Antibiotic resistance and presence of $bla_{PER-1}$, $bla_{VEB-1}$, and $bla_{PSE-1}$ beta-lacamases among clinical isolates of Pseudomonas aeruginosa from ICU settings

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Introduction: Pseudomonas aeruginosa isolates are among the most common pathogens causing nosocomial infections. They are intrinsically resistant to most of antibiotics such as novel β-lactams and therefore, can develop resistance during treatment, culminating in failure in remedy.

Objectives: The aim of this study was to detect the genes encoding class A extended-spectrum beta-lactamases (ESBLs) such as $PER-1$, $VEB-1$ and $PSE-1$ among $P$. aeruginosa isolates from intensive care unit (ICU) patients.

Materials and Methods: A total of 65 isolates were collected from ICU in three hospitals of Tehran in 2016. The antibiotic susceptibility test was conducted according to Clinical and Laboratory Standards Institute (CLSI) guideline. MIC of ceftriaxone was done with agar dilution method. The combine disk test was performed for detection of isolates producing ESBLs. Polymerase chain reaction (PCR) was performed to detect the $PER-1$, $VEB-1$ and $PSE-1$ genes using specific primers.

Results: Fifty-four percent (n=38) of patients were male and 46% (n=27) were female. The majority of ICU isolates were resistant to augmentin (93.8%, n=61) and cefpodoxime (84.8%, n=56). Fifty (77%) isolates were ESBL positive, among which 94% (n=47) harbored $PER-1$ gene followed by 92% (n=26) $VEB-1$ and 16% (n=8) $PSE-1$ genes.

Conclusion: Concomitance presence of $bla_{PSE1}$ and $bla_{VEB1}$ was observed among 10 isolates, and 7 amplified all these three genes. A high number of ICU $P$. aeruginosa isolates were ESBL producers. The frequency of $bla_{PSE1}$ and $bla_{VEB1}$ were relatively high, while $bla_{PER1}$ was detected among a low number of isolates. Moreover, resistance to carbapenemes was low. It is necessary to follow up ICU centers because of drug-resistant $P$. aeruginosa isolates.

Key point
Concomitance presence of blaPER1 and blaVEB1 was observed among $P$. aeruginosa isolates. A high number of ICU $P$. aeruginosa isolates were ESBL producers. The frequency of $bla_{PSE1}$ and $bla_{VEB1}$ were relatively high, while $bla_{PER1}$ was detected among a low number of isolates. The frequency of carbapenem resistance was low.
However, the increasing use of these compounds has culminated in the emergence of carbapenem-resistant *P. aeruginosa* clinical isolates, limiting treatment choices. Detection of isolates producing ESBLs in the clinical laboratory is an important issue for guidance of proper therapeutic schemes and the implementation of the infection control measures. The combined or synergistic methods are phenotypic routes of ESBLs detection (9,10). In *P. aeruginosa* several classes of enzymes such as class A ESBLs have been identified including bla<sub>PER</sub> (mostly from Turkey), bla<sub>VEB</sub> (from South-East Asia, France and Bulgaria), bla<sub>GES/IBC</sub> (France, Greece and South Africa) and other less common types. These six carbapenemase types have low similarity at the genetic level, and yet they have identical hydrolysis profiles. The bla<sub>PER</sub> occurs less frequently, but has clinical importance by conferring resistance to oxy-imino-beta-lactams (11,12). Unfortunately, several antibiotic resistance mechanisms often have been occurred simultaneously, thereby conferring multidrug-resistant phenotypes.

**Objectives**

The aim of this study was to detect the genes encoding class A ESBLs of PER-1, VEB-1 and PSE-1 among clinical isolates of *P. aeruginosa* in ICU patients.

**Materials and Methods**

**Bacterial isolates**

A total of 65 isolates of *P. aeruginosa* were collected from intensive care unit (ICU) patients in several hospitals of Tehran between the years 2015 and 2016. Tests and culture media for identification of these isolates included: catalase and oxidase tests, hydrogen sulfide (H₂S), indol, motility (SIM medium), triple sugar iron agar (TSI), and methyl red (MR), Voges-Proskauer (VP), Simon citrate, urease, oxidative/fermentative (OF), Macconkey agar and cetrimide agar.

**Antibiotic susceptibility test**

The antibiotic susceptibility test was conducted according to the CLSI guidelines. The antibiotic disks used included: aztreonam (30 µg), augmentin (30 µg), amikacin (30 µg), piperacillin (100 µg), ofloxacin (5 µg), ceftriaxone (30 µg), cefoperazone (75 µg), cefotaxime (30 µg), cefpodoxime (10 µg), ciprofloxacin (5 µg), carbenicillin (100 µg), levofloxacin (5 µg), meropenem (10 µg), netilmicin (30 µg), piperacillin-tazobactam (110 µg), imipenem (10 µg), tobramycin (10 µg), ticarcillin (75 µg), gentamicin (120 µg), ceftazidine (30 µg) and cefepime (30 µg).

**Phenotypic detection of ESBL producers**

The combine disk test was performed to detect the isolates producing ESBLs. The ceftazidime and cefotaxime disks with or without clavulanic acid were used in this test. A difference of more than 5 mm between these disks along with clavulanic acid was considered as positive.

**DNA extraction**

One colony of each isolate was suspended in 10 mL Lauria Bertani (LB) broth and incubated an overnight at 37°C. The tubes were centrifuged at 4000 rpm for 10 minutes and the precipitate was used for extraction. DNA was isolated by a boiling method and DNA Extraction kit (DIAtom DNA Prep 100).

**Polymerase chain reaction**

Polymerase chain reaction (PCR) was performed to detect the PER-1, VEB-1 and PSE-1 encoding genes with specific primers (Table 1).

The reaction mixture for these genes was: 10XPCR buffer = 2.5 µL, MgCl₂ (50 mM) = 1.5 µL, dNTP (10 Mm) = 0.75 µL, forward primer (100 µM) = 2.5 µL, reverse primer (100 µM) = 2.5 µL, Taq DNA polymerase (5 U/µL) = 0.2 µL, Template (DNA) = 1 µL and nuclease-free H₂O = 14.05 µL.

**Ethical issues**

The research followed the tenets of the Declaration of Helsinki. This study was approved by AJA University of Medical Sciences, Tehran, Iran (Grant # 9504).

**Data analysis**

Data were analyzed using GraphPad Prism 6 and SPSS 20. The Student’s t-test was applied for analysis, and *P* value of < 0.05 was considered as the significance of differences.

**Results**

Of the total 65 isolates, 46.1% (*n=30*) were collected from urine, followed by 15.9% (*n=11*) in pneumonia, 10.8% in blood and other clinical sites. Fifty-four percent (*n=38*) of patients were male and 46% (*n=27*) were female.

**Antibiotic susceptibility test**

The majority of the isolates were resistant to augmentin (93.8%, *n=61*) and cefpodoxime (84.8%, *n=56*). The frequency of resistance to other antibiotics were as follows: CAZ (60%, *n=39*), CPM (47.6%, *n=31*), PRL (44.6, *n=29*),

**Table 1. The specific primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 3’ to 5’</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaPER-1</td>
<td>F: ATG AAT GTC ATT ATA AAA GCT  R: TTA ATT TCG GCT TAG TG</td>
<td>927</td>
<td>1,12</td>
</tr>
<tr>
<td></td>
<td>F: AAAGCCAATCAGCGCTTC  R: GCCGACGTGATGATTA</td>
<td>699</td>
<td>9,12</td>
</tr>
<tr>
<td>blaPSE-1</td>
<td>F: CGA CTC CCA TTT CCC GAT GC  R: CGA CTC TGC AAC AAA TAC GC</td>
<td>624</td>
<td>9,12</td>
</tr>
</tbody>
</table>
CRO (64.6%, n=42), PY (53.8%, n=35), ATM (57%, n=37), CPZ (46.1%, n=30), CTX (58.4%, n=38), TC (55.3%, n=36), IMI (18.4%, n=12), MEM (20%, n=13), CIP (43.07%, n=28), OFX (26.1%, n=17), LEV (49.2%, n=32), NET (38.4%, n=25), GEN (36.9%, n=24), TN (46.1%, n=30) and AK (41.5%, n=27).

Phenotypic detection of ESBL producers
Fifty (77%) isolates of 
*P. aeruginosa* were ESBL positive (Table 2). The majority of the ESBL positive isolates belonged to urine infection (61%, n=40), followed by wound (23%, n=15) and sputum (15.3%, n=10).

Detection of VEB-1, PER-1 and PSE-1 encoding genes
Fifty (77%) isolates were ESBL positive, among which 94% (n=47) harbored *bla*<sub>PER-1</sub> gene followed by 52% (n=26) *bla*<sub>VEB-1</sub> and 16% (n=8) *bla*<sub>PSE-1</sub> genes (Figures 1 and 2). Concomitance presence of *bla*<sub>PER-1</sub> and *bla*<sub>VEB-1</sub> was observed among 10 isolates, and 7 amplified all these three genes (Table 2). There was a relationship between the presence of these genes and resistance to ceftazidime, ceftriaxone, cefpodoxime and cefotaxime.

Discussion
*Pseudomonas aeruginosa* is a prevalent nosocomial pathogen that causes a wide spectrum of opportunistic and nosocomial infections. Isolates of *P. aeruginosa* are resistant to a variety of antibiotics with mechanisms including intrinsic capabilities and acquired (chromosome or plasmid-mediated) resistance. In the present study, most of isolates were collected from urinary tract infections. Several other previous studies confirm our results (12,13). The urine is the main route for antibiotic excretion from the body and thus, most of resistant isolates were collected from this site. In this study, the majority of the isolates were resistant to augmentin/co-amoxiclav and cefpodoxime. Moreover, most of them were resistant to third generation cephalosporins (aztreonam, ceftriaxone and cefotaxime). These antibiotics are important in the treatment of gram-negative rods, and thus the increasing resistance is a great concern. About 0.3% of the total genes in *P. aeruginosa* are responsible for antibiotic resistance (14). Approximately 77% of ICU isolates were ESBL producers which is comparable with previous studies (15,16). Susceptibility to clavulanic acid is a diagnostic method. Resistance to clavulanic acid indicates the probable presence of AmpC and other enzymes that are not inhibited by this inhibitor (17). On the other hand, de-repression of the chromosomal AmpC β-lactamases can culminate in the decreased susceptibility to β-lactam antibiotics.

Table 2. The relationships of third generation cephalosporins resistance, MIC and the concomitance presence of beta-lactamase genes among ESBL positive isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sex</th>
<th>MIC</th>
<th>Third generation cephalosporins resistance</th>
<th>Concomitance genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>4</td>
<td>CTX, CAZ, CRO, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>4</td>
<td>CTX, CAZ, CRO</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>8</td>
<td>CTX, CAZ, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>8</td>
<td>CTX, CAZ, CRO, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>8</td>
<td>CTX, CAZ, CRO, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>16</td>
<td>CTX, CRO, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>16</td>
<td>CTX, CRO, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>32</td>
<td>CTX, CRO, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>32</td>
<td>CTX, CAZ, CRO, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>32</td>
<td>CTX, CRO, CPM</td>
<td><em>bla</em>&lt;sub&gt;PSE-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;PER-1&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;VEB-1&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

In this study, among ESBL producers, 94% and 52% amplified bla\textsubscript{PER1} and bla\textsubscript{VER1}, respectively, suggesting the presence of other enzymes such as AmpC and MBLs or possibility of efflux pumps interference. In the study by Shacheraghi et al in ESBL positive isolates, the prevalence of bla\textsubscript{VER1} and bla\textsubscript{PER1} was 100% and 68.3%, respectively (18), but a low number (5.8%) of imipenem-resistant isolates contained bla\textsubscript{VER1} in another study. In a study by Davodian et al, among wound isolates the 90% and 88% were resistant to augmentin and cefpodoxime, 40% of them being bla\textsubscript{VER} positive (15). Besides this, in our study, resistance to imipenem and meropenem was low (18.4 and 20%, respectively), suggesting that the low presence of carbapenem-hydrorylizing enzymes in addition to class A enzymes. However, continuous and prolonged antibiotic periods are likely to be a critical factor in the selection of highly antibiotic-resistant \textit{P. aeruginosa} strains. Therefore, combination therapy (usually with a β-lactam and an aminoglycoside) is now important to eradicate infections due to \textit{P. aeruginosa}. The broad-spectrum carbapenems are efficient drugs for the eradication of infections caused by \textit{P. aeruginosa}; however their efficacy is increasingly compromised due to the emergence and the worldwide dissemination of carbapenem-resistant strains. Fortunately, in our study the resistance to carbapenems was low.

A high number isolates of \textit{P. aeruginosa} from ICU were ESBL producers. The frequency of bla\textsubscript{VER} and bla\textsubscript{PER} were relatively high, while bla\textsubscript{PER1} was detected among a low number of isolates. ICU wards are of main sources for infections with drug-resistant strains. Continuous and prolonged antibiotic periods, hospitalization and misuse are pivotal factors in the selection of highly resistant strains. Combination therapy (usually with a β-lactam and an aminoglycoside) is important to treat Pseudomonas infections.

Authors' contribution
AG, SKSM and ME performed the study. FN supervised the work. MV and FH participated in data analysis.

Conflicts of interest
There is no conflict of interest regarding the publication of this paper.

Ethical considerations
Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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References