

## DETECTION AND PREVALENCE OF METALLO-BETA-LACTAMASE ENZYMES PRODUCING PSEUDOMONAS AERUGINOSA ISOLATED FROM VARIOUS CLINICAL SAMPLES.

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### ABSTRACT

**Background:** Pseudomonas aeruginosa is a clinically troublesome gram negative pathogen that causes a wide range of opportunistic infections and nosocomial outbreaks. Nosocomial infections by Pseudomonas aeruginosa are escalating and importantly the production of MBL is a matter of concern. Carbapenems, being the most potent and reserved drug for treating the infections cause by multi-drug resistant bacteria especially Pseudomonas spp is under threat due to the emergence of MBL producing Pseudomonas aeruginosa. Thus, the present study was undertaken to detect MBL producing Pseudomonas aeruginosa isolated from different clinical samples from clinical laboratories. **Methods:** Pseudomonas aeruginosa strains were obtained

by standard isolation and identification techniques from various clinical samples from clinical laboratories were then subjected to susceptibility testing for antipseudomonal drugs as per Clinical and Laboratory Standards Institute (CLSI) guidelines (year 2011). Carbapenems resistant strains were selected for the detection of MBL enzyme production by disc potentiation test. Production of MBL was confirmed by enhancement of inhibition zone around imipenem and meropenem discs impregnated with EDTA, as compared to discs without EDTA. **Results:** Among of 120 samples, ET aspirate (36.66%) wound swab (28%) and blood (28%) showed high percentage of MBL producers. 36 (30%) were found to be carbapenem resistant, 22(18.33%) isolates were positive for MBL production. There was high prevalence of MBL enzyme amongst multidrug resistant P. aeruginosa. Therefore, the reliable detection of the MBL producing strains is essential for the optimal treatment of

infected patients and to control the nosocomial spread of resistance. **Conclusion:** Study indicates that, surveillance for the detection of MBL is necessary.

**KEYWORDS:** Metallo-beta-lactamase, Nosocomial infection, Carbapenems, EDTA.

## INTRODUCTION

*Pseudomonas aeruginosa* is one of the most important pathogens causing nosocomial infections. It is naturally resistant to many antimicrobial agents. It has a distinctive capacity to become resistant to many available antimicrobial agents via multiple mechanisms<sup>[1]</sup>. Acquired resistance is also reported by the production of plasmid mediated AmpC beta ( $\beta$ ) – lactamase, Extended Spectrum Beta ( $\beta$ )–Lactamase (ESBL) and metallo beta ( $\beta$ ) – lactamase (MBL) enzymes.<sup>[2]</sup> With the widespread use of extended-spectrum antibiotics, in a very short time span *P. aeruginosa* has become resistant to a variety of antimicrobial agents, such as  $\beta$ -lactams, aminoglycosides, chloramphenicol, quinolones, tetracycline's and sulphonamides. Emerging resistance to expanded-spectrum cephalosporin's and carbapenems among *P. aeruginosa* has been a major concern<sup>[3]</sup>.

The mechanism of resistance to beta lactam antibiotics includes, the production of beta lactamase, reduced outer membrane permeability, the altered affinity of target penicillin binding proteins, plasmid mediated resistance involving modifying enzymes<sup>[4]</sup>. Metallo beta lactamases are class B beta lactamases. These require zinc or another heavy metal for their catalytic activity and their activities are inhibited by metal chelating agent such as EDTA and thiol based compounds<sup>[5]</sup>. Class B beta lactamases confer resistance to a wide range of beta lactam compounds, including cephalosporin's and carbapenems. Class B beta lactamases are resistant to inactivation by clavulanate, sulbactam and tazobactam<sup>[6]</sup>. Metallo beta lactamases have the ability to hydrolyse a wide variety of beta lactam agents such as penicillin's, cephalosporins and carbapenems. The majority of metallo beta lactamases are chromosomally encoded and their expression may be constitutive and inducible<sup>[7]</sup>.

The genes responsible for MBL production may chromosomally or plasmid mediated and hence poses a threat for spread of resistance by gene transfer among the Gram-negative bacteria.<sup>[8]</sup> MBL producing Gram negative bacilli, specially *Pseudomonas* spp, have been increasingly reported in Asia, Europe, Latin American and the United States<sup>[9]</sup>.

The appearance of MBL genes and their spread among bacterial pathogens is a matter of concern with regard to the future antimicrobial chemotherapy.<sup>[10][11]</sup> Further, due to increase occurrence and types of these multiple  $\beta$  lactamase enzymes, early detection is crucial, the benefits of which includes implementation of proper / optimal antibiotic therapy particularly in critically ill and hospitalized patients, infection control policy and to control the spread of resistance.<sup>[12]</sup> The main objective of the present study was to assess the prevalence of metallo beta lactamase producing strains among multi drug resistant pseudomonas aeruginosa isolated from various samples from clinical laboratories. Therefore the reliable detection of the MBL producing strains is essential for the optimum treatment of infected patients and to control the nosocomial spread of resistance.

## MATERIALS AND METHODS

The present study was carried out on *P. aeruginosa* obtained from various clinical samples from clinical laboratories during the period March 2015 to February 2016. One hundred and twenty MDR *P. aeruginosa* isolates were obtained from 400 clinical samples. The samples from which the strains were isolated include blood, Pus, Urine, Broncho alveolar lavage (BAL) and endotracheal (ET) aspirates and tissues. Repeat isolates were excluded from the study. All the samples were processed for isolation and antibiotic sensitivity. Isolation of organism was done by streaking the samples on MacConkey's agar and Blood agar plates. Further identification was done by gram staining (gram negative bacilli), catalase (positive), oxidase (positive), and pigment production (positive), hanging drop preparation (motile) was done. ATCC *P. aeruginosa* 27853 strain was used as quality control reference strain for all experiments with satisfactory results.

Antibiotic sensitivity testing method was performed by Kirby Bauer method. Antibiotics included in the study are piperacillin (75 $\mu$ g), Piperacillin/Tazobactam (100/10 $\mu$ g), Cefotaxime (30 $\mu$ g), Cefoperazone (75 $\mu$ g), Ceftriaxone (30 $\mu$ g), Imipenem(10 $\mu$ g), Meropenem(10  $\mu$ g), Gentamicin (10 $\mu$ g), Amikacin (30 $\mu$ g), Norfloxacin 10 $\mu$ g), Ciprofloxacin (5 $\mu$ g), Cefoperazone/Sulbactam (75/25 $\mu$ g), Tobramycin (10 $\mu$ g), Netilmicin (30 $\mu$ g), Polymyxin B (300U), gatifloxacin (5ug)

All Carbapenem resistant isolates were tested for MBL enzyme. Various methods such as, the modified Hodge test, Imipenem -EDTA double- disc synergy test (DDST) and Imipenem-EDTA combined disc test (CDT)(13) are described.

Detection of MBL producing *P. aeruginosa* was performed by the Imipenem-EDTA disk potentiation test. **Imipenem- EDTA combined disc test (CDT):** The CDT was performed as described by Yong *et al.*<sup>[14]</sup> each test strains of *P. aeruginosa* were inoculated on Mueller Hinton agar plates as per standard guidelines. Total 18.61g of EDTA was dissolved in 100 ml distilled water to prepare 0.5 M EDTA solutions and its pH was adjusted to 8.0 by using NaOH. This mixture was then sterilized by autoclaving. Two imipenem (10 $\mu$ g) discs were placed on the surface of an agar plate at distance of 30 mm and 4 $\mu$ l EDTA solution was added to one of them to obtain a desired concentration of 750  $\mu$ g. The inhibition zones of imipenem and imipenem- EDTA discs were compared after 16 to 18 h of aerobic incubation at 35 $\pm$ 2C. In the combined disc test, if the increase in inhibition zone with the imipenem and imipenem- EDTA disc was  $\geq$ 7 mm than the imipenem alone, it was considered MBL positive.<sup>[15][16]</sup>

## RESULTS

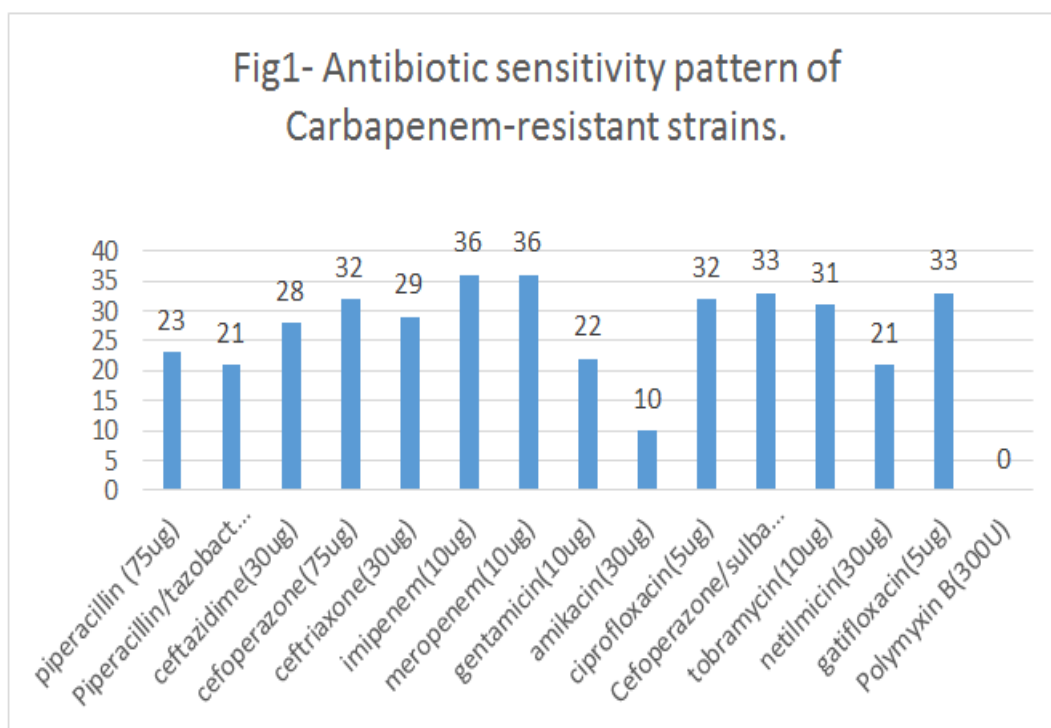
A total of 120 clinical isolates of MDR *Pseudomonas aeruginosa* identified from Clinical Microbiology laboratories for a period of one year were included in this study. Amongst these 120 non-repetitive strains of *P. aeruginosa*, 36 (30%) strains were found resistant to carbapenem and 22 (18.33%) strains were found to be MBL enzyme producer which were confirmed by the disc potentiation method. The ATCC 27853 *P. aeruginosa* did not exhibit any zone size enhance with EDTA-impregnated imipenem disc.

Amongst the 22 MBL enzyme producing isolates, 8(36.66) were from endotracheal (ET) aspirate, 6 (27.27% from) wound swab, 6 were from blood, 1 was from tissue and 1 was from the urine. (**Table 1**)

The antibiotic sensitivity pattern of carbapenem resistant strains of *P. aeruginosa* was found as reflected in (Table 2, Fig-1 ). The antibiotic sensitivity patterns of carbapenem resistant strains of *P. aeruginosa* for MBL-positive and MBL-negative were detailed in (Table 3, Fig-2).

**Table 1- Isolated strains of *P. aeruginosa* from different clinical samples.**

No.	sample	MDR <i>P.</i> isolates (n=120)	Carbapenem Resistant (n=36) (n=30%)	MBL producer (n=22) (n=18.33%)
1	ET aspirate	48	15 (41.66%)	8 (36.66%)
2	BAL	4	0	0
3	Blood	20	9(25%)	6 (27.27%)
4	Wound swab	42	10(27.77%)	6 (27.27%)
5	Tissue	3	1(2.77%)	1 (4.5%)
6	Urine	3	1(2.77%)	1 (4.5%)



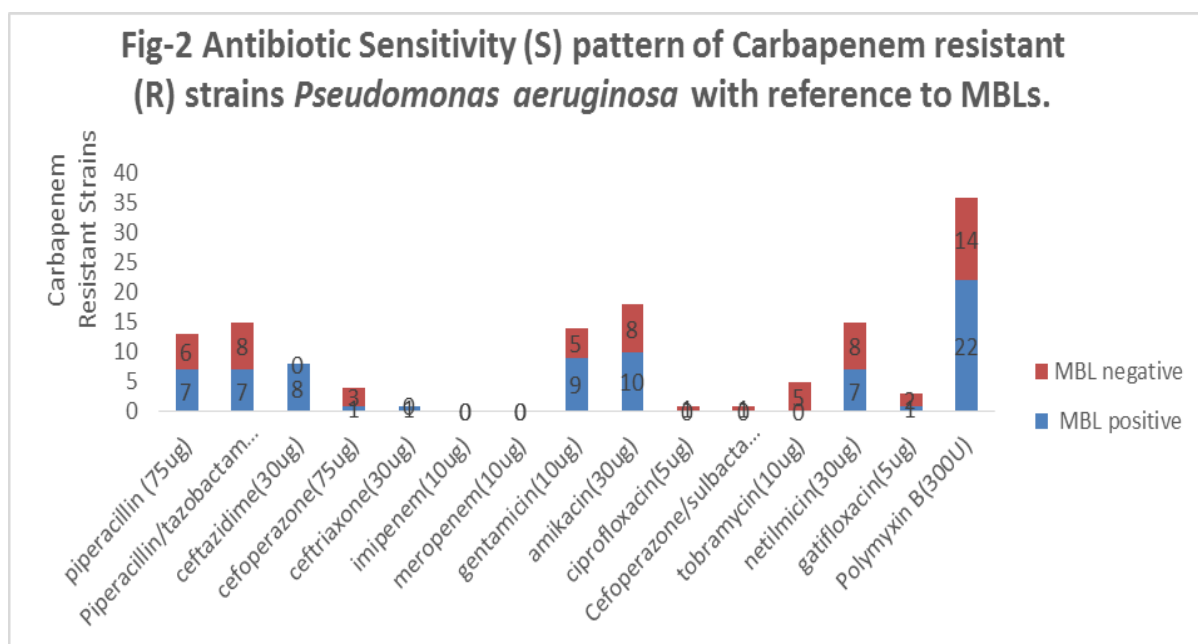
**Table 2 - Antibiotic sensitivity pattern of Carbapenem-resistant strains.**

No.	Antibiotics	n=36 (% sensitivity)S	R
1	piperacillin (75ug)	13 (36.11%)	23
2	Piperacillin/Tazobactam (100/10ug)	15 (41.66%)	21
3	ceftazidime(30ug)	8 (22.22%)	28
4	cefoperazone(75ug)	4 (11.11%)	32
5	ceftriaxone(30ug)	1(2.77%)	29
6	imipenem(10ug)	0	36
7	meropenem(10ug)	0	36
8	gentamicin(10ug)	14 (38.88%)	22
9	amikacin(30ug)	18 (50%)	10
10	ciprofloxacin(5ug)	1 (2.77%)	32
11	Cefoperazone/Sulbactam (75/25ug)	1 (2.77%)	33

12	tobramycin(10ug)	5 (13.88%)	31
13	netilmicin(30ug)	15 (41.66%)	21
14	gatifloxacin(5ug)	3 (8.33%)	33
15	Polymyxin B(300U)	36 (100%)	0

**Table 3 - Antibiotic Sensitivity (S) pattern of Carbapenem resistant (R) strains *Pseudomonas aeruginosa* with reference to MBLs.**

No.	Antibiotics	MBL positive n=22 (18.33%)	MBL negative n=14
1	piperacillin (75ug)	7 (31.81%)	6 (42.85%)
2	Piperacillin/Tazobactam (100/10ug)	7(31.81%)	8 (57.14%)
3	ceftazidime(30ug)	8(36.36%)	0
4	cefoperazone(75ug)	1(4.5%)	3 (21.42%)
5	ceftriaxone(30ug)	1(4.5%)	0
6	imipenem(10ug)	0	
7	meropenem(10ug)	0	
8	gentamicin(10ug)	9(40.9%)	5 (35.71%)
9	amikacin(30ug)	10(45.45%)	8 (57.14%)
10	ciprofloxacin(5ug)	0	1 (7.1%)
11	Cefaperazone/Sulbactam (75/25ug)	0	1 (7.1%)
12	tobramycin(10ug)	0	5 (35.71%)
13	netilmicin(30ug)	7(31.81%)	8(57.14%)
14	gatifloxacin(5ug)	1(4.5%)	2 (14.28%)
15	Polymyxin B(300U)	22(100%)	14 (100%)



## DISCUSSION

MBL enzyme is an emerging threat and cause of concern for nosocomial infections particularly by *Pseudomonas* spp. There are reports on MBL production in *P. aeruginosa* from various countries like Brazil, Korea, Singapore and France.<sup>[13]</sup>

MBL was first reported as a zinc dependent enzyme in *Bacillus cereus* in mid 1960s.<sup>[17]</sup> A few decades later, imipenem-hydrolyzing metallo enzymes were found in *Aeromonas hydrophila*<sup>[18]</sup> and *Bacteroides fragilis*.<sup>[19]</sup> All these enzymes were produced by chromosomal genes and at first were recovered only from single clinical isolates. In 1991 Japan, reported the first plasmid-mediated metallo beta lactamase in *P. aeruginosa*.<sup>[20]</sup> This was soon followed by another report of transferable metallo-enzyme in *B. fragilis*.<sup>[21]</sup> Apart from *P. aeruginosa*, other bacteria like *Serratia*, *Klebsiella pneumonia*, *Escherichia Coli*, *Enterobacter aerogenes*, *E. cloacae*, *Citrobacter freundii*, *Proteus vulgaris*, *P. putida*, *Acinetobacter* and *Alcaligenes xylosoxidans* were also shown to produce MBL.<sup>[22]</sup> There are frequent reports of MBL production in *P. aeruginosa* from the Asian and the Pacific countries, namely Hong Kong, Taiwan and Japan.<sup>[13]</sup>

In various studies across the world, varying resistance (4-60%) has been seen towards imipenem and meropenem.<sup>[23][24]</sup> Where as in our study we found 36(30%) isolates are resistant to imipenem and meropenem. Which is higher than the study conducted by Vipul et al<sup>[32]</sup> showing 19.26% imipenem and meropenem resistant *pseudomonas aeruginosa*.

Peleg et al<sup>[25]</sup> have recently described a two year study from Alfred hospital, showing 55.8% MBL positive isolates<sup>[25]</sup>, Doguen young et al<sup>[26]</sup> from Korea showed 50% of MBL production in *Pseudomonas*. Sarkar et al<sup>[31]</sup> in Uttaranchal used IMP-EDTA disk synergy test for distinguishing MBL producers from non MBL producers reported 54.5% were MBL producers. *P. aeruginosa* producing MBL was first reported from Japan in 1991.<sup>[27]</sup> In 2002 from India, Navneeth et al<sup>[28]</sup>, first reported MBL production in *P. aeruginosa* to be 12 %. Since then, the incidence of MBL production in *P. aeruginosa* has been reported to be 10-30 % from various clinical specimens across the country.<sup>[11]</sup> In a recent reports of Vipul et al<sup>[33]</sup> shows 11.11% MBL production in *P. aeruginosa* and another very recent study conducted by vasundara et al<sup>[33]</sup> shows 36% of MBL production in *P. aeruginosa*. We found 22(18.33%) isolates MBL production in *P. aeruginosa* of which 8(36.66 %) isolates were obtained from endotracheal (ET) aspirate, 6 (27.27%) from blood, 6 (27.27%) and wound swab, 1 (4.5%) each from tissue and urine specimens in our study. Another study conducted by Shashikala et



al<sup>[29]</sup> reported 20.7% carbapenem resistant *P. aeruginosa* isolates from endotracheal (ET) aspirates showing indwelling devices as major risk factors for the development of resistance and by Ami Varaiya et al<sup>[15]</sup> reported 25 % carbapenem resistant *P. aeruginosa* of which 30 % were obtained from respiratory specimen.

A study from European hospital detected multi drug resistant *P. aeruginosa* that were carrying bla-VIM MBL genes, were shown to be wide spread among 20% of all *Pseudomonas* isolates and 70% of Carbapenem resistant isolates.<sup>[30]</sup>

Amongst the MBL positive isolates from various samples maximum sensitivity was observed for polymyxin (300U) 100% followed by amikacin(30ug) 45.45%, gentamicin(10ug) 40.9%, ceftazidime(30ug)36.36%, netilmicin (30ug) 31.81%, Piperacillin/Tazobactam (100/10ug) 31.81%, piperacillin (75ug) 31.81% and 4.5% of gatifloxacin (5ug), ceftriaxone(30ug), cefoperazone (75 ug) respectively. Amongst the MBL negative isolates maximum sensitivity was observed for polymyxin B (300U) 100% followed by 57.14% of netilmicin (30ug), amikacin(30ug), Piperacillin/Tazobactam (100/10ug), respectively, 42.85% of piperacillin(75ug), 35.7% of gentamicin(10ug), 14.28% of gatifloxacin (5ug) and 7.14% of each ciprofloxacin(5ug), Cefoperazone/sulbactam (75/25ug), tobramycin(10ug) respectively.

In the study conducted by Taneja et al<sup>[11]</sup>, piperacillin and amikacin had the best in vitro susceptibility. While the study conducted by Ami varaiya et al<sup>[15]</sup> piperacillin/tazobactam had the best in vitro susceptibility.

*P. aeruginosa* are responsible for 3-7% bloodstream infections and high mortality rates (27-48%) in critically ill patients.<sup>[34]</sup> Early detection of these  $\beta$ -lactamase producing isolates in a routine laboratory could help to avoid treatment failure, as often the isolates producing this enzyme show a susceptible phenotype in routine susceptibility testing. Thus, the rapid dissemination of MBL producers is worrisome and necessitates the implementation of not just surveillance studies but also proper and judicious selection of antibiotics especially carbapenems.

## CONCLUSION

Study indicates that, surveillance for the detection of MBL is necessary. The rapid dissemination of MBL producers is worrisome and necessitates the implementation of proper and judicious selection of antibiotics especially carbapenems.



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