Upstream Region of *OprD* Mutations in Imipenem-Resistant and Imipenem-Sensitive *Pseudomonas Aeruginosa* Isolates

Masoumeh Kiani

Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility

Akram Astani

Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility

Hamed Afkhami

Shahed University Faculty of Medical Sciences

Mansoor Khaledi

Shahed University Faculty of Medical Sciences

Javad Fathi

Shiraz University of Medical Sciences

soodabeh Rostami

Isfahan University of Medical Sciences

Mohadeseh Zarei

Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility

Hengameh Zandi (✉ h.a.university.ac@gmail.com)

Shahid Sadoughi University of Medical Sciences and Health Services

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Abstract

Background: The current study was aimed to investigate the prevalence of the mutations of the *oprD* gene among imipenem-resistant and -sensitive *Pseudomonas aeruginosa* isolated from educational hospitals in Yazd, Iran.

Methods: In this study, 90 *P. aeruginosa* isolates were collected from different clinical samples and transferred to the Department of Microbiology, Shahid Sadoghi University of Medical Science, during 2015 to 2016. All isolates were identified by the conventional biochemical tests and antibiotic resistance was determined using disk diffusion method. E. test was performed to determine the minimum inhibitory concentrations (MIC) of imipenem. The mutations of upstream of the *oprD* coding region and its promoters and 54 primary nucleotide of this gene were recognized by the amplification of this region and subsequently sequenced.

Results: Seventy (77.7%) of isolates had $MIC \geq 16$ and were resistant to imipenem. The results showed that the rate of resistance to imipenem is increasing. Mutations of the upstream of the *oprD* gene and its promoters were seen in 25 (86.2%) of isolates and 4 strains had no mutation. All of the imipenem-resistant isolates had mutations. One isolate had a base substitution A → C at nt 25 in coding region and this isolate had a point mutation leading to an amino acid change at positions 9 (I → L).

Conclusion: The results showed that imipenem resistance is increasing in *P. aeruginosa*, also indicated that the point mutations were the most common cause of the inactivation of upstream of the *oprD* coding region among *P. aeruginosa* isolates, it seems this mechanism is effective in resistance of isolates to imipenem.

1. Background

Pseudomonas aeruginosa is an opportunistic pathogen that causes a variety of infections in immunocompromised patients. In recent years, Antibiotic resistance of *P. aeruginosa* is increasing and the selection of suitable treatments has become difficult and associated with increased morbidity and mortality (1, 2). Carbapenems, mainly imipenem and meropenem, are important and useful antibiotics for the treatment of infections due to multidrug-resistant *Pseudomonas*. Carbapenems are a class of β -lactam antibiotics with good antimicrobial activity against *P. aeruginosa* (3, 4). Carbapenem resistance of *P. aeruginosa* is mainly due to a combination of different factors, including low outer membrane permeability and mutations in the genes encoding *OprD*, the production of the AmpC β -lactamases, overproduction of efflux systems, and producing Carbapenemase (5-7). However, among these mechanisms, the Loss or mutation of outer membrane *OprD* porin and promoter of this gene appears to be the most common mechanisms of intrinsic resistance to imipenem, and a lesser extent to meropenem. This mechanism causes blocking of the entrance of carbapenems particularly imipenem into a bacterium (8, 9). *OprD*-mediated resistance occurs as a result of decreased transcriptional expression of *oprD* and/or loss of function mutations that disrupt protein activity. *OprD*, an outer membrane porin, is a

semipermeable barrier and substrate-specific penetrable protein consisting of 443 amino acids that allows the diffusion of basic amino acids and carbapenems typically imipenem into the cell (6, 10). *OprD* mediated resistance occurs as a result of decreased expression of *oprD* or mutations that hampered protein activity. Specific mechanisms resulting in decreased transcriptional expression of *oprD* include (i) disruption of the *oprD* promoter, (ii) premature termination of *oprD* transcription, (iii) co-regulation with trace metal resistance mechanisms, (iv) salicylate-mediated reduction, and (v) decreased transcriptional expression via co-regulation with the multidrug efflux pump encoded by *mexEF-oprN* (8). The types of mutations in the *oprD* gene and upstream region and promoter of *oprD* gene are various such as nucleotide deletions, insertions and point mutations that have been recognized to be the major mechanisms leading to inactivation of the *oprD* coding region and promoter from imipenem-resistant isolates of *P.aeruginosa* (5, 11). Transcription of *oprD* in *P. aeruginosa* PAO1 initiates with equal frequencies from two start sites, located 23 bases (SS1) and 71 bases (SS2) upstream of the structural gene. The previous investigate, have observed two or three types of imipenem-resistance mutants in clinical isolates. The major type involves deletion and point mutations (12). These well-known alterations are commonly reported, include point mutations or insertion sequences (ISs) inactivating the in resistance to imipenem, especially in Iran. Therefore, the aim of this study was to evaluate the prevalence of mutations in upstream of the *oprD* coding region and its promoters in imipenem resistant and sensitive *Pseudomonas aeruginosa* isolated from educational hospitals.

2. Methods

2.1. Bacterial Isolates

In a descriptive study, 90 isolates of *P. aeruginosa* were collected from June 2015 to April 2016 at Teaching Hospitals of Shahid Sadoghi University of Medical Science, Yazd, Iran. These isolates were originated from different clinical specimens of hospitalized patients, including blood, burn wounds, urine, lungs, etc. The study was approved by Shahid Sadoghi University of Medical Science, Yazd, Iran and ethical code was IR.SSU.REC.1391.24.

2.2. Antimicrobial Susceptibility Testing

After transferring the plate containing Gram-negative rod colonies to the Laboratory of Microbiology, suspected colonies were identified by Gram staining and conventional biochemical tests such as catalase, oxidase, and growth in 42 °C, Oxidative/fermentative test, and differential media such as TSI (Merck, Germany). Isolate identified as *P. aeruginosa* were stored at 70°C in trypticase soy broth (Merck) supplemented with 20°C glycerol unit.

2.3. Minimum Inhibitory Concentration and Phenotypic Confirmatory Tests

Antibiotic susceptibility testing of the isolates was performed using the disk diffusion method (Kirby-Bauer) according to Clinical and Laboratory Standard Institute guideline (CLSI, 2014) using Mueller-Hinton agar (Merck, Germany) and Imipenem, meropenem, ertapenem, ciprofloxacin, ceftazidime,

Cefepime, ceftriaxone, gentamicin, and tobramycin (MAST, UK). *P. aeruginosa* ATCC27853 was used as quality control. The Minimum Inhibitory Concentration (MIC) of imipenem was performed by E. test strips (Liofilchem, Italy) as described in the manufacturer's instructions. MIC breakpoint was defined according to CLSI guideline (CLSI, 2014).

2.4. DNA extraction

DNA extraction was performed using by salting out method and was stored at -20°C until further use (18).

2.5. PCR for detection of *oprD* gene

The occurrence of *oprD* gene was screened in all isolates by PCR. primers were developed for each gene using Primer 3. The primers used for DNA amplification, as follows: 5'- AGACATGCCGTGGATACAAA -3' for the forward and 5'- AGTGCTACCTGCGGAAACC -3' for the reverse primers. The final optimized PCR reaction consisted of 0.5 µl MgCl₂ (100 mM), 0.5 µl dNTP (10 mM), 0.2 µl (1 unit) Taq DNA polymerase (Cinnagen, Iran), 1 µl of each primer (10 pmol) (Alpha DNA, Canada), 2.5 µl PCR buffer (10 X), and 0.5 µl of DNA template (100 µg/ml) in a total volume of 25 µl with double distilled water. DNA amplification was carried out with a thermocycler (Quanta Biotech, England), PCR amplification was performed as follows: one cycle at 95 °C for 300 seconds, then 30 cycles at 95 °C for 45 Sec, 56 °C for 45 Sec, and 72 °C for 60 Sec and a final extension at 72 °C for 10 min using an initial denaturation step for 5 min at 94°C (one cycle), followed by 35 cycles of 1 min at 94°C, 1 min at 50°C and 1min at 72°C. The amplified products were analyzed by 1.5% (w/w) agarose gel electrophoresis and were visualized on an ultraviolet illumination after staining with ethidium bromide. A mutation in the promotor and upstream coding region of *OprD* gene (Table 4) was identified by DNA sequencing.

2.6. DNA sequencing and analyses of sequence data

According to imipenem MIC results, 29 isolates were selected randomly for evaluation of the mutations. For DNA sequencing, upstream regions and fifty four (54) primary nucleotide of *oprD* gene were sequenced. The sequences results were aligned and analyzed using MEGA 6 software and CLUSTAL W2, Vector NTI Advance version9. 0.0 software (InforMax; Invitrogen). Protein alignments were carried out using. ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). In every case, both the nucleotide and the amino acid sequences were compared between the clinical isolates, the PAO1 reference (strain NC 002516.2 and gb AE004091.2)

2.7. Statistical analysis

The data were analyzed using the Statistical Program for Social Sciences version 18. (SPSS Version. 18 IBM, Chicago, IL, USA). For the analysis of data, chi-square tests were employed to calculate the P-value. Statistical significance and levels were set at P<0.05.

3. Results

3.1. Bacterial isolates

Of 90 *P. aeruginosa* isolates, 38.9%, 20% and 13.3% of them were isolated from burn wounds, urine, and wound specimens respectively. The Sources of *P. aeruginosa* isolates according to the hospital ward include Burn (43.3%), ICU (22.2%), Internal (15.6%), Surgery (11.1 %), and other wards (7.7 %).

3.2. Antibiotic resistance patterns

The frequency of resistance to carbapenems was as follows: imipenem 48.9%, meropenem 56.6% and Ertapenem 52.5%. The results of antimicrobial susceptibility testing using the disk diffusion method are shown in table 1. The results of the MIC of Imipenem by an E-test method are shown in table 2.

3.3. PCR and sequencing

The *oprD* gene and upstream regions was amplified by PCR. The electrophoresis agarose gel was performing on PCR products was shown in Figure. The size of the amplified fragment is 570 base pairs. As shown in Figure1. The *oprD* gene was sequenced, including the promoter and

upstream regions including Shine-Dalgarno (GGAG; nucleotides -12 to -9), -10 (TAAGTT; nucleotides -84 to -79), and -35 (TCGCCA; nucleotides -107 to -102) sequences.

Of 30 isolates selected for sequencing, 25 (86.2%) of isolates had mutations that all (100%) of them were resistant to imipenem. Percent of mutations in resistant and sensitive isolates revealed in Table 3. There was a significant relationship ($P<0.05$) between mutations of upstream of the *oprD* coding region and MIC of imipenem.

The frequency of mutations in the upstream region of *oprD* gene based on specimens was as follows: Burn 57.69%, Urine 19.23%, and other specimens 23.07%. Although the most mutations were seen in *P. aeruginosa* isolated from burn specimens and burn ward. The Statistical analysis didn't find any significant correlation between age and sex of the patient. Based on the observed mutations, none of the strains had no mutation in Shine-Dalgarno (GGAG; nucleotides -12 to -9), -10 (TAAGTT; nucleotides -84 to -79), and -35 (TCGCCA; nucleotides -107 to -102) sequences and 25 (86.2%) strains have mutations in the upstream of the structural gene. Six isolates have point mutations in promoter: Five isolates had T→C base substitution at nt -90 and One isolate had a base substitution G→C at nt -120, Also One isolate had a base substitution A→C at nt 25 in coding region and this isolate had a point mutation leading to an amino acid change at positions 9 (I→L). The rest of results sequencing of upstream regions and promoter regions are shown in table 4.

4. Discussion

In medicine, the treatment of community-acquired infections and nosocomial infections caused by *P. aeruginosa* is important. Carbapenem is effective against infectious diseases caused by *P. aeruginosa*. However, carbapenem-resistant *P. aeruginosa* strains are emerging worldwide, and the rate of resistance

in most countries ranges from 10 to 50% (13). In the present study, the prevalence of imipenem resistance in bacteremic *P. aeruginosa* was 48.9 %, and the rate of resistance of *P. aeruginosa* to imipenem was 5.5 % to 62.5% in other studies (14-22). According to studies of antibiotic resistance in different parts of the world and the result of the present study; it can be concluded that resistance rates in *P. aeruginosa* isolates were higher than previous reports, which can be due to a combination of different factors such as the inconsiderate use or the previous use of antibiotics, differences in the type of sample, and the geographical region and care of patients in hospitals and difference in mechanism of resistance. Since the carbapenem are commonly used in the treatment and mutations in *oprD* gene and promoter in upstream regions are the most current reason against resistance to these antibiotics, so identify and assess the prevalence of these mutations in the bacteria population can be very effective in a susceptibility bacteria population. The mutational inactivation of the *oprD* gene and disruption in promoter represents the major cause of *OprD* loss in *P. aeruginosa* strains. In our study alterations were observed in resistant isolates. Mutations of the upstream region *oprD* were seen in all (25) imipenem-resistant isolates. Mutations in SS1 and SS2 were point mutations. The most frequent causes of *oprD* mutational inactivation were point mutations leading to alterations the amino acid profile. In this study, alterations observed, mainly lead to an increase in the MIC and resistance to imipenem. One isolate had a base substitution A→ C at nt 25 in coding region and this isolate had a point mutation leading to an amino acid change at positions 9 (I→L). Also, the insertion of one base was seen in five isolates and the insertion of tree nucleotide were observed in one isolate. In a study performed by Fournier et al (23). Mutations of the *oprD* gene were seen in 86.2% of imipenem-resistant isolates. A similar study conducted by Alain A et al (3). 77 (77%) of isolates had mutations and mutations were observed in both sensitive and resistant isolates. The most isolates showed point mutations and deletion mutations. Reports had done, shows that mutation and inactivation or loss of an *oprD* gene, disruption in promoter and upstream region of *oprD* gene in *P. aeruginosa* strains are the major mechanisms that cause resistance to imipenem. This result was in accordance with previous investigation of clinical isolates of *P. aeruginosa*. In a study performed by Aki Hirabayashi et al (24). There was a direct relationship between the alteration or loss of *oprD* and the increase in MIC, for imipenem but not meropenem and other carbapenems (3, 9, 11). In a study conducted by Yumiko Sanbongi et al (25). The highest mutation was a frame-shift mutation or deletion mutation. Gutiérrez et al (11). Have reported different mutations in the *oprD* gene, the most frequent mutations were frameshift mutations produced by one nucleotide insertions or deletions and point mutations leading to the creation of a premature stop. In a study performed by EL Amin et al (8). Mutation of inactivation, including the insertion or deletion of one and two or more nucleotides and insertion sequences (IS). In investigating Performed by Wolter DJ et al (26). PCR and sequence analysis revealed an interpolation of a large fragment in the *oprD* gene were known as IS elements that are not observed in this study. Jilu Shen et al (9). Reported 96.5% (136/141) of the resistant isolates showed deletions or multi mutations that were driven loss or insertion of the *oprD*-encoding gene, 34 strains had a large deletion in the *OprD* gene, 6 isolates had IS, and 4 strains had no mutation and showed a normal *OprD2* gene. In this study, the insertion of one base was seen in five isolates and four isolates had no mutation. Yoneyama et al (1). Reported the association of a large deletion encompassing the putative promoter and initiation codon that prevented transcription of *oprD* and deletion mutations were observed,

including deletion an11 bp and a large deletion from nucleotides 519-685. Qinghui Sun et al (27). Have reported an insertion sequence element (ISRP10) that causes to disrupt the *oprD* gene and seen in 96% of imipenem-resistant *Pseudomonas aeruginosa* isolates. In a study performed by Yingjun Yan et al (28). The result of the analysis, indicating that the 4-bp insertion in the *oprD* gene resulted in a frameshift in the *OprD* gene and imipenem resistance. A different study conducted by Hussein Chalhoub a Sequencing showed no mutation in the promoter region of the gene, but several in the coding region *oprD* promoter disruptions have occurred as a result of deletions or insertions within the upstream region of *oprD* (29).

5. Results

The results of this study showed, increase the resistance of *P. aeruginosa* to carbapenem family antibiotics such as imipenem. Mutations caused by substitution nucleotide and deletions or insertions within the upstream region of *oprD* and in the *oprD* structural gene can lead to increases of the MICs of imipenem. Usage of imipenem should be controlled to prevent pan-drug resistant. In our country, there is little information about the contribution of different mechanisms to carbapenem resistance in these isolates, especially about *oprD* mutations and upstream region of and promoters mutations in imipenem-resistance isolates. Awareness of resistant mechanisms in *Paeruginosa* isolates could help to regulation infection control strategies and enhances the efficacy of imipenem for treatment of infections due to this bacteria. Thus, there is a need to focus on intrinsic resistance mechanisms, especially Porin alteration which also confers significant imipenem resistance, also suggests in future other mechanisms are evaluated and investigates in other isolates and in other places.

Declarations

Ethics approval and consent to participate

The study was approved by Shahid Sadoghi University of Medical Science, Yazd, Iran and ethical code was IR.SSU.REC.1391.24. In this study, the patient and human samples were not examined directly and the bacterial samples were collected from the Teaching Hospitals of Shahid Sadoghi University of Medical Science, Yazd, Iran.

Consent for publication

There is no limit to the publication.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

We declare that we have no conflict of interest.

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Not applicable

Author's contributions

HZ: Project development, Management and Manuscript writing. MK: Project development; Management and Manuscript writing. AA: Data collection and Manuscript writing. HA: Data collection and Data analysis. MKH: Manuscript writing and Data analysis. JF: Manuscript writing, Manuscript editing and Data analysis. SR: Manuscript writing and Data analysis. MZ: Data collection and Manuscript writing. All Authors read and approved the manuscript.

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Author Information

¹Department of Microbiology, Faculty of Medicine, Shahid-Sadoughi University of Medical Sciences, Yazd, Iran

²Phd Student of Medical Bacteriology, Department of Medical Microbiology, Faculty of Medicine, Shahed University of Medical Science, Tehran, Iran

³ Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

⁴Nosocomial infections Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

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Tables

Table 1- frequency of antibiotic resistance patterns in *P. aeruginosa* isolated from clinical samples

Antibiotic	Sensitive No. (%)	Semi Sensitive No. (%)	Resistant No. (%)
Imipenem (10µg)	44 (48.9)	2 (2.2)	44 (48.9)
Meropenem (10µg)	38 (42.2)	1(1)	51(56.6)
Ertapenem (10µg)	29 (32.2)	14 (15.3)	47 (52/5)
Ciprofloxacin (5µg)	46 (51.1)	4 (4.4)	40 (44.4)
Ceftazidime (30µg)	32 (35.6)	13 (14)	45 (50)
Cefepime (30µg)	40 (44.4)	2 (2.2)	48 (53.3)
Ceftriaxone (30µg)	26 (28.9)	17(18.9)	48 (53.3)
Gentamicin(10µg)	38 (42.2)	7 (7.8)	45 (50)
Tobramycin(10µg)	37 (41.1)	8(8.8)	45 (50)

Table 2: The results of MIC for imipenem by an E. test

Resistant	70 (77.7%)
MIC≥16	
Sensitive	20 (22.2%)
MIC≤4	

Table 3. The relation between mutation and MIC

E-test	Mutation		Total
	Yes	No	
Resistant	25 (86.2%)	0(0%)	25(86.2%)
Sensitive	0 (0%)	4(13.7%)	4 (13.7%)
Total	25(86.2%)	4 (13.7%)	29(100%)

Table 4. The results of the sequencing of upstream region of *oprD* gene and promoter

NO. of isolate	Description of mutation	Resistant/sensitive
115	A→ G base substitution at nt -30 T→C base substitution at nt -297 G→T base substitution at nt -348 C→T base substitution at nt -300 A→ G base substitution at nt -309 A→ T base substitution at nt -384 C→T base substitution at nt -417	R
18	G→A base substitution at nt -302 G→A base substitution at nt -272	R
125	A→C base substitution at nt -273 G→C base substitution at nt -120	R
49	G→T base substitution at nt -252	R
57,58,16, 55,10	T→C base substitution at nt -89	R
31,53,78,72, 68,126,125,91	C→T base substitution at nt -323 C→T base substitution at nt -179	R
200,202,203,31,53,68,80,115,125,126,91,85	A→G base substitution at nt -594 A→ G base substitution at nt -432 A→ G base substitution at nt -426 C→T base substitution at nt -357 C→T base substitution at nt -285	R

		G →A base substitution at nt -270	
		C→ G base substitution at nt -216	
		G →A base substitution at nt-165	
		T→A base substitution at nt -144	
7		Insertion of 3 bp (TCG) at nt -422, -423, -424	R
48,49,17,18,122, 203		G→A base substitution at nt -256	R
17,49,55,10,122		Insertion of 1 bp (C) at nt -420	R

Figures

Figure 1

Agarose gel electrophoresis for amplification analysis of oprD Gene. Lane 1-12,14,15: oprD PCR result, lane 13: negative control, DNA ladder: 50bp.