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VIM-12, a Novel Plasmid-Mediated Metallo-β-Lactamase from *Klebsiella pneumoniae* That Resembles a VIM-1/VIM-2 Hybrid

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A transferable plasmid from *Klebsiella pneumoniae* carried a class 1 integron containing *bla*$_{\text{VIM-12}}$, a novel *bla*$_{\text{VIM}}$-type gene, flanked by two copies of *aacA7*. *bla*$_{\text{VIM-12}}$ was clustered between *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{VIM-2}}$ and differed from *bla*$_{\text{VIM-1}}$ by 18 nucleotides that were all located at the 3′ end and matched the corresponding nucleotides in *bla*$_{\text{VIM-2}}$. The *bla*$_{\text{VIM-12}}$-associated 59-base element was identical to that described in *bla*$_{\text{VIM-2}}$ alleles.

Gram-negative microorganisms producing acquired metallo-β-lactamases (MBLs), including VIM, IMP, GIM, and SPM types, are increasingly implicated in nosocomial infections (12, 21). VIM-type MBLs have been firstly described in *Pseudomonas aeruginosa* and thereafter in other nonfermenters and enterobacterial species. So far, 12 VIM variants clustered into three groups represented by VIM-1, VIM-2, and VIM-7 have been described. The VIM-1 group comprises VIM-4, VIM-5, and VIM-11A. Six VIM variants (VIM-3, VIM-6, VIM-8, VIM-9, VIM-10, and VIM-11B) are considered derivatives of VIM-2, which exhibits 90% amino acid sequence identity with VIM-1. VIM-7 is markedly divergent from the remaining VIM types (21 and www.lahey.org/studies/other.asp#table 1). We report here on the identification of *bla*$_{\text{VIM-12}}$, a novel *bla*$_{\text{VIM}}$ gene classified as intermediate between *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{VIM-2}}$.

Klebsiella pneumoniae 2873 was recovered in March 2005 at the Hippokration Hospital (Thessaloniki, Greece) from blood cultures of a 67-year-old surgical patient. The patient had been treated with multiple courses of antibiotics, including imipenem, prior to the isolation of *K. pneumoniae*. Species identification was performed by the API 20E system (bioMérieux, Marcy l' Étoile, France). *Escherichia coli* K-12 strain 26R793 (Rif^r) was used as the recipient in conjugation experiments. *E. coli* DH5α was used as a host of recombinant plasmids. The plasmids pMON-38201 (7) and pBCSK(+) (Stratagene, La Jolla, CA) were used for cloning of *bla*$_{\text{VIM}}$-containing fragments.

Susceptibility to β-lactams was determined by an agar dilution method (11). Phenotypic detection of MBL production was performed using the E-test MBL containing imipenem and EDTA (AB Biodisk, Solna, Sweden). Susceptibility status to non-β-lactam antibiotics was assessed by disk diffusion (10).

Transfer of resistance by conjugation was performed as described previously (19). *E. coli* transconjugants were selected on MacConkey agar containing rifampin (100 μg/ml) and imipenem (0.5 to 2 μg/ml). Plasmid DNA was extracted with an alkaline lysis procedure (13).

β-Lactamases were extracted by ultrasonic treatment of cell suspensions and clarified by centrifugation. Protein concentration of the extracts was determined with a protein assay kit (Bio-Rad, Richmond, CA). Isoelectric focusing was performed in polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5) (Pharmacia-LKB, Uppsala, Sweden). β-Lactamases were visualized in situ with nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom).

PCR detection of various *bla* gene types, including *bla*$_{\text{VIM}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, and *Citrobacter freundii*-derived *cmy*, was performed using consensus primers and amplification conditions as described previously (1, 22, 23). For integron mapping, PCR assays combining primers specific for 5′ conserved segment (5′CS) and 3′CS sequences (6) with primers specific for *bla*$_{\text{VIM}}$, *aacA*, *dhfrI*, *aadA*, *qacE*Δ*1*, and *sul* genes were performed. PCR products were purified using a Qiaex gel extraction kit (QIAGEN, Chatsworth, CA) and used as templates for sequencing on both strands with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Antibiotic susceptibility testing showed that *K. pneumoniae* 2873 was resistant to penicillin-inhibitor combinations and broad-spectrum cephalosporins and also exhibited reduced susceptibility to carbapenems and aztreonam (Table 1). The isolate was positive by the E-test MBL. *K. pneumoniae* 2873 was also resistant to gentamicin, netilmicin, tobramycin, amikacin, ciprofloxacin, cotrimoxazole, and tetracycline.

Conjugation experiments showed that resistance to β-lactams was readily transferable to *E. coli* at a high frequency (8×10^{-2} per donor cell). Plasmid analysis indicated transfer of a

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TABLE 1. Agar dilution MICs of β-lactams against VIM-12-producing enterobacterial strains

Antibiotic(s)[a]	MIC (μg/ml) for strain:				
	K. pneumoniae 2873 (VIM-12 + CMY + TEM-1 + SHV-1)	*E. coli*(p2873) (VIM-12 + CMY + TEM-1)	*E. coli*(pB-V12) (VIM-12)	*E. coli*(pBCSK)	*E. coli* 26R793
Amoxicillin	>256	>256	>256	2	2
Amoxicillin + CLA	>256	>256	>256	2	2
Piperacillin	>256	>256	>256	1	1
Piperacillin + TAZ	>256	>256	128	0.5	0.5
Cefotaxime	128	64	16	≤0.06	≤0.06
Ceftazidime	>128	>128	>128	0.12	0.12
Cefepime	64	32	16	≤0.06	≤0.06
Aztreonam	16	4	≤0.06	≤0.06	0.12
Imipenem	8	2	1	≤0.06	≤0.06
Meropenem	4	0.5	0.5	≤0.06	≤0.06

[a] CLA, clavulanic acid (2 μg/ml); TAZ, tazobactam (4 μg/ml).

single plasmid of approximately 70 kb, designated p2873. Transconjugants exhibited most of the resistance characters of the donor, although MICs of carbapenems and oxyimino-β-lactams were slightly lower (Table 1). Resistance to cotrimoxazole, amikacin, netilmicin, and tobramycin, but not gentamicin, was also transferred.

Isoelectric focusing showed that *E. coli*(p2873) produced three β-lactamases with pIs of 5.1, 5.4, and 8.9. These bands were also observed in the extracts of *K. pneumoniae* 2873 along with a β-lactamase focusing at 7.6. Plasmid DNA extracts from both strains were positive in the bla_{VIM}-, bla_{CMY}-, and bla_{TEM}-specific PCRs. Types of *bla* genes were confirmed by sequencing of the respective amplicons. These findings, taken together with the β-lactam resistance phenotypes, indicated that p2873 encoded a VIM MBL, a *C. freundii*-originated CMY cephalosporinase, and a TEM-1 β-lactamase. The nontransferable β-lactamase produced by *K. pneumoniae* was SHV-1, as shown by sequencing of the respective PCR product.

Plasmid DNA extracts from *E. coli*(p2873) were subjected to PCR using various primer combinations. Assembly of the nucleotide sequences of overlapping PCR products revealed a class 1 integron named In-h12. The variable region of In-h12 was approximately 2.1 kbp and included a novel bla_{VIM}-type gene cassette (designated bla_{VIM-12}) flanked by two copies of an *aacA7* gene cassette similar to those encountered frequently among VIM-encoding class 1 integrons. A typical 5′ conserved segment (5′CS) containing an *intI1* gene with a strong P1 promoter followed directly by an activated P2 promoter (including a GGG insertion) and an *attI1* site was identified. *qacEΔ1*/*sul1* sequences were also detected at the 3′CS of In-h12. The bla_{VIM-12} gene (798 bp) shared 97.7, 94.5, and 80.2% nucleotide homology with the bla_{VIM-1}, bla_{VIM-2}, and bla_{VIM-7} genes, respectively. This novel MBL differed from bla_{VIM-1} (GenBank accession no. Y18050) by 18 nucleotides. Notably, these changes were all located at the 3′ end and matched exactly the nucleotides found at the corresponding positions in the bla_{VIM-2} gene (GenBank accession no. AF191564). bla_{VIM-12} could therefore be viewed as a bla_{VIM-1}/bla_{VIM-2} hybrid being identical to bla_{VIM-1} from the 5′ end up to nucleotide 663 and to bla_{VIM-2} from nucleotide 614 up to its 3′ end. Furthermore, the 59-base element of the bla_{VIM-12} gene cassette (72 bp in length) was identical to the element commonly found in bla_{VIM-2} cassettes (14–16) and differed significantly from the 59 bp of the bla_{VIM-1} gene cassettes (5, 8, 18). A PCR product that included the promoter sequence, the left-hand *aacA7*, and the bla_{VIM-12} gene cassette was directly cloned to pMON-38201 and subsequently to the polycloning site of pBCSK(+). The resulting recombinant plasmid (pB-V12) was used to transform *E. coli* DH5α-competent cells. The *E. coli*(pB-V12) clone exhibited a β-lactam resistance pattern characteristic of laboratory strains carrying cloned bla_{VIM} genes (Table 1).

The putative VIM-12 polypeptide (266 amino acids; molecular weight, 28,120) shared 97.0 and 93.6% amino acid identity with VIM-1 and VIM-2, respectively. VIM-12 differed from VIM-1 by 8 amino acid residues at the C terminus and by 18 residues from VIM-2 at the N terminus, 8 of which were in the putative leader peptide (Fig. 1). The latter likely comprised 26 amino acid residues (HS-GE). The mature VIM-12 protein (240 amino acids; molecular weight, 25,417) had a calculated pI of 4.77, close to that determined by isoelectric focusing (5.1). A dendrogram based on a CLUSTAL W multiple alignment of the VIM proteins showed that VIM-12 was clustered between the VIM-1 and VIM-2 groups (data not shown).

In this study, VIM-12, a novel plasmid-mediated VIM-type MBL from a clinical isolate of *K. pneumoniae*, is described. Emergence of bla_{VIM-12} in a class 1 integron that is also novel underscores the continuous evolution of these important determinants. It is of interest that the bla_{VIM-12} gene cassette in its 5′ end is identical to bla_{VIM-1}, whereas in its 3′ end it resembles bla_{VIM-2}. The possibility that bla_{VIM-12} belongs to a distinct VIM lineage cannot be excluded. However, the pattern of the nucleotide changes of the bla_{VIM-12} gene, compared with bla_{VIM-1} and bla_{VIM-2}, leads to the hypothesis that this novel variant might have been formed by a recombination event between bla_{VIM-1}- and bla_{VIM-2}-containing sequences. It is probable that bla_{VIM-12} has arisen within our hospital environments, where VIM-1- and VIM-2-producing microorganisms are endemic (4, 17, 20).

VIM-12 differs from VIM-1 by 8 amino acid residues at positions 246, 251, 257, 258, 284, 287, 294, and 299 (standard class B beta-lactamases numbering scheme [3]). All eight residues are located at the protein surface and are distant from the active site (2). Therefore, it is unlikely that the substrate specificity of VIM-12 differs significantly from that of VIM-1. The β-lactam resistance pattern of the VIM-12-producing

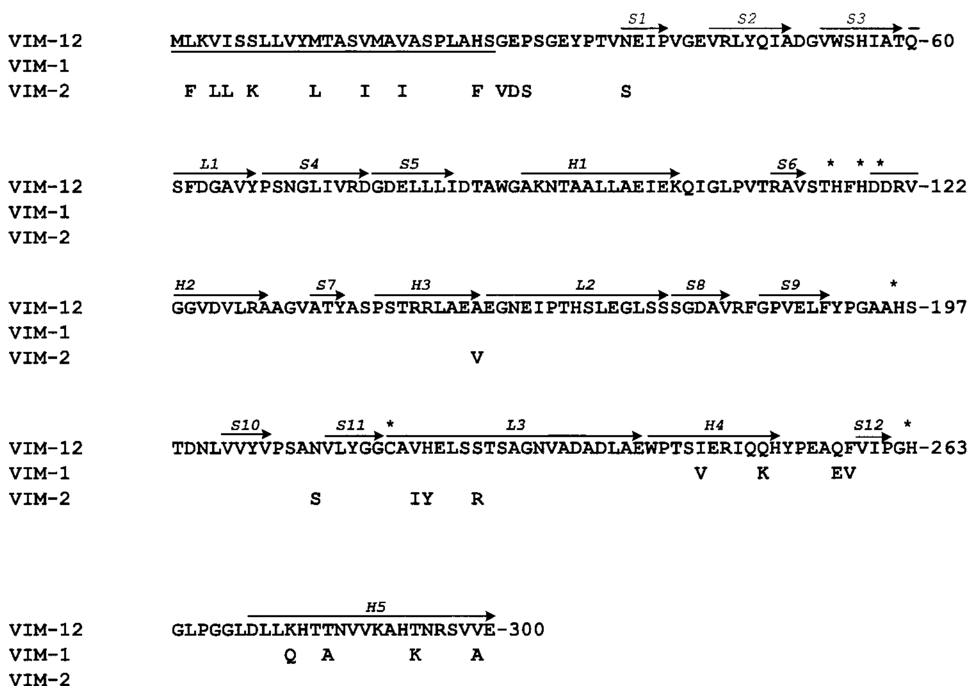

FIG. 1. Amino acid sequence of VIM-12 metallo-β-lactamase and comparison with VIM-1 and VIM-2. Numbering of amino acid residues is according to a standard scheme for MBLs (3). Arrows above sequence regions indicate the secondary structure elements (S, strands; H, helices; L, loops). Asterisks denote zinc-associated amino acid residues. The putative leader peptide is underlined.

strains does not contradict this hypothesis. Nevertheless, slight differences between the hydrolysis spectra of VIM-12 and VIM-1 cannot be excluded.

The simultaneous production of two potent β-lactamases, VIM-12 and a CMY-type cephalosporinase, by a *K. pneumoniae* clinical isolate was also of note. Similar to the case of *E. coli* V541, in a recent clinical isolate from Athens that also produced VIM-1 and CMY-13 (9) the acquired β-lactamases of *K. pneumoniae* 2873 were encoded by a single transferable plasmid. Given that combination of these enzymes inactivates all clinically available β-lactams, spread of the respective strains or plasmids may have serious consequences in the treatment of nosocomial infections.

Nucleotide sequence accession number. The nucleotide sequence described here has been submitted to the EMBL and GenBank nucleotide sequence databases under accession number DQ143913.

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