Prevalence of Imipenemase and Verona Integron–Encoded Metallo–β–Lactamase in Imipenem–Resistant *Pseudomonas aeruginosa*

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Abstract:

**Objective:** To determine the prevalence of metallo–β–lactamase (MBL) including imipenemase (IMP) and Verona integron–encoded metallo–β–lactamase (VIM) in imipenem–resistant *Pseudomonas aeruginosa* (IRPA) isolates and investigate the *in vitro* activities of 9 antimicrobial agents against MBL–positive isolates.

**Material and Methods:** Seventy–eight IRPA isolates were obtained from Songklanagarind Hospital. MBL production was detected by the combined–disk test, and the *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes were determined via Polymerase Chain Reaction (PCR). The *in vitro* activities of amikacin, gentamicin, aztreonam, ceftazidime, meropenem, ciprofloxacin, colistin, piperacillin/tazobactam, and ticarcillin/clavulanate were determined using the E–test method.

**Results:** Of the 78 IRPA isolates, 20 (25.6%, 95% CI 17.2–36.4%) were MBL phenotype–positive, and 30 (38.5%, 95% CI 28.5–49.6%) were MBL genotype–positive (29 Imipenemase (IMP)–type and 1 Verona integron–encoded metallo–β–lactamase (VIM)–type). Ninety percent of the MBL genotype–positive isolates were MDR. Most MBL genotype–positive isolates were susceptible to colistin (susceptibility rate of 96.7% and MIC<sub>50</sub> value of 1.5 µg/mL).

**Conclusion:** Our results showed a high prevalence of MBL–positive isolates (38.5%) in IRPAs, and the IMP–type isolates were dominant among the metallo–β–lactamase–producing imipenem–resistant *P. aeruginosa* tested. Most MBL–positive isolates were susceptible to colistin and had low MIC<sub>50</sub> values.

**Keywords:** *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, imipenem–resistant, metallo–β–lactamase, *Pseudomonas aeruginosa*
**Introduction**

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a serious pathogen that is infamous for its multi-drug resistance (MDR). Carbapenems are potent agents for the treatment of infections caused by MDR *P. aeruginosa* (MDRPA). Metallo-β-lactamases (MBLs) represent one of the most important resistance mechanisms that enables resistance to all β-lactams, including carbapenems, and β-lactamase inhibitors like clavulanic acid, sulbactam, and tazobactam, except monobactams. The infections caused by metallo-β-lactamase–producing *P. aeruginosa* (MBLPA) isolates are associated with high mortality rates. 

Imipenemase (IMP) and Verona integron–encoded metallo-β-lactamase (VIM) are important acquired MBL types. The MBL genes, including *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub>, are carried on mobile genetic elements to a location that eventually facilitates the horizontal spreading among different strains. In addition, the IMP–type and VIM–type metallo-β-lactamases in *P. aeruginosa* isolates have been reported worldwide to associate with a clonal spread and hospital outbreaks.

In Asian countries, recent studies have found a high prevalence of MBL–producing imipenem–resistant *P. aeruginosa* (IRPA) in clinical isolates from tertiary hospitals (68.0–76.0%). In Thailand, the prevalence of MBLPA ranges from 17.0–28.0% of isolates, and the IMP–type and VIM–type MBLs are commonly detected in *P. aeruginosa* clinically isolated from tertiary hospitals. Therefore, the aim of this study was to determine the prevalence of IMP–type and VIM–type MBLIRPA isolates.

**Material and Methods**

Seventy–eight IRPA isolates were collected from clinical samples such as sputum (47), bodily fluid (9), urine (8), tissue (6), pus (4), and blood (4) from patients at Songklanagarind Hospital in Songkhla province, Thailand during the July 2012–October 2013 period. The *P. aeruginosa* isolates were identified by conventional methods, which comprised colony morphology; Gram staining; pyocyanin, pyoverdine, and pyorubin pigment production; and oxidase, citrate, cetrimide, urease, and Triple Sugar Iron fermentation tests. Polymerase Chain Reaction (PCR) with specific primers (forward: 5’ATGGAAATGCTGAAATTCGGC3’, reverse: 5’CTTCTTCAGCTCAGCAGCGACG3’) was used to identify the oprL gene as described by Aghamiri et al. IRPA was defined as an isolate confirmed to be resistant to imipenem. MDR was defined as an isolate that is resistant to three or more classes of antimicrobial agents. The Ethics Committee of the Faculty of Medicine, Prince of Songkla University approved this study (REC 55–340–01–8–3 and REC 60–039–01–8).

All isolates were tested for MBL production using the combined–disk test as described by Franklin et al. as well as for MBL–encoding genes. The deoxyribonucleic acid template was prepared using the boiling method. The *bla*<sub>IMP</sub> gene was detected by PCR with *bla*<sub>IMP</sub>–specific primers (forward: 5’GTTTGAAGAAGTTAACGGGTGG3’, reverse: 5’ATAATTTTGCCGACTTTTGGC3’), and the *bla*<sub>VIM</sub> gene was detected by PCR with *bla*<sub>VIM</sub>–specific primers (forward: 5’TGGGTGGTCGACTGATCG3’, reverse: 5’GAGCAAGTCTAAGCAGCGGC3’) as described by Aghamiri et al. IMP–15–producing *P. aeruginosa* and VIM–producing *P. aeruginosa* Department of Medical Sciences Thailand 41883 were used as positive controls.
The antimicrobial susceptibilities and the minimum inhibitory concentrations (MICs) of amikacin, gentamicin, aztreonam, ceftazidime, meropenem, ciprofloxacin, colistin, piperacillin/tazobactam, and ticarcillin/clavulanate (Liofilchem, Roseto degli Abruzzi, Italy) were determined using the E-test, and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. P. aeruginosa ATCC 27853 was used as the control strain.

The R software 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for the statistical analysis. The normality test was carried out via the Shapiro–Wilk test. The Wilcoxon rank–sum test was employed for continuous variables, and either Pearson’s chi-squared test or Fisher’s exact test was used to analyze categorical variables where appropriate. A p-value of <0.050 was considered to indicate statistical significance.

Results

Of the 78 IRPA isolates, 20 (25.6%, 95% CI 17.2–36.4%) resulted MBL phenotype-positive using the combined–disk test, and 30 (38.5%, 95% CI 28.5–49.6%) were found to be MBL genotype–positive using the PCR method. The bla_imp and bla_vim genes were detected in 29 (96.7%) and 1 (3.3%) of the MBL-positive isolates, respectively (Table 1 and Table 2). In addition, 27 (90.0%) of the 30 MBL–positive isolates were MDR. The IMP–type isolates were obtained mainly from patients with a history of respiratory tract infection (44.8%), and the majority of these isolates were from patients who were admitted in the Respiratory Care Unit (27.6%).

The susceptibility percentages of amikacin, gentamicin, ceftazidime, meropenem, aztreonam, ciprofloxacin, piperacillin/tazobactam, and ticarcillin/clavulanate, except for that of colistin, against MBL–positive isolates were significantly lower than those against MBL–negative isolates (Table 1).

Of the 30 MBL–positive isolates, colistin showed the highest percentages of susceptibility against MBL–positive isolates (96.7%), and its MIC_50 value was 1.5 µg/mL. Meropenem and ticarcillin/clavulanate showed the highest

Table 1 Antimicrobial susceptibilities of 78 imipenem–resistant Pseudomonas aeruginosa isolates

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
<th>Imipenem–resistant P. aeruginosa (n=78)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBL–positive (n=30)</td>
<td>MBL–negative (n=48)</td>
<td>MBL–positive (n=30)</td>
<td>MBL–negative (n=48)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>32.0</td>
<td>3.0</td>
<td>13 (43.3)</td>
<td>36 (75.0)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>96.0</td>
<td>1.5</td>
<td>4 (13.3)</td>
<td>33 (68.8)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>≥256.0</td>
<td>8.0</td>
<td>4 (13.3)</td>
<td>25 (52.1)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>≥32.0</td>
<td>8.0</td>
<td>1 (3.3)</td>
<td>6 (12.5)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>16.0</td>
<td>12.0</td>
<td>3 (10.0)</td>
<td>23 (47.9)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≥32.0</td>
<td>0.1</td>
<td>4 (13.3)</td>
<td>32 (66.7)</td>
</tr>
<tr>
<td>Colistin</td>
<td>1.5</td>
<td>1.5</td>
<td>29 (96.7)</td>
<td>46 (95.8)</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>32.0</td>
<td>16.0</td>
<td>4 (13.3)</td>
<td>24 (50.0)</td>
</tr>
<tr>
<td>Ticarcillin/clavulanate</td>
<td>≥256.0</td>
<td>≥256.0</td>
<td>1 (3.3)</td>
<td>10 (20.8)</td>
</tr>
</tbody>
</table>

MIC breakpoints of amikacin: susceptible ≤16 µg/mL; aztreonam: susceptible ≤8 µg/mL; ceftazidime: susceptible ≤8 µg/mL; ciprofloxacin: susceptible ≤1 µg/mL; colistin: susceptible ≤2 µg/mL; gentamicin: susceptible ≤4 µg/mL; meropenem: susceptible ≤2 µg/mL; piperacillin/tazobactam: susceptible ≤16/4 µg/mL; and ticarcillin/clavulanate: susceptible ≤16/2 µg/mL (CLSI, 2017).

MIC: minimum inhibitory concentration, difference was considered significant at a p–value of <0.050.
Table 2 Antimicrobial susceptibilities of 30 metallo-β-lactamase-producing imipenem-resistant Pseudomonas aeruginosa isolates

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MBL-positive (n=30)</th>
<th>IMP-type (n=29)</th>
<th>VIM-type (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC₅₀ (µg/mL)</td>
<td>S (%)</td>
<td>MIC (µg/mL)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>32.0</td>
<td>13 (44.8)</td>
<td>96.0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>16.0</td>
<td>4 (13.8)</td>
<td>512.0</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;256.0</td>
<td>4 (13.8)</td>
<td>96.0</td>
</tr>
<tr>
<td>Meropenem</td>
<td>&gt;32.0</td>
<td>1 (3.4)</td>
<td>&gt;32.0</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>16.0</td>
<td>3 (10.3)</td>
<td>16.0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;32.0</td>
<td>4 (13.8)</td>
<td>&gt;32.0</td>
</tr>
<tr>
<td>Colistin</td>
<td>1.5</td>
<td>28 (96.5)</td>
<td>1.0</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>32.0</td>
<td>4 (13.8)</td>
<td>&gt;256.0</td>
</tr>
<tr>
<td>Ticarcillin/clavulanate</td>
<td>&gt;256.0</td>
<td>1 (3.4)</td>
<td>&gt;256.0</td>
</tr>
</tbody>
</table>

MIC breakpoints of amikacin: susceptible ≤16 µg/mL; aztreonam: susceptible ≤8 µg/mL; ceftazidime: susceptible ≤8 µg/mL; ciprofloxacin: susceptible ≤1 µg/mL; colistin: susceptible ≤2 µg/mL; gentamicin: susceptible ≤4 µg/mL; meropenem: susceptible ≤2 µg/mL; piperacillin/tazobactam: susceptible ≤16/4 µg/mL; and ticarcillin/clavulanate: susceptible ≤16/2 µg/mL (CLSI, 2017)

S: susceptible, MIC: minimum inhibitory concentration

percentages of resistance against MBL-positive isolates (96.7 and 96.7%, respectively), and their MIC₅₀ values were >32 µg/mL and >256 µg/mL, respectively (Table 1). Concerning the IMP-type isolates, 28 of 29 isolates (96.5%) were susceptible to colistin (MIC₅₀ =1.5 µg/mL); 1 isolate (3.5%) was non-susceptible to colistin (MIC=3 µg/mL). All other antimicrobials tested were active against ≤44.8% of the 29 IMP-type isolates, and their MIC₅₀ values were higher than the CLSI susceptibility breakpoint (Table 2). In addition, 1 VIM-type isolate exhibited resistance against all antimicrobial agents; the MIC values of all antimicrobial agents were higher than the CLSI susceptibility breakpoint, except for colistin (MIC=1 µg/mL) (Table 2).

Discussion

Our study found that most IRPA isolates from the clinical samples tested were MBL-negative isolates (61.5%), which exhibited a high susceptibility level to colistin, amikacin, gentamicin, ciprofloxacin, ceftazidime, and piperacillin/tazobactam (50.0–95.8% susceptibility rates and low MIC₅₀ values), indicating that these antimicrobial agents could be useful in the treatment of infections caused by MBL-negative organisms.

We found that 38.5% of our 78 IRPA isolates were MBL genotype–positive. Furthermore, we observed that 90.0% of the 30 MBL-positive isolates were MDR and exhibited high percentages of drug resistance to most antimicrobials tested. Only colistin had good activity against MBL-positive isolates (susceptibility=96.7% and MIC₅₀ =1.5 µg/mL). Likewise, Huang et al.¹⁴ found that 24.0% of their 72 IRPA isolates were MBL–positive, and colistin was an effective antimicrobial based on in vitro testing. Acharya et al.⁷ reported that 69.0% of their 35 IRPA isolates were MBL–positive and MDR. In addition, their MBL–positive isolates were highly susceptible to colistin (susceptibility=100% and MICS ≤2 µg/mL).

Our results suggest that MBL–negative isolates remained susceptible to commonly used antimicrobial
agents, whereas MBL-positive isolates were susceptible to only colistin.

A previous study also observed that MBL-positive isolates were significantly more resistant to routinely prescribed antimicrobial agents than were MBL-negative isolates.\textsuperscript{15,16}

In addition, we found that 25.6\% of the 78 IRPA isolates were MBL phenotype-positive using the combined-disk test, whereas 38.5\% were MBL genotype-positive. Based on this finding, the underestimation of MBL prevalence in the clinical setting is a real possibility. Isolates harboring MBL-encoding genes might act as silent reservoirs of such resistance determinants with an ability to spread, since MBL-encoding genes are often carried by mobile genetic elements\textsuperscript{1,2}, and this might lead to a possible therapeutic failure in infections caused by MBLPA isolates. Therefore, the PCR confirmation for the detection of MBL-producing isolates should be pursued.

Regarding MBLIRPA isolates, previous studies have reported their prevalence among clinical isolates from tertiary hospitals to range from 17.0\%–76.0\%.\textsuperscript{6–8} In this study, 30 isolates were MBLIRPA (38.5\%), and the IMP-type isolates were predominant among the MBLIRPAs (96.7\%). Similarly, another study from Thailand by Piyakul et al.\textsuperscript{8} reported that 92.0\% of their 13 MBLIRPA isolates were IMP-type, and the remaining 8.0\% were VIM-type. On the other hand, a study from Nepal by Acharya et al.\textsuperscript{7} reported that 25.0\% of their 24 MBLIRPA isolates were IMP-type, and the rest (75.0\%) were VIM-type. We suspect that the reason for these differences in the prevalence of MBLIRPA isolates may be associated with differences in geographic locations, specimen sources, clinical settings, and broad-spectrum antimicrobial consumption.\textsuperscript{17}

A previous study reported that IMP-type isolates were more commonly isolated from respiratory samples (36.7\%), and that the use of mechanical ventilation was associated significantly with the presence of MBL-positive isolates.\textsuperscript{18} In this study, the IMP-type isolates were obtained mainly from patients with a history of respiratory tract infection (44.8\%), and the majority of those isolates were from patients who were admitted in the Respiratory Care Unit (27.6\%). This finding indicated an alarming spread of IMP-type MBL-positive isolates in the Respiratory Care Unit of our hospital, which makes the implementation of effective infection-control strategies crucial for the prevention of the spread of MBLPAs.

Regarding the IMP-type and VIM-type isolates in this study, we found that 89.6\% of the 29 IMP-type isolates and 1 VIM-type isolate were MDR; all of them exhibited high percentages of resistance to most antimicrobials tested, indicating limited treatment options. These findings show colistin to be a viable therapeutic option for infections caused by IMP-type and VIM-type bacteria. However, colistin has been associated with nephrotoxicity, especially in patients with a history of chronic renal failure, neurotoxicity, and colistin resistance.\textsuperscript{19–21} Therefore, close monitoring and caution are often required during colistin therapy.

Generally, MBLs are susceptible to aztreonam.\textsuperscript{1,2} In this study, 26 (89.6\%) of the 29 IMP-type isolates and the 1 VIM-type isolate were MDR; all of them exhibited high percentages of resistance to most antimicrobials tested, indicating limited treatment options. These findings show colistin to be a viable therapeutic option for infections caused by IMP-type and VIM-type bacteria. However, colistin has been associated with nephrotoxicity, especially in patients with a history of chronic renal failure, neurotoxicity, and colistin resistance.\textsuperscript{19–21} Therefore, close monitoring and caution are often required during colistin therapy.

The limitations of this study were the small number of isolates and lack of clonal relatedness information. Therefore, repetitive clones might have been present. In addition, apart from MBL production, the other resistance
mechanisms associated with IRPA isolates were not evaluated. To clarify these other resistance mechanisms, further investigations involving larger numbers of isolates are needed.

**Conclusion**

Our results showed a high prevalence of MBL-positive isolates (38.5%) in IRPAs, and IMP-type was dominant among the MBLIRPAs tested. The majority of MBL-negative and MBL-positive isolates were susceptible to colistin and had low MIC<sub>50</sub> values.

**Acknowledgement**

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**Conflict of Interest**

No conflicts of interests are declared.

**References**


