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Evaluation of the Mutations in Topoisomerase IV Genes and Their Role in Development of Resistance to Quinolones in *Streptococcus pneumoniae*

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ABSTRACT

Background: *Streptococcus pneumoniae* is an important cause of respiratory tract infections. Due to the recent incidence of antibiotic resistance to commonly used antibiotics, the use of quinolones in the treatment of *S. pneumoniae* has been taken into consideration. The aim of this study was to evaluate the molecular mechanisms of quinolone resistance in isolated strains.

Methods: This study was carried out on 45 strains of *S. pneumoniae* isolates from clinical samples. Initially, using biochemical tests, *S. pneumoniae* strains were determined and using *lytA* gene specific primers, these strains were accomplished. Antibiotic resistance was assessed by Clinical & Laboratory Standards Institute (CLSI) criteria and eventually, and then adopting Polymerase Chain Reaction-Restriction Fragment Length polymorphism (PCR-RFLP) techniques were studied with Sau3A and MspI enzymes. Also, point mutations associated with antibiotic resistance was evaluated in *parC* and *parE* genes.

Results: In 45 strains isolated, resistance to nalidixic acid, ciprofloxacin, ofloxacin, norfloxacin and levofloxacin was; 82.22%, 73.43%, 53.33%, 48.88%, and 42.22%, respectively. In clinical samples, 34 (75.55%) strains with mutations in the *parC* genes and 14 (31.11%) strains with mutations in *parE* gene were detected. Using statistical analysis, it was found that there was a significant relationship between mutations in the *parC* genes and resistance to nalidixic acid, ciprofloxacin, norfloxacin and levofloxacin. However, mutations in *parE* genes only showed the significant correlation with resistance to norfloxacin exclusively. On the contrary, unlike other studies, we demonstrated that a mutation in the *parE* gene could be involved in low-level resistance to quinolones.

***Conclusion*:** Due to the considerable resistance to quinolones, evaluation of these mutations is necessary in other parts of the country.

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Introduction

Streptococcus pneumoniae is the primary human pathogen of respiratory infections respiratory tract infections, including community-acquired pneumonia (CAP) (1). Concerns about the global expansion of penicillin-resistant and multidrug-resistant *S. pneumoniae* strains led to the development and consumption of anti-pneumococcal drugs with stronger antimicrobial activity referred to as quinolones (2). The primary targets of quinolones are DNA gyrase (encoded by *gyrA* and *gyrB*) and topoisomerase IV (encoded by *parC* and *parE*) (3, 4, 5).

Topoisomerase IV causes the separation of nucleotides in DNA strand and relieves the DNA supercoiling. Moreover, topoisomerase IV is an ATP dependent enzyme and plays a significant role in replication, transcription and recombination by altering DNA topology (3, 5). Quinolones were widely used to treat Pneumococcal infections. But later quinolone resistant strains were reported in different parts of the world. Following the first report in 1998 in Canada, during the last three decades, the prevalence of resistance to quinolones is increasing rapidly (5). There are two mechanisms suggested for quinolone resistance; (I) point mutations in the quinolone resistance determining region of the DNA gyrase and topoisomerase enzymes, (II) efflux pumps (6, 7). The aim of this study was to investigate the prevalence of quinolone resistance and its molecular mechanism, on topoisomerase IV enzymes in *S. pneumoniae* strains isolated.

Materials and Methods

Bacterial isolates

This cross-sectional descriptive study was implemented on 45 *S. pneumoniae* strains, isolated from Hospitals, in Shiraz (South of Iran). The sample size was determined using Cochran formula. Samples were isolated from patients with pneumonia, meningitis and fever with unknown origin. All patients were in the age range between 10-58 years. Samples were collected from, Sputum, blood and cerebro-spinal fluid. In order to isolate *S. pneumoniae* strains, samples were cultivated on blood agar medium containing 5% sheep blood and then incubated for 24 hours at 37 °C. Identification of pneumococci was confirmed using colony morphology, type of hemolysis, Gram staining and Optochin test. Subsequently, *lytA* gene was amplified by PCR technique using specific primers of *lytA*-F (5'-CAA CCG TAC AGA ATG AAG CGG-3') and lytA-R (5'-TTA TTC GTG CAA TAC TCG TGC-3') (8). The detected 295 bp DNA fragments were corresponded to the presence of *S. pneumoniae*.

PCR-RFLP method

In order to assess mutations in *parC* and *parE* genes, they were amplified using specific primers presented in Table 1 to give 360 and 290 bp DNA fragment (9). PCR reaction was performed using a conventional thermal cycler (Mastercycler Gradient Eppendorf) with the following conditions; 94 °C for 5 min (primary denaturation) , 32 cycles of 94 °C for 1 min (denaturation), 61 °C for one minute (annealing), 72°C for 1 min (extension) and finally 72 °C for 5 min (final extension). The reaction mixture was comprised of, 2.5µl 10X PCR buffer, 1µl Mgcl2 (50 mM), 0.5µl dNTPs (10 mM), 1µl of each primer (10 mM), 17.75µl deionized water, 0.25µl Taq polymerase enzyme and 2µl template DNA. After verification of a successful *parC* and *parE* gene amplification by agarose gel electrophoresis, enzymatic digestion was carried out using Sau3A and MspI enzymes, (Fermentas, Germany) for 24 hours at 37 °C. Eventually RFLP products were visualized on 1.5% agarose gel containing 2µl ethidium bromide, by Gel doc system.

Susceptibility testing

At this step, susceptibility of the detected strains to three generations of quinolone family was estimated by applying antibiogram test with CLSI standard procedures (Clinical and laboratory standards institute). Antibiotic susceptibility rate of the colonies to nalidixic acid (30µg), ciprofloxacin (5µg), norfloxacin (10µg), ofloxacin (5µg) and levofloxacin (5µg) was estimated by measuring the diameter of the zone of inhibition, according to manufacturer's directions (ROSCO, Denmark) and resistant and semi-resistant was determined. The results were analyzed using SPSS software version 15. The significance level was considered at $p<0.05$.

Result

From 45 clinical samples studied in this research, 29 (64.45%) cases were men and 16 (35.55%) were women. In addition, 23 samples (51.11%) were collected from patients with pneumonia, 15 (33.33%) from patients with meningitis and 7 (15.55%) from patients who had had fever with unknown origin (Table 2).

Using the standard disk diffusion method, resistance to nalidixic acid, ciprofloxacin, ofloxacin, norfloxacin, and levofloxacin, were es timated; 82.22%, 73.43%, 53.33%, 48.88% and 42.22%, respectively (Figure 1). PCR-RFLP results indicated that among 45 clinical isolates, 34(75.55%) strains contained mutations in *parC* gene and 14 (31.11%) strains had mutations in *parE* gene. 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 200 bp and 160 bp fragments indicated the absence of mutations in the corresponding gene (Figure 1).

Furthermore, mutant *parE* genes gave three fragments of 130 bp, 130 bp and 30 bp after 290 bp amplicons digested with MspI enzyme, where detection of two fragments with 130 and 160 bp suggests the absence of mutations in this gene (Fig 2). Statistical analysis revealed a significant correlation between mutations in *parC* genes and the resistance to antibiotics; nalidixic acid (P=0.016), ciprofloxacin (P=0.048), Norfloxacin (P=0.039) and levofloxacin (P=0.001). In addition, mutations in *parE* genes, only showed the significant correlation with resistance to norfloxacin, particularly (P=0) (Table 3).

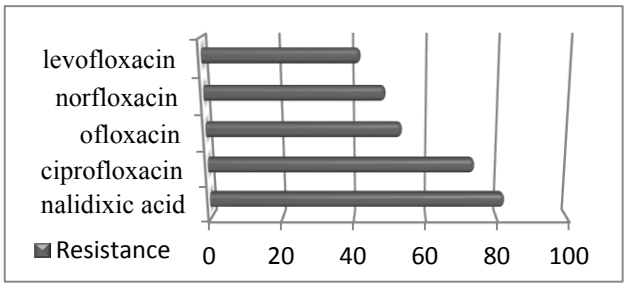

Figure 1. Susceptibility rate of clinical samples to quinolone antibiotics.

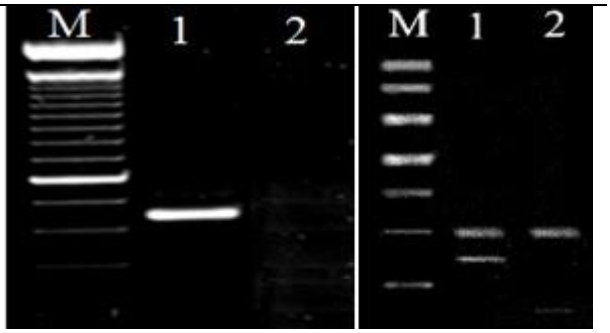

Figure 2. PCR-RFLP of *parC* gene. Right; (M)100 bp DNA marker, (1) products of wild type strain digestion by Sau3A enzyme giving 200 bp and 160 bp fragments, (2) products of mutant strain digestion by Sau3A enzyme with one 200 bp and two 80 bp fragments. Left; (M) 100 bp DNA marker, (1) uncut PCR product (360 bp), (2) negative control.

Table 1. Specific primers for *parC* and *parE* genes amplification.

Primer pair	sequences (5'→3')	Ampilicon size bp
parC –F	5'- TGG GTT GAA	360
parC-R	GCC GGT TCA -3'	
	5'-TGC TGG CAA	
	GAC CGT TGG -3'	
ParE-F	5'-AAG GCG CGT	290
ParE-R	GAT GAG AGC -3'	
	5'-TCT GCT CCA	
	ACA CCC GCA-3'	

Table 2. The demographic information of the samples.

Disease / Sex	Pneumonia (%)	Meningitis (%)	Fever (%)	Total (%)
Female (%)	7(15.55)	7(15.55)	2(4.44)	16(35.55)
Male (%)	16(35/55)	8(17.77)	5(11.11)	29(64.45)
Total (%)	23(51.11)	15(33/33)	7(15.55)	45(100)

Table 3. The frequency of mutations in *parC* and *parE* genes based on quinolone resistance.

Gene	Isolates	Nalidixic acid		Ciprofloxacin		Ofloxacin		Norfloxacin		Levofloxacin	
		Resistant	Semi-resistant	Resistant	Semi-resistant	Resistant	Semi-resistant	Resistant	Semi-resistant	Resistant	Semi-resistant
parC	34 (75.55)	31 (68.89)	2 (4.44)	27 (60)	5 (11.11)	21 (46.67)	10 (22.22)	20 (44.44)	10 (22.22)	19 (42.22)	8 (17.76)
parE	14 (31.11)	12 (26.67)	1 (2.22)	8 (17.76)	4 (8.89)	5 (11.11)	7 (15.56)	4 (8.89)	8 (17.76)	3 (6.67)	7 (15.56)
Total	45 (100)	43 (95.56)	3 (6.67)	35 (77.78)	9 (20)	26 (57.78)	17 (37.78)	24 (53.33)	18 (40)	22 (48.89)	15 (33.33)

Discussion

The intensity of quinolone-resistant strains were variable geographically, and been rising in recent years, so that the level of quinolone resistance in European countries is relatively low. In Canada the resistance level of 0.6% in 1998 rose up to 7.3% in 2006 which is increasing significantly (7). Quinolone resistance levels in Asian countries are higher compared to other parts of the world (10). Also, studies conducted during 1997-2005 in Canada suggested that the highest level of quinolone resistance corresponded to ciprofloxacin and lowest resistance was to levofloxacin (5, 11, 12, 13). The present study similar to other projects indicated that resistance to quinolones is increasing substantially. Nalidixic acid and ciprofloxacin reported to have the highest resistance rate while levofloxacin the least.

De Vecchi et al. in 2009 investigated the prevalence *parC* and *parE* genes in 52 quinolone-resistant strains. According to their results, 40 (70%) strains were carried a mutation in *parC* gene and 14 (27%) strains showed a mutation in *parE* gene (14). Brueggeman et al. in 2002 and Doern et al. in 2005 both studied the mutations in the quinolone resistance-determining region (QRDR) of the topoisomerase IV enzyme of *S. pneumoniae* and reported a 67.3% and 21% prevalence rate of mutation in *parC* gene, respectively (15, 16). Conforming other investigations, it was determined that the prevalence of mutations in the *parC* gene were more common than *parE* gene. The reason of the divergent rate of mutation prevalence in different areas of research could be due to the various geographical locations, the excessive use of antibiotics and the selective pressures which isolates are exposed. De Vecchi et al. in 2009 declared that resistance to ciprofloxacin is significantly higher than levofloxacin. Moreover, regarding to their results, ciprofloxacin-resistant strains possessed single mutations in *parC* gene as well as simultaneous mutations in both, *parC* and *parE* genes (14). Patel et al. in 2010 were suggested that among isolates with mutations, only in the *parC* gene, 17.8% were resistant to ciprofloxacin and 2.2% were semi- resistant to levofloxacin (4). In addition, Balsalobre et al. in 2008 reported *S. pneumoniae* isolates with semi-resistant levofloxacin which only had a mutation in *parE* gene, particularly. But due to lack of evidence and sufficient information, it was not possible to prove the role of *parE* gene in quinolone resistance (17). Also, Ip et al. in 2006 studied the *S. pneumoniae* strains with a single mutation in *parE* gene and deduced that antibiotic susceptibility rate of these strains is similar to that of isolates with no mutation in the examined gene (9). However, Weigel et al. in 2001 detected a slight decrease in levofloxacin and ofloxacin susceptibility rate as a result of mutation in *parE* gene (18). According to the results obtained from the current study, mutations in the *parC* gene were affirmed to play a significant role in quinolone resistance. Despite the contrary impressions on contribution of mutation in *parE* gene in quinolone resistance, the present results indicate that a mutation in this gene could lead to the development of low-level resistance to quinolones.

Conclusion

From results obtained during this study, it is apparent that the quinolone resistance level is rising among *S. pneumoniae* strains isolated. The highest resistance rates pertain was to nalidixic acid (first generation quinolones) and the lowest was to levofloxacin (third generation quinolones). Besides, the mechanism of quinolone resistance and distribution of mutations within the genes in our area of research is similar to the parts of the world. So that the most common mutations occurred in *parC* genes, while, *parE* gene showed the lowest mutation rate. Furthermore, there are different impressions about contribution of *parE* mutation in quinolone resistance. However, our results indicate that a mutation in *parE* gene could develop low-level resistance to quinolones.

Therefore, it seems that further studies are necessary to investigate the detailed sequencing of mutations in *parE* gene, and other mechanisms of resistance.

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Conflict of interest

None declared conflict of interest

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