Molecular Detection of *gyrA*, *parC* and *oprD* Mutation in *Pseudomonas aeruginosa* Isolates from a University Hospital of Isfahan, Iran during 2016

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ABSTRACT

***Background*:** Excessive use of broad-spectrum antibiotics in hospitals has led to the emergence of highly resistant strains of *Pseudomonas aeruginosa*. The main mechanism of resistance of this bacterium to fluoroquinolones and carbapenems are the modification of type II topoisomerases (DNA gyrase and topoisomerase IV) and alterations in the OprD porin, respectively. The aim of this study was to examine for the occurrence of mutations related to fluoroquinolone resistance of *gyrA* and *parC* genes and mutational inactivation of *oprD* gene of clinical isolates using DNA sequencing technique.

***Methods*:** A total of 60 *P. aeruginosa* isolates were collected from the hospitalized patients in the Intensive Care Units (ICUs) of Al-Zahra hospital located in Isfahan, Iran. The pattern of sensitivity to antibiotics was determined using CLSI disk diffusion and MIC methods. The assay was based on a DNA sequencing method using polymerase chain reaction (PCR) for amplification and sequencing of the selected genes.

***Results*:** The results show that replacement of Ile for Thr-83 in *gyrA* was the only replacement, while other substitutions not observed. No mutations were found in *parC*. The most frequent amino acid alterations were E185Q, P186G, and V189T, found in five resistance isolates, However, nucleotide insertions and deletions mutations not observed.

***Conclusion*:** Our study suggested that mutation of *gyrA* and *oprD* genes may play a minor role in fluoroquinolone and carbapenem resistance and other mechanisms may contribute to the fluoroquinolone and carbapenem resistance of *P. aeruginosa.*

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Introduction

Pseudomonas aeruginosa is an important pathogen of nosocomial infections, especially in the Intensive Care Units (ICUs) and is generally resistant to many antibiotics especially in ICUs. Multidrug-resistant (MDR) *P. aeruginosa* "resistance to at least 3 classes of antibiotics" are often isolated from nosocomial infections in ICUs (1-4) and emergence of resistant often seen in the ICUs. MDR *P. aeruginosa* infections are associated with the increased price of care, length of hospitalization and mortality (4, 5). Only a few antibiotics, such as carbapenem and fluoroquinolone, show potent bactericidal activity against MDR *P. aeruginosa* infections (6-9). Therefore, knowing the mechanisms of resistance and developing alternative therapies for these isolates is very important (1). *P. aeruginosa* acquires resistance to antibiotics through chromosomal mutations. These mutations include: mutations in the target genes encoding DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) and mutations causing cell wall impermeability due to loss of *OprD*, a porin that forms narrow transmembrane channels. Resistance to imipenem and reduced susceptibility to meropenem are reported to be associated with loss of OprD porin (4). Mutations in the target genes that encode gyrA and parC are the main mechanisms of resistance to fluoroquinolones. Mutational studies have shown that gyrA mutations in codon 83 (Thr→Ile) and codon 87 (Asp→Tyr or Asp→Asn) have been associated with higher fluoroquinolone resistance. parC mutations in codon 80 (Ser→Leu), and codon 84 (Glu→Lys) are associated with elevated fluoroquinolone resistance. Multiple mutations in both *gyrA* and *parC* genes in codons 80, 83, 84, and 87 are linked to much more elevated resistance in *P. aeruginosa* (4, 10). The important known mechanisms of resistance to carbapenems in *P. aeruginosa* are inactivating mutations in *OprD* have been documented to confer resistance to imipenem and to a lesser extent to meropenem (3, 11, 12). The aim of this study is to determine the antimicrobial resistance rates and point mutations in *oprD* and *gyrA* genes of MDR *P. aeruginosa* strains isolated from the hospitalized patients in ICU of Al-Zahra hospital in Isfahan, Iran.

Material and method

Clinical Isolates

In this cross-sectional study, clinical isolates of *P. aeruginosa* were collected during August 2015 to April 2016 from hospitalized patients in ICU of the main hospital, Isfahan, Iran (Alzahra hospital). Specimens were cultured as described by others (13). The specimens taken from urinary tract (22), tracheal aspirate (20) blood (18) and other sites were cultured.

Identification of P. aeruginosa strains

Bacteria isolates from clinical specimens were identified according to Gram stain and biochemical tests such as growth at 42 °C, catalase, oxidase, pyocyanin production, citrate utilization, triple iron sugar utilization, oxidative-fermentative test with glucose, and methyl red-Voges Proskauer as described in standard bacteriological methods (10).

Antimicrobial susceptibility testing, Disk diffusion

Antimicrobial susceptibility tests were performed by the Kirby-Bauer disk diffusion method based as recommended by National Committee for CLSI, USA with a panel of anti-pseudomonal antimicrobials of standard strengths as follows: ceftazidime (30µg), piperacilin (100µg), piperacilin/-tazobactam (100µg), gentamicin (10µg), amikacin (30µg), imipenem (10µg), meropenem (10µg), ciprofiloxacin (5µg), cefepim (30µg), aztreonam (30µg), levofloxacin (5µg) (MAST Co., UK). Control strains *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 (14) were included in assays. Statistical analyses were performed using WHO net version 5.6.

Determination of MIC

The E-test was performed using Mueller-Hinton agar plates (diameter, 140 mm). The MIC of imipenem and ciprofiloxacin was determined by the E-test method, according to the manufacturer's instructions (Liofilchem, Italy).The MICs of the E-test were rounded up to the next most two-fold dilution for comparison of results with the reference method. Quality control was tested by E. coli ATCC 25922 (11).

PCR amplification and DNA sequencing

Chromosomal DNA was extracted using a DNA extraction kit (Sinaclon, Iran) according to the manufacturer's instructions and were used as template for PCR reactions. PCR amplification of *gyrA*, *oprD* and *parC* genes was performed with whole-DNA extracts from 5 randomly selected FQ and carbapenem resistant clones by using the primers described in table 1. In each case, two independent PCR products were fully sequenced as described above, and the resulting sequences were compared with those of the reference strain PAO1. Multiple sequence aligned by PBIL (PôleBioin-formatique Lyonnais) of *gyrA*, *oprD* and *parC* genes from PAO1 and clinical isolates (15, 16).

Results

Antimicrobial susceptibility testing

In our study the highest resistance ratio was found against meropenem (66.2%) and levofloxacin (66.2%), and the least resistance belonged to amikacin (50.8%) (Table 2). According to the standard breakpoints, our data revealed that 100% of isolates of *P. aeruginosa* had MICs ≥256 µg/ml and MICs ≥32 µg/ml to imipenem and ciprofloxacin, respectively.

Comparison of disk diffusion method and E-test for imipenem and ciprofloxacin

Using the disk diffusion method, 30 of 30 (100%) of the MDR *P. aeruginosa* isolates showed resistance to imipenem and ciprofloxacin. Also E-test using confirmed disk diffusion method revealed that 30 of 30 (100%) of the MDR *P. aeruginosa* isolates were resistant to imipenem and ciprofloxacin. The number of *P. aeruginosa* strains resistant to two drugs, imipenem and ciprofloxacin, were similar in both assays, the disk diffusion method and E-test.

gyrA, oprD and parC mutations

Five clinical isolates of *P. aeruginosa* were examined for the occurrence of mutations related to FQ and carbapenem resistance. To identify point mutations, sequences from clinical isolates were compared with those of wild-type *P. aeruginosa* PAO1. PCR analysis followed by sequencing showed the presence of the *gyrA* and *oprD* gene in clinical isolates (Table 3 and Table 4). The results showed that all of the clinical isolates had a single point mutation in *gyrA* gene. Since alterations in the OprD porin can cause imipenem resistance, four chosen clinical isolates were subjected to sequencing for *OprD* gene. No mutations were found in parC. Amino acid changes found among these carbapenem resistant *P. aeruginosa* strains are displayed in table 4.

Discussion

Fluoroquinolone and Carbapenem resistance mechanisms resulting from chromosomal mutations. The involvement of the classical mutational mechanisms in the fluoroquinolone and carbapenem resistance of the *P. aeruginosa* strains from the Iran hospitals has not investigated (5). Fluoroquinolone resistance in *P. aeruginosa* has been associated with substitutions in the gyrA subunit of DNA gyrase and in the parC subunit of

Table 1. Primers used in this study.

Gene	primer name	sequence5´–3´	reference
gyrA	*gyrA-F* *gyrA-R*	AGTCCTATCTCGACTACGCGAT AGTCGACGGTTTCCTTTTCCAG	12
oprD	*oprD-F* *oprD-R*	TGCTGCTCCGCAACTACTATTTC GTAGGCCAAGGTGAAAGTGTG	13
parC	*parC-F* *parC-R*	CGAGCAGGCCTATCTGAACTAT GAAGGACTTGGGATCGTCCGGA	12

Table 2. Results of antibiotic susceptibility tests of isolated strains of *P. aeruginosa*.

Antibacterial Class	Antibiotic	Sensitive no%	Intermediate no%	Resistant no%
Quinolones	Ciprofloxacin Levofloxacin	26.6 25	5 5	68.3 70
Carbapenems	Meropenem Imipenem	28.3 31.6	1.6 -	70 68.3
Aminoglycosides	Gentamicin Amikacin	30 43.3	3.3 1.6	66.6 55
Cephems	Cefepime Ceftazidime	25 23.3	10 10	65 66.6
β-lactam+Inhibitor	Piperacillin/ Tazobactam	28.3	15	56.6
Penicillins	Piperacillin	23.3	15	61.6

Table 3. Type of point mutations in gyrA and parC genes of *P. aeruginosa* strains

GyrA	3,4,16,20,33	256	83	ACC→ATC	Thr→ Ile
ParC	3,4,16,20,33	256	-	-	-

MIC = minimum inhibitory concentration; CIP = ciprofloxacin

Table 4. oprD amino acid sequence alterations in carbapenem-resistant isolates.

Strain no.	MIC µg/mL	Amino acid position and substitutions											
	IMP	103	115	170	185	186	189	202	210	230	240	262	276
PAO1	0.6	T	K	F	E	P	V	E	I	E	S	N	T
H1	32					Q	G	T	Q	A	K	T	T
A													
H4	32		S		T	L	Q		G	T			
H14	32		S		T	L	Q		G	T			
H16	32		S		T	L	Q		G	T			
H33	32		S		T	L	Q		G	T			

MIC = minimum inhibitory concentration; IMP = imipenem

DNA topoisomerase IV (10, 17, 18). Mutations in *oprD* caused by nucleotide deletions, insertions and point mutations in the *oprD* structural gene have been found to be the main mechanisms leading to inactivation of OprD porin from *P. aeruginosa* (12). In this study sequences of QRDR of *gyrA*, *parC* and as well as *oprD* gene of carbapenem resistant *P. aeruginosa* strains were examined by PCR and sequencing (5). Our results showed that among the five fluoroquinolone resistant *P. aeruginosa* that were randomly selected, only one type mutation in the *gyrA* gene was detected in all isolates. The nucleic acid substitutions in *gyrA* that occurred in these codons changed the amino acid profile from Thr to Ile (ACC:ATC). Common mutations in fluoroquinolone-resistant strains occurred in codon 80 of the *parC* gene but, our results showed the absence of alterations in the *parC* gene (4). There are numerous reports of the correlation between *gyrA* mutations with or without additional alterations in QRDR of *parC* (4, 10, 15, 16, 19).

Multiple studies have evaluated the importance of *oprD* substitutions in clinical isolates of *P. aeruginosa* resistant to carbapenems. Mutations in *oprD* caused by nucleotide deletions, insertions and point mutations in the *oprD* structural gene have been found to be the major mechanisms leading to inactivation of OprD porin from *P. aeruginosa* (12).

Mutational inactivation of *oprD* is the main mechanism of carbapenem resistance in the absence of acquired carbapenemases (8). Since alterations in the OprD porin can cause carbapenem resistance, we sequenced the *oprD* gene for clinical strains. The most frequent causes of *oprD* mutational inactivation were point mutations leading to alterations the amino acid profile (5). These amino acid alterations were frequent in the *P. aeruginosa* strains tested in this study, whereas these alterations probabely lead to carbapenem resistance. The most frequent amino acid alterations were E185Q, P186G, and V189T, found in five resistance isolates. This result was in accordance with previous reports on clinical isolates of *P. aeruginosa*. However, in our study unlike other studies, nucleotide insertions and deletions mutations not observed (3, 12, 20). In this

study, were found also a number of mutations in the third codon that did not affect the protein sequence which confirm other reports (21).

Conclusion

In conclusion, this study show that the primary mechanism of fluoroquinolone resistance in *P. aeruginosa* is mediated through target site substitutions, specifically a Thr-83 to Ile alteration in gyrA and as well as point mutations in the *oprD* structural gene that leading to change the amino acid profile that plays a crucial role in carbapenem resistance. However, we know that other molecular experiments such as quantitative reverse transcription PCR and SDS page analysis showing protein profiles are required to reach comprehensive and valuable results about the carbapenem resistant *P. aeruginosa* strains.

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Ethical Considerations

The study was ethically approved by the Isfahan University of Medical Sciences Ethic Commission and written informed Consent was obtained from all participants.

Conflict of interest

The authors declare no conflicts of interest.

Financial disclosure

The authors declared no financial disclosures.

References

1. Ozer B, Duran N, Onlen Y, Savas L. Efflux pump genes and antimicrobial resistance of *Pseudomonas aeruginosa* strains isolated from lower respiratory tract infections acquired in an intensive care unit. *J Antibiot* 2012; **65**(1): 9–13.
2. Badamchi, A., Masoumi, H., Javadinia, S., Asgarian, R. and Tabatabaie, A., 2017. Molecular detection of six virulence genes in *Pseudomonas aeruginosa* isolates detected in children with urinary tract infection. *Microb Pathog* **107**: 44-47.
3. Farra A, Islam S, Stralfors A, et al. Role of outer membrane protein OprD and penicillin-binding proteins in resistance of *Pseudomonas aeruginosa* to imipenem and meropenem. *Int J Antimicrob Agents* 2008; **31**(5): 427–33.
4. Fazeli H, Havaei SA, Solgi H, et al. Pattern of antibiotic resistance in *Pesudomonas aeruginosa* isolated from intensive care unit, Isfahan, Iran. *Journal of Isfahan Medical School* 2013; **31**(232): 432-438.
5. Gutierrez O, Juan C, Cercenado E, et al. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. *Antimicrob Agents Chemother* 2007; **51**(12): 4329–35.
6. Naenna P, Noisumdaeng P, Pongpech P, et al. Detection of outer membrane porin protein, an imipenem influx channel, in *Pseudomonas aeruginosa* clinical isolates. *Southeast Asian J Trop Med Public Health* 2010; **41**(3): 614–24.
7. Mohammadzadeh M, Tavakoli M, Mohebi A, et al. Phenotypic and genotypic detection of metallo-beta-lactamases among imipenem resistant gram negative isolates. *J Med Bacteriol*, 2016; **5**(1-2): 36-42.
8. Lee JY, Ko KS. OprD mutations and inactivation, expression of efflux pumps and AmpC, and metallo-β-lactamases in carbapenem-resistant *Pseudomonas aeruginosa* isolates from South Korea. *Int J*

Antimicrob Agents 2012; **40**(2): 168–72.
9. Chakraborty D, Basu S, Das S. A study on infections caused by metallo beta lactamase producing gram negative bacteria in intensive care unit patients. *Am J Infect Dis* 2010; **6**: 34–9.
10. Matsumoto M, Shigemura K, Shirakawa T, et al. Mutations in the *gyrA* and *parC* genes and in vitro activities of fluoroquinolones in 114 clinical isolates of *Pseudomonas aeruginosa* derived from urinary tract infections and their rapid detection by denaturing high-performance liquid chromatography. *Int J Antimicrob Agents* 2012; **40**(5): 440–4.
11. Meletis G, Exindari M, Vavatsi N, et al. Mechanisms responsible for the emergence of carbapenem resistance in *Pseudomonas aeruginosa*. *Hippokratia* 2012; **16**(4): 303–307.
12. Ocampo-Sosa AA, Cabot G, Rodriguez C, et al. Alterations of OprD in carbapenem-intermediate and -susceptible strains of *Pseudomonas aeruginosa* Isolated from patients with bacteremia in a spanish multicenter study. *Antimicrob Agents Chemother* 2012; **56**(4): 1703–1713.
13. Rahbar M, Kabeh-Monnavar M, Khadem-Vatan K, et al. Carbapenem resistance in gram negative bacilli isolates in an iranian 1000-bed tertiary hospital. *Pak J Med Sci* 2008; **24**(4): 537-40.
14. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. Wayne, PA: *Clinical and Laboratory Standards Institute* 2012; **32**(3).
15. Aakasaka T, Tanaka M, Yamaguchi A, et al. Type II Topoisomerase Mutations in Fluoroquinolone-Resistant Clinical Strains of *Pseudomonas aeruginosa* Isolated in 1998 and 1999: Role of Target Enzyme in Mechanism of Fluoroquinolone Resistance. *Antimicrob Agents Chemother* 2001; **45**(8): 2263-8.
16. Naenna P, Noisumdaeng P, Pongpech P, et al. Detection of outer membrane porin protein, an imipenem influx channel, in *Pseudomonas aeruginosa* clinical isolates. *Southeast Asian J Trop Med Public Health* 2010; **41**(3): 614-24.
17. Zabihi T, Karbasizade V, Ahadi, AM. Comparative Analysis of Three Methods for Determination of Imipenem Resistance in *Pseudomonas aeruginosa*. *J Med Bacteriol* 2015; **4**(5, 6): 37-41.
18. Salma RM, Dabboussi FA, Kassaa IO, K et al. gyrA and parC Mutations in Quinolone-Resistant Clinical Isolates of *Pseudomonas aeruginosa* from Nini Hospital in North Lebanon. *Intl J Microbiol Res* 2012; **19**(1): 77–81.
19. Wydmuch Z, Skowronek-Ciolec O, Choleva K, et al. gyrA Mutations in Ciprofloxacin-resistant Clinical Isolates of *Pseudomonas aeruginosa* in a Silesian Hospital in Poland. *Pol J Microbiol* 2005; **54**(3): 201–6.
20. Wang J, Zhou J-y, Qu T-t, et al. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Chinese hospitals. *Int J Antimicrob Agents* 2010; **35**(5): 486–91.
21. Lepelletier D, Cady A, Caroff N, et al. Imipenem-resistant *Pseudomonas aeruginosa* gastrointestinal carriage among hospitalized patients: risk factors and resistance mechanisms. *Diagn Microbiol Infect Dis* 2010; **66**(1):1–6.