The frequency of bla Verona imipenemase and bla imipenemase genes in clinical isolates of *Pseudomonas aeruginosa* in therapeutic centers of Zahedan

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Abstract

**Introduction:** Pseudomonads are very important as an opportunistic bacterium among gram-negative bacteria. Class B beta-lactamases such as Verona imipenemase (VIM) and Imipenemase (IMP) are the most important contributors to the resistance of gram-negative bacteria like *Pseudomonas aeruginosa*. These enzymes were isolated from burn wounds in Zahedan due to extended spectrum beta-lactamase (ESBL) activity and high resistance to beta-lactamase antibiotics (IMP and VIM) in *P. aeruginosa* isolates.

**Objectives:** This study was aimed to determine the frequency of VIM, IMP beta-lactamase genes in clinical isolates of *P. aeruginosa* isolated from therapeutic centers of Zahedan.

**Materials and Methods:** In this descriptive cross-sectional study, 125 samples of *P. aeruginosa* from burn wounds were collected from patients who were hospitalized in Zahedan as well as were cultured. Suspected colonies of *P. aeruginosa* were identified using common biochemical methods. The Kirby Bayer method was used to measure antibiotic susceptibility, and then the minimum inhibitory concentration (MIC) of these strains was examined. The genotypes of the beta-lactamase genes of IMP and VIM were investigated in polymerase chain reaction (PCR) strains.

**Results:** In this study, 125 isolates of *P. aeruginosa* were found in 32 isolates (26%) producing ESBL. In general, the resistance of *P. aeruginosa* to the antibiotics of imipenem, piperacillin, ceftazidime, cefotaxime, ceftriaxone, levofloxacin, ciprofloxacin, gentamicin, amikacin, streptomycin, tetracycline, and carbencillin were 88%, 91%, 89%, 98%, 97%, 89%, 84%, 82%, 97%, 91%, 90% and 95%, respectively. According to the PCR test, 32 isolates with ESBL enzymes were 43.75 and 18.75, respectively.

**Conclusion:** The prevalence of ESBLs and antibiotic resistance in the burn injuries of hospital is high and requires such as antibiotic susceptibility test, rational administration of antibiotics and the control of beneficial factors. The results showed that most of the samples were resistant to the drug and the frequency of blaVIM genes was higher than blaIMP among the strains producing ESBLs.

**Key point**

Resistance to antibiotics used in the treatment of patients with infections caused by *Pseudomonas aeruginosa*, especially cefalosporin, is increasing in our country, Iran.
major mechanism of resistance to beta-lactam antibiotics. According to the Ambler classification, β-lactamases are divided into four groups; Groups A, B, C and D, which groups A, C, D are serine-beta lactamases. Class B beta-lactamases are categorized as Ambler metal-beta-lactamase (4). Metal-beta-lactamase enzymes are divided into six groups based on the molecular structure including SPM (Sao Paulo metallo-beta-lactamases), NDM (New Delhi metallo-beta-lactamase), VIM (Verona integron-encoded metallo-beta-lactamase), GIM (German imipenemase), AIM (Adelaide imipenemase) and IPM (imipenem) (5). Class B beta-lactamases including Verona imipenemase (VIM), imipenemase (IMP) and SPM are major problems in the treatment of infectious diseases due to its effects on a wide range of antibiotics including penicillin, expanded-spectrum cephalosporin and carbapenem (exception of monobactams such as aztreonam). Several families of metal-beta-lactamase have been identified among P. aeruginosa strains such as AIM, IMP, VIM, SPM, GIM, SIM, NDM, and KHM (6). VIM and IMP enzymes are the most common metabolic lactamase enzymes found worldwide (7).

Antibacterial resistance in hospital infections is very important. According to data of the Centers for Disease Control and Prevention (CDC), more than 70% of hospital pathogen strains are resistant to at least one common antibiotic in the treatment of infections, and this often results in the use of antibiotics of the second and third choice. In a general condition, 15% of the strains of P. aeruginosa are resistant to ceftazidime in ICU, and ampicillin is an effective drug for the treatment of severe infections of this bacterium (8,9). During 5 years (1997-2001), resistance of P. aeruginosa to imipenem, fluoroquinolones and third generation cephalosporin antibodies has been increased to 32%, 37%, 22% and 28%, respectively (10,11). Regarding the importance of the beta-lactamases in hospitals, rapid detection and tracing of these strains can be considered as an important step in the treatment and control of infections caused by these strains.

Objectives
This study was aimed to determine the frequency of VIM, IMP beta-lactamase genes in clinical isolates of P. aeruginosa isolated from therapeutic centers of Zahedan.

Methods and Materials
Study design
This was a descriptive cross-sectional study. Sampling method was convenience. The studied population of P. aeruginosa genus was isolated from burn wounds of patients hospitalized in the burn injuries section of Zahedan hospitals. A total of 125 clinical isolates of P. aeruginosa were isolated from various clinical specimens in this study. According to the formula for calculating sample size in descriptive studies, 125 sample was obtained.

Isolation and determination of Pseudomonas aeruginosa
During 6 months, referring to educational hospitals in Zahedan, the susceptible isolates of P. aeruginosa were isolated from burn wound specimens. After completing the questionnaire, the isolates were transferred to the Microbiology Laboratory of Islamic Azad University of Zahedan and after re-cultivation on MacConkey agar culture and incubation at 35°C for 24 hours, isolates were tested using standard biochemical tests such as oxidase, culture on the TSI, Simmons citrate agar, examination of movement in the SIM culture, of culture. P. aeruginosa is gram-negative, non-fermented lactose, positive and mobile oxidase (12,13).

Determination of antibiotic sensitivity pattern of P. aeruginosa isolates using disc diffusion method
Purely freeze colonies in 20% BHI-Glycerol were cultured overnight on a MacConkey agar and compared to fresh colonies into the inoculum sterile physiologic serum and compared to the half-McFarland standard to maintain a microbial suspension (with opacity equivalent to the half-McFarland tube). Sterile cotton swabs were immersed in a tube containing bacteria suspension, and after several turns using the wall, the swab impregnated with bacteria swirled in three directions at 60-degree angle on the surface of the Müller-Hinton agar and was cultured. Finally, the plates were transferred to 35°C incubator as upside down and the results were evaluated after 16-18 hours. The results were measured in millimeters using ruler based on the halo diameter of non-growth and reported as sensitive, semi-sensitive and resistant according to the Clinical and Laboratory Standards Institute (CLSI) standard criteria (14). ATCC 27853 was used to control the quality of P. aeruginosa strain.

Test of phenotypic confirmation of expanded-spectrum beta-lactamases by disk combination
To confirm the production of the extended spectrum beta-lactamases (ESBLs), the ESBLs were synthesized using a combination cefotaxime (30 µg), cefotaxime-clavulanic acid (10-30 µg) and ceftazidime (30 µg), ceftazidime-clavulanic acid discs (10-30 µg) (MAST, UK). The isolates that were resistant to at least one third-generation cephalosporin were selected to validate the ESBLs combination. Bacterial suspensions (with opacity equivalent to the half-McFarland tube) were inoculated into the Müller-Hinton agar by sterile swabs, and the cefotaxime, cefotaxime-clavulanic acid, ceftazidime, ceftazidime-clavulanic acid discs were placed on the culture environment. The results were read after 18-24 hours of incubation at 35°C, thus, if the diameter of the halo zone around the disc of cefotaxime-clavulanic acid or the ceftazidime-clavulanic acid disc was 5 mm or more in relation to the cefotaxime or ceftazidime disc, it was considered as the isolate producing the ESBL. ATCC 35218
was used to control the standard strain of Escherichia coli (15).

**Test for determining the minimum inhibitory concentration**
The newly cultured colonies (18 to 24-hour culture) were inoculated on a multicellular MacConkey agar culture into a tube of sterile physiology serum contents. Bacterial suspensions, inoculated with Müller-Hinton agar culture, were performed as disc diffusion method.

To perform the minimum inhibitory concentration (MIC), a sterile 64 microplates (64 samples per plate) was used. To prevent contamination, the test was performed under the hood or in the space between the two flames. Each microplate can be used to determine the 8 isolate of MIC. There were 12 wells in each row. From each of 10 dilutions prepared for each antibiotic, 100 μL per well was poured (serial dilution). Then, 5 μL dilution was added to each well (1×10⁶ CFU/mL). In each row, the last two wells were assigned to positive and negative controls. In one of the wells, 100 μL of the bacterial dilutions was prepared (positive control) and in other wells, about 100 μL of sterile Müller-Hinton broth culture was poured (negative control). Finally, the microplate was placed in an incubator at 37°C for 18-20 hours. In the descending serial dilution (left to right), the first well of a sample that does not have a growth (opacity) is the MIC of that sample. Therefore, subsequent wells should grow and others shouldn’t grow (16).

**Gene multiplication by polymerase chain reaction**
The proliferation of bla IMP and bla VIM genes was performed in a simple way using polymerase chain reaction (PCR). In each serial, in proportion to the samples, 0.2 mL sterile microtube was taken and numbered. The required materials for each multiplication reaction were dispersed in each microtube and the DNA was finally added to produce a final volume of 20 μL for each reaction.

**Ethical considerations**
This research performed based on the Declaration of Helsinki principles.

**Statistical analysis**
Statistical analysis was performed by SPSS version 21. Frequencies and percentages were done for the categorical variables, and measures of central tendency were calculated for the continuous variables. Chi-square was used for the bivariate analysis. P value <0.05 was considered statistically significant

**Results**
According to the results, of a total 125 samples of P. aeruginosa, 73 samples (58.4%) were related to the female population and 52 samples (41.6%) were related to the male population.

**Antimicrobial sensitivity measurement**
Antibiotic sensitivity measurement was performed on 125 isolates of P. aeruginosa. Total resistance of P. aeruginosa isolates to the imipenem, piperacillin, ceftazidime, cefotaxime, ceftriaxone, levofloxacin, ciprofloxacin, gentamicin, amikacin, streptomycin, tetracycline and carbenicillin antibiotics were 88%, 93%, 89%, 98%, 97%, 89%, 84%, 82%, 97%, 91%, 90% and 95%, respectively.

**Determine the minimum inhibitory concentration**
The MIC of antibiotic has been done for 125 isolates of P. aeruginosa using Broth microdilution and based on CLSI guidelines (17). MIC results of P. aeruginosa for the antibiotics of imipenem, piperacillin, ceftazidime, cefotaxime, ceftriaxone, levofloxacin, ciprofloxacin, gentamicin, amikacin, streptomycin, tetracycline, and carbenicillin were reported as numbers in μg/ml and these numbers were compared by the above antibiotics in P. aeruginosa strains with CLSI criteria (Table 1).

**bla IMP and bla VIM genotypes producing expanded-spectrum beta-lactamase enzymes in P. aeruginosa isolates**
As shown in Figure 1, the frequency distribution of bla IMP and bla VIM genes in P. aeruginosa isolates is 18.75 and 43.75, respectively.

**Discussion**
Burn infections as a hospital infection are a major contributor to mortality and disability. P. aeruginosa is a non-fermentative gram-negative bacterium that plays a major role in the development of opportunistic infections and severe infections in burn patients. P. aeruginosa also accounts for 10% to 15% of all hospital infections in the world. Based on the epidemiological studies conducted around the world, it has been proven that the prevalence of various drug resistance patterns in P. aeruginosa strains,

<table>
<thead>
<tr>
<th>Antibiotic (μg)</th>
<th>Sensitive No. (%)</th>
<th>Resistant No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin</td>
<td>91 (75.8)</td>
<td>15 (12.5)</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>82 (68.3)</td>
<td>29 (24.1)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>83 (69.1)</td>
<td>26 (21.6)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>71 (59.1)</td>
<td>38 (31.6)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>68 (56.6)</td>
<td>40 (33.3)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>72 (60)</td>
<td>28 (23.3)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>97 (80.8)</td>
<td>6 (5)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>102 (85)</td>
<td>5 (4.1)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>65 (52)</td>
<td>7 (5.6)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>68 (54.4)</td>
<td>8 (6.4)</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>68 (54.4)</td>
<td>11 (8.8)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>78 (62.4)</td>
<td>25 (20)</td>
</tr>
</tbody>
</table>
from one country to another, from a geographic region to another geographic region, and even between different hospitals, can be different. Therefore, due to the clinical importance of strains produced with multiple resistance to drugs in different hospitals, it is essential to identify these strains, for treatment purposes and for controlling their further release in hospitals.

Due to its genetic nature,  

*P. aeruginosa* has a broad range of antibiotic resistance and can be rapidly resistant to antibiotics. Uncontrolled use of antibiotics, especially fluoroquinolones and carbapenem is one of the risk factors for the resistance of these bacteria to these drugs (18,19). Therefore, due to the high ability of this organism to be resistant to different antibiotics, continuous monitoring of the sensitivity of this bacterium is necessary (20,21). In this study, the frequency of *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes in producing ESBL in  

*P. aeruginosa* isolates and the determination of antibiotic resistance patterns in burn wound samples were studied.

In this study, a total of 125 isolates of  

*P. aeruginosa* isolated from burn wounds were found to be resistant to antibiotics such as imipenem, piperacillin, ceftazidime, cefotaxime, ceftriaxone, levofloxacin, ciprofloxacin, gentamicin, amikacin, streptomycin, tetracycline, Carbenicillin (88%, 93%, 89%, 98%, 97%, 89%, 84%, 82%, 97%, 91%, 90% and 95%, respectively).

In the study of Mirsalehian et al in Tehran, which conducted on 170 isolates of  

*P. aeruginosa* isolated from burn wounds, they were most resistant to aminoglycoside antibiotics such as amikacin (81%), gentamicin (88%), tobramycin (84%) and imipenem (52.9%) (22); whereas in the present study, 88% of isolates were resistant to ampicillin and 93% to meropenem, which could indicate an increase in resistance to carbapenem.

In a study done by Owlia et al (23) in Tehran, 100 isolates of  

*P. aeruginosa* from burn wound was investigated. Resistance to amikacin, gentamicin, cefotaxime, cefazidime, and ciprofloxacin and also ceftriaxone antibiotics was 95%, 96%, 81%, 95%, 89% and 92%, respectively, which was consistent with the present study. In the study of Ullah et al (24) in Pakistan, 106 isolates of  

*P. aeruginosa* were isolated from burn wounds. Resistance to amikacin, gentamicin, ciprofloxacin, doxycycline antibiotics was 70%, 25%, 49% and 21%, respectively (24).

In the study of Akhavan-Tafti et al that was conducted on 54 isolates of  

*P. aeruginosa* at a burn injuries hospital in Yazd, antibiotics resistance to ceftizoxime, imipenem, gentamicin, piperacillin, cefepime, meropenem, and ertapenem were reported as 79%, 74%, 74%, 70%, 66% and 62%, respectively (25).

Analysis of the results of the resistance pattern of the isolates obtained in this study showed the resistance of the strains to the antibiotics of interest. With a glimpse into previous studies, it can be concluded that resistance to different antibiotics was relatively high for  

*P. aeruginosa* isolates, which were different regarding time and location of isolation of strains. On the other hand, these resistance patterns were constantly changing, which should be taken into consideration. In this study, there were 125 isolates of  

*P. aeruginosa* isolated from burn wounds that 32% of them had been produced ESBLs. In the study of Shojapour et al in Shahrekord (26), which samples were isolated from  

*P. aeruginosa* in patients with burn wounds, 37% of samples had been produced ESBLs.

**Conclusion**

According to the results of this study, resistance to antibiotics used in the treatment of patients with infections caused by  

*P. aeruginosa*, especially cephalosporin, is increasing in our country, Iran. In this study, more than 80% of  

*P. aeruginosa* isolates were resistant to ceftazidime, cefepime, ceftriaxone, cefotaxime and tetracycline and the frequency of ESBLs producing  

*P. aeruginosa* was 32%, which indicates an increase in the prevalence of these resistance enzymes. Therefore, in burn wounds, antibiotic susceptibility test and ESBLs producing strains of  

*P. aeruginosa* strains are necessary before administration of the drug. In the present study, the emergence of *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes was reported to indicate high *bla*<sub>VIM</sub> gene compared to *bla*<sub>IMP</sub> gene.
Suggestions
1. Due to the prevalence of several antibiotic resistance isolates in patients with burn wounds, diagnosis and accurate determination of the antibiotic susceptibility pattern of these strains should be done at the early hospitalization times.
2. Since the bacterial drug resistance is constantly changing especially the bacteria that produce hospital infections, therefore it is better to conduct further studies on microorganisms and their drug resistance annually to be used by doctors as a guideline in treating patients.

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Authors' contribution
RGDM; participated in research design, writing of the paper and the performance of the research. JD; contributed to the study design, preparation of manuscript and final revision. NSR; consultant of the performance of the research. RGDM; participated in research design, the writing of the paper andAuthor's contribution

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

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