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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 β-lactamase antibodies (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 β-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 carbenicillin were 88%, 93%, 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.

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Introduction

Pseudomonas aeruginosa is an opportunistic germ-negative pathogen of hospital and obligatory aerobic, which sometimes leads to the creation of lethal infections in its host. *P. aeruginosa* is one of the most important causes of secondary hospital-acquired infections in patients with burns and hospitalized in intensive care units (1). Treatment of infections caused by this bacterium is difficult due to the high resistance. Antibiotic resistance in *P. aeruginosa* can be derived from chromosomal or acquired plasmid origin, in which case it can easily be transmitted between strains even between related strains of bacteria. Recently, strains with multiple drug resistance have been seen (2).

Key point
Resistance to antibiotics used in the treatment of patients with infections caused by Pseudomonas aeruginosa, especially cephalosporin, is increasing in our country, Iran.

Carbapenems (having two β-lactam and carbapenem ring), including imipenem (IMP) and meropenem (MEM) are the most important antimicrobial antibiotics used to treat *P. aeruginosa* strains (3). The production of beta-lactamase enzymes is a

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-β-lactamase (4). Metal-β-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-β-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 Muller-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 (1×10^6 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 1. Determination of the minimum inhibitory concentration of third generation cephalosporin in 125 isolates of *Pseudomonas aeruginosa* by different concentrations

Antibiotic (µg)	MIC (µg/mL)	
	Sensitive No. (%)	Resistant N0. (%)
Piperacillin	91 (75.8)	15 (12.5)
Ceftazidime	82 (68.3)	29 (24.1)
Ciprofloxacin	83 (69.1)	26 (21.6)
Ceftriaxone	71 (59.1)	38 (31.6)
Cefotaxime	68 (56.6)	40 (33.3)
Gentamicin	72 (60)	28 (23.3)
Amikacin	97 (80.8)	6 (5)
Imipenem	102 (85)	5 (4.1)
Streptomycin	65 (52)	7 (5.6)
Tetracycline	68 (54.4)	8 (6.4)
Carbenicillin	68 (54.4)	11 (8.8)
Levofloxacin	78 (62.4)	25 (20)

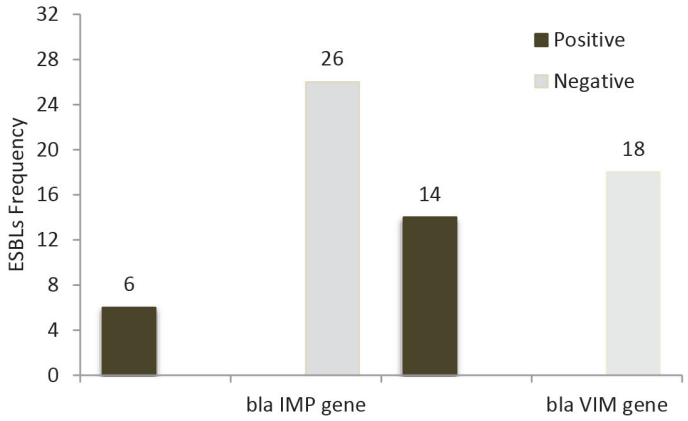

Figure 1. Distribution of bla_{IMP} and bla_{VIM} gene of expanded-spectrum beta-lactamase enzyme in *pseudomonas aeruginosa* isolates.

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_{IMP} and bla_{VIM} 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 carbapenems.

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, ceftazidime, 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_{IMP} and bla_{VIM} genes was reported to indicate high bla_{VIM} gene compared to bla_{IMP} 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, the 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 study. All authors read and approved the paper.

Conflicts of interest

The authors declare no conflict of interest.

Ethical considerations

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

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