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ORIGINAL ARTICLE

Evaluation of efflux pumps gene expression in resistant *Pseudomonas aeruginosa* isolates in an Iranian referral hospital

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ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa* (PA) is one of the most important causes of nosocomial infections and has an intrinsic resistance to many antibiotics. Among all the resistance-nodulation-division (RND) pumps of *P. aeruginosa,* MexAB-OprM is the first efflux pump found to target multiple classes of antibiotics. This study was aimed to evaluate the expression level of genes expressing MexAB-OprM in clinical isolates of *P. aeruginosa.*
Materials and Methods: In this study, 45 *P. aeruginosa* strains were isolated from patients admitted to Children's Medical Center Hospital, an Iranian referral hospital. Disk diffusion and Minimum Inhibitory Concentration (MIC) methods were used for determination of the patterns of resistance to antibiotics. Real-time PCR was used to investigate the expression level of genes of MexAB-OprM efflux pump.
Results: Among 45 resistant PA isolates, the frequency of genes overexpression was as follows: *MexA* (n=25, 55.5%), *MexB* (n=24, 53.3%) and *OprM* (n=16, 35.5%). In addition, in 28 strains (62%) overexpression was observed in one of the studied three genes of MexAB-OprM efflux pump.
Conclusion: In our study 28 isolates (62%) had increased expression level of efflux pumps genes, MexAB-OprM. Although the efflux pumps play important roles in increasing the resistance towards different antibiotics but the role of other agents and mechanisms in evolution of resistance should not be ignored. Since the concomitant overproduction of other Mex efflux systems might have additive effects on antibiotic resistance, the co-expressing of a multicomponent efflux pump is recommended. On the other hand, the concomitant overproduction of two Mex pumps might have additive effects on resistance to antibiotic. Therefore co-expressing of Mex efflux systems is recommended.

Keywords: *Pseudomonas aeruginosa,* Efflux pump, Real-time PCR

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INTRODUCTION

Emergence of bacterial resistance to many different antibiotics is considered as a great concern in human health. *Pseudomonas aeruginosa* (PA) has been recognized as one of the significant pathogens of nosocomial infections (1). Key mechanism of antibiotic resistance in *P. aeruginosa* is the expulsion of antibiotics through multidrug resistance (MDR) efflux systems belonging to the resistance-nodulation-division (RND) family (2). MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK and MexVW contribute the most significantly to antibiotic resistance (3) and play an important role in intrinsic and acquired multidrug resistance (2). Resistance of *P. aeruginosa* to multiple antibiotics is largely attributable to expression of the MexAB-OprM efflux pump (4). Among all the RND pumps of PA, MexAB-OprM was the first efflux pump found to target multiple classes of antibiotics including β-lactam (carboxypenicillins, aztreonam, extended-spectrum cephalosporins, penems, the carbapenems such as meropenem and panipenem except imipenem and biapenem); fluoroquinolones, tetracyclines, chloramphenicol, macrolides, novobiocin, trimethoprim and sulfonamides (5, 6).

The MexAB-OprM efflux pump belongs to the superfamily of ribonucleoproteins and consists of an inner membrane (MexB), a periplasmic membrane fusion protein (MexA) and a channel-forming outer membrane protein, OprM (7).

In this study, we investigated the role of MexAB-OprM efflux pump. Transcription level of efflux pump genes *MexA, MexB, OprM, MexR* and *AmpC* were analyzed using Real-time PCR.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strains were isolated from patients of Children's Hospital Medical Center during 6 months (March and August 2012). All bacterial isolates were identified at microbiology lab using standard biochemical identification methods (8). A total of 45 samples were collected from various clinical specimens such as urine (n=21), exudates (n=11), eye (n=2), ear (n=2), CSF (n=2), blood (n=2), trachea (n=2) and lung secretions (n=3). *P.aeruginosa* PAO1 strain which has entirely sequenced genome was used as the reference wild type strain throughout the study.

Antimicrobial susceptibility tests. Antimicrobial susceptibility tests were performed using disk diffusion and minimum inhibitory concentration (MIC) methods. Antibiotics used in this study were chosen randomly from different antibiotic classes that were used mostly in this hospital. Antibiotics used in the disk diffusion method were cephalothin (30 µg), cefepime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg) (flouroquionolones), meropenem (10 µg), imipenem (10 µg) (carbapenems), piperacillin/tazobactam (10/100 µg) (β-lactams), gentamicin (10 µg) and amikacin (30 µg) (aminoglycosides). All antibiotic disk were purchased from Mast Company (UK).

Determination of MICs for each antibacterial agent was performed by micro broth dilution method. Antibiotics used in this study were cefuroxime, ceftazidim, cefazolin, ceftriaxone (cephems), meropenem (carbapenems), amikacin, tobramycin (aminoglycosides), aztreonam (monobactam), ampicillin, piperacillin (penicillins), colistin (lipopeptides), gatifloxacin, nalidixic acid (flouroquionolones), piperacillin-tazobactam, tazobactam (β-lactams) (Mast, UK).

Isolation of total RNA and cDNA synthesis for RT-PCR. Total RNA was extracted using RNA extraction kit (Fermentas, Lithuania), and converted into cDNA using the cDNA synthesis kit (Fermentas, Lithuania) according to the manufacturer's instruction and the quality and purity of the RNA obtained was evaluated using spectrophotometer.

PCR reaction. The final optimized PCR reaction consisted of 1 µl of each primer (10pmol) (Table 1), 0.5 µl dNTP (10mM), 0.5 µl MgCl (100mM), 0.2 µl (1 unit) Taq DNA polymerase (Metabion, Germany), 2.5 µl PCR buffer (10X), and 0.5 µl of DNA template (100µg/ml) in total volume of 25 µl with double distilled water. The cycling program was adjusted as follows: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 45 sec, 50-72 °C (45 sec), 72 °C (1 min) and a final extension at 72 °C for 10 min (9).

Real-time PCR reaction. Real-time quantification of cDNA was carried out on an ABI step one detection system (Applied Biosystems, UK) using the SYBR green PCR master mix. Real-time PCR was

Table 1. Primers used in PCR and real-time PCR

Genes	Primers (5'-3')	PCR product size (bp)	Reference
MexA	F: 5'acctacgaggccgactaccaga-3' R: 5'- gttggtcaccagggcgccttc-3'	179	24
MexB	F: 5'- gtgttcggctcgcagtactc-3' R: 5'- aaccgtcgggattgaccttg-3'	244	30
OprM	F: 5'- ccatgagccgccaactgtc-3' R: 5'- cctggaacgccgtctggat-3'	205	31
AmpC	F: 5'- ggtgcagaaggaccaggcacagat-3' R: 5'- cgatgctcgggttggaatagaggc-3'	97	26

used to investigate the expression level of each gene in MexAB-OprM efflux pump to expression each of these genes of separately measured by relative quantitation Real-time PCR. The optimized reaction consisted of master mix (10X), 1 µl of each primer (10 pmol), and 0.5 µl of template DNA (100 µg/ml) in a total volume of 20 µl.

Relative expression values (R) were determined using the ΔΔCt method. *P. aeruginosa* strain PAO1 was used as a standard strain for normalization of relative mRNA levels.

AmpC gene was considered as a housekeeping gene and all gene expressions were compared with *AmpC* gene expression. The assay was performed three times for each sample and the mean of three obtained quantities was considered as quantity. Expressions of all genes were calculated using the $2^{-\Delta\Delta Ct}$ method (fold). Cycle of threshold (C_t) was considered as the average threshold cycle number from three independent experiments.

The real-time PCR apparatus was programmed as follows: initial denaturation at 95 °C (15 min) followed by 40 cycles of 95 °C (15 sec), 61 °C (15 sec), 71 °C (20 sec) and melt curve at 61 °C (1 min) and 95 °C for 15 sec. To obtain reproducibility of the reaction, the quantification of standards was run in triplicate. *AmpC* gene was considered as a housekeeping gene and the results were compared with gene expression in a susceptible PA reference strain. Primer dimers and other artifacts were evaluated by melting curve analysis. To confirm that specific amplification had occurred, melting curves of each amplicon were assessed. Relative expression values (R) were determined using the ΔΔCt method. *P. aeruginosa* strain PAO1 was used as a standard strain for normalization of relative mRNA levels.

Real-time PCR efficiencies were acquired by amplification of a standardized dilution series of the template cDNA and were determined for each gene as the slope of a linear regression model. PCR efficiency was determined by measuring the CT Ct to a specific threshold for a serial dilution of cDNA. The corresponding real-time PCR efficiencies were then calculated according to the equation: $E = (10^{-1/slope}-1) \times 100$ (10-12).

RESULTS

Demographic data. Totally, 45 strains of *P. aeruginosa* were isolated from patients aged 2 months to 12 years who were referred to Children's Medical Center Hospital, Tehran, Iran. The majority of isolates were collected from patients hospitalized in PICU (n=18, 39%), NICU (n=5, 11%) followed by emergency ward (n=3, 7%), nephrology (n=3, 7%), surgery (n=5, 11%), neurology (n=3, 7%), urology (n=5, 11%) and infectious ward (n=3, 7%). Most of the strains were isolated from male children aged 10-15 months.

Antibacterial susceptibility testing. The most antibiotic resistance based on CLSI reference guidelines for disk diffusion method was detected for cephalothin (n=41, 92%) whereas MICs should the following frequencies for antibiotic resistance: cefuroxime (n=41, 91%), ceftazidime (n=42, 93%), amikacin (n=38, 84.5%), cefazolin (n=40, 89%), aztreonam (n=42, 93%), piperacillin (n=39, 86.5%), tazobactam (n=41, 91%) and piperacillin-tazobactam (n=42, 93%). Antibiotics such as colistin (15%), ceftriaxone (33%) and tobramycin (22%) showed the highest susceptibility rates against the isolates (Table 2).

PCR and Real-time PCR. The best annealing temperature was obtained at 61 °C. The results of real-time PCR for the genes of MexAB-OprM efflux pump have been shown in Table 3. According to the results of antibiotic resistance via disk diffusion method, overexpression of MexAB-OprM genes was associated to the resistance towards cephalosporin while via MIC method overexpression of MexAB-OprM was seen in those showing resistance towards special antibiotics especially fluoroquinolones, cephalosporin and beta lactams.

The reproducibility of the expression levels of each gene was measured three times. Different expression of MexAB-OprM genes was observed (Fig. 1). *P. aeruginosa* clinical isolates demonstrated increased level of *MexA* (≥ 2 folds), *MexB* (from 2.2 to 12.0 folds), *OprM* (≥ 2 folds) and *AmpC* (≥ 10 folds) at transcriptional mRNA level, respectively.

Among 45 resistant isolates, overexpression of *MexA* gene was observed in 25 isolates (55.5%), *MexB* in 24 isolates (53.3%) and *OprM* in 16 isolates (35.5%). In 28 isolates (62%), overexpression was observed in one of the three genes of MexAB-OprM efflux pump.

The ratio values obtained for each of the four genes in samples showed that the expression levels of *MexA* gene (n=25, 89%) was between 1.8 and 11.7 (mean= 6.7), *MexB* (n=24, 85%) between 1.3 and 14 (mean= 7.6) and *OprM* gene (n=16, 57%) between 1 and 9 (mean= 5).

Primer dimers and other artifacts were evaluated by melting curve analysis. To confirm that specif-

Table 2. Pattern of determined antibiotic resistance among *P. aeruginosa* strains using disk diffusion and MIC methods

Antibiotics	Disk diffusion results		
	Resistant (%)	Intermediate (%)	Sensitive (%)
Cephalothin	41 (92)	1 (1)	3 (7)
Cefepime	27 (60)	3 (7)	15 (33)
Ciprofloxacin	14 (32)	8 (17)	23 (51)
Meropenem	15 (34)	6 (12.6)	24 (53.3)
Ceftazidim	24 (55)	5 (10)	16 (35)
Piperacillin/tazobactam	10 (26)	8 (14)	27 (60)
Imipenem	12 (28)	2 (3)	31 (69)
Gentamicin	35 (78)	5 (11)	5 (11)

Antibiotics	MIC results		
	Resistant (%)	Intermediate (%)	Sensitive (%)
Cefuroxime	41 (91)	4 (9)	0
Cetriaxone	15 (33)	10 (11.2)	25 (55.5)
Ceftazidim	25 (56)	3 (7)	17 (38)
Piperacillin	39 (86.5)	5 (11)	1 (2.5)
Meropenem	16 (35)	10 (23)	19 (42)
Colistin	7 (15)	10 (23)	28 (62)
Amikacin	38 (84.5)	5 (11)	2 (4.5)
Gatifloxacin	40 (89)	4 (9)	1 (2)
Cefazolin	40 (89)	5 (11)	0
Nalidixic acid	41 (91)	4 (9)	0
Aztreonam	42 (93)	3 (7)	0
Piperacillin/tazobactam	12 (27)	5 (11)	28 (62)
Ampicillin	35 (78)	4 (9)	6 (13)
Tazobactam	41 (91)	1 (2)	3 (7)
Tobramycin	10 (22)	10 (22)	25 (56)

Table 3. The expression of MexAB-OprM efflux pump genes in *P. aeruginosa* strains isolated from children

Patients	Gender	Age (Months)	Expression level (folds)			
			AmpC (Ct)	*MexA*	*MexB*	*OprM*
A1	F	20	27.9	9.2	8.7	6.4
F1	M	12	25.7	4.8	5	3.3
A2	M	11	27.6	9.6	10.8	5.4
E1	M	5	28.3	12	14	9.3
C1	F	9	27.9	9.1	10.5	6.3
A3	F	10.5	27.6	8.2	7.3	5.3
G1	M	15	26.1	3.7	3.5	3
D1	M	10	28.0	8.6	5.3	5.8
H1	M	8	25.3	3.9	1.3	2.4
A4	M	65	26.1	4.2	5.1	3.2
E2	F	6	25.1	5.5	6.8	5.2
D2	F	10	24.9	2.7	3	2.2
A5	M	14	26	3.8	4.1	1.8
C2	F	10	24.5	2.2	2.7	1.7
A6	M	12	25.4	3.3	3.1	1.6
A7	M	16	27.9	7.5	8.6	3.1
E3	F	4	25.2	2.7	2.6	1.9
A8	M	50	22.1	1.8	1.9	1.5
A9	F	11	27.1	5.1	4.4	2.1
H2	M	15	27.16	2.7	5.1	1.1
A10	M	15	28	11.3	5	1
B1	F	53	27.1	2.8	5.7	1
D3	M	14	27.1	4.4	4.3	3
A11	F	74	28.1	11.1	7.1	2.3
G2	M	10	25.3	2.7	3.9	1.2
A12	F	7	24.2	1.9	2.7	1.4
H3	M	12	2	2.1	2.2	1.7
B2	M	11	22.5	2	2.1	1.6
A13	F	20	28.1	1	1.5	0.6
B3	M	12	26.0	1.8	1.2	1
H4	F	8	25.1	1.5	1.4	0.8
D4	M	5	2	1.2	1	0.5
A14	M	24	24.9	1.6	1.3	0.7
A15	F	6	27.5	1	1.2	0.8
H5	M	7	26.9	0.8	0.6	0.5
F2	F	14	27.1	0.5	1	0.9
E4	M	10	28.6	0.2	0.5	0.1
D5	F	6	26.5	1.2	0.8	1
A16	M	5	24.1	1.4	1.1	0.8
E5	M	11	27.5	1	0.9	0.2
G3	F	10	28.3	0.5	1	0.8
B3	M	18	26.8	0.7	1.2	1.5
A17	F	12	25.9	1.3	0.8	1
F3	M	15	27.9	0.9	0.5	0.6
A18	M	30	28.7	0.4	0.2	0.2

ic amplification (Fig. 1) had occurred, the melting curves of each amplicon were assessed.

DISCUSSION

P. aeruginosa is one of the most important causes of nosocomial infections due to the presence of various resistant elements (11). During last decades, the emergency of multidrug resistant *P. aeruginosa* has been observed worldwide. In this study, 28-35% of studied *P. aeruginosa* isolates were resistant to carbapenems which was in accordance with previous studies (13, 14). High frequency of cephalosporin resistance was also observed among *P. aeruginosa* isolates similar to the other reports (14, 15). Among all isolates, resistance to piperacillin/tazobactam was low (26-27%) which was in agreement with our previous report (13) and was higher than Ghazi et al. report (16). In this study colistin showed 15% resistance which can be suggested as an effective antibiotic for treatment of PA infections. This finding is similar to the results of study performed by Alekshun et al. in US (17).

At present, the efflux pump has been recognized as one of the significant complexes involved in resistance to most of the classes of antibiotics (11, 18, 19). It has been reported that the prevalence of efflux pump overexpression in clinical *P. aeruginosa* strains of ranged from 14-75% (20, 21). There are rare reports on prevalence of efflux pump overexpression in our country (22, 23) and there is no investigation on PA strains isolated from children. In the present study the increased expression level of *MexAB-OprM* genes of efflux pump simultaneously was 25% which was relatively more than Dumas et al. findings in Switzerland (11) and Mesaros et al. in Belgium (24).

In this study, 28 of 45 patients (62%) showed an increased expression level of efflux pumps *MexAB-OprM* genes that was similar to the studies reported more than 50% overexpression level of these genes (24-27). In Arabestani et al. study all the isolates (n=31; 100%) showed overexpression of efflux pump *MexAB-OprM* genes (23). According to Aghazadeh et al. report, overexpression of *mexA* was 74% among the isolates (28).

Since the concomitant overproduction of two Mex pumps might have additive effects on being resistant to antibiotics (29) evaluating the co-expression of multi-component efflux pumps other than MexAB-OprM is recommended.

CONCLUSION

Development of novel antibiotics that can bypass the effects of efflux pumps is still a challenging task. Further studies on involved mechanisms and structure-function association of bacterial efflux systems as

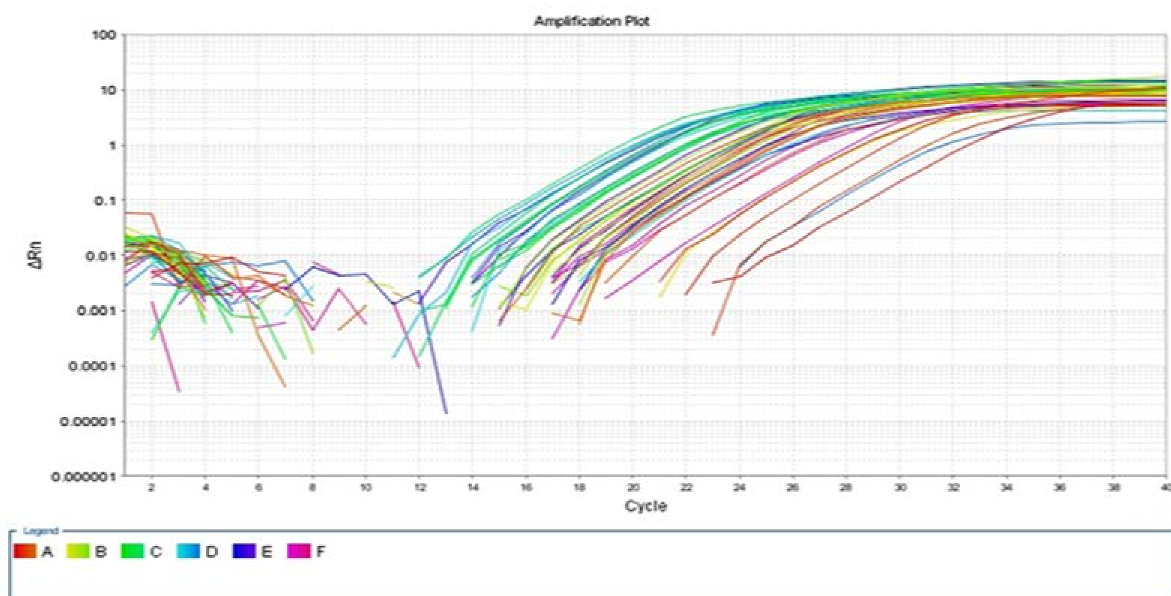

Fig. 1. Confirmation of specific amplifications during real-time PCR on MexAB-OprM genes of efflux pump

well as the interactions between the pumps and other resistance mechanisms are highly recommended.

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