Original Article

Efflux pump regulatory genes mutations in multidrug resistance *Pseudomonas aeruginosa* isolated from wound infections in Isfahan hospitals

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Abstract

Background: Multidrug resistance *Pseudomonas aeruginosa* (MDR-*P. aeruginosa*) is a worldwide threat for public health. Hyperexpression of efflux pump systems (MexAB-OprM and MexCD-OprJ), which is a well-known mechanisms for MDR emerging, is controlled by regulatory genes, *mex*R and *nfx*B, respectively. The aim of this study was to evaluate point mutations in *mex*R and *nfx*B genes in MDR-*P. aeruginosa* isolated from wound infections.

Materials and Methods: A total of 34 *P. aeruginosa* cultures obtained from wound infections were analyzed. Among them eight isolates identified as MDR-*P. aeruginosa* and were subjected to determination of mutations in *mex*R and *nfx*B genes.

Results: We detected eight-point mutations in *mex*R and 12-point mutations in *nfx*B. The most common mutations were common G327-A (eight isolates), G384-A (eight isolates), G411-A (eight isolates). Mutations in A371-C and A372-C were the predominant substitution which was seen in *nfx*B. Amino acid substitutions were also found at position 124 and 126 for NfxB and MexR, respectively.

Conclusions: *P. aeruginosa* isolates with mutation in efflux pump regulatory genes such as *mex*R and *nfx*B could be a main factor contributed to antibiotic resistance and must be considered in antibiotic treatment.

Key Words: Efflux pump, *mex*R, *nfx*B, *Pseudomonas aeruginosa*

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INTRODUCTION

Pseudomonas aeruginosa, which is known as a nonfermentative gram negative opportunistic pathogen, is the leading cause of diverse infections including pneumonia, wound and urinary tract infection (UTI).[1] Because of resistance to many antibiotics, treatment of infections caused by *P. aeruginosa* is difficult.[2] Several mechanisms have been known for antibiotic resistance, for example, inactivation or modification of the antibiotic, alteration, or modification in the target site of the antibiotic, and decreased intracellular drug concentration by overexpression of efflux pump.[3] Until recently, in *P. aeruginosa* five super family of efflux pumps including (i) the ATP-binding cassette (ABC) family, (ii) the small multidrug

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resistance family, (iii) the major facilitator super family, (iv) the resistance-nodulation-division (RND) family, and (v) the multidrug and toxic compound extrusion family have been recognized.[4] Analysis of *P. aeruginosa* genome showed that all five super families are presented but RND family is predominant.[5] RND pumps consist of three subunit including membrane fusion protein (MFP), an outer membrane factor (OMF), and a cytoplasmic membrane transporter.[5,6] MexAB-OprM and MexCD-OprJ, which are members of RND efflux pumps family, have the wide substrate of antibiotics such as, β-lactam class (aztreonam, ceftazidime) and fluoroquinolones.[7] Active efflux pumps decrease intracellular concentration of drug by pumping it to out. Expression of MexAB-OprM and MexCD-OprJ are regulated by *mex*R and *nfx*B, respectively.[7-9] MexR consisting of 147 amino acid residues, negatively regulate MexAB-OprM expression.[8] Mutation in *mex*R (nalB strains) and *nfx*B compromised regulatory functions and therefore resulting in efflux pumps hyperexpression.[8,9] Several independent studies have revealed that overexpression of mentioned efflux pumps contributed to multi-drug resistance (MDR) phenotype.[10-12] MDR *P. aeruginosa* is defined as resistance to at least three antibiotics belonged to different classes especially, carbapenems, fluoroquinolones, and aminoglycosides.[13] Due to limited choice of effective drug, mortality rates in patients infected with MDR *P. aeruginosa* is higher in comparison with multiple drugs susceptible *P. aeruginosa*.[14,15] Therefore, in order to have appropriate therapy for patients infected with *P. aeruginosa*, identification of multidrug resistance isolates is essential. In this study, our aim was to detect *mex*R and *nfx*B mutations in MDR- *P. aeruginosa* isolated from wound infections.

MATERIALS AND METHODS

Sample collection and identification

This study was conducted between January 2013 and march 2013 at three major hospitals of Isfahan, Iran. During the study, a total of 100 wound samples that were obtained from wound infections were analyzed. *P. aeruginosa* identification performed based on standard test such as Gram staining, catalase, oxidase, oxidative-fermentative (OF) test, pigment production, and growth at 42°C.[1,16] Subsequently, primary identification confirmed with PCR by using ITS (16s-23s rRNA internal transcribed spacer) gene specific primer.[17]

Antibiotic susceptibility testing

Antibiotic susceptibility of the isolates was ascertained by Kirby-Bauer disk diffusion method according to CLSI (clinical laboratory standard institute) guidelines.[18] In this study, following antibiotic disks (MAST, UK) were used: ceftazidime (30 μg), cefotaxime (30 μg) aztreonam (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), meropenem (10 μg), and amikacin (30 μg). *P. aeruginosa* ATCC 27853 was used for quality control.

Preparation of genomic DNA

For DNA extraction, two or three colony of fresh culture of *P. aeruginosa* was dissolved in 300 ml of lysis buffer containing (Tris 100 mmol, Nacl 50 mmol, and EDTA 25 mmol, pH = 7.5) completely. Subsequently, suspension was boiled at 95°C for 15 min. Equal volumes of phenol and chloroform (25:24, pH = 7.5) was added, mixed thoroughly, and centrifuged at 9000 g for 5 min. Aqueous–viscous supernatant was transformed to a fresh micro tube, phenol/chloroform (25:24) was added again and centrifuged at 9000 g for 5 min. To DNA precipitation, 600 μl of cold pure ethanol (Merck, Germany) was added and centrifuged at 13000 g (4°C, 20 min). Obtained DNA after washing with 70% ethanol was stored at –20°C.

*mex*R and *nfx*B amplifications and sequencing

PCR for *mex*R (503bp) and *nfx*B (731 bp) amplification were carried out in separate 25 μl reaction mixture consist of 10 pmol of each primer (Metabion, Germany), *mex*R-F (5′-CTGGATCAACCACATTTACA -3′), *mex*R-R (5′-CTTCGAAAAGAATGTTCTTAAA-3′), *nfx*B -F (5′-ACGCGAGGCCAGTTTTCT-3′) and *nfx*B-R (5′-ACTGATCTTCCCGAGTGTCG-3′), 2.5 μl (25 mMol $Mgcl_2$), 200 μM dNTP, 2.5 μl PCR buffer 10X, 1.25 u taq DNApol (Cinna gen, Iran), and 5 μl DNA template.[19,20] Cycling condition performed in 35 cycles including, denaturation at 95°C for 1 min, annealing at 55°C for *mex*R and 60°C for *nfx*B, 45 s, extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR products were visualized on 1.5% agarose gel stained with DNA green viewer dye [Figures 1 and 2]. PCR products were sent for BIO NEER (Korea) company for purification and sequencing on both strands. We used Mega4 as multiple sequence alignment software for sequences analyzing.

RESULTS

Eight of 34 nonrepetitive *P. aeruginosa* were identified as MDR-*P. aeruginosa*. All isolates showed resistance to ceftazidime, cefotaxime, aztreonam, ciprofloxacin, imipenem, and meropenem. Our results showed seven silent mutations including G327-A (eight isolates), G384-A (eight isolates), G411-A (eight isolates), C223-T (five isolates), C264-T (two isolates), G60-A (one isolate), C168-T (one isolate) in *mex*R gene. Only one missense mutation which changed val

126 to Glu was seen [Table 1]. Four isolates had five different point mutations simultaneously [Table 1]. In *nfx*B, 12-point mutations were found. Mutations at position A371-C and A372-C were the predominant substitution. We also found missense mutations in codon 124 that changed Glu to Ala [Table 1]. The GenBank accession numbers of *mex*R and *nfx*B which determined in this work are as follow: KF675781-88.

DISCUSSION

Resistance to multiple antibiotics in *P. aeruginosa* as one of the most common etiological agents of wound infections is well-known property.[21] Treatment of MDR-*P. aeruginosa* is difficult due to limited choice of antibiotics.[22] Generally, antibiotic resistance mechanism in *P. aeruginosa* is divided into two categories, intrinsic and acquired resistance. Low permeability of outer membrane protein, constitutive

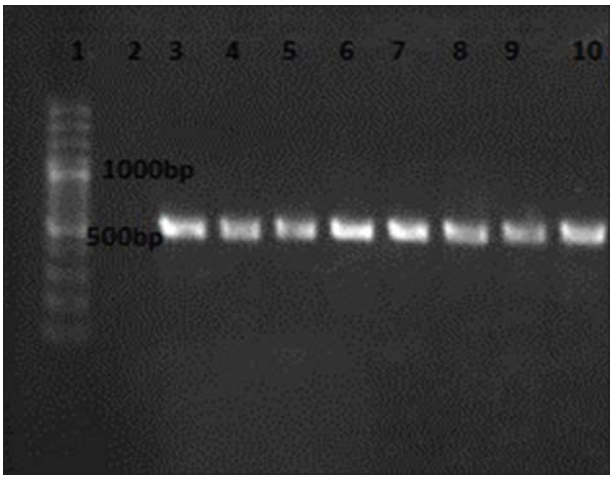

Figure 1: Gel image of representative PCR of mexR gene. Line1 Ladder, Lane 2 negative control, line 3 positive, line 4 to 10 clinical specimens

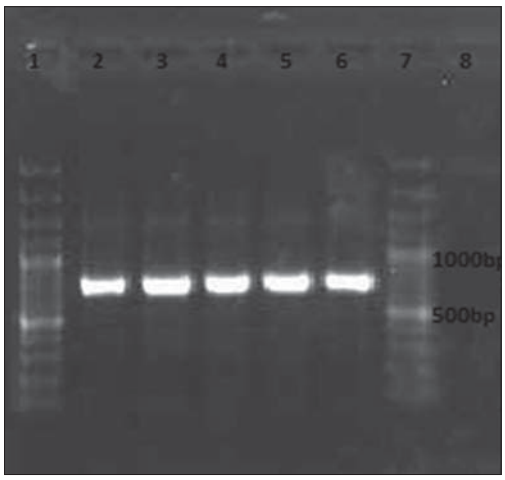

Figure 2: Gel image of representative PCR of nfxB gene. Line1, 7 Ladder, Lane 2 positivecontrol, line 3 to 6 clinical specimens, line 8 negative control

Table 1: Mutations in *mex*R, *nfx*B in MDR-*Pseudomonas aeruginosa* isolated from wound infections

Isolates	*mex*R	*nfx*B	Antibiotic resistance
1	G60-A G327-A T377-A (val 126-Glu) G384-A G411-A	A371-C (Glu 124-Ala) A372-C A387-G T555-G	[a]R: [b]AZ, CZ, CT, CP, MR, IM
2	C223-T G327-A T377-A (val 126-Glu) G384-A G411-A	A371-C (Glu 124-Ala) A372-C G423-A T480-C T537-C C543-T T555-G	R: AZ, CZ, CT, CP, MR IM, AM
3	C223-T G327-A T377-A (val 126-Glu) G384-A G411-A	A371-C (Glu 124-Ala) A372-C G423-A T480-C T537-C C543-T T555-G	R: AZ, CZ, CT, CP, MR IM, AM
4	C223-T G327-A T377-A (val 126-Glu) G384-A G411-A	G39-A A183-G A371-C (Glu 124-Ala) A372-C G423-A T480-C A486-T T537-C C543-T T555-G	R: AZ, CZ, CT, CP, MR IM, AM
5	C223-T G327-A T377-A (val 126-Glu) G384-A G411-A	G39-A A183-G A371-C (Glu 124-Ala) A372-C G423-A T480-C A486-T A536, T537 deletion C543-T T555-G	R: AZ, CZ, CT, CP, MR IM, AM
6	C223-T G327-A T377-A (val 126-Glu) G384-A G411-A	A371-C (Glu 124-Ala) A372-C G423-A T480-C T537-C C543-T T555-G	R: AZ, CZ, CT, CP, MR, IM, AM
7	C168-T C264-T G327-A	A371-C (Glu 124-Ala) A372-C	R: AZ, CZ, CT, CP , MR, IM, AM

Continued

Table 1: Contd...

Isolates	*mex*R	*nfx*B	Antibiotic resistance
	T377-A	T480-C	
	(val 126-Glu)	T537-C	
	G384-A	C543-T	
	G411-A	T555-G	
8	C264-T	A371-C	R: AZ, CZ, CT, CP, MR IM
	G327-A	(Glu 124-Ala)	
	T377-A	A372-C	
	(val 126-Glu)	T480-C	
	G384-A	T537-C	
	G411-A	C543-T	
		T555-G	

a: R: Resistance, b: AZ: Aztreonam, CZ: Ceftazidime, CT: Cefotaxime, CP: Ciprofloxacin, MR: Meropenem, IM: Imipenem, AM: Amikacin

expression of AmpC and efflux pumps is example of intrinsic resistance mechanism.[23-25] MexAB-OprM and MexCD-OprJ are two preponderant efflux pumps that contribute to intrinsic resistance of *P. aeruginosa* to different antibiotics.[3] MexAB-OprM and MexCD-OprJ are negatively regulated by MexR and NfxB, respectively; hence, mutations in these regulatory genes lead to overexpression of mentioned efflux pumps and multiple drug resistance emerging.[19,26] In our study mutations of *mex*R and *nfx*B in MDR-*P. aeruginosa* isolated from wound infections were investigated. In *mex*R, 8-point mutations were detected, seven of them were silent. Only the mutation in thymine 377 and conversion to adenine leads to substitution of Val with Glu in codon 126. This substitution had related to antibiotic resistance, according to similar studies.[19,27,28] Results of several independent studies revealed different mutations, for example, Suman and colleagues reported 24 silent and four missense mutations in 14 clinical isolates of *P. aeruginosa*.[29] Different amino acids substitution with relation to antibiotic resistance was seen in codon 21, 95, 30, 79, 106, 114, 78, 107, 53, and 103 of *mex*R gene.[10,12,29,30] Although mutation in *mex*R is one of the main cause of MexAB-OprM overexpression but its expression and DNA binding activity in order to prediction of antibiotic effectiveness should be evaluated. Based on our results, mutations in *nfx*B were relatively high. The *nfx*B mutations consisted of 11 silent mutations, one missense mutation and a deletion from position 536 to 537. Mutations in position 371 and 372 were the most frequently replacement in all isolates [Table 1]. Analysis of NfxB showed that DNA binding domain is located between amino acid residues 26 and 42.[12] There was not any mutation mentioned region in this study. Missense mutation was seen in Glu 124-Ala of NfxB. Similar study revealed that change in codon 124 was related to ciprofloxacin resistance.[12] Results of another study of Fluoroquinolone resistance *P. aeruginosa* showed that mutation in codon 82 (Arg to Leu) was the most frequently substitution in *nfx*B.[31] Hyperexpression of efflux pumps such as MexAB-OprM and MexCD-OprJ are complicated. Isolates of *nal*C and *nal*D mutants had mutations in PA-3721 and PA-3574 genes showed overexpression of MexAB-OprM independent from *mexR* mutations.[32,33] Therefore, expression of MexAB-OprM is not only regulated by MexR but also affected by PA-3721 and PA-3574 genes. In order to know preponderant mutations, further study for evaluation of expression *mex*B, *mex*C, *opr*M and *opr*J by realtime PCR is necessary.

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