**Abstract:** Introduction: The emergence and rapid spread of multidrug resistant Pseudomonas aeruginosa causing nosocomial infections is a great concern worldwide. Detection of MBL is also a challenge for routine microbiology laboratories, since there are no standardized methods for MBL detection. The present study was conducted at our hospital with an aim to know the prevalence of metallo beta lactamase producing Pseudomonas aeruginosa, to know MIC of various antibiotics, transfer of drug resistance and predisposing factors for infection with MBL strain. Material and Methods: A total of 608 clinical isolates were tested for the presence of metallo beta lactamase enzyme from Oct 2008 to Sept 2013. Imipenem resistant Ps. aeruginosa isolates were selected for the presence of MBL by Imipenem – EDTA combined disc test, Imipenem – EDTA double disc synergy test (DDST), EDTA disk potentiation test using Cefazidime, Cefotaxime, Cefepime, Cefotaxime and MBL E- Test. Result: Out of 608 clinical isolates 91 (14.96%) were resistant to imipenem and 81 (13.32%) were MBL positive by initial two tests. 76(83.51%) were positive by EDTA disk potentiation test using Cefazidime, Cefotaxime, Cefepime, Cefotaxime and 63 (69.23%) were positive by E- test. It is observed that 62/81(76.54%) of MBL producing Pseudomonas isolates could transfer the resistance of imipenem to the recipient E. coli J53 AZR strains at 370C. The prevalence of MBL producing Pseudomonas aeruginosa was 81/608 (13.32%). Conclusion: The early detection of MBL producing Pseudomonas aeruginosa may help in appropriate antimicrobial therapy and avoid the development and dissemination of these multidrug resistance strains. Hence all Pseudomonas aeruginosa isolates resistant to imipenem should be screened for MBL production. Key Words: Pseudomonas aeruginosa, Imipenem, Metallo beta lactamase, Imipenem – EDTA combined disc test, Imipenem – EDTA double disc synergy test, MBL E- Test, EDTA disk potentiation test.

**Introduction:** Pseudomonas aeruginosa isolates are responsible for outbreaks of nosocomial infections in the world. Pseudomonas aeruginosa producing Metallo ß Lactamases (MBLS) was first reported from Japan in 1991 and since then has been described from various parts of the world including Asia, Europe, Australia, South America and North America. (1) A five years longitudinal study involving many centers from Latin America indicated that year after year, Ps aeruginosa resistance has continually risen to the point where approximately 40% are resistance to “antipseudomonal” drugs including carbapenems. While the advent of carbapenems in the 1980s heralded a new treatment option for serious bacterial infections caused by cephalosporin and penicillin resistant bacteria, carbapenems resistance can now be observed in Enterobacteriaceae and Acinetobacter spp. and is becoming common place in Ps. aeruginosa.[2,3] Acquired Metallo –ß- Lactamases (MBLS) have recently emerged as one of the most worrisome resistance mechanisms owing to their capacity to hydrolyze with the exception of aztreonam, all ß lactams including carbapenems and also because their genes are carried on highly mobile elements, allowing easy dissemination.[4,5] The prevalence of imipenem resistance to Ps. aeruginosa has been increasing worldwide. Resistance to carbapenem is due to impermeability via the loss of the Opr D porin, the up regulation of an active efflux pump system of the cytoplasmic membrane or the production of metallo-ß-lactamases (MBLs). MBLs also represent a clinical threat due to their unrivalled spectrum of activity and their resistance to therapeutic serine ß Lactamases inhibitors. The fact that MBL and aminoglycoside resistance genes are genetically linked merely compounds this problem. The problem of an appropriate treatment regimen is also amplified by the lack of new antimicrobials that will possess broad spectrum potency against clinically significant Ps. aeruginosa. It is necessary to identify the prevalence of these strains in hospitals and to characterize their epidemiology to control the spread of these
strains and to determine suitable prevention and treatment policies. Bearing in mind this massive problem of MBL producing Pseudomonas aeruginosa the present study was carried out.

Materials and Methods
A total of 608 Pseudomonas aeruginosa isolates were isolated during the study period. Out of the total 608 Pseudomonas aeruginosa isolates 91 were screen positive that is resistant to Imipenem, they were considered to be positive MBL producers. MBL producers were confirmed by different phenotypic tests i.e. Imipenem – EDTA combined disk Test, Imipenem –EDTA double –disk synergy Test and later on EDTA disk potentiation test using 4 cephalosporins and E-test. Among 91 putative MBL producers, 81(13.32%) were confirmed as MBL producers by Imipenem-EDTA combined disk test and imipenem –EDTA double disk synergy test and further on EDTA disk potentiation test using four cephalosporins and Epsilometer test.

Identification of microorganism was done by standard laboratory technique.3 This figure of 81 confirmed MBL producers is from total sample size of 608. Hence the prevalence rate of MBL producing Pseudomonas aeruginosa is 81/608 i.e.13.32%. 55 (67.90%) of these were from inpatient units and 26 (32.08%) were from outpatient units. Different clinical specimens collected from patients admitted under different clinical disciplines of Krishna Hospital and Medical Research Centre, Karad during 15 Oct 2008 to 15 March 2012, were used in the study.

Metallo Beta Lactamase Detection:
[MBL production was carried out in Imipenem –resistant isolates [1]. MBL detection tests were done by following methods using Ps. aeruginosa ATCC 27853 (Hi-media) as a negative control strain.[6] An inhouse Ps. aeruginosa strain which was repeatedly MBL positive by Imipenem – EDTA combined disc test and Imipenem –EDTA double disc synergy test was used as positive control strain.[7,8]

Imipenem – EDTA combined disc test:
The IMP–EDTA combined disk test was performed as described by Yong et al.[9] Pseudomonas aeruginosa ATCC 27853 (Hi-media) was used as the control strain. The test organisms were inoculated on Muller Hinton agar plates as per CLSI guidelines. A 0.5 M EDTA solution was prepared by dissolving 18.61 g of EDTA in 100 ml of distilled water and adjusting its PH 8.0 by using NaOH. The mixture was autoclaved. The two 10 µg Imipenem disks were placed on Muller Hinton agar plates as recommended by CLSI guidelines. A 0.5 M EDTA solution was added to blank disc and plates were incubated at 37°C for overnight. Then results were recorded.[11] Following incubation period for 16-18 hours at 37°C a clear extension of zone of inhibition in the area between EDTA disc and any one of the cephalosporin disc in comparison with the zone of inhibition on the far side of drug was interpreted as positive for MBL production.

MBL E- Test:
The E- test of MBL strip containing a double sided seven dilution range of Imipenem (4-256 µg/ml) and IMP (1-64 µg/ml) in combination with a fixed concentration of EDTA used for MBL detection. MRC ratio of Imipenem/Imipenem + EDTA of >8, or reduction of Imipenem MIC by =3 log 2 dilutions in the presence of EDTA or appearance of a phantom zone indicates MBL production.

Transfer of resistance by conjugation:
As the recipient strain was E. coli J53AZR, selection was made on MacConkeys agar containing sodium azide 200 µg/ml Plus each drug (8µg/ml) separately to which the donor strain was resistant (i.e. sodium azide + Ceftaxime, sodium azide + Ceftizoxime, sodium azide + Cefepime) and then the mixture was incubated with shaking at 37°C, similarly another set of same mixture was incubated with shaking at room temperature of two hours. [14] Loopulof mixture from both sets of mixture was then streak inoculated on half of the selection plate and donor and recipient strains were also inoculated on MacConkeys agar plate, which served as control.

The transfer of resistance factor from MBL positive Ps. aeruginosa (donor strain) to sodium azide resistant E. coli (recipient strain) i.e. (E. coli J53AZR) was done by conjugation.[15-17]

Results
In our study out of 608 isolates of Pseudomonas aeruginosa 91/608 (14.96%) were resistant to imipenem. We have confirmed that 81/608(13.32%) isolates are MBL producers by imipenem - EDTA combined disk test, Imipenem – EDTA double disc synergy test (DDST). Among these 55(67.90%) were from inpatient units and 26(32.08%) were from outpatient units.

<table>
<thead>
<tr>
<th>Table 1: Distribution of MBL and non MBL producing isolates of Pseudomonas aeruginosa in different specimens.</th>
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<tbody>
<tr>
<td><strong>Specimens</strong></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Urine</td>
</tr>
<tr>
<td>Pus</td>
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<tr>
<td>Sputum</td>
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<td>Blood/ Bone marrow</td>
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<tr>
<td>Body fluids</td>
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<tr>
<td>Others</td>
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<tr>
<td><strong>Total</strong></td>
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</table>
Out of these 81 MBL producing clinical isolates of *Pseudomonas aeruginosa*, 38(46.51%) were from pus, 17(21%) were from urine, 6(7.40%) were from sputum, 5(6.17%) were from body fluids and 2(2.46%) were from Blood/Bone marrow. Likewise MBL producing *Ps. aeruginosa* were more prevalent in surgery ward i.e. 18 isolates (32.72%) followed by medicine ICU 11(20.00%), Orthopaedic and Medicine 10(18.18%).

Majority of MBL producing isolates of *Pseudomonas aeruginosa* had a high MIC in the range of 64 = 64 µg/ml for Ceftriaxone and 75/81 (92.59%) isolates had a MIC in the range of 16 = 64 µg/ml for Ceftazidime. All 81/81(100.00%) isolates had a MIC in the range of 64=512 µg/ml for Cefotaxime, 80/81 (98.76%) isolates had a MIC in the range 128= 512 µg/ml for Pipercillin, 80/81 (98.76%) had a MIC in the range of 4 = 8 µg/ml for ciprofloxacin, 80/81 (98.76%) had a MIC in the range of 16= 256 µg/ml for imipenem.

In India prevalence of MBLs range from 8-87% with a recent study reporting 41% occurrence.[18] In our study the prevalence rate of MBL producing *Ps. aeruginosa* was 13.32% which was similar to studies conducted by Navneeth BV et al (12%)[19], Rajput A et al (12%)[20], Hemalata et al (14%)[21], Attal RO et al. (11.04%)[22] respectively from various parts of India.

All the isolates were 100% sensitive to Aztreonam; they were also sensitive to polymyxin B 96.70% and Colistin 87.91%. Transfer of resistance could be demonstrated from donor to recipient strain. Majority of our MBL isolates are from indoor patients. This is a cause of concern as the percentage of transfer i.e., 76.54% is quite high and according to our literature search this is one of the largest transfers done.

**Discussion:** Since the widespread use of carbapenem in the hospitals, carbapenem –resistance has been detected increasingly worldwide. After the discovery of penicillin and sulfonamides patients were treated empirically and the organisms were mostly susceptible. After the emergence of resistance occurred, it required the development of new beta lactam antibiotics; with new class of antibiotics, a new beta lactamase emerged that caused resistance of third generation cephalosporins and antibiotics. Carbapenem resistance in *Pseudomonas spp.* is an emerging problem and is a cause of concern as many nosocomial*Pseudomonas* are detected to be resistant to most of other antibiotics.[23]

MBLs have been identified from clinical isolates worldwide with increasing frequency over the past few years, and strains producing these enzymes have been responsible for prolonged nosocomial outbreaks that were accompanied by serious infections. The occurrence of an MBL positive isolate in a hospital setting poses a therapeutic problem, as well as a serious concern for infection control practitioners in the spread of this multidrug resistant isolate. (1)

The clinical samples included in the present study were from urine, pus, sputum, blood/bone marrow and body fluids. All the methods for detection of MBL producing bacterial isolates depend on the principle, that MBLs are affected by removal of zinc from their active site. Still, no single screening method has been found to be perfect. Currently, there is no Clinical Laboratory Standard Institute (CLSI) recommended method available. Also, no standard method is recommended by any other international committee for the detection of MBL producers.[22]

A total of 608 *Ps. aeruginosa* clinical samples collected during the three year period of the study, 91/608 (14.97%) were resistant to imipenem were considered to be putative MBL producers, out of which 81/608 (13.32%) isolates were confirmed for positive MBL activity. These isolates were confirmed by using Imipenem – EDTA combined disk test, Imipenem – EDTA double disk synergy test, and also by EDTA disk potentiation test using Ceftazidime, Cefotaxime, Cefepime, Cefotaxime detected 83.51% and Epsilon meter test (E-test) detected 69.23%. (P>0.05)

**Table 2: Comparison of phenotypic tests used for detection of MBL producing *Ps. aeruginosa***

<table>
<thead>
<tr>
<th>Organism</th>
<th>Total number of isolates</th>
<th>Phenotypic tests</th>
</tr>
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<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>81</td>
<td>Imipenem –EDTA combined disk Test; Imipenem –EDTA double disk synergy Test; EDTA disk potentiation test using 4 cephalosporins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epsilometer Test (E-test)</td>
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<tr>
<td>81(89.01 8%)</td>
<td></td>
<td>76 (83.51%)</td>
</tr>
<tr>
<td>81(89.01 8%)</td>
<td></td>
<td>63(69.23%)</td>
</tr>
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</table>

Phenotypic tests i.e., Imipenem –EDTA combined disc test (CDT) and Imipenem –EDTA double disc synergy test (DDST) could detect equal number of MBL producing strains of *Pseudomonas aeruginosa*, i.e., 89.01%, where as EDTA disc potentiation test using Ceftazidime, Cefotaxime, Cefepime, Cefotaxime detected 83.51% and Epsilometer test (E-test) detected 69.23%. (P>0.05)

**Table 3: Resistance transfer experiments of MBL producing isolates of *Pseudomonas aeruginosa***

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates</th>
<th>No. of transfer</th>
<th>Positive percentage of transfer</th>
</tr>
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<tbody>
<tr>
<td><em>Ps. aeruginosa</em></td>
<td>81</td>
<td>62</td>
<td>76.54%</td>
</tr>
</tbody>
</table>

Of MBL producing strains, 62/81 (76.54%) could transfer resistance of third generation cephalosporins and antibiotics to the recipient strain. The frequency of transfer was more at 37% than at room temperature. Predisposing risk factor associated with MBL producing *Pseudomonas aeruginosa* were mainly prolonged hospital stay and the use of Foley's catheter.

The emergence of acquired MBLs among *Pseudomonas aeruginosa* represents an epidemiological risk for at least two reasons. Firstly MBLs confer resistance not only to carbapenem but to virtually all β-lactams and are frequently associated with resistance to aminoglycosides, and secondly genes encoding to MBL enzymes are mostly commonly found on mobile genetic elements (integrons, plasmids, transposons) that can spread horizontally among unrelated strains.[23]

In the present study the prevalence rate of MBL producing strains of *Pseudomonas aeruginosa* was 81/608 i.e., 13.32%.
2008; 70% isolates of Pseudomonas aeruginosa were resistant to cefazidime, 75% to Piperacillin, 59% to Piperacillin/tazobactam, 89% to Ticarcillin/ clavulanic acid, 82% to cepoforazone, 74% to amikacin, 81% to ceftipime, 71% to levofloxacin, 79% to ciprofloxacin, and 69% to aztreonam by disc diffusion method.[25]

In the present study the MIC of Ceftriaxone, Cefazidime, Cefotaxime, Piperacillin, Ciprofloxacin was determined against MBL producing and non MBL producing isolates of Pseudomonas aeruginosa, by agar dilution method. MIC of Imipenem was determined by using E- test as Imipenem powder was not available commercially. The MBL producing isolates of Pseudomonas aeruginosa 80/81 (98.76%) had a MIC in the range of 64—6μg/ml for Ceftriaxone. 75/81 (92.59%) isolates had a MIC in the range of 16—128 μg/ml for Cefazidime. All 81/81(100.00%) isolates had a MIC in the range of 64—512 μg/ml for Cefotaxime. 80/81 (98.76%) isolates had a MIC in range 256—512μg/ml for Piperacillin. 80/81 (98.76%) had a MIC in the range of 4—8 μg/ml for Ciprofloxacin and 80/81 (98.76%) had a MIC in the range of 16—256 μg/ml for Imipenem.

In the present study MIC value for isolates are quite high as compared to other studies. It can be noted that our MIC values are high in comparison to studies carried out abroad. It is also interesting to note that a MIC value of non MBL producers was quite high for most of the antibiotics. There are only very few reports with reference to resistance transfer experiments in respect to Metallo beta lactamase producing isolates. Among the various modes of gene transfer conjugation is most common mode of resistance gene transfer. The transfer of a plasmid carrying a metal– beta lactamase gene suggests the possibilities of clinical spread of plasmid –encoded metal beta lactamases by cell to cell contact because metal beta lactamases confer resistance not only to carbapenems but also to other beta lactams except aztreonam (monobactams), antibiotics are frequently ineffective against organisms carrying this enzyme.[13] Atul Khajuria et al., in 2013 from Pune successfully transferred plasmid carrying blaNDM-1 from Pseudomonas aeruginosa to E. coli J53 recipient strain, only in four strains.[26]

In the present study, 62/81 (76.54%) of MBLs producing Ps. aeruginosa isolates could transfer the resistance of Imipenem, Ceftriaxone, Cefotaxime and Cefazidime to the recipient E.coli J53AR strain at 37°C. By far this is the largest number and percentage of transfer of drug resistance carried out in India as per our literature search.

**Conclusion:**

MBLs have become a wide spread serious problem and several aspects of them are worrying. MBLs compromise the activity of antibiotics creating therapeutic difficulties with a significant impact on the outcome of patients. In the hospital environment plasmids could be transferred easily between patients through healthcare workers due to hand carriage and of selection pressure. The early detection of MBL producing Pseudomonas aeruginosa may help in appropriate antimicrobial therapy and avoid the development and dissemination of these multidrug resistance strains. Hence all Pseudomonas aeruginosa isolates resistant to imipenem should be screened for MBL production. Imipenem–EDTA combined disc test. (CDT) and Imipenem –EDTA double disc synergy test (DDST) which are easy to perform and cheap, should be introduced in every clinical microbiology laboratory to detect MBLs in Pseudomonas aeruginosa and to improve disease management. Also framing of rational antibiotic policy will go a long way in helping the cause.

**References:**


