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The role of *gyrA* and *parC* mutations in fluoroquinolones-resistant *Pseudomonas aeruginosa* isolates from Iran

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ABSTRACT

The aim of this study was to examine mutations in the quinolone-resistance-determining region (QRDR) of *gyrA* and *parC* genes in *Pseudomonas aeruginosa* isolates. A total of 100 clinical *P. aeruginosa* isolates were collected from different university-affiliated hospitals in Tabriz, Iran. Minimum inhibitory concentrations (MICs) of ciprofloxacin and levofloxacin were evaluated by agar dilution assay. DNA sequences of the QRDR of *gyrA* and *parC* were determined by the dideoxy chain termination method. Of the total 100 isolates, 64 were resistant to ciprofloxacin. No amino acid alterations were detected in *gyrA* or *parC* genes of the ciprofloxacin susceptible or ciprofloxacin intermediate isolates. Thr-83 → Ile substitution in *gyrA* was found in all 64 ciprofloxacin resistant isolates. Forty-four (68.75%) of them had additional substitution in *parC*. A correlation was found between the number of the amino acid alterations in the QRDR of *gyrA* and *parC* and the level of ciprofloxacin and levofloxacin resistance of the *P. aeruginosa* isolates. Ala-88 → Pro alteration in *parC* was generally found in high level ciprofloxacin resistant isolates, which were suggested to be responsible for fluoroquinolone resistance. These findings showed that in *P. aeruginosa*, *gyrA* was the primary target for fluoroquinolone and additional mutation in *parC* led to highly resistant isolates.

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Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen[1–3] causing life-threatening infections, especially in immunocompromised patients.[4–6] Often these infections are difficult to treat due to the intrinsic resistance of the species[7] as well as its remarkable ability to acquire resistance to a wide range of antimicrobial agents.[8] Fluoroquinolones are the only accessible antibiotics for effective oral treatment of infections caused by this organism.[9] Among fluoroquinolones, ciprofloxacin and levofloxacin are widely used in the treatment of *P. aeruginosa* infections.[10]

Fluoroquinolones act by inhibiting the action of target enzymes, DNA gyrase and topoisomerase IV, with both enzymes playing a principal role in DNA replication.[11] DNA gyrase and topoisomerase IV are heterotetrameric enzymes that are composed of two subunits encoded by the *gyrA*, *gyrB* and *parC*, *parE*, respectively.[12] The *gyrA* and *gyrB* genes are homologs to *parC* and *parE*, respectively.[13] The mechanisms of fluoroquinolone resistance in *P. aeruginosa* include mutations in the DNA gyrase and topoisomerase IV,[14,15] overexpression of efflux pump system and the innate impermeability of the membrane.[16] Alterations in the so-called quinolone-resistance-determining region (QRDR) within DNA gyrase and topoisomerase IV are the major mechanisms for fluoroquinolone resistance in *P.* aeruginosa.[17–20]

Moreover, isolates with mutations in QRDR of *gyrA* and *parC* show the highest levels of fluoroquinolone resistance.[21] Although amino acid alterations in the *gyrB* and *parE* genes have been described, but the frequency of these mutations is low, with only a complementary role in fluoroquinolone resistance.[18,21]

Several studies from Iran have reported the high prevalence of MDR strains among Iranian hospitals and strains isolated from hospitalized patients; especially, burn patients have show high level resistance to most available antibiotics.[22,23]

The prevalence of mutations in DNA gyrase and topoisomerase IV has not been well studied in Iran. This is the largest analysis of the QRDR of *gyrA* and *parC* in the clinical isolates of *P. aeruginosa* from Iran. The aim of this study was to examine the mutations in *gyrA* and *parC* genes and characterize their correlation with ciprofloxacin and levofloxacin resistance, as well as the evaluation of their effect on ciprofloxacin and levofloxacin Minimum inhibitory concentrations (MICs) among the clinical isolates of *P. aeruginosa*.

Materials and methods

Bacterial isolates

In a prospective study, a total of 100 non-repetitive clinical isolates of *P. aeruginosa* were collected, between December 2013, and July 2014, from four Educational-Health Care Centers of Tabriz University of Medical Sciences in Northwest Iran. The bacterial isolates were recovered from different clinical specimens such as urine (29%), wound discharge (25%), tracheal aspirates (21%), blood (8%) and other clinical specimens (Table 1). All isolates were identified by standard conventional biochemical tests.[24]

Antimicrobial susceptibility testing

Antimicrobial susceptibility to ticarcillin (75 μg), piperacillin-tazobactam (100/10 μg), ceftazidime (30 μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), gentamicin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg) and ofloxacin (5 μg) (Mast, UK) was performed by employing the standard disk diffusion method. MICs of ciprofloxacin and levofloxacin (Sigma-Aldrich) were determined by the standard agar dilution assay. The results of disk diffusion assay as well as MIC were interpreted according to the clinical and laboratory standard institute (CLSI) guidelines.[25] *Pseudomonas aeruginosa* ATCC 27853 was used as the control strain for susceptibility testing.

Table 1 – Distribution of *Pseudomonas aeruginosa* isolates by the site of isolation and hospital origin.

Source of isolates	Hospital origin				Total no. of isolates
	Imam Reza	Sina	Koodakan	Shahid Madani	
Tracheal aspirate	7	4	6	4	21
Wound discharge	7	8	4	6	25
Urine	7	6	9	7	29
Blood	1	4	1	2	8
Throat culture	1	0	3	0	4
Catheter	0	0	2	2	4
Sputum	0	1	1	0	2
Bronchial washing	0	0	0	2	2
Pleural fluid culture	1	0	0	0	1
Urethral discharge	1	0	0	0	1
Peritoneal fluid	1	0	0	0	1
Ear discharge	0	1	0	0	1
Stool	1	0	0	0	1
Total	27	24	26	23	100

Table 2 – Amino acid alterations in *gyrA* and *parC* in ciprofloxacin resistant isolates of *Pseudomonas aeruginosa*.

Groups	No. of isolates	Replacement in QRDR			
		GyrA at position		ParC at position	
		83	87	87	88
PAO1		Thr (ACC)	Asp (GAC)	Ser (TCG)	Ala (GCC)
I	20	Ile (ATC)	–	–	–
II	8	Ile (ATC)	–	–	Pro (CCC)
III	31	Ile (ATC)	–	Leu (TTG)	–
IV	5	Ile (ATC)	Asn (AAC)	Leu (TTG)	–

PCR amplification and DNA sequencing

Chromosomal DNA from the isolates of *P. aeruginosa* was extracted by DNA extraction kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. The PCR reaction was performed in a 50 μL mixture containing 1.5 mM $MgCl_2$, 0.5 pmol of each primer, 0.2 mM dNTPs (Yekta Tajhiz Azma, Iran), 1U of Pfu DNA polymerase (Yekta Tajhiz Azma, Iran), 1X Pfu DNA polymerase buffer and 10–100 ng of the template DNA.

The amplification of *gyrA* and *parC* genes was carried out using the polymerase chain reaction and specific primer sets as described previously.[26] Purified PCR products were sequenced using the Applied Biosystems 3730/3730xl DNA analyzers sequencing (ABI) system, (Bioneer Co., Korea).

Statistical analysis

Categorical variables were compared by the Chi-square test or Fisher's exact test using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL). A statistically significant difference was considered as a *P*-value < 0.05.

Results

Antimicrobial susceptibility testing

Of the 100 *P. aeruginosa* clinical isolates tested for antimicrobial susceptibility, 71 of them were found to show multidrug resistance (MDR). MDR was defined as resistance to three or more unrelated antibiotics.[27] The highest rate of resistance was observed against ticarcillin (84%) and ofloxacin (82%). Moreover, the highest susceptibility rate was obtained against ceftazidime (45%), followed by gentamicin (44%). Of the total isolates, 64 (64%) were resistant, 2 (2%) were intermediate and 34 (34%) were observed to be susceptible to ciprofloxacin. Moreover, 63% of isolates were resistant, 1% were intermediate, and 36% were susceptible to levofloxacin.

DNA sequences analysis

DNA sequences of all *P. aeruginosa* isolates were compared with the corresponding sequences of *P. aeruginosa* PAO1 (Accession: NC_002516.2 GI: 110645304). Aside from ciprofloxacin susceptible, levofloxacin susceptible and ciprofloxacin intermediate isolates which had no amino acid alterations in their *gyrA* or *parC* genes, the amino acid alterations were recognized in *gyrA* and *parC* QRDR of 64 ciprofloxacin resistant, 63 levofloxacin resistant and 1 levofloxacin intermediate isolates, as described in Table 2. The total mutations found in these isolates were classified into 4 distinct groups according to the pattern of amino acid alteration. Group I: isolates contained single mutation Thr-83 → Ile in *gyrA*. Group II: isolates contained one mutation Thr-83 → Ile in *gyrA* and one mutation Ala-88 →Pro in *parC*. Group III: Isolates contained one mutation Thr-83 → Ile in *gyrA* and one mutation Ser-87 → Leu in *parC*. Group IV: isolates contained two mutations Thr-83 → Ile and Asp-87 → Asn in *gyrA* and one mutation Ser-87 → Leu in *parC*.

DNA sequences of QRDR *gyrA* showed Thr-83 → Ile substitution for all 64 ciprofloxacin resistant isolates. Of 64 isolates, 20 (31.25%) had a mutation (Thr-83 → Ile) in *gyrA* alone (group I). A double mutation in *gyrA* (Thr-83 → Ile and Asp-87 → Asn) was detected in 5 of 64 isolates. Amino acid alteration in the QRDR *parC* was observed in 44 (68.75%) of 64 isolates. All of these isolates possessed additional mutations in *gyrA*. No double mutations in *parC* were found. The Ser-87 → Leu substitution was found in 31 (48.4%) of 64 isolates. Moreover, the substitution of Pro for Ala-88 was observed in 8 (12.5%) of 64 isolates.

Correlation between fluoroquinolones MIC and QRDRs mutations

The MIC values of ciprofloxacin and levofloxacin for 64 resistant isolates and their correlation with different types of mutations in *gyrA* and *parC* genes are shown in Table 3. As shown, ciprofloxacin MIC for isolates with a single *gyrA* substitution (Thr-83 → Ile) ranged from 4–64 μg/mL and for levofloxacin, the 4–32 μg/mL range was observed. Isolates with a single *gyrA* (Thr-83 → Ile) substitution and a single *parC* substitution (Ala-88 → Pro or Ser-87 → Leu) had ciprofloxacin MICs ranging from 8 to 128 or 16 to 256 μg/mL and levofloxacin MICs varied from 8 to 64 or 8 to 256 μg/mL. Moreover, the isolates with double *gyrA* substitutions (Thr-83 → Ile and Asp-87 → Asn) and a single *parC* substitution (Ser-87 → Leu) had ciprofloxacin and levofloxacin MICs ranging from 32 to 256 μg/mL. Our results showed that the two concurrent mutations in *gyrA* and *parC* genes were associated with a higher level of ciprofloxacin and levofloxacin MICs, as compared to a single mutation in *gyrA*. (Geometric mean MICs of ciprofloxacin, 29.34 (group 2) and 32 (group 3) versus 16.56 (group 1) μg/mL, $p < 0.05$; geometric mean MICs of levofloxacin, 24.67 (group 2) and 28.6 (group 3) versus 14.42 (group 1) μg/mL $p < 0.05$). Moreover, three concurrent mutations in *gyrA* and *parC* genes were associated with a higher level of ciprofloxacin and levofloxacin MICs, as compared to

Table 3 – Correlation of mutations in *gyrA* and *parC* genes and MICs distribution of ciprofloxacin and levofloxacin.

Group	No. of isolates	Antimicrobial agents	No. of isolates with MICs (μg/mL)									
			0.5	1	2	4	8	16	32	64	128	256
I GyrA (83)	20	Ciprofloxacin Levofloxacin				2 1	2 4	10 12	5 3	1		
II GyrA (83), ParC (88)	8	Ciprofloxacin Levofloxacin					1 1	1 3	5 2	 2	1	
III GyrA (83), ParC (87)	31	Ciprofloxacin Levofloxacin					 4	12 8	11 12	5 4	2 2	1 1
IV GyrA (83 and 87), ParC (87)	5	Ciprofloxacin Levofloxacin							2 2	1 1	1 2	1

two concurrent mutations in *gyrA* and *parC* genes (Geometric mean MICs of ciprofloxacin, 73.5 (group 4) versus 29.3 (group 2) μg/mL, and 32 (group 3) μg/mL $p < 0.05$; geometric mean MICs of levofloxacin, 64 (group 4) versus 24.6 (group 2) and 28.61 (group 3) μg/mL $p < 0.05$) or single mutation in *gyrA*. (Geometric mean MICs of ciprofloxacin, 73.51 (group 4) versus 16.56 (group 1) μg/mL, $p < 0.05$; geometric mean MICs of levofloxacin, 64 (group 4) versus 14.42 (group 1) μg/mL $p < 0.05$).

Discussion

Fluoroquinolones such as ciprofloxacin and levofloxacin are an important class of antibiotics for the treatment of *P. aeruginosa* infections.[26] However, *P. aeruginosa* rapidly becomes resistant to these drugs during antibiotic therapy.[15] The principle mechanism of fluoroquinolones resistance in *P. aeruginosa* involves mutations in the genes of DNA gyrase and topoisomerase IV.[18]

In the present study, the alteration of Thr-83 to Ile in *gyrA* was found in all ciprofloxacin and levofloxacin resistant *P. aeruginosa* isolates. Moreover, a double concomitant mutation in *gyrA* (Thr-83 → Ile and Asp-87 → Asn) was observed in five ciprofloxacin and levofloxacin resistant isolates. More noteworthy, the MIC values of tested fluoroquinolones among these isolates were significantly higher. However, no amino acid change was detected in ciprofloxacin susceptible, levofloxacin susceptible and ciprofloxacin intermediate isolates. So Thr-83 → Ile was shown to be the chief mechanism of fluoroquinolones resistance. This was consistent with the results of other studies.[26,28–30] Furthermore, the amino acid sequences analysis in the QRDR of *parC* showed that more than half of ciprofloxacin and levofloxacin resistant isolates had an alteration in *parC* and the Ser-87 → Leu substitution was the predominant amino acid change (48.4%). Lee et al.[18] and Mouneimne et al.[16] have previously reported this amino acid change in 35.9% and 25% of the ciprofloxacin resistant isolates, respectively. Also, another alteration in *parC*, Ala-88 → Pro substitution, was highly frequent in our isolates, in comparison to the results of Akasaka et al.,[17] and Sekiguchi et al.[31] They found this amino acid change only in one of all studied isolates. More importantly, this substitution was observed generally among high level ciprofloxacin resistant isolates suggested to be responsible for fluoroquinolone resistance. However, we did not detect amino acid alteration at positions Pro-83, Gly-85,[31] Glu-91 and Leu-95[17] in *parC*, as reported in the previous studies.

Based on the analysis of sequencing results, all of the isolates with *parC* mutation had one or two mutations in *gyrA*. This observation confirmed that the DNA gyrase was the primary target for fluoroquinolone resistance in the clinical isolates of *P. aeruginosa*. However, isolates that had double mutation in *gyrA* and *parC* had higher ciprofloxacin and levofloxacin MICs than those with a single mutation in *gyrA*, thereby suggesting that alteration in *parC* occurred after *gyrA*, leading to higher level fluoroquinolone resistance in *P. aeruginosa*. Moreover, the addition of a second *gyrA* alteration to *gyrA* and *parC* mutations had a significant effect on ciprofloxacin and levofloxacin MICs. Our results suggested that there could be a correlation between the number of *gyrA* and *parC* alterations and the level of fluoroquinolone resistance. This has been reported by Lee et al.[18] for the clinical isolates of *P. aeruginosa*. Differences in the MICs of ciprofloxacin and levofloxacin for isolates had a single alteration in QRDR of *gyrA* with or without an alteration in QRDR of *parC*, revealing that other resistance factors were involved in fluoroquinolone resistance. Mechanisms such as overexpression of efflux pumps (MexAB-OprM[32,33] MexCD-OprJ,[28] MexEF-OprN[21] and MexXY-OprM[34]) and mutation in *gyrB* and *parE* have been described for fluoroquinolone resistance in *P. aeruginosa*.[17,18] However, the impact of these mechanisms on MICs of ciprofloxacin and levofloxacin can be clarified by further studies.

To conclude, mechanisms other than mutations in *gyrA* and *parC* (such as active efflux pumps, alterations in *gyrB*, *parE* or innate impermeability of the membrane) may contribute to the level of fluoroquinolone resistance in the clinical isolates of *P. aeruginosa*, but a single amino acid alteration, Thr-83 → Ile, in *gyrA*, is sufficient to cause clinically important levels of resistance to fluoroquinolones, and the simultaneous presence of mutation in *parC* (Ser-87 → Leu or Ala-88 → Pro) mediates significantly higher level fluoroquinolone resistance.

Finally, our results revealed that the mutations in *gyrA* and *parC* were the main mechanism of fluoroquinolone resistance among the clinical isolates of *P. aeruginosa* in Tabriz, Iran. To the best of our knowledge, this study is the largest analysis of

the QRDR of *gyrA* and *parC* in the clinical isolates of *P. aeruginosa* from Iran.

Conflicts of interest

The authors declare no conflicts of interest.

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