Detection of NDM-7 in Germany, a new variant of the New Delhi metallo-β-lactamase with increased carbapenemase activity

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Objectives: This study characterized a new variant of the New Delhi metallo-β-lactamase (NDM).

Methods: A multidrug-resistant Escherichia coli isolate was recovered from the wounds, throat and rectum of a Yemeni patient who presented at the Frankfurt University Hospital in Germany. The presence of β-lactamase genes was analysed by PCR and sequencing. The isolate was further characterized by susceptibility testing, conjugation and transformation assays and plasmid analysis.

Results: The E. coli isolate was resistant to all β-lactams including carbapenems. By PCR analysis, the β-lactamase genes blaCMY-2, blaCTX-M-15, blaTEM-1 and blaNDM were identified. Sequencing revealed a blaNDM gene that differed from blaNDM-1 by two point mutations at positions 388 (G→A) and 460 (A→C) corresponding to amino acid substitutions Asp130Asn and Met154Leu, respectively. This NDM variant was identified as NDM-7. The blaNDM-7 gene was located on a self-transferable IncX3 plasmid of 60 kb. E. coli TOP10 transformants harbouring NDM-7 showed higher MICs of β-lactams including carbapenems compared with transformants harbouring NDM-1. Multilocus sequence typing analysis revealed that the E. coli isolate belonged to a novel sequence type (ST599).

Conclusions: This study identified a novel NDM variant in E. coli, NDM-7, possessing a high ability to hydrolyse β-lactam antibiotics. Given the diversity of NDM variants located on self-transferable plasmids found in different Gram-negative species and isolated in different countries, the blaNDM gene will most likely efficiently disseminate worldwide.

Keywords: NDM-1, antibiotic resistance, carbapenems, Enterobacteriaceae, IncX3

Introduction

Resistance to β-lactam antibiotics in clinically relevant Gram-negative species can be mediated by different β-lactamases, with metallo-β-lactamases (MBLs) being among the most widespread carbapenemases worldwide. MBL genes are mostly located on plasmids and can be transmitted to other species by horizontal gene transfer. The recently identified New Delhi MBL (NDM) is a carbapenemase conferring resistance to all β-lactam antibiotics (penicillins, cephalosporins and carbapenems) except monobactams.1 NDM enzymes have mainly been described in Enterobacteriaceae but have also been detected in non-fermenting bacteria such as Acinetobacter baumannii and Pseudomonas aeruginosa.2–4 NDM is endemic to the Indian subcontinent but has spread worldwide since its first description in 2008.1,3,4 The rapid global dissemination of NDM-producing multidrug-resistant Gram-negatives and the presence on mobile genetic elements in a broad range of species have established NDM as a major public health threat. Currently, there are seven variants of NDM (NDM-1 to NDM-7) that differ by single amino acid substitutions (http://www.lahey.org/studies/). In the present study, we describe the molecular characterization of the novel NDM-7 variant with increased hydrolytic activity for carbapenems.

Materials and methods

Antimicrobial susceptibility testing and phenotypic and molecular detection of β-lactamase production

Antimicrobial susceptibility was evaluated by determining the MIC using MIC strips (Liofilchem, Roseto degli Abruzzi, Italy) following interpretation guidelines set by EUCAST.9 A double-disc synergy test (DDST) using aztreonam and amoxicillin/clavulanate was employed to screen for extended-spectrum β-lactamases (ESBLs). The carbapenemase activity of isolates was assessed by the modified Hodge test (MHT) using imipenem and meropenem discs. Molecular screening for carbapenemase, ESBL and AmpC resistance genes was done by a microarray approach.
Horizontal gene transfer of blaNDM-7 was evaluated by transconjugation using the sodium azide-resistant strain Escherichia coli J53 as a recipient. Transconjugants were selected using lysogeny broth plates containing sodium azide (100 mg/L) and cefoxitin (30 mg/L). Transformation of electrocompetent P. aeruginosa ATCC 27853 and A. baumannii ATCC 19798 was carried out as previously described. Plasmid DNA was extracted using the Plasmid Midi Prep Kit (Qiagen, Hilden, Germany). Estimation of plasmid size was made by restriction fragment length polymorphism analysis using restriction enzymes BamHI, EcoRI and XhoI and PFGE. Plasmid incompatibility groups were determined by two PCR-based replicon typing (PBRT) methods. Partial sequencing of the blaNDM-7-bearing plasmid of the E. coli isolate was carried out by primer walking.

Cloning of blaNDM-7
Analysis of β-lactam resistance mediated by blaNDM-7 in comparison with blaNDM-1 was investigated by amplifying either the entire open reading frame (ORF) or the complete gene with its native promoter using primers preNDM-for and preNDM-rev as previously described. Purified PCR amplicons were cloned into pCR-Blunt II-TOPO vectors and transformed in E. coli TOP10 (Invitrogen, Darmstadt, Germany).

Molecular typing
Multilocus sequence typing (MLST) of the blaNDM-carrying E. coli isolate using eight housekeeping genes was performed as previously described.

Results and discussion
Isolation and characterization of carbapenem-resistant E. coli
In August 2012, an imipenem-resistant E. coli isolate was recovered from the rectum, throat and infected wounds of a Yemeni patient admitted to the Frankfurt University Hospital, Germany. The patient had initially been hospitalized in three different facilities in Mumbai, India, over the period June to August 2012, where he was treated with the carbapenem faropenem. Susceptibility testing of E. coli revealed resistance to all β-lactams tested, including imipenem and meropenem (Table 1), fluoroquinolones and co-trimoxazole. The isolate remained susceptible to aminoglycosides, fosfomycin, tigecycline and colistin. ESBL nolones and co-trimoxazole. The isolate remained susceptible to all tested, including imipenem and meropenem (Table 1). Fluoroquinolones are not a substrate for MBLs. The NDM-7-carrying transconjugants did not show any resistance other than to β-lactams, indicating that other resistance genes were not located on this plasmid, in contrast to the observation that NDM plasmids usually harbour multiple resistance genes for other antibiotics. Transformation assays using P. aeruginosa ATCC 27853 or A. baumannii ATCC 19798 as recipients failed to generate NDM-7-positive transconjugants, suggesting a plasmid with a narrow host range. An ~60 kb plasmid was detected in both the NDM-7 transconjugants and the clinical E. coli isolate. The blaNDM gene has been shown to be carried by different plasmid types (IncA/C, IncF, IncL/M or non-typeable). Using the current PBRT scheme described by Carattoli et al., the plasmid of the NDM-7 transconjugant was non-typeable. However, PBRT following the method published by Johnson et al. revealed that the plasmid belonged to plasmid type IncX3. Interestingly, this plasmid type is the same as that reported by Ho et al., thereby providing further insights into the acquisition and mobility of this gene.

In order to evaluate and compare the spectrum of β-lactamase activity of NDM-7 with that of NDM-1, the corresponding genes were cloned and transformed into TOP10 E. coli. Two different NDM transfortransformants were generated (Table 1): first, transformants containing the complete ORF of NDM, i.e. E. coli TOP10 (NDM-1) and E. coli TOP10 (NDM-7); and second, transformants also including its native promoter, i.e. E. coli TOP10 (pNDM-7) and E. coli TOP10 (pNDM-1). Expression of the blaNDM-1 and blaNDM-7 genes in E. coli TOP10 conferred reduced susceptibility or resistance towards all β-lactams except aztreonam (Table 1). Interestingly, expression of only the ORF of blaNDM in TOP10 (TOP10 NDM) marginally affected the MICs towards carbapenems as observed by Hornsey et al. In contrast, transformants expressing blaNDM under their native promoter (TOP10 pNDM) showed a marked increase in carbapenem resistance.

Genetic localization, transferability and resistance mediated by blaNDM-7
blaNDM-7 was successfully transferred into E. coli J53 by liquid mating assay, suggesting its location on a plasmid. E. coli J53 transconjugants expressing NDM-7 were resistant to all tested β-lactams except aztreonam (Table 1), in accordance with the fact that aztreonam is not a substrate for MBLs. The NDM-7-carrying transconjugants did not show any resistance other than to β-lactams, indicating that other resistance genes were not located on this plasmid, in contrast to the observation that NDM plasmids usually harbour multiple resistance genes for other antibiotics. Transformation assays using P. aeruginosa ATCC 27853 or A. baumannii ATCC 19798 as recipients failed to generate NDM-7-positive transconjugants, suggesting a plasmid with a narrow host range. An ~60 kb plasmid was detected in both the NDM-7 transconjugants and the clinical E. coli isolate. The blaNDM gene has been shown to be carried by different plasmid types (IncA/C, IncF, IncL/M or non-typeable). Using the current PBRT scheme described by Carattoli et al., the plasmid of the NDM-7 transconjugant was non-typeable. However, PBRT following the method published by Johnson et al. revealed that the plasmid belonged to plasmid type IncX3. Interestingly, this plasmid type is the same as that reported by Ho et al., thereby providing further insights into the acquisition and mobility of this gene.

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Identification and genetic context of the blaNDM-7 gene
Sequencing identified a blaNDM gene that differed from blaNDM-1 by two point mutations at positions 388 (G→A) and 460 (A→C). Analysis of the predicted amino acid sequence revealed two substitutions at positions 130 (Asp→Asn) and 154 (Met→Leu). This NDM variant was identified as blaNDM-7 (http://www.lahey.org/studies/). Sequencing of the close genetic environment of blaNDM-7 showed the presence of two insertion elements upstream of blaNDM-7 (Figure 1). It should be noted that the isolation sequence ISAba125 was disrupted by the insertion of IS5, which results in a truncated transposase. A bloemycin resistance gene and genes encoding a putative phosphoribosylanthranilate isomerase and the oxidoreductase DsbC superfamily protein were identified downstream of blaNDM (Figure 1). A similar genetic environment has been observed in many NDM-positive isolates, often with ISAba125 upstream of blaNDM, but without an IS5 insertion. The genetic context of E. coli NDM-7 most closely resembles that of an NDM-1-positive Klebsiella pneumoniae isolate from China described by Ho et al.

The sequence of blaNDM-7 and its genetic environment has been deposited at the NCBI GenBank under accession no. KC567147.

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**Table 1. β-Lactam MICs for the NDM-carrying E. coli clinical isolate, transconjugants and transformants**

<table>
<thead>
<tr>
<th>MIC (mg/L)</th>
<th>E. coli (NDM-7)</th>
<th>E. coli J53</th>
<th>E. coli transconjugant J53&lt;sub&gt;NDM-7&lt;/sub&gt;</th>
<th>E. coli TOP10 (pNDM-1)</th>
<th>E. coli TOP10 (pNDM-7)</th>
<th>E. coli TOP10 (NDM-1)</th>
<th>E. coli TOP10 (NDM-7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>&gt;256</td>
<td>2</td>
<td>&gt;256</td>
<td>4</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>&gt;256</td>
<td>2</td>
<td>&gt;256</td>
<td>4</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Clavulanate</td>
<td>&gt;256</td>
<td>2</td>
<td>&gt;256</td>
<td>4</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;256</td>
<td>1</td>
<td>&gt;256</td>
<td>1</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tazobactam</td>
<td>&gt;256</td>
<td>4</td>
<td>&gt;256</td>
<td>2</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>&gt;256</td>
<td>0.064</td>
<td>&gt;256</td>
<td>0.064</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ceftoxime</td>
<td>&gt;256</td>
<td>0.032</td>
<td>&gt;256</td>
<td>0.032</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;256</td>
<td>0.064</td>
<td>&gt;256</td>
<td>0.064</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cefepime</td>
<td>&gt;256</td>
<td>0.032</td>
<td>&gt;256</td>
<td>0.032</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ertopenem</td>
<td>&gt;32</td>
<td>32</td>
<td>&gt;32</td>
<td>32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
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<tr>
<td>Meropenem</td>
<td>&gt;32</td>
<td>0.016</td>
<td>&gt;32</td>
<td>0.016</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
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<tr>
<td>Doripenem</td>
<td>8</td>
<td>0.016</td>
<td>4</td>
<td>0.016</td>
<td>4</td>
<td>8</td>
<td>8</td>
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<tr>
<td>Aztreonam</td>
<td>&gt;256</td>
<td>0.032</td>
<td>&gt;256</td>
<td>0.032</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

**Figure 1.** Schematic representation of the genetic environment of NDM-7. A 5.9 kb sequence of the genetic context of the NDM-7-harbouring E. coli is shown. bla<sub>NDM-7</sub> is shown in black. A disrupted insertion sequence ISAba125 (ΔISAba125) results from the insertion of an IS5 element. IS5, insertion sequence 5; ble<sub>MBL</sub>, gene encoding a bleomycin resistance protein; trpF, gene encoding a putative phosphoribosylanthranilate isomerase; dsbC, gene encoding the oxidoreductase DsbC superfamily protein.

MICs (Table 1), indicating that the wild-type promoter contributes significantly to carbapenem resistance.

Since the sequences of the cloned promoter in both the NDM-1 and NDM-7 transformants were identical, the differences in carbapenem MICs are most likely to have been caused by mutations outside the promoter region. Thus, the amino acid changes at positions 130 and 154 have to be responsible for the higher carbapenemase activity, even though they are located outside the active centre. Molecular modelling applying the software tool Molecular Operating Environment (Chemical Computing Group, Montreal, Canada) revealed that the presence of Leu154 in NDM-7 increases the distance to Gln123, which is part of the binding pocket, from 3.90 Å to 4.61 Å (data not shown). It is most likely that Leu154 influences the flexibility of the binding pocket, thereby leading to lower substrate specificity and finally elevated carbapenem MICs.

**MLST**

MLST analysis revealed a novel combination of alleles and thus an as yet undescribed sequence type (ST) that was designated ST599. The closest related STs were ST462 and ST463. In a recent study, ST462 has been shown to cause a significant proportion of ESBL-producing E. coli bacteraemia in a study reported from Israel, but no association with NDM has been described until now.17

**Conclusions**

This study identified a novel NDM-type β-lactamase, NDM-7, possessing a high ability to hydrolyse carbapenems and cephalosporins. The occurrence of new NDM variants in Enterobacteriaceae is of great concern, especially in low-prevalence countries such as Germany. It further illustrates the rapid evolution of the NDM gene and the role of intercontinental travel and healthcare tourism in the global dissemination of multiresistant organisms. Since NDM-expressing Gram-negatives will most likely further disseminate and treatment options are very limited, rigorous screening and the prophylactic isolation of at-risk patients seem vital.

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**Transparency declarations**

T. A. W. has accepted speaking invitations from various pharmaceutical companies, although none poses a conflict of interest with the work presented here. S. G., A. G. H. and S. C. and V. A. J. K. declare that they have no competing interests.

**References**


