Original Article

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Microbiology Section

Phenotypic Tests for Detecting Incidence of Metallo-B-Lactamase (MBL) Producing *Pseudomonas aeruginosa* in Jaipur

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ABSTRACT

Background: Metallo-beta-lactamase (MBL) mediated resistance to carbapenems is an emerging threat in hospital isolates of *Pseudomonas aeruginosa*.Prompt detection and recognition of MBLs is important for timely implementation of infection control interventions and treatment with alternative antimicrobials. There is limited information from the state of Rajasthan regarding the prevalence of such enzymes.

Objectives: A study was conducted to determine the prevalence of metallo – β – lactamases (MBLs) in clinical strains of Psuedomonas aeruginosa and to compare three phenotypic tests as a method for MBL detection.

Material And Methods: *Pseudomonas aeruginosa* strains isolated from various clinical specimens at a tertiary care private hospital in Jaipur city, which tested resistant to imipenem, were tested for MBL production using three phenotypic tests: double disc synergy test, disc potentiation test and thiol based compound test.

Result: Of the 95 *Pseudomonas aeruginosa* isolates obtained during the study period, 69 (72.63%) were resistant to imipenem. All the three phenotypic methods were equally effective in demonstrating that 100% of the imipenem resistant strains were harboring MBLs. Maximum MBL producer isolates were found in patients admitted to the ICUs (56.52%) with majority of isolates from the Medical ICU (47.82%). Maximum MBL producers were obtained from lower respiratory secretions (39.13%) followed by urine (27.52%). 100% sensitivity to Colistin and Polymixin B was observed in these MBL harboring *Pseudomonas aeruginosa*.

Conclusion: The three phenotypic methods proved to be equally effective as a method to detect MBL production in *Pseudomonas aeruginosa* isolates and any one of these can be easily incorporated in routine lab procedures to detect MBLs.

Keywords: MBL, *Pseudomonas aeruginosa*

INTRODUCTION

The introduction of carbapenems into clinical practice represented a great advance for the treatment of beta -lactam resistant bacteria. The carbapenems have been the drugs of choice for treatment of infections caused by penicillins or cephalosporin –resistant gram negative bacilli because of their broad spectrum of activity and stability to hydrolysis by most beta lactamases [1]. However, this scenario has changed with the emergence of carbapenem resistant strains especially in the non- fermenting gram negative bacilli (NFGNB) such as *Pseudomonas aeruginosa* and Acinetobacter spp [2].

Pseudomonas aeruginosa producing Metallo-β- lactamases was first reported from Japan in 1991 and since then these have been described from various parts of the world including Asia, Europe, Australia, South America and North America [3,4] Metallo –β-lactamases belong to the Ambler class B and have the ability to hydrolyze a wide variety of beta lactam agents, such as penicillins, cephalosporins and carbapenems but not monobactams [5]. MBLs can be divided into six categories according to their molecular structure namely IMP, VIM, GIM, SIM, SPM and AIM. These enzymes require zinc for their catalytic activity and are inhibited by metal chelators, such EDTA and thiol compounds [6].

MBL producing *Pseudomonas aeruginosa* isolates have been reported as important causes of nosocomial infections and have been associated with clonal spread [7]. Thus, early detection of MBL producing organisms is of great importance for the prevention of their inter and intra- hospital dissemination.

Several phenotypic methods are available for the detection of MBL producing bacteria. All these methods are based on the ability of metal chelators, such as EDTA and thiol

based compounds to inhibit the activity of MBLs. These tests include the double disk synergy tests using EDTA with ceftazidime(CAZ) or Imipenem(IMP),2-mercapto propionic acid with IMP or CAZ, Hodge test, a combined disk test using EDTA with CAZ or IMP, the MBL test E test and a microdilution method using EDTA and 1,10-phenanthroline with IMP [8].

The present study was undertaken to detect the prevalence of MBL in clinical strains of *Pseudomonas aeruginosa* isolated at a tertiary care private hospital in Jaipur, Rajasthan and to evaluate the accuracy of three phenotypic tests -disc potentiation test, double disc synergy test and thiol based compound test for the detection of MBLs in *P. aeruginosa* isolates.

MATERIALS AND METHOD

After due approval from the Institutional Review Board, a study was carried out in Microbiology lab of a tertiary care private hospital in Jaipur to detect the production of Metallo beta lactamase (MBL) in clinical isolates of Psuedomonas aeruginosa. No duplicate isolates were included in this study. These isolates were collected over a period of 8 months from April to November 2010.

Identification of bacterial isolates: All samples (except urine) received in the Microbiology lab were initially cultured on Sheep blood agar, Mac Conkey agar and Thioglycollate broth and incubated for 24-48 hours at 37°C under aerobic conditions. Urine samples were cultured on to CLED (Cystine Lactose Electrolyte Deficient) agar. Blood samples were collected in commercial BACTEC vials for automated culture on the BACTEC 9050 system (Sparks, Maryland, U.S.A). Oxidase test was performed to detect the isolates suspicious of being Pseudomonas species. Microscan auto Scan- 4 identification and susceptibility system (Siemens, West Sacramento, California, USA) was used for organism identification and sensitivity testing. *Pseudomonas aeruginosa* ATCC 25923 was used as control strain. All imipenem resistant isolates of *P. aeruginosa* were tested for production of Metallo – β - lactamase production by three phenotypic methods- (i) Disc potentiation test (ii) Double disc synergy test (iii) Thiol based compound test.

Test for detection of Metallo Beta Lactamase:

(I) Disc Potentiation test [9]

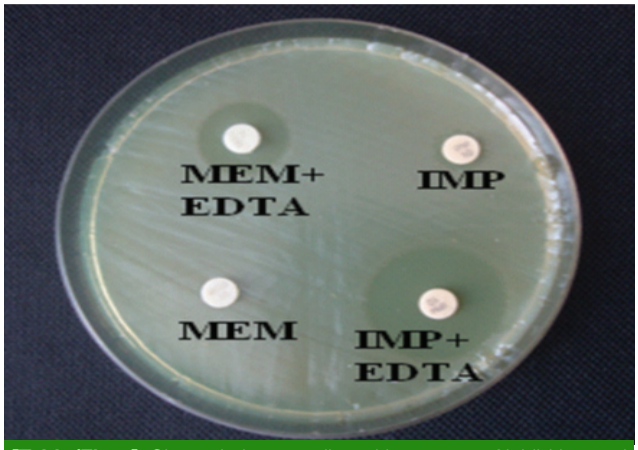
A 0.5 M EDTA solution (by dissolving 186.1 g of disodium EDTA.2H2O in 1000 ml of distilled water and adjusting it to pH 8.0 using NaOH) was prepared and was sterilized by autoclaving. The test organism was swabbed onto plates of Mueller Hinton agar plate. Two 10 µg imipenem discs and two 10 µg meropenem discs were placed on inoculated plates and 5 µl of EDTA solution was added to one imipenem and one meropenem disc. The zone of inhibition around imipenem and meropenem discs alone and those with EDTA was recorded and compared after 16-18 hour incubation at 35°C. An increase in zone size of at least 7 mm around the imipenem-EDTA disc and meropenem-EDTA discs was recorded as a positive result [Table/Fig-1].

(II) Double Disc synergy test [10]

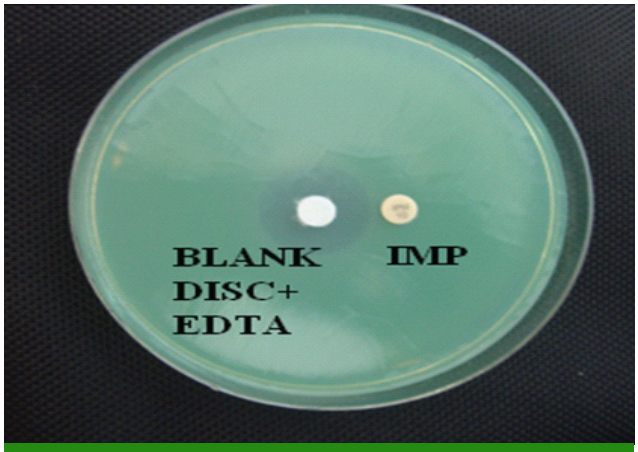
Test strain was suspended to the turbidity of a McFarland no. 0.5 tube and used to swab inoculate a Mueller–Hinton agar plate. After drying, a 10-µg imipenem disc and a blank filter paper disc were placed 10 mm apart from edge to edge, and 10 µL of 0.5 M EDTA solution was then applied to the blank disc, which resulted in approximately 1.5 mg/disc. After overnight incubation, the presence of an enlarged zone of inhibition around the blank disc was interpreted as EDTA-synergy test positive [Table/Fig-2].

(III) Thiol based compound test [11]

A colony of each bacterial strain was suspended and diluted with Mueller-Hinton (MH) broth to 106 CFU/ml and spread on

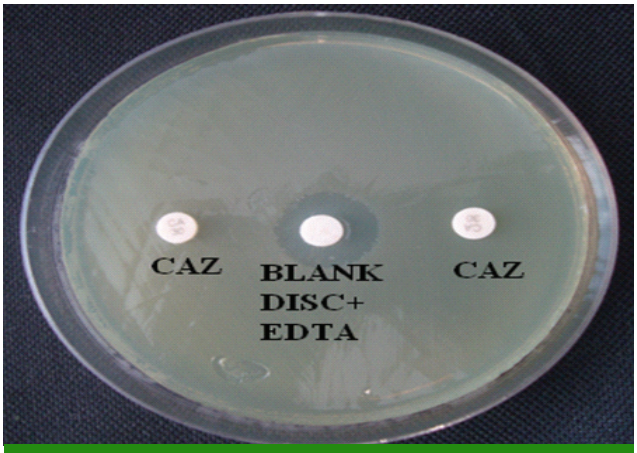


[Table/Fig-1]: Shows Imipenem disc with no zone of inhibition and imipenem with EDTA disc with an inhibition zone of 19 mm. The enhancement of zone size of zone size of imipenem+EDTA ≥ 7mm indicates the test strain is positive for metallo-β -lactamase by disc potentiation test.



[Table/Fig-2]: Shows Imipenem disc with no zone of inhibition and the blank disc with EDTA with zone size of 18 mm. The enlargement of inhibition zone of ≥7mm around blank disc indicates the test strain is positive for metallo-β- lactamase by double disc synergy test.

Mueller Hinton agar plate with a cotton swab according to the protocol recommended by the National Committee for Clinical Laboratory Standards. Two commercially supplied discs, each containing 30 µ g of CAZ (Himedia), were then placed on the plates. The distance between the two CAZ discs was kept at about 4 to 5 cm, and a filter disc was placed near one of the CAZ discs within a center-to-center distance of 1.0 to 2.5 cm. Two to five micro liters of 0.5 M EDTA solution was added to the filter disc on the agar, and each agar plate was incubated at 37°C overnight. The zone of inhibition between CAZ and blank disc with EDTA were compared, the presence of an enlarged zone of inhibition in blank disc with EDTA was interpreted as positive result [Table/Fig-3].

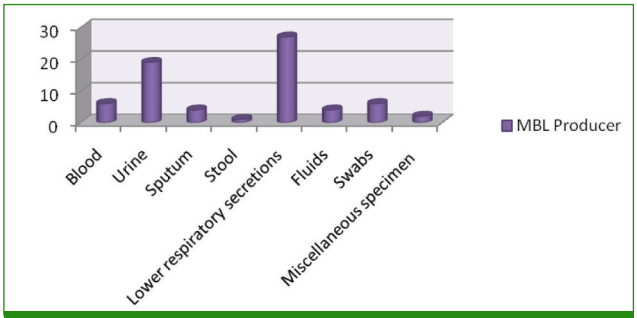


[Table/Fig-3]: Shows the test strain is resistant to ceftazidime (CAZ) discs and show a zone of inhibition of 15mm around a blank disc impregnated with EDTA (a thiol compound).This indicates the test strain is positive for metallo-β- lactamase by thiol based compound test.

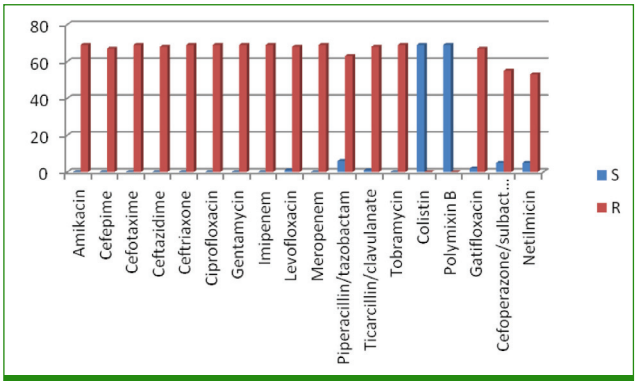
RESULTS

A total of 95 isolates of P.aeruginosa were collected from various clinical specimens over the study period. 69(72.63%) isolates were found to be resistant to imipenem. These 69 imipenem resistant isolates were tested for MBL production using three different phenotypic tests: double disc synergy test, disc potentiation test and thiol based compound test. MBL production was detected in all 69 (100%) Imipenem resistant *Pseudomonas aeruginosa* strains by each of the three phenotypic test methods.

OPD (outpatient department) specimens shared 2.90% of MBL producers and IPD (in patient department) specimens shared 97.10 % MBL producer P.aeruginosa isolates. In the IPD, maximum MBL producer *P. aeruginosa* isolates were found in patients admitted to the ICUs-Intensive care units (56.52%). [Table/Fig-4] shows the gender wise distribution of patients yielding *Pseudomonas aeruginosa* in their clinical specimens. 81.05% of the total *Pseudomonas aeruginosa* isolates were obtained from male patients and 18.95% from female patients. [Table/Fig-5] shows the age distribution of patients yielding *Pseudomonas aeruginosa* in their clinical



[Table/Fig-4]: Frequency distribution of MBL producer P.aeruginosa from various clinical specimens



[Table/Fig-5]: Antibiotic susceptibility pattern of MBL producer *P.aeruginosa.*

Gender	MBLproducer (No. and %)	MBL non producer (No. and %)	Total isolates (No. and%) (n=95)
Male	56 (72.72)	21 (27.27)	77 (81.05)
Female	13 (72.22)	5 (27.77)	18 (18.95)
Total	69 (72..63)	26 (27.36)	95 (100)

[Table/Fig-6]: Gender wise distribution of patients with MBL producer and MBL non producer P.aeruginosa isolates.

Age Group (in years)	MBL Producer (No. and %)	MBL Non producer (No. and %)	Total isolates (n=95) (No. and %)
1-10	1 (1.45)	1 (3.85)	2 (2.10)
11-20	3 (4.35)	1 (3.85)	4 (4.21)
21-30	10 (14.50)	0	10 (10.52)
31-40	9 (13.04)	4 (15.38)	13 (13.70)
41-50	3 (4.35)	1 (3.85)	4 (4.21)
51-60	16 (23.19)	8 (30.76)	24 (25.26)
61-70	9 (13.04)	5 (19.23)	14 (14.75)
71-80	15 (21.74)	6 (23.08)	21 (22.10)
81-90	3 (4.35)	0	3 (3.15)
Total	69 (100)	26 (100)	95 (100)

[Table/Fig-7] : Age wise distribution of patients with MBL producer and MBL non – producer *P. aeruginosa* isolates.

specimens. Maximum MBL production was found in isolates obtained from patients in the age group 51-60 years.

[Table/Fig-6] demonstrates the frequency distribution of MBL producer *Pseudomonas aeruginosa* from various clinical specimens. Maximum MBL producer P.aeruginosa were isolated from lower respiratory secretions (39.13%) followed by urine (27.52%).

[Table/Fig-7] shows the antibiotic sensitivity profile of MBL producer *Pseudomonas aeruginosa* isolates. 100% sensitivity to Colistin and Polymixin B was observed.

DISCUSSION

Carbapenems are often used as last resort antibiotics for treating infections caused by multidrug resistant gram negative bacilli as they are stable and respond only to extended spectrum and Amp C- β lactamases [12]. However, there has been increasing reports of resistant to this life saving antimicrobials in *Pseudomonas aeruginosa* [13,14]. Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin binding proteins and the production of carbapenem hydrolyzing enzymes i.e. carbapenemases. The resistance due to the production of carbapenem hydrolyzing enzymes such as metallo- beta- lactamases (MBL) may be chromosomally encoded or plasmid mediated and hence poses a threat of spread of resistance by gene transfer among gram negative bacteria [14]. 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.

Pseudomonas aeruginosa producing MBL was first reported in India in 2002 [15]. In this study we observed MBL production in 72.63% of the *Pseudomonas aeruginosa* isolates. Goel et al., (2013) have reported 53.85% of *Pseudomonas aeruginosa* isolates from ICU of a tertiary care hospital to be MBL producers [16] 11.42% of *Pseudomonas aeruginosa* were found to harbor MBLs in another study from Gujarat [17] and 32.04% MBL production in *Pseudomonas aeruginosa* isolates from burn and surgical wards was noted in a recent study from Mumbai, Maharashtra [18]. MBL prevalence ranging between 8-97% in *Pseudomonas aeruginosa* has been reported in various other Indian studies [1,8,15,19,20].

We found 100% of the imipenem resistant *Pseudomonas aeruginosa* strains to be MBL producers. Our findings are consistent with those reported at a tertiary care centre in Kenya [6]. Based on observations of other researchers, [21, 22,23] a likely explanation of such a finding is that other mechanisms of resistance to the carbapenems such as impermeability which arises via the loss of the Opr D porin or the up regulation of an active efflux pump system present in the cytoplasmic membrane of these organisms were totally absent at our centre.

Currently there are no recommendations available from Clinical laboratory standards Institute (CLSI) or elsewhere for the detection of MBLs in *Pseudomonas aeruginosa* [24]. A few phenotypic methods have been published for MBL detection; however, the results have shown that no method alone is able to detect all these enzymes, probably due to the genetic variability of these enzymes [25]. PCR gives specific and accurate results; its use is limited to only a few laboratories because of its high cost and because of the different types of MBLs which are present worldwide [19].

We evaluated three phenotypic methods: double disc synergy test, disc potentiation test (combined disc test) and thiol compound test to detect the MBL production in carbapenem resistant *Pseudomonas aeruginosa* isolates. All the three methods were equally effective in identifying MBL production. A study from New Delhi compared four phenotypic methods for screening of MBL production and reported that the combined disc method using Imipenem+EDTA was superior to DDST using Imi-EDTA [20]. Another study has also reported that the Imp-EDTA combined disc diffusion test has the highest sensitivity in detecting MBLs [8]. A comparative analysis of phenotypic tests for detection of MBLs reported that Combined disc test (IMP+EDTA), EDTA –Impenem Microbiological assay, imipenem-EDTA + sodium mercaptoacetic acid Double Disc Synergy test (IPM-EDTA+SMA DDST) and extended EDTA disc synergy test (eEDST) to be equally efficient for detection of MBLs in Indian clinical isolates of *P. aeruginosa* [26]. However, Tomasz et al., reported the highest percentage of positive results for detection of MBLs in P.aeruginosa was obtained using double disk synergy tests with Imipenem and EDTA [27]. Similar to our findings, few studies have found that both combined disc test and double disc synergy test were equally effective in detecting MBL in imipenem resistant *Pseudomonas aeruginosa* [19,28]. Khosravi et al., compared the Double-disk synergy test (DDST), combined disk test (CDT), and imipenem/imipenem-inhibitor (IP/IPI) E-test to evaluate their MBLs detection capability and found all three methods were shown to have a sensitivity of 100%. However, DDST was found to be the most specific of the three followed by IP/IPI E-test and CDT was the least specific [29].

In this study MBL production in imipenem resistant *Pseudomonas aeruginosa* was observed in 18.84% from females and 81.15% from males. Our findings are consistent with an earlier study from coastal Karnataka which reported MBL production in 81% isolates from males and 19% isolates from females [28]. Maximum MBL production was found in isolates obtained from patients in the age group 51-60 years followed by patients in the age group 71-80 years. Shobha et al., reported maximum MBL production in the isolates obtained from age group 40-75 years [28]. In our study maximum MBL producer *P. aeruginosa* were isolated from lower respiratory secretions (39.13%) followed by urine (27.52%). Similar preponderance of MBL production in *P. aeruginosa* isolates from lower respiratory tract secretions has been noted by few other Indian studies [2,30]. 100% sensitivity to Colistin and Polymixin B was observed in MBL producer *Pseudomonas*

aeruginosa isolates in our study. Similar to our findings it has been reported that in addition to resistance to all β-Lactams the MBL producing strains are frequently resistant to aminoglycosides and flouroquinolones [31].

CONCLUSION

Emergence of MBLs producing *Pseudomonas aeruginosa* in our clinical strains is alarming and reflects excessive use of carbapenem. Therefore early detection and prompt instillation of infection control measures is important to prevent spread of MBLs to other gram negative rods. Additionally it is also important to follow antibiotic restriction policies to avoid excessive use of carbapenem and other broad spectrum antibiotics. Finally to understand the epidemiology, there is a need of genetic analysis and also typing of metallo-β-lactamase enzymes.

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