Sources of diversity of carbapenem resistance levels in *Klebsiella pneumoniae* carrying *bla*<sub>VIM-1</sub>

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**Objectives:** To elucidate the mechanisms responsible for the diversity of β-lactam resistance phenotypes among isolates of a VIM-1-producing *Klebsiella pneumoniae* (VPKP) strain that is endemic in Greek hospitals.

**Methods:** Five VPKP clinical isolates were studied. MICs of β-lactams were determined by agar dilution. PFGE of *Xba*I-digested genomic DNA was used for typing. Profiles of outer membrane proteins (OMPs) were determined by SDS–PAGE. Selected isolates were transformed with a plasmid encoding the Omp36K porin. β-Lactamase activities were analysed by IEF and imipenem hydrolysis was assessed by spectrophotometry. VIM-1-encoding, self-transmissible plasmids were characterized by replicon typing, RFLP and hybridization with *bla*<sub>VIM</sub>- and IS<sub>26</sub>-specific probes. Characterization of integrons was performed by PCR, cloning and sequencing.

**Results:** Isolates exhibited highly similar PFGE patterns. Imipenem MICs were 2, 4, 16, 32 and 64 mg/L. The isolate with the highest imipenem MIC (Vipm-64) lacked a 36 kDa OMP. Expression of a cloned OmpK36 in this isolate reduced the imipenem MIC to susceptibility levels. Imipenem-hydrolysing activity was significantly higher in Vipm-16 as compared with the other isolates that expressed similar amounts of VIM-1. All isolates transferred β-lactam resistance to *Escherichia coli* through conjugative, IncN plasmids that exhibited differences in the RFLP and hybridization patterns with *bla*<sub>VIM</sub>- and IS<sub>26</sub>-specific probes. The Vipm-16 plasmid, mediating the higher imipenem MICs among transconjugants, carried two copies of *bla*<sub>VIM-1</sub>. Cloning and sequencing showed in-e541-like integrons truncated at the 5′CS by insertion of IS<sub>26</sub> elements at two different positions.

**Conclusions:** A VIM-1-producing strain of *K. pneumoniae* has evolved through OMP alterations and rearrangements in the *bla*<sub>VIM-1</sub>-carrying plasmid probably mediated by IS<sub>26</sub>, generating isolates with imipenem MICs ranging from susceptibility to resistance.

**Keywords:** *K. pneumoniae*, metallo-β-lactamases, β-lactam resistance

**Introduction**

Various enterobacterial species carrying metallo-β-lactamase (MBL) genes of the VIM and IMP types have been described mostly in southern Europe and the Far East. VIM-1-producing *Klebsiella pneumoniae* (VPKP) strains have been established in Greek hospitals and have also spread to other European countries via colonized patients. Previous studies indicated a complex situation involving apparently different strains and plasmids as well as clonal outbreaks. Typing by PFGE of VPKP isolates systematically collected in the major teaching hospitals in Athens since 2003, however, has indicated a wide spread of a single VPKP strain (V. Miriagou, unpublished data). While the majority of isolates of this endemic strain appear susceptible to carbapenems according to the current CLSI criteria, isolates with imipenem MICs ≥16 mg/L and varying susceptibilities to...
other β-lactams such as cefepime have been noted. In the present study we attempted to elucidate the mechanisms responsible for this diversity of β-lactam resistance phenotypes.

Materials and methods

Strains and plasmids

Five VPKP isolates from the collection (2003–2005) of the National School of Public Health exhibiting a wide range of imipenem MICs (2–64 mg/L) were studied. MICs of β-lactams were determined by an agar dilution technique as recommended by the CLSI. Isolates were typed by PFGE of XbaI-restricted genomic DNA as described previously. An Escherichia coli K12 strain resistant to rifampicin was used as recipient in conjugation experiments. E. coli XL-1 Blue was used as host for recombinant plasmids. The pBCSK(+) phagemid (Stratagene, La Jolla, CA, USA) was utilized as a cloning vector. Plasmid pSHA2, a pACYC84 derivative encoding the porin OmpK36, was used to transform VPKP isolates by electroporation. Plasmid-containing clones were selected with potassium tellurite (30 mg/L).

Outer membrane proteins (OMPs)

OMP preparations were obtained after sonication of bacterial cells grown in nutrient broth followed by selective solubilization of cytoplasmic material with sodium N-lauroyl sarcosinate (2% w/v) and ultracentrifugation. The preparations were run on discontinuous SDS-polyacrylamide gels (11%) and stained with Coomassie Blue.

Characterization of plasmids

Conjugal transfer of VIM-1-encoding plasmids was performed in mixed broth cultures. Transconjugants were selected in medium containing rifampicin (300 mg/L) plus ampicillin (40 mg/L). Plasmids were purified using a commercial kit (Qiagen Inc., Valencia, CA, USA). Replicon typing was performed by a PCR-based method. Plasmid pVipm-64 was used to transform VPKP isolates by electroporation. Plasmid-containing clones were selected with potassium tellurite (30 mg/L).

β-Lactamase studies

Cell-free extracts were obtained by ultrasonic treatment of bacterial cultures. Extracts were clarified by ultracentrifugation and analysed by isoelectric focusing (IEF) in polyacrylamide gels (pH range 3.5–9.5). Imipenem hydrolytic activity of the β-lactamase extracts was determined by spectrophotometry essentially as described previously in the presence of Zn2+ (50 μM) and expressed as units (U) (1 U was the amount of enzyme hydrolysing 1 nmol of substrate per min per mg of protein).7

PCR assays, cloning and sequencing

blaVIM-1 and blaðSHV-5 genes were detected by PCR as described previously. Mapping of VIM-1-encoding integrons was performed by PCR using primers based on the sequence of In-e541 (GenBank accession no. AY339625). Plasmid-generated fragments of plasmids were cloned into pBCSK(+). Recombinant plasmids were used to transform E. coli XL-1 Blue cells. Nucleotide sequences of blaVIM-1 carrying inserts as well as selected PCR products were determined on both strands with an ABI Prism 377 sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA).

Nucleotide sequence accession numbers

The described nucleotide sequences have been assigned accession numbers AY339625 and DQ489717 in the EMBL-GenBank database.

Results and discussion

Genetic relatedness of the studied isolates was documented by PFGE (not shown). blaVIM-1 carriage was confirmed by PCR and sequencing. IEF experiments indicated production of the VIM-1 MBL with an isoelectric point of 5.3 by all isolates. Isolate Vipm-32 also expressed an SHV-5-like β-lactamase focusing at a pH of 8.2. Identity of the respective β-lactamase gene was confirmed by PCR and sequencing. The isolates were resistant to penicillins, penicillin/inhibitor combinations, cefotaxime and ceftazidime (MICs > 128 mg/L). MICs of imipenem, meropenem and cefepime varied (2–64, 0.5–64 and 16–128 mg/L, respectively). Aztreonam was active against all isolates apart from Vipm-32 due to SHV-5 production (Table 1).

Electrophoresis of OMPs showed that all preparations contained a protein with a MW of 32 kDa that was probably OmpA. Protein bands with apparent MWs corresponding to the main porins of K. pneumoniae were seen in the OMP preparations from Vipm-2 (36 kDa), Vipm-4 (36 kDa), Vipm-16 (36 kDa) and Vipm-32 (35 kDa). Isolate Vipm-64 did not produce detectable amounts of OMPs in this MW range (Figure 1, part a). Transformation of Vipm-64 with pSHA2 and expression of the cloned porin, as confirmed by SDS–PAGE, caused a significant reduction in the MICs of carbapenems and cefepime (Table 1). It was not possible to obtain stable pSHA2 transformants from Vipm-32.

Imipenem hydrolytic activity was similar in the extracts derived from four VPKP isolates, including two of the imipenem-resistant ones, Vipm-64 and Vipm-32. Isolate Vipm-16, however, exhibited significantly higher carbapenemase activity (Table 1). VIM-1 production was readily transferred by all VPKP isolates to E. coli by conjugation at high frequency (10–4–10–5 per donor cell). β-Lactam resistance phenotypes of the transconjugants were characteristic of MBL-producing enterobacteria but the range of carbapenem MICs was narrower than that of the donors. The higher carbapenem and cefepime MICs were observed among transconjugants derived from Vipm-16 (Table 1). VIM-1 encoding plasmids were 50–60 kb in size and, as determined by replicon typing, belonged to the incompatibility group IncN. Also, PCR mapping showed that these plasmids all carried integrons with variable regions similar to that of In-e541, including, from 5’ to 3’, the blaVIM-1, aacA7, dfrA1, ada and sul1 gene cassettes (from nt 8936 to 13 121 in AY339625, GenBank). However, digestion of purified plasmids with PstI produced three RFLP types, I (plasmids from Vipm-2, Vipm-32 and Vipm-64), II (Vipm-4) and III (Vipm-16) (Figure 1, part b1). Hybridization with a blaVIM-1 specific probe occurred in PstI fragments of ~5.7 kb (type I) and 5.0 kb (type II). In the RFLP type III plasmid, hybridization occurred in both fragments (Figure 1, part b2). Cloning of PstI digests from pVipm-64, as a RFLP type I representative, resulted in recombinant plasmids with a blaVIM-1-containing insert of 5638 bp in which an IS26 element was inserted in the 5’ end of In-e541, 120 bp downstream from the stop codon of intI.
In contrast, an In-e541-like sequence of 4988 bp in which an IS\textsubscript{26} was inserted within the coding sequence of int\textsubscript{I} deleting 570 bp from the 3' end was found in recombinants from pVipm-4 (RFLP type II). Finally, sequencing of recombinant plasmids derived from pVipm-16 (RFLP type III) showed that the latter plasmid carried both these IS\textsubscript{26}-associated In-e541-like sequences. These experiments confirmed dual carriage of \textit{bla}\textsubscript{VIM-1} in two In-e541 integrons, differently modified by IS\textsubscript{26}, in pVipm-16.

Given the two IS\textsubscript{26} copies in this plasmid, we investigated the possibility that more copies of this element might be present on the plasmid backbone. Indeed, hybridization with an IS\textsubscript{26}-specific probe confirmed the presence of multiple copies of this element in all three plasmid variants (Figure 1, part b3). In addition, it was evident that at least five IS\textsubscript{26}-hybridizing fragments of indistinguishable MWs were present in all three plasmid variants, suggesting that the remaining variant-specific fragments may have arisen from IS\textsubscript{26} activity.

The presence of \textit{bla}\textsubscript{VIM-1} plasmid variants of the same incompatibility group, generated by IS\textsubscript{26} activity in a putative common progenitor, with harbouring isolates of highly related PFGE patterns, suggests evolution from a single VPKP progenitor, with respect to both chromosomal and plasmid DNA. Nevertheless, this genetic diversity only partly explained the phenotypic diversity observed. Indeed, though the amount of \(\beta\)-lactamase is expected to significantly influence resistance to its respective substrates, a straightforward association between \textit{carbapenemase} activity and imipenem MICs could not be established for all isolates. Nevertheless, imipenem resistance

### Table 1. Susceptibility to \(\beta\)-lactam antibiotics and \(\beta\)-lactamase content of five \textit{K. pneumoniae} clinical isolates producing VIM-1 (Vipm), the respective \textit{E. coli} transconjugants and a \textit{K. pneumoniae} isolate transformed with the porin-encoding plasmid pSHA2

<table>
<thead>
<tr>
<th>Strain</th>
<th>CAZ</th>
<th>IPM</th>
<th>MEM</th>
<th>FEP</th>
<th>ATM</th>
<th>(\beta)-Lactamases</th>
<th>Carbenapenase activity (U)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vipm-2</td>
<td>&gt;128</td>
<td>2</td>
<td>0.5</td>
<td>32</td>
<td>0.25</td>
<td>VIM-1</td>
<td>49.0</td>
</tr>
<tr>
<td>\textit{E. coli} (pVipm-2)</td>
<td>&gt;128</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>&lt;0.12</td>
<td>VIM-1</td>
<td>51.5</td>
</tr>
<tr>
<td>Vipm-4</td>
<td>&gt;128</td>
<td>4</td>
<td>1</td>
<td>16</td>
<td>0.12</td>
<td>VIM-1</td>
<td>78.1</td>
</tr>
<tr>
<td>\textit{E. coli} (pVipm-4)</td>
<td>&gt;128</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>&lt;0.12</td>
<td>VIM-1</td>
<td>45.2</td>
</tr>
<tr>
<td>Vipm-16</td>
<td>&gt;128</td>
<td>16</td>
<td>4</td>
<td>64</td>
<td>0.5</td>
<td>VIM-1</td>
<td>78.1</td>
</tr>
<tr>
<td>\textit{E. coli} (pVipm-16)</td>
<td>&gt;128</td>
<td>4</td>
<td>4</td>
<td>16</td>
<td>&lt;0.12</td>
<td>VIM-1</td>
<td>47.9</td>
</tr>
<tr>
<td>Vipm-32</td>
<td>&gt;128</td>
<td>32</td>
<td>8</td>
<td>128</td>
<td>&gt;128</td>
<td>VIM-1, SHV-5</td>
<td>45.2</td>
</tr>
<tr>
<td>\textit{E. coli} (pVipm-32)</td>
<td>&gt;128</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>&lt;0.12</td>
<td>VIM-1</td>
<td>–</td>
</tr>
<tr>
<td>Vipm-64</td>
<td>&gt;128</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>0.25</td>
<td>VIM-1</td>
<td>47.9</td>
</tr>
<tr>
<td>Vipm-64 (pSHA2)</td>
<td>&gt;128</td>
<td>2</td>
<td>4</td>
<td>16</td>
<td>0.25</td>
<td>VIM-1</td>
<td>47.9</td>
</tr>
<tr>
<td>\textit{E. coli} (pVipm-64)</td>
<td>&gt;128</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>&lt;0.12</td>
<td>VIM-1</td>
<td>–</td>
</tr>
<tr>
<td>\textit{E. coli} K12</td>
<td>0.25</td>
<td>&lt;0.12</td>
<td>&lt;0.12</td>
<td>&lt;0.12</td>
<td>&lt;0.12</td>
<td>VIM-1</td>
<td>–</td>
</tr>
</tbody>
</table>

CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; FEP, cefepime; ATM, aztreonam.

*aMean of three measurements not differing more than 10%.

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mediated by pVipm-16 seemed to be related to an increased production of VIM-1 that, most probably, resulted from the duplication of \( \text{bla}_{\text{VIM-1}} \). The relatively high imipenem MIC for the respective transconjugant is compatible with this hypothesis. Association of the \( \text{bla}_{\text{VIM-1}} \) integron with IS26, an element not requiring a specific target sequence and frequently implicated in mobilization of resistance genes,\(^6\) might have promoted this duplication. On the other hand, the possibility that the IS-mediated rearrangements of the plasmid structure affected copy number and, consequently, the rate of VIM-1 synthesis cannot be excluded.

The similarity of the RFLP type I plasmids, which was also reflected in the comparable \( \beta \)-lactam resistance levels of the respective transconjugants, suggested that most of the phenotypic diversity in the clinical isolates was due to chromosomal mutations. However, a clear indication for such changes was obtained for only one isolate: Vipm-64 lacked an OMP that was most probably a porin. Porin loss in \( K. \) pneumoniae strains producing either an MBL such as IMP-1 or \( \beta \)-lactamases without significant activity against carbapenems may increase the MICs of these antibiotics.\(^5\)\(^-\)\(^11\) Therefore, amongst these isolates, the highest level resistance to carbapenems seems to have been achieved by synergy between hydrolysis and decreased diffusion rate. The same explanation can also be applied to cefepime resistance. Nevertheless, in a different isolate, Vipm-32, cefepime and aztreonam were also affected by the simultaneous production of SHV-5, as has been described previously.\(^2\)\(^-\)\(^4\)\(^12\)

In conclusion, we have described VPKP isolates, with carbapenem MICs from susceptibility to high-level resistance, that appear to have been derived from a common progenitor strain. Clinical use of carbapenems and, to a lesser extent, cefepime and aztreonam, against the phenotypically susceptible isolates of this group may therefore have provided precisely the pressure that led to selection of the high-level resistance isolates.

Transparency declarations

None to declare.

References