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MECHANISMS OF RESISTANCE

A Novel VIM-Type Metallo-β-Lactamase Variant, VIM-60, with Increased Hydrolyzing Activity against Fourth-Generation Cephalosporins in *Pseudomonas aeruginosa* Clinical Isolates in Japan

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ABSTRACT A novel VIM-type metallo-β-lactamase variant, VIM-60, was identified in multidrug-resistant *Pseudomonas aeruginosa* clinical isolates in Japan. Compared with VIM-2, VIM-60 had two amino acid substitutions (Arg228Leu and His252Arg) and higher catalytic activities against fourth-generation cephalosporins. The genetic context for bla_{VIM-60} was *intI1-*bla_{VIM-60}*-aadA1-aacA31-qacEdeltaI-sulI* on the chromosome.

KEYWORDS *Pseudomonas aeruginosa*, fourth-generation cephalosporins, metallo-β-lactamase

The emergence of metallo-β-lactamases (MBLs) and the increased carbapenem resistance among Gram-negative pathogens have become serious problems worldwide (1). MBLs, which are produced by many Gram-negative bacterial species (1) and by Gram-positive *Bacillus* spp. (2, 3), confer resistance or reduce bacterial susceptibility to carbapenems, cephalosporins, and penicillins, except for monobactams (1). Since the identification in 1997 of Verona integron-encoded metallo-β-lactamase-1 (VIM-1) in a strain of *Pseudomonas aeruginosa* in northern Italy (4), at least 59 other VIM variants have been identified in *Enterobacteriaceae*, *Acinetobacter baumannii*, and *P. aeruginosa* in several countries (ftp://ftp.ncbi.nlm.nih.gov/pathogen/betalactamases/Allele.tab). This study describes a novel VIM-type MBL, VIM-60, produced by two clinical isolates of *P. aeruginosa* in a medical setting in Japan.

P. aeruginosa NCGM3661 and NCGM3750 were isolated in 2017 from the urine samples of two inpatients. MICs were determined using the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute. The genomic DNA of these isolates were extracted and sequenced by a next-generation sequencer (MiSeq; Illumina, San Diego, CA). Multilocus sequence typing (MLST) was deduced, as described by the protocols of the PubMLST database (http://pubmlst.org/paeruginosa/). Sequences of drug resistance genes, including genes encoding β-lactamases (www.lahey.org/studies); aminoglycosides, chloramphenicol, and fosfomycin resistance genes registered in GenBank (https://www.ncbi.nlm.nih.gov/nuccore/); and quinolone resistance genes, were determined using CLC Genomics Workbench version 9.5. The contig sequence constructed by CLC Genomics Workbench was used as the genetic environment surrounding bla_{VIM-60}.

The bla_{VIM-2} and bla_{VIM-60} genes were amplified using the primer sets EcoRI-VIM-2-F (5′-ATGAATTCATGTTCAAACTTTTGAGTAAGT-3′) and PstI-VIM-2-R (5′-ATCTGCAGCTACTCAACGACTGAGCGATTT-3′). The PCR products ligated into pHSG398 (TaKaRa Bio, Shiga, Japan) were used to transform *Escherichia coli* DH5α (TaKaRa Bio, Shiga, Japan).

The open reading frames of VIM-2 and VIM-60 without signal peptide regions were

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TABLE 1 MICs of β-lactams for *P. aeruginosa* NCGM3661 and NCGM3750 and *E. coli* transformants expressing VIM-2 and VIM-60

Antibiotic	MIC (μg/ml) for:				
	P. aeruginosa		*E. coli* transformant		
	NCGM 3661	NCGM 3750	pHSG398/ VIM-2	pHSG398/ VIM-60	pHSG398
Ampicillin	>1,024	>1,024	32	64	2
Ampicillin-sulbactam	>1,024	>1,024	16	32	2
Penicillin G	>1,024	>1,024	64	128	32
Aztreonam	16	16	0.125	0.06	0.06
Cefepime	1,024	1,024	0.125	0.5	0.06
Cefmetazole	>1,024	>1,024	1	1	1
Cefotaxime	>1,024	>1,024	1	4	≤0.03
Cefoxitin	1,024	1,024	16	16	8
Cefozopran	>1,024	>1,024	0.5	4	0.06
Cefpirome	>1,024	1,024	0.125	1	0.06
Cefsulodin	>1,024	>1,024	512	1024	256
Ceftazidime	256	128	1	4	0.125
Ceftriaxone	512	512	1	4	≤0.03
Cefuroxime	>1,024	>1,024	32	64	4
Cephradine	>1,024	>1,024	32	64	8
Doripenem	512	512	≤0.03	0.06	≤0.03
Imipenem	512	512	0.25	0.25	0.125
Meropenem	1,024	1,024	≤0.03	0.125	≤0.03
Panipenem	256	512	0.125	0.25	0.06
Moxalactam	>1,024	>1,024	8	16	0.125

cloned into the pET28a expression vector (Novagen, Inc., Madison, WI) using the primer sets BamHI-VIM-2 (TEV) 79F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCGTAGATTCTAG CGGTGAGTATCC-3′) and XhoI-VIM-2 R (5′-ATCTCGAGCTACTCAACGACTGAGCGATTT-3′), as previously described (5). The plasmids were transformed into *E. coli* BL-21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA). Recombinant VIM proteins were purified using Ni-nitrilotriacetic acid (NTA) agarose. His tags were removed by digestion with TurboTEV protease (Accelagen, San Diego, CA), and untagged proteins were purified by an additional passage over the Ni-NTA agarose. The purities of VIM-2 and VIM-60 were >90%, as estimated by SDS-PAGE. The yields of VIM-2 and VIM-60 proteins were 1.105 and 1.209 mg/liter of culture, respectively. During the purification process, β-lactamase activity was monitored using nitrocefin (Oxoid Ltd., Basingstoke, UK). The initial rate of hydrolysis in 50 mM Tris-HCl (pH 7.4), 0.3 M NaCl, and 5 μM $Zn(NO_3)_2$ at 37°C was determined by UV-visible spectrophotometry (V-530; Jasco, Tokyo, Japan), with the reaction initiated by the addition of substrate into spectrophotometer cells and UV absorption measured during the initial phase of the reaction. K_m, k_{cat} and the k_{cat}/K_m ratio were determined using a Lineweaver-Burk plot, with K_m and k_{cat} determined using triplicate analyses. To determine whether bla_{VIM-60} is located on the plasmids or chromosomes, the DNA plugs of the isolates digested with S1 nuclease were separated by pulsed-field gel electrophoresis (PFGE), followed by Southern blotting and hybridization with a labeled bla_{VIM-60} probe (6).

Both strains, *P. aeruginosa* NCGM3661 and NCGM3750, were resistant to all β-lactamases tested (Table 1). In NCGM3661 and NCGM3750 isolates, the MICs of other antibiotics were 64 and 16 μg/ml for amikacin, 32 and 16 μg/ml for arbekacin, 4 and 8 μg/ml for gentamicin, 1,024 and 512 μg/ml for kanamycin, 32 and 16 μg/ml for tobramycin, 64 and 16 μg/ml for ciprofloxacin, 32 and 32 μg/ml for levofloxacin, <0.25 and 0.25 μg/ml for colistin, and >1,024 and >1,024 μg/ml for fosfomycin, respectively.

Both isolates were positive for VIM-type MBL. Whole-genome sequencing revealed that these isolates had a novel bla_{VIM} variant with two nucleotide substitutions (G614T and A686G) compared with bla_{VIM-2}, and the novel bla_{VIM} variant was designated bla_{VIM-60} (GenBank accession no. NG_061404). Its predicted amino acid sequence revealed that VIM-60 had two amino acid substitutions (Arg228Leu and His252Arg)

TABLE 2 Kinetic parameters of β-lactamases VIM-2 and VIM-60 with various substrates

Substrate	K_m (μM)[a]		k_{cat} (s−1)[a]		k_{cat}/K_m (μM s−1)[a]	
	VIM-2	VIM-60	VIM-2	VIM-60	VIM-2	VIM-60
Ampicillin	62 ± 6	160 ± 5	214 ± 8	345 ± 11	3.49	2.15
Penicillin G	72 ± 7	262 ± 9	252 ± 15	481 ± 10	3.51	1.83
Aztreonam	NH[b]	NH	NH	NH	NH	NH
Cefepime	200 ± 3	804 ± 83	7.9 ± 0.1	85 ± 8	0.040	0.11
Cefmetazole	51 ± 2	74 ± 7	1.9 ± 0.01	2.6 ± 0.2	0.037	0.035
Cefotaxime	41 ± 5	69 ± 1	121 ± 4	134 ± 2	3.00	1.93
Cefoxitin	67 ± 3	40 ± 3	4.1 ± 0.1	2.8 ± 0.02	0.061	0.069
Cefozopran	270 ± 8	133 ± 10	52 ± 1	195 ± 7	0.19	1.47
Cefpirome	246 ± 5	190 ± 2	88 ± 1	234 ± 2	0.36	1.23
Ceftazidime	71 ± 5	179 ± 6	2.1 ± 0.1	7.1 ± 0.2	0.030	0.040
Ceftriaxone	87 ± 2	54 ± 1	58 ± 1	33 ± 0.1	0.66	0.61
Cephradine	33 ± 0.5	94 ± 8	102 ± 1	68 ± 3	3.11	0.72
Doripenem	27 ± 1	27 ± 1	2.8 ± 0.01	2.3 ± 0.03	0.10	0.084
Imipenem	81 ± 7	40 ± 3	46 ± 1	13 ± 1	0.57	0.31
Meropenem	51 ± 2	68 ± 4	9.2 ± 0.1	16 ± 1	0.18	0.24
Panipenem	27 ± 3	39 ± 7	11 ± 0.2	3.6 ± 0.2	0.40	0.094
Moxalactam	79 ± 10	67 ± 1	87 ± 9	49 ± 0.2	1.10	0.73

[a]K_m and k_{cat} were calculated as means ± SD from three independent experiments.
[b]NH, no hydrolysis detected with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

compared with VIM-2. In addition to bla_{VIM-60}, several other drug resistance genes were present in both isolates, including *aadA1*, *aac(6′)-Ia*, *aac(6′)-31*, bla_{PAO}, bla_{OXA-50}, *cat*, *fosA*, and *sulI*. Both NCGM3661 and NCGM3750 had amino acid substitutions in the quinolone resistance-determining regions (QRDR) of GyrA (T83I) and ParC (S87L) but did not have the plasmid-mediated quinolone resistance factors, such as AAC(6′)-Ib-cr, Qep, and Qnr (7–9). Moreover, the two isolates had efflux pump systems associated with ciprofloxacin resistance, including MexA, MexC, and MexE (10).

These isolates belonged to sequence type (ST) 1816 (allelic profile: 40, 5, 11, 3, 4, 40, 2) and were related to ST3002 (allelic profile: 40, 101, 11, 3, 4, 40, 2). *P. aeruginosa* ST1816 was first isolated in Mexico in 2011 (11). Until now, *P. aeruginosa* ST1816 was isolated in 2014 in Japan (id-2248 in *P. aeruginosa* MLST database [https://pubmlst.org/paeruginosa/]).

The contig of 5,865 bp, including bla_{VIM-60}, was constructed after assembling the raw read data (GenBank accession no. DRA008130). The genetic environment surrounding bla_{VIM-60} was *intI1-*bla_{VIM-60}*-aadA1-aacA31-qacEdeltaI-sulI* (GenBank accession no. LC434516). The novel class 1 integron structure was deposited in INTEGRALL (http://integrall.bio.ua.pt/) under the number In1610. PFGE and Southern blotting and hybridization revealed that the *P. aeruginosa* isolates NCGM3661 and NCGM3750 had no plasmids harboring bla_{VIM-60}, indicating that bla_{VIM-60} is located on their chromosomes.

E. coli DH5α expressing bla_{VIM-60} was resistant to all cephalosporins, moxalactams, and penicillins and showed reduced susceptibility to carbapenems (Table 1). A comparison of the MICs of *E. coli* DH5α expressing bla_{VIM-2} and bla_{VIM-60} showed that bla_{VIM-60} was equally or more resistant to all antibiotics except aztreonam. In particular, the MICs to cefepime, cefotaxime, cefozopran, cefpirome, ceftazidime, and ceftriaxone of *E. coli* expressing bla_{VIM-60} were >4-fold higher than the MICs to bacteria expressing bla_{VIM-2}.

Recombinant VIM-type enzymes hydrolyzed all β-lactams tested except aztreonam. VIM-60 had significantly greater enzymatic activities than VIM-2 toward cefepime, cefozopran, and cefpirome, due primarily to their differences in k_{cat} values against these substrates (Table 2). In contrast, VIM-60 had significantly lower enzymatic activities against cephradine and panipenem than VIM-2, due primarily to differences in K_m values. The amino acid substitutions Arg228Leu and His252Arg seem to have a significant impact on the ability of VIM-60 to efficiently hydrolyze cefepime, cefozopran, and cefpirome. The amino acid residue at position 228 is located in the L3 loop and is

associated with the binding of substrates or inhibitors (12). VIM-24, with the amino acid substitution Arg228Leu, enhances resistance to ceftazidime and cefepime (13, 14). In contrast, the amino acid residue at position 252 is situated on the α4 helix, distant from the active site of the VIM enzyme. The His252Arg mutation may also contribute to tuning VIM activities and to resistance to fourth-generation cephalosporins.

To our knowledge, this is the first report describing VIM-60-producing Gram-negative pathogens in Japan. These findings indicate that VIMs have evolved in response to the use of fourth-generation cephalosporins, such as cefepime and cefpirome, in medical settings in Japan. Careful monitoring of VIM-producing pathogens is required.

Accession number(s). The whole-genome sequences of NCGM3661 and NCGM3750 have been deposited in GenBank under accession number DRA008130. The genetic environment surrounding bla_{VIM-60} has been deposited in GenBank under accession number LC434516.

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REFERENCES

1. Bush K. 2001. New β-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin Infect Dis 32:1085–1089. https://doi.org/10.1086/319610.
2. Queenan AM, Bush K. 2007. Carbapenemases: the versatile β-lactamases. Clin Microbiol Rev 20:440–458, table of contents. https://doi.org/10.1128/CMR.00001-07.
3. Schlesinger SR, Kim SG, Lee JS, Kim SK. 2011. Purification development and characterization of the zinc-dependent metallo-β-lactamase from *Bacillusanthracis*. Biotechnol Lett 33:1417–1422. https://doi.org/10.1007/s10529-011-0569-9.
4. Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, Rossolini GM. 1999. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob Agents Chemother 43:1584–1590. https://doi.org/10.1128/AAC.43.7.1584.
5. Tada T, Miyoshi-Akiyama, K Shimada T, Kirikae T. 2014. Biochemical analysis of the metallo-β-lactamase NDM-3 from a multidrug-resistant *Escherichia coli* strain isolated in Japan. Antimicrob Agents Chemother 58:3538–3540. https://doi.org/10.1128/AAC.02793-13.
6. Barton BM, Harding GP, Zuccarelli AJ. 1995. A general method for detecting and sizing large plasmids. Anal Biochem 226:235–240. https://doi.org/10.1006/abio.1995.1220.
7. Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC. 2006. Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob Agents Chemother 50:3953–3955. https://doi.org/10.1128/AAC.00915-06.
8. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC. 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med 12:83–88. https://doi.org/10.1038/nm1347.
9. Martínez-Martínez L, Pascual A, Jacoby GA. 1998. Quinolone resistance from a transferable plasmid. Lancet 351:797–799. https://doi.org/10.1016/S0140-6736(97)07322-4.
10. Adabi M, Talebi-Taher M, Arbabi L, Afshar M, Fathizadeh S, Minaeian S, Moghadam-Maragheh N, Majidpour A. 2015. Spread of efflux pump overexpressing-mediated fluoroquinolone resistance and multidrug resistance in *Pseudomonas aeruginosa* by using an efflux pump inhibitor. Infect Chemother 47:98–104. https://doi.org/10.3947/ic.2015.47.2.98.
11. Morales-Espinosa R, Delgado G, Espinosa LF, Isselo D, Méndez JL, Rodriguez C, Miranda G, Cravioto A. 2017. Fingerprint analysis and identification of strains ST309 as a potential high risk clone in a *Pseudomonas aeruginosa* population isolated from children with bacteremia in Mexico City. Front Microbiol 8:313. https://doi.org/10.3389/fmicb.2017.00313.
12. Yamaguchi Y, Jin W, Matsunaga K, Ikemizu S, Yamagata Y, Wachino J, Shibata N, Arakawa Y, Kurosaki H. 2007. Crystallographic investigation of the inhibition mode of a VIM-2 metallo-β-lactamase from *Pseudomonas aeruginosa* by a mercaptocarboxylate inhibitor. J Med Chem 50:6647–6653. https://doi.org/10.1021/jm701031n.
13. Montealegre MC, Correa A, Briceño DF, Rosas NC, De La Cadena E, Ruiz SJ, Mojica MF, Camargo RD, Zuluaga I, Marin A, Quinn JP, Villegas MV, Colombian Nosocomial Resistance Study Group. 2011. Novel VIM metallo-β-lactamase variant, VIM-24, from a *Klebsiella pneumoniae* isolate from Colombia. Antimicrob Agents Chemother 55:2428–2430. https://doi.org/10.1128/AAC.01208-10.
14. Mojica MF, Mahler SG, Bethel CR, Taracila MA, Kosmopoulou M, Papp-Wallace KM, Llarrull LI, Wilson BM, Marshall SH, Wallace CJ, Villegas MV, Harris ME, Vila AJ, Spencer J, Bonomo RA. 2015. Exploring the role of residue 228 in substrate and inhibitor recognition by VIM metallo-β-lactamases. Biochemistry 54:3183–3196. https://doi.org/10.1021/acs.biochem.5b00106.