Antibiotic Resistance and Molecular Analysis of *Streptococcus pyogenes* Isolated from Iranian Patients

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**ABSTRACT**

Macrolide-Lincosamide-Streptogramin (MLSb) is of growing concern in Iran. A total of 828 clinical isolates of *S. pyogenes* were collected from several Sanandajian laboratories in Iran between September to March 2014. Strain identification was confirmed with bacitracin disks and by a latex agglutination. The absence of a significant zone of inhibition around the two disks was taken to indicate constitutive resistance (cMLS phenotype), blunting of the clindamycin zone of inhibition proximal to the erythromycin disk was taken to indicate inducible resistance (iMLS phenotype) and susceptibility to clindamycin with no blunting of the zone of inhibition around the clindamycin disk was taken to indicate the M phenotype. Macrolide resistance among isolates was 16 (40%), 4 (50%) cases expressed the M phenotype (erythromycin resistant and clindamycin susceptible). There was one sample (12.5%) that displayed the inducible (MLS,sub.B) phenotype. The remaining three samples (37.5%) displayed the constitutive (MLS,sub.B) phenotype. The M phenotypes contained the mef A gene (100%). In addition, of the remaining macrolid resistant isolates were, 1 (6.25%) strain carriers mef E and 4 (25%) isolate carriers mef A gene and 7 (43.75%) isolates and didn’t have any mef A/E genes. A sample was carrier mef A gene among the 24 isolates of none-macrolide resistance. The correlation results of PCR assay genotyping in the present study compared with those of conventional PCR genotyping and resistance phenotyping was 100%. This highly sensitive and specific PCR assay to detect macrolide resistance has the potential to provide sufficiently rapid results to improve antibiotic treatment of streptococcal infections.

**Key words:** Beta-hemolytic, streptococi group A, macrolide resistance, mef gene, MLSb

**INTRODUCTION**

*Streptococcus pyogenes* is the most frequent bacterial cause of pharyngitis; this bacterium also causes impetigo, crysipelas and less frequently, sever’s invasive disease (Bisno, 1995). Penicillin has long been regarded as the treatment of choice for streptococcal tonsillitis (Bisno et al., 1997). In patients with known or suspected allergy to penicillin, clinicians avoid penicillin and β-lactam antibiotics in general. Erythromycin and other macrolides are considered alternative treatments for streptococcal pharyngitis and other non-serious infections caused by *S. pyogenes*; they have been demonstrated to be effective and safe as penicillins (Adam et al., 1996; Hooton, 1991; Stein et al., 1991).
Therefore, the emergence and spread of resistance to macrolides in *S. pyogenes* constitute an important problem in the management of streptococcal infection. In recent years, a variety of studies in different areas and countries have been published, showing great diversity in rates of resistance to macrolides (D’Humieres *et al.*, 2012; Ardanuy *et al.*, 2010; Michos *et al.*, 2009; Ayatollahi and Bahroolomi, 2006; Brooks *et al.*, 2004). Recent data from various parts of Iran indicate an increasing prevalence of resistance to macrolide over the years (Brook and Dohar, 2006; Cochet et al., 2005; Cohen, 2004; Klaassen and Mouton, 2005). The mechanisms of macrolide resistance have been elucidated and involved target modification mediated by a methylase (encoded by *erm* genes) which modifies an adenine in 23S rRNA (Granizo *et al.*, 2000) and an efflux mechanism encoded by the *mef* gene (Seppala *et al.*, 1993). The gene is a novel one with sequence homology to membrane-associated pump proteins. Target site modification due to methylase activity confers resistance to macrolides, lincosamides and streptogramin B (*MLS*₂) antibiotics and is expressed constitutively or inducible. The efflux mechanism selectively pumps 14 and 15 membered macrolides out of the cell but not 16-membered macrolides or lincosamides. The efflux determinant in *streptococci* seems to be distinct from the multicompartment macrolide efflux system in coagulase-negative staphylococci (Seppala *et al.*, 1993). These (*MLS*₃) phenotypes can be easily distinguished by utilizing the D-test or double disk diffusion test with a macrolide and a lincosamide. The inducible (*MLS*₃) requires the presence of a specific antimicrobial to induce the production of the enzyme. In the case of *S. pyogenes*, the macrolide erythromycin can elicit the production of the enzyme whereas the lincosamide clindamycin cannot. The bacterium is resistant to the lincosamide only in the presence of the enzyme inducing macrolide. Inducible (*MLS*₃) correlates with the *erm A* gene. The constitutive (*MLS*₃) mechanism continually produces the enzyme and can therefore continually resist any ribosome targeting antimicrobial. Constitutives (*MLS*₃) express resistance to erythromycin and clindamycin and is associated with the *erm B* gene (Adam *et al.*, 1996). While previous studies have used different methods (agar diffusion, agar dilution, broth micro dilution), different interpretations of the results and different populations. Moreover, in several studies the genes responsible for antibiotic resistance were not determined. The aim of the present study was to determine the prevalence of this organism and investigate the molecular mechanisms of macrolide resistant strains isolated from clinical samples and to compare the phenotype and genotype of the *mef* gene carriers isolated from patients in a Sanandaj city of Iran in 2014. The different susceptibility phenotypes and the genetic basis for the antibiotic resistance were also determined.

**MATERIALS AND METHODS**

**Bacterial strains:** A total of 828 clinical isolates of *S. pyogenes* (the vast majority of which were from cultures of throat, ear, skin, blood and urine swabs from patients) were collected from several sanandajian laboratories between September to March 2014. Strain identification was confirmed in Sanandaj Islamic Azad University laboratory with bacitracin disks and by a latex agglutination assay (Streptex; Welcome, Dart ford, United Kingdom) and erythromycin resistance (MIC, $\leq$1 mg mL⁻¹) was confirmed by the broth microdilution method. The strains were maintained in glycerol at 270°C until all isolates were collected and were subculture twice on blood agar before susceptibility testing.

**Macrolide susceptibility:** The macrolide susceptibility test was carried out by the disk diffusion method on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, using 15 μg erythromycin, 2 μg clindamycin and 15 μg azithromycin, 15 μg clarithromycin and 10 μg penicillin disks (Laboratories Iranian, Padtan Tab).
The disks were placed 15-20 mm apart on Mueller-Hinton II agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood which had been inoculated with a swab dipped into a bacterial suspension with a turbidity equivalent to that of a 0.5 McFarland standard. After 16-24 h of incubation at 37°C, the absence of a significant zone of inhibition around the two disks was taken to indicate constitutive resistance (cMLS phenotype), blunting of the clindamycin zone of inhibition proximal to the erythromycin disk was taken to indicate inducible resistance (iMLS phenotype) and susceptibility to clindamycin with no blunting of the zone of inhibition around the clindamycin disk was taken to indicate the M phenotype. The presence of a D shape around the susceptible region of clindamycin was the inducible (MLS.sub.B) phenotype. Resistance to erythromycin (less than or equal to 15 mm) and clindamycin (less than or equal to 15 mm) was recorded as the constitutive (MLS.sub.B) phenotype. After incubation, the zones of inhibition to each disk were measured using a micrometer. The size of the zone was used to determine if the strain was resistant (less than or equal to 15 mm), intermediate (16-20 mm) or susceptible (greater than or equal to 21 mm) using the zones of inhibition listed by the antimicrobial manufacturer (Fig. 1). Isolates were kept frozen in skim milk at -30°C.

**Detection resistance genes:** DNA was extracted from all of *S. pyogenes* isolates. The extraction was performed using DNA CinnaPure (COO, Sinagen, Iran), follows the manufacturer’s protocol for DNA extraction from bacterial colonies. The MLS resistance efflux pump mechanism was determined by PCR with amplification of mef genes, using specific primers for mefA (F-5’-GGGCTTATTATTAGGGTT-3’; R-5’-TACCTGATACTAAAAACCAATG-3’) and specific primers for mefE (F-5’-AGGACCTTATTATTAGGAAG-3’; R-5’-TACCTGATACTAAAAACCAATG-3’) (CO, Takapoozist, Iran). Each extracted sample was tested for each of the two genes separately. The PCR reaction concentrations consisted of the following: 250 μL each of deoxynucleotide, 1 mM MgCl₂, 10 Mm Tris HCl (pH 9), 30 μL KCL, 1U DNA polymerase, 1 μL of forward and revers primers and 0.5 μL of extracted DNA and 17.5 μL distilled water. For the mef A/E PCR, cycling comprised initial denaturation for 10 min at 95°C and 35 amplification cycles for 95°C, at 61°C, at

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72°C amplification products were separated by electrophoresis on a 1.5% agarose gel (100 V for 30 min) and stained with ethidium bromide. The presence of the gene was determined by the presence of bands with the expected molecular sizes of 339 and 340 base pairs for mef A and mef E genes, respectively. Control strains were used to confirm the accuracy of the PCR amplification: S. pyogenes PTCC 1447 for mef A, S. pyogenes, PTCC 104030 for erm A and S. pyogenes (provided courtesy of the Institute Pasteur, Iran).

Genomic DNA for PCR reaction was obtained with the Acc Power PCR Premix (CoBioNEER, Korea). The asymptotic standard error assuming the null hypothesis Cramer’s V, phi and Contingency Coefficient test was used for statistical analysis.

RESULTS

During the study period, 825 strains of beta-hemolytic streptococci group A were collected and analyzed. Out of 825 patients, 40 (4.84%), S. pyogenes isolated and confirmed. Most of S. pyogenes isolates were collected concerning the throat (Fig. 2). Of the 16 (40%) cases, found resistant to macrolide showed that among the 40 strains of GAS, 8 (20%) were resistant to erythromycin, 4 (10%) to azithromycin, 3 (7.5%) to clindamycin and 1 (2.5%) to clarithromycin and all the isolates were susceptible to penicillin (Fig. 3).

**Fig. 2:** Frequency of S. pyogenes isolates of the location sampling

**Fig. 3:** Frequency of isolates with macrolide resistance in antibiogram test
Fig. 4: Frequency of macrolide resistance phenotype in D-test

Fig. 5: Frequency of macrolide genotype carrier's mef A/E genes

Antimicrobial susceptibility by disk diffusion method and induction of D-TEST resistance was assessed by PCR using specific primers for all isolates of the two mef A and mef E. Prevalence of group A streptococcus in the study population was 4.84% and macrolide resistance among isolates was 16 (40%).

Phenotypes measured and recorded on 8 of the erythromycin resistant showed the induction of resistance (D-TEST). There were 4 (50%) cases that expressed the M phenotype (erythromycin resistant and clindamycin susceptible). There was one sample (12.5%) that displayed the inducible (MLS.sub.B) phenotype (erythromycin resistant and inducible clindamycin resistance) (Fig. 4).

The remaining three samples (37.5%) displayed the constitutive (MLS.sub.B) phenotype (resistant to both erythromycin and clindamycin). All of samples with M phenotype contained the mef A gene (100%) that included the positive correlation between the phenotype and genotype in these samples.

In addition, of the remaining macro lid resistant isolates were, 1 (6.25%) strain carriers mef E and 4 (25%) isolate carriers mef A gene and 7 (43.75%) isolates and didnt have any mef A/E genes (Fig. 5 and 6).

An sample was carrier mef A gene among the 24 isolates of none-macrolide resistance.
Fig. 6(a-d): Gel electrophoresis of PCR products, (a) Among of without macrolide resistance isolates, 1 isolate carriers mef A gene, (b) Repeat 8 positive macrolide resistance isolates carrier mef A gene and 1 positive macrolide resistance isolate carriers mef E gene, (c) Carrier mef A gene and (d) Carrier mef E gene

**DISCUSSION**

Our findings indicates that the erythromycin could induce resistance to clindamycin in streptococcus group A strains. Therefore, we recommend the test routinely be used for correct determination of resistance to clindamycin. Penicillin based antimicrobials should still be the first drug of choice when prescribing antimicrobials for treatment against this organism. The most common mechanism responsible for the resistance in the population of patients surveyed in this study was the efflux pump due to the presence of mef A gene (M phenotype) (Lopardo et al., 2004). Coupling of mef A and erm B genes (efflux with constitutive ribosomal modification) suggests a greater presence of constitutive (MLS.sub.B) phenotype will be seen in clinical settings when testing for mechanisms using the D-test. Knowing the mechanisms of resistance is useful in determining how to counteract the resistance and will influence the development and future administration of antimicrobials (Howard et al., 2001).

A significant finding from this study was that all *S. pyogenes* isolates were susceptible to penicillin, verifying its continued effectiveness against the organism. Previous studies have also reported little to no penicillin resistance in *S. pyogenes*. The results show that the efflux pump...
(mef A gene) was the most prevalent mechanism (48.1%) of the erythromycin resistant strains. This is higher than the percent found in a previous published study done nationwide (Yoon et al., 2008). Typically, all of these mechanisms for macrolide resistance are represented in a given patient population. However which mechanism is predominant varies from place to place. The efflux pump (mef A gene) is the most common in Spain (Michos et al., 2009). A French pediatric study found 69.4% of the erythromycin resistance to be caused by the constitutive MLS.sub.B (erm B gene) (Ardanuy et al., 2010). In the United States, the efflux pump (mef A) has been previously reported to account for 43% while inducible (MLS.sub.B) (erm A) accounts for 46%, with constitutive (MLS.sub.B) (erm B) being much less at 8.5%. The purpose of this study is to determine the rates of each mechanism of erythromycin resistance in northern Utah (Rowe et al., 2009).

A similar low rate of resistance to erythromycin has been also reported from Serbia (6.8%) (Pavlovic et al., 2010), Romania (5%) (Luca-Harari et al., 2008), Korea (4.6%) (Koh and Kim, 2010) and Chile (3.5%) (Rodriguez et al., 2011). In the USA, several multicenter studies have shown a stable rate of macrolide resistance (about 6%), with a majority of M phenotypes (Green et al., 2005). In contrast, high rates of resistance to erythromycin have been reported in China (96.8%) (Chang et al., 2010), Hong Kong (28%) and Italy (25%) (Canton et al., 2002) identified 3 German clones that accounted for more than 50% of macrolide-resistance GAS isolates, namely mef A-positive emm type 4 isolates (ST 89), an erm A-positive clone of emm type 77 (ST 63) and mef A-positive emm type 12 isolates (ST 36). Contrary to our study, these latter authors detected the mef A emm77 clone. In Germany, the rate of erythromycin resistance in pediatric GAS isolates fell significantly from 13.8% (Seppala et al., 1997) to 2.6% (2005-2009) (Canton et al., 2002). Our results are in keeping with these data, as the rate of erythromycin resistance fell from 24% in 2002-2003 to 3.2% in 2009-2011. This decline has been attributed both to lesser macrolide consumption in France (since 2002) and Germany and to the decline of the resistant emm 28 clone (46% in 2003, versus 15% in this study) (Farmand et al., 2012). In 1995, found a relationship between macrolide consumption and the GAS resistance rate: Logistic regression analysis showed that the proportion of isolates resistant to erythromycin increased clearly with increasing local erythromycin consumption (Sabuncu et al., 2009). Then, after nationwide reductions in the use of macrolide antibiotics for out-patient therapy, there was a significant decline in the frequency of erythromycin resistance among GAS isolates (Malhotra-Kumar et al., 2007). In contrast, found that new-generation macrolides such as azithromycin and clarithromycin were prone to induced resistance (Bingen et al., 2004). The high level of GAS resistance to macrolide antibiotics in 2003 was associated with very high macrolide consumption in France. The current fall in antibiotic consumption has led to a very low rate of GAS macrolide resistance, with no predominant clone.

In contrast, S. pyogenes isolates included in the present study showed a macrolide resistance rate of 8.2% which is significantly lower than the resistance rates reported from Spain (34%) (Goossens et al., 2005) and Italy (35%) (Rubio-Lopez et al., 2012) and even somewhat lower than in earlier reports from Germany, with a 14% resistance level reported amongst children up to 16 years of age with community-acquired infections in 2004 (Reinert et al., 2004) or a rate of 19.1% (adults) and 11.8% (children) reported from Bavaria in 2003 (Durmez et al., 2003).

This study demonstrated that the M phenotype and mef A gene were the most common among ERY-resistant strains in our geographic region. These findings are in agreement with prior studies that have taken place in Greece (Ioannidou et al., 2003).

Group A beta-hemolytic streptococci as potential causes of sore throat in children, especially among patients admitted to hospitals of Sanandaj.
Isolates to detect inducible resistance to erythromycin and avoid hidden iMLSB by these isolates, it is suggested D-TEST next anti-biogram test as a diagnostic test, be included in addition to viewing and interpretation of standard tests a like PCR test.

In addition to viewing and interpretation of standard tests, PCR testing as a routine test in clinical laboratories be included.

REFERENCES


