Original Article

Expression of Efflux Pump *MexAB-OprM* and *OprD* of *Pseudomonas aeruginosa* Strains Isolated from Clinical Samples using qRT-PCR

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Abstract

Background: *Pseudomonas aeruginosa* is one of the most common nosocomial pathogens with high mortality rates. Organisms such as *Pseudomonas aeruginosa* have the ability to develop high level MDR (Multi drug resistance). The *MexAB-OprM* system is one of the largest multi-drug resistant efflux pumps with high levels of expression and the first finding of the RND (Resistance-nodulation-division) family in *P. aeruginosa*.

Aim: For better understanding of the antibiotic resistance mechanism in *P. aeruginosa,* this study was conducted to determine the expression of the genes encoding these efflux pumps in 100 strains of *P. aeruginosa* isolated from patients admitted to various hospitals in Hamadan using quantitative Real-Time PCR (qRT-PCR).

Methods: This study examined 100 strains of *P. aeruginosa* isolated from patients admitted to various hospitals in Hamadan. Then, 31 samples were selected based on collected specimen type and their antibiotic susceptibility pattern; i.e., the samples with reduced susceptibility to antibiotics, particularly carbapenems. Antibiotic disk diffusion method was performed for aminoglycoside, quinolone and carbapenem. Furthermore, MIC method was performed for ciprofloxacin, gentamicin and imipenem. Finally, qRT-PCR was used for determining the efflux pump genes expression.

Results: Among eight selected antibiotics, the greatest resistance was to levofloxacin (61.2%, n = 19) and the lowest one to imipenem (9.6%, n = 3). All isolates (100%, n = 31) exhibited efflux pump *MexAB-OprM* genes but different expression was observed in different strains. The result of gene expression indicated that significant differences in expression of *MexR* (*P* value = 0.003), *OprD* (*P* value < 0.001), and *MexB* (*P* value = 0.026) genes. In addition, there was high level of *MexR* gene expression in bacteria that leads to reduced expression of *MexA*, *MexB*, and *OprM*. The *OprD* gene was presented in all strains but different expression has been observed.

Conclusion: Identifying the bacterial resistance mechanisms is very complicated. Although efflux pump *MexAB-OprM* plays an important role in antibiotic resistance in *P. aeruginosa,* because of acting the efflux pumps on antibiotics in a non-specific manner, it is elusive to consider or describe an antibiotic resistance based on the presence or absence of an efflux pump.

Keywords: Antibiotic, gene expression, MIC, *Pseudomonas aeruginosa*, qRT-PCR

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that has been recognized as one of the significant pathogenesis agents of nosocomial infections in recent years.[1] Involvement of *P. aeruginosa* has been mostly reported in cases of urinary tract and particularly burn infections as well as pulmonary diseases such as cystic fibrosis (CF).[2] One of the main problems regarding prevention and/or treatment of infections resulting from this bacterium is the capability for developing antibiotic resistance. In fact, *P. aeruginosa* can develop multidrug resistance which has been demonstrated by several antibiotics resistance mechanisms such as efflux pump.[3]

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At present, the efflux pump has been recognized as one of the significant bacterial resistance complexes to most antibiotic classes. The pump is located across bacterial cytoplasmic membrane rising to active transport removing the toxic substances, e.g., antibiotics from bacterial cell. Initially in 1980, the efflux pumps were found and identified associated with mechanism providing antibiotic resistance to tetracycline antibiotic class for *Enterobacteriaceae* family; several years later, the association of these structures with bacterial resistance to most antibiotics were shown by several studies.[4] Regarding the fact that efflux pumps act on various substrates, these structures are significant in developing bacterial multidrug resistance. The efflux pumps not only increase minimum inhibitory concentrations (MIC) for a given bacterium, but also by reducing antibiotic concentration from inside the bacterial cell, lead to development of antibiotic resistant mutant strains.[5]

Among gram negative bacteria, *MexAB-OprM* system is one of the largest multi-drug resistant efflux pumps with high levels of expression and the first finding of the RND (Resistance-nodulation-division) family in *P. aeruginosa*, which mostly discharge lipophilic and ambiphilic drugs (antibiotics) out of the bacterium

and lead to antibiotic resistance. The *MexR* protein regulates expression of *MexAB-OprM* efflux pump genes. Exposure of bacteria with mutant *MexR* gene to given antibiotic enhances the expression of *MexA*, *MexB*, and *OprM* proteins, thereby developing higher antibiotic resistance in comparison to wild strains.[6,7]

Another well-known system conferring antibiotic resistance in *P. aeruginosa* is *OprD* protein. The *OprD* is a purine located on the cytoplasmic membrane of *P. aeruginosa*. Due to extensive antibiotic resistance in *P. aeruginosa*, only a few antibiotic classes can be used for treatment of caused infections. Therefore, according to previous studies, carbapenems antibiotic classes are the most important antibiotics used for this purpose. In fact, carbapenems are considered as one of the last alternatives for treating infections caused by gram negative bacteria, particularly *P. aeruginosa* after most antibiotics fail.[8] It has been shown that the main reason for resistance to carbapenems is reduced diffusion of these antibiotics to bacterial cell caused by alteration in *OprD* gene expression. In fact, *P. aeruginosa* by reducing expression of *OprD* gene leads to resistance to carbapenems antibiotic classes.[9]

Considering that the efflux pumps play a significant role in antibiotic resistance for *P. aeruginosa*, and the different expression levels in efflux pump genes for various strains conferring different antibiotic susceptibility pattern, the aim of this study was to determine the expression of the genes encoding these efflux pumps using quantitative Real-Time PCR (qRT-PCR).

Materials and Methods

Identification of isolates

Conventional phenotypic methods including gram staining, oxidase and catalase tests, pyocyanin production, growth in 41–42°C, oxidation/fermentation (OF) determination, and pigmentation test were used for microbiological presumptive identification of *P. aeruginosa* isolates collected during approximately 1 year from February 2012 through March 2013. Furthermore, PCR assay targeting PA-*16S rRNA* gene specific for *P. aeruginosa* species was used in order to confirm performed presumptive phenotypic tests, as explained in the following. A total of 31 samples were selected based on collected specimen type and their antibiotic susceptibility pattern; i.e., the samples with reduced susceptibility to antibiotics particularly carbapenems. These samples included 8 tracheal tube samples from hospitalized patients, 7 blood culture samples from hospitalized patients, 5 wound samples [bedsore (2), hand wound (2), and corneal wound (1)], 4 sputum samples, 4 synovial fluid samples, and 3 urine culture samples from hospitalized patients. The Brain Heart Infusion (BHI) broth containing glycerol was inoculated by the confirmed isolates and used for preservation of the isolates in deep freeze (-70°C) condition, finally.

DNA extraction and confirmation of identified isolates by PCR assay

The Phenol-Chloroform procedure was used for DNA extraction. The obtained DNA was used for PCR assay or frozen at -20°C until further use.

PCR assay targeting 956-bp *PA-16S rRNA* (PA-ss) gene was performed for confirmation or definitive identification of *P. aeruginosa* species. The forward and reverse primer sequences were GGG GGA TCT TCG GAC CTCA and TCC TTA GAG TGC CCA CCCG, respectively.[10] The PCR reaction volume was 20 µL. The *P. aeruginosa* PAO1 (ATCC 27853) and distilled water (as blank template) were used as positive and negative controls, respectively. PCR products and 100-bp DNA size marker (Kiagen Co., Iran) were run simultaneously on 1.5% agarose gel (SinaClon Co., Iran) stained with DNA safe stain (SinaClon Co., Iran) at 80 V for 1 hour. Finally, the agarose gel was visualized and photographed by UV transilluminator.

Antimicrobial susceptibility testing

All isolated strains were tested by Kirby-Bauer disk diffusion and MIC methodology for determining antimicrobial susceptibility pattern according to the Clinical and Laboratory Standards Institute (CLSI 2013) recommendations.[11] The antibiotic disks (Himedia Co., India) utilized in this study for disk diffusion method were amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ofloxacin (5 µg), meropenem (10 µg), and imipenem (10 µg). MIC was performed using imipenem, ciprofloxacin, and gentamicin (Himedia Co., India), separately. The micro-plates containing Mueller Hinton broth were used for this propose. Different serial dilution for each antibiotic including 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/mL were prepared and bacterial standard suspension was added to them. The micro-plates were incubated for 18 hour at 37°C. The well with no turbidity was considered as used antibiotic minimum inhibitory concentration forgiven strain

Detection of MexAB-OprM, MexR and OprD genes by PCR assay

After DNA extraction, multiplex PCR assay was performed for detecting three genes including *MexA*, *MexB*, and *MexR*; likewise, two separate PCR assays were performed for detecting two *OprM* and *OprD* genes. The *rPSL* gene was used for each reaction as house keeping control gene. Printer sequences used in this study are listed in Table 1.[12] The *P. aeruginosa* PAO1 (ATCC 27853) and distilled water (as blank template) were used as positive and negative controls, respectively. PCR products and 100-bp DNA size marker (Kiagen Co., Iran) were run simultaneously on 1.5% agarose gel stained with DNA safe stain (SinaClon Co., Iran) at 80 V for 1 hour. Finally, the agarose gel was visualized by using UV transllluminator.

RNA extraction and synthesis of cDNA

RNA was extracted using RNA extraction kit (Tous Biotech Co., Iran) in order to determine the expression of *MexA*, *MexB*, *MexR*, *OprM*, and *OprD* genes of different isolated strains in a semi-quantitative manner using quantitative Real Time PCR (qRT-PCR). Reverse transcription reaction was performed for cDNA synthesis using hexamer primer (Kiagen Co., Iran).

Amplification of *MexA, MexB, MexR* and *OprD* genes by PCR assay using cDNA

This assay was performed to verify whether or not the mentioned genes were expressed in the studied strains. The primers listed in Table 1 were used for this purpose. Similar to previous assay, the *rPsl* gene was used as control. The multiplex PCR assay procedure for five genes was the same as the previously mentioned procedure (Figure 1). The volume of reaction was 20 µL.

Determining expression of efflux pump genes (*MexA, MexB, MexR, OprM and OprD*) using quantitative Real Time PCR (qRT-PCR)

Real-time PCR was performed as multiplex PCR assay using Fermentase kit (Fermentase Co., USA) and primers presented in Table 1 for quantitative assessing of efflux pump genes expres-

Table 1. Primers used for PCR assay in this study.

Target gene	Sequences	Product (bp)	Annealing temperature (°C)
Mex-A	F: CTCGACCCGATCTACGTC R: GTCTTCACCTCGACACCC	503	57
Mex-B	F: TGTCGAAGTTTTTCATTGAG R: AAGGTCAC GGTGATGGT	280	57
Mex-R	F: GAACTACCCCGTGAA TC R: CACTGGTCGAGGAGATGC	411	57
OprM	F: GATCCCCGACTACCAGCGCCCCG R: ATGCGGTACTGCGCCCGGAAGGC	247	57
OprD	F: ATCTACCGCACAAACGATGAG R: GCCGAAGCCGATATAATCAAACG	156	57
rPsL	F: GCAAGCGCATGGTCGACAAGA R: CGCTGTGCTCTTGCAGGTTGTGA	201	57

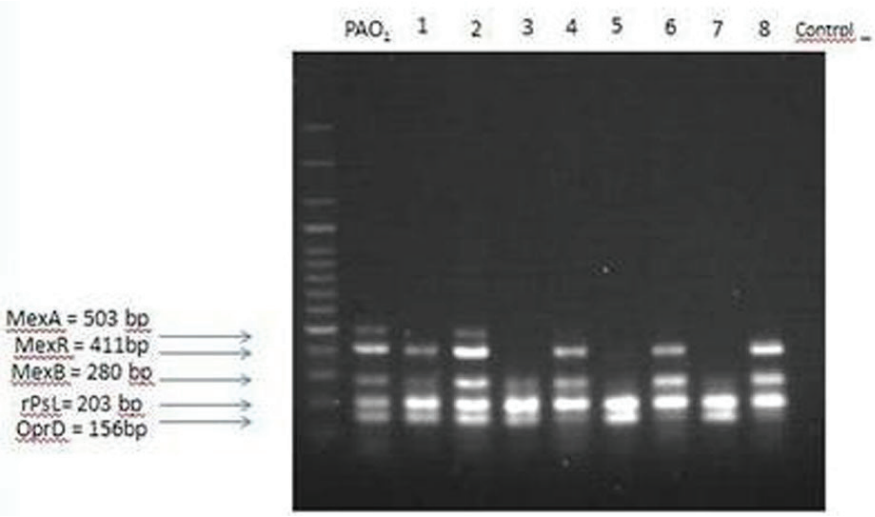

Figure 1. Results of multiplex PCR for amplification of *MexA*, *MexB*, *MexR OprD*, and *rPsL* genes using cDNA. *Pseudomonas aeruginosa* PAO1 (ATCC 27853) has expressed all five genes; lanes 1, 4, 6, and 8: clinical strains that have expressed all four genes except for *MexA* gene; lane 2: clinical strain that has expressed all five genes; lanes 3, 5, and 7: clinical strains that have expressed only three *MexB*, *OprD*, and *rPsL* genes.

sion. According to protocols, Master Mix, the primers and synthesized cDNA were mixed in proper volume and examined by Bio-Rad Real-time PCR. The *P. aeruginosa* PAO1 (ATCC 27853) was used as positive control. In addition, all gene expressions were compared with *rPsL* gene expression as the housekeeping gene. The assay was performed three times for each sample and the mean of three obtained quantities was considered as quantity of given gene expression for that sample. The ΔΔCt was used for determining gene expression. The ΔΔCt was earned by subtracting ΔCt of sample from ΔCt of standard strain (PAO1). The ΔCt values for all gene expression from standard strain were 1.

Statistical analysis

Antibiotic susceptibility pattern for disk diffusion and MIC method were compared using Chi-Square test. One-Sample Student's *t*-test was used to compare the mean values of quantitative variables. i.e., comparison of quantities of efflux pump genes expression obtained for all clinical strains with those of obtained for standard (control) *P. aeruginosa* PAO1 (ATCC 27853) strain. Besides, the Pearson Chi-square (χ^2) test was used to assess the association of antibiotic susceptibility pattern with quantities of efflux pump genes expression for clinical strains. Finally, correlation between *MexR* gene expression and *MexAB-OprM* genes expression was analyzed using Pearson Chi-square test. For all mentioned tests, the significance test was two-tailed and a *P* value < 0.05 was considered statistically significant. All tests were performed with SPSS version 20.

Results

Antimicrobial susceptibility pattern of strains

The results of antimicrobial susceptibility testing indicated that among the eight selected antibiotics, the greatest resistance was to levofloxacin (61.2%, n = 19) and the lowest to imipenem (9.6%, n = 3) (Table 2). Moreover, the results showed that there was partial resistance to the antibiotics used, as 22% of the studied strains showed no resistance to any of the eight antibiotics; 16% of strains showed resistance to only one antibiotic, 6.5% to two antibiotics, 12.5% to three antibiotics, 12.5% to four antibiotics, 6.5% to six antibiotics, 17.5% to seven antibiotics, and 6.5% to eight antibiotics. Resistance to all eight antibiotics was observed by antibiogram method. The results of MIC for three mentioned antibiotics showed that 18 samples (58.08%) were resistant, 8 samples (25.8%) were intermediate, and 5 samples (16.12%) were sensitive to gentamicin; 28 samples (90.33%) were resistant, and 3 samples (9.67%) were intermediate to ciprofloxacin; 12 samples (38.7%) were resistant, 13 samples (41.93%) were intermediate, and 6 samples (19.35%) were sensitive to imipenem.

Table 2. Rates of antibiotic susceptibility patterns determined in *Pseudomonas aeruginosa* strains.

Antibiotics	Disk diffusion results				MIC results			*P* value*
	Resistance No (%)	Intermediate No (%)	Sensitive No (%)	Total (%)	Resistance No (%)	Intermediate No (%)	Sensitive No (%)	
Imipenem	3(9.6)	1(3.40)	27(87.09)	100	12(38.70)	13(41.93)	6(19.35)	< 0.001
Meropenem	11(35.48)	2(6.45)	18(58.06)	100	—	—	—	—
Gentamicin	14(45.16)	2(6.45)	15(48.38)	100	18(58.06)	8(25.81)	5(16.13)	< 0.001
Amikacin	4(12.9)	2(6.45)	25(80.65)	100	—	—	—	—
Tobramycin	11(35.48)	1(3.22)	19(61.29)	100	—	—	—	—
Ciprofloxacin	12(38.07)	6(19.35)	13(41.93)	100	28(90.32)	3(9.68)	0	< 0.001
Ofloxacin	12(38.07)	0	19(61.29)	100	—	—	—	—
Levofloxacin	19(61.29)	0	12(38.07)	100	—	—	—	—

* Comparison between results of disk diffusion method and MIC method for CIP, GM, IPM antibiotics based on one-way analysis of variance (ANOVA). Correlation is significant at the 0.05 level (2-tailed).

Table 3. Results of analysis of efflux pump genes expression in some strains.

Samples name	Efflux pump genes				
	MexA	*MexB*	*OprM*	*MexR*	*OprD*
PAO1	1	1	1	1	1
Isolate 33	0.09	0.21	1.7	426	0.05
Isolate 8	0.002	0.17	0.65	152	99.9
Isolate 12	0.002	0.18	0.447	131	0.009
Isolate 5	0.04	185	0.35	338	58.9
Isolate 24	0.14	2.3	13.8	3543	1.9
Isolate 9	0.005	0.3	1.007	393	0.007
Isolate 35	0.001	0.2	0.62	80.7	0.05
Isolate 66	0.71	0.71	0.65	410	164
Isolate 30	0.008	0.2	1.06	7.2	1.2

Table 4. Results of *t* test and analysis of correlation between efflux pump genes expression and antibiotic susceptibility pattern of studied strains based on Pearson Chi-Square test.

Genes	*P*-value* for correlation of antibiotic susceptibility pattern with efflux pump genes											Test Value = 1	
	Imipenem (MIC)	Imipenem (DD)	Meropenem (DD)	Gentamicin (MIC)	Gentamicin (DD)	Tobramycin (DD)	Amikacin (DD)	Ciprofloxacin (MIC)	Ciprofloxacin (DD)	Ofloxacin (DD)	Levofloxacin (DD)	*T*-test	*P*-value*
MexA	0.244	0.395	0.851	0.462	0.548	0.784	0.936	0.483	0.240	0.383	0.911	1.239	0.225
MexB	0.242	0.402	0.880	0.442	0.568	0.812	0.958	0.480	0.251	0.369	0.923	2.348	0.026
OprM	0.014	<0.001	0.194	<0.001	0.316	0.207	0.020	0.001	0.402	0.273	0.585	0.442	0.661
MexR	0.041	0.001	0.196	0.001	0.292	0.175	0.010	0.001	0.268	0.298	0.549	3.195	0.003
OprD	0.125	0.509	0.005	0.021	0.008	<0.001	0.721	0.071	0.028	0.099	0.084	3.977	<0.001

DD = disk diffusion method; *Correlation is significant at the 0.05 level (2-tailed).

PCR and qRT-PCR assay results

All isolates had efflux pump *MexAB-OprM* genes but different expressions were observed in different strains (Figure 1). In addition, there was high level of *MexR* gene expression in bacteria that leads to reduced expression of *MexA*, *MexB*, and *OprM*. The *OprD* gene was present in all strains but different expression was observed. The strains with over-expression of *OprD* gene showed high sensitivity to carbapenems family antibiotics, especially to imipenem. The results of analysis of efflux pump genes expression in some strains are shown in Table 3.

Results of Statistical analysis

The results of One Sample *t*-test done for analysis of differences between standard strain efflux pump genes expression and clinical strains as well as the results of correlation analysis between efflux pump genes expression and antibiotic susceptibility pattern using Pearson Chi-square test are shown in Table 4.

Discussion

Pseudomonas aeruginosa is a common human pathogen considered as the third agent of nosocomial infections after *Staphylo-*

coccus aureus and *Escherichia coli*.[13] One of the main problems regarding treatment and prevention of *P. aeruginosa* infections is the development of antibiotic resistances.[3] At present, the efflux pump has been recognized as one of the significant complexes that result in resistance to most antibiotic classes.[4]

Many investigations have been performed on antibiotic resistance in *P. aeruginosa* with different results depending on time and region. The results of this study showed the rates of antibiotic resistance to gentamicin as 51.6% (n = 16), amikacin 19.3% (n = 6), tobramycin 38.7% (n = 12), meropenem 41.9% (n = 13), imipenem 9.6% (n = 3), ciprofloxacin 58% (n = 18), ofloxacin 38.7% (n = 12), and levofloxacin 61.2% (n = 19); which are partly similar to reported antibiotic resistance values for ciprofloxacin 49%, tobramycin 68%, levofloxacin 25%, gentamicin 63%, and imipenem 14% by Rajat Rakesh, et al., in India.[14,15] Also, in the study performed by Chander Anil, et al., in Nepal in 2012, resistance to amikacin was shown as 25% and 75% for ciprofloxacin,[16] which are similar to this study. In a study performed in 2012, resistance to antibiotics was reported as follows: imipenem 23.3%, gentamicin 37.2%, amikacin 48.8%, tobramycin 58.2%, and ciprofloxacin 63.3%[17]; in which antibiotic resistance rates to imipenem and amikacin were higher than our study in Hamadan. In the study performed by Kianpour, et al., in 2010, resistance rates was reported as 58.14% for amikacin, and 42.85% and 14.8% for ciprofloxacin and imipenem, respectively.[18] In another study performed by Shahcheraghi, et al., in 2003, the rates of resistance to gentamicin, amikacin, and ciprofloxacin were 93.7%, 93.4%, and 86.7%, respectively;[19] it seems that the reason for higher resistance rates in comparison to the present study may be associated with the type of clinical samples that was only burn samples which according to previous studies have usually higher antibiotic resistance rates than other clinical samples. Based on a recent study performed by Vijaya Haudhari, et al., in 2013, the rates of resistance to ciprofloxacin and meropenem were reported as 59% and 11%, respectively.[20]

The antibiotic resistance rates of *P. aeruginosa* to Imipenem have been reported as 8.3% (Japan), 12% (Canada), 13.4% (Russia), 18.5% (France), and 14% (Spain) in 2001[21,22]; except for the study performed in Japan, the other reported values are higher than the present study in Hamadan (Iran) with 9.6%.

The rates of antibiotic resistance to ciprofloxacin have been reported as 9.20% by Imani, et al., in 2011.[23] In addition, resistance to this antibiotic has been reported as 9% (France), 61% (Zimbabwe), 91.7% (Russia), 18% (Canada), and 20.7% (USA) in 2002, as well as 23% in 1999 (Spain).[22,24,25] In comparison to the mentioned studies, the resistance rate of 58% to ciprofloxacin indicates enhanced resistance to this antibiotic in Hamadan. The observed difference in antibiotic rates can be explained by increased prescription of this antibiotic for treatment of diseases caused by *Pseudomonas*.

The rate of resistance to amikacin was estimated as 19% that is similar to other reported resistance rates for this antibiotic, that is, 25% (Russia), 13.1% (USA), 9% (Spain), 9% (France), and 4% (Turkey).[24,26,27]

Genotypic investigation using PCR for surveying efflux pump *MexAB-OprM* genes was performed and showed that all 31 isolates had the *MexA*, *MexB*, and *OprM* genes. Similar results were obtained in a study by Auda Al-Grawi Ibtesam Ghadban, et al., in 2012.[28] One-Sample Student's *t* test comparing the quantities of efflux pump genes expression for all strains with those of standard *P. aeruginosa* PAO1 (ATCC 27853) strain indicated significant differences in the expression of *MexR* (P value = 0.003), *OprD* (P value < 0.001), and *MexB* (P value = 0.026) genes. In addition, statistical analysis to determine the correlation between efflux pump genes expression and antibiotic susceptibility pattern in clinical strains indicated that antibiotic susceptibility pattern of at least one utilized antibiotic from each three classes (aminoglycosides, fluoroquinolones, and carbapenems) had significant correlation (P value < 0.05) with at least one of the efflux pump genes expression level. Therefore, it is elusive to precisely describe the fact that efflux pumps expression leads to development of antibiotic resistance. In fact, it seems there is an association between multidrug resistance and efflux pump complexes; in other words, the efflux pumps may confer and/or develop resistance to various antibiotic classes.

In our study, the quantity of *MexR* gene expression was high due to raised *MexR* gene expression accompanied by decreased level of *MexAB-OprM* genes expression. This finding was similar to the study performed in 2000 by Kohjiro Saito, et al., revealing that the rising of *MexR* gene expression led to decreased *MexAB-OprM* gene expression, resulting in decreased Aztreonam and quinolones MIC. In addition, they demonstrated that by suppressing *MexR* gene expression, *MexAB-OprM* genes expression level enhanced and MIC of antibiotics particularly quinolones would be increased. In this study the MIC of ciprofloxacin and ofloxacin increased up to 256 µg/mL and 128 µg/mL, respectively.[7] Likewise, in the present study, strains with increased *MexR* gene expression had decreased *MexA*, *MexB*, and *OprM* genes expression (P value < 0.001). In addition, this finding was consistent with another study performed by Jean-Luc Dumas, et al., in 2005 which demonstrated the strains with high efflux pump genes expression increased MIC for antibiotics in the way that the strains with higher efflux pump genes expression, had higher MIC of ciprofloxacin up to 256 µg/mL. In addition, rising of efflux pump genes expression led to increased MIC of aztreonam up to 128µg/mL.[12] Recent study by Sacha, et al., in 2014 in different clones of multiresistant *P. aeruginosa* strains collected at two intensive care units (ICU), regulatory and structural genes *MexB*, *MexR*, and *MexA* were found in 99%, 98%, and 94% of tested strains, respectively and *MexB* gene expression exhibited higher level,[29] the results of which are the same as the present study, and another study by Aghazadeh, et al., in 2014 among 21 CF isolates, *MexA* overexpression was the most prevalent mechanism (47.6%) followed by *MexX* (42.8%), *AmpC* (9.5%), and *OprD* down regulation (4.7%) among 27 burn isolates, the prevalence of *MexX*, *MexA*, *AmpC* overexpression, and *OprD* down regulation was 62.9%, 74%, and 11.1%, 14.8%, respectively.[30]

According to Yoneyama, et al., concurrent occurrence of the three MexA, MexB, and OprM proteins are essential for function of MexAB-OprM efflux.[31] In our study, several strains had increased *MexB* gene expression but decreased *MexA*, and *OprM* gene expression, thereby all three parts of efflux pump were not completely expressed and functioned; thus the strains were susceptible to antibiotics. In 2011, Burcin Ozer, et al., investigated survey of efflux pump gene expression of *P. aeruginosa* on 50 clinical strains isolated from ICU patients. Their investigation included multiplex PCR assay for determining the four known genes expression of efflux pump as well as *OprD* gene. The results of this study showed that efflux pumps are in relation with multidrug resistance. In addition, other mechanisms such as car-

bapenemases can develop resistance in *P. aeruginosa* strains.[32] In our study, some strains had extensive resistance to many antibiotics but efflux pump genes expression of these strains were very low; therefore, the related occurred resistance can be attributed to mechanisms other than the efflux pump.

Based on the study performed by Jean-Luc Dumas, et al., in 2006, strains with decreased *OprD* purine gene expression had increased resistance to Carbapenems (imipenem and meropenem) and on the other hand, strains with increased *OprD* gene expression were much susceptible to this antibiotic class; in addition, it was demonstrated that the strains with mutation in *OprD* gene region were fully resistant to Imipenem and meropenem.[12] In another similar study by Quale, et al., in 2006 aiming to determine the *OprD* purine gene expression using RT-PCR and its correlation with resistance to carbapenems, it was shown that *OprD* is the most significant protein for developing resistance to carbapenems.[9]

In the present study, we found several strains with significant *OprD* gene expression. The strain with decreased *OprD* gene expression had full resistance to carbapenems (imipenem and meropenem). Moreover, the strains with increased susceptibility to carbapenems showed increase in *OprD* gene expression. However, in some strains, we could not find a significant correlation between *OprD* gene expression and resistance to carbapenems, as the strain with low *OprD* gene expression showed susceptibility to carbapenems. It was notable that one strain (isolate 8) with high *OprD* gene expression showed full resistance to imipenem and meropenem; thus, it is thought that other mechanisms such as carbapenemases have caused this resistance pattern.

In conclusion, considering the present study and also other performed similar studies, it can be concluded that *P. aeruginosa* use various mechanisms to avoid antibiotics and other toxic molecules. *MexAB-OprM* is house-keeping genes and the most significant pump among the known efflux pumps mechanisms. However, the expression level of these genes is not always the same. Although the function of efflux pump plays an important role in reducing the susceptibility to various antibiotics, the roles of other involved agents and mechanisms in resistance development should not be ignored.

Conflict of Interests

The authors have no competing interests.

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