Phenotypic and Molecular Characterization of Imipenem Resistant pseudomonas Isolates

Sherin A.S. ElMasry, Rania A. Ammar and Sally M. Saber

Clinical and Chemical Pathology Department, Faculty of Medicine, Ain Shams University. sallysaber@ymail.com

Abstract: Background: Pseudomonas spp. is one of the most frequent nosocomial pathogen that is often difficult to treat due to a multi-drug resistant pattern to a wide range of antibiotics like carbapenems. Imipenem-resistant Pseudomonas aeruginosa (IRP) resulting from metallo-b-lactamases has been reported to be an important cause of nosocomial infection and is a critical therapeutic problem worldwide, especially in the case of bacteremia. Objectives: In this work we aimed to detect Carbapenemase and MBL production as well as molecular detection of bla IMP gene in imipenem resistant nosocomial isolates of Pseudomonas spp. Material and Methods: A total number of 150 Pseudomonas isolates were collected from clinical specimens submitted to Ain Shams University microbiology Laboratory for culture and antibiotic susceptibility testing. We used the Imipenem E-test strips to determine the MIC of the IPM resistant isolates. Screening for Carbapenemase and Metallo-β-lactamase was done phenotypically using modified hodge test (MHT) and Imipenem-EDTA Double disk Synergy test (DDST), also molecular detection of bla IMP gene was performed. Results: It was found that 38 out of 150 isolates (25.4%) were resistant to imipenem by disc diffusion method which confirmed by E test that showed only 27 out of 150 (18%) were resistant. Ten Out of 27 isolates (37%) were expressed blaIMP gene. However, The Modified Hodge Test Positive isolates were 13 out of 27 E-test resistant isolates (48.1%). While the DDST positive isolates were 21 out of 27 (77.8%) of E-test resistant isolates. Conclusion: Metallo-β-lactamases among imipenem-resistant Pseudomonas were detected in 37% by PCR. This number might have been higher if other genes were included. Phenotypic tests could be misleading when testing for metallo-β-lactamases. Polymerase Chain Reaction detection remains the gold standard.

Keywords: Pseudomonas; imipenem resistance; metallo-b-lactamases; phenotypic tests

1. Introduction

During the past decade, nosocomial outbreaks of Pseudomonas spp. have been described with increasing frequency (10-20% of hospital-acquired infections), occurring mostly in surgical wards, intensive care units and internal medicine wards (Fine et al.; 2005). Carbapenems, such as imipenem and meropenem, remain one of the best drugs to treat infections caused by Pseudomonas spp. Increasing usage of these drugs and other expanded-spectrum antibiotics has resulted in the development of carbapenem-resistant Pseudomonas spp. With treatment failure even with combination therapy (Giamarellos et al., 2006)

MBL, an Ambler class B enzyme, is characterized by its ability to hydrolyze carbapenems, its resistance to all commercially available b-lactamase inhibitors and its inhibition by metal ion chelators as EDTA, Mercapto acetic acid (MAA). The substrate spectrum of this enzyme is quite broad, as it can hydrolyze penicillins, cephalosporins and carbapenems, but it lacks the ability to hydrolyze aztreonam (francoetal., 2010).

Clinical Laboratory Standard Institute (CLSI) documents do not yet contain a phenotypic method for detection of metallo-β-lactamase production in clinical isolates, and hence methods can be standardized and could be of use as it contributes towards the optimal treatment of patients and control of the spread of resistance and infection control (Jesudasonetal., 2005 and francoetal., 2010).

The implementation of a simple reliable phenotypic method to detect carbapenemase and metallo-β-lactamate production is useful particularly in situations where carbapenem and other β-lactams are indicated or preferred as therapeutic regimen (francoetal., 2010).

Objectives To determine the frequency of metallo-b-lactamases and Carbapenemase among imipenem-resistant Pseudomonas isolates by phenotypic and molecular detection methods.

2. Materials and Methods

The current study was conducted on 150 Pseudomonas spp. isolated from clinical specimens submitted to Ain Shams University Hospital Microbiology Central Laboratory for culture and sensitivity.
All selected isolates of *Pseudomonas spp* in this study were subjected to:

**I) Routine standard culture & sensitivity testing:**
- Routine subculture on blood agar and MacConkey agar media No. (Oxoid, England) for 24 to 48 hours incubation at 37 °C to obtain separate colonies. Identification of the isolates using: Gram stains morphology & Conventional biochemical tests.
- Antibiotic susceptibility test by disc diffusion method according to (CLSI, 2011). For the following antibiotics: Imipenem, Meropenem, Piperacillin+Tazobactam, Cefepime, Amikacin, Ciprofloxacin, Cefotaxime, Ceftazidime.

**II) Imipenem resistant isolates were subjected to:**

**A-Determination of MIC by E-test strips:**

The inoculums were prepared and the media was inoculated, with sterile forceps, the E-test strips were placed on the agar plate. Plates were incubated in an inverted position for 16-18 hours at 35-37°C. If MIC ≤ 4μg/ml, the organism was considered susceptible. While if MIC ≥ 16μg/ml the organism was considered resistant (CLSI, 2011).

**B-Screening for Carbapenemase and Metallo-β-lactamase according to franco et al., 2010:**

**Modified Hodge test (MHT):** The surface of a Mueller-Hinton agar plate was inoculated with a culture suspension of quality control reference strain E. coli ATCC 25922 which was susceptible to imipenem. An imipenem disc 10 μg was placed at the center and the strains to be tested were streaked from the edge of the disc to the periphery. Plates were incubated at 35-37°Covernight. MHT Positive test showed a clover leaf-like indentation of the E.coli indicating that this isolate was producing a carbapenemase, or MBL Fig 1.

**Imipenem-EDTA Double disk Synergy test (DDST):** To differentiate metalloenzymes from other carbapenemases. Test strains were adjusted to the McFarland 0.5 standard and used to inoculate Mueller-Hinton agar plates. A 10 μg IMP disc was placed on the plate and a 10 μl EDTA solution disc was placed at a distance of 10 mm (edge to edge). After overnight incubation, the presence of a synergistic inhibition zone between both discs was interpreted as a positive result Fig 2.

**C- Molecular identification of imipenem resistant gene, (MBLs gene IMP type, *blaIMP* gene) using conventional PCR,** according to Senda et al.; (1996).

**Template DNA extraction:** each isolate were heated in a hot block at 96°C up to 10 minutes, then were placed on ice for 5min

**PCR amplification:** Reaction mixture contained 1 ml (each) primer, Specific primer designed from Metallo-β-Lactamase gene (*blaIMP*) Forward primer: 5'-CTA CCG CAG CAG AGT CTT ACC AT (Operon Co., Germany, 200 mM (each)deoxynucleoside triphosphate (Promega Co., Ltd.), 1X reaction buffer containing 1.5 mM Mg²⁺Cl₂ (Promega Co., Ltd.), 2.5 U of PromegaTaqpolymerase (Promeg Co., Ltd.), and approximately 25 ng of template DNA. Amplification was performed in a 25-ml volume with the Biozyme PCR system PTC-200 (Perkin-Elmer, USA) thermal cycler. Cycles were designed after an initial denaturation step (2 min at 94°C), 30 cycles of amplification were performed, as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and DNA extension at 72°C for 1.5 min.

**Detection of the amplification product on agarose gel electrophoresis**

The positive control gave a sharp band at 500bp. Samples that gave a band at the same band of the positive control were considered positive for *blaIMP* gene as shown in the following figure (3).
3. Results

In this study, 150 Pseudomonas spp. isolates were collected and antibiotic resistance were determined. The carbapenem resistant isolates (Imipenem, Meropenem) represented 38 out of 150 (25.4%) by disc diffusion method.

However, using the Imipenem E-test strips, 27 isolates out of 38 (71.1%) was confirmed as resistant to Imipenem 27/150 (18%) (MIC ≥ 16 mg/L). The rate of detection among wound swabs was the highest rate (48.2%) as shown in Table (1).

Table (1): Distribution of confirmed Imipenem resistant Pseudomonas isolates among different clinical specimens.

<table>
<thead>
<tr>
<th>Types of specimens</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound swab</td>
<td>13(48.2%)</td>
</tr>
<tr>
<td>Sputum</td>
<td>7(25.9%)</td>
</tr>
<tr>
<td>Blood culture</td>
<td>3(11.1%)</td>
</tr>
<tr>
<td>Pus</td>
<td>3(11.1%)</td>
</tr>
<tr>
<td>CSF</td>
<td>1(3.7%)</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
</tr>
</tbody>
</table>

Most of those 27 (IRP) were isolated from the surgical units (51.9%) (Table 2).

Table (2): Distribution of confirmed IRP isolates in our hospital.

<table>
<thead>
<tr>
<th>Distribution of isolates</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical units</td>
<td>14 (51.9%)</td>
</tr>
<tr>
<td>ICU</td>
<td>11 (40.7%)</td>
</tr>
<tr>
<td>Internal medicine wards</td>
<td>2 (7.4%)</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
</tr>
</tbody>
</table>

The IRP isolates with MIC ≥ 16 mg/L were subjected to modified Hodge test (MHT) and double disc synergy test (DDST). The Modified Hodge Test Positive isolates were 13 out of 27 E-test resistant isolates (48.1%). While the Double Disc Synergy Test Positive isolates were 21 out of 27 E-test resistant isolates (77.8%). As regards the association between the results of two tests, it was found that 11/27 (40.8%) were positive by the two tests (MBL positive), while 10/27 where positive only by DDST (MBL positive), however, 2/27 (7.4%) where positive only MHT Carbapenemase production due to non MBL production (Table 3).

Table 3 the association between results of MHT and DDST.

<table>
<thead>
<tr>
<th>Modified Hodge test</th>
<th>Double disc synergy test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>11(40.8%)</td>
</tr>
<tr>
<td></td>
<td>2(7.4%)</td>
</tr>
<tr>
<td>Negative</td>
<td>10(37%)</td>
</tr>
<tr>
<td></td>
<td>4(14.8%)</td>
</tr>
<tr>
<td></td>
<td>21(77.8%)</td>
</tr>
<tr>
<td></td>
<td>6(22.2%)</td>
</tr>
</tbody>
</table>

As regards the results of (PCR), it was found that 10 out of 27 (37%) isolates of IRP expressed bla\textsubscript{IMP} gene. Most of these isolates were from wound 6 out of 10 (60%) and sputum: 4 out of 10 (40%). Interpretation of antibiotic resistance pattern of the confirmed IRP isolates was summarized in Table (4). PCR confirmed IRP isolates were resistant to Ceftazidime, cefepime, cefotaxime, tazocin and Meropenem.
As regards the diagnostic performance of phenotypic tests in detection of MBL gene, DDST was more sensitive while MHT was more specific than MHT was more specific.

Table 5 : Diagnostic performance of phenotypic methods for detecting IMP gene (PCR gold standard):

<table>
<thead>
<tr>
<th>Phenotypic test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHT</td>
<td>70%</td>
<td>64.7%</td>
<td>53.8%</td>
<td>46.1%</td>
<td>66.6%</td>
</tr>
<tr>
<td>DDST</td>
<td>100%</td>
<td>35.2%</td>
<td>47.6%</td>
<td>100%</td>
<td>59.2%</td>
</tr>
</tbody>
</table>

3. Discussion

Imipenem-Resistant *Pseudomonas* (IRP) is a current and significant concern, especially because of the limited therapeutic options for this pathogen. MBL enzymes demonstrated that a large proportion of MBL genes are associated with one or more aminoglycoside- or b-lactam resistant genes, partially explaining multi-drug-resistant Cases. (Agrawal et al., 2008 and Franco et al., 2010).

In this study a total number of 150 *Pseudomonas* isolates were collected from clinical specimens submitted to Ain Shams University Microbiology Laboratory for culture and antibiotic susceptibility testing. The resistance was (25.4%) to both Imipenem and meropenem by disc diffusion. In a study by Altoparlak et al., (2005) in Turkey, the resistance rates of *Pseudomonas* strains collected only from burn wounds were as: (30.8%) to imipenem, (32.5%) to meropenem. In another study by Sarkar et al., (2006) in India showed resistance (36.36%) to Imipenem. On the other hand, Carvalhoet al., (2005) in Portugal. The antibiotic susceptibility tests revealed high resistance to most antibiotics tested, Imipenem (96.3%) and Meropenem (84%). Astudy by Behera et al. (2008) in India showed (69%) resistance to Imipenem. This may be due to the wild use of those antibiotics regardless of the severity of infection.

A study conducted in India by Agrawal et al., (2008), the resistance pattern was (8.05%) to Imipenem. Another study by Hui Wang et al. (2010) from China, The resistance was (13.7%) to Imipenem and (13.9%) to meropenem. The resistance to carbapenems in our study was higher than the previous two studies. This may be due to the extensive use of carbapenems in our hospital.

We used the Imipenem E-test strips to determine the MIC of the IPM resistant isolates and to confirm their resistance to IPM. 27 out of 38 (71.1%) were confirmed as resistant with MIC ≥ 16 mg / l.

In a study by Japoni et al. (2006) from Iran, (37%) of resistant isolates by antibiotic susceptibility test was confirmed as resistant by E-test. Another one by Behera et al., (2008) in India, (100%) of resistant isolates by antibiotic susceptibility test was confirmed as resistant by E-test. In another study by Hemalatha et al. (2008) 87.5% of resistant isolates by antibiotic susceptibility test was confirmed as resistant but they used the agar dilution method. It has been established that although all MBLs hydrolyze imipenem, they vary considerably in their rate of hydrolysis which may or may not correlate with the bacterium’s level of resistance to carbapenems resulting in imipenem sensitive phenotypes (Agrawal et al., 2008).

In our study, the distribution of confirmed IRP isolates according to the type of specimens were (48.2%) from wound, (25.9%) from sputum, (11.1%) from pus and blood culture and (3.7%) from CSF. They were collected from surgical units (51.9%). The high percentage of resistance in surgical units explains the high percentage of resistance from wound.

In a study by Bisiklis et al., (2005), the confirmed resistant isolates were distributed as: sputum (47.8%) which was higher than our results, pus (13%) which was close to our results, wound (17.4%) which was lower than our results, blood...
culture (8.7%) and CSF (4.4%). Those confirmed resistant strains were collected from ICU (61%), surgical units (26%) and internal medicine wards (13%). The high percentage of resistance in ICU explains the high percentage of resistance from sputum.

In another study by Pitout et al., (2008) from Kenya, the confirmed resistant isolates were distributed as: sputum (53%), pus (7%), wound (30%) and blood culture (7%). The majority of them were collected from ICU (58%).

In our study, the MHT detected (48.1%) as carbapenemase and MBL producers. The DDST detected (77.8%) as MBL producers. These results are close to results of a study by Jesudason et al., (2005) from India as the modified Hodge test detected (56%) as carbapenemase and MBL producers. The double disc synergy test detected (72%) as MBL producers. Another study by Noyal et al., (2009) from India found that the modified Hodge test detected (28.1%) as carbapenemase and MBL producers and the double disc synergy test detected (50%) as MBL producers. A study by Lee et al., (2003) from Korea, used the modified Hodge test which detected (83.7%) as carbapenemase and MBL producers and the double disc synergy test detected (91.8%) as MBL producers. The DDST in the previous studies detected more positives than MHT with their results similar to our results. This may be due to false negative results frequently produced by the MHT.

On the other hand, Sevillano et al., (2006) from Spain used the MHT and detected (15.15%) as carbapenemase and MBL producers. However, the double disc synergy test detected (0%) as metallo-β-lactamase producers, they may be resistant to carbapenem due to mechanisms other than metallo-β-lactamase production.

In a study by Mendiratta et al., (2005), the modified Hodge test did not give an appreciable distorted zone of inhibition and therefore was not found suitable when compared to DDST which detected (93.3%) as metallo-β-lactamase producers.

In a study performed in Portugal by Pena et al., (2005), they used only the DDST with 52.8% detected as metallo-β-lactamase producers. Similar studies by Grfan et al., (2008), Behera et al., (2008) and Renata et al., (2008). They used only the DDST to detect metallo-β-lactamase producers with 100%, 57.14%, 96.4% respectively as metallo-β-lactamase producers.

As regards the performance of phenotypic tests in detecting MBL in our study, the DDST detected additional 10 isolates which were negative by MHT. This was similar to studies by Jesudason et al., (2005) and Noyal et al., (2009) in which the DDST detected 8 and 9 additional MβL producing isolates respectively not detected by MHT. In the current study, DDST was more sensitive than MHT (100%, 70%) respectively, this result was in accordance to Franco et al., 2010. They reported that the DDST was a better method for screening MBL detection than MHT, the sensitivity was (100%, 82.4%) respectively. They reported that they don’t rely on MHT as a good test for carbapenemase production due to the high rate of false negative results. However, the evaluation of the performance of DDST depends mainly on inhibitor-substrate combinations being better in mercapto acetic (MAA) or propionic acid (MPA) than EDTA – Imipenem combinations.

In our work, 10 out of 27 IRP isolates (37%) expressed blaIMP gene which was detected by PCR. Our results were close to that in Japan conducted by Zhao et al., (2008) in which blaIMP gene expression was (33%). But in other studies from France conducted by Johann et al., (2005), from Poland conducted by Sacha et al., (2009) and from China conducted by Wang and Huang, (2004) the prevalence of blaIMP gene was lower than in our study (2%), (28.5%) and (29%) respectively.

Inspite that PCR was the gold standard in detection of MBL, The low blaIMP expression in our study may be explained by the presence of different mechanisms of resistance to carbapenems such as reduced uptake as a result of loss of the porins or the presence of other untested genes, as reported by many previous studies (Hanso et al., 2006, Franco et al., 2010). They all had predominance of another genes blaVIM or blaSPM in their strains which was not tested in our study.

The main source of blaIMP producing isolates in our study was from wound (60%), sputum (40%), they were mainly from surgical units (70%) and ICUs (30%). In comparison to a study from France conducted by Johann et al., (2005), the isolates were from urine (43%), wound (21%), sputum (9%) and blood (7%), this difference may be due to the fact most of the isolates were from ICU.

In our study the resistance pattern ofblaIMP expressing pseudomonas isolates was (100%) to fortum, tazocin, cefepime and cefotaxime (70%) to amikin, (90%) to ciprofloxacin. This was in agreement with Franco et al., 2010, they reported that large proportion of MBL gene are associated with one or more aminoglycosides or b lactam resistance gene partially explaining multi drug resistant pattern.

However, in a study from India conducted by Varaiya et al., (2008) the resistance pattern of blaIMP expressing pseudomonas isolates was (34%) to quinolones, (50%) to fourth generation cephalosporins, (67%) to third generation
Cephalosporins and (84%) to aminoglycosides. In another study in Canada Gregson et al., (2006) found that the resistance pattern was (59%) to third generation cephalosporins, (64%) to quinolones and (72%) to aminoglycosides.

**CONCLUSION:**

A high prevalence of IRP is a critical problem representing a practical therapeutic challenge. Even early recognition of MBL or Carbapenemase through routine laboratory testing is desirable; care should be taken when phenotypic tests are interpreted. In such cases, PCR should also be done to validate such results. The best method for MBL screening should be based on inhibitor synergy with respect to local factors such as bacterial type, MBL enzyme prevalence and the technical abilities of relevant facilities. Further genetic investigations for genes responsible for carbapenem resistance are recommended and strict infection control procedures should be followed.

**References**


