Molecular characterization of blaNDM-1 in an Acinetobacter baumannii strain isolated in Germany in 2007

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Objectives: To investigate the genetic environment of the metallo-β-lactamase gene blaNDM-1 in an Acinetobacter baumannii isolated in 2007 in a German hospital.

Methods: Antimicrobial susceptibility testing was performed and resistance genes were characterized by PCR amplification and sequencing. Transferability of β-lactam resistance was tested by broth mating assays and transformation of plasmids. The genetic background of blaNDM-1 was analysed by primer walking. Typing of the A. baumannii strain was performed by repetitive extragenic palindromic sequence-based PCR (rep-PCR) using the DiversiLab system.

Results: The multidrug-resistant A. baumannii isolate harboured β-lactamase genes blaNDM-1 and intrinsic blaOXA-64, but without the insertion sequence ISAba1 often located upstream. Transfer of carbapenem resistance by conjugation and transformation failed. Hybridization of isolated plasmid DNA with blaNDM probes was not successful. Shotgun cloning of whole genomic DNA and sequence analyses revealed that blaNDM-1 was located between two insertion elements of ISAba125. Furthermore, this blaNDM-containing transposon structure was integrated into a chromosomal gene encoding a putative A. baumannii major facilitator superfamily (MFS) metabolite/H+ symporter.

Conclusions: The metallo-β-lactamase gene blaNDM-1 in this A. baumannii strain was integrated in the chromosome on a new transposon structure composed of two copies of insertion sequence ISAba125. The variability of the genetic environment of blaNDM-1 likely facilitates the rapid dissemination of this gene within many Gram-negative bacterial species.

Keywords: multidrug resistance, metallo-β-lactamases, carbapenemases, transposons, DiversiLab

Introduction

In recent years an increasing number of reports on the emergence of multidrug-resistant Gram-negative pathogens have been published and in particular it is the emergence of blaNDM-1 that has created the most concern. Indeed, since its first description in 2009, this metallo-β-lactamase (MBL) has now been found worldwide. NDM-1 has been identified in various Enterobacteriaceae due to localization of blaNDM-1 on conjugative plasmids, enabling transfer and rapid dissemination of multidrug resistance. In 2010 blaNDM-1 and the related blaNDM-2 gene were also found in Acinetobacter baumannii, whereby either plasmid transfer of these genes was confirmed or the genetic location was not further investigated in detail. Infections with carbapenemase-producing A. baumannii are a serious threat because colistin is often the only treatment option. Carbapenem resistance in A. baumannii is mainly due to expression of various OXA β-lactamases, such as OXA-23, OXA-58, OXA-40 and OXA-143-related enzymes, as well as overexpression of the intrinsic OXA-51-like enzyme. Non-OXA-mediated carbapenem resistance is still rare. Here we report the molecular characterization of the genetic environment of blaNDM-1 detected in a clinical A. baumannii strain from a German hospital.

Materials and methods

Bacterial strains
A. baumannii strain 161/07 was isolated from a patient who had been repatriated to Germany from Serbia in 2007. The case history surrounding

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this bla<sub>NDM-1</sub>-positive A. baumannii strain 161/07 has been described previously. Standard A. baumannii ATCC 19606 and ATCC 17978 were used as recipients for plasmid transformation. Sodium azide-resistant Escherichia coli J53 was used as the recipient for transformation and broth mating assays.

**Antimicrobial susceptibility**

Antimicrobial drug susceptibilities were determined according to the guidelines of the CLSI by broth microdilution (Table S1, available as Supplementary data at JAC Online) and Etest (bioMérieux, Nürtingen, Germany).<sup>10</sup> The MBL phenotype was confirmed with MBL-Etest and a combined disc diffusion test CDDT (KPC-4-MBL Confirm ID Kit; Alere GmbH, Cologne, Germany).

**Presence of carbapenem resistance genes**

Detection of OXA-type carbapenemases and associated insertion elements was performed by PCR and sequence analyses were performed as previously described.<sup>11,12</sup> Detection of the bla<sub>NDM-1</sub> gene was performed by PCR using primers ndm1_F (5′-CTGAGACCCGCTATGACC-3′) and ndm_1_R (5′-GGGCGCTATGAGTGTAGTC-3′). For detection of other common bla<sub>OXA</sub> genes (bla<sub>OXA-2</sub>, bla<sub>OXA-10</sub>), the following primers were used: oxa1_ F (5′-TATCTACAGCCGCGCCATG-3′); oxa1_ R (5′-TAAATTGCGCCAAGTTTCTC-3′); oxa2_ F (5′-GCAAAGAAGGGCAGCTAGAC-3′); oxa2_ R (5′-CAGCGTCGAGTGTACGTC-3′); oxa9_ F (5′-TTTGGCTGTGCTATGGTG-3′); oxa9_ R (5′-CCATCAACAGGTGTAATC-3′); oxa10_ F (5′-TTCGAGTACGGCCATTAGG-3′); and oxa10_ R (5′-CAATGGCCCTCATCTTCC-3′).

**Molecular typing**

Molecular typing was performed by rep-PCR using the DiversiLab system (bioMérieux) and results were compared with our database of worldwide clonal lineages.<sup>9</sup> In addition, multiplex PCR-based typing in combination with ApaI PFGE was performed.<sup>13</sup>

**Characterization of bla<sub>NDM-1</sub>**

Transfer of β-lactam resistance was tested by broth mating assays with E. coli J53 as the recipient. Selection of transconjugants was performed on Mueller–Hinton agar plates that contained sodium azide (200 mg/L) and ampicillin (100 mg/L). Plasmid DNA was isolated using the QIAgen Plasmid Mini Kit (QIAgen, Hilden, Germany). Transformation of plasmids into an electrocompetent E. coli J53 and A. baumannii ATCC 19606 and ATCC 17978 recipients was performed using standard procedures.<sup>14</sup> Plasmid size was determined by performing S1 nuclease restriction of whole genomic DNA combined with PFGE.<sup>15</sup>

**Results and discussion**

Strain typing by rep-PCR (DiversiLab) revealed that the NDM-1-producing multidrug-resistant A. baumannii strain 161/07 clustered with isolates of the clonal lineage WW7. Previous work has shown that A. baumannii strains belonging to the WW7 cluster harboured the carbapenemase genes bla<sub>OXA-58</sub> or bla<sub>OXA-23</sub> and were from different countries in South America, Europe and Asia.<sup>9</sup> Furthermore, all A. baumannii of the WW7 cluster harboured bla<sub>OXA-64</sub>, a variant of the intrinsic bla<sub>OXA-51</sub>.<sup>17</sup> It was confirmed by PCR that A. baumannii 161/07 was positive for the chromosomally located bla<sub>OXA-64</sub>, but this gene was not associated with insertion element ISAbA1. Other bla<sub>OXA</sub> genes were not detected. PCR-based A. baumannii typing in combination with ApaI PFGE analysis confirmed that A. baumannii 161/07 was not related to European clonal lineages 1–3.<sup>13</sup>

Transfer of resistance genes by in vitro conjugation and transformation of plasmids into E. coli or A. baumannii recipients was not successful. Analysis of S1-digested DNA fragments revealed the presence of two plasmids (125 kb, 75 kb) in the NDM-1-producing A. baumannii 161/07 strain. However, hybridization signals with a bla<sub>NDM-1</sub>-probe were not detected for these plasmids, and PCR with bla<sub>NDM-1</sub>-specific primers failed to amplify a PCR product, suggesting a chromosomal location of bla<sub>NDM-1</sub>.

Using naked whole genomic DNA from A. baumannii 161/07 to transform the naturally competent ampicillin-susceptible A. baumannii recipient 102/07, the gene bla<sub>NDM-1</sub> was successfully transferred, as confirmed by PCR. All β-lactams tested against the transformant 161/07–102 showed an increase in MIC, with imipenem and meropenem MICs increasing from 0.25 mg/L to &gt;32 mg/L, and 0.5 mg/L to &gt;32 mg/L, respectively (Table S1). No other class of antibiotic was affected.

Sequence analysis of shotgun cloned A. baumannii 161/07 genomic DNA revealed a 3.9 kb insert containing the bla<sub>NDM-1</sub> gene and parts of the plasmid sequence pkpANDM-1 described previously (Figure 1C).<sup>3</sup> However, the IS26 transposase upstream of bla<sub>NDM-1</sub> in previously characterized plasmids pkpANDM-1 and pNDM-HK was not present in A. baumannii 161/07. Instead, the insertion sequence ISAba125 was identified (Figure 1B). Interestingly, pkpANDM-1 includes a partial sequence of ISAba125 adjacent to bla<sub>NDM-1</sub>. BLAST analysis of ISAba125 revealed seven copies of ISAba125 on the chromosome of A. baumannii strain AC1675 in the GenBank database, and ISAba125 was found disrupting the carO gene, leading to carbapenem resistance.<sup>18</sup>

Furthermore, ISAba125 can also be plasmid located, and has been described recently upstream of the bla<sub>OXA-58</sub> gene.<sup>19</sup>

To determine whether bla<sub>NDM-1</sub> was located in a transposon, PCR was performed with inverse primers to ISAba125 (TRANSIS_R (5′-AAACAACGGATGCCTCAAC-3′) and TRANSIS_F (5′-CGAGCAT TACCAAGGGTG-3′)) using genomic DNA of A. baumannii 161/07 as template. Two products of 2 kb and 9 kb were amplified. Sequencing of the 2 kb amplicon revealed aphA-6, an

**Nucleotide sequence accession number**

The nucleotide and protein sequences of the bla<sub>NDM-1</sub>-containing transposon and gene aphA-6 have been registered in GenBank under accession numbers HQ857107 and JF949760, respectively.
aminoglycoside resistance gene bracketed between two copies of ISAba125.

Primer walking from both ends of the 9 kb amplicon revealed a composite transposon structure containing blaNDM-1 bracketed between two copies of ISAba125 (Figure 1B). Both copies of ISAba125 were flanked by 17 bp inverted repeats. Both 5' inverted repeats and both 3' inverted repeats were identical, respectively, but there were two nucleotide differences between 5' and 3' inverted repeats. The two ISAba125 transposase genes differed by four nucleotides whereby one resulted in amino acid substitution, Arg-41 → Gln. Including both ISAba125 insertion elements, the composite transposon was 10093 bp in length. Furthermore, it was integrated into a chromosomal gene encoding a putative A. baumannii major facilitator superfamily (MFS) metabolite/H+ symporter (Figure 1A) that has previously been disrupted by ISAba125-blanoxASA-23.20 Further PCR and sequencing with primers for ISAba125 and the disrupted MFS transporter confirmed the chromosomal location of the blanoxASA-1-containing transposon. Evidence of this being a transposition event was an 8 bp target site duplication at the point of insertion in the gene encoding MFS (Figure 1E). Adjacent to the MFS gene we identified a chromosomal homoserine lactone synthase gene (cepI). In addition, inside the blanoxASA-1-containing transposon there was a 4 kb element 93% similar to that described in E. coli plasmids pEH4H and pAR060302, encoding the chaperonin subunits groS and groL, and the transposase insE, which was not associated with inverted repeats (Figure 1D). The 8 bp target site duplication was also present at the 5'-end of the insE gene (Figure 1E). Further BLAST analysis revealed a 297 bp sequence, including 41 bp of the 3'-end of insE and extending towards ISAba125, that showed 99% identity to insertion sequence ISCR19-like and oriIS previously described in a Pseudomonas aeruginosa isolate.13 Deletions were found in gene trpF (79 bp, 3'-end) and gene groS (154 bp, 5'-end). No significant DNA homology or open reading frames were detected in the 1.6 kb region between the truncated trpF and groS genes.

In conclusion, analysis of the genetic environment of blanoxASA-1 in A. baumannii 161/07 revealed a transposon structure composed of two copies of insertion sequence ISAba125 that is integrated into the bacterial chromosome. However, since blanoxASA-1 is flanked by these insertion elements, we cannot discount the possibility of integration into a plasmid and subsequent horizontal spread. The variability of the genetic environment of blanoxASA-1, as evidenced by the transposon structure described here, with DNA seemingly originating from Enterobacteriaceae, P. aeruginosa and A. baumannii, may explain the observed rapid dissemination of this gene within many Gram-negative bacterial species and across genera. Thus there is an urgent need for further investigations to find the origin of this gene and its mechanisms of spread.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References

Figure 1. Schematic diagram showing the genetic environment of blanoxASA-1 in A. baumannii 161/07. (A) Chromosomal genes cepI and the truncated mfs; (B) ISAba125; (C) blanoxASA-1 and truncated trpF; (D) truncated chaperonin subunit groS, chaperonin groL and the transposase insE; (E) 8 bp target duplication.