

Original Research Article

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Characterization of VIM and IMP Metallobetalactamases (MBL) in *Pseudomonas aeruginosa* Isolated in a Tertiary Care Hospital

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ABSTRACT

Keywords

Carbapenem resistance, *Pseudomonas aeruginosa*, Metallo-betalactamases (MBL), MBL genes.

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Resistance to carbapenems are because of various mechanisms like mutations in the outer membrane proteins like Opr D causing decreased permeability of the drugs, the carbapenem hydrolyzing enzymes—carbapenemases and the efflux mechanisms. Carbapenem resistance was studied in 140 non-repeat clinical isolates of *Pseudomonas aeruginosa* in a tertiary care hospital. Imipenem resistance was detected using Kirby Bauer's standard disc diffusion method. MIC for Imipenem was detected in all resistant isolates using E-test strips. Production of Metallo-betalactamases (MBL) was detected using phenotypic MBL E-test method. MBL producing genes were tested in *Pseudomonas* using conventional PCR for IMP, VIM-type genes. Out of 140 isolates, 68 (48.5 %) showed imipenem resistance. MIC in majority (58 %) of imipenem resistant isolates was $\geq 32\mu\text{g/ml}$. MBL E-test, showed 54 (79.4%) as positive for MBL. The PCR analysis of the 54 isolates which were detected to have MBL showed VIM type (43 %), IMP-1 (12%), IMP-2 (2%) like genes. There is high level of prevalence of imipenem resistance in the *Pseudomonas aeruginosa* strains isolated. The carbapenem resistance in *Pseudomonas aeruginosa* in our hospital setting is mainly because of MBL gene that is of VIM type.

Introduction

The early eighties saw the introduction of a new group of antibiotics known as the carbapenems, which exhibited broader spectrum of activity as compared to cephalosporins. These group of antibiotics were active against Gram negative bacilli including non-fermenters like *Pseudomonas* producing extended spectrum betalactamases (ESBLs) and AmpC betalactamases (Deshpande *et al.*, 2004; Gladstone *et al.*, 2005). However, reports of resistance to these antibiotics amongst non-fermenting Gram negative bacteria (NFGNB) particularly

Pseudomonas aeruginosa have been published in the last decade. The strains of carbapenem resistant *Pseudomonas aeruginosa* can cause serious outbreaks in immunocompromised and hospitalized patients especially those admitted to intensive care and acute care units (Gowda and Marie, 2014; Maltezou *et al.*, 2013).

Resistance to carbapenems are because of various mechanisms such as mutations in the outer membrane proteins like Opr D causing decreased permeability of the drugs, the

carbapenem hydrolyzing enzymes—carbapenemases, and efflux mechanisms. Out of these, the resistant mechanism because of Carbapenem hydrolyzing enzymes is commonest and has gained much importance because of their horizontal spread to other Gram negative bacteria by plasmid mediated transfer (Maltezou *et al.*, 2013; Walsh *et al.*, 2005). Of the Carbapenemases, the Metallo-beta-lactamases (MBLs) are the commonest. Their activity is dependent on divalent cations like zinc or cadmium, so they can be inhibited by metal chelators such as ethylene diamine tetra acetic acid (EDTA), 2- mercapto propionic acid.

MBLs are broad-spectrum enzymes that hydrolyse most beta-lactam antibiotics, except monobactams and are not inhibited by conventional beta--lactamase inhibitors such as clavulanic acid or sulbactam. MBLs fall under group B of the Ambler classification. This group is further sub- divided into three subclasses: B-I, B-II, and B-III. The commonly occurring subclass is B-I and it comprises IMP (Imipenemase), VIM (Verona integron encoded metallo-beta-lactamase), GIM (Germany Imipenemase), and SPM (Sao Paulo metallo-beta-lactamase), FIM (Florence Imipenemase) types based on the their molecular structure (Walsh *et al.*, 2005).

The newest type detected recently in India is New Delhi metallo beta lactamase-1 (NDM-1) and the prevalence of NDM-1 carbapenemase in *Pseudomonas* in India is low (Khajuria *et al.*, 2013; Shanthi *et al.*, 2014). Of all the MBL types the most widely prevalent types are the IMP and VIM like MBL genes. The present study was done to detect the Carbapenem resistance due to Ambler class B carbapenemases which are known as MBLs by a phenotypic method along with the present gold standard (molecular detection of incriminating genes) for detection of two of the metallo-beta-lactamases, VIM and IMP as they are of most

predominant type.

Materials and Methods

The study was carried out in the department of microbiology, of a tertiary care hospital for one year. A total of 140 consecutive, non-repeat isolates of *Pseudomonas aeruginosa* were collected from various clinical samples like tracheal aspirate, bronchoalveolar lavage, sputum, blood etc. All the isolates were identified based on colony morphology, biochemical characteristics, and oxidative fermentation tests as per standard identification methods. *Pseudomonas aeruginosa* ATCC27853 was used as a control.

Antibiotic susceptibility testing for Imipenem and other antimicrobial agents was carried out using Kirby Bauer's standard disc diffusion method as per CLSI (Clinical and Laboratory Standards Institute, 2012) standard tables. For the antimicrobial susceptibility testing the antibiotic disks (HiMedia, Mumbai, India) used were imipenem (10 µg), ciprofloxacin (5 µg), norfloxacin (10 µg) ceftazidime (30 µg), cefipime (30 µg), aztreonam (30 µg), amikacin (30µg), ticarcillin (75µg), and piperacillin (100 µg), piperacillin-tazobactam (100/10 µg), colistin (10 µg), polymyxin B (300 U). *Pseudomonas aeruginosa* ATCC27853 was used as control.

Screening for Carbapenem group susceptibility was performed using Imipenem (IPM) disc of 10µg. Isolates were considered Imipenem resistant if the inhibitory zone diameters were as per CLSI guidelines (2012). Minimum Inhibitory Concentrations (MIC) for Imipenem was determined for all the resistant isolates using Epsilon meter (E-test) strips (Biomérieux, France). According to CLSI guidelines the breakpoint MIC was taken as ≥ 8 µg/mL, Sensitivity to Imipenem was taken as ≤ 2 µg/ml.

Screening for MBL production amongst the imipenem resistant isolates was carried out using a phenotypic MBL E-test method (Behera *et al.*, 2008; Walsh *et al.*, 2002; Yong *et al.*, 2002). The MBL E Test strip contains a seven dilution range of IPM (4 to 256 µg/mL) on one end and IPM (1 to 64 µg/mL) in combination with fixed concentration of EDTA on the other end.

The MBL E-test was performed based on manufacturer’s instructions. The strain was considered as an MBL producer if the MIC ratio of IPM/IPI (Imipenem and Imipenem along with an Inhibitor-EDTA) ≥ 8 or ≥ 3 log dilutions. Formation of a phantom zone or deformation of the ellipse was also interpreted as positive for MBL irrespective of the IPM/IPI ratio. MBL Negative control used was *Pseudomonas aeruginosa* ATCC 27853 with MIC of IPM ≤ 4 µg/ml and that of IPI 1-4 µg/ml.

All Carbapenem resistant *Pseudomonas* isolates, were screened for presence of MBL producing genes IMP, VIM using conventional PCR technique (Marchiaro *et al.*, 2005). Nucleic acid extraction was done from the resistant isolates using QIAmp DNA extraction Kit (Qiagen, USA) according to the manufacturer’s protocol. The reaction was performed in a volume of 25 µl with 18 µl of 2x Mastermix (Thermo Fisher Scientific, India), 2 µl of the primers (Sigma-Aldrich, India) and 5 µl of template DNA. Primer sequences, Cycling parameters and the

expected amplicon size are summarized in table 1.

Results and Discussion

Sixty eight (48.5%) of the 140 isolates were found to be Imipenem resistant by Kirby Bauer’s standard disc diffusion method. The Imipenem resistant isolates were mainly isolated from ICU and acute care wards (70 %) and the sample distribution is as given in figure 1.

The antibiogram of the Imipenem resistant isolates is depicted in figure 2. Of the 68 total resistant isolates of *Pseudomonas aeruginosa*, 100% were resistant to piperacillin, ceftazidime and cefepime, 31 (45.5 %) to piperacillin/ tazobactam, 45 (66 %) to amikacin, 26 (38%) to Aztreonam 64 (94%) to ciprofloxacin, but all (100 %) were sensitive to polymyxin B and colistin. Majority 39 of 68 (58 %) of the Imipenem resistant isolates exhibited an MIC of ≥ 32µg/ml by E-test, 21 of 68 exhibited MIC of ≥ 24 µg/ml and 8 of 68 exhibited MIC of ≥ 16 µg/ml.

MBL E test that was used to screen for the production of MBL showed 54 (79.4%) of 68 imipenem resistant isolates of *Pseudomonas aeruginosa* positive for MBL production (Fig. 3). The PCR analysis of the 54 isolates which were detected to have MBL showed VIM type (43 %) (Fig. 4), IMP-1 (12%), IMP-2 (2%) like genes.

Table.1 Summary of the primers, cycling conditions and amplicon size

GENE	PRIMER SEQUENCE Sequence (5'→ 3')	AMPLICON SIZE (bp)	CYCLING CONDITIONS (30 cycles)		
			Denaturation	Annealing	Extension
IMP 1	(F): GAGCAAGTTATTTGTATTCT	630	95°C	49°C	72°C
	(R): ACCAGTTTTGCCTTACTATA				
IMP 2	(F): TGTTTTATGTGTATGCTTCC	708	95°C	54°C	72°C
	(R): AGTACTTGGCTGTGATGGT				
VIM	(F): ATTGGTCTATTTGACCGCGT	780	95°C	58°C	72°C
	(R): CTAICTCAACGACTGAGCGAT				

Fig.1 Antibiogram of the imipenem resistant isolates of *Pseudomonas*

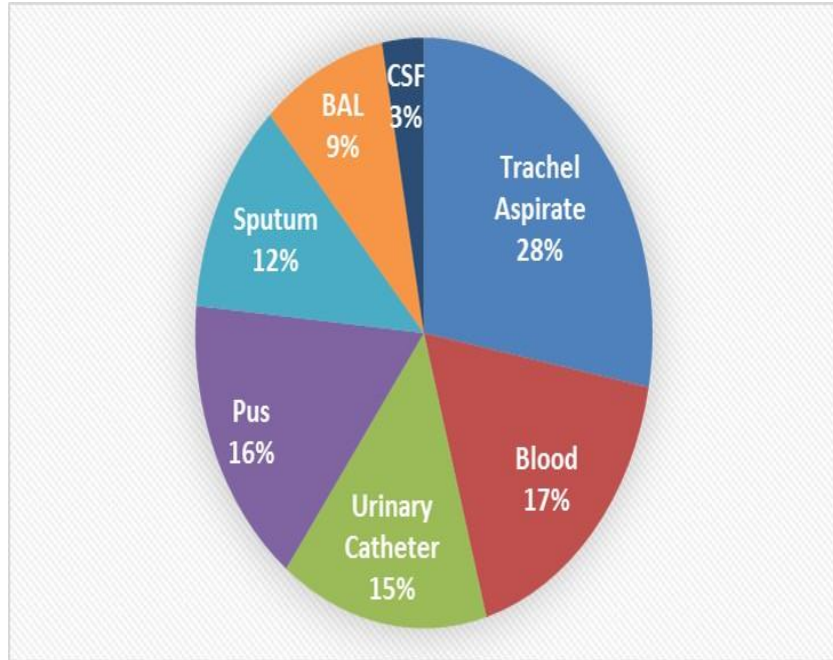


Fig.2 Antibiogram of the Imipenem resistant isolates of *Pseudomonas*

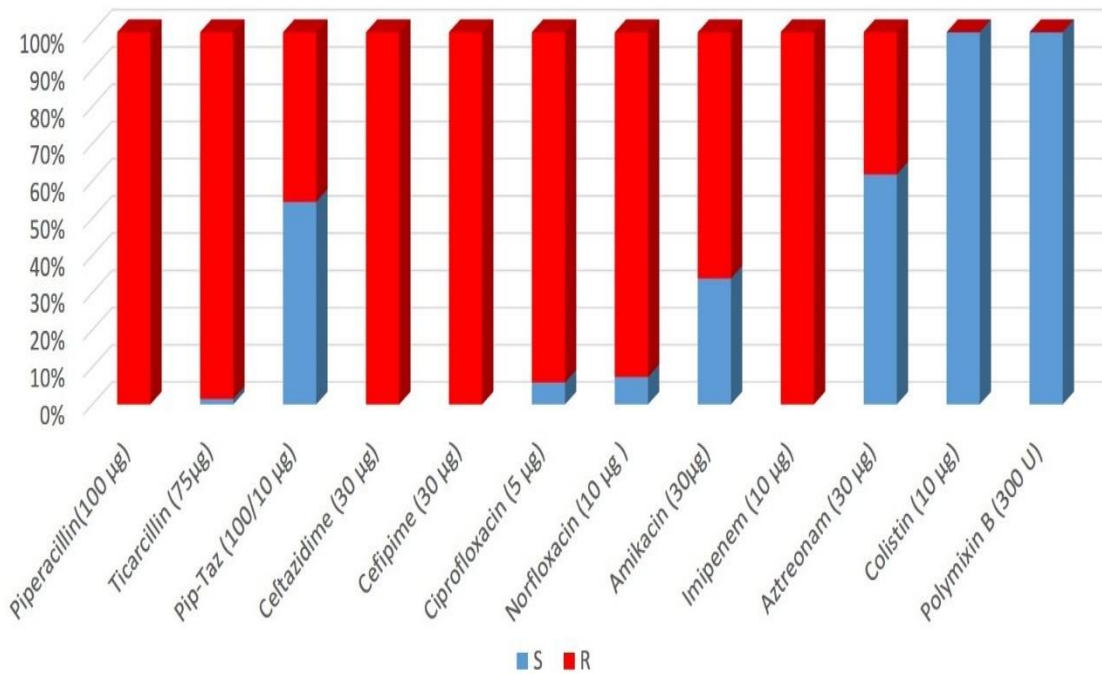


Fig.3 Panel A and B: Detection of Metallo-beta-lactamase (MBL) by E test. Panel C: Imipenem sensitive strain. Panel D: Imipenem resistant but non MBL producing *Pseudomonas spp.*

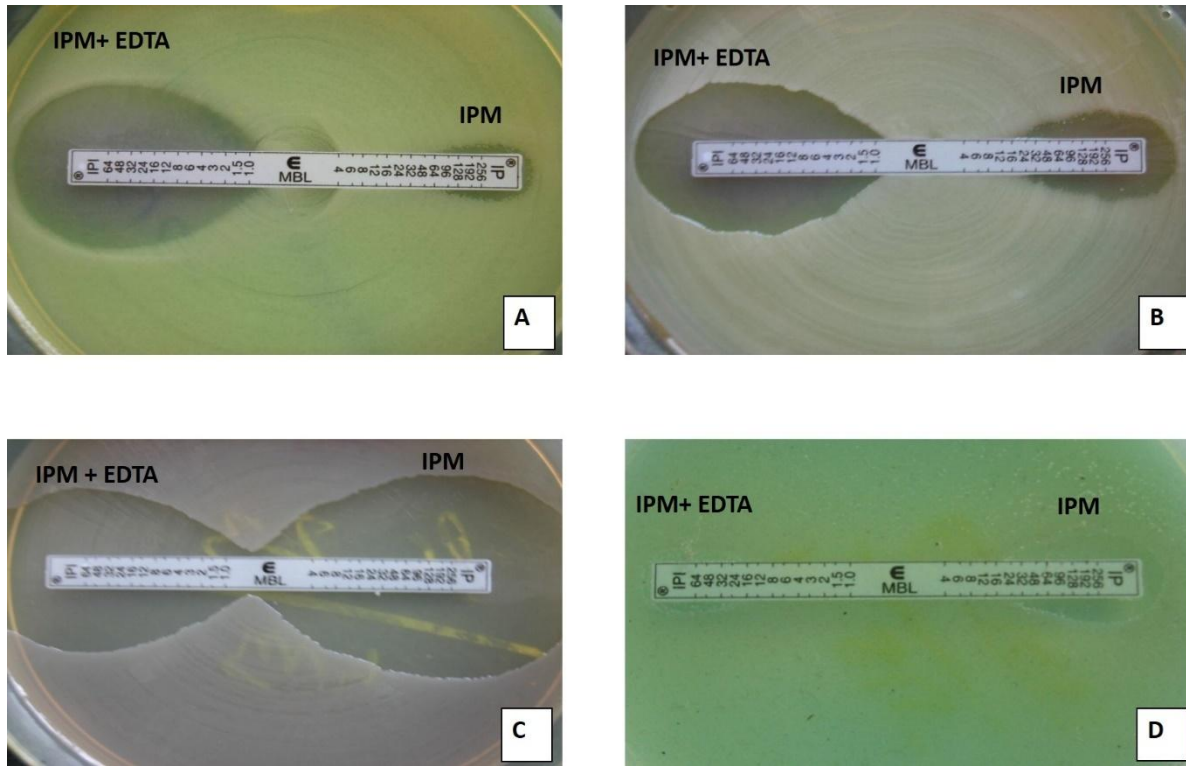
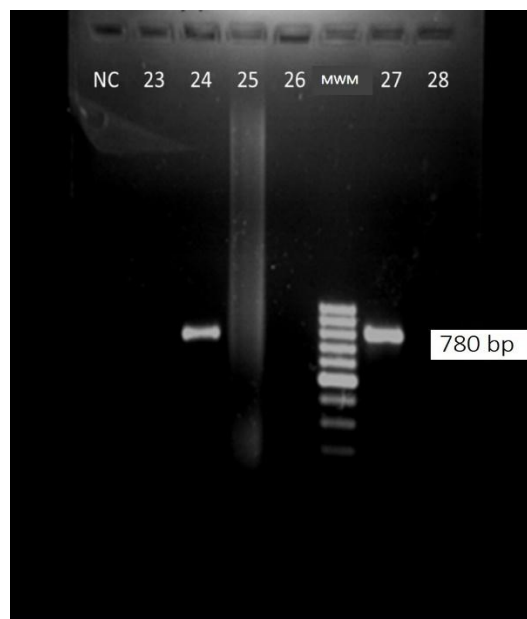


Fig.4 Agarose gel electrophoresis for detection of VIM gene. NC – Negative control, 23-28 samples, MWM – 100bp DNA ladder. Samples 24 and 28 show band for VIM type gene at 780bp



There is high level of prevalence of Imipenem resistance (48.5 %) in the strains of *Pseudomonas aeruginosa* isolated from our Hospital. The Imipenem resistant strains of *Pseudomonas* were predominantly isolated from blood, pus, urine and tracheal aspirate. Majority of resistant strains isolated were mainly from ICU (45%) and Acute Medical Care unit (25%). The antibiogram demonstrates that all these isolates are fully resistant to piperacillin, majority to ciprofloxacin and third/fourth generation cephalosporins like ceftazidime and cefipime. These strains were sensitive to only polymyxin B (100%), colistin (100%) and aztreonam (94%). This multi drug resistance amongst the Imipenem resistant isolates severely limits our therapeutic options in treating these infections. The MBL positive strains are, at present, sensitive to polymyxin B and polymyxin E (Colistin). Colistin may be combined with tigecycline as combination therapy for better results (Betts *et al.*, 2014; Datta and Wattal, 2010). The strains isolated from ICU showed same antibiogram profile indicating the possibility of origin from the same clone, but it needs to be confirmed by DNA sequencing. The infection control measures have been adequately instituted in the hospital to further contain the spread of this strain.

The PCR analysis for the MBL genes showed predominantly VIM type (43%) genes and IMP (14%) type in our setting, similar prevalence was seen ranging from 18 % to 56% for VIM and 22 % to 48% for IMP type in other studies (Doosti *et al.*, 2013; Irfan *et al.*, 2008; Kim *et al.*, 2012; Lee *et al.*, 2002). In another study, there was prevalence of 17.8% VIM type MBLs in the *Citrobacter* spp (Praharaj *et al.*, 2016). Reports of these kinds of MBL genes are presently less in number as far as India is concerned. The presence of MBL genes in only 57 % of the resistant strains of *Pseudomonas* in this study indicates the possibility of multiple mechanisms responsible for resistance to Imipenem like mutations in the outer membrane proteins Opr D, the efflux mechanisms such as Mex A and Mex-B with Opr M which need to

be tested and confirmed. In conclusion, continuous surveillance and strict infection control measures are warranted to control carbapenem resistance in all health care settings.

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Conflict of Interests: None to declare.

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