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ANALYSES OF *parC* AND *gyrA* MUTATIONS IN CIPROFLOXACIN-RESISTANT AND SUSCEPTIBLE *pseudomonas aeruginosa* ISOLATED FROM SOIL BY PCR-RFLP AND SSCP METHOD

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ABSTRACT

The aims of this study were to assess the prevalence of *gyrA* and *parC* mutations in ciprofloxacin-resistant and susceptible *Pseudomonas aeruginosa* isolated from soil and to evaluate the suitability of the restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) techniques as screening methods for molecular characterizations of ciprofloxacin resistance. From the examined 21 *P. aeruginosa* isolates 9 strains were resistant to ciprofloxacin. These 21 P. aeruginosa isolates and one control strain were analyzed for alterations in the ciprofloxacin resistance determining region of *gyrA* and *parC* by RFLP and SSCP analyses. The PCR reaction confirmed the presence of the *gyrA* and *parC* genes in all of the strains. PCR-RFLP analyses with *Sac*II for gyrA and *Hin*fI for *parC* were performed as a screening method. We found that 18 and 17 out of 22 isolates have *SacII* and *HinfI* restriction site and 4 and 5 strains did not have the site recognized by these enzymes, respectively. Seven for *gyrA* and fourteen for *parC* PCR products were electrophoresed for SSCP. By SSCP analysis, 21 (in *parC*) and 15 (in *gyrA*) different band patterns were detected, and each pattern corresponded to a distinct mutation. As a result, the RFLP and SSCP methods are suitable for a molecular screening of ciprofloxacin resistant and susceptible *P. aeruginosa* isolates. SSCP analysis can also provide advantage for the detection of novel and multiple mutations**.** In addition, we can say that environmental monitoring followed by clinical surveillance can be successful in uncovering previously unrecognized cases of infection.

Keywords: *gyrA*, *parC*, PCR-RFLP, *Pseudomonas aeruginosa*, SSCP

INTRODUCTION

Pseudomonas aeruginosa is a common environmental microorganism and can be found in faeces, soil, water and sewage. It can multiply in water environments. *P. aeruginosa* is a recognized cause of hospital-acquired infections with potentially serious complications (**Hardalo and Edberg, 1997**). *P. aeruginosa* can cause a wide range of infections, and is a leading cause of illness in immunocompromised individuals. It predominanly colonizes damaged sites such as burn and surgical wounds, the respiratory track of human with underlying disease and physically damaged eyes (**Mena and Gerba, 2009**). Multidrug-resistant Gram-negative bacteria represent important nosocomial pathogens and are responsible for a significant proportion of infections in patiens in hospital (**Daugherty *et al*., 2014**). The Gram-negative bacterium *P. aeruginosa*, which can infect a wide range of animal and plant hosts, has become resistant to practically all antimicrobial drugs available on the market (**Breidenstein *et al*., 2011**). Ciprofloxacin has emerged as one of the most effective quinolones against *P. aeruginosa*. The mechanisms of quinolone resistance described in *P. aeruginosa* are mutations in the DNA gyrase *gyrA* and *gyrB* genes (**Sugino *et al*., 1980**) and in the topoisomerase IV *parC* and *parE* genes (**Peng and Marians, 1993**), decreased permeability of the cell wall, and multidrug efflux systemes (**Mauneimne *et al*., 1999**). Resistance to fluoroquinolones in *P. aeruginosa* has mostly been attributed to mutations in these genes.
Commonly, sequencing is a common technique for the identification of mutations in the fluoroquinole-resistance determining regions of *gyrA* and *parC*. Although the widespread use of this method, it is slow and expensive. Various alternative methods to replace sequencing have been proposed, including single-stranded conformation polymorphisim (SSCP) and polymerase chain reaction- restriction fragment length polymorphism (PRC-RFLP) (**Kim *et al*., 2012**).
The aim of this study was to evalute a rapid method for detection of *gyrA* and *parC* mutations associated with ciprofloxacin resistance in *P. aeruginosa* strains isolated from soil samples.

MATERIAL AND METHODS

Collection of the soil samples and isolation of *P. aeruginosa* strains

17 soil samples (from Sinop province, Turkey) were collected from different sampling sites. The samples were collected at depth of 5-10 cm below the soil surface. The samples were transported in cooling bags until processing at the laboratory. One gram of each soil sample was homogenized in 9 ml of 0.85% NaCl in sterile test tubes. A 0.1 ml aliquot of the dilution was spread aseptically on King's B agar (KBA- Merck) medium and incubated at 30°C for 48 hours. After incubation, representative colonies were selected on the basis of distinct morphological characterictics (**Atlas, 1995**). The selected bacteria were identified by their morphological, physiological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (**Brenner *et al*., 2005**).

Antibiogram

Antimicrobial susceptibility testing of ciprofloxacin (CIP- 5 µg- OXOID, UK) antibiotic was carried out through Kirby-Bauer disk diffusion method on Mueller-Hinton medium according to Clinical and Laboratory Standarts Institute (CLSI) recommendation. MHA plates were inoculated with a bacterial suspension equivalent to a 0.5 McFarland standard and antibiotic susceptibility disks were applied. Zones of growth inhibition (in millimeters) were recorded after 24 h incubation at 37°C.

Genomic DNA isolation

Chromosomal DNA extraction from *P. aeruginosa* strains was carried out according to the method of **Sambrook *et al*.** (1989) with some modifications. The *P. aeruginosa* samples were activated with incubation at 37°C for 24 h. Recovered bacteria were centrifuged at 3.000 xg for 5 min and cell pellets were resuspended in 500 µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Pellets were then incubated at 55°C for 30 min following the addition of 50 µl SDS

(10%) and 25 µl proteinase K (20 mg/ml). Total DNA was recovered by sequential extractions with 575 µl phenol/chloroform (1:1). Then, tubes were inverted, incubated on ice for a few minutes and centrifuged at 14.000 xg for 10 min. Upper layer (500 µl volume) was transferred into a new eppendorf tube and treated with 50 µl sodium acetate (3 M, pH:5.2) and 330 µl isopropanol (100%) (Overnight). Tubes were inverted and DNA was precipitated by centrifugation at 14.000 xg for 10 min followed by washing with 70% (v/v) ethanol, dried, and resuspended in 100 µl TE buffer containing 2 µl RNase. Then, DNA samples were stored at -20°C until further molecular analysis.

PCR amplification

Nucleotide sequence from Gen Bank, *P. aeruginosa* accession numbers L29417 (*gyrA*) and AB003428 (*parC*) were used for amplification (**Lee *et al*., 2005**). The target regions were amplified by PCR using specific primers described in Table 1. PCR was carried out in a reaction volume of 50 µl containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphates (dNTPs), 50 pmol of each primer, 2.5 U of Taq DNA polymerase (Thermo Scientific, USA) and 1 µl genomic DNA. The reactions were performed in a Techne TC-5000 thermal cycler (California, USA) for 35 cycles, each consisting of a denaturation for 1 min 94°C, annealing for 1 min at 65°C (*gyrA*) or 55°C (*parC*), and extension for 1 min at 72°C. PCR products were analyzed by 1.8% agarose gel (Sigma-Aldrich, St. Louis, MO, USA) electrophoresis with a molecular size marker (O' Gene Ruler, 1 kb DNA Ladder, ready-to-use, Thermo Scientific, USA). The gels were stained with ethidium bromide and bands were visualized under UV light.

Table 1 Primer sequences for detection of the ciprofloxacin-resistant region in gyrA and parC genes

Gene	Nucleotide sequence (5' to 3')	Nucleotide position
gyrA	GACGGCCTGAAGCCGGTGCAC	115-135
	GCCCACGGCGATACCGCTGGA	531-511
parC	CGAGCAGGCCTATCTGAACTAT	63-84
	GAAGGACTTGGGATCGTCCGGA	366-344

PCR-RFLP for mutation detection in the gyrA and parC genes

PCR products of *gyrA* and *parC* genes were digested with *Cfr*42I (*Sac*II) and *Hin*fI to screen for mutations at positions 83 in the *gyrA* and 87 and 109 in the *parC*, respectively. Enzymes digestion was performed in a 31 µl mixture containing 10 µl of the PCR product, 1 µl (10U) of enzyme, 2 µl buffer and 18 µl of nuclease free water at 37°C for 15 h. After digestion with enzymes, the presence of PCR products were analyzed by 2.5% agarose gel and visualized under UV light.

SSCP for mutation detection in the gyrA and parC genes

Two µl of PCR products were added to 10 µl of denaturation solution (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 10 mM NaOH). Mixture was heated at 95°C for 5 minutes and immediately cooled on ice. The mixture was analyzed by non-denaturing polyacrylamide gel-electrophoresis (39:1 acrylamide:bisacrylamide) (16x18 cm) in 0.5X TBE in a Hoefer electrophoresis system (Hoefer Inc. Holliston, MA, USA) at constant power of 5 mA, 18°C for 27 h (Hongyo *et al*., 1993). The gels were silver stained according to the procedure of **Byun *et al*.** (2009) and dried at room temperature.

Statistical analysis

The molecular weight of each band was calculated with Total Lab 1D Manual R11.1, UK programme. Following installation of the gel images into the programme, the bands were determined with their pixel positions and their molecular weights were scored according to the molecular size marker. The correlation between antibiotic resistance and ciprofloxacin was analysed by Pearson correlation test. Analysis was performed using the PASW statistics 18 (IBM SPSS Inc., Chicago, IL, ABD) program.

RESULTS AND DISCUSSION

In the study, the 21 *P. aeruginosa* strains were isolated from soil samples collected from Sinop, Turkey. All isolates were screened on account of the morphological and phenotypic characteristics, and according to biochemical tests, the isolates were members of P. aeruginosa species. Our results indicated that the strains were Gram-negative, rod shaped and positive for motility, catalase, oxidase, urea, pyocyanin fluorescence, glucose, xylose and capable of growth in 42°C. However, they were negative for lactose, maltose, esculin, DNase, indole, H_2S and capable of growth in 46°C and 8.5% NaCl.

The results of disc diffusion test showed that the 9 (42.8%) out of 21 isolates were resistant to ciprofloxacin with an average diameter of inhibition zone <15 mm (Table 2). According to **Ali *et al*.** (2010), 29 (38.6%) out of 75 *P. aeruginosa* isolates were resistant to ciprofloxacin. **Henwood *et al*.** (2001) reported that 177 (8%) out of 2194 *P. aeruginosa* strains were resistant to ciprofloxacin. Similarly, **Biswal *et al*.** (2014) reported that 7 (12%) out of 58 *P. aeruginosa* isolated from inpatients and environmental sources were resistant to ciprofloxacin. Our findings are in agreement with these data. The resistance to flouroquinolones is chromosomal. Thus, increasing intraspecies resistance to flouroquinolones is a reflection of mutation, which is a result of selective pressure created by the use of flouroquinolones, such as ciprofloxacin, norfloxacin and ofloxacin (**Sheng *et al*.,** 2002).

The 22 (including control strain) isolates were analyzed for alterations in the ciprofloxacin resistance determining region of gyrA and parC by RFLP and non-radioactive SSCP methods. PCR amplification of *gyrA* and *parC* was successful for all 22 strains and generated products with the expected sizes of 417 and 304 bp, respectively.

A single mutation in the gyrA and parC was associated with a decrease in susceptibility of the P. aeruginosa isolates to ciprofloxacin. A single mutation at codon 83 and 87 is the most frequent single mutation site in gyrA and parC gene, respectively.

Table 2 Ciprofloxacin resistance of *P. aeruginosa* environmental isolates and mutations in *gyrA* and *parC* genes

Strains	*gyrA*	*parC*	CIP
1	-	-	S
2	-	-	R
3	-	-	S
4	-	-	R
5	-	-	S
6	-	-	S
7	M	M	R
8	-	M	R
9	-	-	S
10	M	-	R
11	-	-	S
12	-	-	I
13	-	-	S
14	-	M	R
15	-	-	S
16	-	-	S
17	M	M	R
18	M	M	R
19	-	-	S
20	-	-	R
21	-	-	S
P	-	-	I

(M): Mutant, (-): No mutant, CIP: Ciprofloxacin, R: Resistant, S: Susceptibility, I: Intermediate; P: *P. aeruginosa* ATCC 27853

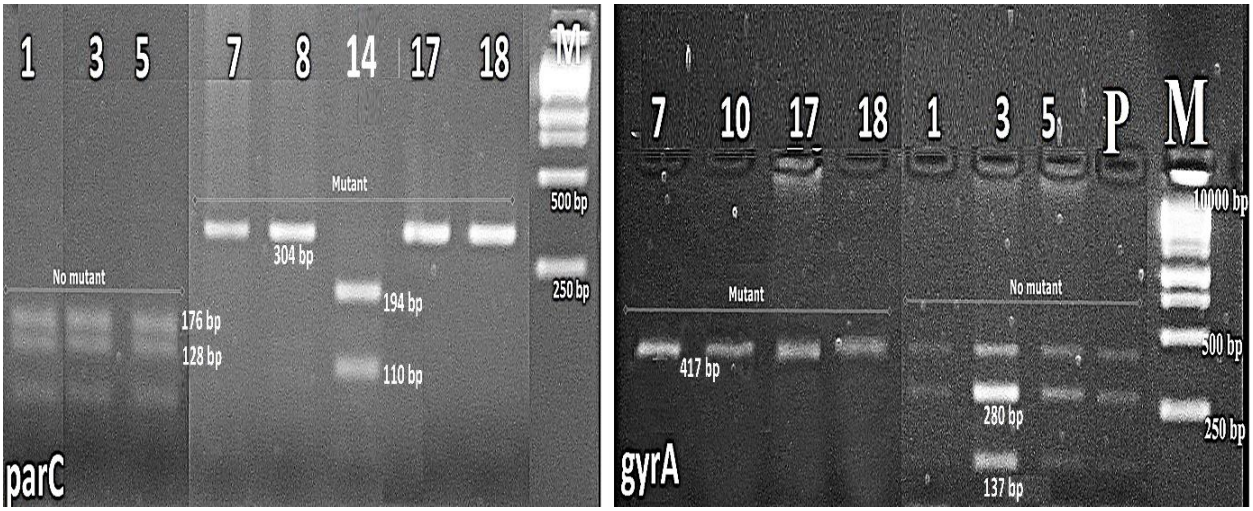

Figure 1 RFLP analyses of selected isolates. *parC* – RFLP. 7, 8, 14, 17 and 18: Mutant strains, the others not mutant; *gyrA* – RFLP. 7, 10, 17 and 18: Mutant strains, the others not mutant; M: Marker

After digestion by SacII restriction enzyme, the gyrA PCR products amplified from wild type strains generated two fragment of 280 and 137 bp; whereas, the mutant strains remained 417 bp. Mutation at position 83 in gyrA was observed in 4 (18.1%) isolates (Figure 1 and Table 2). Similarly, it was observed that the parC non-mutant strains formed two bands of 176 and 128 bp after cutting with *Hinf*I, while mutant strains were expected to form a band of 304 bp, but a mutant strain was found to have two bands of 194 and 110 bp (Figure 1 and Table 2). Of the 5 strains with mutations in the *parC* gene, all of them had a change at the 87th codon, and four of them remained as 304 bp. Hovewer, only one was digested in the 109th codon by *Hinf*I restriction enzyme (Figure 1). Three strains which had resistant to ciprofloxacin, had no mutations in either the gyrA and parC gene. In addition, one and two strains had mutations in the *gyrA* and *parC*, respectively, and 3 strains had mutations in both the *gyrA* and *parC* genes (Table 2). Morever, we could find no close corelation between antibiotic resistance and mutation in *gyrA* gene (*P*=0.56) and *parC* gene (*P*=0.65).

In previous studies, sequencing confirmed the presence of mutations in the quinolone resistance determining-regions (QRDRs) in most of the clinical and environmental *P. aeruginosa* isolates. In accordance with several previous studies (**Mauneimne *et al.*,** 1999; **Deguchi *et al.*,** 2000; **Lee *et al.*,** 2005; **Gorgani *et al.*,** 2009; **Bruchmann *et al.*,** 2013), the most frequenly observed mutations, positions 83 and 87, were encoded in QRDR of *gyrA* and *parC*, respectively. The RFLP method of the PCR products digested with *Sac*II and *Hinf*I has been used successfully to screen for mutations in the *gyrA* and *parC* gene of *P. aeruginosa*, respectively (**Takenouchi *et al.*,** 1999; **Deguchi *et al.*,** 2000).

The mutations in codon 83 (for *gyrA*) and 87 (for *parC*) were recognized in our study in only 4 and 5 of ciprofloxacin resistant *P. aeruginosa* isolates, respectively and SSCP analysis was not suitable to detect these mutations too. However, each band pattern detected by SSCP method indicates a different mutation.

In present study, the PCR products were analysed by non-radioactive SSCP to identify different alleles of *gyrA* and *parC*. Several distinct SSCP patterns were determined, which were shown in Figure 2. The 7 isolates for *gyrA* and 14 isolates for *parC* PCR products were electrophoresed for SSCP. Twenty-one for *parC* and fifteen for *gyrA* different band patterns were detected, and each pattern corresponded to a distinct mutation. It was also found that 2 strains (17 and 18) with mutation in the *parC* gene gave the same patterns. Morever, the strain 14 which had a different the RFLP pattern in the *parC* gene gave different dominant band patterns from the other strains.

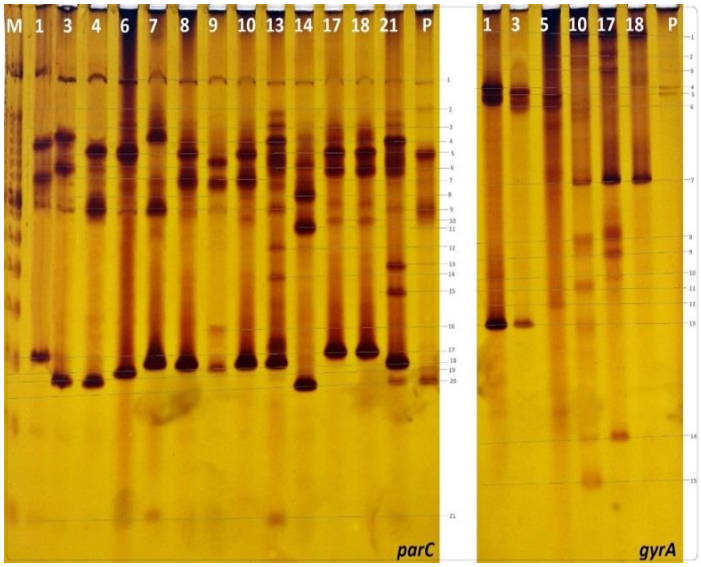

Figure 2 SSCP analyses of selected isolates. M: Marker; P: *P. aeruginosa* ATCC 27853

CONCLUSION

Our data suggest the PCR-RFLP and SSCP analyses provide simple, rapid and inexpensive detections of significant ciprofloxacin resistance mutations. Also, SSCP analysis can be advantageous for the detection of novel and multiple mutations. Therefore, obtained band pattern by SSCP method requires DNA sequence analyses to identify the mutations. Finally, we can say that the knowledge of epidemiology of P. aeruginosa isolates in soil samples may allow the establishment of preventive measures to decrease bacterial infections (**Stout *et al.*,** 2007).

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