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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