxacin, minocycline and tigecycline, to which the other three isolates remained susceptible. All isolates were susceptible to colistin, except ABC4289 (Table 2). All the isolates were positive by the MBL Etest.

Detection of resistance genes

ABCA207, ABC4289 and ABWA7 were negative when tested by PCR for additional resistance genes and insertion elements except blaOXA-51-like, which occurs naturally in A. baumannii. ABC3229 was positive for the β-lactamase gene blaTEM-1 and for the 16S rRNA methylase gene armA.

Table 1. Characteristics of the four NDM-1-positive A. baumannii

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Year of isolation</th>
<th>Patient age</th>
<th>Patient sex</th>
<th>Specimen</th>
<th>Underlying disease</th>
<th>Ward</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA207</td>
<td>2010</td>
<td>14 days</td>
<td>male</td>
<td>sputum</td>
<td>pneumonia</td>
<td>neonatal</td>
<td>Xinjiang</td>
</tr>
<tr>
<td>ABC3229</td>
<td>2010</td>
<td>60 years</td>
<td>male</td>
<td>blood</td>
<td>fever of unknown origin</td>
<td>haematology</td>
<td>Jilin</td>
</tr>
<tr>
<td>ABC4289</td>
<td>2010</td>
<td>20 days</td>
<td>female</td>
<td>secretions</td>
<td>intestinal perforation</td>
<td>neonatal</td>
<td>Shaanxi</td>
</tr>
<tr>
<td>ABWA7</td>
<td>2009</td>
<td>26 days</td>
<td>male</td>
<td>sputum</td>
<td>pneumonia</td>
<td>neonatal</td>
<td>Hubei</td>
</tr>
</tbody>
</table>
### Molecular typing and plasmid analysis of NDM-positive A. baumannii

PFGE of Apal-digested DNA was performed for NDM-positive A. baumannii. The four isolates from four provinces had different PFGE profiles (Figure 1). Genomic DNA restricted with S1 nuclease in agarose blocks was separated by PFGE, and then the gel was blotted and hybridized with blaNDM-1. The results showed that each of these four isolates carried one to three plasmids and that blaNDM-1 was located on diverse plasmids with sizes from ~30 to 50 kb (Figure 1).

The plasmids carrying blaNDM-1 from A. baumannii ABC3229, ABC4289 and ABWA7 were successfully transferred to E. coli J53 and DH5α. The transconjugants J53-ABC3229, J53-ABC4289 and J53-ABWA7 were resistant to cefotaxime and ceftazidime, but remained susceptible to aztreonam and cefepime. The three transconjugants remained susceptible to imipenem and meropenem, although the MICs of these carbapenems for the transconjugants were relatively high compared with those for E. coli J53 (Table 2). However, all the transconjugants and transformants were prone to lose the transferred plasmids and became blaNDM-1-negative after one passage in the absence of ampicillin selection.

### Discussion

Since the wide dissemination of NDM-producing Enterobacteriaceae (mostly K. pneumoniae and E. coli) in India, Pakistan and the UK reported by Kumarasamy and colleagues, more cases have been detected from other countries in all continents. Here, we report blaNDM-1-positive clinical isolates in China. In contrast to in other countries where blaNDM-1 was mostly carried by Enterobacteriaceae, this gene was not detected in the 3439 E. coli and 2840 K. pneumoniae tested in our study. All the blaNDM-1-positive isolates were A. baumannii, which suggests that this species, which has a robust survival capability, can easily acquire foreign resistance genes such as blaNDM-1.

These four positive isolates were recovered from four provinces located in distinct regions of China. The earliest positive isolate was collected in 2009. Though the prevalence of the blaNDM-1 gene was extremely low (4/2109 isolates, 0.18%), it alerts us to an emerging problem that has the potential to spread. There is epidemiological evidence that travel to the Indian subcontinent is a significant risk factor for infection with a blaNDM-1-producing bacterium. Although China borders India and Pakistan, we have no evidence that the infected patients had any connection with these two countries. The cases from whom the four isolates were obtained had no known epidemiological association and the isolates showed various pulsotypes, indicating a lack of clonal relatedness. This demonstrates the current sporadic nature of blaNDM-1 in China. The possibility could not be ruled out that this resistance gene was selected out from the environmental resistome.

The amA-positive isolate A. baumannii ABC3229 was highly resistant to β-lactams, fluoroquinolones and aminoglycosides, suggesting the co-existence of multiresistance mechanisms. The other isolates were recovered from neonates, for whom fluoroquinolones and aminoglycosides are rarely used, which may go some way towards explaining their susceptibility to these antibiotics. Although A. baumannii is not a common pathogen of neonates, infection and colonization with A. baumannii in neonatal units has been reported.

Previous studies revealed that the genes encoding NDM-1 were mostly located on plasmids, usually adjacent to insertion elements, which may facilitate the intra- or interspecies transmission of blaNDM-1. Karthikeyan et al. reported the first blaNDM-1-positive A. baumannii in India; however, no detail about the location of blaNDM-1 was provided. In present study, we confirmed that the blaNDM-1 genes of each of four NDM-positive A. baumannii isolates were located on various plasmids that ranged from ~30 to 50 kb in size.

Filter mating showed the plasmids carrying the blaNDM-1 gene in ABC3229, ABC4289 and ABWA7 were conjugative to E. coli. However, these resistance plasmids were easily eliminated from their new hosts in the absence of selection pressure, implying the instability of Acinetobacter-derived plasmids in Enterobacteriaceae. Nonetheless, it displays the potential for the spread of blaNDM-1 through plasmid transmission from A. baumannii to Enterobacteriaceae in the nosocomial environment, where strong antibiotic pressure is likely to be manifest.

In recent years, A. baumannii have emerged as one of the most troublesome pathogens for healthcare institutions globally. Despite being less commonly identified in A. baumannii than the OXA-type carbapenemases, MBLs have significantly

### Table 2. Antibiotic susceptibilities of blaNDM-1-positive A. baumannii and transconjugants (mg/L)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>IPM</th>
<th>MEM</th>
<th>FEP</th>
<th>CTX</th>
<th>CAZ</th>
<th>ATM</th>
<th>SAM</th>
<th>CPS2/1</th>
<th>TGP</th>
<th>MIN</th>
<th>TGC</th>
<th>GEN</th>
<th>AMK</th>
<th>CIP</th>
<th>CST</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC207</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>32</td>
<td>256</td>
<td>256</td>
<td>&gt;256</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>ABC3229</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>32</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>32</td>
<td>8</td>
<td>&gt;256</td>
<td>256</td>
<td>&gt;32</td>
<td>1</td>
</tr>
<tr>
<td>ABC4289</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>32</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>16</td>
<td>0.125</td>
<td>4</td>
</tr>
<tr>
<td>ABWA7</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>32</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.25</td>
<td>0.5</td>
<td>1</td>
<td>8</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

IPM, imipenem; MEM, meropenem; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; SAM, ampicillin/sulbactam; CPS2/1, cefoperazone/sulbactam 2:1; TGP, piperacillin/tazobactam; MIN, minocycline; TGC, tigecycline; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; CST, colistin.
more potent hydrolytic activities toward carbapenems. Antibiotic resistance is considered to be a major future challenge in China. It is no doubt the emergence and spread of NDM-1 in A. baumannii will further limit clinical therapeutic options and threaten the public health of China. A national monitoring and control system aimed at this new type of ‘superbug’ should be established.

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Transparency declarations
None to declare.

References
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Figure 1. PFGE of ApaI-digested DNA (a), S1-digested plasmid DNA (b) and Southern blot hybridization with blaoX-1 of A. baumannii isolates (c). The bands with white arrows showed positive signals by Southern blot hybridization with the NDM-1 probe. M, Salmonella serotype Braenderup strain H9812 molecular marker; 1, A. baumannii ABCA207; 2, A. baumannii ABC3229; 3, A. baumannii ABC4289; and 4, A. baumannii ABWA7.


