Antibiotic Resistance Pattern and Evaluation of bla_{OXA-10}, bla_{PER-1}, bla_{VEB}, bla_{SHV} Genes in Clinical Isolates of *Pseudomonas aeruginosa* Isolated from Hospital in South of Iran in 2014-2015

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Background: *Pseudomonas aeruginosa* is one of the main causes of nosocomial infections with a mortality rate up to 40-50%. Resistance to antibiotics is a global challenge in the treatment of infections caused by this bacterium. The Class A beta-lactamases genes, including bla_{SHV}, bla_{PER}, bla_{VEB}, are the most common causes of resistance in this microorganism. This study was conducted to determine antibiotic resistance pattern and the presence of bla_{per}, bla_{veb}, bla_{shv} and bla_{oxa-10} genes in clinical isolates of *P. aeruginosa* isolated from patients in a hospital in Bandar Abbas.

Materials and Methods: This cross-sectional study was conducted on 72 *P. aeruginosa* clinical isolates. Antibiotic susceptibility testing was performed by disk diffusion method according to the clinical Laboratory Standard Institute. MIC (Minimum inhibitory concentration) of ceftazidime was performed by E-Test. Polymerase chain reaction (PCR) was performed to identify bla_{shv}, bla_{veb-1}, bla_{oxa-10}, and bla_{per-1} genes.

Results: Most of the isolates were detected from intensive care unit and urine samples. The highest resistance rate which was observed to sulfamethoxazole and ceftazidime, were 68 (94.44%) and 44 (61.11%), respectively. About 27.8% of these isolates were multidrug resistant. Among 44 ceftazidime resistant isolates, 15 isolates (34%) showed MIC $\geq$32 µg.mL in the E-test. The prevalence rates of genes were 4.16, 12.5, 8.33, and 1.38% for bla_{Oxa-10}, bla_{Shv}, bla_{Veb-1}, and bla_{Per-1} genes, respectively.

Conclusion: The ceftazidime resistance rate and the prevalence rate of resistance genes in the present study were lower than other Iranian studies. However, isolation of these genes is alarming that excessive use of antibiotics can lead to over expression of resistance genes and bacterial efflux pumps and the emergence of MDR phenotypes.

Keywords: Pseudomonas aeruginosa, Beta-Lactamase, Genes, Multidrug resistance

1. Background

Pseudomonas aeruginosa is an opportunistic gram-negative, non-fermentative, oxidase positive bacterium which is inherently resistant to various antibiotics. This microorganism is one of the most important bacterial pathogen causing health care-associated infections (1). The ability of the bacterium to survive and cause infection in the hospital, can be a threat for the patients, especially for those who are in the ICU with ventilator-associated pneumonia (2), and those with problems such as burns, cystic fibrosis, and immune suppression (3). *P. aeruginosa* is responsible for 10% of all nosocomial infections and the second cause of these infections among Gram-negative bacteria. The prevalence rate of multiple drug-resistant (MDR) *P. aeruginosa* isolates is enhancing. This fact can be resulted in increasing the morbidity and mortality rates, and economic losses (4). Resistance to antibiotics in *P. aeruginosa* is accomplished through several mechanisms including the 12-100 fold lower permeability in its outer-membrane, compared with *Escherichia coli*, along with an inducible cephalosporinase and antibiotic efflux pumps with the ability to export the amphipathic antibiotics (5). Extended-spectrum beta-lactamases (ESBLs) are enzymes with hydrolyzing activity against beta-lactam antibiotics, which mediate resistance to a wide range of cephalosporins, including ceftriaxone, ceftazidime, and aztreonam. These enzymes were originally isolated from *Klebsiella pneumoniae* and *E. coli,* but recently, it has been reported that they are isolated from *P. aeruginosa.* Several classes of ESBLs consisting of Class A, B, and C have

been reported to be in *P. aeruginosa* (6). ESBL belonging to the Class A beta-lactamase, mediates a high level of resistance to cefepime, aztreonam, and ceftazidime (7). ESBLs have been classified based on their deduced amino acid sequences to families of TEM, SHV, CTX-M, PER, VEB, GES, TLA, BES, and OXA (8). SHV ESBLs are point mutants of either narrow-spectrum SHV-1 or SHV-11 b -lactamases. So far 143 variants of SHV ESBLs have been identified, which have activity against penicillins and broad-spectrum cephalosporins; they are more active against cefotaxime than ceftazidime, and almost ineffective against cephamycins and carbapenems (9). Per-beta lactamase enzyme of Class A, firstly isolated from Turkish patient in France hospital, is able to hydrolyze penicillins and cephalosporins and responsible for resistance to the ceftazidime, cefotaxime, and aztreonam. This enzyme has spread in Europe and Asia (10). Although this enzyme is common in Turkey, it has also been reported in France, Italy, and Belgium (11). PER-3, PER-4, PER-5, and PER-7 are members of PER-1-like and derived by point mutation. PER-2 and PER-6 are different from each other in 22 amino acids and have 85% amino acid homology with PER-1(9). Group A beta-Lactamase, bla_{Veb-1}, encodes resistance to cephalosporins such as ceftazidime, cefotaxime, and ceftriaxone (12). VEB-1 revealed 38% amino acid homology with PER-1. So far 6 point mutant derivatives of VEB-1 have been described (9).

Bla_{OXA}, with the ability to hydrolyze oxacillin, is responsible for resistance to penicillin and penicillin carboxy

(13). OXA ESBLs are structural derivatives of OXA-2 and OXA-10 (subgroup 2d) (8). Assessing antibiotic susceptibility of nosocomial infections agents can lead to the selection of appropriate treatment, and the prevention and control the emergence of antibiotic resistance in hospital environments. However, in recent years, the use of molecular techniques such as PCR for detection of microbial resistance genes has highly developed.

2. Objective

The aim of the present study was to investigate antibiotic susceptibility pattern and the presence of bla_{oxa-10}, bla_{shv}, bla_{Per-1} and bla_{Veb-1} genes, in the various wards and different clinical samples of the referral Hospital in Bandar Abbas in 2014-2015.

3. Materials and methods

3.1. Bacterial strains

In this cross-sectional study, from December 2014 to June 2015, 72 clinical isolates of *P. aeruginosa* were examined, taken from 72 patients with various infections, including urine, blood, burns, sputum, and tracheal tube. Standard differential biochemical tests were performed to identify bacteria in level of genus and species. These tests were consisted of growth at 42°C, oxidase and catalase positive, Pyocyanin production, utilization of carbohydrates through oxidation testing (OF), the reaction of glucose and lactose fermentation in the TSI, Simon citrate test agar. Then isolates were stored in Tripticase Soy Broth (TSB) containing 20% glycerol in the freezer at -70°C.

3.2. Antibiotic Sensitivity

Susceptibility testing was performed by disk diffusion method (Kirby- Bauer) based on CLSI standards using 13 antibiotics (14). Antibiotics were as follows: imipenem (IPM, 10μg), meropenem (MEM, 10μg), gentamicin (GM, 10μg), ciprofloxacin (CP, 5μg), amikacin (AK, 30μg), cotrimoxazole (SXT, 25μg), cefepime (FEP, 30μg), ofloxacin (OFX, 5μg), Doripenem (DOR, 10 μg), Ticarcillin (TC, 75μg), Aztreonam (ATM, 30μg), piperacillin (PIP, 10μg), ceftazidime (CAZ, 30μg), (Mast Co. Ltd, UK). The minimum inhibitory concentration (MIC) of 44 strains that were resistant to the antibiotic ceftazidime, was determined by E-test method (BIOTEST, TURKEY) according to CLSI protocols. MIC ≥ 32 μg.mL was considered as resistant. The strains of *P. aeruginosa* ATCC 27853 were used as a quality control.

3.3. Extraction of DNA

In order to extract genomic DNA for PCR, boiling method was used (15-16). After mixing five pure and fresh colonies in 200 μL of TE buffer, suspension was centrifuged in 8000(rpm) for 4 minutes, supernatant was discarded. Then DNA was extracted by boiling five colonies in 250 μL of sterile distilled water for 10 minutes, followed by cooling in ice for 10 minutes and centrifuging for 1 min at 14,000 rpm. Supernatants were placed at -20°C.

3.4. PCR

Polymerase chain reaction was carried out for detection of beta-lactamase genes of bla_{Oxa-10}, bla_{Shv}, bla_{Per-1}, and bla_{Veb-1} using primers (Cinnagen, Iran) listed in (Table 1). Mixture contained 25 μL reaction volumes with 50 mg of extracted DNA, 10 pmol of each primer, and 1 U of Taq DNA polymerase in 10x PCR buffer containing 1.5 mM Mgcl2 and 200 μM of each deoxynucleoside triphosphate. Program is

described in the thermocycler polymerase chain reaction (bioRad, usa) for 35 times cycle in Table 1.

Statistical analysis: Data were analyzed using Fisher test and SPSS software (version 22); P ≥0.05 was considered to be significant.

Table 1. Sequencing primers for genes bla_{Shv-1}, bla_{per-1}, bla_{oxa-10} and bla_{veb-1} in *P. aeruginosa*.

Primers	Sequence (5' to 3')	Ref.
Per1-f	5'-ATGAATGTCATTATAAAAGCT-3'	(33)
Per1-r	5'-TTAATTTGGGCTTAGGG-3'	(33)
Oxa10-f	5'-TATCGCGTGTCTTTCGAGTA-3'	(34)
Oxa10-r	5'-TTAGCCACCAATGATGCCC-3'	(34)
Shv-1-f	5'-TCAGCGAAAAACACCTTG-3'	(35)
Shv-1-r	5'-TCCCGCAGATAAATCACCA-3'	(35)
Veb-1-f	5'-CGACTTCCATTTCCCGATGC-3'	(36)
Veb-1-r	5-'GGACTCTGCAACAAATACGC-3'	(36)

4. Results

In this study, 72 *P. aeruginosa* clinical isolates were isolated from different clinical specimens including urine (32 isolates, 45%), Tracheal tube (28%), Wounds (14%), Sputum (10%), Chest tubes and blood cultures (1%). Most of the samples were taken from the intensive care unit (33 samples) (46%). From a total of 72 samples, 24 (33%) samples were taken from females, and 48 (67%) samples were taken from males.

Table 2 shows the antibiotic resistance *P.aeruginosa* isolates.

Table 2. Frequency of antibiotic resistance among *P. aeruginosa* isolates in the study.

Antibiotic	Number/Frequency
Co-trimoxazole	68 (94.44%)
Ceftazidime	44 (61.11%)
Cefepime	35 (48.61%)
Ofloxacin	32 (44.44%)
Aztreonam	27 (37.5%)
Ticarcillin	25 (34.72%)
Imipenem	23 (31.94%)
Ciprofloxacin	20 (27.77%)
Meropenem	18 (25%)
Gentamicin	18 (25%)
Piperacillin	16 (22.22%)
Amikacin	14 (19.44%)
Doripenem	11 (15.27%)

In 15 (34%) ceftazidime resistance isolates, MIC was ≥ 32 μg.mL, and in 11(25%) isolates out of 44 ceftazidime resistance isolates, MIC was 16 μg.mL. From a total of 72 isolates, 20 (27.77%) isolates were multiple drug resistance (MDR). These isolates showed resistance to carbapenems, cephalosporins, and aminoglycosides. The intensive care unit has the highest number of samples with multiple resistance phenotypes (11 samples) (55%). In urine samples, the strains of multidrug resistance were detected more than the other samples. Table 3 shows the characteristics of MDR *P.aeruginosa* isolates isolated from different wards of hospital.

Table 3. Characteristics of MDR *P.aeruginosa* isolated from different wards of hospital.

Isolate	Sex	Site of isolation	Site of clinical samples	Pattern of resistance	Beta-lactamase gene
190	M	Tracheal tube	ICU	IMI-PTZ-FEP-CAZ-MEM-CP-TC-SXT-ATM-OFX-DOR	Bla_{per-1}
8	M	W.C	Burn	IMI-PTZ-FEP-CAZ-MEM-CP-GM-AK-TC-SXT-ATM-OFX	Bla_{oxa-10}
102	M	Urine	ICU	FEP-CAZ-SXT-OFX	Bla_{oxa-10}
116	M	Tracheal tube	ICU	IMI-FEP-CAZ-MEM-SXT-OFX-DOR	Bla_{oxa-10}
52	M	Urine	EM	FEP-CAZ-SXT-ATM-OFX-	Bla_{veb-1}
73	F	W.C	Burn	IMI-FEP-CAZ-CP-GM-AK-TC-SXT-ATM-OFX	Bla_{veb-1}
80	M	W.C	Burn	IMI-FEP-CAZ-CP-GM-AK-TC-SXT-ATM-OFX	Bla_{veb-1}
85	M	W.C	Burn	IMI-FEP-CAZ-CP-GM-AK-TC-SXT-ATM-OFX	Bla_{veb-1}
161	M	W.C	Burn	IMI-FEP-CAZ-MEM-CP-GM-AK-TC-SXT-ATM-OFX	Bla_{veb-1}
209	F	W.C	Burn	FEP-CAZ-CP-SXT-ATM-OFX-	Bla_{veb-1}
65	M	Tracheal tube	ICU	IMI-FEP-CAZ-GM-TC-SXT	Bla_{shv-1}
90	F	ascites	EM	FEP-CAZ-SXT	Bla_{shv-1}
126	F	Urine	EM	FEP-SXT	Bla_{shv-1}
180	F	Urine	NSW	FEP-SXT	Bla_{shv-1}
191	M	Tracheal tube	ICU	FEP-CAZ-SXT	Bla_{shv-1}
199	M	Urine	Internal	SXT	Bla_{shv-1}
153	M	Urine	ICU	CAZ-TC-SXT	Bla_{shv-1}
218	F	Tracheal tube	ICU	IMI-TC-CAZ	Bla_{shv-1}
102	M	Urine	ICU	FEP-CAZ-SXT-OFX	Bla_{shv-1}

IPM=imipenem, MEM=meropenem, GM=gentamicin, CP=ciprofloxacin, AK=amikacin, SXT=cotrimoxazole, FEP=cefepime, OFX=ofloxacin , DOR=Doripenem, TC=Ticarcilin , ATM=Aztreonam , PIP=piperacillin , CAZ=ceftazidime, M=male, F=female

The prevalence rate of beta lactamase genes was found to be 6 (8.33%), 3 (4.16%), 9 (12.5%), 1 (1.38%) for bla_{Veb-1}, bla_{Oxa-10}, bla_{Shv-1}, and bla_{Per-1}, respectively.

By using the SPSS software version 21, and employing the chi-square test, and p-values more than .05, the statistical analysis showed that there was no significant relationship between the presence of beta-lactamase genes and antibiotic resistance.

5. Discussion

Antibiotic resistance is a serious challenge in the treatment of infections caused by *P. aeruginosa* in different wards of the hospital, especially in intensive care units due to weak immune system and severe illnesses in patients (6). According to our knowledge, the present study was the first report on the prevalence rate of the beta-lactamase genes responsible for advanced generation of cephalosporins resistance in *P.aeruginosa* in the south east of Iran. In this study, the most strains were isolated from urine (32 strains, 44.44%) and intensive care unit while in other studies conducted in Turkey, respiratory tract specimen had the most samples infected with *P. aeruginosa* in intensive care unit (14). Presumably the patient's inability to pass urine and the use of a urinary catheter play an important role in this phenomenon. Moreover, this may indicate that *Acinetobacter* is gradually replaced with *P.aeruginosa* in respiratory infections in intensive care unit in this hospital. The most effective antibiotics in the present study were amikacin, piperacillin. Amongst the 72 isolates, 20 (27.77%) isolates were multiple drug resistance (MDR). These isolates were resistant to carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), and aminoglycosides (amikacin, gentamicin) (17-18). This finding was less than the finding of other studies conducted by Alikhani (88.7%) (7) and Mirsalehian (87%) (19) in Tehran. However, in these studies, all or more of the samples were isolated from burn wards, and the presence of MDR phenotype could mainly be due to excessive use of antibiotics. Reports from around the world show a significant increase in the resistance rate of *P. aeruginosa* to ceftazidime so that in America during the years 1998-2003, ceftazidime resistance rate was reported to be 11.8% (20), and in Korea during the years 2002-2006, it was reported to be 18.8% (21), which are less than the present study's finding (61.11%). Also, in Iran during the years 2010-2014, resistance to ceftazidime in different cities indicated a significant increase. In 2010 (25.5%) (22), 2009 (53.57%) (23), 2013 (92%) (24), and 2014 (51%)(7), resistance to ceftazidime was reported to be increasing. Also, resistance to cefepime was similarly reported to be increasing so that during the years 2013-2014 in Tehran (96%) (24) and the year 2014 in the Hamadan (97%) (7), it was reported to be higher than the present study's finding (48.61%). Also, increase in the frequency of resistance to aztreonam has been reported to be more than the finding of the present study so that in 2013 and 2014, the frequency of resistance to aztreonam has been reported as 98% (24) and 27% (7), respectively, compared with the current study's finding (37.5%). Also, based on the studies from around the world, resistance to imipenem was reported as follow: in Spain 14%, Italy 13.8%, Saudi Arabia 68%, Brazil 58.8%, and Turkey 32.9% (25).

In Iran during the years 2010-2014 in different regions, resistance rate to imipenem was reported between 42.28% in

Esfahan (23), and 84.9% in Hamadan (7), compared with 31.94% of the current study. The prevalence rate of *bla* VEB-1 gene in various studies around the world was as follow: in Thailand 94.44% in 2001 (26), and in Italy 24.61% in 2006 (27). The prevalence rate of *bla* VEB-1 gene in various studies conducted in Iran was as follow: in Hamedan 15% in 2010 (7), 24% in 2009 (28), 25.5% in 2010 (19), and 10.9% in 2012 (11), which were more than the present study's finding (8.33%). The prevalence rate of *bla*PER-1 in the present study (1.38%) was less than the findings of other studies conducted around the world and in Iran. The prevalence rate of *Bla* PER-1 in Turkey was reported as 11% in 1997(29) and 55.4% in 2005 (30). In several studies conducted in Iran, the prevalence rate of *bla* PER-1 has been reported more than the present study's finding, for example, Alikhani, 2014 (26.6%)(7); Shahcheraghi, 2009 (17%)(28); and Mirsalehian, 2010 (31.4%) (19). Also, in the current study, the prevalence rate of BlasHV-1, (12.5%) was less than the findings of the other studies conducted in Iran during the years 2010-2009 such as pakbaten, 2010 (36%)(31); Imani, 2010 (37.5%)(22). In the current study, the prevalence rate of Bla Oxa-10 (4.16%) was also less than the findings of other studies conducted in Saudi Arabia in 2012 (56%)(32), in Iran in 2008 by Nakhjavanian (29%)(6), in Shiraz in 2013 by Conani (96.2%)(32), and in Tehran in 2009 by Pakbaten (36%)(31). It is expected that different regions show different prevalence rate of resistance determinant genes that is partly due to the antibiotic utilization policy in each region or the distribution of dominant clones of bacteria carrying resistance genes.

6. Conclusion

The results of this study show that although the prevalence rate of antibiotic resistance and also ceftazidime resistance were higher than the findings of other studies conducted in Iran and other parts of the world, but the prevalence rate of beta-Lactamase genes was lower than the findings of other studies conducted in other parts of Iran and other countries. This finding is not consistent with the pattern of antibiotic resistance. A possible explanation for this phenomenon is that antibiotics exposure in healthcare settings is resulted in the over expression of bacterial efflux pumps and resistance to several classes of antibiotics and the emergence of MDR phenotypes, which were detected in this study (27.77%). It is recommended that further studies to be carried out in order to evaluate over expression of efflux pumps in these isolates, to investigate the epidemiology of agents causing health care associated infection, and to consider the prevention strategies.

Conflict of interests

There is no conflict of interest to be declared.

Authors' Contribution

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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