

Detection and Characterization of Metallo- β -lactamases in *Pseudomonas aeruginosa* by Phenotypic and Molecular Methods from Clinical Samples in a Tertiary Care Hospital

K Arunagiri¹, B Sekar¹, G Sangeetha¹, J John²

ABSTRACT

Aims: The aim of this study was to detect and characterize the presence of metallo- β -lactamase (MBL) production in multidrug resistant (MDR) *P aeruginosa* collected from clinical samples in a tertiary care hospital.

Methods and materials: A total of 67 non-repetitive isolates of MDR *P aeruginosa* recovered from various clinical specimens were screened for MBL production by IPM/MEM-EDTA combined disc test. Polymerase chain reaction was performed on all isolates using *bla*_{IMP} and *bla*_{VIM} consensus primers to characterize them genotypically.

Results: Among 67 *P aeruginosa* isolates, 62.7% (42/67) and 70.1% (47/67) were resistant to imipenem and meropenem respectively and 47 (70.1%) were found to be MBL producers. Among this 47 MBL-producing isolates, 41 (61.1%) strains carried the *bla*_{VIM} gene and 2 (3%) strains carried the *bla*_{IMP} gene. Three strains were phenotypically negative but positive genotypically for *bla*_{VIM} gene. One strain was resistant to both imipenem and meropenem but did not show phenotypic positivity.

Conclusion: This study confirms the dissemination of *bla*_{VIM} genes among MDR *Pseudomonas aeruginosa* and hence it is indispensable to identify and aptly control the threat of horizontal and vertical transfer.

Keywords: Combined disc test, *bla*_{IMP}, *bla*_{VIM}, IPM/MEM-EDTA, metallo-beta-lactamase, *Pseudomonas aeruginosa*

Detección y Caracterización de la Metallo-betalactamasa en la *Pseudomonas aeruginosa* Mediante Métodos Fenotípicos y Moleculares a Partir de Muestras Clínicas en un Hospital de Atención Terciaria

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RESUMEN

Objetivo: El objetivo de este estudio es descubrir y caracterizar la presencia de producción de metallo-betalactamasa (MBL) en *P aeruginosa* resistente a los multifármacos (RMF), recogida de muestras clínicas de un hospital de atención terciaria.

Método: Un total de 67 aislados no repetitivos de *P aeruginosa* RMF obtenidos de varios especímenes clínicos, fueron tamizados en busca de producción de MBL, mediante una prueba de disco combinado IPM/MEM-EDTA. Se efectuó una reacción en cadena de la polimerasa sobre todos los aislados, usando iniciadores de consenso *bla*_{IMP} y *bla*_{VIM} para la caracterización genotípica.

Resultados: Entre los aislados de *P aeruginosa*, 62.7% (42/67) y 70.1% (47/67) fueron resistentes al Imipenem y al Meropenem respectivamente, mientras que se halló que 47 (70.1%) eran productores de MBL. De los 47 aislados productores de MBL, 41 (61.1%) cepas eran portadoras del gen *bla*_{VIM} en tanto que 2 (3%) cepas eran portadoras del gen *bla*_{IMP}. Tres cepas fueron fenotípicamente negativas, pero genotípicamente positivas con respecto al gen *bla*_{VIM}. Una cepa fue resistente tanto al Imipenem como al Meropenem, pero no mostró positividad fenotípicamente.

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Conclusión: El presente estudio confirma la diseminación de los genes *bla_{VIM}* entre las *Pseudomonas aeruginosa* RMF. Es importante identificar así como controlar adecuadamente la amenaza de la transferencia horizontal y vertical.

Palabras claves: gen *bla_{IMP}*, gen *bla_{VIM}*, prueba de disco combinado, IPM/MEM-EDTA, metallo-betalactamasa, *Pseudomonas aeruginosa*

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INTRODUCTION

Pseudomonas aeruginosa is one of the most important pathogens causing nosocomial infections; it is naturally resistant to many antimicrobial agents. It has a distinctive capacity to become resistant to many available antimicrobial agents via multiple mechanisms (1). With the widespread use of extended-spectrum antibiotics, in a very short time span *P aeruginosa* has become resistant to a variety of antimicrobial agents, such as β -lactams, aminoglycosides, chloramphenicol, quinolones, tetracyclines and sulphonamides. Emerging resistance to expanded-spectrum cephalosporins and carbapenems among *P aeruginosa* has been a major concern (2).

Carbapenems such as imipenem, panipenem and meropenem are the most potent agents for treatment of gram-negative infections due to stability of these agents against the majority of β -lactamases including extended-spectrum β -lactamases (ESBLs) and their high rate of permeation through bacterial outer membranes (3). On a molecular level, these β -lactamases belong either to class A, C and D which includes those with serine at their active site, whereas molecular class B β -lactamases are all metalloenzymes with an active-site zinc (4). The carbapenem-hydrolyzing enzymes were identified as metallo- β -lactamases (MBLs) belonging to Ambler's class B or to group 3 (4). Most of these MBLs confer resistance not only to carbapenems but also to other β -lactams and are hardly blocked by suicide β -lactamase inhibitors, such as clavulanate, sulbactam and tazobactam (5).

Nosocomial outbreaks of carbapenem-resistant *P aeruginosa* due to MBLs production of the IMP, VIM, SPM, SIM and GIM families are being increasingly reported. To date, five genotypes of MBL-producing *P aeruginosa* have been reported in many countries (6). New subtypes of IMP and VIM are constantly appearing. To date, 21 subtypes of IMP, 11 of VIM and single members of the SPM, GIM and SIM families has been identified (7).

Among the class B metallo enzymes, two carbapenem-hydrolyzing β -lactamases have been widely genetically characterized in *Pseudomonas aeruginosa*: IMP and VIM. Both enzymes possess the broadest substrate of hydrolysis range. IMP-1 production demonstrated a wide range of resistance to various broad-spectrum β -lactams including the oxyimino cephalosporins, cephamycins, and carbapenems and hence early recognition of IMP-1 producers is very important for rigorous infection control (8, 9). Similarly,

VIM-1 production was associated with a significant decrease in the *in vitro* susceptibility of the host to ampicillin, carbenicillin, piperacillin, mezlocillin, cefotaxime, ceftazidime, cefoperazone, ceftazidime, imipenem and meropenem but not to aztreonam indicating that the enzyme has a very broad substrate specificity and can contribute to broad-spectrum β -lactam resistance in the microbial host (10). This study primarily identifies the prevalence of carbapenem resistance in *P aeruginosa* and the associated genotype. This will aid in choosing appropriate therapy and in formulation of anti-microbial policy.

MATERIALS AND METHODS

Bacterial isolates and testing

A total of 67 non-repetitive isolates of multi-drug resistant (MDR) *P aeruginosa* were recovered from various clinical specimens in a tertiary care hospital in Chennai.

All the isolates were tested against various antibiotics such as ceftazidime, ceftazidime, cephoxitin, piperacillin/tazobactam, amikacin, imipenem, and meropenem (HiMedia, Mumbai) by Kirby Bauer disc diffusion test as per the Clinical and Laboratory Standards Institute (CLSI) guidelines. Plates were incubated at 37 °C for 18–20 hours. Diameter of zone of inhibition was noted and compared with CLSI standards.

Screening for MBL Production

IPM-EDTA/MEM-EDTA combined disc test (CDT)

A 0.5 M EDTA solution was prepared by adding 1.86 g of disodium EDTA in 10 mL of distilled water and pH was adjusted to 8.0 by using NaOH and sterilized by autoclaving. Test organisms were inoculated on plates of Muller Hinton agar (HiMedia, Mumbai). Opacity was adjusted to 0.5 McFarland opacity standards.

Two imipenem (10 μ g) and meropenem (10 μ g) discs were placed on inoculated plates and 5 μ L of EDTA solution was added to one of each imipenem and meropenem disc. The zone of inhibition around individual imipenem and meropenem discs and those with EDTA was recorded and compared after 16–18-hour incubation at 37 °C. An increase in zone size of at least 7 mm around the imipenem-EDTA disc and meropenem EDTA discs was considered as a positive result [Fig. 1] (11).

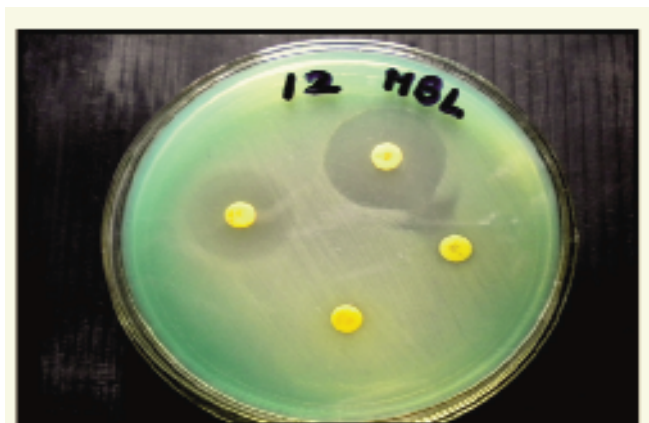


Fig. 1: Metallo- β -lactamase positive test (sample no. 12): synergy seen with imipenem and imipenem.

Extraction of DNA

The DNA was extracted as described earlier (12, 13). Briefly, one loopful of fresh bacterial growth from a blood agar plate was suspended in 300 μ l of 0.85% NaCl and heated for 15 minutes at 70 $^{\circ}$ C. The samples were centrifuged and resuspended in 50 μ l TE buffer (10 mM Tris, 1 mM EDTA, pH 8) supplemented with 10 μ l (3000 U/ml) of mutanolysin and 8 μ l (30 mg/mL) of hyaluronidase (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The mixture was then incubated for 30 minutes at 37 $^{\circ}$ C, heated at 100 $^{\circ}$ C for 10 minutes and briefly clarified by centrifugation. The supernatant containing the genomic DNA was used as template for polymerase chain reaction (PCR).

Method for DNA amplification

Commercially synthesized oligonucleotide primers were used for amplification (Sigma Aldrich, Bangalore). Primers were reconstituted in TE buffer (pH 8.0) to get the required concentration of oligonucleotide. Reaction mixture consists of the following: MilliQ water 38 μ l, 10X Reaction buffer - 5.0 μ l (1x), dNTP's mix (10 mM)-1 μ l (200 μ M), primer # 1 x -0.5 μ l (100 pM/reaction), primer # 2 x -0.5 μ l (100 pM/reaction), Taq DNA polymerase (1 U/ μ l) 1 μ l (1 U/reaction), Template DNA-3 μ l (~ 100 ng). The contents were mixed gently and the reaction tubes were placed into the thermal cycler (MJ Research - 150 minicyler) and it was covered with hot bonnet.

PCR for *bla*_{IMP} and *bla*_{VIM} genes

The presence of various *bla*_{IMP} and *bla*_{VIM} MBLs among all 67 MDR *P aeruginosa* isolates were detected by PCR with the consensus primers specifically recognizing all known IMP and VIM alleles (14). The primer sequences were as follows:- *bla*_{IMP} forward primer (5'-GTT TAT GTT CAT ACW TCG -3'), *bla*_{IMP} reverse primer (5'-GGT TTA AYA AAA CAA CCA C-3') (W = A or T; Y = C or T); *bla*_{VIM} forward primer (5' - TTT GGT CGC ATA TCG CAA CG -3'), *bla*_{VIM} reverse primer (5'-CCA TTC AGC CAG ATC

GGC AT -3'). Polymerase chain reaction was carried out in 50 μ l of a reaction mixture and 200 mM of dNTPs, 1U Taq polymerase, 100 pmoles of each set of primers and 100 ng of DNA. Samples were then subjected to initial denaturation at 95 $^{\circ}$ C for 3 minutes followed by 30 cycles of 95 $^{\circ}$ C for 30 seconds, 66 $^{\circ}$ C for 1 minute for *bla*_{IMP} gene and 45 $^{\circ}$ C for 1 minute for *bla*_{VIM} gene, 72 $^{\circ}$ C for 1 minute and a final extension at 72 $^{\circ}$ C for 10 minutes to complete the elongation of the PCR intermediate products. The amplified products were detected on 1.5% agarose gel in TBE buffer for the presence of the 432 bp and 500 bp by comparing with 100 bp ladder and photographed with a BioRad gel documentation system (Figs. 2, 3).

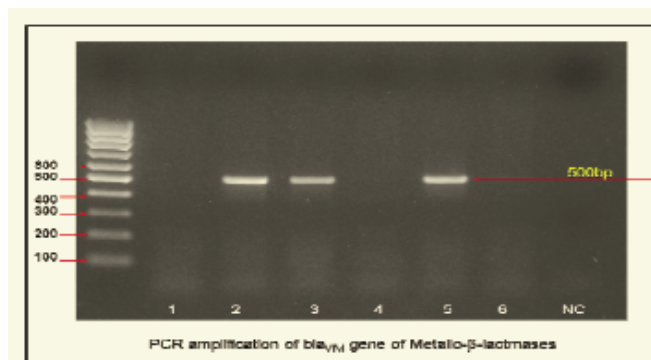


Fig. 2: Polymerase chain reaction amplification of *bla*_{VIM} gene of metallo- β -lactamase.

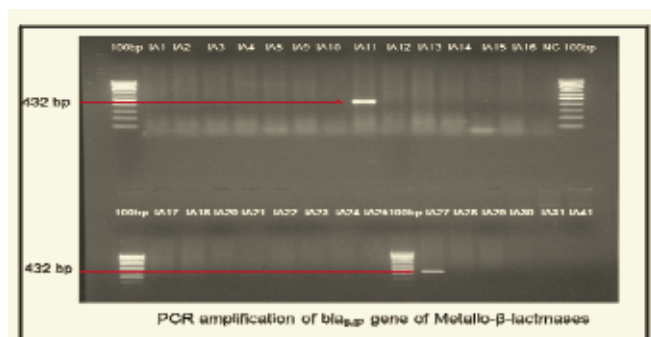


Fig. 3: Polymerase chain reaction amplification of *bla*_{VIM} gene of metallo- β -lactamase.

RESULTS

Among 67 MDR *P aeruginosa* isolates, 22 (33%) were isolated from urine, 16 (23.9%) from pus, 2 (3%) from blood, 6 (9%) from sputum, 7 (10.4%) from endotracheal aspirations, 6 (9%) from non-broncho-alveolar lavage and the remaining 13 (19.4%) from various other specimens.

All these isolates were 100% resistant to cefotaxime and ceftazidime by Kirby-Bauer disc diffusion method. Among 67 MDR *P aeruginosa* isolates, 62.7% (42/67) and 70.1% (47/67) were resistant to imipenem and meropenem, respectively and 59.7% (40/67) showed resistance to both imipenem and meropenem (Fig. 4).

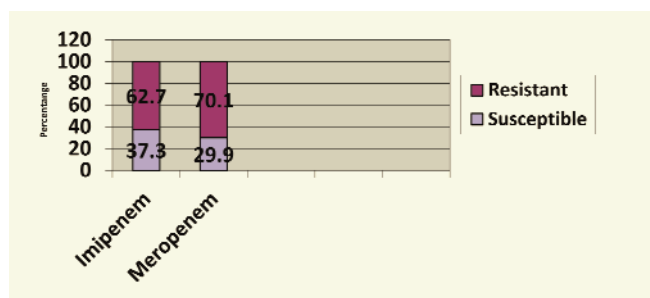


Fig. 4: Antibiotic resistance to imipenem and meropenem.

Out of 67 MDRP isolates, 47 (70.1%) were positive for MBL production by IPM/MEM-EDTA combined disc tests. Among these 47 MBL positive isolates, 41 (87.2%) strains showed the presence of the bla_{VIM} gene and 2 (4.3%) strains had the bla_{IMP} gene. Of the 47 combined disc tests positive strains, four were genotypically negative for PCR amplification of both bla_{IMP} and bla_{VIM} gene. Three, among 20 combined disc tests negative strains, were positive genotypically for bla_{VIM} gene. Polymerase chain reaction amplification revealed that the remaining 17 (25.3%) MDR *P aeruginosa* isolates were non-MBL producers and negative for both bla_{VIM} and bla_{IMP} genes. One strain was resistant to both imipenem and meropenem but did not show phenotypic positivity.

Several phenotypic methods have been proposed for the MBL detection. In 2005, from India, it was reported that the combined disc test was highly sensitive in detecting

Table: Comparison of IMP/MEP-EDTA combined disc test with polymerase chain reaction (PCR)

IMP/MEP-EDTA combined disc test	Genotyping (PCR) results		Total
	bla _{VIM/IMP} positive	bla _{VIM/IMP} negative	
Positive	43	04	47
Negative	03	17	20
Total	46	21	67

Sensitivity = 94%, Specificity = 81%, PPV = 92%, NPV = 85%

MBL-producing *Pseudomonas aeruginosa* (15). In the present study, the test used for MBL screening included IPM-EDTA and/or MEM-EDTA combined disc test, which was based on MBL inactivation by EDTA, the MBL inhibitor. The IMP-EDTA and MEM-EDTA combined disc tests showed 94% sensitivity and 81% specificity using PCR as a gold standard (Table). This is in accordance with the previous reports which found combined disc tests to be one of the most sensitive methods for phenotypic characterisation of MBL (16).

DISCUSSION

This study emphasized that bla_{IMP} harbouring strains accounted for only 3% of clinical isolates compared to 61% of bla_{VIM} harbouring strains. Among 67 MDR *P aeruginosa* clinical isolates, three *P aeruginosa* isolates positive for MBL production by EDTA combined disc tests were negative for both bla_{VIM} and bla_{IMP} genes by PCR. This may be due to the presence of genes other than bla_{IMP} and bla_{VIM} such as SPM-1, SIM-1 and GIM-1 enzymes which are confined to Brazil and Germany (17), and primers for these enzymes were not included in this study. Furthermore, this phenotypic positivity may be due to the presence of bla_{NDM-1} gene which is not included in this study but prevalent in India (18).

One isolate was resistant to imipenem and meropenem but MBL production was found to be negative. This may be due to the alteration of the outer membrane porin OprD2 as previously reported (19). Three strains of *P aeruginosa* were phenotypically negative for MBL production by combined disc test but genotypically positive for bla_{VIM} gene. This may be due to the presence of cryptic bla_{VIM} genes which were not expressed thus did not show phenotypic positivity. Prolonged exposure to carbapenems may cause mutations in bla_{VIM} promoter regions of these cryptic strains and emerge as a carbapenem resistant strain through elevated expression of the enzyme (20). Nevertheless, these isolates were sensitive to imipenem and meropenem which made the interpretation difficult. Discs containing 10 µg of imipenem or meropenem are not suitable for screening all strains that produce MBL, because the MICs of imipenem and meropenem for several MBL producers are lower than 8 µg/m (9). Moreover, this phenotypic contradiction may also be due to the production of large amounts of ESBL/Amp C enzymes as well as bacterial membrane alterations (21, 22). Thus, the results of susceptibility testing of IPM/MEM-EDTA were not enough for identification of MBL-producing strains of *P aeruginosa* since EDTA can give false positive results due to altered OprD levels.

Metallo-β-lactamases have been identified from clinical isolates worldwide with increasing frequency over the past few years, and strains producing these enzymes have been responsible for prolonged nosocomial outbreaks that were accompanied by serious infections. The occurrence of an MBL-positive isolate in a hospital setting poses a therapeutic problem, as well as a serious concern for infection control management. The accurate identification and reporting of MBL-producing *P aeruginosa* will aid infection control practitioners in preventing the spread of these multidrug-resistant isolates (23). In the present study, the MDR *P aeruginosa* isolates from the clinical samples showed the highest rate of drug resistance to third generation cephalosporins such as ceftazidime and cefotaxime (100%), with the second highest rate being to carbapenems (59.7%). Thus, the growing rate of carbapenem resistance is due to the widespread clinical use of these antibiotics which induce

MBL production amidst the nosocomial pathogens. Varying prevalence rates of the MBLs producing *P aeruginosa* have been reported worldwide (16).

In 2002 from India, MBL production in *P aeruginosa* was recorded as 12 per cent (24). In India, few reports have been carried out in different parts of the country in the past. The prevalence rates of MBL reported by these workers ranged from 4.5 to 14%. Since then, the incidence of MBL production in *P aeruginosa* has been reported to be 10–30 per cent from various clinical specimens across the country (16).

High numbers of MBL producers among MDR *P aeruginosa* is a worrisome trend that compromises the treatment options with the carbapenems. In another previous study, it has been identified that 30% MBL-producing *P aeruginosa* were obtained from respiratory specimens (25). Another study reported 20.7% carbapenem resistant *P aeruginosa* isolates from endotracheal aspirates showing indwelling devices as major risk factors for the development of resistance (26).

In Asian countries and regions, two genotypes, IMP and VIM, are prevalent. The former is mainly found in Japan, while the latter predominates in Korea, Taiwan and China. Furthermore, it is reported that IMP-1 type of MBL predominates at 89.7%, while the VIM-2 type has a frequency of only 10.3% in a paediatric clinic in China (7). In contrast, the PCR results of the present study demonstrated the presence of bla_{IMP} and bla_{VIM}, of which bla_{VIM} was the most abundant gene among the MDR *P aeruginosa* clinical isolates.

The present study suggests that there should be routine screening of third generation cephalosporin resistant *P aeruginosa* isolates for MBL production, since three imipenem susceptible *P aeruginosa* were evidenced to be positive for VIM gene by PCR amplification. Research has recommended the use of IPM non-susceptible or resistant isolates to *P aeruginosa* be routinely screened for MBL production using the EDTA disc screen test (23). Metallo- β -lactamase producers may lead to treatment failures with increased morbidity and mortality. The early detection of MBL-producing *P aeruginosa* may avoid the future spread of these multidrug-resistant isolates.

Prolonged clinical use of carbapenems such as imipenem and meropenem for the treatment of MDR *P aeruginosa* clinical infections has been selected for carbapenamase producing strains. The phenotypic method described here in this study may be helpful for screening the resistant strains for potential MBL production in routine clinical laboratory testing. Moreover, the predominance of bla_{VIM} gene among these isolates represents an emerging threat. Hence, early recognition of MBL producers is indispensable and necessitates rigorous infection control measures and restricted use of carbapenems for treatment of clinical infections. The dissemination of this gene to clinically rele-

vant species may endanger patients and it may have adverse implications on treatment.

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