

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 *ampC* 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), 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-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 synthesis) 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 *rpsL* 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 ceftazidime. 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
<i>oprD</i>	F-CGCCACAAGAAGAACTAGC	1413	[10]	Amplification and Sequencing of <i>oprD</i>
	R-GTCGATTACAGGATCGACAG			
	F-TCGATGCCTTCGGCTACTT	191	[13]	Used for quantitative RT-PCR
	F-GCGCTTGATCACCCGGAATG			
	F-GGTAGCCGTATACACTCCG			
F-TCCGCAGGTAGCACTCAGTTC	230	[14]		
R-AAGCCGGATTCATAGGTGGTG				
<i>rpsL</i>	F-GCAACTATCAACCAGCTGGTG	230	[14]	
	R-GCTGTGCTTTCAGGTTGGT			

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