Section A-Research paper
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Detection and Characterization of Class B Metallo-β-lactamase Producing Pseudomonas aeruginosa from various clinical samples in Tertiary care Hospital, Bhopal

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ABSTRACT:
Introduction: MBL producing P. aeruginosa is an emerging threat and a cause of concern as they have emerged as one of the most feared resistance mechanisms. MBL belongs to Ambler Class B owing to their capacity to hydrolyze all β-lactams including penicillins, cephalosporins and carbapenems with the exception of aztreonam. **Aim& Objective:**The aim of the present investigation was to characterize the prevalence of metallo β-lactamases and to study the antibiotic susceptibility profile among 102 clinical isolates of *Carbapenem resistant Pseudomonas aeruginosa.* **Material & Methods:** A total of 102 nonduplicate,consecutive, carbapenem resistant *Pseudomonas aeruginosa* isolated from patients hospitalized for 48 hours or more were included in the study.AST to imipenem and meropenem were determined and interpreted according to CLSI guidelines. The Combined disc test and MBL E test were used for screening of carbapenamases and MBL production respectively.PCR were performed for the detection of MBL (*bla*IMP,*bla*VIM and NDM) genes. **Result:**Among 102 Carbapenem (resistant to either or both Imipenem and Meropenem) non-susceptible isolates of Pseudomonas 27 (26.47%) were found to be MBL producers. Of 27 MBL-producing isolates, and 81.48% carried the blaVIM gene and 18.51% carried the blaIMP gene. All MBL-producing isolates were multidrug resistant.**Conclusion:** In our study the prevalence of MBL in P.aeruginosa which we found to be 26.47% of total carbapenem resistant isolates. Colistin and Polymixin B drug that showed 100% susceptibility towards MBL producing isolates. Timely identification of non-fermenters and monitoring their antibiotic susceptibility patterns are suggested for effective management of infections and limitation of the emergence of MDR.

Keywords: Pseudomonas aeruginosa,Metallo-beta-lactamase,blaIMP,blaVIM.

Introduction:

Pseudomonas aeruginosa is a Gram-negative, non-fermentative
organism found in diverse environmental settings. It is an opportunistic pathogen, causing serious infection in patients with weakened immune systems.[1] Pseudomonas aeruginosa was 2nd among the critical pathogens which are multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes, and among patients whose care requires devices such as ventilators and blood catheters, was published by WHO in 2017.[2]

Resistance to Carbapenem is predominantly mediated by MBL, Ambler molecular class B type of beta lactamases that recognize bivalent metal ions, having capacity to hydrolyze all β lactams including carbapenems.[3]Metallo-β-lactam genes are usually part of an integron structure and are carried on transferable plasmids but can also be part of the chromosome. Because of the integron associated gene cassettes, MBLs-producing *P. aeruginosa* isolates are often resistant to different groups of antimicrobial agents, which can be transferred to various types of bacteria. Therefore, MBLs-producing strains are important from an infection-control perspective.[4]

The different forms of MBL genes have been discovered in gram-negative bacteria including Verona integron- coded MBL (VIM), imipenemase (IMP),Seoul imipenemase (SIM), Germany imipenemase (GIM), Spaulo MBL (SPM), New delhi MBL (NDM). VIM and IMP are the most common inherited MBL genotypes in India.[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.Though several methods are employed in many studies, Clinical Laboratory Standards International (CLSI) guidelines do not recommend a standardised method for the detection of MBL producing isolates.[6]The aim of this study to detect and characterized the metallo-beta-lactamases producing genes of *Pseudomonas aeruginosa*.

MATERIAL & METHODS:

The study was conducted in a SRK university teaching hospital, Bhopal from July 2021 to August 2022. It included 102 clinical isolates of Carbapenem resistant *Pseudomonas aeruginosa* recovered from various clinical specimens of patients hospitalized for 48 hours or more. The isolates were obtained from clinical samples such as pus, urine, sputum, lower respiratory secretions(bronchoalveolar lavage, and endotracheal secretions), blood, and other body fluids from inpatients admitted to the different wards and ICUs of our tertiary care centre. The samples were processed in the Microbiology laboratory as per standard protocol.

Antibiotic Susceptibility Testing:

Antimicrobial susceptibility testing was done in Mueller Hinton agar by Kirby Bauer disc diffusion method and the result was interpreted as per the 2021 CLSI [7]guidelines.The following antibiotics were tested by disc diffusion method, Imipenem(10ug),Meropenem(10ug)(Himedia Laboratories, Mumbai, India),Ceftazidime(30ug),Cefepime(30ug),Piperacillin(100ug),Piperacillin/Tazobactum(100ug),Gentamicin(10ug),Tobramicin(10ug),Amikacin(10ug),Levofloxacin(5ug),Ciprofloxacin(5ug),Azteronam(30ug), Polymixin B(10ug) and Tigcycline(15ug).

PHENOTYPIC METHODS FOR DETECTION OF MBL:-

1.Imipenem-EDTA Combined disc Test: This test was performed as described by *Sondakar et al.*2020[8]. A lawn culture of test isolate was prepared. Allowed to dry for five minutes. Two imipenem (10 mg) discs, one with 0.5 M EDTA and other a plain imipenem disc, were placed on the surface of agar plates approximately 30mm apart. The plates were incubated overnight at 37*o*C for 16-18h. An increase in zone diameter of >7mm around the imipenem-EDTA disc in comparison to imipenem disk alone indicates the production of MBL.

2. MBL E-Test: The E test MBL-strip (Hi Media) contain a double sided seven dilution range of imepenem (IMP 4 to 256 µg/ml) gradient at one end & imipenem (1 to 64 µg/ml) in combination with a fixed concentration of EDTA at the other end. Mueller-Hinton agar plates were inoculated with the isolates equivalent to 0.5 McFarland standards, read after 24 hrs of incubation. If ratio of the MIC of Imepenem/ Imepenem-EDTA was >8 dilutions, it considered as an indicator of MBL production.[3]

Control strains: P. aeruginosa ATCC 27853

Preparation of Glycerol nutrient broth: Glycerol nutrient broth is prepared by mixing 16 ml of glycerol in 84 ml of nutrient broth and autoclaving it at 115^0C for 20 min(Cheesbrough *et al*.2006)[9]. The isolates of (27/102) MBL producing were non duplicate. The strains were stocked in 16% glycerol broth at -20 ^{0}C.

Fig. Primers used for the Pseudomonas aeruginosa.

Target Gene	Primer Sequence		Aplicon size	Referenc es
	Forward	Reverse		
IMP	5'CCA GAT TTA AAA ATA GAG AAG CTTG-3'	5'TGG CCA AGC TTC TAC ATT TGC GTC-3'	587	10
VIM	5'TCTACATGACCGCGTCTGTC -3'	5'TGTGCTTTGACAACGTTCGC-3'	748	10
NDM	5'GGTTTTGGCGATCTGGTTTT C-3'	5'CGGAATGGCTCATCACGATC-3'	522	10

STEPS OF PCR:

DNA Extraction: The PCR products of representative isolates were then purified by using a PCR DNA purification kit (QIA prep spin Miniprep Kit, Qiagen).

Detection of Metallo-β-Lactamase genes by PCR: The presence of bla IMP,bla VIM and bla NDM gene was tested in all the 27 test isolates.

Preparation of Master Mix- The working solution of the PCR were prepared by adding 12.5µl of the prepared Master Mix(Genei Laboratory Pvt.Ltd, Bangalore), than add 1 µl of each primer (the forward and reverse primers for NDM

genes), and 1µl of DNA and Finally 9.5 µl of the free Dnase/Rnase water. Each PCR reaction was carried out using a final volume of 25µl.

Amplification: PCR conditions included 35 cycles of amplification under the following conditions: denaturation at 94°C for 3 min., 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 5 minutes and then cooled at 4°C. For detection of NDM gene amplification was carried out under the following thermal cycling conditions: 10 min at 94°C; 35 cycles of amplification consisting of 3min. at 94°C, 1min. at 52°C, and 1min. at 72°C; and 5 min at 72°C for the final extension. Isolates positive for metallo beta lactamase production was found to be carrying either VIM or IMP. NDM was not detected in any of the isolates.

Gel Electrophoresis:
The PCR products were analyzed by electrophoresis in 1.0% agarose gel to detect specific amplified product of 587bp, 748bp and 522bp by comparing with standard molecular weight marker of 100 base pair (DNA ladder). The amplified products of the study samples were visualized by trans-illuminator, photographed by a digital camera and transferred to computer data for labeling and storage.

RESULT:

Table1.Sex wise Distribution of MBL.

	Total no. of Male	No. of MBL producer	Percentage(%)	Total no. of Female	No. of MBL producer	Percentage(%)
P.aeruginosa	68	17	25%	34	10	29.41%

In the present study, the above table no.1 is showing, MBL producers were found in lesser number in female patients but the percentage of MBL production among the Carbapenem resistant isolates is more in male patients having Pseudomonas aeruginosa infection (29.41%).

Table2. Sample wise distribution of MBL and Non MBL isolates.

Specimen Name	MBL	NON-MBL	Total
Pus	12	32	44
Urine	09	16	25
Sputum	01	07	08
ET aspirates	04	15	19
Blood	00	02	02
Others	01	03	04
Total	27	75	102

In this study(Table no.2) maximum isolates were obtained from pus sample(44) followed by, urine(25), sputum(8), Endotracheal aspirate(19), blood(02) and other(04).

Table3.Antibiotic Susceptibility Pattern of MBL (n=102)

Name of Antibiotics	**MBL Positive(27)**	**MBL Negative(75)**
Imipenem	26(96.29%)	72(96%)
Meropenem	25(92.59%)	72(96%)
Ceftazidime	27(100%)	75(100%)
Cefepime	27(100%)	74(98.66%)
Piperacillin	27(100%)	73(97.33%)
Piperacillin/Tazobactum	27(100%)	64(85.33%)
Gentamicin	27(100%)	75(100%)
Tobramycin	27(100%)	75(100%)
Amikacin	20(74.07%)	73(97.33%)
Ciprofloxacin	27(100%)	75(100%)
Levofloxacin	27(100%)	75(100%)
Aztreonam	27(100%)	75(100%)
Colistin	00(00%)	00(00%)
Polymixin B	00(00%)	00(00%)

Among the MBL producer Pseudomonas aeruginosa the highest sensitivity was found towards Colistin and Polymixin B (100% Sensitivity) followed by Amikacin (Resistance 74.07%).100% resistance was shown against, Ceftazidime,Cefepime, Piperacillin,Piperacilin/Tazobactum,gentamicin,Tobramycin,Ciprofloxacin.levofloxacin and Aztreonam.

Table4. Distribution of Metallo-β- Lactamase Genes(n=27)

Isolates Name	blaIMP	blaVIM
Pseudomonas aeruginosa	05	22
Total	05(18.51%)	22(81.48%)

blaVIM Gene (81.48%) was found to be the most abundant in the present study followed by blaIMP(18.51%). No other gene were detected.(Table no. 4).

DISCUSSION:

Carbapenemases are enzymes that are capable of hydrolyzing nearly all β-lactam antibiotics, including carbapenems. They are divided into three separate molecular groups (A, B, and D), and in recent years, epidemiological significance has increased in many regions of the globe, including Europe. Most carbapenemases are plasmid-mediated and have been mainly seen in Enterobacteriaceae, *Acinetobacter baumannii* as well as *Pseudomonas aeruginosa*.[11] The production of most MBLs is chromosomally encoded and does not pose a serious threat to other bacteria. However, in 1991, the first plasmid-mediated MBL, IMP-1 from Pseudomonas aeruginosa, was reported in Japan, while another type of acquired MBL, VIM-1, was first reported in Italy in 1999.[12]

In India the studies done on metallo beta lactamase producing nonfermentors are numerous.The results vary all over the country. In the present study,the prevalence of metallo beta lactamase producers among carbapenem resistant isolates (Resistant to either or both Imipenem and Meropenem) was found to be 26.47%.

Similarly,One research study published from Maharashtra, L.T.M. Medical College, by Kumar, *et al*.2012[13] reported 26.9% of carbapenem-resistant isolates to be metallo beta-lactamase producers. Some other studies published by Ajana Rai,2018[14], Wesam Hatem Amer, 2010[15], Goel, *et al*.2013[16] who reported 35.2%, 80%, 53.85% MBL producers, which is much higher compared to the present study.

In the present study pus comprised for the majority of specimen followed by, urine, ET aspirates, sputum, other, blood sample. Similarly,a study published by Ranjan *et al*.2014[17] where the majority of specimen included was pus (48.28%) and the study done by Wankhede *et al*,2011[18] where the majority of specimen was wound swab(44.11%).

The isolates included in this study were taken from 68 male patient and 34 female patient. In the present study, The percentage of MBL production in female is 29.41% which is slightly higher than in male patients (25%).as Compared to other studies have reported a lower prevalence of MBL-PA conducted by Kali *et al*. (16.32%)[19], Gupta *et al*.(14.3%)[20], Ranjan *et al*.(16.72%)[21], Chaudhary *et al*. (16.89%), and Chauhan *et al*.(19.15%)[22].

In Pseudomonas aeruginosa colistin and Polymixin B was found to be 100% effective and next most effective drug were Amikacin20(74.07%). 100% resistance was shown against,Ceftazidime,Cefepime,Piperacillin,Piperacilin/Tazobactum,gentamicin,Tor-amycin,Ciprofloxacin.levofloxacin and Aztreonam.

A study done by John *et al*, 2011[23] and Smita Sood *et al*.2014[24],the sensitivity towards Colistin and Polymixin B was reported to be 100%. The present study is in accordance to this findings. However it differed in case of Gentamycin, John *et al*.2011[23], reported 100% resistance and Amikacin 56.7% resistance. In this study it was seen that all MBL producing P.aeruginosa isolates showed aminoglycoside resistance. In Pseudomonas isolates the effective aminoglycoside was Amikacin. All the MBL producers showed some kind of resistance mechanism towards aminoglycosides. This hints that these organisms carry multiple resistance genes which makes the therapeutic options very limited.

In the present study only bla IMP and blaVIM genes were detected. No other gene were detected. Among 27 MBL producer majority of the isolates had 22(81.48%) VIM gene and only 05(18.51%) IMP gene were detected. 20 MBL-producing isolates by Ramakrishnan, *et al*.2014[25] from Punducherry, reported the *bla*VIM-2 gene in 13% of MBL producers in *Pseudomonas* isolates and only one (1.33%) was positive for *IMP* 1 gene. A study done by R. Mahesh Reddy from Maharashtra, India in 2017[26] reported, VIM type (43%), IMP-1 (12%) in *P.aeruginosa* isolates. One study from Nepal published by Mahesh Acharya in 2017,who reported blaVIM-2 gene in 75% isolates and blaIMP-1 in 25% isolates respectively.

In India, mostly the VIM gene has been reported in various studies. IMP gene has been reported in much lesser numbers. Very few cases of the NDM gene, to be specific isolated cases of the NDM gene in non-fermenters have been reported which indicates a limited spread of the NDM gene.

CONCLUSION:

In this study was intended to find out the prevalence of MBL in P.aeruginosa which we found to be 26.47% of total carbapenem resistant isolates. MBL producing isolates carry multidrug resistant integrons. Infact the only Colistin and Polymixin B drug that showed 100% susceptibility towards MBL producing isolates. Timely identification of non-fermenters and monitoring their antibiotic susceptibility patterns are suggested for effective management of infections and limitation of the emergence of MDR.

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