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High Carbapenem Resistance Among *Pseudomonas aeruginosa* from India: A National Epidemic of Multiple Metallo-ß-Lactamase Clones (VIM-2, -5, -6, -11 and new VIM-18)

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SENTRY ANTIMICROBIAL SURVEILLANCE

AMENDED ABSTRACT

Objectives: We assessed the prevalence of metallo-ß-lactamase (MßL)-encoding genes among carbapenem-resistant *P. aeruginosa* isolates recovered in India during 2006. In addition, class 1 integrons harbouring MßL genes were amplified and compared, and MßL-positive isolates were molecular typed to evaluate clonal dissemination.

Methods: A total of 282 *P. aeruginosa* were consecutively collected in 10 Indian medical centers and tested by CLSI broth microdilution methods. Isolates resistant to imipenem or meropenem (MIC, ≥8 mg/L) were screened for MßL-encoding genes by real-time PCR. Positive samples were tested with primers targeting the class 1 integron conserved structures anchoring in the MßL genes. Amplicons generated were sequenced on both strands and MßL isolates ribotyped for possible clonality.

Results: Among the 282 isolates, 96 (34%) showed carbapenem resistance and MßL genes were detected in 53 strains (19% of total; 55% of carbapenem-resistant). MßL-producing *P. aeruginosa* were detected in 9 of 10 hospitals. Five bla_{VIM} genes were found, including a new variant named bla_{VIM-18}. This new MßL gene showed a 12-bp deletion (position 428) when compared to bla_{VIM-2} and was carried as a single gene cassette in a class 1 integron. VIM-2 producing isolates were most common (38 strains) and were detected in 8 medical centers. VIM-6 was identified in 12 isolates from 4 sites. VIM-5, VIM-11 and VIM-18 were found only once. Two medical centers had 3 distinct MßL types and other 2 had 2 MßL types. Wide genetic diversity was noted among MßL-carrying *P. aeruginosa* with 21 and 10 ribotypes seen among VIM-2- and -6-producers, respectively. Seven clones were found in ≥1 participant hospital and 6 clones were noted within institutions. bla_{VIM-6}-carrying integrons of 3.5 and 5 Kb (5 and 7 isolates, respectively) were detected and 2 sites had both bla_{VIM-6} integron types.

Site	CARB-R[a]/ MßL (%)	MßL types (No. of isolates)	MßL clonality: Intra	Inter	No. of clones
Kolkatta	30/22	VIM-2 (3), -5 (1), 18 (1)	0	2	2
Indore	42/42	VIM-2 (3), -6 (5)	1	2	3
New Delhi	34/18	VIM-2 (8)	1	3	4
Trivandrum	11/11	VIM-6 (2)	1	0	1
Hyderabad	51/17	VIM-2 (3), -6 (2)	1	3	4
Chennai[b]	46/0	None	-	-	-
Kochi	30/20	VIM-2 (5)	0	2	2
Manipal	30/20	VIM-2 (10)	2	3	5
Mumbai	30/22	VIM-2 (3), -6 (2), -11 (1)	0	3	3
Rajkot	25/25	VIM-2 (4)	0	1	1

a. CARB-R: carbapenem-resistant
b. Clones of non-MBL CARB-R *P. aeruginosa* were documented and VIM-2 by Toleman et al. (2007) from a 2003 isolate.

Conclusions: MßL-producing isolates have recently been reported in India, but limited data exists on the prevalence and characterization of MßL-encoding genes. In this study, we show that MßL-producing *P. aeruginosa* are epidemic in India with a great diversity of MßL-types (5 VIM types, including new VIM-18). In addition, different MßL-carrying integrons were observed, suggesting widespread dissemination of MßL-carrying mobile elements. Carbapenem resistance rates among *P. aeruginosa* were elevated and were principally caused by MßL production (55%).

INTRODUCTION

Metallo-ß-lactamases (MßLs) constitute one of the most important carbapenem resistance mechanisms found in *Pseudomonas aeruginosa*. These enzymes can hydrolyze the vast majority of available ß-lactam agents available for clinical use and are not inhibited by ß-lactamase inhibitors currently marketed or in development. Additionally, the acquired genes encoding these enzymes are carried in mobile genetic structures that usually harbor other resistance elements and facilitate the dissemination of the MßL genes.

Among the six types of MßLs reported to date (IMP, VIM, SPM-1, GIM-1, SIM-1 and AIM), IMP- and VIM-variants are the most prevalent and have been described in numerous geographic locations. Among the VIM-types, VIM-2 appears to be the most dominant genotype and has been described in 23 countries, including India. Only small numbers of isolates, however, have been evaluated and limited data on the prevalence and characterization of MßL-encoding genes is available from this country.

In this study, initiated to characterize MßL-producing *P. aeruginosa* isolates collected during 2006, we describe a high prevalence of VIM-type enzymes and the dissemination of these genes in numerous Indian medical centers.

MATERIALS AND METHODS

Bacterial isolates. During 2006, 10 medical centers located in India were recruited to participate in the SENTRY Antimicrobial Surveillance Program. *P. aeruginosa* isolates were consecutively collected from bloodstream, respiratory tract, skin and soft tissue, and urinary tract infections according to defined protocols. Only clinically significant isolates were included in the study; one per patient episode. Species identification was confirmed by standard biochemical tests and use of Vitek System (bioMérieux; Hazelwood, Missouri, USA), when necessary.

Susceptibility testing. All isolates were susceptibility tested against more than 25 antimicrobials by the broth microdilution procedure described by the Clinical and Laboratory Standards Institute (CLSI, 2006) using validated panels manufactured by TREK Diagnostics (Cleveland, Ohio, USA). Interpretations of susceptibility testing results were by CLSI (2008) breakpoint criteria. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were concurrently tested for quality assurance.

MßL detection. *P. aeruginosa* isolates non-susceptible to imipenem and meropenem (MIC, ≥8 mg/L) were tested with multiplex PCR using MßL generic primers in a Real-time platform, recently described. Amplicons obtained were sequenced on both strands. The nucleotide sequences and deduced amino acid sequences were analyzed using Lasergene software package (DNASTAR, Madison, WI) and compared with the sequences available through the internet using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

Class 1 integron characterization. bla_{VIM-6} and bla_{VIM-18}-carrying integrons were further analyzed. Primers designed in the 5' and 3' conserved sequence (CS) regions of class 1 integrons were used in combination with the MßL primers to determine the size and structure of the integron. One integron of each type was fully sequenced. Restriction patterns generated with *DraI* and *XbaI* were used to confirm the integron arrays in the bla_{VIM-6} remaining strains.

Molecular Typing. MßL-producing isolates were ribotyped using the Riboprinter Microbial Characterization System® (Qualicon, Wilmington, Delaware). Overnight cultures were treated with lysis buffer and placed into the automated system. In brief, this automated process includes bacterial cell lysis, cleavage of DNA using the restriction enzime *PvuII*, size separation using gel electrophoresis and modified Southern blotting. Results were analyzed by the Riboprinter and isolates were considered to have the same ribotype if the similarity coefficient was ≥0.93.

RESULTS

- Ninety-six of 282 (34.0%) *P. aeruginosa* isolates collected in 2006 from medical center sites participating on the SENTRY Program (India) showed elevated MIC values for carbapenems (MIC, ≥8 mg/L).
- MßL genes were detected in 53 (55.2%) carbapenem-resistant strains (18.8% overall). All MßL-carrying strains possessed VIM-encoding genes. These isolates were detected in 9 of 10 participating institutions (Table 1).
- Sequencing of the MßL gene showed five bla_{VIM} variants. bla_{VIM-2} was the most common, being detected in 38 (71.7% of the MßL-producers) isolates collected from 8 of 10 medical centers sampled.
- bla_{VIM-6} was detected in 12 (22.7%) strains from 4 Indian hospitals. bla_{VIM-5} and bla_{VIM-11} were detected in one isolate each (1.8%).
- A new variant, named bla_{VIM-18}, was identified in one strain. This MßL gene showed a 12-bp deletion (position 428) when compared to bla_{VIM-2} and was carried as a single gene cassette in a class 1 integron.
- Medical centers located in Kolkata and Mumbai had 3 VIM-types and the isolates from Indore and Hyderabad had 2 distinct VIM-variants (Figure 1).
- Thirty-one different ribotypes and 12 clones (Figure 2) were identified among the VIM-producing *P. aeruginosa*. Six of these clones had isolates carrying distinct bla_{VIM} genes. Furthermore, 7 of 12 VIM-producing *P. aeruginosa* clones were detected in multiple institutions (Figure 2), indicating inter-hospital dissemination.
- Integrons carrying bla_{VIM-6} of 3.5 and 5 Kb (5 and 7 isolates, respectively) were characterized further. Both integron types were identified in Indore and Hyderabad (Figure 1).
- VIM-encoding genes were detected in other *Pseudomonas* species in three medical centers (Indore, Mumbai and Rajkot). Two *P. putida* carrying bla_{VIM-2} and two *P. stutzeri*, one harboring bla_{VIM-2} and the other carrying the bla_{VIM-11} were detected.

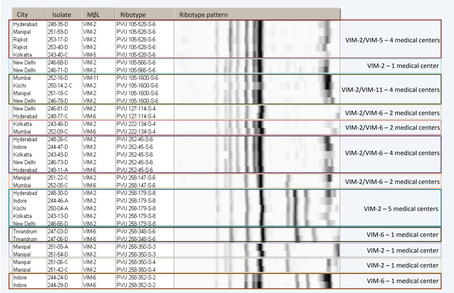
Table 1. Distribution of carbapenem-resistant *Pseudomonas* spp. in medical centers located in different Indian cities participating in the SENTRY Program for 2006.

	Number and species of VIM-producing *Pseudomonas* isolates in Indian medical centers (total number of carbapenem-resistant isolates)									
MßL enzymes	Kolkatta (30)	Indore (42)	New Delhi (34)	Trivandrum (11)	Hyderabad (51)	Chennai (46)	Kochi (30)	Manipal (30)	Mumbai (30)	Rajkot (25)
VIM-2	3 PSA	3 PSA 1 PPU	8 PSA		3 PSA		6 PSA	10 PSA	2 PSA 1 PST	4 PSA 1 PPU
VIM-5	1 PSA									
VIM-6		5 PSA		2 PSA	3 PSA				2 PSA	
VIM-11									1 PSA	1 PST
VIM-18	1 PSA									
PSA with negative MßL result	25	34	26	9	45	46	24	20	25	21

PSA= *P. aeruginosa*
PST= *P. stutzeri*
PPU= *P. putida*

Figure 1. Geographic dissemination of acquired VIM-producing *Pseudomonas* spp. recovered from medical centers in India. Indicated cities represent the location of the participating medical centers and the VIM-types detected. Locations with VIM-6 underlined had isolates with different bla_{VIM-6} carrying integrons (3.5 and 5 Kb).

Figure 2. Ribotyping patterns of clonal bla_{VIM}-carrying *P. aeruginosa* isolated in 2006 from Indian medical centers. Twelve clones were detected, six involving distinct VIM-enzymes and medical centers (each clone is indicated in a different color). Eighteen bla_{VIM}-carrying isolates showing unique ribotypes are not presented here.



CONCLUSIONS

- A high prevalence of MßL-producing isolates was observed in Indian medical centers (approximately 20% of all *P. aeruginosa*), with only one medical center having no MßL-producing strains.
- *P. aeruginosa* isolates from India were found to harbor distinct bla_{VIM}-types; interestingly, the amino acid sequences of VIM-2, VIM-6, VIM-11 and VIM-18 are very similar, suggesting that this enzymes could be derived from a unique ancestor.
- Despite genetic diversity recognized among the MßL-producing isolates, inter-hospital dissemination was observed with several clones presenting distinct VIM-types.
- This initial nationwide MßL survey for India suggests a complex dissemination pattern and evolution of VIM-enzymes. The high prevalence and continuous dissemination of these resistance determinants in this region is a serious therapeutic dilemma that compromises the utility of carbapenem-class agents and many other classes of ß-lactams.

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