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Original Article

Carbapenem and Fluoroquinolone Resistance in Multidrug Resistant *Pseudomonas aeruginosa* Isolates from Al-Zahra Hospital, Isfahan, Iran

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Introduction: The major resistance mechanisms of *Pseudomonas aeruginosa* to fluoroquinolones and carbapenems are associated with the mutations in the genes *gyrA* and *oprD* encoding type II topoisomerases (DNA gyrase) and OprD porin, respectively. **Method:** In this cross-sectional study, sixty five clinical samples were collected from patients hospitalized in Al-Zahra Hospital of Isfahan, Iran. Susceptibility testing was performed by using disk diffusion and minimum inhibitory concentration (MIC) by E-test methods as recommended by Clinical Laboratory Standards Institute (CLSI). The assay was based on a DNA sequencing method using polymerase chain reaction (PCR). **Results:** The disk diffusion and E-test methods showed significant concordance in determining the in-vitro activity of the meropenem and ciprofloxacin against *P. aeruginosa* isolates. The mutations associated with antibiotic resistance were detected in the codon 83 of the *gyrA* gene, and various codons of the *oprD* gene. **Conclusion:** Our results showed that the main mechanism of fluoroquinolone resistance in *P. aeruginosa* is mediated primarily through mutations in *gyrA* and carbapenem resistance was driven mainly by the mutational inactivation of *oprD* gene. *J Med Microbiol Infec Dis, 2014, 2 (4): 147-152.*

Keywords: *Pseudomonas aeruginosa*, *gyrA*, *oprD,* Carbapenems, Fluoroquinolone resistance.

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen commonly associated with nosocomial infections occurring in intensive care units (ICUs). Drug resistance of *P. aeruginosa* is a major concern in the ICUs, where resistance rates are more than other hospital wards [1, 2]. This organism is considered as the most common cause of death among patients with nosocomial infections and the first cause of death in ICUs [3]. Patients in ICUs are mostly infected with multidrug resistant (MDR) isolates, which is associated with high morbidity and mortality [4]. The increasing outbreaks of nosocomial infections caused by MDR *P. aeruginosa* strains, including isolates resistant to aminoglycosides, fluoroquinolones (FQs) and broad-spectrum β-lactams, compromise the selection of the appropriate treatment [5]. Carbapenems and FQs remains the choice drugs used in the treatment of *P. aeruginosa* nosocomial infections [6].

Carbapenems are the major drugs for treating infections caused by MDR *P. aeruginosa*, but the development of carbapenem resistant strains may significantly reduce their efficacy and has become an important public health issue [5-7]. However, resistance to ciprofloxacin (CIP) and levofloxacin (LVX), two of the major FQs currently in use, also easily found in clinical isolates of *P. aeruginosa* [8].

Disk diffusion method is a suitable tool, but it yields only categorized qualitative results and does not demonstrate minimum inhibitory concentration (MICs). The E-test is a relatively new agar diffusion-based method for the quantitative evaluation of bacterial susceptibilities. We compared the sensitivities of the results obtained from the disk diffusion method with those obtained from E-test method approved by the Clinical and Standards Institute (CLSI). E-test strips containing ciprofloxacin and imipenem were purchased from a commercial company (Liofilchem, Italy). For the agar disk diffusion method, antimicrobial drug-impregnated disks were supplied by a commercial company **(**MAST, Merseyside, U.K) as well. The effective mechanism of E-test are similar to those of the widely used disk diffusion method; contrary to the disk diffusion method, prediffusion, preincubation and inoculums size does not affect E-test results, because antimicrobial gradient generated by the E-test remains stable during the test [9].

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P. aeruginosa acquires resistance to the above mentioned antibiotics through chromosomal mutations. These include 1) mutations resulting in cell wall impermeability due to loss of OprD, a porin that forms narrow trans-membrane channels, 2) mutations in the genes encoding DNA gyrase *(gyrA)* and topoisomerase IV *(parC)*, [3] mutations in the genes that encode *gyrA* and *parC* that are the major mechanisms of resistance to FQs. Mutational studies have shown that *gyrA* mutations in codon 83 and 87 are associated with higher FQ resistance [3, 9]. The major known mechanism of resistance to carbapenems in *P. aeruginosa* is inactivating mutations in *OprD*, which has showed to confer resistance to imipenem [10, 11].

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 METHODS

Clinical isolates. In this cross-sectional study, clinical isolates of *P. aeruginosa* were collected over a 6-month period (September 2012 to February 2013) from hospitalized patients in ICU of Al-Zahra hospital, Isfahan, Iran. This study included all clinical specimens submitted for bacterial culture at the microbiology laboratory of the hospital. Specimens obtained from urinary tract (26), tracheal aspirate (19), blood samples (6) and other sites. They were cultured as described by others (15). The data of the collected samples is reflected in table 1.

Table 1. Data of the collected samples from ICU of Al-Zahra hospital in Isfahan, Iran

Variable	Number of patients (%)	Type of sample	Number of sample (%)
Gender		Urine	25 (38.5)
Male	49.2	Tracheal aspirate	19 (29.2)
Female	50.8	Blood	7 (10.8)
		Wound	5 (7.6)
Age		Bronch	3 (4.7)
≤1 year	9.3	Cerebral spinal fluid	3 (4.7)
>1-20 year	7.7	Abscess	2 (3)
>20-50 year	43	Abdominal fluid	1 (1.5)
>50 year	40	**Total**	**65 (100)**

Table 2. Primers used in this study

Gene	Primer name	Sequence 5´–3´	Product size (bp)	Reference	Use
gyrA	*gyrA-F*	AGTCCTATCTCGACTACGCGAT	382	[12]	Sequencing of *gyrA*
	gyrA-R	AGTCGACGGTTTCCTTTTCCAG			
oprD	*oprD-F*	TGCTGCTCCGCAACTACTATTTC	752	[13]	Sequencing of *oprD*
	oprD-R	GTAGGCCAAGGTGAAAGTGTG			

Identification of *P. aeruginosa* strains. Bacteria recovered from clinical specimens were identified by standard biochemical methods. The samples were cultured on Mac Conkey agar, Blood agar and Cetrimide agar (Himedia Company). The cultured media were incubated at 35°C for 18-24 h and the pure colonies were characterized and 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 Company, England). Control strains *P. aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922 [11] 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 MIC was read where inhibition of growth intersected the E-test strip. When the small colonies grew within the zone of inhibition or a haze of growth occurred around the MIC end-point, the maximum MIC crossing was recorded. 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* ATCC25922 [11].

PCR amplification and DNA sequencing. Chromosomal DNA was extracted using a DNA extraction kit (Sinaclon) according to the manufacturer's instructions and were used as template for PCR reactions. PCR amplification of *gyrA* and *oprD* genes was performed with whole-DNA extracts from 5 randomly selected FQ and carbapenem resistant clones by using the primers described in table 2. 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 (PôleBioinformatique Lyonnais) of *gyrA* and *oprD* genes from PAO1 and clinical isolates.

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 3). 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 and oprD* 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 (Figure 1 and Figure 2). 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. Amino acid changes found among these carbapenem resistant *P. aeruginosa* strains are displayed in figure 3 and table 4.

Table 3. The resistance rates of *P. aeruginosa* strains based on the disk diffusion method

Antibacterial Class	Antibiotic	Sensitive %	Intermediate %	Resistant %
Carbapenems	Meropenem	32.3	1.5	66.2
	Imipenem	36.9	0	63.1
Quinolones	Ciprofloxacin	29.2	7.7	63.1
	Levofloxacin	27.7	6.2	66.2
Aminoglycosides	Gentamicin	33.8	4.6	61.5
	Amikacin	47.7	1.5	50.8
Cephems	Cefepime	27.7	12.3	60
	Ceftazidime	24.6	12.3	63.1
β-lactam + Inhibitor	Piperacillin/Tazobactam	27.7	18.5	53.8
Monobactams	Aztreonam	15.4	20	64.6
Penicillins	Piperacillin	23.1	16.9	60

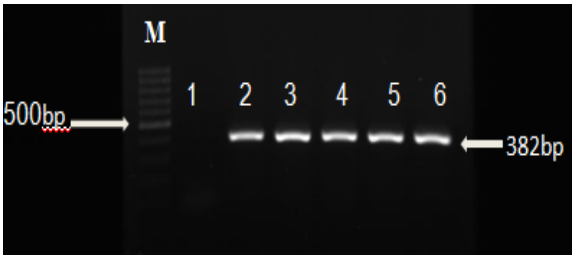

Fig. 1. Agarose gel electrophoresis of *gyrA* gene of *P. aeruginosa* stains isolated from clinical samples.

M, 100 bp DNA size marker; lane 1, negative control; lane 2, positive control; lanes 3-6, clinical isolates.

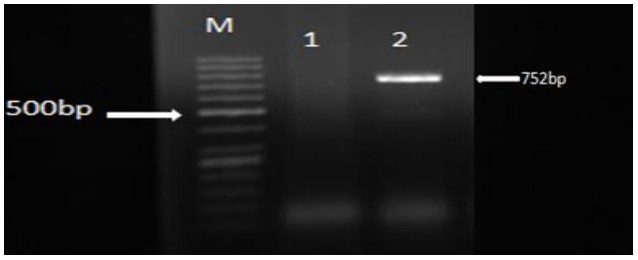

Fig. 2. Agarose gel electrophoresis of *oprD* gene of *P. aeruginosa* stains isolated from clinical sample.

M, 50 bp DNA size marker; lane 1, negative control; lane 2, clinical isolate.

Table 4. *OprD* and *gyrA* amino acid sequence alterations in MDR-resistant isolates

N[1]	MIC[2] (µg/mL)		Amino acid position and substitutions												
	CIP	IMP	*gyrA*	*oprD*											
			83	103	115	170	185	186	189	202	210	230	240	262
PAO1 (wt)[3]	<0.06	0.6	T	T	K	F	E	P	V	E	I	E	S	N
1	256	32	I	-	-	-	Q	G	T	Q	A	K	T	T
3	256	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	32	I	S	T	L	Q	G	T	-	-	-	-	-
14	-	32	-	S	T	L	Q	G	T	-	-	-	-	-
16	256	32	I	S	T	L	Q	G	T	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	256	32	I	S	T	L	Q	G	T	-	-	-	-	-
57	256	-	I	-	-	-	-	-	-	-	-	-	-	-

[1]N, number of strains; [2]MIC, minimum inhibitory concentration; IMP: imipenem, CIP: ciprofloxacin; [3]wt, wild-type

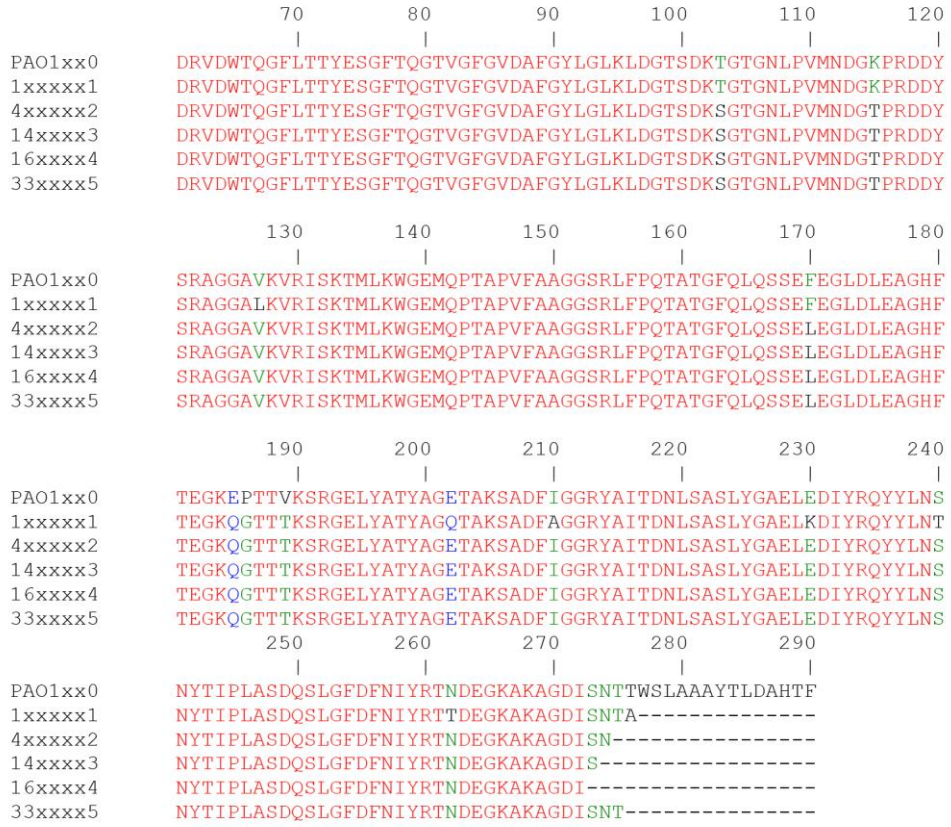

Fig. 3. Multiple sequence alignment by PBIL (Pôle Bioinformatique Lyonnais) of the OprD from PAO1 and the clinical isolates belonging to different porin types.

DISCUSSION

P. aeruginosa is a major pathogen causing serious nosocomial infections. The prevalence of nosocomial infections develops by MDR strains. The characteristics of ICU isolates play an important role in the anti-bacterial therapy of the life-threatening infections in ICUs [3]. Emergence of MDR *P. aeruginosa* in ICUs is increasingly reported as an obstacle in patients treatment [14]. In Iran, carbapenems and FQs are used widely because of their low rate of side effects. Regarding the lack of adherence to approved infection control practices by hospitals, routine use of these antibiotics may lead to the increased risk of drug resistance. Results of the present study are indicative of high resistance rates in *P. aeruginosa* isolates. The resistance ratio to carbapenems and FQs among *P. aeruginosa* isolates obtained in this study was higher than previous reports [15, 16].

We found strong correlation between E-test and disk diffusion methods. All the isolates showed resistant to tested antibiotics by the disk diffusion method and E-test (specificity 100%).

The high percentage of similarity showed that both methods (E-test and disk diffusion) have good concordance in determining the in vitro activity of the drugs on *P. aeruginosa* isolates. Therefore, although the quantitative E-test method is a rapid, easy to use, labor-efficient and accurate method for MIC determination on an agar medium, the disk diffusion method is equally reliable and more cost-effective for routine hospital use [7, 17, 18].

One of the important mechanisms of resistance to carbapenem and FQ is chromosomal mutations. In Iran, there is not much data on mutations leading to Carbapenem and FQ-resistance in *P. aeruginosa* strains [19]. 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* [20]. FQ resistance in *P. aeruginosa* has been associated with substitutions in the *gyrA* subunit of DNA gyrase and in the parC subunit of DNA topoisomerase IV [21-23].

We found similar type of mutation in *gyrA* gene at codon 83 in addition to point mutation in *gyrA* gene followed by a single amino acid substitution (Thr-83→ Ile) in 10 strains.

This result was in accordance with previous reports on clinical isolates of *P. aeruginosa* [12, 21, 22, 24, 25]. The most frequent causes of *oprD* mutational inactivation were point mutations leading to substitutions in the amino acid profile [19].

Amino acid substitutions in OprD porin are frequently seen in the examined *P. aeruginosa* strains and also these may lead to carbapenem resistance. The $E_{185}Q$, $P_{186}G$, and $V_{189}T$ mutations were the most frequent, while other mutations were rare. Our results in this study confirmed previous reports on clinical isolates of *P. aeruginosa*. Unlike

previous reports [2, 5, 20], nucleotide insertions and deletions were not observed in our clinical isolates. Our findings also showed a number of mutations in the third codon which did not affect the protein sequence consistency compared with previous reports [26].

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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