

A Study of Phenotypic & Genotypic Assays For Detection of Metallo β -Lactamase Producing *Pseudomonas Aeruginosa* With Special Reference to New Delhi Metallo β -Lactamase

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Abstract:

Introduction: Carbapenems are among the few useful antibiotics against multidrug resistant Gram negative bacteria particularly those with extended spectrum beta-lactamase. Resistance to carbapenems is mediated by loss of outer membrane proteins and production of beta lactamase that is capable of hydrolyzing carbapenems.

Methods: A total of 200 clinical isolates of *Pseudomonas aeruginosa* were tested for the presence of MBL. Detection of MBL was performed by various phenotypic methods like Modified Hodge Test, Imipenem(IMP)-EDTA Combined disc test and MBL E-test. The polymerase chain reaction (PCR) test was used for the detection of 3 genes encoding MBLs (IMP, VIM, NDM).

Results: 21 out of 62 IMP resistant *P. aeruginosa* isolates were found to be MBL producers by MBL E-Test method. However, all 21 of these MBL E-Test positive strains contained the MBL gene where, *bla*_{VIM} and *bla*_{IMP} genes was observed in 12 and 7 isolates respectively. 2 isolates harboured *bla*_{NDM} gene.

Conclusion: The study emphasizes the high prevalence of *P. aeruginosa* producing metallo-beta-lactamase enzymes. Thus proper antibiotic policy and all IMP resistant *P. aeruginosa* isolates should be routinely screened for MBL production using phenotypic detection methods and that PCR confirmation be performed at a regional laboratory to minimize the emergence of this multiple beta-lactamase producing pathogens.

Keywords: E-test, Modified Hodge test, MBL, Imipenem, *bla*_{IMP}, *bla*_{VIM} and *bla*_{NDM}.

I. Introduction

Treatment of infectious diseases is becoming more challenging with each passing year. This is especially true for infections caused by the opportunistic pathogen *Pseudomonas aeruginosa*, due to its ability to rapidly develop resistance to multiple classes of antibiotics.[1] Carbapenems are the antibiotics of choice for severe *Pseudomonas* infections, however, in recent years resistance to this novel antibiotic is increasing worldwide.[3] The most common mechanism for carbapenem resistance is production of metallo-beta-lactamases (MBLs), which are broad-spectrum enzymes that hydrolyze most beta-lactam antibiotics, and are not inhibited by conventional beta-lactamase inhibitors like clavulanic acid or sulbactam.[2]

Carbapenem hydrolyzing enzymes can be divided into two main sub types; Serine β -lactamases (Ambler class A and D) and metallo- β -lactamases (Ambler class B) [4,5,6]. The emergence of MBLs in Gram negative bacilli is becoming a therapeutic challenge as these enzymes render all penicillins, cephalosporins and carbapenems ineffective[5,7].

New Delhi metallo beta lactamase-1 (NDM-1), the recently discovered transferable molecular class B β -lactamase is a growing threat worldwide. First reported in 2008 in Sweden from a patient previously hospitalized in India, NDM-1-producing *Enterobacteriaceae* are now the focus of attention globally[8,9]. Till date, reports of NDM-1 in *Pseudomonas aeruginosa* are scarce. Though reports are scarce and sporadic, knowledge of its prevalence is essential because *P. aeruginosa* is an environmental pathogen with intense colonization capacity and ability to persist for indefinite periods in the hospital environment[1,10].

Currently, no standardized method for MBL detection has been proposed. Polymerase Chain Reaction (PCR) is highly accurate and reliable but its accessibility is often limited to reference laboratories [11]. Several non molecular techniques have been studied, all taking advantage of the fact that MBLs require zinc or another heavy metal for their action and their activities are inhibited by chelating agents e.g. EDTA, dipicolinic acid and thiol compounds[11,12,13,14]. Various phenotypic methods for MBL detection are combined disc method, double disc synergy method and Etest. MBL E-test is considered the phenotypic standard method for MBL detection but the test is expensive [15]. Double disc synergy and combined disc tests are economical and simple to perform but double disc test is observer dependent while CD test is measureable with lesser chances of subjective error [15].

The lack of antibiotic policy in a hospital and sale of antibiotics over the counter, without prescription, leads to indiscriminate and injudicious use of antibiotics, further increasing the burden of antimicrobial resistant organisms. Therefore, there is a need to have a strong antibiotic policy, which is revised from time-to-time, with a stringent check over the sale of antibiotics, and incorporation of an antibiotic stewardship program. Hence, this study was conducted to detect the presence of MBLs in *P. aeruginosa* isolates obtained from the clinical sample, from a tertiary care hospital in North India, and to compare the antibiograms of MBL-producing and non-MBL-producing *P. aeruginosa* isolates, to guide clinicians in prescribing proper antibiotics and controlling hospital infection.

II. Material And Methods

The study was carried out in the Department of Microbiology Rama Medical College and Hospitals for a period of one years, Overall, 200 *Pseudomonas aeruginosa* from sputum, urine, blood, pus, wound swab, and aspirated fluids were collected from the IPD and OPD patients (both males and females of all age groups), sent to the microbiology laboratory for the culture identification and sensitivity testing were included in the study. All samples were routinely cultured on MacConkey and blood agar plates. The organisms were identified by their colony characteristics, staining procedures, pigment production, motility and other relevant biochemical reactions as per standard laboratory methods for identification of bacteria.

0.5 McFarland Turbidity Bacterial suspension was processed on Muller-Hinton agar medium (MHA) and antibiogram disks containing piperacillin(100µg), ticarcillin(75µg), ampicillin/sulbactam(10/10µg), piperacillin/Tazobactam(100/10µg), ticarcillin-clavulanic acid(75/10µg), ceftazidime(30µg), cefepime(30µg), cefotaxime(30µg), ceftriaxone(30µg), meropenem(10µg), imipenem(10µg), colistin(10µg), tamycin(10µg), Tobramycin (10µg), amikacin(30µg), netillimycin, tetracycline(30µg), ciprofloxacin(5µg), levofloxacin(5µg), doxycycline(30µg), cotrimoxazole (1.25/23.75µg), tigecycline were placed on the medium. The results were interpret according to the recommendations of the Clinical and Laboratory Standards Institute-2015. Quality control strains that were used are *Escherichia coli*- American type culture collection(ATCC) 25922 and *Pseudomonas aeruginosa* – ATCC 27853 .

MBL Screening methods: Antimicrobial susceptibility of *Pseudomonas aeruginosa* was performed by the disc- diffusion method (Modified- Kirby baur disc diffusion method) as per CLSI guidelines.[16] Isolates resistant to imipenem, meropenem and third generation cephalosporin were considered as screening positive.

Phenotypic confirmatory test

Modified Hodge Test: The modified Hodge Test (MHT) detects carbapenemase production in gram negative isolates. An overnight culture suspension of *Escherichia coli* ATCC 25922 adjusted to 0.5 McFarland was inoculated using a sterile cotton swab on the surface of Muller-Hinton agar(MHA). After drying, 10 µg imipenem disk(Hi-Media, Mumbai, India) was kept at the centre of the MHA plate and the test strains suspension was inoculated by streaking method from the edge of the imipenem disc to the periphery of the petriplate in four different directions. The plates was incubated overnight at optimum temperature. If the test strain that are carbapenemase producing there will be the presence of “cloverleaf shaped” zone of inhibition. The test organism was considered as Metallo-beta lactamase (MBL) positive.[17]

Imipenem(IMP)- EDTA Combined disc test: The test organisms are inoculated by lawn culture technique on the plates of Muller-Hinton agar(MHA) as recommended by CLSI. [16] 10 µg Imipenem Disk and 750 µg Imipenem-EDTA Disk(Hi-media SD281) are placed on the plate. The inhibition zones of the imipenem and imipenem-EDTA disks are compared after 16 to 18 hours of incubation at 37°C. In the combined disc test, if the increase in inhibition zone with the imipenem and EDTA disc will be ≥ 7 mm than the imipenem disc alone, it is considered as MBL positive. [18]

MBL E-test: The E-test MBL Strip contains a double sided seven-dilution range of IP(Imipenem) (4 to 256 µg/ml) and Imipenem (1 to 64µg/ml) in combination with a fixed concentration of EDTA is considered as the most sensitive method for MBL detection]. The E-test was done according to manufacturer’s instructions. MIC ratio of IP/ IPI(Imipenem+EDTA) of >8 or >3 log dilutions indicates MBL production. [19]

Genotypic detection of Metallo β - lactamase

Extraction of bacterial DNA and polymerase chain reaction was performed according to the manufactures guidelines. (Chromous biotech DNA).The sequence of primers and size of amplification products are listed in Table 1.

Target gene	Primer sequence		Amplicon size(bp)	Reference
	Forward	Reverse		
IMP	GTTTATGTTTCAT ACWTCG	GGTTTAAAYAAAA CAACCAC	432	Srinivasan et al,2009 [20]
VIM	TTTGGTCGCATA TCGCAACG	CCATTCAGCCAG ATCGGCAT	500	Srinivasan et al,2009 [20]
NDM	GGTGCATGCCCG GTGAAATC	ATGCTGGCCTTG GGGAACG	264	Nordmann et al,2011 [9]

Table-1

Figures and Tables

Table-2 MBL screening test

Organism	Positive
<i>Pseudomonas aeruginosa</i>	62/200(31%)

Table-3 Phenotypic confirmatory test

Organisms	Modified hodge test	Imipenem(IMP)- EDTA Combined disc test:	E-test
<i>Pseudomonas aeruginosa</i>	24/62	21/62	21/62

Table-4 : Distribution of various genes in the MBL producers (n=21).

Distribution of various genes in the MBL producers (n=21).			
Organisms	<i>blaIMP</i>	<i>blaVIM</i>	<i>bla NDM</i>
<i>Pseudomonas aeruginosa</i>	7	12	2

Table-5: Detection of MBL Production by Different Phenotypic Tests and Genotypic tests amongst Isolated Strains of *Pseudomonas aeruginosa* from different Clinical Samples

S. no	SAMPLE	TOTAL SAMPLE	SCREENING TEST POSITIVE	PHENOTYPIC CONFIRMATORY TEST			GENOTYPIC METHOD		
				Modified hodge test	IMP AND IMP-EDTA CDT	E-test	IMP	VIM	NDM
1	Pus	75	22	11	7	7	2	4	1
2	Urine	44	18	7	8	8	4	4	-
3	Sputum	14	3	-	1	1	-	1	-
4	Endotracheal tube	38	12	3	2	2	-	1	1
5	Blood	9	1	1	1	1	1	-	-
6	Pleural fluid	5	-	-	-	-	-	-	-
7	BAL	4	-	-	-	-	-	-	-
8	Folley's catheter tip	3	2	2	1	1	-	1	-
9	Vaginal swab	2	-	-	-	-	-	-	-
10	Nasal swab	3	-	-	-	-	-	-	-
11	Ascitic fluid	1	1	-	1	-	-	-	-
12	Bile	1	-	-	-	1	-	1	-
13	Throat swab	1	3	-	-	-	-	-	-
	TOTAL	200	62	24	21	21	7	12	2



Image 1: Modified hodge test



Image 2: Imipenem(IMP)- EDTA
Combined disc test



Image 3: E-Test

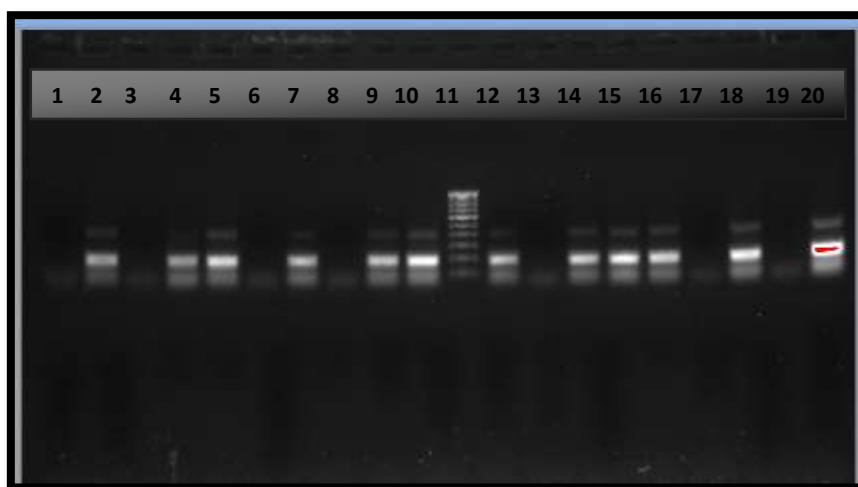


Image 4: Detection of bla VIM gene. 11 th lane is DNA ladder, 1 st lane is Negative control and 2, 4, 5, 7, 9, 10, 12, 14, 15, 16, 18, 20 lane is sample positive for VIM gene.

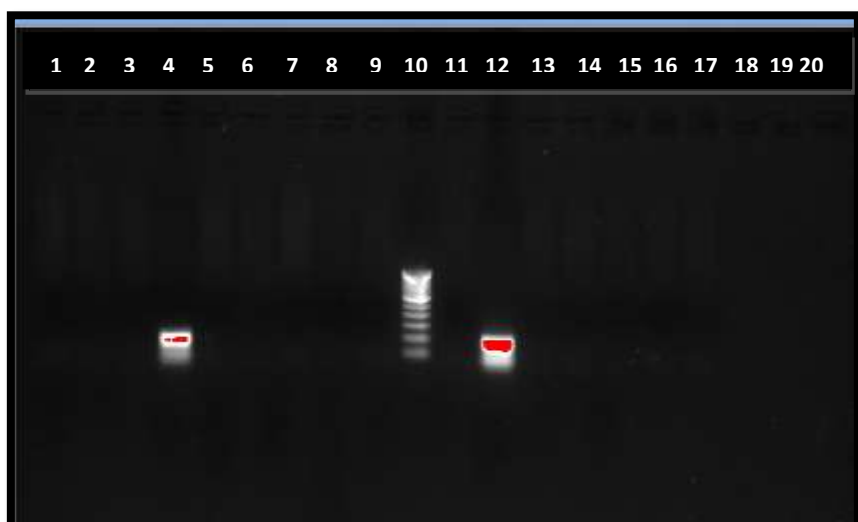


Image 5: Detection of bla NDM gene. 10 th lane is DNA ladder, 1 st lane is Negative control and 4th and 12 th lane is sample positive for NDM gene.

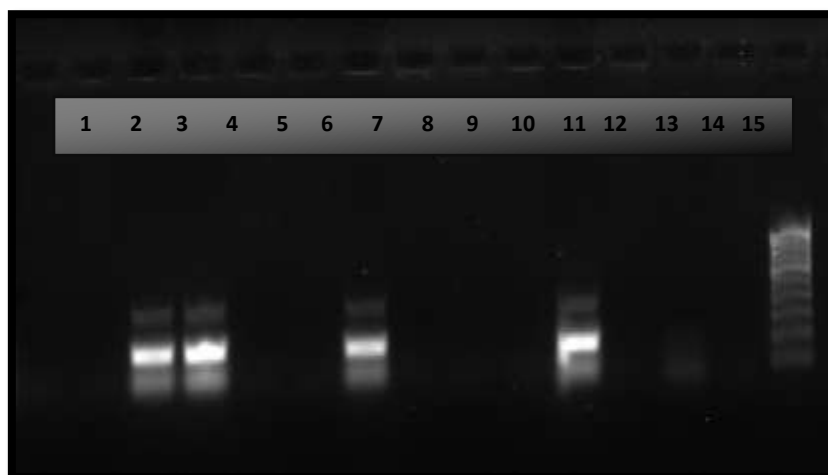


Image 6: Detection of bla IMP gene. 15 th lane is DNA ladder,1st lane is Negative control and 3, 4, 7 and 11 th lane is sample positive forIMP gene.

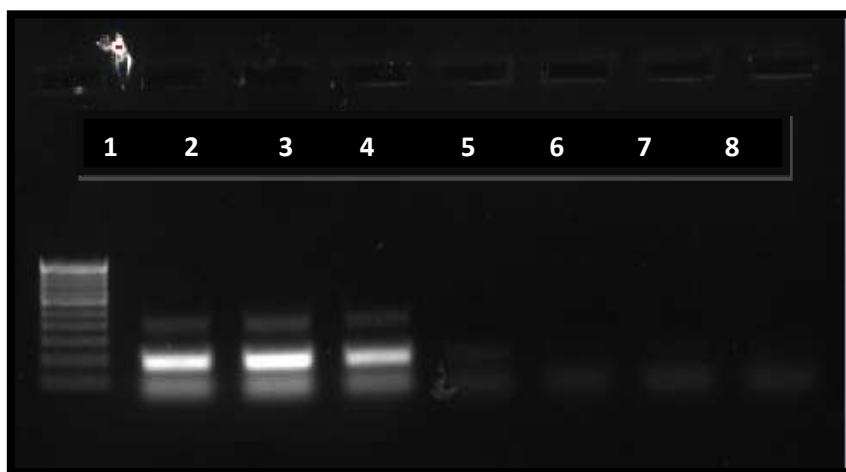


Image 7: Detection of bla IMP gene. 1 st lane is DNA ladder and 2, 3 and 4 rth lane is sample positive for IMP gene.

III. Results And Discussion

Carbapenems have a broad spectrum of antibacterial activity. Hence, they are often used as a last resort in treatment. These are resistant to hydrolysis by most β -lactamases including extended spectrum β -lactamases (ESBL) and AmpC β -lactamases. There has been an increase in reports of Carbapenem resistance in *P. aeruginosa* worldwide. In India the first report of metallo beta lactamase was published from Bangalore, MS Ramaiah Medical College by Navaneeth et al.12% of the isolates included in the study were resistant to both beta lactamase inhibitors and Carbapenem.100% of these isolates were found to be metallo beta lactamase producer (Navaneeth et al, 2002)[21]. In India the studies done on metallo beta lactamase producing non fermenters are numerous. The prevalence of metallo beta lactamase producers among Carbapenem resistant isolates (Resistant to either or both Imipenem and Meropenem) in the present study was found to be 21/200. The results vary all over the country. The rate of metallo beta lactamase production in our study is much lower compared to most of the other studies done in India It has been reported as low as 7.5%(Gupta et al, 2006) [22] to as high as 100%(Navaneeth et al, 2002) [21].

In the present study pus comprised for the majority of specimen followed by urine, sputum endotracheal aspirations, blood, and other sample. This study is similar to the study by Ranjan et al, 2014[23] where the majority of specimen included was pus (48.28%).This study is different from the study done by Wankhede et al [24] where the majority of specimen was wound swab (44.11%).

The criteria for choosing the isolates for MBL screening are varied. Some studies have chosen Ceftazidime resistant strains for screening MBL(Hemalatha et al,2005,Arakawa et al,2000 ,Franklin et al,2006).[25,12,26] Most of the studies have chosen Imipenem resistant strains for screening of MBL. In the present study strains resistant towards either or both Imipenem and Meropenem were included. However all the

isolates included in the study was resistant to Ceftazidime. 62/200(31%) *Pseudomonas aeruginosa* showed screening test positive. The similar finding were seen by Buchunde et al,2012 and Renu et al,2010.[27,28]

For screening of Metallo beta lactamase in the present study Double disc synergy test, Modified hodge test and Etest was done. Modified hodge test(MHT) shows more positive MBL then that of Imipenem(IMP)-EDTA Combined disc test. Modified hodge test is recommended by CLSI for confirmation of MBL production. One study by Picao et al,2008 ,shows that MHT had better sensitivity compared to CDT. Where as, Buchunde et al,2012 reported Meropenem EDTA CDT to be a better test compared to MHT.[27]In the present study E-test could detect MBL in the Imipenem resistant isolates correctly. E-test showed high sensitivity as compared to Double disk synergy test and Modified hodge test. Modified Hodge test showed 4 false positive results. This is in accordance with the study done by Behera et al, 2008, where all the isolates that screened positive for CDT where also positive by Etest.[11]

Many bacterial species require specific pH or temperature to grow and produce biofilm, *Pseudomonas* has been reported to produce EPS wherever conditions are appropriate for bacterial colonization. 24/68 *Pseudomonas* isolates produced biofilm *in vitro* in our study. Out of the 24 biofilm producing pseudomonas 8 were MBL positive by E-Test method, in our study it shows high rate of Biofilm production. (Nagaveni S et al. 2010) reported biofilm formation in *P. aeruginosa* in 83% of clinical strains & that biofilm formation was prevalent among isolates with a MDR phenotype.[29] PCR for 3 genes i.e. IMP, VIM, NDM was done in all the 62 screening positive isolates. 12 of the isolates were carrying blaVIM and 7 were carrying blaIMP gene.NDM was detected in only 2 isolates. None of the isolates were found to be carrying multiple MBL genes.The genes detected in the present study are bla IMP, bla VIM and bla NDM. The study done by Buchunde et al, 2012 from Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra reported all the MBL producing isolates carrying VIM gene. From Christian Medical College, Vellore Manoharan et al,2010 reported 15 VIM producing isolates among 20 MBL producer .Purohit et al,2012 from Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra reported bla-VIM MBL gene only in 7 (16.28%) of the 43 screen test positive *Acinetobacter* isolates. None of the isolates showed presence of bla-IMP gene. Whereas Uma et al, 2009 of Pondicherry University from Puducherry reported only bla-IMP-1 in 42% (23 isolates) of *A. baumannii*. Amudhan et al, 2001 from Chennai reported bla-VIM in 46.55% with and both bla-IMP and bla-VIM in only one isolate of *A. baumannii*. However all these studies had found single gene responsible for metallobeta lactamase production. A study from Delhi by Niranjana et al,2013 had reported presence of multiple genes responsible for MBL production in *Acinetobacter baumannii*.They also detected NDM-1 in the isolates .[15,30]

IV. Conclusion

Pseudomonas aeruginosa should be considered to be a very important nosocomial pathogen, which may have far-reaching effects. It is a crucial to provide information on the current status of *Pseudomonas aeruginosa* in hospitals, especially in view of the incidence of multi drug resistance in the organism. When used appropriately by doctors, health administrators and the commercial pharmaceutical industry, surveillance data can offer economic as well as health benefits to the health care system. Considering this, it is crucial to investigate the prevalence as well as susceptibility patterns of *P.aeruginosa* in hospitals. The detection of Metallo beta lactamase producing *Pseudomonas aeruginosa* needs screening and molecular identification for early initiating of the treatment, which would be helpful for identification of better Metallo β -actamase(MBL) producing screening methods and shows genetic variability in contrast to prevalence and mutational changes, in different parts of India.

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