Detection of Metallo-Beta-Lactamase Enzymes Producing Pseudomonas Aeruginosa Isolated from Various Clinical Samples.

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Abstract: Background: Acquired drug resistance is reported in Pseudomonas spp by production of plasmid mediated AmpC beta (β)-lactamase, Extended Spectrum (β)-lactamase (ESBL) and Metallo beta (β)-lactamase (MBL) enzymes. 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 Psedumonas aeruginosa isolated from different clinical samples of hospitalized patient. Methods: Psedumonas aeruginosa strains were obtained by standard isolation and identification techniques from various clinical samples of hospital. Strains were then subjected to susceptibility testing for anti-pseudomonas 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: Amongst the 135 strains of P. aeruginosa isolated, 26 (19.26%) were found to be carbapenem resistant and 15 (11.11%) were found to be MBL producers. There was high prevalence of MBL enzyme amongst multidrug resistant P. aeruginosa. **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 carbapenem[Khakhkhar V NJIRM 2012; 3(4) : 4-9].

Key-words: Metallo-beta (β)-lactamase, Nosocomial MBL, Carbapenems, disc potentiation test, EDTA

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Introduction: Infection caused by *Pseudomonas aeruginosa* is frequent amongst Hospital Acquired Infections (HAI). Further, acquired drug resistance is common in nosocomial isolates of Pseudomonas spp. [1] Acquired resistance is also reported by the production of plasmid mediated AmpC beta (β)–lactamase, Extended Spectrum Beta (β)–Lactamase (ESBL) and metallo beta (β)–lactamase (MBL) enzymes. [2, 3] Carbapenems are often used as antibiotics of last resort for treating infections due to multi-drug resistant Gram-negative bacilli as they are stable against ESBL and AmC β-lactamase. [4] However, Acquired MBL in *Pseudomonas spp* have recently emerged as one of the most worrisome resistance mechanism because of their capacity to hydrolyze all beta (β)–lactam antibiotics including penicillins, cephalosporins and carbapenems, with the exception of aztreonam. [5]

The carbapenems available for use in India are imipenem and meropenem.[6] However, carbapenem resistance has been observed frequently in non fermenting bacilli *Pseudomonas aeruginosa* and *Acinetobacter* spp. Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes-carbapenemase.[7] Based on molecular studies, cabapenem-hydrolyzing enzymes are classified into four groups A, B, C and D. The MBLs belong to group B.[1,8] These carbapenemase are class B metallo β-lactamases (IMP, VIM) or class D-oxacillinases (OXA 23 to OXA 27) or class A - clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC).[7] MBL belongs to a group B - lactamase which requires divalent cations of Zinc as cofactors for enzyme activity. These have potent hydrolysing activity not only against carbapenem but also against other β-lactam antibiotics. [9]

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.[9] MBL producing Gram negative bacilli, specially Pseudomonas spp, have been increasingly reported in Asia, Europe, Latin American and the United States. [4]

The appearance of MBL genes and their spread among bacterial pathogens is a matter of concern with regard to the future antimicrobial chemotherapy. [10, 11, 12] Further, due to increase occurrence and types of these multiple β-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. [2, 13] Thus, our study was undertaken to detect the MBL (metallo-β-lactamase) positive isolates of *Pseudomonas aeruginosa* from different clinical samples received in our tertiary care hospital.

Subjects and Methods: One hundred thirty five, non-repetitive strains of *Pseudomonas aeruginosa* were isolated during June 2011 to July 2012 from various clinical specimens, i.e. pus / wound swab, urine, blood, respiratory secretion, pleural fluid, ear discharge, ICD catheter and ocular discharge that were received from different wards of the hospital. Samples were collected, transported and processed in Microbiology Department without delay and using the standard protocols and with universal safety precautions. Identification of organisms was done by the standard laboratory technique.[14] Strains of *Pseudomonas aeruginosa* were enrolled for the MBL production study.

The routine antimicrobial sensitivity testing was performed on Muller-Hinton agar plates with commercially available discs by Kirby-Bauer disc diffusion method and interpreted as per CLSI-2011 recommendations. [15] *P.aeruginosa* ATCC 27853 (β-lactamase negative) strain was used as a control. Antibiotic sensitivity tests were put up for aminoglycosides [amikacin (30 µg), gentamicin (10 µg), netilmicin (30 µg), tobramicin (10 µg)], cephalosporin's [cefoperazone (75 µg), cefepime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), floroquinolones [ciprofloxacin (5 µg), gatifloxacin (5 µg)], carbapenems [imipenem (10 µg), meropenem (10 µg)], chloramphenicol (30 µg), piperacillin (100 µg), piperacillin/tazobactam (100/10 µg) and colistin (10 µg).

P. aeruginosa strains were considered carbapenem resistant, when the zone size around imipenem and meropenem was ≤ 13 mm, intermediate 14-15 mm and sensitive ≥ 16 mm (CLSI-2011). MBL production by *P. aeruginosa* was suspected when the strain found resistant to meropenem and imipenem.

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) [1] are described.

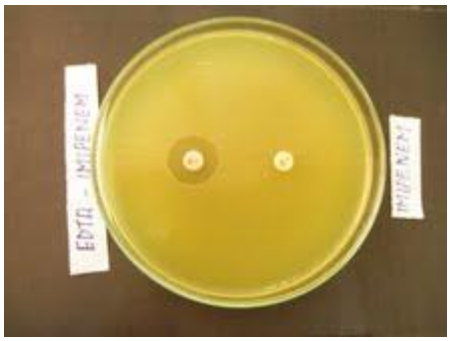

Figure 1: Imipenem & EDTA-Imepenem disc diffusion test

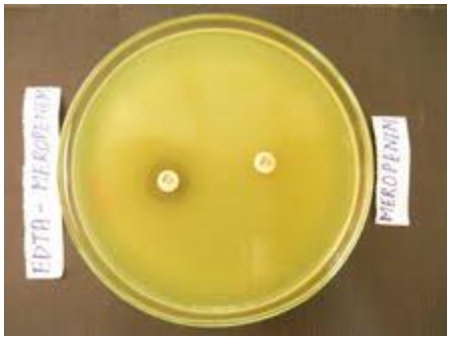

Figure 2: Meropenem & EDTA-Meropenem disc diffusion test

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.[16] Each test strains of *P. aeruginosa* were inoculated on Mueller Hinton agar plats 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 (10µg) discs were placed on the surface of an agar plate at distance of 25 mm and 4µl EDTA solution was added to one of them to obtain a desired concentration of 750 µg. The inhibition zones of imipenem and imipenem- EDTA discs were compared after 16 to 18 h of aerobic incubation at 35±2C. In the combined disc test, if the increase in inhibition zone with the imipenem and imipenem- EDTA disc was ≥7 mm than the imipenem alone, it was considered MBL positive. [17, 18]

Results: Amongst the 135 non-repetitive strains of *P.aeuroginosa,* 26 (19.25%) strains were found resistant to carbapenem and 15 (11.11%) strains were found to be MBL enzyme producer which were confirmed by the disc potentitiation method. The ATCC 27853 *P. aeruginosa* did not exhibit any zone size enhance with EDTA-impregnated imipenem disc.

Amongst the 15 MBL enzyme producing isolates, 10 were from pus / wound swab, 3 were from urine, 1 was from respiratory secretion and 1 was from the ICD catheter (Table 1)

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

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

No.	Sample	Pseudomonas Isolates (n=135)	Carbapenem Resistant (n=26) (19.26%)	MBL producer (n=15) (11.11%)
1	Pus / Wound Swab	65	16	10
2	Urine	28	5	3
3	Blood	7	1	0
4	Respiratory secretion	19	2	1
5	Pleural fluid	4	1	0
6	Ear discharge	2	0	0
7	Ocular discharge	2	0	0
8	ICD catheter	8	1	1

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. [1]

MBL was first reported as a zinc dependent enzyme in *Bacillus cerus* in mid 1960s. [19] A few decades later, imipenem-hydrolyzing metallo enzymes were found in *Aeromonas hydrophila* [20] and *Bacteroides fragilis.* [21] 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.* [22] This was soon followed by another report of transferable metallo-enzyme in *B.fragilis.* [23] Apart from *P. aeruginosa*, other bacteria like *Serratia, Klebsiella pneumonia, Escherichia Coli, Enterobacter aerogenes, E.clocae, Citrobacter freudii, Proteus vulgaris, P. putida, Acinetobacter* and *Alcaligenes xylosoxidans* were also shown to produce MBL. [24] There are frequent reports of MBL production in *P. aeruginosa* from the Asian and the Pacific countries, namely Hong Kong, Taiwan and Japan. [1]

Table 2 Antibiotic sensitivity pattern of Carbapenem-resistant strains.

No.	Antibiotics	n=26 (% sensitivity)
1	Amikacin	7 (26.92%)
2	Gentamicin	3 (11.53%)
3	Netilmicin	4 (15.38%)
4	Tobramycin	3 (11.53%)
5	Cefepime	3 (11.53%)
6	Ceftazidime	2 (7.69%)
7	Cefoperazone	3 (11.53%)
8	Ceftriaxone	2 (7.69%)
9	Ciprofloxacin	2 (7.69%)
10	Gatifloxacin	9 (34.61%)
11	Chloramphenicol	1 (3.84%)
12	Piperacillin	6 (23.07%)
13	Piperacillin/ Tazobactam	7 (26.92%)
14	Imepenam	0
15	Meropenam	0
16	Colistin	16 (61.53%)

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

Antibiotics	MBL-positive (n=15)	MBL-negative (n=11)
Amikacin	3 (20%)	4 (36.36%)
Gentamicin	0	3 (27.27%)
Netilmicin	1 (6.66%)	3 (27.27%)
Tobramycin	0	3 (27.27%)
Cefepime	0	3 (27.27%)
Ceftazidime	0	2 (18.18%)
Cefoperazone	0	3 (27.27%)
Ceftriaxone	0	2 (18.18%)
Ciprofloxacin	0	2 (18.18%)
Gatifloxacin	4 (26.66%)	5 (45.45%)
Chloramphenicol	0	1 (9.09%)
Piperacillin	2 (13.33%)	4 (36.36%)
Piperacillin / Tazobactam	2 (13.33%)	5 (45.45%)
Imepenam	0	0
Meropenam	0	0
Colistin	9 (60%)	7 (63.63%)

In various studies across the world, varying resistance (4-60%) has been seen towards imipenem and meropenem.[25, 26] We found 19.26% resistance to imipenem and meropenem. *P. aeruginosa* producing MBL was first reported from Japan in 1991. [27] In 2002 from India, Navneeth *et al* [28] 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. [12] We found 11.11% MBL production in *P. aeruginosa* of which 66.66 % were obtained from pus / wound swab specimens in our study. Another study conducted by Shashikala *et al* [29] reported 20.7% carbapenem resistant *P. aeruginosa* isolates from endotracheal aspirates showing indwelling devices as major risk factors for the development of resistance and by Ami Varaiya et al [17] reported 25 % carbapenem resistant *P. aeruginosa* of which 30 % were obtained from respiratory specimen.

Amongst the MBL positive isolates from various samples of patient's admitted to hospital in our study, maximum sensitivity was observed for colistin followed by gatifloxacin, amikacin, piperacillin and piperacillin / tazobactam. Amongst the MBL negative isolates maximum sensitivity was observed for colistin followed by piperacillin/tazobactam, gatifloxacin, piperacillin, and amikacin. In the study conducted by Taneja *et al* [12], piperacillin and amikacin had the best in vitro susceptibility. While the study conducted by Ami varaiya et al [17] piperacillin/tazobactam had the best in vitro susceptibility.

P. aeruginosa are responsible for 3-7% blood-stream infections and high mortality rates (27-48%) in critically ill patients.[30] Early detection of these β-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. Furthermore, strict antibiotic policies and measures to limit the indiscriminative use of cephalosporins and carbapenems in the hospital environment should be undertaken. This will minimize the multiple β-lactamase producing pathogens whose spread would leave no other option to treat Gram-negative nosocomial infections.

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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