Original Article:

Phenotypic detection of Metallo-β-lactamase (MBL) producers among multidrug resistant (MDR) strains of \textit{P. aeruginosa} in Himachal Pradesh.

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Abstract

**Introduction:** The increasing number of Metallo-β-lactamase producing \textit{Pseudomonas aeruginosa} is a cause of concern worldover. In India also, this organism is emerging as a threat for clinicians. The present study has been undertaken to detect Metallo-β-lactamase producing strains among multidrug resistant clinical isolates of this organism in the state of Himachal Pradesh.

**Methods:** Clinical isolates of \textit{Pseudomonas aeruginosa} (141 in number) were examined for their susceptibility to different antibiotics including carbapenems and the proportion of MDR strains worked out. Metallo-β-lactamase (MBL) producing strains were detected by Combined disc and Ezy MBL strip tests.

**Observations and Results:** A total of 98 (69.50 %) out of 141 examined isolates were recorded as multidrug-resistant, of these 58 (59.18%) MDRs were resistant to Carbapenems. Among the Carbapenem (Imipenem and Meropenem) resistant strains, 17 (29.31%) were positive for MBL production by Ezy MBL strip and 18 (31.3%) by combined disc test. However, only 8 were positive for MBL production by both the tests. Precisely, a total of 27 strains out of 58 Carbapenem resistant MBL producers were detected. Presence of MBL producing \textit{P. aeruginosa} is a cause of health concern.

**Conclusion:** High resistance to commercial available antibiotics which are generally used to treat infections was observed amongst \textit{P. aeruginosa} strains. 46.55% of Carbapenem resistant isolates produced Metallo-β-lactamases. Carbapenems have been considered as most potent agents for treating infections due to multi-drug resistant gram-negative bacilli but resistance to carbapenems has been observed among \textit{P. aeruginosa} strains which can disseminated in the community. Hence, strategies to minimize the emergence of multiple β-lactamase producing pathogen need to be developed.

**Keywords:** Multidrug resistance, Metallo-β-lactamase

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**Introduction**

\textit{Pseudomonas aeruginosa}, an important opportunistic pathogen causing a wide range of acute and chronic infections is one of the most common gram-negative pathogen involved in nosocomial infections. Infections due to this organism are prevalent among patients with burn wounds, cystic fibrosis, acute leukaemia, organ transplants and intravenous drug-addiction$^{1,2}$. The pathogenicity of \textit{P. aeruginosa} is caused by multiple bacterial virulence factors and genetic flexibility enabling it to survive in varied environments. Condition worsens due to the rise of resistance against multiple antimicrobial drugs, which complicates the treatment of \textit{Pseudomonas aeruginosa} infection leading to high morbidity and mortality$^{3,4}$. 

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Therefore, the increasing number of infections due to multidrug resistant (MDR) *P. aeruginosa* producing metallo-β-lactamases (MBL) is an issue of public health concern. MBL producing strains are resistant to broad spectrum β-lactams, aminoglycosides and fluoroquinolones, which are used as major anti-therapeutic agents. MBL producing *P. aeruginosa* was first reported from Japan in 1991 and since then its incidence has been reported from various parts of the world including India. Metallo-β-lactamases are metalloenzymes of Ambler class B and are clavulanic acid resistant enzymes. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by ethylenediamine tetra acetic acid (EDTA). These enzymes are characterized by their ability to hydrolyze Carbapenems, which are β-lactam antibiotics considered as most potent agents for treating multi-drug resistant gram-negative bacilli infections.

The pattern of antimicrobial resistance among *Pseudomonas aeruginosa* strains is required to be determined in order to choose appropriate therapy for control of infection due to this organism and for prevention of further spread. Not much information is available on MBL producing *P. aeruginosa* isolates in the State of Himachal Pradesh, we therefore undertook this study to detect MBL producing *P. aeruginosa* strains in this State.

**Materials and Methods**

**Collection and confirmation of clinical isolates of *P. aeruginosa***

A total of 153 isolates recovered from suspected cases of *P. aeruginosa* infections at the Department of Microbiology, Indira Gandhi Medical College (IGMC) Shimla, Himachal Pradesh (H.P.) were collected in batches over a period of 18 months i.e. during September 2011 to February 2013. Of these, 134 originated from pus (burns, Diabetic foot, wounds), 10 from urine, 6 from sputum and 5 from blood. The bacterial isolates were transported on ice to the research laboratory at Shoolini University, Solan and cultured on Pseudomonas isolation agar, a selective medium, incubated at 37°C overnight. The organisms were identified on the basis of colony characteristic, pigment production, Gram’s staining, hemolysis on blood agar and other biochemical tests such as nitrate reduction, gelatin liquification, motility test, citrate utilisation, catalase and oxidase tests.

**In Vitro Antibiotic Cultural Sensitivity Assay**

The *in vitro* antibiotic sensitivity of *P. aeruginosa* was determined by Disc diffusion method in vitro as described by Kirby-Bauer *et al* (1966), modified and updated by Clinical and Laboratory Standards Institute guidelines (2012). The following antibiotics were used in the test: Amkacin, Gentamicin, Cefepime, Ceftazidine, Ciprofloxacin, Piperacillin, Piperacillin/Tazobactum, Imipenem, Meropenem, Levofoxacin, Aztreonam, Cefoperazone, Colistin, tobramycin, Carbencillin, Polymixin B, Gatifloxacin, Cefazolin, Azthromycin, Tigecycline, Ticaracillin/Clavulanic acid, Ofloxacin, Netilllin, Cefotaxime from Himedia Laboratories Pvt Ltd, India.

**MBL screening of Imipenem Resistant *P. aeruginosa* isolates**

The screening was done by the following methods:

**Imipenem-EDTA combined disc test**

The IMP-EDTA combined disc test was performed as described by Yong *et al* (2002). Precisely, the test organisms were inoculated on to Mueller Hinton agar
plates with the help of sterile cotton swabs. Imipenem disc (10 µg) and Imipenem/EDTA disc (10µg/750µg) (Himedia) were then placed 24mm apart on the surface of agar in the plate. Zones of inhibition around the two types of discs as mentioned above were recorded after 16 to 18 hours of incubation at 35°C and compared. In the combined disc test, the results were interpreted as follows: if the increase in inhibition zone with the Imipenem and EDTA disc was ≥ 7mm as compared to the Imipenem disc alone, the isolate was considered as MBL positive or MBL producer.

**Ezy MBL strip**

An inoculum (0.5 Mc Farland standard) was prepared from 24 hour old culture of the test strain, inoculated on Mueller Hinton agar plate with the help of sterile cotton swabs. The Ezy MBL strip containing a twofold seven-dilution range of IPM alone i.e. (4µg/ml to 256µg/ml) and IPM+EDTA (1µg/ml to 64µg/ml) were then placed over the surface of agar, incubated at 37°C for 18 to 24 hours. A ratio of MICs of the Imipenem (IP) to IP + EDTA (IPI) of ≥ 8 was interpreted as MBL positive.

**Observations and Results**

**Confirmation of P. aeruginosa isolates**

Of the 153 isolates examined, 141 were confirmed as P. aeruginosa. The confirmation was based on growth characteristics, Gram’s staining and biochemical characteristics.

**Susceptibility of P. aeruginosa strains to different antibiotic groups**

Of the 141 isolates of P. aeruginosa 70 (49.65%) were found resistant to 4 or more than four different groups of antibiotics. Such isolates were regarded as multidrug resistant. This number further increased to 98 (69.50%) when the intermediate resistance was also included (Fig. 1, Table 1). No resistance was seen against Polymixin B and 100% resistance was seen against Cefazolin. P. aeruginosa ATCC 27853 was included as quality control strain in the assay. The susceptibility pattern of P. aeruginosa strains to different antibiotics and pattern of MBL resistance is presented through Fig.1, Fig.2 and Table 1

**MBL Screening**

MBL screening was done by Combined Disc and Ezy MBL tests. The results of phenotypic detection of Metallo-β-lactamase producing strains are presented in Table 2 and through Fig. 3.

**Imipenem-EDTA combined Disc Test**

Of the 54 Imipenem and/or Meropenem resistant strains and 4 with intermediate resistance (total 58), 18 (31.03%) exhibited a ≥ 7mm zone size enhancement in the combined disc test.

**Ezy MBL Strip Test**

Of the 58 isolates, 17 (29.31%) were MBL positive by Ezy MBL Strip test. Both these tests gave discordant results for most of the isolates, only 8 isolates gave positive results for MBL production by both Imipenem-EDTA combined disc test and Ezy MBL strip test (Table 2). Stenotrophomonas maltophilia ATCC 13636 and Pseudomonas aeruginosa ATCC 27853 were used as quality control strains in these assays.

**Discussion**

P. aeruginosa is a pathogen associated with many nosocomial infections in immunocompromised patients. Carbapenems are drugs of choice for treating multidrug resistant P. aeruginosa. However, there is a tremendous increase in reports of Carbapenem resistance among P. aeruginosa strains. The first MBL producing P. aeruginosa strain was first
isolated in Japan in 1991 and reported in India in 2002. In the present study, among all the clinical isolates, multidrug resistant (resistant to 4 or more classes of antimicrobials including Carbapenems) were screened for phenotypic detection of Metallo-β-lactamase producing strains. A similar study was done by Goyal et al (2010) where they used the same criteria for declaration of MDR isolates as we describe here. However, some workers have used different criteria for multi drug resistance. For example, Irfan et al (2008) and Saderi et al (2010) define MDR isolate as the one which is resistant to two or more drugs or drug classes of therapeutic relevance whereas Souli et al (2008) regarded those strains as MDR which were resistant to three or more classes of antimicrobial agents. We report high prevalence 98/141 (69.50%) of multidrug resistant (MDR) strains of *P. aeruginosa* among hospital patients, outdoor as well as indoor, at IGMC Shimla, H.P. In the present study, we included drugs of different antibiotic classes namely Aminoglycosides, Carbapenems, Cephalosporins, Macrolides, Monobactams, Penicillins, Glycopeptides, Quinolones and Tigecycline. We found 38.29% (54/141) isolates resistant to Imipenem and 20.57% (29/141) isolates resistant to Meropenem and 15.60% (22/141) and 4.96% (7/141) showed intermediate resistance for Imipenem and Meropenem respectively. However all the Imipenem and Meropenem resistant isolates were not MBL producers but some isolates with intermediate zones were found to produce Metallo-β-lactamases. For this reason, such isolates were included in the resistant category. In various studies across the world, varying resistance has been seen towards Imipenem and Meropenem. The highest resistance of the 58
however, been observed against other three Aminoglycosides (Netillin (75.86%), Tobramycin (60.34%), Gentamicin (37.93%)). The latter observation is consistent with others34,35,36,37. High resistance to Ofloxacin and Gatifloxacin was also found as compared to other Quinolones (Ciprofloxacin and Levofloxacin) used in this study. Gad et al (2007)28 reported 23% resistance to Ofloxacin and 29% and 31% resistance of P. aeruginosa isolates towards Ciprofloxacin and Levofloxacin and whereas Khan et al (2008)30 reported 68.4% resistance against Ofloxacin which is in accordance to our study. We observed higher resistance to Cefotaxime (79.31%) and Cefoperazone (43.10%). In a similar study done by Gad et al (2007)28 68% and 36% resistance was shown against Cefotaxime and Cefoperazone respectively whereas to the tune of 100% resistance was observed by Deshmukh et al (2011)27. A very high resistance of 94% and 93.4% for cefotaxime and Cefoperazone respectively was reported by Vahdani et al (2012)33 and Varaiya et al (2008)29.

As the standard guidelines for detection of MBL producers are not clearly defined, different workers have employed different methods of detection such as Modified Hodge Test and Spectrophotometrically.35,36. Some workers have used screening methods which utilize metal chelators such as EDTA18,38,39. We detected MBL production in 27 isolates (19.15%) of the entire collection of 141 isolates and 58 Carbapenem resistant strains of which 27 (46.55%) were recorded as positive for MBL production. Other workers in different studies have reported MBL production in 69.5%, 72%, 83.33% and 85.7% of Imipenem resistant isolates39,40,41,42. The comparative analysis of 27 MBL positive and 17 MBL negative have reflected more or less similar or comparable patterns of resistance against different antibiotics used in the study except in case of MBL negative strains more resistance was recorded as compared to MBL positive strains against Cefepime, Ciprofloxacin, Piperacillin and Piperacillin/Tazobactam.

We screened MBL producers among MDRs by two methods, the combined disc method and Ezy MBL strip test for MBL screening and found disconcordance in the results obtained by these methods as only 8 (29.6%) of the Carbapenem resistant isolates were MBL producers by both the tests (Table 2). Out of 27 MBL positive isolates, 10 (37.04%) had a positive combined disc test and were negative by Ezy MBL strip test and 9 (33.33%) had a positive Ezy MBL Strip Test which were negative by the other test. In a similar study from India by Patwardhan et al (2013)43, 39 (28.89%) and 30 (22.22%) isolates were positive for MBL production by Combined disc test and Ezy MBL strip respectively43. Several studies report combined disc test as more sensitive test for MBL detection7,44,45,41 but several workers have employed E test strip test also for the detection of MBL producing P. aeruginosa strains26,46.

**Conclusion**

The present study pinpoints high prevalence of multidrug-resistant P. aeruginosa producing metallo-β-lactamase enzyme in the State of Himachal Pradesh. Ezy MBL test strip was a rapid and sensitive method for screening MBL producing P. aeruginosa. These phenotypic tests can be used effectively in clinical settings lacking molecular biology tools until confirmation by a reference centre. Further studies are required to confirm the accuracy of these simple detection tests in other clinical settings with strains harbouring enzymes other than...
MBL also for designing appropriate prevention and control strategies. The Phenotypic tests need to be correlated to the genetic markers of resistance. The work in this direction is underway in our laboratory. Furthermore, strict antibiotic policies may be framed and measures to limit the indiscriminative use of Carbapenems in the hospital environment be implemented in order to minimize the emergence of multiple β-lactamase producing *P. aeruginosa* strains whose spread might complicate the control the nosocomial infections due to this or related organisms.

**Fig 1.** The susceptibility pattern of *P. aeruginosa* isolates to different antibiotics.

**Fig 2.** *In vitro* cultural antibiotic sensitivity assay. Significant zones of inhibition are visible around most antibiotics.

**Fig 3.** MBL production in *P. aeruginosa* isolate recovered from pus (Isolate no.Pa138) is observed by both phenotypic tests (Ezy MBL strip and Combined disc test).
Table 1. Antibiotic sensitivity patterns of MBL positive (n=27) and MBL negative (n=17) isolates

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MBL positive</th>
<th>MBL negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Amikacin</td>
<td>26</td>
<td>1 (3.7 %)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>14</td>
<td>7+6=13(48.15 %)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>22</td>
<td>2+3=5 (18.52 %)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>23</td>
<td>3+1=4 (14.81 %)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>12</td>
<td>1+4=5(18.52 %)</td>
</tr>
<tr>
<td>Piperacillin/Tazobactum</td>
<td>26</td>
<td>1*(3.7 %)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>19+7=26(96.29 %)</td>
<td>2</td>
</tr>
<tr>
<td>Meropenem</td>
<td>13+1=14(51.85 %)</td>
<td>5</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>26</td>
<td>1+1=2(7.40 %)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>2+8=10(37.04 %)</td>
<td>10</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>22</td>
<td>1+4=5(18.52 %)</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>17</td>
<td>3+7=10(37.04 %)</td>
</tr>
<tr>
<td>Colistin</td>
<td>23</td>
<td>4(14.81 %)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>19(70.37 %)</td>
<td>4</td>
</tr>
<tr>
<td>Carbencillin</td>
<td>22</td>
<td>2+3=5(18.52 %)</td>
</tr>
<tr>
<td>Polymixin B</td>
<td>27</td>
<td>0(0 %)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>19+4=23(85.18 %)</td>
<td>12</td>
</tr>
<tr>
<td>Meropenem</td>
<td>10</td>
<td>5(29.41 %)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>22</td>
<td>3+2=5(18.52 %)</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>9</td>
<td>18(66.67 %)</td>
</tr>
<tr>
<td>Ticarcillin/Clavulanic acid</td>
<td>20+6=26(96.29 %)</td>
<td>0</td>
</tr>
<tr>
<td>Orloxicin</td>
<td>4</td>
<td>22+1=23(85.15 %)</td>
</tr>
<tr>
<td>Netilin</td>
<td>4</td>
<td>23(85.18 %)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>2</td>
<td>19+6=25(92.59 %)</td>
</tr>
</tbody>
</table>

*Intermediate Resistance

Table 2. Phenotypic detection of Metallo-β-lactamase producing strains

<table>
<thead>
<tr>
<th>Total number of isolates</th>
<th>Screening positive</th>
<th>Confirmatory Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDT positive, Ezy MBL negative</td>
<td>Ezy MBL negative, CDT negative</td>
</tr>
<tr>
<td>58</td>
<td>27</td>
<td>10</td>
</tr>
</tbody>
</table>

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References:


