Detection of \textit{bla}_{SPM-1} and \textit{bla}_{SIM-2} Metallo-β-Lactamases Genes in Imipenem-Resistant \textit{Pseudomonas aeruginosa} Clinical Isolates Recovered from Two University Hospitals in Egypt

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ABSTRACT

Objectives: Unraveling mechanisms whereby \textit{Pseudomonas aeruginosa} becomes resistant to carbapenems through testing 114 non-duplicate \textit{P. aeruginosa} clinical isolates for their susceptibility to various classes of antibiotics and scrutinizing the production of metallo-β-lactamases (MBLs) by tested isolates. Methods: Susceptibility testing of \textit{P. aeruginosa} to different antibiotics was determined by Kirby-Bauer disk diffusion method and MBLs production by tested isolates was studied phenotypically and genotypically and PCR products were confirmed by sequencing. Results: All tested clinical isolates showed eminent resistance to the majority of tested antibiotics and 14 isolates were imipenem (IPM) resistant. Furthermore, IPM-resistant isolates were verified to be MBLs-producers. MBLs-encoding genes \textit{bla}_{SIM} and \textit{bla}_{SPM} genes were detected by PCR where four isolates were found to harbor \textit{bla}_{SIM} gene while only one isolate harbored \textit{bla}_{SPM} gene. The correct size of PCR products of \textit{bla}_{SIM} and \textit{bla}_{SPM} genes were sequenced and sequences were submitted to the GenBank databases and assigned the accession numbers \texttt{KX452682} and \texttt{KX452683} for \textit{bla}_{SIM-2} and \textit{bla}_{SPM-1}, respectively. Conclusion: Here, we report the emanation of \textit{P. aeruginosa} clinical isolates harboring \textit{bla}_{SPM-1} and \textit{bla}_{SIM-2} genes. This may reflect the substantial increase in the rate of imipenem resistance due to MBL in \textit{P. aeruginosa} clinical isolates from Egypt. Early detection and infection-control practices are of the best antimicrobial strategy for combating this organism.

Keywords: PCR; MDR; Sequencing; Antimicrobial susceptibility tests; Carbapenemase

INTRODUCTION

\textit{Pseudomonas aeruginosa}, a notorious primary opportunistic pathogen, is the leading cause of hospital-acquired infections worldwide and commonly associated with high fatality and morbidity rates¹. Among the most common Gram negative bacteria, \textit{P. aeruginosa} is the most frequently isolated worrisome
pathogen that causes life threatening respiratory tract infection, urinary tract infections in patients admitted to intensive care units2,3. Infections caused by this pathogen are often difficult to be treated because of both its intrinsic resistance and its remarkable ability to acquire further resistance mechanisms to a plethora of antibiotics4,5. Despite improvements in antibiotic therapy, there is still an escalating difficulty in finding new antibiotics and search for alternative approaches to combat drug resistance6,7.

The acquired resistance of P. aeruginosa to beta lactam antibiotics has been attributed to several mechanisms including beta-lactamase production, upregulation of efflux systems and reduced outer membrane permeability7,8. However, acquired extended spectrum beta lactamas (ESBL) and metallo-beta-lactamases (MBLs)-mediated resistance are the most important emerging mechanisms of resistance in P. aeruginosa since carbapenemases often jeopardize chemotherapy for infectious diseases8. Despite the abundant literature on those mechanisms in P. aeruginosa, there is no data regarding the prevalence of such novel MBLs (especially blasIM and blasSPM) harbored by P. aeruginosa in Egypt.

MBLs are particularly worrisome in the clinical setting due to their broad range, robust carbapenemases activity, and resistance to beta-lactamase inhibitors9. Various types of MBLs have been identified so far, including Imipenemase (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), Sao Paolo metallo-beta-lactamase (SPM), Germany imipenemase (GIM), New Delhi metallo-beta-lactamase (NDM) and Florence imipenemase (FIM)10-14.

The ultimate goal of current work was to study the susceptibility of P. aeruginosa clinical isolates (obtained from two University hospitals in Egypt) to the most common clinically-used antibiotics including carbapenems and to investigate the production of MBLs (blasIM and blasSPM) by these isolates.

MATERIAL AND METHODS

Bacterial strains, antibiotic susceptibility testing and MIC determination

Bacterial isolates were recovered from inpatients hospitalized in two main referral hospitals at Tanta and Mansoura universities. A total of 114 consecutive, clinical samples were collected from various sources including urine (n=38), wound swab (n=14), sputum (n=20) and blood (n=42) using proper sampling techniques15,16 and inoculated onto Pseudomonas P agar. Susceptibility of P. aeruginosa to various classes of antibiotics was tested by Kirby-Bauer disk diffusion method as recommended by Clinical Laboratory Standards Institute guidelines (CLSI-2014)17 and were ascertained by automated methods using VITEK 2 Compact System18. The tested antibiotics included imipenem, meropenem, ticarcillin, ticarcillin/clavulanic acid, ciprofloxacin, tobramycin, ceftazidime, piperacillin, piperacillin/tazobactam and colistin.

The MIC values of the aforementioned 10 antimicrobial agents for 114 isolates were determined as described by Clinical and Laboratory Standard Institute-CLSI 17. P. aeruginosa ATCC 27853 was used as the control reference strain.

Molecular detection and sequencing of MBLs genes in P. aeruginosa isolates

The genomic DNA was extracted from selected isolates using Gene JET genomic DNA extraction kit following the manufacturer instructions (Fermentas, #K0721). The extracted DNA was subjected to PCR for the detection the MBLs genes namely, blasSPM and blasIM using specific primer sets targeting those genes as previously described19. The primers sequences used were: blasSPM gene forward primer 5’AAAATCTGGGTACGCAAAAGC3’, reverse primer 5’ACATTATCCGCTGGAAACAGG3’ and blasIM gene forward primer 5’TAC-AAGGGATTCGGCATCG3’, reverse primer 5’TAATGGCCTGTTCATCGTG3’.

The PCR reaction was performed in a final volume of 50 µl comprising 5 µl of DNA template (5-20 ng/µl); 25 µl of PCR master mix; 2.5 µl of forward primer (0.1-0.5 µM); 2.5 µl of reverse primer (0.1-0.5 µM) and 15 µl nuclease-free water and the DNA was amplified using thermal cycler (Applied Biosystem, USA). The amplification was carried out using the following protocol: initial denaturation (95°C, 5 min), followed by 35 cycles of denaturation (94°C, 30s), annealing (52°C, 40s), extension (72°C, 30s) and final cycle of amplification (72°C, 5min). Positive and a negative controls for each PCR were included. The DNA fragments were separated by agarose gel electrophoresis and amplified products were visualized and analyzed under UV light. P. aeruginosa ATCC 27853 was used as the control strain.

Samples were purified using PCR purification kit (Jena Bioscience, # PP-201S, Germany) according to the manufacturer's catalogue20. The purified PCR products were sent to MacroGen Company (South Korea) for sequencing (ABI 3730XL DNA sequencer, Applied Biosystem, USA). The Sequences were analyzed using the ChromasLite 2.1 program (http://technelysium.com.au/?page_id=13) and the identity of the sequenced PCR products were investigated using Blast search against Genbank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignments, annotations and assembly of the sequences were performed using Geneious 4.8.4 software http://www.geneious.com/web/geneious/home.
Statistical analyses
Pearson χ2 or Fisher’s exact tests were performed to compare the number of isolates resistant and sensitive to antibiotics. Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA); P ≤ 0.05 was considered as level of significance for the two-tailed test.

RESULTS

Identification of clinical isolates and antimicrobial susceptibility testing
A total of 114 non-duplicate clinical isolates of P. aeruginosa, collected from different clinical sources, were identified based on their morphological, cultural and biochemical characteristics as well as by automated VITEK2 system. Susceptibility of all tested P. aeruginosa clinical isolates to the 10 tested antibiotics was examined and tested isolates were then classified according to their resistance profile into XDR and MDR. Totally, 43.8% (n=50) of the isolates were MDR, 17.5% (n=20) were XDR and 12.3% (n=14) were Imipenem-resistant (MIC>8mg/L). Interestingly, colistin was the most effective antibiotic (no resistant isolates were detected). MIC values obtained for all tested antibiotics are listed in Table 1 and the prevalence of blaSIM and blaSPM genes among XDR, MDR and IPM-resistant isolates is shown in Table 2.

Molecular detection of MBLs encoding genes

Isolation of Bacterial DNA
DNA was extracted from P. aeruginosa clinical isolates as revealed by gel electrophoresis which showed intact bands indicating pure genomic DNA. The DNA concentration and purity were determined by Nanodrop (uv-vis spectrophotometer Q5000, Quawell, USA) by measuring the absorption at 260 and 280 nm. The ratio of 260/280 and 230/260 ranged between 1.8 and 2.2 confirming the purity of extracted DNA.

Detection of MBLs encoding genes
To confirm phenotypic results, PCR was done to detect MBLs encoding genes. PCR amplification and sequencing confirmed the presence of blaSIM gene in one isolate and blaSPM in 4 isolates out of 114 P. aeruginosa isolates. Of note, one MDR isolate was found to harbour both blaSIM and blaSPM.

The MBLs-encoding blaSIM and blaSPM genes that were detected in the current study were consistent with those of the positive controls with PCR products matching the predicted sizes of blaSIM= 570bp and blaSPM= 271bp. PCR results revealed 4 isolates harbored blaSIM gene and only one isolate harbored blaSPM gene. Data obtained also revealed that blaSIM and blaSPM genes were detected in isolates collected from blood samples only.

Table 1. Antibiogram of tested P. aeruginosa isolates

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No of sensitive isolates</th>
<th>MIC for sensitive isolates (mg/L)</th>
<th>No of resistant isolates</th>
<th>MIC for resistant isolates (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>64</td>
<td>≤1</td>
<td>50</td>
<td>≥8</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>95</td>
<td>≤4</td>
<td>19</td>
<td>≥32</td>
</tr>
<tr>
<td>Imipenem</td>
<td>100</td>
<td>≤1</td>
<td>14</td>
<td>≥16</td>
</tr>
<tr>
<td>Meropenem</td>
<td>105</td>
<td>≤1</td>
<td>9</td>
<td>≥16</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>59</td>
<td>≤4</td>
<td>55</td>
<td>≥64</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>84</td>
<td>≤8</td>
<td>30</td>
<td>≥256</td>
</tr>
<tr>
<td>Ticarcillin/ Clav</td>
<td>84</td>
<td>≤8</td>
<td>30</td>
<td>≥256</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>89</td>
<td>≤4</td>
<td>25</td>
<td>≥256</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>90</td>
<td>≤4</td>
<td>24</td>
<td>≥256</td>
</tr>
<tr>
<td>Colistin</td>
<td>114</td>
<td>≤1</td>
<td>0</td>
<td>-------</td>
</tr>
</tbody>
</table>
Table 2. Prevalence of blaSIM-2 and blaSPM-1 genes among isolates and their related antimicrobial resistance profile

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>blaSIM-2</td>
<td>1/114 (0.8%)</td>
<td>1/20 (5%)</td>
<td>1/8 (12.5%)</td>
<td>0/14 (0%)</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>blaSPM-1</td>
<td>4/114 (3.5 %)</td>
<td>2/20 (10%)</td>
<td>2/8 (25%)</td>
<td>2/14 (14%)</td>
<td>2/6 (33.3%)</td>
</tr>
</tbody>
</table>

IPM; imipenem, MDR; multiple drug resistant and XDR; extensively drug resistant

**Figure 1.** Ethidium bromide stained gel shows PCR products of blaSIM gene with size of 570bp in one positive isolate (lane 1); negative isolates (lanes 2-8); M100 bp marker (ladder).

**Figure 2.** Ethidium bromide stained gel shows PCR products of blaSPM gene with size of 271bp in four positive isolates (lanes 1, 2, 3 and 4); negative isolates (lanes 5 and 6); M 100bp marker (ladder).

DNA sequencing and alignments of MBLs encoding genes

PCR products of MBLs genes [blaSPM and blaSIM] were sequenced in both directions to search for any potential polymorphism among MBLs genes of tested isolates. These sequences were submitted to the GenBank databases and assigned the accession numbers KX452682 and KX452683 for blaSIM-2 and blaSPM-1, respectively. Data analysis of these sequences showed 100% identity of nucleotide sequences of MBLs encoding genes among tested Egyptian isolates (see supplementary data).

**DISCUSSION**

*P. aeruginosa* is a notorious pathogen that poses a serious problem to the human health sector. This organism is mainly associated with multiple dangerous disorders including ventilator-associated pneumonia, urinary tract infections, meningitis, cystic fibrosis, catheter-associated infections, corneal infections and soft tissue infections.

The emanation of MBLs-mediated carbapenems resistance in *P. aeruginosa* and dissemination of carbapenemases via horizontal gene transfer represent a major rising public health hazard. Globally, there is a substantial increase in MBLs production by carbapenem-resistant *P. aeruginosa*. In recent years, Egypt was among the countries that reported high rates of antimicrobial resistance, however, data regarding the prevalence of MBLs (blaSIM and blaSPM) production by *P. aeruginosa* isolates in Egypt is so limited. Therefore, this work aimed at studying the prevalence of MBLs-producing *P. aeruginosa* clinical isolates from Egypt. Investigating the MBLs production by *P. aeruginosa* is a crucial step towards large scale monitoring of these emerging resistant determinants and could pave the way towards finding new interventions to the threatening problem of massive emergence of MDR pathogens.

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Although comparison between studies is difficult since the patient populations of the centers and the methods of studying differ, here we reported 14 IPM-resistant \textit{P. aeruginosa} (12\%) which was higher (5.7\%) than that was previously published in Iran\textsuperscript{26}. On contrary, another study from Iran reported a higher prevalence percentage (51.4\%) of IPM-resistant \textit{P. aeruginosa}\textsuperscript{37}.

Colistin belongs to the polymyxin family and has a wide range of activity against most clinically-relevant Gram-negative bacteria. Colistin is currently used for clinical application and represents the mainstay for treatment of carbapenem-resistant \textit{P. aeruginosa}. Based on the breakpoint reported by CLSI\textsuperscript{28} for colistin against \textit{P. aeruginosa}, a susceptible breakpoint of \(\leq 2\)mg/L and a resistant breakpoint of \(\geq 4\)mg/L were applied to all isolates. Interestingly, colistin was the most effective antimicrobial agent in study since all of our tested isolates \((n=114)\) were found to be sensitive to colistin.

Various MBLs have previously been reported in Egypt including \textit{bla}NDM-1 in Gram negative bacteria\textsuperscript{39}, \textit{bla}NDM-2 in \textit{Acinetobacter baumannii}\textsuperscript{30}, \textit{bla}OXA-48 and \textit{bla}VIM-1 in Enterobacteriaceae\textsuperscript{31}, \textit{bla}OXA-48 and \textit{bla}NDM-1 in \textit{Klebsiella pneumoniae}\textsuperscript{32}, \textit{bla}VIM-4 in \textit{Enterobacter cloacae}\textsuperscript{33}, \textit{bla}NDM-1 in \textit{P. aeruginosa} \textsuperscript{25}, \textit{bla}NDM-1 in \textit{Klebsiella pneumoniae}\textsuperscript{34,35}, \textit{bla}GIM in \textit{P. aeruginosa}\textsuperscript{46}, \textit{bla}VIM-2 in \textit{P. aeruginosa}\textsuperscript{24}, \textit{bla}VIM-28 in \textit{P. aeruginosa}\textsuperscript{37}, \textit{bla}SIM and \textit{bla}VIM in \textit{P. aeruginosa}\textsuperscript{38} and \textit{bla}GGM in \textit{Acinetobacter baumannii}\textsuperscript{39}. Very recently, metallo-beta-lactamas producing \textit{Pseudomonas aeruginosa} were isolated from hospitals in Minia, Egypt\textsuperscript{49}. Furthermore, a recent work from our laboratory has shown the emergence of \textit{P. aeruginosa} clinical isolates that coproduce \textit{bla}VIM and \textit{bla}SIM carbapenemases\textsuperscript{41}. Moreover, we have recently reported the emergence of Imipenem-resistant \textit{Pseudomonas aeruginosa} clinical isolates from Egypt that harbor \textit{bla}NDM-1 gene\textsuperscript{42}.

The MBL gene \textit{bla}SIM was first identified in \textit{Acinetobacter baumannii}\textsuperscript{43}. Our data showed the emergence of one \textit{bla}SIM-2-producing \textit{P. aeruginosa} clinical isolate out of 114 total isolates with a prevalence of (0.8\%). As per literature, \textit{bla}SIM-producing \textit{P. aeruginosa} was previously reported in China\textsuperscript{44}. Further investigations are required to identify possible reasons whereby \textit{bla}SIM gene was transferred to Egypt. Sequence analysis showed 100\% identity with the \textit{bla}SIM gene of \textit{P. aeruginosa} (accession number KX452683).

Interestingly, the DNA sequence analysis of our \textit{bla}SPM and \textit{bla}SIM showed 100\% identity with the sequence of global genotypes. The results, further confirmed that, MBL genes might have been evolved from the same clone and can spread to another population by selective pressure of antibiotic which is described in that local region.

Therefore, the periodic investigation of antibiotic resistance profiles and the resistance genes among clinical \textit{P. aeruginosa} strains in Egypt is so crucial to gauge the level of activity among commonly prescribed antipseudomonal drugs. Moreover, local epidemiology patterns need to be actively evaluated as the majority of endemic cases usually occurs through the clonal dissemination of notorious MBL-producing clones.

**CONCLUSION**

Altogether, the present study reports the emanation of \textit{bla}SIM-2- and \textit{bla}SPM-1-producing \textit{P. aeruginosa} in Egypt which raises the concern about its potential spread to other pathogens infecting hospitalized patients.

**Conflict of interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.

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