Carbapenem Resistance among *Pseudomonas aeruginosa* Strains from India: Evidence for Nationwide Endemicity of Multiple Metallo- β -Lactamase Clones (VIM-2, -5, -6, and -11 and the Newly Characterized VIM-18)[∇]

Mariana Castanheira,^{1*} Jan M. Bell,² John D. Turnidge,² Dillip Mathai,³ and Ronald N. Jones^{1,4}

JMI Laboratories, North Liberty, Iowa 52317¹; Women's and Children's Hospital, Adelaide, Australia²; Christian Medical College, Vellore, India³; and Tufts University School of Medicine, Boston, Massachusetts 02110⁴

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Among 57 metallo- β -lactamase (M β L)-producing *Pseudomonas* spp. detected in India during 2006, five *bla*_{VIM} genes were found, including a newly characterized *bla*_{VIM} gene. *Pseudomonas aeruginosa* strains were clustered in 33 ribotypes with clones found in multiple hospitals. Several types of *bla*_{VIM-2}-carrying integrons were detected. The newly characterized variant VIM-18 showed a 4-amino-acid deletion compared to other VIM variants. In this study, we show that VIM-producing *Pseudomonas* spp. were highly prevalent in India with a great diversity of *bla*_{VIM} types and M β L-carrying integrons.

Among the types of metallo- β -lactamases (M β Ls) reported to date, IMP and VIM variants are the most prevalent and have been described from numerous geographic locations (9). VIM-2 was first reported from a *Pseudomonas aeruginosa* strain recovered during 1996 in a hospital located in Marseille, France (5). Among VIM enzymes, VIM-2 appears to be the most dominant allelic variant and isolates carrying this enzyme have been described in more than 23 countries, including India (7).

In this study, initiated to characterize M β L-producing *Pseudomonas* isolates, we observed that such strains are endemic in India, with a great diversity of M β L types (five VIM variants) and different M β L-carrying integrons. In addition we characterized a VIM variant, herein named VIM-18.

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A total of 301 *Pseudomonas* isolates were consecutively collected as previously described (3) in 10 Indian hospitals. The isolates were susceptibility tested against 28 antimicrobial agents according the Clinical and Laboratory Standards Institute (1) broth microdilution procedure and interpretation criteria. Among those isolates, 107 (35.5%) showed elevated MICs for imipenem or meropenem (MIC, ≥ 8 μ g/ml). These isolates belonged to five different species: *P. aeruginosa* (97/107), *Pseudomonas putida* (7/107), and *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, and *Pseudomonas mendocina* (1/107 each). Isolates nonsusceptible to imipenem or meropenem (MIC, ≥ 8 μ g/ml) were tested with a multiplex PCR strategy, as recently described (4). Amplicons obtained were sequenced on both strands and analyzed.

M β L genes were detected in 57 (53.2% [18.9% of overall

Pseudomonas spp.]) of the carbapenem-nonsusceptible strains. Five *bla*_{VIM} variants were detected, and *bla*_{VIM-2} was detected in 38 (66.7% of the M β L producers) isolates, including 35 *P. aeruginosa* isolates, 2 *P. putida* isolates, and 1 *P. stutzeri* isolate. The second most common VIM-encoding gene was *bla*_{VIM-6}, found in 12 (21.0%) *P. aeruginosa* isolates. *bla*_{VIM-11} was detected in one *P. aeruginosa* isolate and one *P. stutzeri* isolate, while *bla*_{VIM-5} was observed in one *P. aeruginosa* isolate. Additionally, one *P. aeruginosa* isolate was found to carry a new VIM variant, named VIM-18.

VIM-producing isolates were detected in 9 of 10 participating medical institutions, and VIM-2-producing strains were observed in 8 of the 10 hospitals (Table 1). In 5 of 10 hospitals, isolates harbored multiple M β L types (Table 1). VIM-encoding genes were detected in non-*P. aeruginosa* *Pseudomonas* isolates in three medical centers (Indore, Mumbai, and Rajkot). The VIM-2-producing *P. putida* and *P. stutzeri* isolates (two isolates and one isolate, respectively) were detected in hospitals where *P. aeruginosa* isolates carrying the same *bla*_{VIM} gene were present; however, in one hospital (Rajkot) only a *P. stutzeri* isolate carrying *bla*_{VIM-11} was detected.

M β L-producing isolates were ribotyped using the Riboprinter microbial characterization system (Qualicon, Wilmington, DE). Isolates were clustered in 33 ribogroups, and 12 clones were detected. Seven of these clones were detected in multiple institutions. Six clones had isolates carrying distinct *bla*_{VIM} genes and were further evaluated by pulsed-field gel electrophoresis, as previously described (6). The results obtained demonstrated that these isolates were not genetically related. Pulsed-field gel electrophoresis is usually considered to have a better discriminatory power for *Pseudomonas* spp.(8); however, ribotyping has been shown to be a valuable tool to identify long-term epidemiological events for other bacterial species, such as isolates from geographically distinct sources, like in this study, or over a broad time frame (2). Thus, clonal strains showing different VIM alleles could have had the same ancestor, but these isolates could have more recently

* Corresponding author. Mailing address: 345 Beaver Creek Centre, Suite A, North Liberty, IA 52317. Phone: (319) 665-3370. Fax: (319) 665-3371. E-mail: mariana-castanheira@jmlabs.com.

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TABLE 1. Distribution of carbapenem-resistant *Pseudomonas* spp. in medical centers in various Indian cities participating in the SENTRY Program in 2006

Site (no. of spp./ carbapenem- nonsusceptible strains)	MβL type (no. of isolates)	<i>Pseudomonas</i> sp.	Ribotype(s) (no. of clonal isolates within medical center) ^a
Kolkatta (23/7)	VIM-2 (3) VIM-5 (1) VIM-18 (1)	<i>P. aeruginosa</i> <i>P. aeruginosa</i> <i>P. aeruginosa</i>	<u>222-134-4</u> , <u>252-45-6</u> , <u>258-179-8</u> 105-528-6 258-347-2
Mumbai (27/9)	VIM-2 (2) VIM-6 (2) VIM-11 (1)	<i>P. aeruginosa</i> <i>P. stutzeri</i> <i>P. aeruginosa</i> <i>P. aeruginosa</i>	258-350-8, 258-351-2 <u>222-134-4</u> , 258-147-6 <u>105-1600-6</u>
Indore (23/12)	VIM-2 (3) VIM-6 (5)	<i>P. aeruginosa</i> <i>P. putida</i> <i>P. aeruginosa</i>	<u>252-45-6</u> , 258-179-8, 258-347-7 258-347-6, 258-347-8, 258-352-2 (3)
Hyderabad (37/18)	VIM-2 (3) VIM-6 (2)	<i>P. aeruginosa</i> <i>P. aeruginosa</i>	<u>105-528-6</u> , <u>252-45-6</u> , <u>258-179-8</u>
Rajkot (24/8)	VIM-2 (4) VIM-11 (1)	<i>P. aeruginosa</i> <i>P. putida</i> <i>P. stutzeri</i>	<u>105-528-6</u> (2), 258-231-1, 258-351-3
New Delhi (44/15)	VIM-2 (8)	<i>P. aeruginosa</i>	105-566-6 (2), <u>105-1600-6</u> , 127-114-4, <u>252-45-6</u> , 258-179-8, 258-380-1, 258-380-2
Kochi (31/10)	VIM-2 (5)	<i>P. aeruginosa</i>	<u>105-1600-6</u> , 258-179-8, 258-349-6, 258-349-7, 258-349-8
Manipal (47/14)	VIM-2 (10)	<i>P. aeruginosa</i>	105-528-6, 105-774-2, 105-781-7, <u>105-1600-6</u> , 127-277-1, 258-147-6, 258-350-3 (2), 258-350-4 (2)
Trivandrum (20/3)	VIM-6 (2)	<i>P. aeruginosa</i>	258-348-6 (2)
Chennai (25/11) ^b	None		

^a The ribotypes underlined were found in multiple medical centers.^b No MβL-producing *Pseudomonas* spp. isolates were found in Chennai.

evolved and acquired distinct *bla*_{VIM}-carrying genetic elements.

MβL-carrying integrons were evaluated, and PCR experiments using primers annealing into *bla*_{VIM-2} and the *qacEΔI* gene showed amplicons in 12 isolates that ranged from 2.0 to 2.2 kb in size. In several isolates, reactions failed to amplify *bla*_{VIM-2}-carrying class 1 integrons when primers annealing to the 3' conserved sequence (3'CS) were used. However, reactions using *bla*_{VIM} primers in combination with primers for *tniC*, which was found to be associated with *bla*_{VIM-2}-carrying integrons in India (7), amplified 21 of the 38 VIM-2-producing isolates. Amplicons of six molecular sizes were detected (1.2, 1.8, 2, 2.2, 3.5 and 4 kb). Sau96I restriction digestions of these amplicons generated identical patterns for all of the 2-kb integrons (11 isolates from five hospitals), indicating that they possess an identical gene arrangement. Additionally, integrons of 1.8 kb detected in two isolates from the same hospital also showed identical Sau96I patterns. Only one isolate showed a Sau96I restriction profile, as expected for the *bla*_{VIM-2}-carrying integron previously reported in India (GenBank accession no. AM296017) (7). *bla*_{VIM-2} was carried in the first or second position of the integrons harboring the integrase gene (*intI1*) in the 5'CS. All *bla*_{VIM-6} strains possessed the 5'CS and 3'CS typical of class 1 integrons. Integrons harboring *bla*_{VIM-6} genes of 3.5 and 5 kb (five and seven isolates, respectively) were

detected. Both integron types were identified in Indore and Hyderabad.

The gene encoding VIM-18 was identified in a *P. aeruginosa* (243-31C) strain isolated from a patient sputum specimen (Kolkatta). The susceptibility profile for isolate 243-31C is presented in Table 2. *P. aeruginosa* isolate 243-31C was genetically distinct from other MβL-producing strains from India (Table 1). *bla*_{VIM-18} was carried as a single gene cassette in a class 1 integron that contained the integrase gene in the 5'CS and the *qacEΔI/sul1* gene in the 3'CS. This integron was flanked upstream by a resolvase gene (*tnpR*) from a Tn5051-like transposon. Isolate 243-31C appeared to be without plasmid DNA in preparations carried out with the Plasmid DNA Midi kit; Qiagen, Hilden, Germany).

VIM-18 showed the highest homology to VIM-6 (99.6%), followed by VIM-2, -3, and -11 (99.2%). Amino acid sequence analysis demonstrated that VIM-18 possessed 262 amino acids and contained a deletion of 4 amino acids in position 140 compared with other VIM enzymes. *bla*_{VIM-18} cloned into pPCRScriptCam SK+ (Stratagene Cloning Systems, La Jolla, CA) and expressed in XL10-Gold Kan ultracompetent *Escherichia coli* cells showed lower MICs for ampicillin, cefoxitin, cefazolin, and ertapenem (>3-, >6-, 4-, and 9-fold, respectively) compared with VIM-2 expressed in the same background (Table 2). MICs for imipenem, meropenem, piperacil-

TABLE 2. Antimicrobial susceptibility profile of the VIM-18-producing *P. aeruginosa* clinical isolate and β -lactam susceptibility profiles of the *E. coli* strain carrying the recombinant plasmid vector pPCRScrip with or without *bla*_{VIM-18} and *bla*_{VIM-2} inserts

Antimicrobial agent	MIC (μ g/ml) for ^a :			
	<i>P. aeruginosa</i> 243-31C	<i>E. coli</i>		
		pPCRScrip carrying <i>bla</i> _{VIM-18}	pPCRScrip carrying <i>bla</i> _{VIM-2}	pPCRScrip
Imipenem	8	0.5	0.5	0.25
Meropenem	8	0.064	0.125	0.032
Ertapenem	>8	0.016	0.25	0.006
Doripenem	4	NT	NT	NT
Ampicillin	>16	64	>256	4
Piperacillin	>64	8	4	2
Cefoxitin	>16	8	>256	4
Cefazolin	>16	32	>256	2
Cefotaxime	NT	2	4	0.064
Ceftriaxone	>32	NT	NT	NT
Ceftazidime	>16	16	4	0.125
Cefepime	>16	0.125	0.5	0.032
Aztreonam	16	0.032	0.032	0.032
Piperacillin-tazobactam	>64	NT	NT	NT
Amikacin	>32	NT	NT	NT
Gentamicin	8	NT	NT	NT
Tobramycin	>16	NT	NT	NT
Ciprofloxacin	0.06	NT	NT	NT
Tetracycline	>8	NT	NT	NT
Polymyxin B	1	NT	NT	NT

^a *P. aeruginosa* isolate 243-31C was tested by broth microdilution, and *E. coli* strains carrying pPCRScrip with and without the inserts were tested with Etest strips. NT, antimicrobial agent was not tested.

lin, cefotaxime, ceftazidime, and cefepime were similar for both M β L enzymes (Table 2).

This initial nationwide M β L survey for India suggests a complex dissemination pattern and evolution of VIM enzymes.

The high prevalence, continuous dissemination, and expansion of these resistance determinants in this region pose a serious therapeutic challenge.

Nucleotide sequence accession number. The nucleotide sequence of *bla*_{VIM-18} has been submitted to GenBank nucleotide database and assigned accession no. AM778091.

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