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, cefazidime, 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, cefazidime, 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 *S. pneumoniae* were detected 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*.

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 is a 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 ribosomal mutations in the 23S rRNA gene and 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...
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), ototrrhea (1), pharynx (8), ear (1) and eye (1). Clinical isolates were grown on 5% sheep blood agar. *S. pneumoniae* isolates were 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% CO₂ 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 of lysis solution and 1 µl of 10x buffer: (2.5 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).

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, ceftazidime, 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 McFarland 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 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

| Table 1. Sequences of primers |
|------------------------------|
| Name (gene)                  | Sequence (5' to 3') | Position | Product length (bp) |
| LytA                         | Forward: TGAAAGGGATTATCATGGG | 694-713  | 273                 |
|                              | Reverse: GCTAAATCCCATGATCAAGG | 966-945  |                     |
| ermB                         | Forward: GTACCTTGATATCAACGG  | 721-740  | 224                 |
|                              | Reverse: GTAAACAGTGGATGATATCC | 944-922  |                     |
| mefA                         | Forward: CTGATAGGATCATTGTCTGG | 288-309  |                     |
|                              | Reverse: GCGAAGTTAGTATACGTAC  | 581-562  | 294                 |

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

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**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 performed for 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 pneumoniae (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).
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 *mef* 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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**References**

1. Van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet 2009; 374:1543-1556.
2. Zignoi M, van Gemert W, Falzon D, Jaramillo E, Blanc L, Raviglione M. Modernizing surveillance of anti-tuberculosis drug resistance: from special surveys to routine testing. Clin Infect Dis 2011; 52:901-906.
3. Song JH, Chang HH, Suh JY, Ko KS, Jung SI, Oh WS, et al. Macrolide resistance and genotypic characterization of *Streptococcus pneumoniae* in Asian Countries: ANSORP Study. J AntimicrobChemother 2004; 53:457-463.
4. Littauer P, Sangvik M, Caugant DA, Holby EA, Simonsen GS, Sundsford A, et al. Molecular epidemiology of macrolide-resistant isolates of *Streptococcus pneumoniae* collected from blood and respiratory specimens in Norway. J Clin Microbiol 2005; 43:2125-2132.
5. Garau J. Treatment of drug-resistant pneumococcal pneumonia. Lancet Infect Dis 2002; 2:404-415.
6. Harwell JI, Brown RB. The drug-resistant pneumococcus: clinical relevance, therapy, and prevention. Chest 2000; 117:530-541.
7. Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Roof J, Seppala H. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob Agents Chemother 1999; 43:2823-2830.
8. Taït-Kamradt A, Davies T, Appelbaum PC, Depardieu F, Courvalin P, Petitpas J, et al. Two new mechanisms of macrolide resistance in clinical strains of *Streptococcus pneumoniae* from Eastern Europe and North America. Antimicrob Agents Chemother 2000; 44:3395-3401.
9. Edelstein PH. Pneumococcal resistance to macrolides, lincosamides, ketolides, and streptogramin B agents: molecular mechanisms and resistance phenotypes. Clin Infect Dis 2004; 38:S22–S27.
10. Reinert RR, Filimonova OY, Al-Lahham A, Grudinina SA, Lina EN, Weigel LM, et al. Mechanisms of macrolide resistance among *Streptococcus pneumoniae* isolates from Russia. Antimicrob Agents Chemother 2008; 52:2260-2262.
11. Taït-Kamradt A, Davies T, Cronan M, Jacobs MR, Appelbaum PC, Sutcliffe J. Mutations in 235 rRNA and ribosomal protein L4 account for resistance in pneumococcal strains selected in vitro by macrolide passage. Antimicrob Agents Chemother 2000; 44:2118-2125.
12. Ubukata K, Muraki T, Igarashi A, Asahi Y, Konno M. Identification of penicillin and other beta lactam Resistance in *Streptococcus pneumoniae* by polymerase chain reaction. J Infect Chemother 1997; 3:190-197.
13. Ubukata K, Asahi Y, Yamane Y, Konno M. Combinational detection of autolysin and penicillin-binding protein 2B genes of *Streptococcus pneumoniae* by PCR. J Clin Microbiol 1996; 34:592-596.
14. García P, García JL, García E, López R. Nucleotide sequence and expression of the pneumococcal autolysin gene from its own promoter in *Escherichia coli*. Gene 1986; 43:265-272.
15. Jorgensen JH, Howell AW, Maher LA. Quantitative antimicrobial susceptibility testing of haemophilus influenzae and *Streptococcus pneumoniae* by Using the E-Test. J ClinMicrobial 1991; 29:109–114.
16. Baker CN, Stocher SA, Culver DH, Thornsberry C. Comparison of the E-Test to agar dilution, broth microdilution, and agar diffusion susceptibility testingtechniques by using a special challenge set of bacteria. J Clin Microbiol 1991; 29:533-538.
17. Ubukata K, Ivata S, Sunakawa K. *In vitro* activity of new ketolide, telithromycin, and eight other macrolide antibiotics against *Streptococcus pneumoniae* having mefA and ermB genes that mediate macrolide resistance. J Infect Chemother 2003; 9:221-226.
18. Jorgensen JH, Maher LA, Howell AW. Use of Haemophilus test medium for broth microdilution. antimicrobial susceptibility testing of *Streptococcus pneumoniae*. J Clin Microbiol 1990; 28:430–434.
19. National Committee for Clinical Laboratory Standards (NCCLS). Performance Standards for Antimicrobial Disk Susceptibility Test: Approved Standards. 4th ed. Villanova, PA: NCCLS; 2001.
20. Farajnia S, Azhari F, Aliharni MY, Hosseini MK, Peymani A, Sohrabi N. Prevalence of PER and VEB type extended spectrum beta lactamases among multidrug resistant acinetobacter baumannii isolates in north-west of Iran. Iran J Basic Med Sci 2013; 16:751-755.
21. Zamani A, Yousefi Mashouf R, Ebrahimzadeh Namvar AM, Aliharni MY. Detection of magA gene in Klebsiella spp isolated from clinical samples detection of magA. Iran J Basic Med Sci 2013; 16:173-176.
22. Pourahmad Jaktaji R, Jazayeri N. Expression of acrA and acrB genes in *escherichia coli* mutants with or without marK or acrR or acrB mutations. Iran J Basic Med Sci 2013; 16:1254-1258.
23. Ubukata K, Chiba N, Hasegawa K, Kobayashi R, Ivata S, Sunakawa K. Antibiotic susceptibility in relation to penicillin-binding protein genes and
serotype distribution of strains responsible for meningitis in Japan, 1999 to 2002. Antimicrob Agents Chemother 2004; 48:1488–1494.
24. Karlowsky JA, Thornsberry C, Jones ME, Evangelista AT, Critchley IA, Sahm DF, et al. Factors associated with relative rates of antimicrobial resistance among Streptococcus pneumoniae in the United States: results from the TRUST surveillance program (1998–2002). Clin Infect Dis 2003; 36:963–970.
25. Whitney CG, Farley MM, Hadler J, Harrison LH, Lexau C, Reingold A, et al. Increasing prevalence of multidrug-resistant Streptococcus pneumoniae in the United States. N Engl J Med 2000; 343:1917–1924.
26. Chen DK, McGeer A, de Azavedo JC, Law DE. Decreased susceptibility of Streptococcus pneumoniae to fluoroquinolones in Canada. N Engl J Med 1999; 341:233–239.
27. Davidson R, Cavalcanti R, Brunton JL, Bast DJ, de Azavedo JCS, Kibsey P, et al. Resistance to levofloxacin and failure of treatment of pneumococcal pneumonia. N Engl J Med 2002; 346:747–750.
28. Noguchi N, Tano J, Nasu Y, Koyama M, Narui K, Kamishima H, et al. Antimicrobial susceptibilities and distribution of resistance genes for beta-lactams and macrolides in Streptococcus pneumoniae isolated between 2002 and 2004 in Tokyo. Int J Antimicrob Agents 2007; 29:26–33.
29. Okamoto H, Tateda K, Ishii Y, Matsumoto T, Kobayashi T, Miyazaki S, et al. High frequency of erythromycin A resistance and distribution of mefE and ermB genes in clinical isolates of Streptococcus pneumoniae in Japan. J Infect Chemother 2002; 8:28–32.
30. Hiramatsu K, Ohama M, Miyajima Y, Kishi K, Mizunoe S, Tokimatsu I, et al. Antimicrobial susceptibilities and analysis of genes related to penicillin or macrolide resistance in Streptococcus pneumoniae. Int J Antimicrob Agents 2004; 24:125-129.