Multiplex PCR study of nosocomial *Pseudomonas aeruginosa* for genes of Metallo-Beta-Lactamases: could such enzymes bring us to end of antibiotics?

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Metallo-beta lactamases (MBLs) have been increasingly recognized from bacterial isolates worldwide where they are considered to be one of the most worrisome antibiotic resistance mechanisms. They differ from other beta-lactamases in their need of metallic cofactor for enzymatic activity which could be inhibited by metallic chelators, in their ability to resist beta-lactamase inhibitors and in being able to hydrolyze all beta-lactams including carbapenems. In the present study, the most common MBLs-genes (the VIM and IMP genes) were detected using multiplex polymerase chain reaction (PCR) in nosocomial isolates of 40 Imipenem (IPM) resistant and 20 Imipenem sensitive *Pseudomonas aeruginosa*. The results revealed that none of the Imipenem sensitive isolates had any MBLs-gene while 16/40 (40%) of the Imipenem resistant isolates were positive for MBL-VIM gene and none of them had MBL-IMP gene. So, MBL-VIM gene was found to be significantly associated with IPM resisting than IPM sensitive isolates (P<0.001). Among the IPM resisting *P. aeruginosa*, isolates which had the VIM gene were found to be more prevalent in blood (87.5 %) and sputum (60 %) than urine (20 %) and infected post operative wounds or burns (8.3 %). It was also found that VIM positive isolates were more resistant to Amikacin (75% versus 66.7%, P 1.000), Ceftazidime (93.8 % versus 45.8 %, P 0.034), Ciprofloxacin (100% versus 29.2%, P 0.000), Gentamycin (87.5% versus 50%, P 0.020) and Tobramycin (81.3% versus 41.7%, P 0.022), and less resistant to Piperacillin (50% versus 75%, P 0.205) than VIM negative isolates. Moreover, case fatality rate was found to be significantly higher in patients infected with VIM positive isolates than those infected with VIM negative isolates (50 % versus 8.4 %, P 0.005). It could be concluded that isolates of IPM – resistant *P. aeruginosa* which had MBL-gene (MBL-producers) were more resistant to antibiotics and more associated with serious infections and higher mortality rate than those which have other mechanisms of beta lactam resistance. So, as it is thought that emergence of such enzymes could bring us to feared end of antibiotics, it has to be recommended that all IPM-resistant isolates should be tested for MBLs production. This will ensure the early recognition of an outbreak and introduction of appropriate infection control measures to prevent further nosocomial spreading of such multiresistant highly virulent strains.

INTRODUCTION

Beta-Lactams are among the first choice agents for chemotherapy of *Pseudomonas aeruginosa* (*P. aeruginosa*). However, several mechanisms can contribute to acquired beta-lactam resistance, including impermeability due to loss of certain porin (1), the up regulation of an active efflux pump system present in the cytoplasmic membrane (2), or the production of beta-lactamase enzymes (3).

Metallo-beta lactamases (MBLs) are considered to be the most worrisome resistance mechanisms owing to their capacity to hydrolyze, with the exception of aztreonam, all beta-lactam agents, including the carbapenems (such as imipenem and meropenem), penicillins and cephalosporins. Moreover, they are not susceptible to therapeutic beta lactamase inhibitors, such as clavulenate (4).

These enzymes differ from Extended spectrum beta lactamases (ESBLs) discovered in 1980s which could be inhibited by beta lactamase inhibitors and could only hydrolyze penicillins and extended spectrum cephalosporins (3rd generation) while not carbapenems (5). MBLs were also found to be different from ESBLs in requiring divalent cation, usually zinc, as metal cofactor for enzyme activity, which could be inhibited by metal chelators, such as EDTA (3).

MBLs were divided into four categories according to their molecular structures, namely the IMP (6), VIM (7), SPM (8) and GIM (9). IMP and VIM types are the most commonly distributed world wide, while GIM and SPM-MBLs
are suggested to be confined only to Germany and Brazil, respectively \((9, 10)\).

The production of MBLs can potentially lead to treatment failure if \(\beta\) lactams are used. Moreover, MBL-producing strains are of additional importance from an infection control perspective, because they may be responsible for horizontal transmission of the resistance gene(s) carried on highly motile elements such as plasmids and transposons \((11)\). So, the emergence of these enzymes compromises effective treatment of nosocomial infections, bringing us closer to the much feared 'end of antibiotics' \((12, 13, 14)\).

*Pseudomonas aeruginosa* producing MBLs was first reported from Japan in 1991 \((15)\). It has been responsible for several nosocomial outbreaks of serious infections, and has been associated with failure of therapy with carbapenems \((16, 17)\).

Several phenotypic methods (screening tests) are available for the detection of MBL-producing bacteria based on the ability of metal chelators, such as EDTA, to inhibit the activity of these enzymes. These methods included a MBL-E test and a double-disk synergy test using EDTA with imipenem (IPM) or ceftazidime (CAZ), which are known to be hydrolyzed by all types of MBLs \((18)\). These tests differ from the double-disc synergistic test used for detection of ESBL production which depends on synergistic effect of \(\beta\) lactamase inhibitors (such as clavulanate) on the action of extended spectrum cephalosporins \((3^{rd}\) generation). These screening tests are thought to be difficult to interpret and time-consuming, since optimal disk spacing and re-incubation of plates are sometimes required to obtain ideal results. So, a multiplex polymerase chain reaction (PCR) was developed in 2005 for the detection of MBLs genes \((19)\).

In the present study, a multiplex PCR was used for detection of the most common 2 types of MBLs genes, the VIM and IMP genes, in selected nosocomial isolates of Imipenem-resistant *P. aeruginosa*, where Imipenem is known to be hydrolysed by all types of MBLs. The results were correlated with the sensitivity pattern of the isolates and the mortality rate of the patients.

**MATERIALS AND METHODS:**

**Clinical specimens:**

This study was performed on 40 IPM-resistant nosocomial isolates of *P. aeruginosa* selected from patients who acquired infection after being admitted to In-patient of Tanta University Hospital during the period from November 2005 to August 2006. A control group of 20 IPM-sensitive isolates was also included. Different clinical specimens included urine, sputum, blood and swabs taken from patients with catheter-related urinary tract infection (UTI), respiratory tract infection (RTI), pyrexia of unknown origin (PUO) and post-operative wound (POW) or burn infection, respectively. Patients were followed up from the first isolation of *P. aeruginosa* to discharge from hospital or to death. Patients who had major causes of death other than infection were excluded from the study, such as those with heart failure, respiratory failure, hepatorenal failure, major surgery and massive burns.

**Identification and Antimicrobial susceptibility testing:**

Conventional microbiological methods were used for *P. aeruginosa* identification \((20)\), and susceptibility test was performed using disk-diffusion method on Muller Hinton agar according to National Committee for Clinical Laboratory Standards guidelines \((21)\). The following antibiotics discs \((\text{Oxoid, England})\) were chosen to be used as previously recommended \((19)\):

- Imipenem \((15\mu g)\)
- Amikacin \((30\mu g)\)
- Ceftazidime \((30\mu g)\)
- Ciprofloxacin \((5\mu g)\)
- Piperacillin \((30\mu g)\)
- Gentamicin \((10\mu g)\)
- Tobramycin \((10\mu g)\)

Forty IPM-resistant isolates were selected for the present study, as Imipenem was known to be hydrolysed by all types of MBLs \((19)\), while 20 IPM-sensitive isolates were included as a control group.

The bacterial isolates were subjected for detection of MBL-VIM and IMP genes using multiplex PCR.
**MBLs gene identification using multiplex PCR (19):**

DNA template preparation was performed as follows: The isolates were inoculated into 5 ml of Trypticase soy broth (Difco, Detroit, Mich.) and incubated for 24 h at 37°C. Cells from 1.5 ml of an overnight culture were harvested by centrifugation for 5 min, where the pellet was resuspended in 500 µl of distilled water. The cells were lysed by heating them at 95°C for 10 min, and cellular debris was removed by centrifugation for 5 min. The supernatant was used as a source of template DNA for amplification.

Multiplex PCR amplification for the simultaneous detection of *bla*IMP and *bla*VIM metallo beta-lactamase genes was carried out using Taq DNA polymerase, deoxynucleoside triphosphate and gene-specific primers (Invitrogen Co, CA).

The composition of the reaction mixture was as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.25 mM each of the deoxynucleoside triphosphates (ATP, GTP, CTP and TTP), the four primers (one pair for each gene) at a concentration of 0.16 µM each, and 2 U of Taq polymerase in a total volume of 23 µl with addition of 2 µl of sample lysate. The PCR program was performed in a Thermal Cycler and it consisted of an initial incubation of 10 min at 37°C and an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and primer extension at 72°C for 1.5 min.

The multiplex PCR products were analyzed by electrophoresis with 1.4% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.5]). The gels were stained with ethidium bromide and visualized with UV light.

Sequences of the primers used for multiplex PCR were as follows (22):

**For MBL-IMP gene:**
IMP-A (5’-GAAGCGTTTATGTCATAC-3’)
and
IMP-B (5’-GTACGTTTCAAGAGTGATGC-3’),
which give an amplified product of 587-bp.

**For MBL-VIM gene:**
VIM2004A (5’-GTT TGG TCGCAT ATC GCA AC-3’) and
VIM2004B (5’-AAT GCG CAG CAC CAG GATAG-3’), which give an amplified product of 382-bp.

**Statistical analysis:**
The collected data was organized, tabulated and statistically analyzed using SPSS software statistical computer package version 12. For qualitative data, the number and percentage distribution were calculated and statistically analyzed using Chi square test. The Odds ratio and 95% confidence interval were calculated to estimate the risk. Significance was adopted at <0.05 for interpretation of results of test of significance.

**RESULTS**
Out of the 40 IPM resistant *P. aeruginosa* isolates, 16 (40%) were positive for *bla*VIM gene (Fig. 1), while none of them was positive for *bla*IMP gene. Moreover, none of the 20 IPM sensitive isolates had any of the MBLs-genes. So, MBLs gene was found to be significantly associated with IPM-resistant isolates (Odds ratio: 1.833, CI: 1.400-2.40, P-value<0.001).
The Imepinem (IPM) resistant *P. aeruginosa* included 10 isolates (25%) from urine of patients with catheter related - UTI, 10 (25%) from sputum of patients with RTI, 8 (20%) from blood of patients with PUO, and 12 (30%) from infected POW and burns. Isolates positive for *bla*VIM gene were found to be more prevalent in blood (7/8, 87.5 %) and sputum (6/10, 60 %) than infected wounds or burns (1/12, 8.3 %) and urine (2/10, 20 %). There was a significant decrease in the VIM positive isolates in urine (P= 0.05), a non significant difference between positive and negative isolates in sputum (P= 0.52) , a significant increase in the positive isolates in blood (P = 0.03) , a highly significant decrease in the positive isolates in swabs from POW or burns (P=0.004) and a non significant difference between total positive and negative isolates (P=0.20) as shown in table 1 and figure 2.
Table (1): Multiplex PCR detection of MBLs genes in IPM – resistant *P. aeruginosa* isolated from different nosocomial specimens :

<table>
<thead>
<tr>
<th>Source of nosocomial <em>P. aeruginosa</em></th>
<th>IPM-resistant isolates +ve for <em>blaVIM</em> gene</th>
<th>IPM-resistant isolates -ve for <em>blaVIM</em> gene</th>
<th>Chi square and P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Urine (UTI) $n=10$</td>
<td>2</td>
<td>20.00</td>
<td>8</td>
</tr>
<tr>
<td>Sputum (RTI) $n=10$</td>
<td>6</td>
<td>60.00</td>
<td>4</td>
</tr>
<tr>
<td>Blood (PUO) $n=8$</td>
<td>7</td>
<td>87.50</td>
<td>1</td>
</tr>
<tr>
<td>Swabs (POW&amp;burns) $n=12$</td>
<td>1</td>
<td>8.33</td>
<td>11</td>
</tr>
<tr>
<td>Total $n=40$</td>
<td>16</td>
<td>40.00</td>
<td>24</td>
</tr>
</tbody>
</table>

P1: Significant decrease in the VIM positive isolates in relation to negative isolates.
P2: Non Significant difference between positive and negative isolates.
P3: Significant increase in the positive isolates in relation to negative isolates.
P4: Highly significant decrease in the positive isolates in relation to negative isolates.
P5: Non significant difference between total positive and negative isolates.

Figure 2: Multiplex PCR results for detection of VIM gene in IPM resistant *P. aeruginosa* from different clinical specimens

Out of the 40 IPM resistant *P. aeruginosa* clinical isolates, 28 (70%) were resistant to Amikacin (AMK), 26 (65 %) to Ceftazidime (CAZ), 23 (57.5%) to Ciprofloxacin (CIP), 25 (62.5%) to Piperacillin (PIP), 26 (65%) to Gentamycin (GEN), and 23 (57.5 %) to Tobramycin (TOB). The isolates positive for *blaVIM* gene were more resistant to Amikacin (75 % versus 66.7% , P=1.000), Ceftazidime (93.8 % versus 45.8 % , P=0.034), Ciprofloxacin
(100% versus 29.2%, P=0.000), resistant to Piperacillin (50% versus Gentamycin (87.5% versus 50%, 75%, P=0.205) than VIM negative (81.3% versus 41.7%, P=0.022) and less resistant to Tobramycin (81.3% versus 41.7%, P=0.022) as shown in Table 2.

Table 2 : Comparison of the antimicrobial resistance pattern in bla\_VIM gene - positive and negative isolates of Imipenem resistant P. aeruginosa:

<table>
<thead>
<tr>
<th>Resistance to antimicrobial agents</th>
<th>bla_VIM gene +ve isolates N=16</th>
<th>bla_VIM gene -ve isolates N=24</th>
<th>OR</th>
<th>(95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>12 (75.00)</td>
<td>16 (66.67)</td>
<td>0.857</td>
<td>0.243</td>
<td>3.024</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>15 (93.75)</td>
<td>11 (45.83)</td>
<td>4.432</td>
<td>1.133</td>
<td>17.341</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>16 (100.00)</td>
<td>7 (29.17)</td>
<td>3.429</td>
<td>1.838</td>
<td>6.396</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>8 (50.00)</td>
<td>17 (70.83)</td>
<td>0.412</td>
<td>0.110</td>
<td>1.537</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>14 (87.50)</td>
<td>12 (50.00)</td>
<td>7.000</td>
<td>1.300</td>
<td>37.704</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>13 (81.25)</td>
<td>10 (41.67)</td>
<td>6.067</td>
<td>1.361</td>
<td>27.049</td>
</tr>
</tbody>
</table>

*Significant increase in the resistance in VIM positive than VIM negative isolates.

OR : Odds ratio
CI: confidence interval (L: lower value, U: upper value)

None of the patients infected with the IPM- sensitive isolates has died in the hospital during the study. As regard patients infected with the IPM- resistant isolates, eight out of the 16 patients (50%) who were infected with VIM positive isolates were died in hospital, including 6 patients with PUO (37.5%) and 2 with RTI (12.5%). On the other hand, two patients out of 24 (8.4%) were died from those infected with VIM negative isolates, and they both had burn infection. Case fatality rate was found to be significantly higher in patients infected with VIM positive than VIM negative isolates (50% versus 8.4%, P=0.005) as shown in Table 3 and Figure 3.

Table 3 : Comparison between the case fatality rate in patients infected with bla\_VIM gene-positive and negative isolates of Imipenem resisting P. aeruginosa:

<table>
<thead>
<tr>
<th>Clinical disease</th>
<th>Patients infected with isolates n=16</th>
<th>VIM +ve</th>
<th>Patients infected withVIM -ve isolates n= 24</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of deaths</td>
<td>%</td>
<td>Number of deaths</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>PUO</td>
<td>6</td>
<td>37.50</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>RTI</td>
<td>2</td>
<td>12.50</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>UTI</td>
<td>0</td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Infected POW &amp;burns</td>
<td>0</td>
<td>0.00</td>
<td>2</td>
<td>8.33</td>
</tr>
<tr>
<td>Total deaths</td>
<td>8</td>
<td>50.00</td>
<td>2</td>
<td>8.33</td>
</tr>
<tr>
<td>OR</td>
<td>11.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(95%CI)</td>
<td>1.915-63.177</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.005*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant increase in total deaths in VIM positive than VIM negative isolates.

OR: Odds ratio
CI: confidence interval
DISCUSSION

The presence of MBL-producing bacteria in a hospital is usually associated with a therapeutic problem, as well as a serious concern for infection control management. They are responsible for prolonged nosocomial outbreaks of serious infections and so, their early identification will aid in preventing the spread of such multidrug-resistant highly virulent isolates (16, 23).

Clinical microbiological laboratories must be able to distinguish MBL-producing strains from those with other mechanisms responsible for beta-lactam resistance as it is considered to be the most worrisome resistance mechanism. In the absence of novel agents for treatment of infections caused by multidrug-resistant bacteria in the near future, the uncontrolled spread of MBL producers may lead to treatment failure with increased morbidity and mortality (19).

These enzymes differ from Extended spectrum beta lactamases (ESBLs) discovered in 1980s which could be inhibited by β lactamase inhibitors and could only hydrolyze penicillins and extended spectrum cephalosporins (3rd generation) while not carbapenems. MBLs were also found to be different from ESBLs in requiring divalent cation, usually zinc, as metal cofactor for enzyme activity (5) which could be inhibited by metal chelators, such as EDTA (3).

In the present study, a multiplex PCR was used for detection of the most commonly distributed MBLs genes, *bla*IMP and *bla*VIM genes, in 40 IPM-resistant nosocomial isolates of *P. aeruginosa*. The results revealed that *bla*VIM gene was detected in 40% of them with a significant association of MBL-gene to IPM-resistant than IPM-sensitive isolates, in which no MBL-gene could be found (P < 0.001). Similar results were obtained by Laupland et al., (24) who examined 228 IPM resistant *P. aeruginosa*, where 39% were positive for *bla*VIM gene, and 2% were positive for *bla*IMP gene. Moreover, Pitout et al., (19) studied 241 *P. aeruginosa* isolates, where 43% were positive for *bla*VIM gene and 2% were positive for *bla*IMP gene. The present results agreed also with both Hemalatha et al., (20), who found *bla*VIM gene in the imipenem resisting *P. aeruginosa* but not in sensitive strains, and Giakkoupi et al., (25) who could not detect *bla*IMP gene in any of his isolates.

Much higher incidence of VIM gene was found in IPM resistant *P. aeruginosa* in some studies [88.7% (26), 76.56 % (27) and 62% (25)] while lower
incidence was detected in others [18.1% (28) and 0.48% (29)].

This controversy in the incidence of strains carrying MBL-genes may be due to differences in number of cases studied, source of samples, or variations in the prevalence between different hospitals and geographic areas.

It was suggested that isolates with blaVIM gene have replaced those with blaIMP gene that were identified in the 1990s (30). This agrees with the results of the present work that could not find blaIMP gene in any of the IPM resistant isolates. On the other hand, no isolate was found to have the two genes together, where it was suggested that the simultaneous occurrence of two different types of MBLs in a single hospital or region was very rare and has been previously described only in Canada and Brazil (19, 31).

It has to be reported that SPM-1 and GIM-1 types of MBLs were not examined in this study as they were noticed to be confined only to Brazil (10, 32) and Germany (9). This suggests that the evolution, maintenance, and dissemination of MBL resistance genes among P. aeruginosa populations in larger geographic regions is a complex and dynamic field that needs to be studied in detail (32).

In the present study, the VIM-positive isolates were found to be more resistant to 5 out of 6 tested antimicrobial agents (Amikacin, Ceftazidime, Ciprofloxacin, Gentamicin, and Tobramycin) than VIM-negative ones, which is in accordance with the results previously obtained (10,33,34). Moreover, it has been reported that the majority of IPM-resistant isolates demonstrated cross-resistance to many other antibiotics (35).

Moreover, the VIM-positive isolates were found to be more prevalent in blood and sputum (87.5% and 60% respectively) and less prevalent in urine and wound or burn (20% and 8.3% respectively) than those lacking the gene. This could be explained by the previously reported studies which suggested that MBL producing isolates induced more serious and fatal infections because they are highly virulent (19, 24, 36, and 37). This could also explain the significantly higher case fatality rate noticed in this study among patients infected with VIM-positive isolates than those infected with VIM-negative isolates (50% versus 8.4%, P 0.005). In similar studies, mortality rate was found to be 48% and 57.1% among MBL-producing P. aeruginosa versus 10% and 29.6% in isolates not producing MBLs, respectively (38, 17).

Conclusion and recommendation: It could be concluded that isolates of IPM-resisting P. aeruginosa which had MBL-gene (produce MBL) are more resistant to antibiotics and more associated with serious infection and high mortality rate than those which have other mechanisms of beta lactam resistance. So, as it is thought that emergence of such enzymes could bring us to feared end of antibiotics, it has to be recommended that all IPM-resistant nosocomial isolates should be tested for MBLs production, at least by using simple screening tests. This will ensure the early recognition of an outbreak and introduction of appropriate infection control procedures to prevent further nosocomial spreading of such multi-resisting highly virulent strains. Moreover, case fatality rate should be calculated on larger scale in such patients with respect to other co-morbid diseases in the studied groups.

REFERENCES


19-Pitout JD, Gregson DB, Poirel L, McClure JA, Le P, Church DL (2005): Detection of Pseudomonas aeruginosa producing


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