Epidemiological Characteristics of \( \textit{bla}_{\text{NDM-1}} \) in \textit{Enterobacteriaceae} and 
\textit{Acinetobacter calcoaceticus} – \textit{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 \( \textit{bla}_{\text{NDM-1}} \) in \textit{Enterobacteriaceae} and \textit{Acinetobacter calcoaceticus}–\textit{Acinetobacter baumannii} complex (ABC) in China from July 2011 to June 2012.

**Methods:** All organisms studied were screened for the presence of \( \textit{bla}_{\text{NDM-1}} \) using PCR. For those \( \textit{bla}_{\text{NDM-1}} \)-positive strains, 16S rRNA along with API strips were performed to validate the bacterial genus and species. The ABCs were confirmed by PCR detection of \( \textit{bla}_{\text{NDM-1}} \). 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 \( \textit{bla}_{\text{NDM-1}} \).

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

**Conclusions:** Compared with the previous reports, the numbers and species of the \( \textit{bla}_{\text{NDM-1}} \) in \textit{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 \( \textit{bla}_{\text{NDM-1}} \) among gram-negative clinical isolates as well.

Keywords: New Delhi metallo-\( \beta \)-lactamase 1 (NDM-1); \textit{Enterobacteriaceae}; \textit{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 worsed, New Delhi metallo-\( \beta \)-lactamase 1(NDM-1), a carbapenemase in clinical isolates as well.

NDM-1, a new type of Ambler class B metallo-\( \beta \)-lactamasas (MBLs), encoded by \( \textit{bla}_{\text{NDM-1}} \), was first reported in \textit{K. pneumoniae} and \textit{Escherichia coli} derived from a Swedish patient of Indian origin who was admitted to hospital in New Delhi, India in 2009 [1]. Since then, \( \textit{bla}_{\text{NDM-1}} \)-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 \( \textit{bla}_{\text{NDM-1}} \) gene was identified in \textit{K. pneumoniae}, \textit{E. coli}, \textit{Klebsiella oxytoca}, \textit{Enterobacter cloacae}, \textit{Enterobacter aerogenes}, \textit{Proteus spp.}, \textit{Citrobacter freundii}, \textit{Morganella morganii}, \textit{Providencia spp.}, \textit{Acinetobacter spp.} and \textit{Raoultella ornithinolytica} [3-23]. The \( \textit{bla}_{\text{NDM-1}} \) gene was mostly on different large plasmids and partly on chromosome [24]. Those plasmids carrying \( \textit{bla}_{\text{NDM-1}} \) 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 \( \textit{bla}_{\text{NDM-1}} \) in \textit{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 \textit{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:TGC 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 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 blaNDM-1-positive organisms were affirmed for bacterial genus by the sequence analysis of the 16S rRNA, 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 DNA fragments were visualized by electrophoresis in a 1.5% agarose gel at 110 V for 45 min in 0.5xTBE (45 mM Tris-HCl, 45 mM boric acid, 1mM EDTA) followed by ethidium bromide staining. The PCR products were sequenced and compared with the published sequence in the Genbank database by multiple sequence alignment. To make sure the species, a Kliger Iron Agar assay was carried out first to detect whether the bacteria were ferment or not. The distinction determined using API (bioMe´rieux, Craponne, OH [pH 8.0], 45 mM boric acid, 1mM EDTA) followed by ethidium bromide staining. The PCR products were sequenced and compared with the published sequence in the Genbank database by multiple sequence alignment.

**Antimicrobial susceptibility**

Susceptibility testing for blaNDM-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 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 visible. After that, the DNA fragments were transferred to nylon membranes (GE, China), hybridized with digoxigenin-labelled blaNDM-1-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 agarase 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 blaNDM-1-specific probes and detected using an NBT/BCIP colour detection kit as above.

**Results**

The identification of blaNDM-1-positive bacteria

All PCR detection for blaNDM-1 results was positive. The sequencing

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**Table 1:** The MICs of blaNDM-1-positive bacteria.

| Strains | PRL | TSP | CTX | CRU | CAZ | CFP | SFC | SFE | FEP | ATM | IMP | MEM | PAN | ETP | GEN | AMK | TCG | MNO | TGC | CIP | LVP | NIT | POL | POS |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| M186    | 512 | 512 | 256 | 512 | 512 | 512 | 256 | 32  | 512 | 4   | 8   | 16  | 32 | 0.5 | 1   | 0.5 | 1   | 0.5 | 0.031| 0.031 | 128 | 2   |
| M187    | 512 | 512 | 256 | 512 | 512 | 512 | 64  | 512 | 4   | 8   | 16  | 16 | 16 | 0.5 | 1   | 1   | 0.5 | 0.031| 0.031 | 128 | 4   |
| M194    | 512 | 512 | 256 | 512 | 512 | 64  | 512 | 8   | 16  | 32  | 16 | 0.5 | 1   | 2   | 1   | 0.5 | 0.031 | 0.062 | 128 | 4   |
| U901    | 512 | 512 | 256 | 512 | 512 | 512 | 512 | 32  | 64  | 4   | 8   | 8   | 8   | 1   | 0.25 | 256 | 64   | 0.25 | 0.5 | 32  | 8   |
| Q297    | 512 | 512 | 256 | 512 | 512 | 512 | 64  | 256 | 4   | 8   | 64  | 32 | 128 | 2   | 128 | 64  | 0.5 | 2   | 64  | 2   |
| Q442    | 512 | 512 | 256 | 512 | 512 | 512 | 512 | 64  | 256 | 4   | 8   | 16  | 0.25 | 1   | 256 | 128 | 4   | 4   | 128 | 4   |
| X122    | 512 | 512 | 256 | 512 | 512 | 512 | 512 | 64  | 256 | 4   | 2   | 8   | 8   | 32 | 0.5 | 128 | 16 | 0.5 | 8   | 8   | 16  | 0.3 |
| G113    | 256 | 256 | >256 | >256 | >256 | 128 | 256 | 128 | 256 | 128 | 256 | 128 | 256 | 1   | 2   | 0.062 | 0.1   | 0.062 | 0.062 | 1   |
| X231    | 256 | 256 | 512 | 512 | 512 | 512 | 256 | >256 | >256 | >256 | 128 | 128 | 256 | 1   | 2   | 0.062 | 0.1   | 0.062 | 0.062 | 1   |

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*<sub>OXA-51-like</sub> detection of the two ABCs was negative, indicating that both G113 and X231 were *A. calcoaceticus*.

### The MICs of *bla*<sub>NDM-1</sub>-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*<sub>NDM-1</sub>-positive bacteria

Except U091 on chromosome and X122 not succeed, the other seven strains all displayed the *bla*<sub>NDM-1</sub> 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*<sub>NDM-1</sub> 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*<sub>NDM-1</sub> 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*<sub>NDM-1</sub> 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*<sub>NDM-1</sub> 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 blaNDM-1-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 blaNDM-1-positive bacteria unconsciously. Hence, the bacteria possessing the blaNDM-1 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 efflux pump mechanism [29]). The most common mechanism of binding protein with low affinity for β-lactams; 3. over expression of 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 efflux pump mechanism [29]).

Our study reported nine blaNDM-1-producing strains in all. Except the U091 strain the blaNDM-1 gene was on chromosome, other eight Southern blot hybridization results showed that blaNDM-1 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

Figure 3: Plasmid profiles and Southern blot results. M:ADNA/HindIII.


