Original Article

bla$_{IMP}$ and *bla*$_{VIM}$ mediated carbapenem resistance in *Pseudomonas* and *Acinetobacter* species in India

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Abstract

Introduction: The emergence and rapid spread of *bla*$_{IMP}$ and *bla*$_{VIM}$ metallo-beta-lactamase (MBL) producing Gram-negative bacteria causing nosocomial infections are of concern worldwide due to limited treatment options.

Methodology: A total of 179 nonreplicate, consecutive, carbapenem resistant *Pseudomonas aeruginosa* (61), *Acinetobacter baumannii* (116), *Acinetobacter lwoffii* (1) and *Pseudomonas stutzeri* (1) isolated from patients hospitalized for 48 hours or more were included in the study. The minimum inhibitory concentrations (MIC) to imipenem and meropenem were determined and interpreted according to Clinical Laboratory Standards Institute guidelines. The Modified Hodge Test (MHT) and inhibitor potentiated disk diffusion tests with ethylenediaminetetraacetic acid (EDTA) were used for screening of carbapenamases and MBL production respectively. Polymerase chain reaction (PCR) was performed for the detection of MBL (*bla*$_{VIM}$ and *bla*$_{IMP}$) genes. Gene sequencing was performed for representative isolates.

Results: MHT was positive in 94.4% (n = 169). MBL screening with EDTA was positive in 80.4% (n = 144). MBL genes *bla* $_{VIM}$ and *bla* $_{IMP}$ were detected in 92 (51.4%) isolates. *Bla* $_{VIM}$ alone was detected in 89 isolates while two isolates had *bla* $_{IMP}$ alone. One isolate had both *bla* $_{VIM}$ and *bla* $_{IMP.}$ Among the *P. aeruginosa*, 36 carried the MBL gene. In *A. baumannii*, 54 carried the MBL gene. *Bla* $_{VIM}$ was found in *P. stutzeri* and *A. lwoffii* isolates.

Conclusion: Carbapenem resistance in *P. aeruginosa* and *A. baumannii* is chiefly mediated by MBL production. The common MBL gene is the *bla*$_{VIM}$.

Key words: carbapenems; metallo-beta-lactamases; polymerase chain reaction

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Introduction

Infections caused by metallo-beta-lactamase [MBL] producing organisms are associated with high rates of mortality, morbidity and rising health-care costs. MBL producing *Pseudomonas aeruginosa* was first reported in Japan in 1991 and since then they have disseminated worldwide [1]. MBL that belong to class B require divalent cations as cofactors for optimal enzyme activity, and are inhibited by the action of a metal ion chelator. They hydrolyse all the beta lactams including carbapenems except the monobactams such as aztreonam. Acquired MBL is encoded by integron borne mobile gene cassettes; hence MBL producing strains are often resistant to different classes of antimicrobial agents with transferable properties to various types of bacteria [2,3]. Thus the detection of MBL producing Gram-negative bacilli is necessary to aid in appropriate treatment and infection control measures, and to prevent their dissemination. The most common MBLs include the VIM, IMP, GIM, SPM, SIM enzymes and the recently identified NDM-1. In particular, *bla* $_{VIM\ -2}$ has emerged as a dominant MBL variant worldwide [4,5].

Inhibitor-based tests have been employed for the detection of MBL producers using carbapenem as indicator beta lactam. The inhibitors used are metal ion chelators such as ethylene diaminetetraacetic acid (EDTA) or thiol based compounds [6,7]. Though several methods are advocated in many studies, Clinical Laboratory Standards International (CLSI) guidelines do not recommend a standardised method for the detection of MBL producing isolates.

This study was undertaken to detect the prevalence of metallo-beta-lactamases (*bla*$_{VIM}$ and *bla*$_{IMP}$) in carbapenem-resistant nosocomial isolates of *Pseudomonas* and *Acinetobacter* species.

Table 1. PCR Primers for the detection of genes encoding metallo-beta-lactamases (*bla* $_{VIM}$ and *bla*$_{IMP}$)

Primer	Primer sequence (5'-3')	Product size (bp)
Bla $_{VIM}$-F	TTTGGTCGCATATCGCAACG	500
Bla $_{VIM}$-R	CCATTCAGCCAGATCGGCAT	
Bla $_{IMP}$-F	GTTTATGTTCATACWTCG	432
Bla $_{IMP}$-R	GGTTTAAYAAAACAACCAC	

Methodology

Bacterial strains

The study was conducted in a 1,600-bed university teaching hospital from April 2010 to October 2010. It included 179 clinically significant, non-duplicate, carbapenem-resistant Pseudomonas and Acinetobacter species recovered from clinical specimens of patients hospitalized for 48 hours or more. The isolates were obtained from clinical specimens such as blood, urine, exudative specimens, and lower respiratory secretions (bronchoalveolar lavage, bronchial wash and endotracheal secretions). The exudative specimens included pus, wound swabs, cerebrospinal fluid (CSF) and other body fluids. The organisms were identified up to species level using Microscan WalkAway-96 using Gram-negative panels (Seimens Healthcare Diagnostics Inc, Sacramento CA, USA). Commensals were differentiated from pathogens for isolates obtained from nonsterile sites (respiratory tract, urinary tract, and wound swabs) by ascertaining their significance based on clinical history, presence of the organism in the Gram stain, presence of intracellular forms of the organism, and pure growth in culture with significant colony count.

Antimicrobial Susceptibility Testing

Susceptibility to various classes of antibiotics was determined by the disc diffusion method in accordance with CLSI guidelines. [8]. The antibiotics tested were amikacin (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), piperacillin-tazobactam (100/10 µg), imipenem (10 µg), and meropenem (10 µg) (Himedia Laboratories, Mumbai, India). Susceptibility to aztreonam (30 µg) and polymyxin B (300 units) were determined by disc diffusion methods for the Pseudomonas spp. Disc diffusion susceptibility testing was also performed for all the Acinetobacter spp. using tigecycline disks (15µg) (BBL, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Interpretation of zone diameters was performed using the United States Food and Drug Administration tigecycline susceptibility breakpoint criteria listed for

Enterobacteriaceae (susceptible ≥19 mm, intermediate 15-18 mm, resistance ≤14 mm)[9]. MIC to imipenem and meropenem was performed by the agar dilution method (range: 0.008-256 µg/ml) in accordance with CLSI guidelines [8].

Phenotypic methods

Modified Hodge Test (MHT): Detection of carbapenamases production was performed using the Modified Hodge Test on Mueller-Hinton agar (Himedia Laboratories, Mumbai, India) as described by Lee et al. [6].

MBL screening: Zone enhancement with EDTA impregnated imipenem and ceftazidime discs was performed according to the methods described previously by Hemalatha and colleagues [10].

Detection of carbapenem resistance genes by Polymerase Chain Reaction (PCR)

A 1:10 dilution of an overnight culture was boiled for 10 minutes. Amplification was then performed with 10 µl of this dilution as the DNA template. Primers used are given in Table 1. PCR conditions included 30 cycles of amplification under the following conditions: denaturation at 95°C for 30 seconds, annealing for 1 minute at specific temperatures (blaVIM-66°C and bla IMP -45°C), and extension at 72°C for 1 minute/kb product. Cycling was followed by a final extension at 72°C for 10 minutes. The PCR product of 500 bp (blaVIM) and 432 bp (blaIMP) was visualised by agarose gel electrophoresis [11].

Table 2. Distribution of *bla*$_{VIM}$ and *bla* $_{IMP}$ among the organisms isolated from various clinical specimens

Specimen (n = 179)	Blood n = 34		Exudative specimens n = 37		Respiratory secretions n = 83		Urine n = 25	
bla$_{VIM}$/ *bla*$_{IMP}$	Positive(n = 16)	Negative (n = 18)	Positive (n = 18)	Negative (n = 19)	Positive (n = 43)	Negative (n = 40)	Positive (n = 15)	Negative (n = 10)
Acinetobacter baumannii (n = 116)	11	14	10	15	32	30	1	3
Pseudomonas aeruginosa (n = 61)	4	4	7	4	11	10	14	7
Pseudomonas stutzeri (n = 1)	-	-	1	-	-	-	-	-
Acinetobacter lwoffii (n = 1)	1	-	-	-	-	-	-	-
Total (n = 179)	16	18	18	19	43	40	15	10

Table 3. Results of phenotypic tests in *bla*$_{VIM}$/*bla*$_{IMP}$ negative *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

Organism	Phenotypic tests in *bla*$_{VIM}$/ *bla*$_{IMP}$ negative isolates			
	MHT-positive MBL screen test – positive	MHT-positive MBL screen test – negative	MHT-negative MBL screen test – positive	MHT-negative MBL screen test – negative
Pseudomonas aeruginosa (n = 25)	12	6	2	5
Acinetobacter baumannii (n = 62)	40	19	-	3

DNA sequencing

The PCR products of representative isolates were then purified by using a PCR DNA purification kit (QIA Quick Gel Extraction Kit, Qiagen, Valencia, CA, USA) and subjected to automated DNA sequencing (ABI 3100, Genetic Analyser, Applied Biosystems, Foster City, CA, USA). The aligned sequences were then analyzed with the Bioedit sequence program and similarities searches for the nucleotide sequences were performed with the BLAST program (http://www.ncbi.nlm.nih.gov). The sequences were submitted to the GenBank.

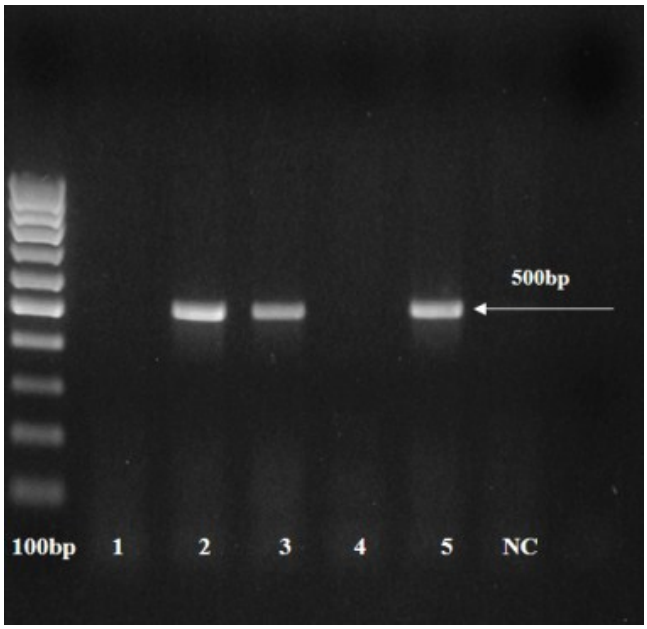
Results

The following isolates were included in the study: *A. baumannii* (n = 116), *P. aeruginosa* (n = 61), *A. lwoffii* (n = 1) and *P. Stutzeri* (n = 1). They were obtained from clinical specimens such as respiratory secretions (n = 83), blood (n = 34), urine (n = 25) and exudates (n = 37). The exudative specimens included CSF (n = 5), pus (n = 6), wound swabs (n = 23) and other body fluids (n = 3). The majority were from patients in intensive care units (ICU) of the hospital (n = 164) and the rest (n = 15) were from non-critical units of the health-care facility.

Antimicrobial susceptibility testing

All the study isolates were resistant to amikacin, ciprofloxacin, ceftazidime, piperacillin-tazobactam, imipenem and meropenem. *P. aeruginosa* and *P. stutzeri*isolates were also resistant to aztreonam. The MIC to imipenem and meropenem ranged from 8-128mg/L. The MIC $_{50}$ and MIC $_{90}$ values for imipenem were 16mg/L and 32mg/L respectively. For meropenem, the MIC $_{50}$ and MIC $_{90}$ values were32mg/L and 64 mg /L, respectively. Among the

Figure1. Detection of *bla* VIM and *bla* IMP



61 *P. aeruginosa* isolates, 91.8% (n = 56) were susceptible to polymyxin B as tested by the disc diffusion method. *P. stutzeri* was also susceptible to polymyxin. Susceptibility to tigecycline was seen in 93.1% (n = 108) of the *A. baumannii* and in the lone *A. lwoffii* isolate.

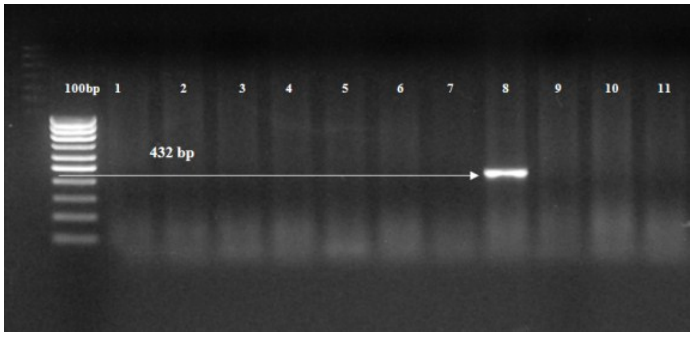
Phenotypic tests

Modified Hodge Test: MHT was positive in 94.4% (n = 169). MBL screening with EDTA was positive in 80.4% (n = 144).

PCR: The metallo-beta-lactamase genes *bla* VIM and *bla* IMP were detected in 92 (51.4%) isolates (Figure 1). Among these metallo-beta-lactamase producers, 89 isolates carried *bla* VIM alone, 2 carried *bla* IMP alone, and 1 carried both *bla* VIM and *bla* IMP. The distribution of the *bla* VIM and *bla* IMP among the organisms isolated from various clinical specimens included in the study is given in Table 2.

Figure 2 shows the results of the Modified Hodge Test (MHT), MBL screen test, and PCR (*bla* VIM and *bla* IMP) for *Pseudomonas aeruginosa* and *Acinetobacter baumannii.* Among the *P. aeruginosa* (n = 61), *bla* VIM was detected in 34 isolates and 2 isolates carried the *bla* IMP gene. MBL screen tests were positive in all these isolates. MHT was positive in 34 isolates and negative in 2. On subsequent testing of these isolates on Mueller-Hinton agar supplemented with zinc sulphate at a concentration of 70mg/L, the test turned positive, thus emphasising the importance

Figure 2. Results of Modified Hodge Test (MHT), MBL screen test, and PCR (*bla* VIM and *bla* IMP) for *Pseudomonas aeruginosa* and *Acinetobacter baumannii*



Molecular mass marker (100bp DNA ladder)
Lane 1: Negative control; Lanes 2 and 3 *bla* VIM – Positive (500bp); Lane 5*bla* IMP -Positive (432bp) ; Lanes 4, 6 and 7 – Negative

of zinc concentration in the medium on the test outcome.

The results of phenotypic tests in bla_{VIM}/bla_{IMP} negative *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are shown in Table 3. The lone *P. stutzeri* isolate carried the *bla* VIM gene. MBL screen test and MHT were positive in the isolate.

Among the *A. baumannii* isolaes (n = 116), MHT and MBL screen tests were positive in 113 and 92 isolates respectively. MBL genes (*bla* VIM /bla_{IMP}) were detected in 54 isolates, of which 53 carried the *bla* VIMand one isolate harboured both *bla* VIM and bla_{IMP}. In all the PCR-positive isolates MHT and MBL screen tests were positive.

A. lwoffii was MHT and MBL screen test positive and harboured the *bla* VIM gene.

Discussion

The advent of carbapenems in the 1980s heralded a new treatment option for serious bacterial infections. They have a broad spectrum of activity and are stable to hydrolysis by most of the beta lactamases, including the extended spectrum beta lactamases (ESBL) and the Amp C beta lactamases. In recent years there has been an increase in carbapenem resistance among Gram-negative bacteria in the Indian subcontinent [12,13,14]. Resistance mechanisms include lack of drug penetration (*i.e.*, porin mutations and efflux pumps) and/or carbapenem hydrolysingbetalactamase enzymes [2,15,16]. Over the past few years MBL producing isolates have emerged worldwide and are associated with outbreaks in health-care settings. They cause serious infections such as bacteremia and

ventilator associated pneumonia, particularly in patients admitted to the ICU [14,17].

The CLSI has not recommended any standardized phenotypic methods for screening MBL in clinical isolates. We screened for carbapenamases production by Modified Hodge Test and MBL production by inhibitor-based methods using EDTA as the inhibitor. Of the 179 isolates studied, MHT was positive in 167, indicating the production of carbapenamases. For the 12 isolates that were carbapenem resistant but MHT negative, the test was performed on Mueller-Hinton agar supplemented with zinc sulphate (70mg/L) [6], out of which 2 *P. aeruginosa* isolates were positive. Hence MHT was positive in 169 isolates (94.4%). The remaining 10 were MHT negative, thereby suggestive of other mechanisms such as loss of porins or upregulation of efflux pumps [15].

Among the 179 study isolates, the MBL screen test was positive in 80.4% (n = 144). PCR detected the MBL genes *bla* $_{VIM}$/*bla*$_{IMP}$ in 51.4% (n = 92) and the MBL screen test was positive in all these isolates. In 52 isolates that were MBL screen test positive, *bla* $_{VIM}$/*bla*$_{IMP}$ were not found, suggesting the presence of other MBL genes such as SPM-1, GIM-1, SIM-1 or NDM-1. Despite the good performance of inhibitor-based methods for the detection of MBL by using EDTA, it is not a specific test. False positive results have been reported in *P. aeruginosa* as EDTA acts on the membrane of the bacterial cell and increases the cell permeability. Presence of OXA carbapenamases in *A. baumannii* may also lead to false positive results [7,16]. Hence the results of the MBL phenotypic tests must be interpreted cautiously. The overall *bla* $_{VIM}$/*bla*$_{IMP}$ production among the study isolates was 51.4%. Of the 61 *P. aeruginosa* isolates, 36 produced the above enzymes. Fifty-four out of the 116 *A. baumannii* isolates were *bla* $_{VIM}$/*bla*$_{IMP}$ producers. These results indicate that carbapenem resistance in *P. aeruginosa* is mainly due to MBL production whereas in *A. baumannii* it is due to the presence of multiple betalactamases, which may include the OXA carbapenamases and other MBLs.

The common MBL genotype was the *bla* $_{VIM}$ (n = 89). *Bla* $_{IMP}$ was found in three isolates of which one carried both.

In Asia, bla$_{IMP}$ and *bla*$_{VIM}$ are prevalent. *bla*$_{IMP}$ is found mainly in Japan, Korea, China, Taiwan, and Iran [18,19,20]. The prevalence of MBL in India has ranged from 7% to 65% among carbapenem-resistant *P. aeruginosa*. In a study from India, the rate of MBL production was 24.5% among 61 *P. aeruginosa* isolates, and *bla* $_{VIM}$ type was the most common [21]. Another study from India also reported *bla*$_{VIM-2}$ from *P. aeruginosa* [22]. In a nation-wide survey conducted to characterise 301 MBL producing *Pseudomonas* species in 10 medical centres from India, the MBL genes were detected in 18.9% of the isolates and 5 VIM variants were reported with VIM-2 being the most common. The others were VIM-6, VIM-11, VIM-5 and VIM-18. [23]. In India, MBL production among *A. baumannii* isolates has been reported at 42%. The most prevalent MBL gene was *bla*$_{IMP-1}$ [15]. There is limited data on the prevalence and distribution of metallo-beta-lactamases among Indian isolates.

Regarding resistance profiles, all isolates were resistant to other classes of antimicrobial agents such as aminoglycosides and fluoroquinolones. All the *P. aeruginosa* and the lone *P. stutzeri* were resistant to aztreonam, indicating the concomitant presence of other beta lactamases. Polymyxins predominate as the mainstay of treatment for *P.aeruginosa* with susceptibility of 91.8%. Susceptibility to tigecycline was seen in 93.1% (n = 108) of *A. baumannii* isolates.

In this study, *bla*$_{VIM}$ /bla$_{IMP}$ production contributes to 51.4% of carbapenem resistance. Hence early detection of MBL producing organisms is important to guide in the treatment of infections caused by them and also to arrest their spread. In the clinical microbiology laboratory, all clinical isolates that are resistant to carbapenems must be screened for carbapenamase and MBL production by using simple phenotypic tests.

To conclude, carbapenem resistance in *P. aeruginosa* and *A. baumannii* is chiefly mediated by MBL production. The common MBL gene is *bla*$_{VIM}$. The development of simple and inexpensive screening methods to detect carbapenamases and MBL production in microbiology Laboratories is crucial for optimal treatment of patients, particularly critically ill and hospitalized patients, and to control the spread of resistance.

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