Novel VIM Metallo-Beta-Lactamase Variant from Clinical Isolates of *Enterobacteriaceae* from Algeria.

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Running title: Characterization of VIM-19, Algeria

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Abstract

Five different strains of Enterobacteriaceae were isolated from two patients hospitalized in the intensive care unit of the Central Military Hospital of Algiers, Algeria. All five strains, one Providencia stuartii, two Escherichia coli and two Klebsiella pneumoniae, were intermediate or resistant to all β-lactams, including carbapenems. Synergy was observed for all the strains between imipenem and EDTA. PCR experiment confirmed the presence of a blavIM in all five strains. The blavIM genes were located as part of a class 1 integron on a 180 kb conjugative plasmid. They encoded a novel metallo-β-lactamase designated VIM-19, which differed from the parental enzyme VIM-1 by only two substitutions: Ser228Arg, previously observed in the closely related enzyme VIM-4, and Asn215Lys, not previously observed in other VIM-type carbapenemases. VIM-19 was further characterized after purification through determination of its kinetic constants. This enzyme was inhibited by EDTA, and hydrolyzed penicillins, cephalosporins and carbapenems, as observed for other VIM-carbapenemases but with greater catalytic efficiency against penicillins than VIM-1. VIM-19 is the first carbapenemase enzyme identified in Algeria. These results confirm the emergence of VIM-4-like enzymes in Enterobacteriaceae from Mediterranean countries.
Introduction

Carbapenems are the most active molecule in β-lactams with 98 % of worldwide susceptibility among Enterobacteriaceae (24). However, a growing number of carbapenemase-producing strains have been identified since the beginning of the 90s. They displayed variable in vitro levels of resistance to carbapenems, including susceptibility (24), but were clinically resistant to these molecules (35). The possible low level of resistance makes reliable detection of such strains difficult (35).

The geographical distribution of these strains differs: those producing class A KPC-type carbapenemase have been more frequently observed in the United States and in Israel whereas those producing VIM- and IMP-type class B β-lactamases are generally encountered in Asia and in the north of the Mediterranean basin (24). Among these enzymes metallo-carbapenemases are especially worrying because they virtually hydrolyze all classes of β-lactams except aztreonam (35). Initially observed in Pseudomonas aeruginosa and Acinetobacter spp., they spread to Enterobacteriaceae during the late 1990s and the 2000s (24). The most frequently acquired MBLs are the IMP and VIM types (24). Four other types of acquired MBLs in P. aeruginosa isolates from Brazil (SPM-1) (34) and Germany (GIM-1) (5), in Acinetobacter baumannii isolates from Korea (SIM-1) (17) and in a Citrobacter freundii isolate from Japan (KHM-1) (30) have recently been described.

VIM-producing Enterobacteriaceae have been involved in different outbreaks in particular in Italy and Greece during the 2000s (4, 23, 33). The south of the Mediterranean basin seemed to be spared by such strains until 2006 when VIM-4-producing strains of Klebsiella pneumoniae were identified during a nosocomial outbreak in a Tunisian hospital (14), suggesting a possible dissemination in northwest Africa.

Between January and May 2008, five imipenem-resistant strains of Enterobacteriaceae were recovered from two patients in the intensive care unit of the Central Military Hospital of
Algiers, Algeria. We identified a novel metallo-carbapenemase VIM-19 in these strains. This is the first report of VIM-producing strains in Algeria.
Material and methods

Bacterial isolates and plasmids. The strains used in this study were *Escherichia coli* 138, *E. coli* 2603, *K. pneumoniae* 2878, *K. pneumoniae* 6828 and *Providencia stuartii* 6858 isolated from two different patients hospitalized in the intensive care unit of the Central Military Hospital of Algiers, Algeria. *E. coli* DH5α (Novagen, Darmstadt, Germany) and *E. coli* BL21 (DE3) (Novagen) were used for cloning experiments and *E. coli* C600 for mating-out assays. Plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) was used for the initial cloning experiments and a modified pET9a plasmid harbouring a *Not*I restriction site (18) for the over-expression of the β-lactamase-encoding genes.

Phenotypic detection of Metallo-β-lactamases. EDTA-combined disk method was performed with imipenem-containing disks as described by Picão *et al.* (20). Two imipenem-containing disks were placed on a Mueller-Hinton agar plate (Biomérieux, Marcy l'Etoile, France) and 10 µl of a 100 mM EDTA solution were added to one of the disks. After overnight incubation at 37°C, an inhibition diameter extension of at least 5 mm for EDTA-containing disk indicated a synergy.

Genomic typing. The clinical isolates *Escherichia coli* 138, *E. coli* 2603, *K. pneumoniae* 2878 and *K. pneumoniae* 6828 were compared by enterobacterial repetitive intergenic consensus sequence PCR (ERIC2-PCR), as previously described (8).

Susceptibility to β-lactams. Antibiotic-containing disks were used for antibiotic susceptibility testing by the disk diffusion assay (MAST Diagnostic, Amiens, France). Results of susceptibility testing were interpreted according to the recommendations of the Comité de
l’Antibiogramme de la Société Française de Microbiologie (CA-SFM) (7). MICs were determined by a microdilution method on Mueller-Hinton agar (Bio-Rad, Richmond, USA) with an inoculum of $10^4$ CFU per spot and were interpreted according to guidelines of the CA-SFM (7). The antibiotics were provided as powders by Glaxo Smith Kline (amoxicillin, ticarcillin, cefuroxime, ceftazidime and clavulanic acid), Wyeth Laboratories (piperacillin and tazobactam), Eli Lilly (cephalothin), Roussel-Uclaf (cefotaxime and cefpirome), Bristol-Myers-Squibb (aztreonam and cefepime), Merck Sharp and Dohme-Chibret (cefotetan, imipenem and ertapenem) and AstraZeneca (meropenem).

**Isoelectric focusing.** Isoelectric focusing of β-lactamases was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10.0, as previously described (2), with TEM-39 [pI 5.2], TEM-1 [pI 5.4], TEM-2 [pI 5.6], CTX-M-15 [pI 8.6] and CMY-4 [pI 9] as standards.

**Plasmid analysis.** Mating experiments were performed with in vitro-obtained rifampicin resistant mutants of *E. coli* C600 as previously described (27). Transconjugants were selected on agar containing rifampicin (300 µg/ml) and cefotaxime (0.5 µg/ml). Plasmid DNA extraction of the different clinical strains was made with the Qiagen Plasmid DNA Maxi Kit (Qiagen, Courtaboeuf, France). The extracted plasmids were electroporated onto *E. coli* DH5α with selection on agar containing cefotaxime (0.5 µg/ml). Plasmid DNAs were extracted from the clinical strains, transconjugants and transformants by the method of Kado and Liu (13). Plasmid size was determined by comparison with that of plasmids Rsa (39 kb), TP114 (61 kb), pCFF04 (85 kb) and pCFF14 (180 kb) as previously described (19). The plasmids of the transformants corresponding to the five clinical strains were extracted by the alkaline lysis...
method (1) and were digested with EcoRI, HindIII and PstI restriction endonucleases according to the manufacturer’s recommendations (Boehringer Mannheim, Meylan, France).

**Sequencing and analysis of bla genes.** Molecular screening for bla\textsubscript{VIM}, bla\textsubscript{TEM}, bla\textsubscript{CTX-M} and bla\textsubscript{CMY} was performed using PCR with primers VIM-F, VIM-B, CitMF, CF2, CTX-M A and CTX-M B (3), (9), (21), (26). VIM-1 specific primers VIM-1A NdeI 5’-GGAATTCCATATGTTAAAAGTTATTAGT-3’ and VIM-1B NotI 5’-ATAGTTTACGCCGCCCCTACTCGGCGACTGAGCGATT-3’ were used to amplify and sequence the bla\textsubscript{VIM} gene. Direct sequencing was performed on PCR products, which were obtained from the five clinical strains, the transconjugant E. coli C600 and the different recombinant E. coli. These PCR products were sequenced by dyeoxy chain termination on both strands with an Applied Biosystems sequencer (ABI 377) (29). Primer INT/5CS targeting the 5’ conserved sequence of class 1 integron previously described by Riccio et al. (25) (table 1) was used with VIM-1B NotI to amplify and sequence a part of the bla\textsubscript{VIM} genetic environment. The nucleotide and deduced protein sequences were analysed using software available at the website of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov). The ClustalW program (http://infobiogen.fr) was used for the alignment of amino acid sequences (32).

**Cloning experiments.** Recombinant DNA manipulation and transformations were performed as described by Sambrook et al. (27). The PCR products obtained with primers VIM-1A NdeI and VIM-1B NotI were cloned into SmaI (Roche Diagnostics, Meylan, France) restriction site of the pBK-CMV plasmid. Recombinant E. coli DH\textsubscript{5}\alpha were selected on Mueller-Hinton agar plates supplemented with 30 µg kanamycin and 0.5 µg cefotaxime. The plasmid was then digested by NdeI and NotI (Roche Diagnostics, Meylan, France) and ligated into the
corresponding restriction sites of a modified pET-9a plasmid. Recombinant *E. coli* BL21 were selected on Mueller-Hinton agar plates supplemented with 30 µg/ml kanamycin and 0.5 µg/ml cefotaxime. All the cloned fragments were subjected to confirmatory sequencing.

**Over-production and purification of β-lactamases.** VIM-19-producing *E. coli* BL21 (DE3) clone was used to overproduce the VIM-19 β-lactamase, as previously described (6). Bacteria were disrupted by sonication. The supernatant was loaded onto a Q Sepharose column (10 mL; Amersham Pharmacia Biotech., Orsay, France) equilibrated with 20 mM Tris-HCl (pH 7.4). The bound proteins were eluted with a linear NaCl gradient (0 to 500 mM). The β-lactamase-containing elution peak was loaded onto a Superose 12 (Amersham Pharmacia Biotech.) and eluted with the buffer 5 mM Tris-HCl 100 mM NaCl (pH 7.4). The β-lactamase-containing elution peak was dialyzed against NaCl 100 mM, concentrated and stored at -20°C until use. The total protein concentration was estimated by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.), with bovine serum albumin (Sigma Chemical Co., St Louis, Mo.) used as a standard.

**Determination of β-lactamase kinetic parameters** $k_{cat}$, $K_m$. Michaelis constant ($K_m$) and catalytic activity ($k_{cat}$) were determined with purified extracts using a computerized microacidimetric method (15) at pH 7 and 37°C in 0.1 M NaCl solution.

**Nucleotide sequence accession number.** The nucleotide sequence has been assigned accession number FJ499397 in the GenBank database.
Results

Clinical strains and resistance phenotype. *E. coli* 138 was isolated in January 2008 from the urinary tract of a patient hospitalized for acute renal failure in the intensive care unit of the Central Military Hospital of Algiers, Algeria. He was treated with imipenem and amikacin and died from septic shock one day later. *E. coli* 2603 was isolated in February 2008 from the urinary tract of a second patient hospitalized in the same ward. *K. pneumoniae* 2878 and 6828 and *P. stuartii* 6858 were isolated in May 2008 from two different urinary samples of this second patient. *P. stuartii* 6858 was isolated from the two samples, whereas *K. pneumoniae* 2878 and 6828 were only isolated from the first and second sample respectively. She was treated with cefotaxime and died from a pulmonary embolism.

Both patients had been previously treated with different broad spectrum β-lactams, the first with cefotaxime and the second with cefotaxime, ceftazidime and imipenem. These five strains were the first strains of *Enterobacteriaceae* intermediate or resistant to carbapenems isolated in this hospital.

All the five strains were intermediate or resistant to all β-lactams including carbapenems (Table 1). They were resistant to sulfonamides, trimethoprim, tetracycline, and chloramphenicol. They were intermediate or resistant to kanamycin, tobramycin, netilmicin, amikacin and gentamicin. They were also susceptible to all quinolones and to fosfomycin.

The two *E. coli* and the two *K. pneumoniae* strains presented different ERIC2-PCR patterns confirming they were not clonally related (data not shown). EDTA-combined disk method suggested the production of a metallo-β-lactamase by the five strains.

Molecular β-lactamase identification. Isoelectric focalisation experiments showed that the five clinical strains produced different β-lactamases of pI 5.4 and 9 in addition to their natural respective β-lactamases. The PCR-based analysis of β-lactamase-encoding genes revealed
that they all harbored a \textit{bla} gene encoding a VIM-type carbapenemase, and a \textit{bla} gene encoding a CMY-type cephalosporinase corresponding to the enzyme of pI 9. Partial sequencing of the \textit{bla}_{CMY} genes showed that it encoded for a CMY-4-like enzyme. The two \textit{E. coli} strains 138 and 2603 also presented a \textit{bla}_{TEM}, which was identified as \textit{bla}_{TEM,1}. No CTX-M-type enzyme was detected in any of these strains unlike in the Tunisian VIM-4-producing strains (14).

The five clinical strains harbored the same novel \textit{bla}_{VIM} gene, designated \textit{bla}_{VIM,19}. This gene differed from \textit{bla}_{VIM,4} by only one substitution at position 1034 from A to C (numbering according to the sequence AM181293 (14)) which led to the substitution Asn215Lys according to the MBL scheme (11). VIM-19 was therefore a new VIM enzyme which differed from VIM-1 by two amino acid substitutions: Asn215Lys and Ser228Arg (previously observed in VIM-2 and VIM-4).

**Genetic support.** Different transconjugants and transformants were obtained from the five clinical strains by mating-out and electroporation. Specific PCR experiments confirmed the presence of the \textit{bla}_{VIM} and the \textit{bla}_{CMY} genes in all of them. No \textit{bla}_{TEM} was detected in any of the transconjugants or transformants. Plasmid content analysis of the clinical strains and their respective transformants and transconjugants revealed the transfer of a 180-kb plasmid that encoded both VIM-type and CMY-type \(\beta\)-lactamases, as previously observed in the Tunisian VIM-4-producing strains (14). The restriction profiles of the transformants were identical suggesting the dissemination of a single plasmid among the different isolates (data not shown).

PCR experiments and sequencing of \textit{bla}_{VIM,19} genetic environment showed that this gene was the first gene cassette of a class 1 integron as previously observed for other \textit{bla}_{VIM} genes (14).
The transconjugant TC *E. coli* C600 2878 and the transformant TR *E. coli* DH₅α 2878 of the clinical strain *K. pneumoniae* 2878 were chosen for further characterization.

**β-lactams MICs (Table 1).** All the clinical strains, TC *E. coli* C600 2878, TR *E. coli* DH₅α 2878, and the clone *E. coli* DH₅α (pBK-VIM-19) were highly resistant to all penicillins (alone or in association with clavulanic acid or tazobactam), cephalothin and cefuroxime (MICs, 1,024 to > 2,048 µg/ml). MICs of cefoxitin, cefotaxime and ceftazidime were slightly lower, ranging from 32 to 512 µg/ml. Carbapenems and cefepime were more active with MICs ranging from 1 to 64 µg/ml. Aztreonam was only active against *E. coli* DH₅α (pBK-VIM-19), which only produced VIM-19. The other strains, which also produced a CMY-4-like enzyme, had MICs of aztreonam between 2 and 128 µg/ml. In presence of EDTA, the activity of the different carbapenems was restored (MICs, <0.12 to 0.5 µg/ml). The MICs of ceftazidime and cefotaxime decreased two-fold to eleven-fold but aztreonam MICs were not significantly modified.

**Kinetic constants (Table 2).** The two steps of purification yielded approximately 1.6 mg of pure enzyme per liter of culture. The specific activity of the extract against benzylpenicillin was 419.6 µmol/min/mg. The level of purity was estimated > 97% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (2, 16).

Table 3 lists the steady-state kinetic parameters $k_{cat}$, $K_m$ and $k_{cat}/K_m$ of VIM-19 and compares these data with previously published values of VIM-1 and VIM-2 (10, 22, 29). These data have been measured under different experimental conditions; the comparison should be taken with caution.

Like other VIM enzymes, VIM-19 has a broad hydrolysis profile. However, it was more active against penicillins than against cephalosporins and carbapenems whereas the $K_m$ values
remained lower than 100 µM for all of the tested substrates. Greatest catalytic efficiency was observed for benzylpenicillin, cloxacillin, amoxillin, piperacilline and cephalothin. Among the oxyimino cephalosporins, VIM-19 was especially active and efficient against cefotaxime. Finally, VIM-19 was 6-fold to 14-fold more active against imipenem than against ertapenem and meropenem. As previously observed for other metallo-β-lactamases, VIM-19 had no detectable activity against aztreonam.

In contrast with VIM-1, VIM-19 was slightly more efficient against penicillins than against cephalosporins, as observed for VIM-2, because of lower $K_m$ values. Both VIM-2 and VIM-19 harbored an arginine residue at position 228 instead of a serine in VIM-1. The crystallographic structure of VIM-2 revealed that the side chain of this residue was close to one of the two Zn atoms (Zn 2) and interacted, in the oxidised form of VIM-2, with the residues of the active site of VIM-2 located at positions 224 and 263 (12). In addition, in the reduced form of VIM-2, Arg228 contributed to the creation of a positively charged environment for substrate binding (12). These structural features, which are shared by VIM-2 and VIM-19, could be involved in the differences of hydrolysis profile observed between VIM-1 and VIM-19. The role of substitution Asn215Lys of VIM-19 remained unclear but this residue seemed to have less impact because its side-chain was oriented toward the solvent in the structure of VIM-2.
Concluding remarks

Infections caused by metallo-β-lactamase-producing Enterobacteriaceae are currently a worrying threat because the antibiotic options available are extremely limited and the infections are associated with a high risk of mortality (31). We report here the first documented cases of infections caused by VIM-producing Enterobacteriaceae strains in Algeria. The five strains produced a novel VIM-type metallo-β-lactamase VIM-19. This enzyme is closely related to VIM-4 and VIM-1 which are so far the most frequently observed VIM-enzymes in Enterobacteriaceae strains (14, 22, 24). As in a recent outbreak of VIM-4-producing Klebsiella pneumoniae in Tunisia (14), the bla\textsubscript{VIM-19} was located among a class 1 integron on a large plasmid and was associated with a bla\textsubscript{CMY} gene. Our study confirms the emergence of VIM-producing Enterobacteriaceae in northwest Africa and their dissemination all around the Mediterranean basin. A large multicentric study would be necessary to assess the dissemination of such strains in the different countries of the region.

Acknowledgments

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**TABLE 1.** MICs of β-lactam antibiotics for *E. coli* 138, *E. coli* 2603, *K. pneumoniae* 2878, *K. pneumoniae* 6828 and *Providencia stuartii* 6858, TC *E. coli* C600 2878, TR *E. coli DH5α* 2878 and recombinant *E. coli DH5α* (pBK-VIM-19), and *E. coli DH5α* (pBK-CMV).

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</tr>
<tr>
<td>Ertapenem + EDTA</td>
<td>0.25</td>
<td>&lt; 0.12</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>&lt; 0.12</td>
<td>&lt; 0.12</td>
</tr>
</tbody>
</table>

*a* CLA. clavulanic acid at 2 µg/ml.

*b* TZB. tazobactam at 4 µg/ml.

*c* EDTA. EDTA at 0.4 mM.
TABLE 2. Kinetic parameters\(^a\) of \(\beta\)-lactamases VIM-1, VIM-2 and VIM-19.

<table>
<thead>
<tr>
<th>(\beta)-lactam</th>
<th>VIM-19</th>
<th>VIM-1(^b)</th>
<th>VIM-2(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{cat}) (s(^{-1}))</td>
<td>(K_m) (µM)</td>
<td>(k_{cat}/K_m) (µM(^{-1}).s(^{-1}))</td>
<td>(k_{cat}) (s(^{-1}))</td>
</tr>
<tr>
<td>Benzylopenicillin</td>
<td>206</td>
<td>57</td>
<td>3.6</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>79.2</td>
<td>14</td>
<td>5.7</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>112</td>
<td>34</td>
<td>3.3</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>45.8</td>
<td>68</td>
<td>0.7</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>160</td>
<td>37</td>
<td>4.3</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>21.2</td>
<td>15</td>
<td>1.4</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>4.0</td>
<td>53</td>
<td>0.08</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>5.3</td>
<td>13</td>
<td>0.4</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&lt;0.1</td>
<td>&lt;0.01</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>6.3</td>
<td>88</td>
<td>0.07</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>34.3</td>
<td>36</td>
<td>0.95</td>
</tr>
<tr>
<td>Cefepime</td>
<td>12.5</td>
<td>55</td>
<td>0.23</td>
</tr>
<tr>
<td>Imipenem</td>
<td>8.2</td>
<td>22</td>
<td>0.37</td>
</tr>
<tr>
<td>Meropenem</td>
<td>1.3</td>
<td>9</td>
<td>0.14</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>0.6</td>
<td>21</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^a\)The standard deviation for analysis was \(\leq 10\%\).

\(^b\) Kinetic parameters for VIM-1 are from Franceschini \textit{et al}. (10) and those for VIM-2 from Poirel \textit{et al}. (21) except for cloxacillin, and ertapenem, which are from Samuelsen \textit{et al}. (28).

ND, Not determined.