

Evaluation of *oprD* Gene Expression in Carbapenem-Resistant *Pseudomonas aeruginosa* Strains Isolated From Severe Burn Patients With Secondary Infection

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Background: *Pseudomonas aeruginosa* is the most common pathogen isolated from severe burn patients with secondary infection. Since high resistance to most types of antibiotics is common among these bacteria, the treatment of infections caused by these agents is very difficult. Loss of *oprD* proteins from the outer membrane of bacterial cells causes a significant decrease in the sensitivity of *Pseudomonas aeruginosa* to carbapenems.

Objectives: This study was performed to investigate the prevalence of carbapenem-resistant strains of *Pseudomonas aeruginosa* in burn patients with secondary infection and also to evaluate *oprD* gene expression as a possible resistance mechanism to carbapenem in isolated carbapenem resistant *P. aeruginosa* strains.

Patients and Methods: One-hundred and eighty-nine clinical isolates of carbapenem resistant *Pseudomonas aeruginosa*, isolated from burn patients, were identified by microbiological methods followed by determination of antibiotic resistance pattern by the Kirby-Bauer procedure. The expression of *oprD* gene was determined by quantitative real-time polymerase chain reaction (PCR).

Results: Our study showed that 94.2% of the isolates were resistant to imipenem, 99.5% to meropenem, and all were resistant to ertapenem. The *OprD* gene expression among carbapenem resistant *Pseudomonas aeruginosa* isolates showed a 2×10^{-3} to 0.5 times decrease compared to the standard sensitive strain ($P < 0.05$).

Conclusions: The results of this study indicated that a decrease in *oprD* gene expression is an important mechanism of resistance in carbapenem resistant *Pseudomonas aeruginosa* isolated from severe burn patients with secondary infection.

Keywords: Drug Resistance; *OprD*; *Pseudomonas aeruginosa*

1. Background

Pseudomonas aeruginosa are gram negative bacteria distributed in soil, water, skin flora, and the majority of man-made environmental sources, worldwide (1). Regarding the presence of widespread antibiotic resistance among the clinical isolates of *P. aeruginosa*, this organism is considered as one of the most important bacterial agents associated with nosocomial infections, for which the process of successful treatment is frequently hard to achieve (2, 3). These bacteria are known as the second most common bacterial agents in burn infections (4, 5) and are also ranked third, after *Staphylococcus aureus* and *Escherichia coli*, amongst the most frequent agents of nosocomial infections (6). *Pseudomonas aeruginosa* is amongst common causes of mechanical ventilation-associated pneumonia, surgical site infection, hospital-acquired urinary tract infection and bacteremia in patients admitted to intensive care units (ICUs) (7, 8). This organism is reported to be the most prevalent pathogen isolated from patients

with secondary infection following severe burn injury (9). Currently, the prevalence of hospital Multi-Drug Resistance (MDR) strains of *P. aeruginosa* is increasingly on the rise because of widespread clinical application of antibiotics throughout the world (10), making the treatment of infections caused by these MDR organisms very complicated (2, 3).

Carbapenems such as imipenem and meropenem are amongst the most important antibacterial agents routinely used in treating infections caused by MDR *Pseudomonas aeruginosa* strains (11-13). Generally, controlling the prevalence of these MDR organisms is difficult as *P. aeruginosa* has intrinsic antibiotic resistance against different antibacterial agents (10). There are several mechanisms through which resistance against diverse antibacterial agents occurs by this organism (14). Resistance to different antibacterial drugs generally occurs either as the result of the combination of different mechanisms in a

given strain or the function of a specific mechanism (15).

At present, three different resistance mechanisms against carbapenems have been reported, including decreased carbapenem penetration due to a change in the expression of outer membrane protein (*oprD*), release of carbapenemases from the pathogen, and the expression of efflux pump (16). There are different resistance patterns resulting from each resistance mechanism. While a decrease of *oprD* in *P. aeruginosa* causes an intermediate level of resistance against imipenem, a combination of increased efflux pump expression accompanied with the loss of the *oprD* protein not only induces a high-level of resistance against imipenem but also against meropenem and imipenem (17, 18). The majority of carbapenem-resistant *Pseudomonas aeruginosa* strains are defective in expression of *oprD* (19). The *oprD* proteins are members of the outer membrane specific channel super-family in gram-negative bacteria (20). *Pseudomonas aeruginosa oprD* is a specific porin regarded as a gateway that facilitates the uptake of carbapenems antibiotics. Loss of *oprD* in the bacterial outer membrane causes a significant reduction in the susceptibility of *P. aeruginosa* against carbapenems (21). In the past, Western blot analysis was used to measure the level of efflux pump protein expression, *oprD* porin and AmpC followed by performance of the polymerase chain reaction (PCR) protocol to detect the occurrence of mutations in *oprD* gene and eventually the application of sequencing method to determine the amino acid sequence of the target protein. Since the application of methods mentioned earlier requires a long duration of time and usually with less sensitivity, compared to the real-time PCR (RT-PCR), the application of RT-PCR technique has become a common standard procedure in studies carried out within recent years to investigate the expression of antibiotic resistance genes in *P. aeruginosa*. This technique needs less time with high sensitivity and the potential to be used in clinical laboratories (22).

2. Objectives

The aim of the present study was to investigate the antibiotic resistance pattern and identify the clinical strains of carbapenem-resistant *P. aeruginosa* isolated from burn patients in hospitals of Tehran and Qazvin, and also to examine the expression of *oprD* gene as one of the important factors of drug resistance.

3. Patients and Methods

This was a descriptive study in which a total of 189 clinical strains of carbapenem-resistant *P. aeruginosa* were isolated from burn patients at Shahid Motahhari hospital, Tehran, Iran. These patients were admitted to burn units during March 2011 to November 2012.

The isolates collected were confirmed by standard bacteriologic methods performed at the microbiology laboratory of the medical school of Qazvin university

of medical sciences. The antibiotic-resistance profile of isolated organisms was evaluated by the Kirby Bauer disk diffusion assay according to the clinical and laboratory standards institute (CLSI) guideline. The antibiotics used were imipenem (10 µg), meropenem (10 µg) and er-tapenem (10 µg); all were obtained from the MAST company (England). A standard strain of *P. aeruginosa* (ATCC 27853) was used as the control organism. For RNA extraction and performance of RT-PCR, *P. aeruginosa* isolates were cultured in 1.5 mL Lysogeny Broth (LB) and left in a shaker incubator (180 rpm) at 37°C for 18 - 24 hours. Later, following overnight incubation, the cultures were centrifuged (8000 rpm) at 4°C for seven minutes. The sediment obtained was used for extraction of total RNA using a commercial kit (RNeasy kit, Qiagen, Germany), according to the manufacturer's instructions. The elimination of genomic DNA was achieved using a commercial RNase-free DNase kit (DNase, RNase-free, Fermentas, Thermo Scientific, USA), according to the manufacturer's instructions. To prepare cDNA, 5 µL of extracted RNA was added to 15 µL distilled, deionized water and converted to cDNA in a total volume of 20 µL, using a commercial kit (cDNA kit, BioNeer Pacific, Australia), according to the manufacturer's instructions. The quality and quantity of extracted RNA was assessed by electrophoresis on agarose gel. Real-time PCR was performed on an Applied Biosystems 7500 real-time PCR instrument (Fort Collins, Colorado, USA) using a commercial kit (SYBR® Green2X Master Mix; Applied Biosystems, USA). Briefly, using a standard 96-well microplate (Applied Biosystems; USA), a volume of 0.5 µL of each primer, 5 µL of cDNA, 4 µL distilled deionized water, and 10 µL of Master Mix were added to each well. All PCR reactions were performed in a 20-µL total volume for 45 cycles. After PCR performance, a melting curve was drawn to evaluate the specificity of the PCR reaction (Figures 1 and 2) followed by electrophoresis of PCR products on 2% agarose gel (Figure 3). Specific primers of *oprD* (Table 1) were used to evaluate changes of *oprD* gene expression in carbapenem-resistant *P. aeruginosa* isolates. Housekeeping gene *rpoD* was used as an internal standard for normalizing the level of transcription of target genes, and the standard *P. aeruginosa* strain (ATCC 27853) for comparative evaluation of the gene mentioned above.

3.1. Method for Evaluating Gene Expression

Once the PCR reaction was over, the threshold line was determined and the data were analyzed by descriptive statistics using the Microsoft Excel software. To determine the output and correlation coefficient of the target gene (*oprD*) and the housekeeping gene (*rpoD*) from extracted RNA, serial dilutions were prepared and following performance of RT-PCR, a standard curve was drawn for each segment of the gene. Later, the Ct of samples was used to calculate the increase or decrease in gene expression using the $2^{-\Delta\Delta CT}$ method.

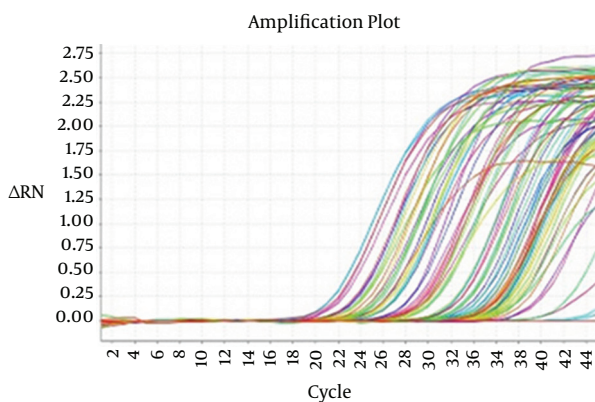


Figure 1. *OprD* Gene Linear Amplification Plot

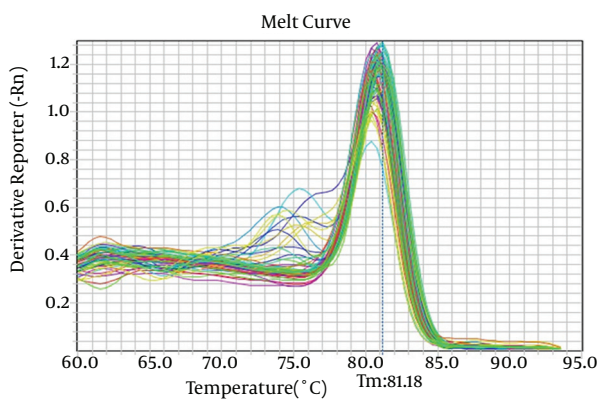
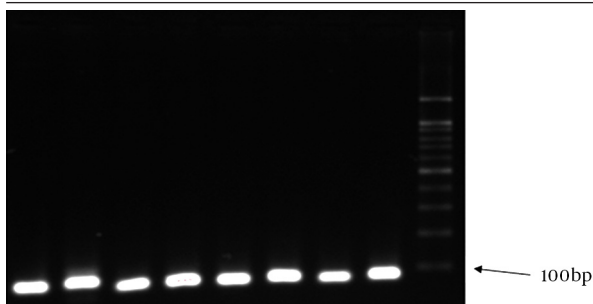


Figure 2. Melting Curve Plot for the Isolates With *oprD* Gene

Figure 3. Polymerase Chain Reaction Product



Lines 1 to 8 (positive samples); line 9 (100 bp DNA molecular marker).

Table 1. Primers List Used to Evaluate the *oprD* Gene Expression

Gene	Primer Sequence	Reference
<i>oprD</i>	For 5'-CTACGGTACTCGGCGAGGAT-3'	(16)
	Rev 5'-GACCGGACTGGACCACGTACT-3'	
<i>rpoD</i>	For 5'-GGGCTGTCTCGAATACGTTGA-3'	(16)
	Rev 5'-ACCTGCCGGAGGATATTCC-3'	

4. Results

The findings of drug susceptibility assay for imipenem, meropenem and ertapenem by the Kirby-Bauer disk diffusion technique on 189 isolates of carbapenem-resistant *P. aeruginosa*, isolated from burn wounds, indicated 94.2%, 99.5%, and 100% resistance against the drugs used, respectively. The *oprD* gene expression among carbapenem resistant *Pseudomonas aeruginosa* isolates showed 2×10^{-3} to 0.5 times decrease compared to the standard sensitive strain ($P < 0.05$).

5. Discussion

Carbapenem antibiotics are amongst important antibacterial agents for treating hospital-acquired infections associated with *Pseudomonas aeruginosa* (17). These agents are usually used as the last therapeutic strategy against infections caused by MDR organisms (23), in which the spread of resistance makes successful treatment extremely complicated (17). Loss of *oprD* in the bacterial outer membrane of *P. aeruginosa* is one of the most important resistance mechanisms against carbapenems (24). In the present study the CT for *oprD* and *rpoD* genes in carbapenem-sensitive *P. aeruginosa* isolates was 37.527 ± 2.9343 and 24.5312 ± 0.4943 , respectively, with a ΔCT equal to -12.9958 ; while the CT of *oprD* and *rpoD* genes in the carbapenem-resistant *P. aeruginosa* clinical strains, isolated from burn patients, was 30.2848 ± 5.6786 and 25.8296 ± 6.1574 with a ΔCT of -4.4552 , respectively.

Considering the $2^{-\Delta\Delta CT}$ formula, it is evident that the degree of *oprD* gene in the clinical isolates of carbapenem-resistant *P. aeruginosa*, compared to the control strain of carbapenem-sensitive *P. aeruginosa*, has decreased by 2×10^{-3} to 0.5 times. In a study by Wang et al. (2010), out of 258 clinical isolates of imipenem/meropenem-resistant *P. aeruginosa*, 79.8% (206/258) of the isolates showed different degrees of reduction (from partial to complete loss) in the *oprD* gene expression (25). Riera et al. reported that the inactivation of *oprD* gene is the major mechanism that causes resistance against imipenem (26). In another study by Quale et al. a reduction of $\geq 30\%$ in the *oprD* gene expression, compared to the reference strain of *P. aeruginosa* (PAO1), was observed (17). A similar report by Rodriguez-Martinez et al. also emphasized on the reduction of *oprD* gene expression in all 32 isolates of imipenem and meropenem-resistant *P. aeruginosa*, with 24 isolates showing a reduction of $\geq 10\%$ in *oprD* gene expression, compared to the PAO1 reference strain (27). Hammami et al. selected a limited number (18) of non-metallo- β -lactamase-producing clinical isolates of carbapenem-resistant *P. aeruginosa* with minimum inhibitory concentrations (MICs) equal to 16 - 32 $\mu\text{g}/\text{mL}$ (from a total 144 clinical isolate of carbapenem-resistant *P. aeruginosa*) and randomly tested for the expression of *oprD*, *mexA* and *mexE* genes. The expression of *oprD* gene was diminished in all selected isolates compared to the wild type strain PAO1.18. Previous studies have shown that the

transcription of *oprD* gene in carbapenem-resistant isolates of *P. aeruginosa* is enormously reduced (28).

The present study, similar to other studies published so far, also showed the reduction of *oprD* gene expression in the clinical isolates of carbapenem-resistant *P. aeruginosa* isolated from burn patients, compared to the standard strain of *Pseudomonas aeruginosa* ATCC 27853; indicating that a reduction in *oprD* gene expression could be one of the important causes of resistance to carbapenems and in particular imipenem. This is the first report from Iran on one of the resistance mechanisms, i.e. reduction of *oprD* gene expression, against carbapenem in *P. aeruginosa*, especially on clinical isolates obtained from severely burned patients. Understanding the mechanisms associated with resistance against carbapenems in clinical isolates of *P. aeruginosa* will undoubtedly be crucial in expanding new strategies for antimicrobial treatment (17).

Change in the strategy of antibiotic administration and the use of appropriate infection control measures in hospital wards, in particular those in which patients stay for a long time such as burn units, are of vital importance which could, in part, play important roles in controlling the spread of carbapenem-resistant organisms.

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Authors' Contributions

Study concept and design: Akram Azimi and Taghi Naserpour. Acquisition of data: Akram Azimi and Fariba Bazmi. Analysis and interpretation of data: Akram Azimi and Amir Peymani. Drafting of the manuscript: Akram Azimi, Taghi Naserpour and Masumeh Aslanimehr. Critical revision of the manuscript for important intellectual content: Akram Azimi, Taghi Naserpour and Saman Saadat. Statistical analysis, administrative, technical and material support: Akram Azimi, Taghi Naserpour. Study supervision: Akram Azimi.

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