Detection of the new metallo-β-lactamase VIM-19 along with KPC-2, CMY-2 and CTX-M-15 in Klebsiella pneumoniae

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Objectives: To report the identification of the metallo-β-lactamase (MBL) variant VIM-19 in a Klebsiella pneumoniae clinical strain co-producing KPC-2 carbapenemase, CMY-2 cephalosporinase and CTX-M-15 extended-spectrum β-lactamase.

Methods: MICs were determined by agar dilution. Phenotypic tests were performed to detect carbapenemase production. PCR and nucleotide sequencing were used for the identification of bla gene types and mapping of the integron carrying the MBL gene. The location of the MBL and KPC alleles was investigated by mating experiments, plasmid analysis and PCR assays.

Results: Imipenem, meropenem and ertapenem MICs for the study strain were 32, 16 and 64 mg/L, respectively. The strain carried blaTEM-1, blaCMY-2, blaKPC-2 and blaCTX-M-15 genes along with the gene blaVIM-19, which was located in a class 1 integron as the first gene cassette, followed by aacA6, dfrA1 and adaA1 cassettes. Mating experiments, plasmid analysis and PCR assays revealed that blaVIM-19 and blaCMY-2 were carried on an ~150 kb self-transferable plasmid, while blaKPC-2 and blaTEM-1 were on an ~70 kb self-transferable plasmid; blaCTX-M-15 was non-transferable.

Conclusions: The detection of the new MBL, VIM-19, which has enhanced carbapenemase activity, along with KPC-2, CMY-2 and CTX-M-15 is of concern. Further spread of the respective strains or plasmids may have serious consequences for antimicrobial chemotherapy.

Keywords: blaVIM-19, blaKPC-2, plasmids, transferable, conjugation, carbapenemases, MBLs

Introduction

During the last few years, carbapenem resistance has been increasingly reported in Klebsiella pneumoniae and is largely attributed to the production of carbapenem-hydrolysing enzymes, such as metallo-β-lactamase (MBL) and K. pneumoniae carbapenemase (KPC) types.1,2 K. pneumoniae isolates producing KPC enzymes have become increasingly prevalent on the East Coast of the USA since 2001.2 They have also caused outbreaks in Israel, and have recently become emerging public health concerns in several regions worldwide, such as China, Latin America and Greece.2 The dissemination of KPC producers poses a significant threat for carbapenem activity, considering the increasing rates also of MBL-producing K. pneumoniae. MBLs of the VIM group have been identified in different countries as a source of several nosocomial K. pneumoniae outbreaks.3,2 The VIM group currently includes 23 variants, clustered into three evolutionary lineages, represented by VIM-1, VIM-2 and VIM-7 (www.lahey.org/studies). In 2009, a new blaVIM gene variant, designated blaVIM-19, was detected in enterobacterial pathogens from Algiers and characterized by two research groups.3,4 In both descriptions, the gene blaVIM-19 was located as the first gene cassette in a class 1 integron with an unidentified 3′ extremity.3,4 In addition, Rodriguez-Martinez et al.4 have shown that the amino acid substitutions by which VIM-19 differs from the closely resembled VIM-1 and VIM-4 enzymes confer increased carbapenem hydrolytic activity. During the same year, K. pneumoniae producing this potent VIM-19 carbapenemase, along with KPC-2, CMY-2 and CTX-M-15 enzymes, was identified in another Mediterranean region, Greece (GenBank accession number FJ 915116). The present report documents this worrisome evolution.
**Materials and methods**

**Bacterial isolates and phenotypic testing**

*K. pneumoniae* strain KP1935 was recovered in May 2008 from a urinary tract infection of a 64-year-old female patient hospitalized at Serres General Hospital, Greece. MICs for the clinical and transconjugant strains of several β-lactams, aminoglycosides, ciprofloxacin, trimethoprim, tetracycline, tigecycline and colistin were determined by agar dilution. The isolate was phenotypically screened for carbapenemase production by performing combined tests using four discs of meropenem, one without and the other three with EDTA, phenylboronic acid (PBA) or both EDTA and PBA. K. pneumoniae ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853 and isolates from our laboratory collection that produce KPC, VIM and extended-spectrum β-lactamas (ESBLs) were used as controls in susceptibility assays.

**PCR assays and DNA sequencing**

The detection of broad-spectrum β-lactamase genes (*KPC*, *CMY* and other plasmidic AmpC-like genes, OXA-48 carbapenemase, MBLs, and ESBLs of TEM, SHV and CTX-M types) was sought by PCR using consensus primers that were specific for each enzyme group. Isolates from our collection (TEM, SHV and CTX-M types) was sought by PCR using consensus primers that were previously characterized were used as controls in the PCR assays. For integron mapping, PCR assays combining primers specific for conserved 5′-CS and 3′-CS sequences with primers for *bla*<sub>TEM</sub>, *aacA*<sup>+</sup>, *qacE*<sup>+</sup> and *sulI* were performed. PCR products of the genes tested and also of the *bla*<sub>VIM</sub>-carrying integron were sequenced on both strands. The *bla*<sub>VIM</sub>-flanking region PCR was mapped using a series of successive primers.

**Conjugation assays and plasmid analysis**

Filter mating experiments were performed using *Escherichia coli* 26R793 (lac<sup>+</sup> rif<sup>+</sup>) as recipient. Selection of the transconjugants was made on MacConkey agar plates containing rifampicin (100 mg/L) and either ampicillin (40 mg/L) or ertapenem (0.5–1 mg/L). Plasmid isolation was performed with the QIAfilter Plasmid Maxi Kit (Qiagen) and with a standard alkaline lysis protocol, using *E. coli* 39R861 as the standard plasmid control. The plasmid DNA bands of the transconjugants were extracted from the agarose gel, and used as templates in PCR for *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, *bla*<sub>CMY</sub> and *bla*<sub>TEM</sub> genes.

**Results**

The strain KP1935 was resistant or intermediate to all β-lactams, including carbapenems, with imipenem, meropenem and ertapenem MICs being 32, 16 and 64 mg/L, respectively. It also exhibited resistance to almost all alternative antimicrobials, including colistin (MIC 16 mg/L), and was susceptible to only tigecycline (MIC 2 mg/L) (Table 1). The simultaneous presence of both MBL and KPC carbapenemases was indicated by a positive combined-disc test using meropenem with both EDTA and PBA, while meropenem discs containing EDTA or PBA were negative.

PCR for β-lactamase genes showed that KP1935 was positive for *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>CMY</sub>, *bla*<sub>VIM</sub> and *bla*<sub>KPC</sub> genes, and negative for the remaining genes tested, including all *bla*<sub>SHV</sub> variants. Sequencing analysis identified *bla*<sub>TEM</sub>-1, *bla*<sub>CTX-M-15</sub>, *bla*<sub>CMY-2</sub>, *bla*<sub>VIM-19</sub> and *bla*<sub>KPC-2</sub> alleles. PCR and sequencing using primers 5′-CS and 3′-CS in various combinations with primers for *bla*<sub>CMY</sub>, *aacA*<sup>+</sup>, *aadA*<sup>+</sup> and *dfrA1* genes revealed a new class 1 integron with a structure similar to that carrying the close variant gene *bla*<sub>VIM-4</sub> in an *Enterobacter cloacae* isolate from Greece. The *bla*<sub>VIM-19</sub> gene cassette is located downstream of the *attI* recombination site, followed by an *aacA6* cassette, a *dfrA1* cassette, an *aadA1* cassette and the 3′-CS, containing *qacE*<sup>+</sup> and *sulI* (Figure 1). PCR mapping of the *bla*<sub>KPC</sub>-flanking region showed that this gene was located in a Tn4401 transposon similar to that found in *K. pneumoniae* isolates previously.

**Table 1. MICs of antibiotics for KP1935 and the respective transconjugants**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>KP1935 (VIM-19+KPC-2+CTX-M-15)</th>
<th>Tcs E. coli 26R793 (pVIM-19+CMY)</th>
<th>Tcs E. coli 26R793 (pKPC-2)</th>
<th>E. coli 26R793</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>32</td>
<td>8</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Meropenem</td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>64</td>
<td>8</td>
<td>2</td>
<td>0.06</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>4</td>
</tr>
<tr>
<td>Amoxicillin+CLA</td>
<td>&gt;256</td>
<td>256</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Piperacillin+TZB</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>256</td>
<td>1</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&gt;256</td>
<td>4</td>
<td>32</td>
<td>0.12</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>16</td>
<td>0.12</td>
</tr>
<tr>
<td>Ceferpine</td>
<td>128</td>
<td>64</td>
<td>8</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;32</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>64</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Amikacin</td>
<td>16</td>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>&gt;256</td>
<td>128</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>2</td>
<td>0.25</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Colistin</td>
<td>16</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

CLA, clavulanic acid (2 mg/L); TZB, tazobactam (4 mg/L); Tcs, transconjugant strain.
Repeated mating experiments yielded transconjugant colonies with MICs that, compared with the MICs for the recipient strain E. coli 26R793, were elevated for penicillins, cephalosporins and carbapenems. Phenotypic testing of many transconjugant colonies showed that some of them were positive in the combined-disc test using meropenem and boronic acid, while others were positive using meropenem and EDTA. PCR assays showed that all boronic acid-positive transconjugant colonies tested were positive for bla_VIM and bla_CMY and negative for bla_VIM, bla_CMY and blaCTX-M, while all EDTA-positive colonies tested were positive for blaVIM and blaCMY and negative for blaKPC, blaCTX-M and blaTEM. Transconjugant colonies that were PCR-positive for blaCTX-M were not detected after repeated mating experiments and testing of numerous colonies. Plasmid analysis of the clinical strain KP1935 revealed two plasmids of ~70 and 150 kb, while the blaVIM- and blaCMY-bearing transconjugants had a single plasmid of ~150 kb and the blaKPC- and blaTEM-bearing transconjugants had a single plasmid of ~70 kb. PCR, using as template the gel-extracted plasmid DNA band of the transconjugants, confirmed the carriage of the bla_VIM and bla_CMY genes on the ~70 kb plasmid, and of the bla_VIM and bla_CMY genes on the ~150 kb plasmid. The MICs for the bla_KPC-positive and the bla_TEM-positive transconjugant colonies of the antibiotics tested are shown in Table 1.

Discussion
In the present report, the novel MBL _bla_VIM-19_ was detected in a clinical strain of _K. pneumoniae_ that also harboured _bla_VIM-1, bla_CMY-2, bla_KPC_ and _blaCTX-M-15_. The gene _bla_VIM-19_ differs from _bla_VIM-4_ by one amino acid residue at position 215 (Asn215Lys, standard class B β-lactamase numbering scheme) and from _bla_VIM_ by two amino acid residues (Asn215Lys and Ser228Arg) that increase the carbapenemase activity of the VIM-1 enzyme compared with VIM-1. It could be postulated that VIM-19 has arisen under carbapenem pressure in our hospitals, where VIM-1- or VIM-4-producing microorganisms are common. Alternatively, this novel variant may have arisen independently and be common in our regions. The latter possibility is supported by the detection of _bla_VIM-19_ in a distantly located Mediterranean country and also by the wide occurrence of the close variant gene _bla_VIM-4_ in remote countries. Indeed, the emergence of _bla_VIM-19_ in a class 1 integron with a gene content similar to that of an integron detected previously in Greece, but including _bla_VIM-4_ instead of _bla_VIM-19_, underlines the continuous evolution of these important determinants. It should be noted that a similar integron carrying _bla_VIM-4_ in an _E. cloacae_ isolate was harboured in a different plasmid (40 kb) that also included the ESBL gene _blaCTX-M-9_ in contrast with the _bla_VIM-19_-carrying integron, indicating that the dissemination of a single plasmid is probably not the cause of VIM-4 or VIM-19 spread.

The study strain KP1935 had higher carbapenem MICs compared with most _K. pneumoniae_ clinical strains from Greece that produce VIM or KPC as single carbapenemases. Furthermore, the VIM-19-producing transconjugants had MICs of imipenem (8 mg/L) and meropenem (2 mg/L) that were similar to those of other VIM-19-producing laboratory strains, being higher than those of VIM-1-producing laboratory strains. This observation further supports the enhanced carbapenemase activity of VIM-19 relative to VIM-1.

In tertiary care Greek hospitals, _K. pneumoniae_ isolates that produce MBLs or, more recently, KPC carbapenemases are commonly recovered from clinical infections. The current detection of VIM-19 along with KPC-2 in our strain is only the second description of a _K. pneumoniae_ strain that produced two different carbapenemases after the appearance of VIM-1- and KPC-2-producing _K. pneumoniae_. Considering that the combination of a potent VIM variant along with KPC and CTX-M-15 ESBL inactivates all clinically available β-lactams, a further spread of the respective strains or plasmids may have even more serious consequences in the treatment of nosocomial infections.

**Nucleotide sequence accession number**
The nucleotide sequence of the gene _bla_VIM-19_ has been assigned accession number FJ 915116 in the EMBL/GenBank/ DDBJ databases.

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**Transparency declarations**
None to declare.

**References**


