Detection of metallo-beta-lactamase producing *Pseudomonas aeruginosa* in intensive care units

Arunava Kali, Sreenivasan Srirangaraj, Shailesh Kumar, Hema. A Divya, Akhila Kalyani, Sivaraman Umadevi

Department of Microbiology, Mahatma Gandhi Medical College and Research Institute, Pondicherry, India

**Research**

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**Corresponding Author:**
Arunava Kali
Dept. of Microbiology, Mahatma Gandhi Medical College and Research Institute, Pondicherry, India.
Email: ak.arunava@gmail.com

**Abstract**

**Background**

Metallo-beta-lactamase (MBL) producing *Pseudomonas aeruginosa* has emerged as a threat to hospital infection control, due to its multi-drug resistance, especially in intensive care units (ICUs).

**Aims**

This study was carried out to detect MBL producing *P. aeruginosa* isolates from medical and surgical ICUs, to compare and evaluate different phenotypic methods currently in use and to determine antibiograms.

**Method**

A prospective study was undertaken to detect MBLs in *P. aeruginosa* isolates obtained from various clinical samples. A total of 49 strains were recovered from patients admitted in inpatient wards and ICUs, and screened for imipenem resistance by Kirby Bauer disk diffusion method. Detection of MBLs was further done by imipenem-EDTA disk synergy test and combined disk test.

**Results**

Out of 49 isolates, 11 isolates (22.4 per cent) were imipenem resistant. All 11 imipenem resistant *P. aeruginosa* strains, when further tested, were positive for MBL production by combined disk test, but, only eight showed positive results by imipenem-EDTA disk synergy test.

**Conclusion**

MBL production was the main resistance mechanism in the 11 carbapenem resistant *P. aeruginosa* isolates collected, with multidrug resistance associating significantly with MBL production in *P. aeruginosa* from our institution.

**Key Words**

Metallo-beta-lactamases; infection control; *Pseudomonas aeruginosa*; Multidrug resistance.

**What this study adds:**

1. *P. aeruginosa* is a well-established nosocomial pathogen and MBL production in *P. aeruginosa* is associated with treatment failure, longer hospital stay and significant morbidity and mortality.
2. Out of 11 MBL producing *P. aeruginosa*, 10 isolates displayed multidrug resistance. Imipenem disk diffusion test and combined disk test are simple cost-effective phenotypic tests for detection of MBL production in *P. aeruginosa*.
3. Routine screening of *P. aeruginosa* using inexpensive phenotypic tests like imipenem and imipenem + EDTA combined disk test should be an essential component of infection control in both ICU and non-ICU patients.

**Background**

*Pseudomonas aeruginosa* is a well-known isolate in hospital settings and has been frequently associated with nosocomial outbreaks among susceptible patients.\(^1\) Owing to its persistence in the hospital environment, as a survival strategy an array of multidrug resistance mechanisms are often seen in such hospital isolates.\(^2\) Consequently, treatment options are narrowed down to only few antibiotics. Carbapenems are the antibiotics of choice for severe pseudomonas infections. However, resistance to this novel antibiotic is increasing worldwide.\(^3\)

Carbapenem resistance in *Pseudomonas aeruginosa* is most commonly due to production of metallo-beta-lactamases (MBLs).\(^4\) MBLs are broad–spectrum enzymes that hydrolyse
most beta lactam antibiotics, except monobactams, and are not inhibited by conventional beta-lactamase inhibitors like clavulanic acid or sulbactam.\textsuperscript{5} Emergence of MBL producing \textit{P. aeruginosa} in intensive care units (ICUs) is alarming and reflects overuse of carbapenems.\textsuperscript{5} There is intense selection pressure, due to high usage of broad spectrum antibiotics in ICUs. This results in eradication of competitive flora and subsequent selection of multidrug-resistant strains.\textsuperscript{6}

In recent years, MBL genes have spread from \textit{P. aeruginosa} to members of Enterobacteriaceae.\textsuperscript{7} Notably, high morbidity and mortality rates (ranging between 27 per cent to 48 per cent) have been observed in critically ill patients.\textsuperscript{8,9} Furthermore, mortality rates are significantly higher in MBL producing \textit{P. aeruginosa} (MBL-PA) compared to non-MBL-PA.\textsuperscript{10} Currently, no standardised method for MBL detection has been proposed and despite polymerase chain reaction (PCR) being highly accurate and reliable, its accessibility is often limited to reference laboratories.\textsuperscript{4} The aim of this study was to detect MBL producing \textit{P. aeruginosa} isolates from medical and surgical ICUs, compare and evaluate different phenotypic methods currently in use, and determine antibiograms to guide clinicians in prescribing proper antibiotic and controlling hospital infection.

Method

A prospective study was carried out in a tertiary care hospital in South India over a period of six months (from May 2012 to October 2012). Institutional Ethical Committee approval was obtained (Reference ID 2012-02083). Consecutive non-duplicate isolates of \textit{P. aeruginosa} from various clinical samples received in the microbiology laboratory for bacterial culture and sensitivity from patients admitted in medical and surgical wards and ICUs were included in this study. Patients from outpatient departments were excluded. Patients with \textit{P. aeruginosa} were followed up for the duration of their hospital stay to determine risk factors, response to treatment and mortality rates.

Identification of \textit{P. aeruginosa} was done as per standard laboratory procedures. Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates by Kirby-Bauer disk diffusion method, according to Clinical Laboratory Standards Institute (CLSI) guidelines.\textsuperscript{11} The following antibiotics (Hi-Media, Mumbai, India) were tested by disk diffusion method: piperacillin/ tazobactam (100μg/ 10μg), ceftazidime (30μg), amikacin (30μg), ciprofloxacin (5μg) and imipenem (10μg), gentamicin (10μg), netilmycin (30μg), polymyxin-B (300 units) and colistin (10μg).

Imipenem disk diffusion method was employed as a screening test to select suspected MBL-PA strains showing resistance to imipenem which were further confirmed by imipenem-EDTA combined disk method and imipenem-EDTA double disk synergy test. 0.5M EDTA (Hi-Media, Mumbai, India) was prepared with distilled water and sterilized by autoclaving. Imipenem disks were supplemented with EDTA by dispensing 10μl of this solution to each imipenem disk.

Imipenem-EDTA combined disk method (CDT) was performed as described by Yong et al.\textsuperscript{12} A lawn culture of test isolates was prepared. After allowing it to dry for five minutes, two imipenem discs, one with 0.5 M EDTA and the other a plain imipenem disc, were placed on the surface of agar plates approximately 30mm apart. The plates were incubated overnight at 37°C. An increase in zone diameter of ≥ 7mm around imipenem+EDTA disk in comparison to imipenem disk alone indicated production of MBL (Figure 1).

**Figure 1: Combined disk test using imipenem and imipenem + EDTA. Imipenem + EDTA disk (on the right) produced ≥ 7mm larger zone of inhibition than the imipenem disk (on the left)**

Imipenem-EDTA double disk synergy test (DDST) was performed as described by Lee et al.\textsuperscript{13} Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by CLSI. An imipenem (10μg) disk was placed 20mm centre to centre from a blank disk containing 10μL of 0.5 M EDTA (750μg). Enhancement of the zone of inhibition in the area between imipenem and EDTA disk in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result for MBL production (Figure 2).\textsuperscript{13}
Figure 2: Imipenem-EDTA double disk synergy test (DDST). Imipenem disk (on the left) produced large synergistic zone of inhibition towards the imipenem + EDTA disk (on the right).

Antibiotic susceptibility of three randomly selected imipenem resistant isolates was done by VITEK 2 advanced expert system (bioMerieux Vitek Systems Inc, Hazelwood, France), by which minimum inhibitory concentrations (MICs) were determined for the following antibiotics: piperacillin, piperacillin/tazobactam, ticarcillin/clavulonic acid, ceftazidime, cefepime, cefoperazone, ceftriaxone, cefotaxime, amikacin, levofloxacin, ciprofloxacin and imipenem. International reference strain P. aeruginosa ATCC 27853 was used for quality control.

All findings were entered in an MS Excel data sheet and on completion of the study data was statistically analysed in SPSS software version 17.0. The data was expressed as mean ±SD and percentage.

Table 1: Age distribution of patients infected with P. aeruginosa

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Total cases (n=49)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5 year</td>
<td>1</td>
<td>2%</td>
</tr>
<tr>
<td>6-18 year</td>
<td>5</td>
<td>10.2%</td>
</tr>
<tr>
<td>19-40 year</td>
<td>15</td>
<td>30.6%</td>
</tr>
<tr>
<td>41-65 year</td>
<td>22</td>
<td>44.9%</td>
</tr>
<tr>
<td>&gt;65 year</td>
<td>6</td>
<td>12.2%</td>
</tr>
</tbody>
</table>

Results

Out of 320 samples examined during the study period, 49 P. aeruginosa strains were recovered from 42 (85.7 per cent) males and 7 (14.3 per cent) females admitted in inpatient wards and ICUs. These were screened for MBL production. Table 1 shows the number of patients with P. aeruginosa infections across different age groups. The mean age of patients with P. aeruginosa infection was 43.3 ± 18.9 years.

Among all inpatients the highest number of cases with P. aeruginosa infections were from the surgical ward (42.8 per cent), followed by orthopaedic (14.3 per cent), ENT (12.2 per cent), ICU (8.1 per cent), medicine (8.1 per cent), pulmonary medicine (6.1 per cent) and other wards (8 per cent). Among seven orthopaedic patients, six developed fracture site infections. Out of 21 general surgery patients, 16 had ulcerative lesion viz. diabetic ulcer (n=5), non-healing ulcer (n=8), traumatic ulcer (n=1) and varicose ulcer (n=2). The distribution of P. aeruginosa in clinical samples is summarised in Table 2. Pus swab and aspirate were most common source (67.3%), followed by respiratory specimens (26.4%). Among other samples, P. aeruginosa was recovered from 2 urine samples and 1 vaginal swab.

Table 2: Distribution of P. aeruginosa in clinical samples

<table>
<thead>
<tr>
<th>Sources of P. aeruginosa isolates</th>
<th>Number of isolates (n=49)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pus swab</td>
<td>27</td>
<td>55.1%</td>
</tr>
<tr>
<td>Sputum</td>
<td>8</td>
<td>16.3%</td>
</tr>
<tr>
<td>Pus aspirate</td>
<td>6</td>
<td>12.2%</td>
</tr>
<tr>
<td>ET secretion</td>
<td>4</td>
<td>8.1%</td>
</tr>
<tr>
<td>Urine</td>
<td>2</td>
<td>4.0%</td>
</tr>
<tr>
<td>Bronchial aspirate</td>
<td>1</td>
<td>2.0%</td>
</tr>
<tr>
<td>Vaginal swab</td>
<td>1</td>
<td>2.0%</td>
</tr>
</tbody>
</table>

Out of total 49 isolates of P. aeruginosa screened for MBL production by imipenem disk diffusion test, 11 isolates (22.4 per cent) were imipenem resistant and the remaining 38 (77.6 per cent) were sensitive to imipenem. All 11 P. aeruginosa strains showing resistance to imipenem 10µg disk in the screening test were further tested by imipenem-EDTA disk synergy test and combined disk test. Although all 11 strains were MBL producers by combined disk test, only eight showed positive results in imipenem-EDTA disk synergy test. Detection of MBL production in P. aeruginosa by minimum inhibitory concentration (MIC) for imipenem was done by VITEK-2 system using N090 AST card for three randomly selected imipenem resistant isolates due to cost constrains. Two out of three strains were identified as IMPER phenotype with high level resistance to beta-lactams and carbapenem by AES (Advanced Expert System). The susceptibility pattern of imipenem resistant P. aeruginosa strains is shown in Table 3. P. aeruginosa with resistance to three or more antibiotic groups are considered multidrug resistant strains. Table 4 compares the prevalence of multidrug resistance among imipenem sensitive and imipenem resistant P. aeruginosa.
Table 3: Susceptibility pattern of imipenem resistant *P. aeruginosa* strains to commonly used anti-pseudomonal antibiotics

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>11 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>8 (72.7%)</td>
<td>0</td>
<td>3 (27.2%)</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>7 (63.6%)</td>
<td>0</td>
<td>4 (36.3%)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>9 (81.1%)</td>
<td>2 (18.1%)</td>
<td>0</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>7 (63.6%)</td>
<td>1 (9%)</td>
<td>3 (27.2%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>8 (72.7%)</td>
<td>2 (18.1%)</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>0</td>
<td>0</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>Colistin</td>
<td>0</td>
<td>0</td>
<td>11 (100%)</td>
</tr>
</tbody>
</table>

Table 4: Comparison of multi drug resistance among imipenem sensitive and resistant *P. aeruginosa* isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Multi drug resistant (resistant to 3 or more antibiotic groups)</th>
<th>Non-multi drug resistant (resistant to &lt; 3 antibiotic groups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem sensitive strains (n=38)</td>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>Imipenem resistant strains (n=11)</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

Two-tailed P value (Fisher’s Exact Test) = 0.177360
Odd ratio = 0.337500

Table 5: Comparison of duration of hospital stay among imipenem sensitive and resistant *P. aeruginosa*

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Hospital stay &gt;8 days</th>
<th>Hospital stay &lt;8 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem sensitive strains (n=38)</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Imipenem resistant strains (n=11)</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 6: Comparison of other risk factors among imipenem sensitive and resistant *P. aeruginosa*

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Imipenem resistant strains (n=11)</th>
<th>Imipenem sensitive strains (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Alcoholic</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>History of prior antibiotic use</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

Discussion

*P. aeruginosa* is a pervasive pathogen in hospital acquired infections, especially among critically ill patients. Multidrug resistance in *P. aeruginosa* has appeared as an issue of great concern with emergence of MBL-PA. Although simple phenotypic tests are available, these strains often escape detection during routine laboratory processing. We compared different phenotypic detection methods currently in use and elucidated risk factors and prevalence of MBL-PA infections in our hospital and its impact in terms of mortality.

In this study, most cases of *P. aeruginosa* were from surgical inpatients, in contrast to medical wards. The most common specimens received were pus swab (55.1 per cent) and pus aspirates (12.2 per cent) followed by respiratory specimens. The nature of samples can be easily correlated from the lesions in patients of these wards. Except one post-operative wound infection case, all orthopaedic patients had fracture site infections. Ulcerative lesions were predominant in surgery cases where diabetic ulcer, non-healing ulcer, traumatic ulcer and varicose ulcer accounted for most cases. Similarly, all medicine, ICU and chest medicine patients had either primary lung disease or developed respiratory co-morbidity. These findings are in keeping with other studies where *P. aeruginosa* was found frequently to cause respiratory and suppurative skin
P. aeruginosa infection was predominantly found among males (85.7 per cent) and in young and middle aged adults of 19-65 year age group (75.5 per cent). The mean age of patients with P. aeruginosa infection was 43.3 ± 18.9 years while patients with MBL-PA infection had 44.6 ± 21.2 year mean age. In this study, mean age of patients is much lower than the mean age commonly reported.\textsuperscript{15, 16} The preponderance of males can be explained by the greater number of cases from surgery and orthopaedic wards having more male patient admissions. Other authors also had similar findings.\textsuperscript{15, 16} Tsakris et al, found 93.3 per cent of patients with MBL-PA were males and concluded that male gender was an independent high risk association.

The imipenem disk diffusion screening divided 49 study isolates into two groups: 11 isolates (22.4 per cent) of imipenem resistant and 38 (77.6 per cent) isolates of imipenem sensitive P. aeruginosa. This test was employed as a screening test for selecting probable MBL producing strains for further testing. Ceftazidime resistance is more significant in the case of Enterobacteriaceae where MBL producing strains can have low MIC for carbapenems and may appear sensitive on disk diffusion, as reported in other studies.\textsuperscript{2, 13} Since this study is only focused on P. aeruginosa isolates, ceftazidime resistance was not considered for the initial screening.\textsuperscript{13} However, we found ceftazidime resistance in 9 out of 11 imipenem resistant P. aeruginosa isolates and the remaining two isolates had intermediate sensitivity. In this group, all 11 strains were uniformly sensitive to polymyxin and colistin. Polymyxin and colistin are peptide antibiotics\textsuperscript{17} and are the last resort of therapy in MBL-PA with additional resistance to aztreonam.\textsuperscript{14} However, the high incidence of nephrotoxicity and neurotoxicity that is associated with these drugs limits their use.\textsuperscript{18} Polymyxin resistance is uncommon among P. aeruginosa and several studies have reported Multi Drug Resistant (MDR) strains being uniformly sensitive to polymyxin.\textsuperscript{2, 19}

Diverse resistance patterns have been described by different authors.\textsuperscript{6, 16, 20, 21} Tsakris et al, reported 100 per cent resistance to ceftazidime, cefepime, carbapenems, amikacin, netilmicin and ciprofloxacin in VIM-2 type MBL-PA which showed only 44 per cent and 47 per cent resistance to gentamicin and piperacillin-tazobactam, respectively.\textsuperscript{16} In a recent Indian study, imipenem, gentamicin, ciprofloxacin, netilmicin, piperacillin and amikacin resistance amongst MBL-PA were 77.5 per cent, 77 per cent, 72.1 per cent, 67.3 per cent, 57.7 per cent and 56.1 per cent, respectively.\textsuperscript{20} While a further study by De et al, found 100 per cent resistance to all aminoglycosides, beta-lactam and quinolones.\textsuperscript{6} These regional variations in susceptibility patterns reflect the antibiotic practices prevailing in regional hospitals. Our study shows lower resistance to most non-beta lactam agents compared to others which can be attributed to rational antibiotic usage.

In contrast to the common observation of high prevalence P. aeruginosa with multidrug resistance in ICU in different studies,\textsuperscript{6} only four P. aeruginosa isolates were recovered from the ICU during the study period and all of them showed sensitivity to imipenem and most anti-pseudomonal drugs. Furthermore, no mortality was reported in these four ICU patients or the remaining 45 patients from other wards. High mortality and multidrug resistance among ICU patients with P. aeruginosa infection has been frequently reported by several authors. This may be related to excessive use of broad spectrum antibiotics, invasive procedures, associated septicaemia and higher co-morbidities among ICU patients.\textsuperscript{6, 8} However, in our case the study duration was very short and all P. aeruginosa isolates were sensitive to commonly used anti-pseudomonal drugs. Hence, these P. aeruginosa isolates were amenable to treatment. Moreover, good infection control measures and rational use of antibiotics in therapy in our hospital could explain zero mortality rates in these patients.

All 11 imipenem resistant P. aeruginosa isolates were tested for MBL production by DDST and CDT using imipenem and EDTA. Positive results were detected in all strains in CDT (100 per cent) and eight strains in DST (72 per cent). Although carbapenem resistant non-MBL-producing P. aeruginosa is not very rare, no such isolate was detected in this study. The prevalence of MBL production among P. aeruginosa in our hospital was 22.4 per cent, which is in accordance with other Indian studies.\textsuperscript{6, 20} Currently, phenotypic tests like modified Hodge test, E-test, DDST and CDT as well as molecular methods (PCR) are available for detection of MBL. PCR is the gold standard test with high sensitivity and specificity. However, its use is limited by the cost and infrastructural as well as technical requirements. EDTA based resistance reduction tests viz. CDT, DDST and E-test are most commonly practiced phenotypic tests for MBL which differentiate metal dependence of carbapenemase enzymes by using chelating agents. However, reliability of the test is questionable since EDTA was found to have some inhibitory effect on bacterial growth which may result in false-positive results.\textsuperscript{2} On the other hand, modified Hodge test which precludes the use of EDTA, detects only carbapenemase activity. It does not confirm the metal dependence of the carbapenemase.\textsuperscript{22} Despite conflicting observations of different authors, none of these phenotypic tests were optimal due to low sensitivity or specificity.\textsuperscript{23} Behera et al. reported equal efficacy of both combined disk...
test and E test. Some workers reported CDT to be satisfactory for screening despite its low specificity as it is an easy procedure and is simple to interpret. Others, however, found DST superior to and more reliable than CDT or modified Hodge test. We found that CDT showed better correlation with the imipenem disk diffusion screening method. However, sensitivity and specificity of these tests could not be calculated since PCR was not done. CDT has less chance of subjective variation, since it measures the increase in inhibition zone above a cut off value. On the other hand, interpretation of DDST is more subjective. CDT and DDST both are limited by factors like temperature, aeration, pH and thickness of media. However, the synergy between imipenem and imipenem+EDTA disks are influenced by diffusion. EDTA must diffuse close to the imipenem disk and achieve a concentration with effective chelating activity to demonstrate a synergy. This may explain the difference in results of CDT and DDST found in our study.

Multidrug resistance is an emerging problem in P. aeruginosa. MDR strains have been reported to cause outbreaks and greater mortality morbidity. Resistance to three or more antibiotic classes of anti-pseudomonal antibiotics viz. β-lactams, aminoglycosides and fluoroquinolones is considered as MDR in P. aeruginosa. We detected 17 MDR strains of which 10 (58.8%) were MBL producing isolates. High-level resistance to beta-lactams and carbapenem resistant phenotype was identified by AES (Advanced Expert System) in two of these isolates.

Several risk factors have been described in MBL-PA infection in literature. Duration of hospital stay of more than eight days was found to be significantly associated with MBL-PA infections in various studies. We found 8 out of 11 cases of MBL-PA infection had hospitalisation of more than eight days. However, for this study length of stay was found not to be significant (p value = 0.177360 and odd ratio = 0.337500) in determining risk of MBL-PA infection. Among 11 patients with MBL-PA infection, four (36.3 per cent) were diabetic, one (9 per cent) was alcoholic and five (45.4 per cent) patients had history of prior antibiotic use. Similar findings were reported by others. De et al. reported prolong hospitalisation > eight days, mechanical ventilation and endotracheal intubation were common risk factors of MBL-PA in ICU patients. Others, have shown that prior exposure of a β-lactam or fluoroquinolone, neurological disease, urinary tract infection, renal failure and ICU stay were significant risk factors for MBL-PA infections. Among drugs, prior uses of fluoroquinolones or carbapenems were associated with resistance to imipenem resistance. This is in accordance with our observation. We found 45.4 per cent patients had prior exposure to antibiotic use, especially ciprofloxacin, metronidazole and cefotaxime.

The main limitation of this study was the short duration of the study period and small sample size. The small number of cases may not be representative of hospital patient population under study. Because of the short duration of the study our observations might have been affected by seasonal trends of patient admission in different wards of hospital. Hence, positive associations found in our study may not be conclusive and need further evaluation. Imipenem MIC was not detected for all isolates. Molecular gold standard tests for detection of MBL genes were not done. Consequently, sensitivity and specificity of phenotypic methods could not be computed.

**Conclusion**

MBL production was the main resistance mechanism in carbapenem resistant P. aeruginosa and multidrug resistance was frequently detected among MBL producing P. aeruginosa in our institution. Prevalence of MBL producing P. aeruginosa was similar to other Indian hospitals, but these MBL producing strains were more sensitive to non-beta lactam drugs. Imipenem resistance detected by disk diffusion test was a reliable screening test that correlated well with the combined disk test method.

**References**


**PEER REVIEW**
Not commissioned.

**CONFLICTS OF INTEREST**
The authors declare that they have no competing interests

**ETHICS COMMITTEE APPROVAL**
Institutional Ethical Committee, Mahatma Gandhi Medical College & Research Institute. Reference ID 2012-02083