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Antimicrobial susceptibility and analysis of macrolide resistance genes in *Streptococcus pneumoniae* isolated in Hamadan

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ABSTRACT

Objective(s): Macrolide resistant *Streptococcus pneumoniae* pose an emerging problem globally. The aim of this study was to investigate the prevalence of *ermB* and *mefA* genes (macrolide resistant genes) by polymerase chain reaction (PCR) method and to detect drug resistance patterns of *S. pneumoniae* isolated from clinical samples to macrolides and other antibiotic agents by E-test method.

Materials and Methods: Fifty five isolates of *S. pneumoniae* were obtained from clinical samples with microbial tests. The antibiotic susceptibility of isolates for erythromycin, azithromycin, clarithromycin, ceftazidime, ciprofloxacin and vancomycin were determined by E-test method. Genotypic antibiotic resistance pattern was determined by PCR with primer designed for *ermB* and *mefA* genes.

Results: The number of *S. pneumoniae* isolates resistance to erythromycin, azithromycin, clarithromycin, ceftazidim, ciprofloxacin were 25.5%, 18.2%, 16.4%, 21.8% and 10.9%, respectively while no resistance to vancomycin was observed. The macrolide resistance genes of *ermB* and *mefA* were found in 10.9% and 18.2% of the isolates, respectively.

Conclusion: The result of the current study suggests the necessity of evaluation the changes in MIC (minimum inhibitory concentration) values as well as genetic mutations to estimate the prevalence of the resistance antimicrobial agents in *S. pneumoniae*.

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Introduction

Streptococcus pneumoniae is a common etiologic agent of serious invasive infections, with high 22 morbidity and mortality in children and adults, such as meningitis and septicemia (1). Antimicrobial resistance isa global issue and several countries have implemented surveillance systems in recent years (2). The outbreak of macrolides resistance in *S. pneumoniae* among clinical isolates has increased worldwide (3). The macrolide-resistant *S. pneumoniae* has been reported to be moderately low in northern Europe; however, it has considerably increased in some European countries (4). Data confirmed that macrolide resistance in pneumococci is a main problem in many Asian countries. In addition, resistance to other classes of antibiotics traditionally used as alternatives in the treatment of pneumococcal infections has also been increased markedly during recent years (5-7). Macrolide resistance in *S. pneumoniae* occurs by three mechanisms: ribosomal methylase encoded by the *ermB* gene which causes a specific adenine residue on the 23S rRNA to be methylated, macrolide efflux encoded by *mefA* gene and ribosomalmutations in the 23S rRNAgene or in the ribosomalprotein L4 or L22 (8-10). According to previous studies, the first two mechanisms are predominant forms of macrolide resistance in *S. pneumoniae* (8, 11).

Molecular epidemiological information on antimicrobial susceptibility and analysis of resistance genes is crucial in prevention and therapy of infectious diseases such as drug-resistant *S. pneumoniae*. Consequently, clinical laboratories should consider screening-selected isolations to determine the susceptibility to macrolides, β-lactams, vancomycin and clindamycin. Hence, assessment of changes in MIC as well as genetic mutations could be regarded as an alternative for

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Table 1. Sequences of primers

Name (gene)	Sequence(5′ to 3′)	Position	Product lengh (bp)
LytA	Forward: TGAAGCGGATTATCATGGC	694-713	273
	Reverse: GCTAAACTCCCTGTATCAAGCG	966-945	
ermB	Forward: CGTACCTTGGATATTCACCG	721-740	224
	Reverse: GTAAACAGTTGACGATATTC	944-922	
mefA	Forward: CTGTATGGAGCTACCTGTCTGG	288-309	294
	Reverse: CCCAGCTTAGGTATACGTAC	581-562	

The amplified DNA fragment were analysed by electrophoresis on 3% agarose gels (13)
S. pneumoniae strains PTCC 1240 were used as control

evaluation and medication of *S. pneumoniae*-related diseases. Thus, this study was aimed to survey the true prevalence of macrolide resistance among clinical isolates of *S. pneumoniae.* The samples were tested by E-Test and assessed with PCR and the relationship between the obtained results using two methods was compared.

Materials and Methods

Bacterial strains

Clinical isolates of *S. pneumoniae* (n=55) were obtained from outpatients samples (n=400) at Medical Center of Hamadan. Clinical samples were CSF (6), sputum (38), otorrhea (1), pharynx (8), ear (1) and eye (1). Clinical isolates were grown on 5% sheep blood agar. *S. pneumoniae* was identified by the alpha-haemolytic and microbial tests (optochin test, inulin test, and bile solubility test) and was confirmed by PCR amplification of autolysin gene (*lytA*) (12-14). Isolates were stored in 15% glycerol at -80˚C until used for PCR and E-Test (15-16).

PCR analysis

Isolates were grown on sheep blood agar at 37˚C incubator with 5% CO_2 for 48 hr (Merck, Darmstadt, Germany). A single colony of *S. pneumoniae* grown on a blood agar plate was suspended in a 0.05 ml microtube containing 30 µl of lysis solution as reported previously (13, 17). The tubes were placed in thermal cycler and the bacterial cells were lysed for 10 min at 60˚C and for 5 min at 94˚C. 1 µl of the bacterial lysate was added to tubes containing 19.1 µl solutions (10x buffer: (2 µl), $MgCl_2$ (25 Mm): (0.4 µl), dNTPs (10 Mm): (0.8 µl), Distilled Water: (13.7 µl), Taq (5U/µl): (0.2 µl), Primer1 (10 pm): (1 µl), Primer 2 (10 pm): (1 µl)). Detection of *S. pneumoniae* was confirmed by amplification of *lytA* using PCR with specific primers as reported by Ubukata and co-workers (12).

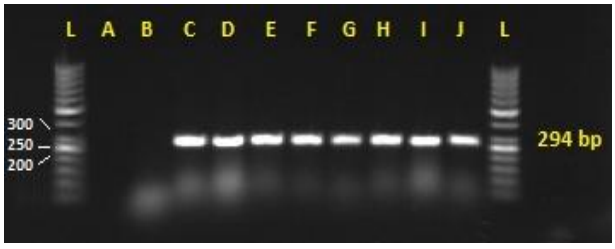

Figure 1. Gel electrophoresis with *mefA* gene (L=Marker 50 bp, Agarose LE 3% A=Negative control with distilled water; B=Negative control with *Escherichia coli*; C=positive control with Streptococcus *pneumoniae* (PTCC NO. 1240); D-J= Positive sample (*mefA* gene $^{+}$, (294 bp))

Presence of the macrolide resistance genes, *ermB* and *mefA*, was assessed by PCR using the following sets of primers (17). Specific Primers were designed to amplify the genes were indicated in Table 1.

Antimicrobial test

The drug susceptibility of confirmed samples with microbial tests was determined using antibiotic E-test strips (Himedia Laboratories pvt. Ltd, India). Antibiotics employed in this study were: erythromycin, azithromycin, clarithromycin, ceftazidim, ciprofloxacin, and vancomycin. Inocula were prepared by direct suspension in Muller-Hinton broth of colonies grown overnight on sheep blood agar to achieve turbidity equivalent to a 0.5 Mc-Farland opacity standard. The 100-mm-diameter agar plates were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. After application of the E-test strips, pneumococcal test plates were incubated in ambient air at 35˚C for 20 to 22 hr (15, 16, 18, 19).

Results

Fifty five out of 400 taken samples were positive for *S. pneumoniae* with microbial test and PCR. Based on the results, 21.8% were resistant to macrolide with the following genotypes: $ermB^{+}$ (n=6, 10.9%), $mefA^{+}$ (n=10, 18.2%), $ermB^{+}$ $mefA^{+}$ (n=4, 7.3%), $ermB^{-}$ $mefA^{-}$ (n=35, 63.6%) (Figure 1).

The strains have only *mefA* gene that were specific only for 14- and 15-membered macrolides (M phenotype) and the strains have *ermB* gene that conveys crossresistance to macrolides, lincosamides and streptogramin B compounds (MLSB phenotype). The isolates lacking macrolide resistance genes were susceptible to macrolide.

According to the E-Test, 25.5% of the isolates were resistant to erythromycin, 18.2% to clarithromycin, 16.4% to azithromycin, 21.8% to ceftazidime and 10.9% to ciprofloxacin while no resistance to vancomycin was observed. The most frequent macrolide resistance genes were related to *mefA* and only 7.3% of the samples harbored both macrolide resistance genes. Figure 2 shows the relationship between distributions of antimicrobial minimum inhibitory concentrations (MIC) and macrolide resistance genes *(ermB* and *mefA)* for

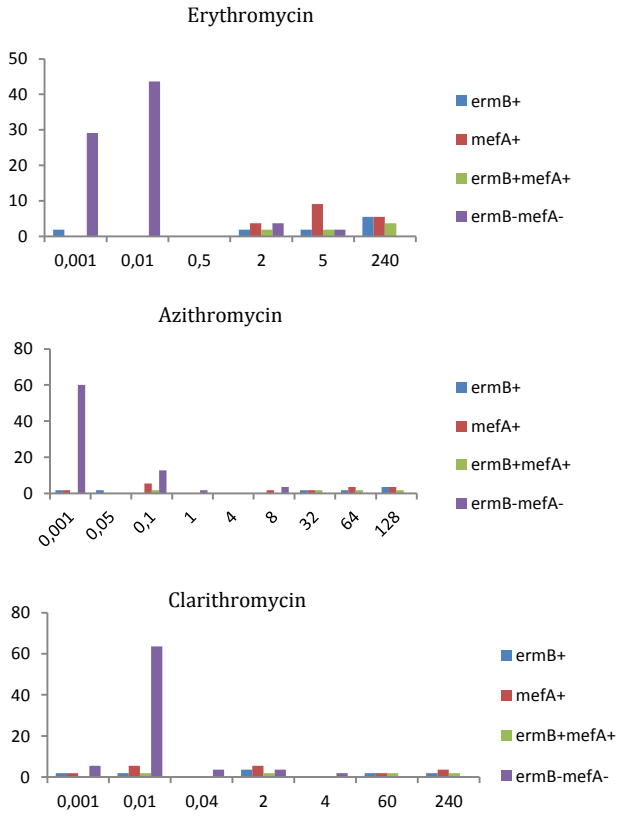

Figure 2. Correlation between MICs of three macrolide antibiotic and resistance genes (*ermB*, *mefA*) for 55 *S. pneumoniae* isolates from patients

S. pneumoniae in which those isolates with both macrolide resistance genes (*ermB*, *mefA)* showed higher MIC than others whichindicating high resistance to macrolide. In addition, the existence of *mefA* gene had greater effect on MIC in studied isolates.

Discussion

PCR provides a rapid and accurate mean of amplifying DNA with high specificity and sensitivity for correct diagnosis of genetic, infectious, oncologic diseases, drug resistance pattern in bacteria and many other fields (20-22). Using the method many studies have been conducted in different fields. The present study was undertaken for identification of *S. pneumoniae* by PCR and microbial tests. Furthermore, macrolide resistance was determined by PCR and E-test method was performedfor isolates of *S. pneumoniae*.

Pneumococci have remained important human pathogens despite the introduction of macrolides and the new generation of antibiotics. Since the first cases of invasive pneumococcal infections caused by penicillin resistant Streptococcus pneumonia (PRSP) were reported in 1977, penicillin-non susceptible strains have become a global concern (23). Several reports have determined a high prevalence of pneumococcal resistance to penicillin as well as other antibiotics, such as cephalosporins and macrolides. A recent national survey in the U.S. showed that 27.5% of isolates were resistant to azithromycin (24). According to Whitney *et al* (2000), a significant increase in proportion of isolates resistant to antimicrobial agents such as erythromycin (from 11 to 15%) and penicillin (21–25%) was reported between 1995 and 1998. Furthermore, the overall proportion of the isolates which were resistant to three or more classes of drugs was reported to be increased (25). Some studies have documented the emergence of decreased susceptibility of *S. pneumoniae* to fluoroquinolones and a failure in therapy of some cases of pneumococcal pneumonia treated with oral levofloxacin (26, 27).

In this study, resistance to macrolides, fluoroquinolone and cephalosporins were observed even though it seemed to be relatively low. Hence, cephalosporins and macrolides after β-lactams can be used to treat pneumococcal diseases. For those strains which are more resistance, fluoroquinolone and vancomycin can be correctly applied if necessary. The result of current study suggests the necessity of the surveillance of the antimicrobial susceptibility due to the rapid changes in distribution of antimicrobial susceptibility in *S. pneumoniae*.

In current work, the genetic changes in *mefA* and *ermB* were showed to be associated with resistance to macrolides. The incidence of *mefA*-or *ermB*-positive strains showed to be increased according to previous studies (28) which in turn resulted in the increase in macrolide-resistant strains. Our results which are in line with previous finding (29) have shown that *ermB* is related to high MIC of macrolide. The existence of *mefA* and *ermB* genes can affect on MIC of macrolide resistance due to the increase of these genes in resistant isolates compared to sensitive ones (Figure 2). In addition, a high correlation of *ermB* genes with erythromycin was shown. Thus, for fast screening of macrolides resistance in *S. pneumoniae*, the *ermB* can be used instead of E-tapes of erythromycin.

Regarding to the macrolides resistance among the samples undergone PCR, *mefA* gene frequency was higher than that of *ermB* gene. Some isolates contained both *mefA* and *ermB* genes which according to an accomplished study (30), these isolates were highly multi-drug resistance. Among those macrolide resistance strains isolated by antimicrobial strips, there were no strains to be negative for both *mefA* and *ermB genes*, suggesting that other macrolide resistance mechanisms, such as mutations in the 23S rRNA or alterations in ribosomal proteins L4 and L22 seem to be less important determinants in pneumococci.

Conclusion

The current work suggests the necessity of evaluation the changes in MIC values as well as genetic mutations in order to estimate the prevalence

of the resistance of *S. pneumoniae* to antimicrobial agents. With a range of macrolides, those strains with intermediate resistance to penicillin can be treated successfully in serious infections. However, due to the increased macrolides-resistant strains observed in this family, macrolides must be correctly applied if necessary. For those strains with high resistance to penicillin and macrolides, vancomycin and fluoroquinolones including sparfloxacin should be selected as an antimicrobial agent. Hence, based on the pattern of pneumococcal drug resistance in the area, selected antibiotics should be administrated to treat pneumococcal bacterial infection.

In conclusion, for those clinical isolates assumed to be *S. pneumoniae*, examination of *ermB* and *mefA*genes together with *lytA* would be valuable strategy for susceptibility prediction within 2 hours and could be regarded as a remarkable approach in *S. pneumoniae*-related infection diseases.

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