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Determination of carbapenem resistance mechanism in clinical isolates of *Pseudomonas aeruginosa* isolated from burn patients, in Tehran, Iran

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ABSTRACT

Carbapenems are the most important therapeutic options that effect against serious infections caused by multidrug resistant *Pseudomonas aeruginosa* (MDR-PA) isolates. Carbapenems resistant isolates of *P. aeruginosa* are increasing worldwide. The aim of this study was to determine the carbapenem resistance mechanisms in clinical *P. aeruginosa* isolates from burn patients, in Tehran, Iran. A total of 53 non-duplicated isolates of carbapenem-resistant *P. aeruginosa* were collected from burn patients. The presence of carbapenemase genes were determined by PCR. AmpC overproducer isolates were detected by phenotypic method. The mutation and transcription level of *oprD* were determined by PCR-sequencing and quantitative Real-time PCR (RT-PCR), respectively. Twenty-seven (50.9%) isolates were positive for carbapenemase (bla_{VIM} = 25 and bla_{IMP} = 2) and showed high-level resistance to imipenem and meropenem. Twenty-eight isolates were AmpC overproducers. All isolates had a mutation in the *oprD* gene and down-regulation of *oprD* was found in 56.6% of MDR-PA isolates. Although the presence of carbapenemase is the common mechanism of resistant to carbapenem, but carbapenem resistance was found by *oprD* mutation-driven and the AmpC overproducing isolates in Tehran, Iran.
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1. Introduction

Pseudomonas aeruginosa is one of the most important causes of nosocomial infections especially in patients with secondary infection associated with burns and cystic fibrosis [1]. Infections caused by multidrug-resistant *P. aeruginosa* (MDR-PA) can be fatal for involved patients [2]. Carbapenems are among the last-line antibiotics for infections caused by MDR-PA [3]. Carbapenem-resistant isolates are gradually increasing worldwide and in Iran [2,3]. The mechanisms of resistance to carbapenems are multifactorial, including production of carbapenemase such as metallo beta-lactamase (MBLs), AmpC overproducer, mutation in *oprD* gene and over-expression of efflux-pumps [3,4]. MBLs are important groups of β-lactamases, which can hydrolyze carbapenems and extended spectrum cephalosporins [5,6]. AmpC β-lactamases are weakly able to hydrolysis the carbapenems and usually, are expressed constitutively at a low level in *P. aeruginosa* [6,7]. Mutations in *amp*C locus may lead to overexpression of AmpC β-lactamases in *P. aeruginosa* isolates and cause resistance to carbapenems [2,6]. Porin proteins in outer membranes such as OprD is a channel for entrance of amino acids and carbapenems especially imipenem in *P. aeruginosa* [1,2]. Therefore, lose of OprD or reduced OprD protein expression could cause a resistance to imipenem in *P. aeruginosa* [1,2]. Active efflux or overexpression of efflux-pumps is a responsible mechanism for moving antibacterial agents such as antibiotics from inside to outside of the cell. The aim of this study was to determine the carbapenem resistance mechanisms in carbapenem-resistant isolates of *P. aeruginosa* collected from burn patients, in Tehran, Iran.

2. Materials and methods

2.1. Bacterial isolates

Totally, fifty-three non-duplicated carbapenem resistant *P. aeruginosa* were collected from 130 burn patients who were admitted to Shahid Motahari Hospital during June 2011 to March 2012 in

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Tehran, Iran. Bacterial identification was performed by standard biochemical tests [8] such as Gram staining, colony morphology, non-lactose fermentation on MacConkey agar, oxidase and catalase tests, Triple Sugar Iron agar (TSI) and oxidative-fermentative (OF) test.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of isolates were determined using the disk diffusion method according to the Clinical and Laboratory Standards [9]. The following antibiotics (MAST, UK) were tested: Imipenem (IMI: 10 μg), meropenem (MEM: 10 μg), aztreonam (ATM: 30 μg), carbenicillin (CB: 30 μg), amikacin (AK: 10 μg), gentamicin (GM: 10 μg), ciprofloxacin (CIP: 5 μg), cefepime (CPM: 30 μg), ceftazidime (CAZ: 30 μg) and polymyxin B (PB: 300unit). The minimum inhibitory concentrations (MICs) of IMI and MEM (Jaber Ebne Hayyan Pharmaceutical Co. Iran) were determined by the microbroth dilution method. Susceptibility breakpoints were defined according to CLSI recommendations [9]. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains in antimicrobial susceptibility testing and MICs.

2.3. AmpC overproducer isolates

Isolates were considered as AmpC overproducers when there was at least a twofold dilution difference between each one of the followings: MICs of IMI or MEM, MICs of IMI or MEM plus 250 μg cloxacillin (Sigma-Aldrich, Product Number: 27555) [10].

2.4. DNA extraction and amplification of carbapenemas genes

The genomic DNA from isolates was prepared using genomic DNA extraction kit (tip 100; Bioneer, Korea) according to the manufacturer's instructions. Detection of bla_{VIM}, bla_{IMP}, bla_{SMP}, bla_{SIM}, bla_{GIM}, bla_{NDM}, bla_{AIM}, bla_{KPC}, $bla_{OXA\text{-}48}$, and bla_{GES} was done by PCR as described previously [3,11]. *P. aeruginosa* strain psa1 (GenBank accession No. KT313641.1) and *P. aeruginosa* strain PAFSHIRAN (GenBank accession No. JX644173.1) were used as the positive control for bla_{VIM} and bla_{IMP} [12].

2.5. PCR amplification and sequencing of the oprD gene

PCR amplification and sequencing of *oprD* were performed using primers listed in Table 1. PCR conditions were as follows: 95 °C for 5 min and 30 cycles of 95 °C for 45 s, 61 °C for 45 s, and 72 °C for 2 min. A final extension step was performed at 72 °C for 10 min. Sequences of both strands of the amplicons were determined at Macrogen (Seoul, South Korea) and were compared with *P. aeruginosa* PAO1 as a reference strain (GenBank accession No. CAA78448) (http://www.ncbi.nih.gov/BLAST).

2.6. RNA extraction and quantitative reverse transcription PCR (quantitative RT-PCR)

The quantitative RT-PCR was applied for determination the transcription level of *oprD* gene in carbapenem resistant isolates except in those with *oprD* gene disruption by insertion sequence (IS elements), according to sequencing results . Briefly, strains were grown in LB broth by using shaker incubator at 37 °C and 180 rpm to the late log phase (optical density at 600 nm [OD 600] = 0.8–1) and collected by centrifugation at 10,000 RPM in 5 min. Total RNA was extracted with Hybrid-R™ (GeneALL, Cat. No. 305-101), according to the manufacturer's recommendations and then RNase-Free DNase I (Thermo Scientific™) was used for elimination of DNA contaminations. Concentrations and quality of RNA were determined by spectrophotometer (NanoDrop 2000; Thermo Scientific). Reverse transcription (cDNA synthesize) was performed by the 2-step RT-PCR kit (Vivantis Co, Malaysia), according to the manufacturer's recommendations. The transcription level of the *oprD* gene was determined by relative quantitative RT-PCR as described using the standard curve method and using the RealQ Plus 2x Master Mix Green Kit (Ampliqon, Danmark) in Line-Gene K Thermal Cycler FDQ-48A BIOER (Hangzhou Bioer Technology Co., Ltd, China). Expression of the 30S ribosomal gene *rps*L was assessed in parallel to normalized transcriptional levels of target gene. Decreased expression of *oprD* genes was considered as relevant if expression of *oprD* was ≤30%, in compared with that of *P. aeruginosa* PAO1 [15]. The quantitative RT-PCR of *oprD* and *rpsL* was performed using primers listed in Table 1.

2.7. Statistical analysis

Statistical analysis of data was carried out using the SPSS 16 statistical software. We used the χ^2 and T-test analysis for comparison of data. A difference was considered statistically significant at P-value of ≤0.05.

3. Results

Among 53 *P. aeruginosa* isolates, 71.6% (38/53) were from males and 28.4% (15/53) from females. All isolates were recovered from wounds of burns' patients. All isolates were resistant to imipenem, meropenem, aztreonam, carbenicillin, amikacin, gentamicin, ciprofloxacin and cefepime. Twenty-six percent (14/53) of isolates were susceptible to ceftazidime and all isolates were susceptible to polymyxin B. All carbapenem resistant isolates showed a high-level resistance to carbapenems. The range of MIC to IMI and MEM were 16–512 μg/ml and 4–128 μg/ml, respectively (Table 2). Fifty-two percent (28/53) of isolates were AmpC overproducers and AmpC overproduction caused an increase in the MICs of imipenem, but not meropenem (Table 2). The PCR assay of all carbapenem resistant isolates revealed that 47.1% (25/53) and 3.7% (2/53) of isolates were positive for bla_{VIM} and bla_{IMP}, respectively and all isolates

Table 1
Primers used in this study.

Primer name	Primer sequence (5′-3′)	Amplicon size (bp)	Reference	Use
oprD	F-CGCCGACAAGAAGAACTAGC R-GTCGATTACAGGATCGACAG	1413	[10]	Amplification and Sequencing of *oprD*
	F-TCGATGCCTTCGGCTACCT F-GCGCTTGATCACCCGGAATG F-GGTAGCCGTATACACCTCCG		This study	Used for sequencing of IS elements in *oprD*
	F-TCCGCAGGTAGCACTCAGTTC R-AAGCCGGATTCATAGGTGGTG	191	[13]	Used for quantitative RT-PCR
rpsL	F-GCAACTATCAACCAGCTGGTG R-GCTGTGCTCTTGCAGGTTGTG	230	[14]	

Table 2
Distribution of susceptibility patterns, level of MICs to IMI and MEM, AmpC overproducer and carbapenemase genes (bla_{VIM} and bla_{IMP}) in 53 *P. aeruginosa* isolates resistant to carbapenem with *oprD* mutation.

Susceptibility patterns									MIC* (μg/ml)				AmpC	bla_{VIM}	bla_{IMP}	Mutations in *oprD*	No of isolates
IMI¥	MEM	ATM	CB	AK	GM	CIP	CPM	CAZ	IMI	IMI/COL	MEM	MEM/COL					
R#	R	R	R	R	R	R	R	**S**	16	4	16	16	+	–	–	IS*Ppu21*	3
R	R	R	R	R	R	R	R	**S**	16	4	32	16	+	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	S	16	4	32	32	+	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	**S**	16	8	16	16	–	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	R	16	16	8	8	–	–	–	98n Deletion	1
R	R	R	R	R	R	R	R	**S**	32	2	32	32	+	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	**S**	32	8	16	16	+	–	+	23n Deletion	1
R	R	R	R	R	R	R	R	**S**	32	8	32	16	+	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	**S**	32	8	32	32	+	–	+	75n Deletion	1
R	R	R	R	R	R	R	R	S	32	8	32	32	+	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	R	32	8	32	32	+	–	–	IS*Ppu21*	2
R	R	R	R	R	R	R	R	R	32	16	4	4	–	+	–	3n Deletion	1
R	R	R	R	R	R	R	R	R	32	16	8	8	–	–	–	15 n Deletion	1
R	R	R	R	R	R	R	R	R	32	16	16	16	–	+	–	13n Deletion	1
R	R	R	R	R	R	R	R	R	32	16	16	16	–	+	–	16n Deletion	1
R	R	R	R	R	R	R	R	R	32	16	64	64	–	–	–	7n Deletion	1
R	R	R	R	R	R	R	R	R	64	8	8	8	+	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	**S**	64	8	64	64	+	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	R	64	16	8	4	+	+	–	4 n Deletion	1
R	R	R	R	R	R	R	R	R	64	16	8	4	+	–	–	7n Deletion	1
R	R	R	R	R	R	R	R	R	64	16	8	8	+	+	–	Negative**	1
R	R	R	R	R	R	R	R	**S**	64	16	16	16	+	–	–	IS*Ppu22*	1
R	R	R	R	R	R	R	R	**S**	64	16	16	16	+	+	–	89n Deletion	1
R	R	R	R	R	R	R	R	R	64	16	32	16	+	–	–	4n Deletion	1
R	R	R	R	R	R	R	R	R	64	16	32	32	+	–	–	14n Deletion	1
R	R	R	R	R	R	R	R	**S**	64	16	32	32	+	+	–	3n Deletion	1
R	R	R	R	R	R	R	R	S	64	16	32	32	+	+	–	4n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	32	16	–	+	–	13n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	32	32	–	–	–	IS*Ppu21*	1
R	R	R	R	R	R	R	R	R	64	32	32	32	–	–	–	5n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	32	32	–	–	–	6n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	32	32	–	+	–	4n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	64	32	–	–	–	22n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	64	64	–	–	–	4n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	64	64	–	+	–	62n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	64	64	–	+	–	Stop codon	1
R	R	R	R	R	R	R	R	R	64	32	64	64	–	+	–	5n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	64	64	–	+	–	7n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	64	64	–	+	–	20n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	64	64	–	+	–	31n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	128	128	–	–	–	1n Deletion	1
R	R	R	R	R	R	R	R	R	64	32	128	128	–	+	–	17n Deletion	1
R	R	R	R	R	R	R	R	R	128	32	64	64	+	+	–	1n Deletion	3
R	R	R	R	R	R	R	R	R	128	32	64	64	+	+	–	3n Deletion	1
R	R	R	R	R	R	R	R	R	128	32	64	64	+	+	–	4n Deletion	1
R	R	R	R	R	R	R	R	R	128	64	64	64	–	+	–	1n Deletion	1
R	R	R	R	R	R	R	R	R	256	256	64	64	–	+	–	13n Deletion	1
R	R	R	R	R	R	R	R	R	512	256	64	64	–	+	–	19n Deletion	1

¥ IMI; Imipenem, MEM; Meropenem, COL; Cloxacillin, ATM; Aztreonam, CB; Carbenicillin, AK; Amikacin, GM; Gentamicin, CIP; Ciprofloxacin, CPM; Cefepime, CAZ; Ceftazidime, n; nucleotides.
R, resistant; S, susceptible.
* MIC; Minimum inhibitory concentration.
** *oprD* in this isolate was not detectable by PCR.

were negative for $bla_{OXA\text{-}48}$, bla_{KPC}, bla_{GES}, bla_{GIM}, bla_{AIM}, bla_{SPM}, bla_{NDM} and bla_{SIM} genes. All isolates had a mutation in the *oprD* gene and down-regulation of *oprD* was found in 56.6% of the carbapenem resistant isolates of *P. aeruginosa*. In 67.9% (36/53) of the isolates, *oprD* was defective by deletion mutations (by 1-98 bp deletion) and these deletion mutations were occurred in upstream of the *oprD* gene. The *oprD* gene in 26.4% (14/53) of carbapenem-resistant *P. aeruginosa* isolates was disrupted by IS*Ppu21* insertion sequence (KM053284.1: GenBank accession number for IS*Ppu21*) and in one isolate *oprD* was disrupted by IS*Ppu22* insertion sequence (KJ825703.1: GenBank accession number for IS*Ppu22*). No carbapenemase genes were detected in isolates for which *oprD* was disrupted by insertion sequence. One isolate had a premature stop codon by single point mutation and in one isolate *oprD* was not detectable by PCR (Table 3). The distribution of antibiotic resistance, level of MICs to imipenem and meropenem, carbapenemase genes and AmpC overproducer isolates in *P. aeruginosa* isolates with *oprD* mutation have been summarized in Table 2. Eighty percent (30/38) of carbapenem-resistants had a relevant decrease in *oprD* expression (≤30%) compared with that of the reference PAO1 strain. Expression of *oprD* could not be determined in isolates that *oprD* was disrupted by IS elements (n = 15).

4. Discussion

Several mechanisms such as carbapenemase production, *oprD* mutation, AmpC and efflux pumps overexpression are involved in carbapenems resistance among *P. aeruginosa* strains [2,3].

Table 3
Distribution of mutation and expression in the *oprD* gene in *P. aeruginosa* isolates resistant to carbapenems.

Type of mutation		Number of isolates	Decrease in *oprD* expression
Frameshift	1-bp deletion	5	4
	Deletion of 2–98 bp	31	26
	IS*Ppu21* insertion	14	Not determined
	IS*Ppu22* insertion	1	Not determined
Premature stop codon	Single mutation	1	Normal
Non detectable by PCR	Unknown, large change	1	Not determination

Deletion of 1–98 bp (nt 81–179).
IS*Ppu21*: 1194-bp IS(IS*Ppu21*) at nt 603, encodes a 326-aa putative type transposase.
IS*Ppu22*: 1232-bp IS(IS*Ppu22*) at nt 81, encodes a 293-aa putative type transposase.

Although, all isolates in this study were carbapenem resistant and MDR, nevertheless 13 (24%) isolates were susceptible to ceftazidime, indicating the importance of antimicrobial susceptibility testing in choosing of antibiotics for treatment of infections. In the present study, similar to several studies in Iran, bla_{VIM} and bla_{IMP} have been only carbapenemase genes that were identified in carbapenem-resistant isolates of *P. aeruginosa* [3,16,17]. Also, our study, was not in agreement with Hakemi Vala et al. and Bagheri Bejestani studies in Tehran, Iran, that reported no bla_{VIM} positive *P. aeruginosa* isolates from different clinical samples such as burns' patients [18,19]. However, bla_{VIM} rates vary greatly among different studies [16–19] which may reflect differences in infection control policies and other factors.

In our study, MIC to of imipenem in bla_{IMP} and bla_{VIM} positive isolates was ≥ 32 μg/ml but the MIC to imipenem was ≤ 16 μg/ml in bla_{IMP} and bla_{VIM} negative isolates. This finding, suggests that carbapenemase genes increase the resistance to carbapenems in bla_{IMP} and bla_{VIM} positive isolates compared to bla_{IMP} and bla_{VIM} negative isolates (Table 2).

AmpC overproducer and mutational inactivation of *oprD* are known to be the main mechanisms of carbapenem resistance especially to imipenem in the absence of acquired carbapenemases [2].

AmpC overproducer, usually when is combined with efflux systems over-expression and/or down-regulation *oprD*, has been proven to lead to carbapenem resistance or increase of 2–4 folds MIC to carbapenems [2,20,21]. As it is shown in table 2, AmpC overproducer was correlated with increase of resistance to imipenem and because of the 18 (64.2%) AmpC overproducer isolates, the MIC of imipenem (MICs: 16–512 μg/ml) was 2–4-folds higher than of meropenem (MICs: 4–128 μg/ml) ($P < 0.0001$).

In this study similar to other reports from Korea and Spain and other countries, *oprD* gene was defected by deletion mutation in 71.7% of isolates [10,21,22]. In our study, all mutation in *oprD* were occurred in the parts of the gene that were encoding loop 2 and 3 in OprD protein. The OprD porin has 8 loop structures on the external surface of outer membrane in *P. aeruginosa* [1]. Loop 2 and loop 3 on external surface have important roles in the entrance of carbapenems especially imipenem [1]. Therefore, mutations (substitution or deletion) within loop 2 and loop 3 have a potential to show an imipenem resistance phenotype [1].

In our study, *oprD* was disrupted by two IS elements including IS*Ppu22* and IS*Ppu21*. IS*Ppu22* is a novel insertion sequence in the *oprD* porin gene of a carbapenem-resistant *P. aeruginosa* and it was reported, for the first time, from Iran [23]. IS*Ppu21* was previously reported in *Pseudomonas putida* and possibly has been transferred from *P. putida* to *P. aeruginosa.* Also IS*Ppu21* was previously reported in *P. aeruginosa* by Estepa et al. in GenBank (GenBank accession number: JX440360).

In this study, transcriptional levels of *oprD* were decreased in 56.6% of imipenem and meropenem resistant isolates. Similar to our study, down regulation of *oprD* gene in carbapenem resistant *P. aeruginosa* isolates has been reported by several studies. Several studies in 2009 and 2012, have reported that down regulation of *oprD* was responsible for reduced susceptibility to imipenem among carbapenem resistant *P. aeruginosa* [10,13,24].

Finally, our results showed that OprD defective and AmpC overproducer are two important mechanisms that involved in carbapenem-resistant among *P. aeruginosa* isolated from burn patients in Tehran, Iran.

5. Conclusions

Although the production of carbapenemases is the common mechanism of resistance to carbapenems in *P. aeruginosa* but resistance to carbapenems can also be driven by *oprD* mutation and AmpC in *P. aeruginosa* isolated from burn patients, in Tehran, Iran.

Conflict of interests

There is no conflict of interests between authors for this work.

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