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ORIGINAL ARTICLE

Molecular Investigation of Quinolone Resistance of Quinolone Resistance-Determining Region in *Streptococcus pneumoniae* Strains Isolated from Iran Using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism Method

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Abstract
Objectives: The resistance of *Streptococcus pneumoniae* to the recently available antibiotic treatment has been a growing problem. The aim of the study was to determine the quinolone-resistant strains and detect the presence of mutations in the quinolone resistance-determining regions of the *gyrA*, *parE*, and *parC* genes.
Methods: In this study, for the first time in Iran, the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was used to investigate the presence of mutations at quinolone resistance-determining regions of topoisomerase IV and DNA gyrase on 82 *S. pneumoniae* strains, among them 45 clinical samples were from patients and 37 from healthy carriers (control group).
Results: In clinical samples, 34 (75.56%) strains contained mutations in the *parC* gene, 31 (68.89%) carried mutations in the *gyrA* gene, and 14 (31.11%) had *parE* gene mutations. Antibiotic susceptibility test was performed using the CLSI (Clinical and Laboratory Standards Institute) criteria on three different generations of quinolone family, with nalidixic acid (82.22%) showing the highest resistance and levofloxacin (42.22%) the least resistance.
Conclusion: Results indicated that there is a significant correlation between quinolone resistance development and mutations in the *parE* gene as well as in the *parC* and *gyrA* genes.

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1. Introduction

Streptococcus pneumoniae is an important human pathogen that causes many diseases such as bacteremia, acute sinusitis, pneumonia, meningitis, and otitis [1,2]. Development of antibiotic resistance in this species has become a worldwide problem with serious effects on the treatment of diseases within the past 20 years [3,4]. Therefore, it is necessary to apply new therapeutic alternatives, such as newer fluoroquinolones that include levofloxacin and moxifloxacin [5,6]. Fluoroquinolones such as ciprofloxacin can be a suitable antibiotic to treat Gram-negative infections [7]. The mechanism of fluoroquinolone action involves inhibition of DNA gyrase and topoisomerase IV. Topoisomerase IV and DNA gyrase are heterotetramer proteins composed of two subunits: DNA gyrase, encoded by the genes *gyrA* and *gyrB*, and topoisomerase IV, encoded by the genes *parC* and *parE*. Fluoroquinolone resistance most commonly develops as a result of a stepwise mutational process in quinolone resistance-determining regions (QRDRs) of either the *parC/E* or the *gyrA/B* gene [8]. Pneumococcal resistance to fluoroquinolones is due to mutations in either *gyrA* or *parC* or in both of them. These strains usually become completely fluoroquinolone resistant with the addition of a mutation in other target genes such as *parE* and *gyrB* [9]. Several studies have shown that a significant proportion of isolates harboring mutations in *parC* or *gyrA* have low or no phenotypic expression, but they have the potential to develop higher levels of resistance to fluoroquinolones when they suffered a mutation in both *gyrA* and *parC* resulting in treatment failure [1,9]. Obviously, rapid detection of a resistance mechanism in a molecular test would allow clinicians to initially avoid potentially unsuitable treatment. However, bacteria that give positive results in genotypic tests may remain phenotypically susceptible. Therefore, microbiologists concluded that the risk of resistance developing is adequately probably to warrant continuation of alternative therapy [10].

The aim of the study was to determine the quinolone-resistant strains and detect the presence of mutations in the QRDRs of the *gyrA*, *parE*, and *parC* genes.

2. Materials and methods

2.1. Bacterial and clinical specimens

A total of 82 clinical specimens containing *S. pneumoniae* were collected during 2011−2012 from the patients admitted to the intensive care units of two hospitals in Shiraz, Iran. Among them, 45 samples were isolated from patients with pneumonia, meningitis, and fever, and 37 samples were related to healthy controls. Samples were collected from the sputum, blood, and cerebrospinal fluid. Samples were divided into five groups, with the highest abundance being observed in the age group of 31−40 years. Basic identification of the colonies was performed based on colony characteristics, type of hemolysis, Gram staining, bile solubility, and the optochin test. Then in order to confirm the exact isolates, the *lytA* gene was amplified by *lytA-F* (5′-CAA CCG TAC AGA ATG AAG CGG-3′) and *lytA-R* (5′-TTA TTC GTG CAA TAC TCG TGC-3′) primers [11]. These primers were provided by Cinnagen Company (Tehran, Iran). DNA was extracted using the boiling method. Polymerase chain reaction (PCR) was performed in 25 μL reaction mixtures containing 1 μL of DNA template, 1 μL of each primer, 1 μL of $MgCl_2$, 0.5 μL of deoxynucleotide triphosphates, and 0.25 μL of Taq polymerase. The following steps were followed while performing PCR: initial denaturation at 94°C for 5 minutes, followed by 32 cycles consisting of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 8 minutes. Products were analyzed by electrophoresis on 1% agarose gels and visualized under UV light after staining with ethidium bromide. After identification, each strain was subcultured in 20% glycerol in Tryptone soy broth (TSB) (Oxoid, Basingstoke, UK), and all isolated strains were stored at −70°C as frozen stock.

2.2. Antibiotic susceptibility test

The antibiogram test was performed in order to measure the susceptibility rate of the strains to quinolones, adopting the CLSI standard method. Initially, the bacteria were cultivated in Nutrient broth (Merck, Darmstadt, Germany) and incubated at 37°C for 2 hours until the turbidity reached § McFarland standard (approximately 1.5 × 10 cells/mL bacteria). Then the grown bacteria were transferred to Mueller−Hinton agar plates (Merck) containing 5% defibrinated sheep blood. Antibiotic disks, including nalidixic acid (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), ofloxacin (5 μg), and levofloxacin (5 μg), were placed on the plates and then incubated under microaerophilic conditions at 37°C for 16−18 hours. Susceptible, intermediate, and resistant colonies were determined by measuring the diameter of the growth inhibition ring (in millimeters), according to the manufacturer's (Rosco, Teardrop, Denmark) instructions. *Escherichia coli* Top 10 strain was used as the standard strain.

2.3. QRDR determination

Polymerase chain reaction−restriction fragment length polymorphism (PCR-RFLP) was used to amplify fragments of the *gyrA*, *parC*, and *parE* genes. Primers used for the detection of mutations are shown in Table 1. These primers were provided by Cinnagen Company (Tehran, Iran). PCR amplification was carried out in 25 μL reaction mixture containing 1 μL of DNA template, 1 μL of each primer, 1 μL of $MgCl_2$, 0.5 μL of deoxynucleotide triphosphates (dNTP), and 0.25 μL of Taq polymerase. After

Table 1. PCR primers for QRDRs of the fluoroquinolone resistance genes.

PCR conditions	Amplicon size (bp)	Primer (5′−3′)	Gene
One cycle of 5 min at 94°C; 32 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 72°C; one cycle of 8 min at 72°C	360	F: 5′-TGG GTT GAA GCC GGT TCA-3′ R: 5′-TGC TGG CAA GAC CGT TGG-3′	*parC*
One cycle of 5 min at 94°C; 32 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 72°C; one cycle of 8 min at 72°C	290	F: 5′-AAG GCG CGT GAT GAG AGC-3′ R: 5′-TCT GCT CCA ACA CCC GCA-3′	*parE*
One cycle of 5 min at 94°C; 32 cycles of 1 min at 94°C, 1 min at 57°C, 1 min at 72°C; one cycle of 8 min at 72°C	380	F: 5′-CCG TCG CAT TCT TTA CG-3′ R: 5′-AGT TGC TCC ATT AAC CA-3′	*gyrA*

PCR = polymerase chain reaction; QRDR = quinolone resistance-determining region.

amplification of the *parC*, *parE*, and *gyrA* genes, they were digested with *Sau3A*, *MspI*, and *AluI* enzymes (Fermentas, St. Leon-Rot, Germany), respectively. Then RFLP products were separated by electrophoresis on 1.5% (wt/vol.) agarose gel and visualized under UV light after staining with ethidium bromide.

2.4. Statistical analysis

The results were analyzed by SPSS software version 15 (SPSS Inc., Chicago, IL, USA), and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Bacteria and antibiotic susceptibility

S. pneumoniae was isolated from 45 patients included in this study. Of these patients, 16 (35.33%) were male and 29 (64.45%) were female. In addition, 23 (51.11%) samples were from patients with pneumonia, 16 (35.55%) were from those with meningitis, and seven (15.55%) were related to patients having fever. The resistance percentages of all strains to tested antibiotics were as follows: nalidixic acid 82.22%, ciprofloxacin 73.33%, ofloxacin 53.33%, norfloxacin 48.89%, and levofloxacin 42.22% (Table 2). The highest resistance was observed in patients in the age group of 31−40 years.

3.2. Analysis of PCR-RFLP patterns of QRDR

The results indicated that 34 (75.55%) out of 45 clinical isolates contained mutations in the *parC* gene, 31 (68.89%) had mutations in the *gyrA* gene, and 14 (31.11%) had mutations in the *parE* gene. Moreover, from 37 control samples, nine (24.32%) strains with mutations in the *parC* gene, seven (18.99%) with mutations in the *gyrA* gene, and eight (21.52%) with mutations in the *parE* gene were observed. Detection of three DNA fragments of 200 bp, 80 bp, and 80 bp, as a result of digestion of the 360 bp *parC* gene amplicons with *Sau3A* enzyme, represented the existence of mutations, whereas the observation of two fragments of 200 bp and 160 bp indicated the absence of mutations in the correspondent gene (Figure 1). Furthermore, the mutant *parE* gene gave three fragments of 130 bp, 130 bp, and 30 bp after digestion of the 290 bp amplicons with MspI enzyme, where detection of two fragments of 130 bp and 160 bp suggested the absence of mutations in this gene (Figure 2). Detection of three DNA fragments of 180 bp, 140 bp, and 60 bp as a result of digestion of the 380 bp *gyrA* gene amplicons with *AluI* enzyme represented the existence of mutations, whereas the observation of two fragments of 200 bp and 180 bp indicated the absence of mutations in the correspondent gene (Figure 3).

3.3. Statistical analysis

Using statistical analysis, it was illustrated that there is a significant association between mutations in the *gyrA* gene and resistance to all quinolones (nalidixic acid ($p = 0.048$), ciprofloxacin ($p = 0.002$), norfloxacin ($p = 0$), ofloxacin ($p = 0$), and levofloxacin ($p = 0$)). Statistical analysis revealed a significant correlation between mutations in the *parC* gene and the

Table 2. Antibiotic susceptibility of *S. pneumoniae*.

Susceptible (%)	Intermediate (%)	Resistance (%)	Antibiotic
4 (8.89)	4 (8.89)	37 (82.22)	Nalidixic acid
5 (11.11)	7 (15.56)	33 (73.33)	Ciprofloxacin
6 (13.33)	15 (33.33)	24 (53.33)	Ofloxacin
8 (7.78)	15 (33.33)	22 (48.89)	Norfloxacin
14 (31.11)	12 (26.67)	19 (42.22)	Levofloxacin

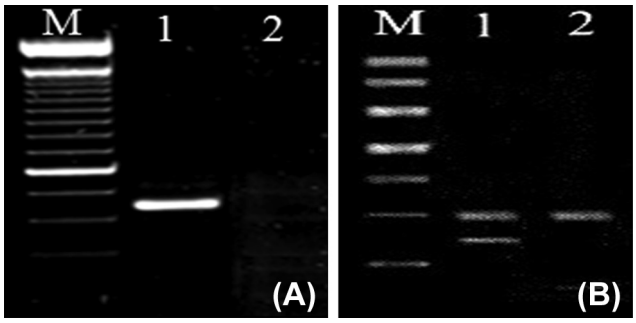

Figure 1. PCR-RFLP, *parC* gene. (A) M, represents 100 bp DNA marker; 1, uncut PCR product (360 bp); and 2, negative control. (B) M, represents 100 bp DNA marker; 1, products of wild-type strain digestion by *Sau3A* enzyme giving 200 bp and 160 bp fragments; and 2, products of mutant strain digestion by *Sau3A* enzyme with 200 bp, 80 bp, and 80 bp fragments. PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

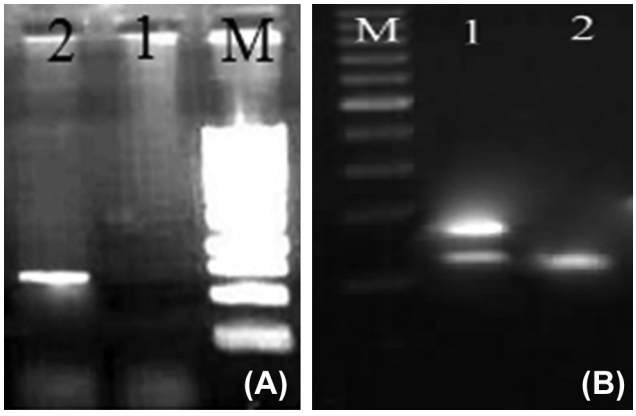

Figure 2. PCR-RFLP, *parE* gene. (A) M, represents 100 bp DNA marker; 1. negative control; and 2, uncut PCR product (290 bp). (B) M, represents 100 bp DNA marker; 1, *MspI* restriction enzyme digestion products of wild-type strain giving 160 bp and 130 bp fragments; and 2, products of mutant strain digestion by *MspI* enzyme representing 130 bp, 130 bp, and 30 bp fragments. PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

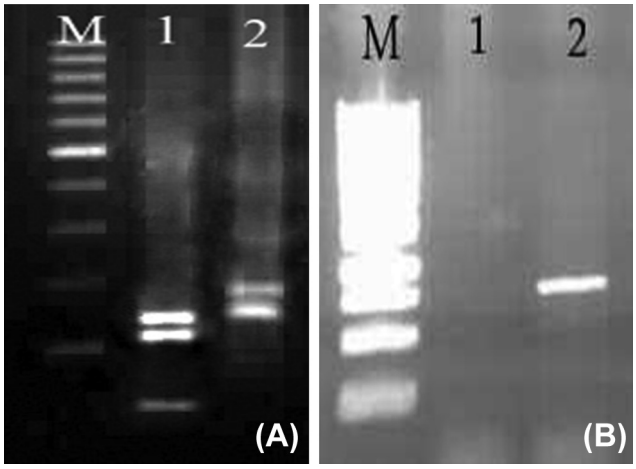

Figure 3. PCR-RFLP, *gyrA* gene. (A) M, represents 100 bp DNA marker; 1, products of mutant strains digestion by *AluI* enzyme representing 180 bp, 140 bp, and 60 bp fragments; and 2, *AluI* restriction enzyme digestion products of wild-type strain giving 200 bp and 180 bp fragments. (B) M, represents 100 bp DNA marker; 1, negative control; and 2, uncut PCR product (380 bp). PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

resistance to antibiotics; nalidixic acid ($p = 0.016$), ciprofloxacin ($p = 0.048$), norfloxacin ($p = 0.039$), and levofloxacin ($p = 0.001$). However, mutations in the *parE* gene indicated a considerable correlation only with resistance to norfloxacin ($p = 0$).

4. Discussion

Considering the seriousness of pneumococcal infections, prevention and treatment of such infections play a substantial role in social healthcare management. Unfortunately, drug abuse led to a worldwide spread of quinolone-resistant *S. pneumoniae* strains, especially in developing countries. Determination of antimicrobial susceptibility is important, particularly when treatment has failed. According to studies, the level of resistance to quinolones in Asian countries, especially in the Middle East, is significantly higher than that in other parts of the world; quinolone resistance has been reported in Korea (6.1%), the Philippines (9.1%), and Hong Kong (14.3%) was reported, and the development of strains resistant to levofloxacin in Qatar, Kuwait, Lebanon raised serious concerns [12,13]. In addition, a research in southern Nigeria confirmed that the level of resistance to first-generation quinolones is relatively higher than that to third-generation quinolones, i.e., the highest resistance is to nalidixic acid and the least resistance is to levofloxacin [14]. In this study, we investigated the prevalence of mutations in the *parE*, *parC*, and *gyrA* genes and their role in the development of quinolone resistance. Similar to the findings of other investigations, our results showed the highest prevalence of mutations regarded to the *parC* gene with 75.56%. The prevalence of this gene was reported to be 67.3% in 2001 in the United States [15], and 21.9% in 2005 [16] and 70% in 2009 in Italy [5]. We studied both mutations and their effects on the rate of quinolone resistance (Table 3). Similar to the findings of Credito et al [17] and Jorgensen et al [18], our findings imply that only mutations in the *parC* or *gyrA* gene were resistant to ciprofloxacin, susceptible to ofloxacin, and semisusceptible to levofloxacin. However, the isolates that had simultaneous mutations in both genes were completely resistant to ofloxacin and levofloxacin [9]. However, there are various opinions about the *parE* gene and its role in the development of resistance to quinolones; according to research by Kawamura et al [19] in Japan and Credito et al [17] in the United States, isolates that had *parE* gene mutations, along with mutations in the *parC* or *gyrA* gene, had higher resistance to ciprofloxacin, ofloxacin, norfloxacin, and lorfloxacin than mutants that did not have mutations in the *parE* gene. Our finding is contrary to the results of Ip et al [20], which showed that the strains possess mutations in the *parE* gene are susceptible to quinolones and have no difference from the wild strains. In contrast to Ip et al's [20] results, for the first time in Iran, we illustrated that there is a significant

Table 3. Mutation pattern in clinical samples based on quinolone resistance.

LEV		OFL		NOR		CIP		NA		Mutation
SR	R	SR	R	SR	R	SR	R	SR	R	
1 (2.22)	0 (0)	2 (4.44)	1 (2.22)	2 (4.44)	0 (0)	2 (4.44)	2 (4.44)	1 (2.22)	3 (6.66)	$parC^{+}/parE^{-}/gyrA^{-}$
1 (2.22)	0 (0)	4 (8.88)	1 (2.22)	3 (6.66)	1 (2.22)	1 (2.22)	4 (8.88)	2 (4.44)	3 (6.66)	$parC^{-}/parE^{-}/gyrA^{+}$
2 (4.44)	16[a] (35.55)	1 (2.22)	17[a] (37.77)	1 (2.22)	17[a] (37.77)	0 (0)	18[a] (40)	0 (0)	18[a] (40)	$parC^{+}/parE^{-}/gyrA^{+}$
4[a] (8.88)	0 (0)	6[a] (13.33)	0 (0)	6[a] (13.33)	0 (0)	2 (4.44)	4 (8.88)	1 (2.22)	6 (13.33)	$parC^{+}/parE^{+}/gyrA^{-}$
3[a] (6.66)	0 (0)	1 (2.22)	2 (4.44)	2 (4.44)	1 (2.22)	2 (4.44)	1 (2.22)	0 (0)	3 (6.66)	$parC^{-}/parE^{+}/gyrA^{+}$
0 (0)	3 (6.66)	1 (2.22)	3 (6.66)	1 (2.22)	3 (6.66)	0 (0)	4 (8.88)	4 (8.88)	4 (8.88)	$parC^{+}/parE^{+}/gyrA^{+}$
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	$parC^{-}/parE^{-}/gyrA^{-}$

[a]*Significant correlation.* CIP = ciprofloxacin; LEV = levofloxacin; NA = nalidixic acid; NOR = norfloxacin; OFL = ofloxacin; R = resistance; SR = semiresistance.

correlation between mutations in the *parE* gene and resistance to norfloxacin, as strains that had mutations in the *parC* or *gyrA* gene along with mutations in the *parE* gene showed higher levels of resistance to this antibiotic.

In this study, in contrast to the previous thinking about the role of mutation in the *parE* gene and resistance to quinolones, the effect of mutations in the *parE* gene that consequently cause low-level resistance to quinolones has been determined. These results are arguable, and more accurate studies investigating precise sequencing of a gene are required. In addition, it is shown that mutations in the *parC* or *gyrA* gene play the most effective role in the development of resistance to quinolones, as mutations in either the *parC* or the *gyrA* gene alone can lead to low-level quinolone resistance, while simultaneous mutations in both *parC* and *gyrA* genes will bring about high-level resistance. The prevalence of mutations in QRDRs in *S. pneumoniae* strains and the subsequent development of antibiotic resistance to different generations of quinolones may lead to the outbreak of antimicrobial resistance, complicating the treatment of infections in the future. Therefore, precautionary measures for the treatment and control of resistance must be adopted to prevent the spread of resistant mutant strains.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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