Detection of the carbapenemase GIM-1 in Enterobacter cloacae in Germany

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Objectives: To characterize the mechanisms involved in reduced susceptibility to carbapenems in two Enterobacter cloacae clinical isolates.

Methods: Two E. cloacae isolates recovered from different regions in Germany and showing reduced susceptibility to carbapenems were analysed. Susceptibility testing, conjugation, transformation assays, plasmid analysis, sequencing and molecular typing using rep-PCR were performed.

Results: The two clinical isolates carried the blaGIM-1 gene and showed resistance to ertapenem, with variable MIC values of imipenem and meropenem. The isolates were clonally unrelated. The blaGIM-1 gene was located on self-transferable and non-typeable plasmids. Both isolates harboured distinct plasmids and integron structures containing the blaGIM-1 gene cassette. Interestingly, one of the two plasmids was able to replicate in Pseudomonas aeruginosa, demonstrating its broad host range.

Conclusions: This is the first identification in E. cloacae of the blaGIM-1 gene, which is responsible for reduced susceptibility to carbapenems. We showed that this gene, previously identified in P. aeruginosa, was located in a different genetic background in E. cloacae. The blaGIM-1 gene might spread quite efficiently in Enterobacteriaceae and P. aeruginosa, as it is difficult to detect and in addition is located on conjugative plasmids.

Keywords: Enterobacteriaceae, resistance, β-lactams, carbapenems

Introduction

GIM-1 (German imipenemase) is a carbapenemase that was first identified in five clonally related Pseudomonas aeruginosa clinical isolates recovered in North Rhine-Westfalia, Germany, in 2004.1 Five other GIM-1-producing P. aeruginosa isolates were then reported in the same region in 2012.2 GIM-1 is an Ambler class B metallo-β-lactamase (MBL) that is distantly related to other acquired MBLs, with the most closely related being DIM-1 (55% amino acid identity). The blaGIM-1 gene was found to be located on plasmids, however, their transferability (either by conjugation or by transformation) has not been demonstrated.1,2,3 The blaGIM-1 gene cassette was found to be embedded in a class 1 integron structure, together with the β-lactamase gene blaOXA-2, and the aminoglycoside resistance genes aacA4 and adaA1.1 Whereas other MBL genes such as blaIMP, blaVIM, and blaNDM have been found either in P. aeruginosa or Enterobacteriaceae, the occurrence of blaGIM-1 had only been described in P. aeruginosa and then recently in a single Serratia marcescens isolate.3

We report here the identification of blaGIM-1 in two Enterobacter cloacae isolates and describe its plasmid and genetic context.

Materials and methods

Bacterial isolates

The first isolate was obtained in 2011 from a 78-year-old man who was admitted to the University Hospital of Cologne for the treatment of a partial middle cerebral artery infarction. The patient had a urethral infection that grew E. cloacae 1 that was resistant to ertapenem and meropenem. The infection subsided after local antiseptic treatment without antibiotic therapy. The second isolate was obtained in 2012 from a 78-year-old patient who had been admitted for the treatment of a
myocardial infarction at the University Hospital Frankfurt. *E. cloacae* 2 showing reduced susceptibility to ertapenem was recovered from a rectal swab taken for surveillance purposes. *P. aeruginosa* 7312198 harbouring the *bla*<sub>GIM-1</sub> gene, kindly provided by T. Walsh, Cardiff University, UK, was used as a reference strain.

**Susceptibility, phenotypic and molecular testing for **β**-lactamase production**

Production of extended-spectrum β-lactamases (ESBLs) was investigated by a double disc synergy test (DDST) using ticarcillin/clavulanate and aztreonam (which is not a substrate of GIM-1). Production of carbapenemases was evidenced by using the Carba NP test as described. MBL production was investigated by a DDST using a meropenem disc and a blank disc impregnated with 10 μL 0.1 M EDTA. Detection of the *bla*<sub>IMP</sub>, *bla*<sub>GIM-1</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub>-48 and *bla*<sub>OXA</sub>-23 carbapenemase genes was performed by PCR, followed by sequencing. A similar approach was used to search for the ESBL genes *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, and for the class D β-lactamase genes *bla*<sub>OXA-1</sub> and *bla*<sub>OXA-2</sub>. Susceptibility testing by agar diffusion was performed and interpreted according to CLSI guidelines. MICs of carbapenemases were evaluated by Etest (bioMérieux, La Balme-les-Grottes, France) and interpreted according to the CLSI; colistin MIC was interpreted according to EUCAST in the absence of a CLSI breakpoint.

**Clonal relatedness and outer membrane analysis**

The clonal relationship of the two *E. cloacae* isolates was investigated by rep-PCR using the DiversiLab system dedicated for Enterobacter (bioMérieux, La Balme-les-Grottes, France) following the manufacturer’s recommendations. The outer membrane protein (OMP) profiles of the two *E. cloacae* isolates were analysed to explain the higher carbapenem resistance level observed for isolate 1. OMP studies were performed using SDS–PAGE as described previously.

**Conjugation and transformation assays**

The transferability of the *bla*<sub>GIM-1</sub> gene was evaluated by a mating-out assay using sodium azide-resistant *E. coli* J53 as recipient as described previously. Transconjugants were obtained after plating on tryptic soy agar containing sodium azide (100 mg/L) and cefoxitin (30 mg/L) as selective agents. Transformation of the plasmid from isolate 1 into *P. aeruginosa* strain PU21 was done by electroporation as described. Transformants were selected on ticarcillin (100 mg/L)-containing agar plates.

**Plasmid analysis**

Plasmid DNA was extracted using the Plasmid Maxi Prep Kit and the Large Construct Kit (Qiagen, Hilden, Germany). The estimation of plasmid size was done by S1 nuclease treatment of plasmid DNA and agarose gel electrophoresis. Restriction fragment length polymorphism (RFLP) analysis of plasmids was performed using restriction enzymes KpnI, ApaI, HindIII, BamHI and NotI. Partial sequencing of the *bla*<sub>GIM-1</sub>-bearing natural plasmid of isolate 1 was done by primer walking. Plasmid compatibility groups were determined by a PCR-based replicon typing (PBRT) method.

**Results**

**Susceptibility, molecular and phenotypic testing for **β**-lactamases**

Both isolates were resistant to ceftazidime and ertapenem, but remained susceptible to imipenem, cefepime and aztreonam (Table 1), and to the non-β-lactam antibiotics amikacin, gentamicin, fosfomycin, and colistin. Isolate 2 was additionally susceptible to meropenem, ciprofloxacin and co-trimoxazole. In isolate 1, the MICs of ertapenem and meropenem, but also of some other β-lactams, were higher than in isolate 2 (Table 1). This discrepancy was probably the result of two features of isolate 1: the production of β-lactamase OXA-2 in combination with a decreased outer membrane permeability linked to the loss of an OMP (data not shown).

ESBL testing by DDST was negative for both isolates, and PCR analyses confirmed the absence of ESBL-encoding genes. In contrast, modified Hodge test (MHT) and DDST with meropenem and EDTA was positive for both isolates, thus strongly suggesting production of an MBL. PCR screening for carbapenemase genes identified the *bla*<sub>GIM-1</sub> gene in both isolates, which was confirmed by subsequent DNA sequencing. Molecular typing by rep-PCR showed that the two isolates had a similarity of only 75.9%, thereby showing that they were non-clonal.

**Plasmid background and genetic context of the **bla**GIM-1**

Analysis of the plasmid content of the two *E. cloacae* isolates revealed a plasmid of ~24 kb in isolate 1 and of >50 kb in isolate 2. Both plasmids were self-transferable to *E. coli* J53 by conjugation. Interestingly, the 24 kb plasmid was successfully transferred and replicated in *P. aeruginosa* PU21, but the same experiment failed with the plasmid extract obtained from isolate 2.

*E. coli* J53 transconjugants expressing GIM-1 showed a 2-fold increase in MICs of imipenem and meropenem, and a >60-fold MIC increase for ertapenem (Table 1). The MIC of aztreonam was not modified, in accordance with the lack of hydrolysis of that substrate by GIM-1. MICs of the *P. aeruginosa* transformant (TF PU21) were significantly higher than those of *E. coli* J53 transconjugants. As expected, this transformant exhibited resistance to carbapenems, but noticeably the MICs of cefepime and cefpiramide were considerably increased, with only the aztreonam MIC remaining unchanged (Table 1). These discrepancies with what was observed in *E. coli* might be explained by the intrinsic weak permeability of *P. aeruginosa* for β-lactams playing a synergistic role with the MBL production.

PBRT analysis did not give any positive amplicon for the two *E. coli* transconjugants, thus suggesting that the plasmid carrying the *bla*<sub>GIM-1</sub> gene did not correspond to the common and widespread plasmid types usually spreading antibiotic resistance genes among Enterobacteriaceae. RFLP analysis of the two plasmids that had a similar size (isolate 1 and *P. aeruginosa* 7312198) showed that the plasmid structures were different, thus ruling out any relationship between them.

**Genetic context of the **bla**GIM-1**

Sequencing of the close genetic environment of *bla*<sub>GIM-1</sub> revealed a class 1 integron structure. In isolate 1, the class 1 integron contained four gene cassettes, namely *bla*<sub>GIM-1</sub>, *aac*<sub>A4</sub>, *aad*A1 and *bla*<sub>OXA-2</sub>, similar to the integron previously identified in *P. aeruginosa* (Figure 1). Interestingly, insertion sequence IS1394

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**JAC**

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identified inside the integron in *P. aeruginosa* was absent here. In isolate 2, the *bla*GIM-1 gene was also embedded in a class 1 integron structure, but contained only an additional gene cassette downstream of *bla*GIM-1, namely *aadB*, encoding resistance to aminoglycosides (Figure 1).

**Table 1.** MICs of β-lactams for *E. cloacae* isolates and recipient strains *E. coli* J53 and *P. aeruginosa* PU21

<table>
<thead>
<tr>
<th>β-Lactam(s)</th>
<th>E. coli J53</th>
<th><em>E. cloacae</em> isolate 1</th>
<th>E. coli J53 isolate 1</th>
<th><em>E. cloacae</em> isolate 2</th>
<th>E. coli J53 isolate 2</th>
<th><em>P. aeruginosa</em> PU21</th>
<th><em>P. aeruginosa</em> TF PU21 isolate 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>4</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Amoxicillin/clavulanate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>&gt;256</td>
<td>128</td>
<td>&gt;256</td>
<td>128</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>1</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>1</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>128</td>
<td>32</td>
<td>4</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.06</td>
<td>&gt;256</td>
<td>16</td>
<td>32</td>
<td>16</td>
<td>2</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.03</td>
<td>&gt;256</td>
<td>256</td>
<td>64</td>
<td>8</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Cefepime</td>
<td>0.03</td>
<td>2</td>
<td>0.12</td>
<td>0.25</td>
<td>0.12</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Cefpirome</td>
<td>0.03</td>
<td>4</td>
<td>0.25</td>
<td>1.5</td>
<td>0.25</td>
<td>4</td>
<td>128</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0.03</td>
<td>2</td>
<td>0.03</td>
<td>0.25</td>
<td>0.03</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.25</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>0.004</td>
<td>&gt;32</td>
<td>1</td>
<td>2</td>
<td>0.25</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.25</td>
<td>8</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Doripenem</td>
<td>0.016</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.06</td>
<td>0.25</td>
<td>&gt;32</td>
</tr>
</tbody>
</table>

TC, transconjugant; TF, transformant; NT, not tested (intrinsic resistance).

<sup>a</sup>Clavulanic acid at a fixed concentration of 4 mg/L.

**Figure 1.** Class 1 integron structures identified in the *bla*GIM-1-carrying isolates: (a) isolate 1; (b) isolate 2; (c) *P. aeruginosa* reference strain.

Discussion

This study constitutes the first report of the *bla*GIM-1 gene in *E. cloacae*. This gene was located on different self-transferable plasmids. One plasmid was ~24 kb in size, which is similar to that reported in *P. aeruginosa* (~22 kb).<sup>1</sup> That plasmid harboured a very similar integron structure, and replicated in *P. aeruginosa*, thus suggesting that such a broad-host-range plasmid could be the vehicle responsible for spreading the *bla*GIM-1 gene among distantly related Gram-negative species. In contrast, the second isolate harboured a different type of plasmid, probably of narrow host range.

Interestingly, while this work was in progress, a *bla*GIM-1-positive *Serratia marcescens* isolate was identified in North Rhine-Westfalia,<sup>3</sup> thus highlighting the current emergence of this carbapenemase in Enterobacteriaceae at least in that region. In that latter strain, the integron structure was identical to the one we identified in isolate 1. In contrast to what has been hypothesized by Rieber et al.,<sup>3</sup> we do not consider those plasmids found in Enterobacteriaceae to directly originate from *P. aeruginosa*. If this was the case, one identical plasmid would be expected to be present in all strains. Rather, we speculate that the *bla*GIM-1 gene cassette has successfully targeted different class 1 integrons located on different plasmid scaffolds.

The isolation of two unrelated *E. cloacae* strains from different parts of Germany indicates that GIM-1 is no longer confined to a small geographical area as previously thought. Considering that the laboratory recognition of GIM-positive isolates as carbapenemase producers is challenging (especially when ertapenem is not routinely tested), the *bla*GIM-1 gene might be more widespread among Enterobacteriaceae than currently known.
Funding

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Transparency declarations

None to declare.

References