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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 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 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, streptococci group A, macrolide resistance, mef gene, MLS$_B$

INTRODUCTION

Streptococcus pyogenes is the most frequent bacterial cause of pharyngitis; this bacterium also causes impetigo, erysipelas 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 pharengitis 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 is 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; Ayatolahi 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; Cochetti *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_B) 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 multicomponent macrolide efflux system in coagulase-negative staphylococci (Seppala *et al.*, 1993). These (MLS.sub.B) phenotypes can be easily distinguished by utilizing the D-test or double disk diffusion test with a macrolide and a lincosamide. The inducible (MLS.sub.B) 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.sub.B) correlates with the erm A gene. The constitutive (MLS.sub.B) mechanism continually produces the enzyme and can therefore continually resist any ribosome targeting antimicrobial. Constitutives (MLS.sub.B) 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, $1 mg mL^{-1}$) 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).
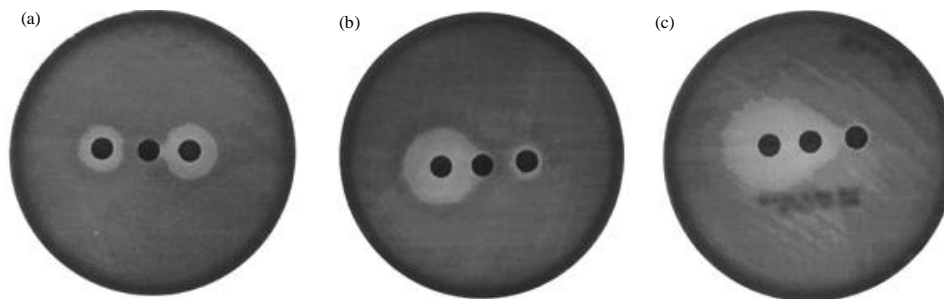
Fig. 1(a-c): Examples of triple disk testing of rRNA or ribosomal protein mutants, (a) Clindamycin, (b) Erythromycin and (c) Streptogramin B. All bacteria are *S. pneumonia*. Plate A shows ML (rRNA mutation); plates B and C show MSB (Both L4 mutations). Taken from AAC 2000; 44:3395

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 (CO0. 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 mef A (F-5'-GGGGTCTATTATTAGGGTT-3'; R-5'-TACCTGATAGTAAAAACCAATG-3') and specific primers for mef E (F-5'-AGGAGGCTTATTATTAGGAAG-3'; R-5'-TACCTGATAGTAAAAACCAATG-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

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 Inistitue 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 the16 (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 (5/2%) to clarithromycin and all the isolates were susceptible to penicillin (Fig. 3).
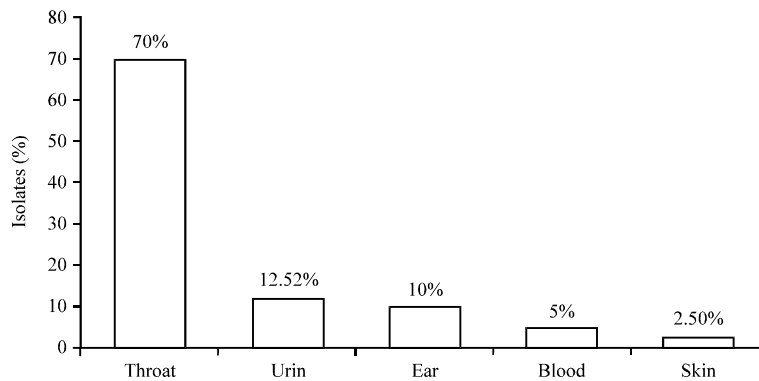

Fig. 2: Frequency of *S. pyogenes* isolates of the location sampling
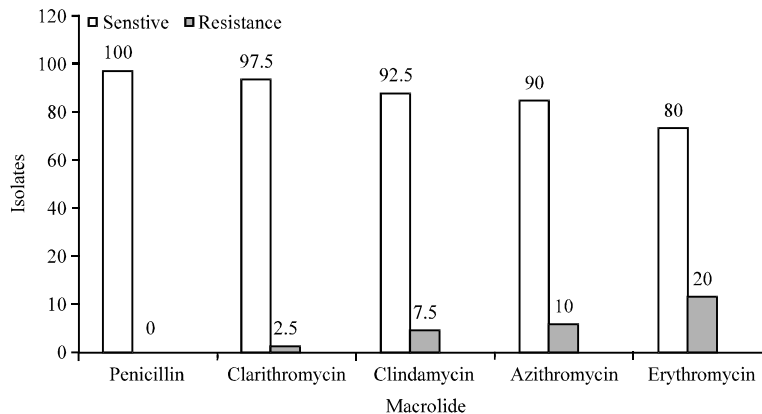

Fig. 3: Frequency of isolates with macrolide resistance in antibiogram test
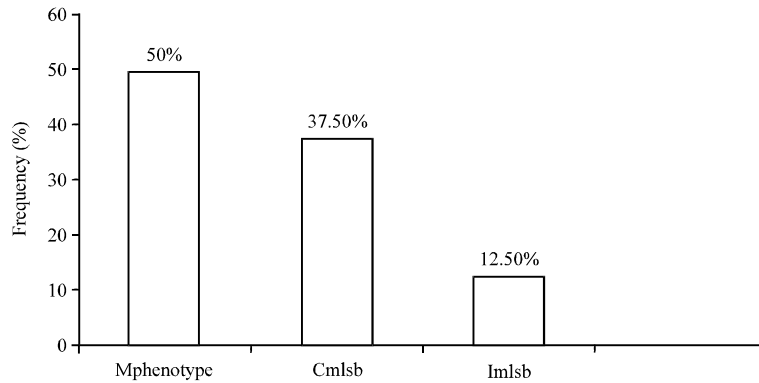
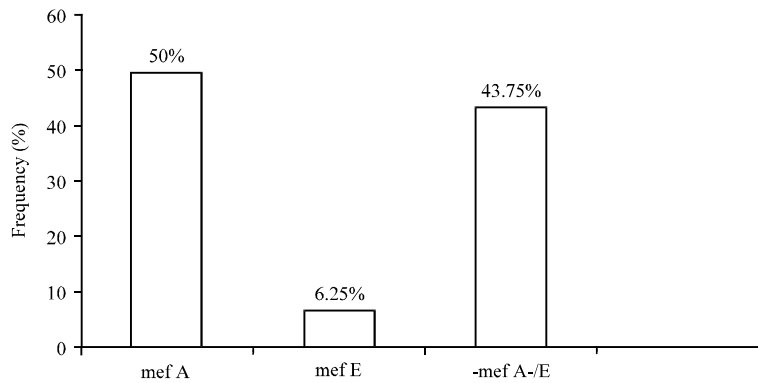Fig. 4: Frequency of macrolide resistance phenotype in D-test

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 didn't have any mef A/E genes (Fig. 5 and 6).

An sample was carrier mef A gene amomg the 24 isolates of none-macrolide resistance.
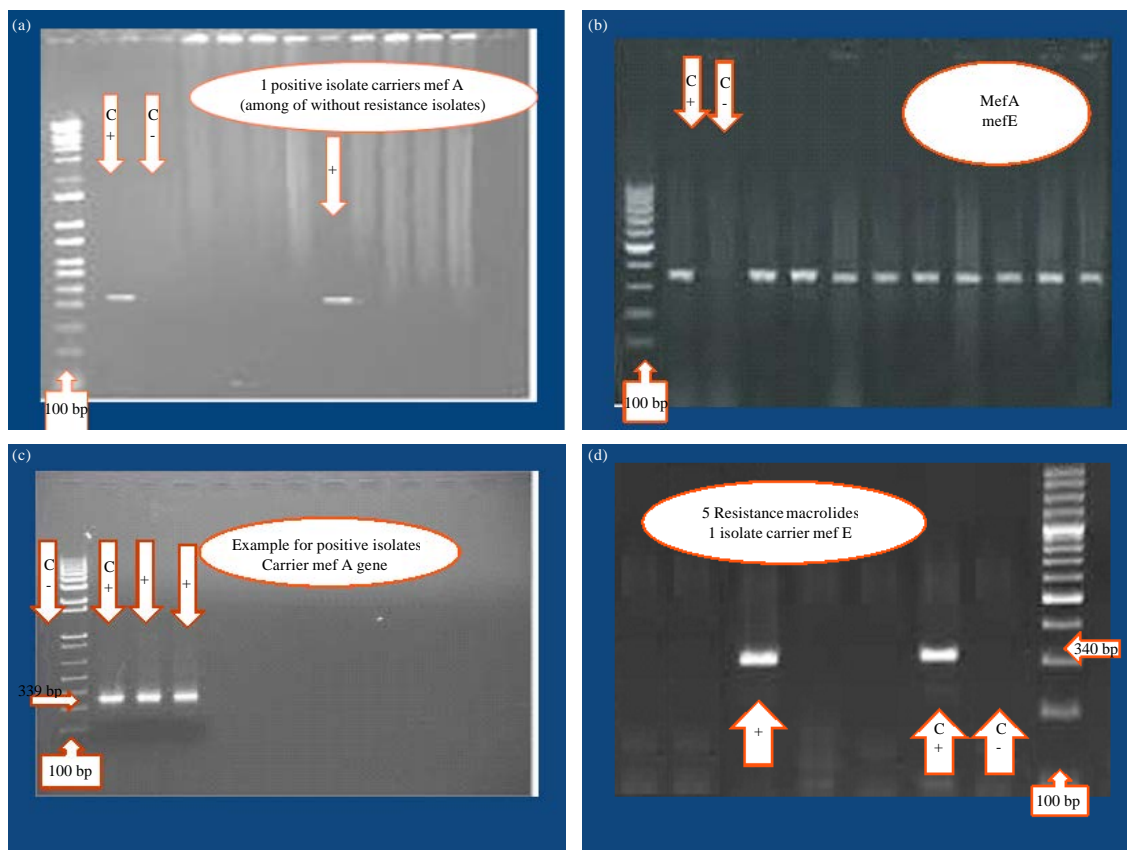
Fig. 6(a-d): Gel electrophoresis of PCR products, (a) Among of without macrolide resistance isolates, 1 isolate carriers mef A gene, (b) Repead 8 positive macrolide resistance isolates carrier mef A gene and 1 positive macrolide resistance isolate carriers mef E gene, (c) Carrier mef A gene and (d) Carrier 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 39), 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 pediatricm GAS isolates fell significantly from 13.6% (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 asazithromycin 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 upto 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 (Durmaz *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 streptococcic 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

Adam, D., H. Scholz, C. Aspe, H.G. Berzel and D. Bulle *et al.*, 1996. Five days of erythromycin estolate versus ten days of penicillin V in the treatment of group A streptococcal tonsillopharyngitis in children. Eur. J. Clin. Microbiol. Infect. Dis., 15: 712-717.

Ardanuy, C., A. Domenech, D. Rolo, L. Calatayud and F. Tubau *et al.*, 2010. Molecular characterization of macrolide- and multidrug-resistant *Streptococcus pyogenes* isolated from adult patients in Barcelona, Spain (1993-2008). J. Antimicrob. Chemother., 65: 634-643.

Ayatolahi, J. and R. Bahroolomi, 2006. Survey onpharyngitis. Sci. J. Shahid Sadoughi Univ. Med. Sci., 14: 76-83.

Bingen, E., P. Bidet, L. Mihaila-Amrouche, C. Doit and S. Forcet *et al.*, 2004. Emergence of macrolide-resistant *Streptococcus pyogenes* strains in French children. Antimicrob. Agents Chemother., 48: 3559-3562.

Bisno, A.L., 1995. *Sterptococcus pyogenes*. In: Mandell, Douglas and Bennetts Principles and Practice of Infectious Diseases, Mandell, G.L., J.E. Bennett and R. Dolin (Eds.). 4th Edn., Churchill Livingstone, New York, pp: 1786-1799.

Bisno, A.L., M.A. Gerber, E.L. Kaplan, J.M. Gwaltney and R.H. Schwartz, 1997. Diagnosis and management of group A streptococcal pharyngitis: A practice guideline. Clin. Infect. Dis., 25: 574-583.

Brook, I. and J.E. Dohar, 2006. Management of group A â-hemolytic streptococcal pharyngotonsillitis in children. J. Family Pract., 55: S1-S11.

Brooks, G.F., J.S. Butel, S.A. Morse and J.M. Adebergs, 2004. Medical Microbiology. 23rd Edn., McGraw-Hill, New York, pp: 235-240.

Canton, R., E. Loza, M.I. Morosini and F. Baquero, 2002. Antimicrobial resistance amongst isolates of *Streptococcus pyogenes* and *Staphylococcus aureus* in the PROTEKT antimicrobial surveillance programme during 1999-2000. J. Antimicrob. Chemother., 50: 9-24.

Chang, H., X. Shen, Z. Fu, L. Liu and Y. Shen *et al.*, 2010. Antibiotic resistance and molecular analysis of *Streptococcus pyogenes* isolated from healthy schoolchildren in China. Scand. J. Infect. Dis., 42: 84-89.

Cochetti, I., M. Vecchi, M. Mingoia, E. Tili and M.R. Catania *et al.*, 2005. Molecular characterization of pneumococci with efflux-mediated erythromycin resistance and identification of a novel mef gene subclass, mef (I). Antimicrob. Agents Chemother., 49: 4999-5006.

Cohen, R., 2004. Defining the optimum treatment regimen for azithromycin in acute tonsillopharyngitis. Pediatr. Infect. Dis. J., 23: S129-S134.

D'Humieres, C., R. Cohen, C. Levy, P. Bidet, F. Thollot, A. Wollner and E. Bingen, 2012. Decline in macrolide-resistant *Streptococcus pyogenes* isolates from French children. Int. J. Med. Microbiol., 302: 300-303.

Durmaz, R., B. Durmaz, M. Bayraktar, I.H. Ozerol, M.T. Kalcioglu, E. Aktas and Z. Cizmeci, 2003. Prevalence of group A streptococcal carriers in asymptomatic children and clonal relatedness among isolates in Malatya, Turkey. J. Clin. Microbiol., 41: 5285-5287.

Farmand, S., P. Henneke, M. Hufnagel and R. Berner, 2012. Significant decline in the erythromycin resistance of group A streptococcus isolates at a German paediatric tertiary care centre. Eur. J. Clin. Microbiol. Infect. Dis., 31: 707-710.

Goossens, H., M. Ferech, R. Van der Stichele and M. Elseviers, 2005. Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. Lancet, 365: 579-587.

Granizo, J.J., L. Aguilar, J. Casal, R. Dal-Re and F. Baquero, 2000. *Streptococcus pyogenes* resistance to erythromycin in relation to macrolide consumption in Spain (1986-1997). J. Antimicrob. Chemother., 46: 959-964.

Green, M., C. Allen, J. Bradley, B. Dashefsky and J.R. Gilsdorf *et al.*, 2005. *In vitro* activity of telithromycin against macrolide-susceptible and macrolide-resistant pharyngeal isolates of group A streptococci in the United States. Antimicrob. Agents Chemother., 49: 2487-2489.

Hooton, T.M., 1991. A comparison of azithromycin and penicillin V for the treatment of streptococcal pharyngitis. Am. J. Med., 91: S23-S26.

Howard, A.J., J.T. Magee, K.A. Fitzgerald and F.D.J. Dunstan, 2001. Factors associated with antibiotic resistance in coliform organisms from community urinary tract infection in Wales. J. Antimicrob. Chemother., 47: 305-313.

Ioannidou, S., P.T. Tassios, L. Zachariadou, Z. Salem and M. Kanelopoulou *et al.*, 2003. *In vitro* activity of telithromycin (HMR 3647) against Greek *Streptococcus pyogenes* and *Streptococcus pneumoniae* clinical isolates with different macrolide susceptibilities. Clin. Microbiol. Infect., 9: 704-707.

Klaassen, C.H. and J.W. Mouton, 2005. Molecular detection of the macrolide efflux gene: To discriminate or not to discriminate between *mef* (A) and *mef* (E). Antimicrob. Agents Chemother., 49: 1271-1278.

Koh, E. and S. Kim, 2010. Decline in erythromycin resistance in group A streptococci from acute pharyngitis due to changes in the *emm* genotypes rather than restriction of antibiotic use. Korean J. Lab. Med., 30: 485-490.

Lopardo, H.A., C. Hernandez, P. Vidal, M. Vazquez and L. Rosaenz *et al.*, 2004. Erythromycin-resistant *Streptococcus pyogenes* in Argentina. Medicina, 64: 143-145.

Luca-Harari, B., M. Straut, S. Cretoiu, M. Surdeanu, V. Ungureanu, M.V.D. Linden and A. Jasir, 2008. Molecular characterization of invasive and non-invasive *Streptococcus pyogenes* isolates from Romania. J. Med. Microbiol., 57: 1354-1363.

Malhotra-Kumar, S., C. Lammens, S. Coenen, K. Van Herck and H. Goossens, 2007. Effect of azithromycin and clarithromycin therapy on pharyngeal carriage of macrolide-resistant streptococci in healthy volunteers: A randomised, double-blind, placebo-controlled study. Lancet, 369: 482-490.

Michos, A.G., C.G. Bakoula, M. Braoudaki, F.I. Koutouzi and E.S. Roma *et al.*, 2009. Macrolide resistance in *Streptococcus pyogenes*: Prevalence, resistance determinants and *emm* types. Diagn. Microbiol. Infect. Dis., 64: 295-299.

Pavlovic, L., E. Grego and S. Sipetic-Grujicic, 2010. Prevalence of macrolide resistance in *Streptococcus pyogenes* collected in Serbia. Jpn. J. Infect. Dis., 63: 275-276.

Reinert, R.R., C. Franken, M. Van Der Linden, R. Lutticken, M. Cil and A. Al-Lahham, 2004. Molecular characterisation of macrolide resistance mechanisms of *Streptococcus pneumoniae* and *Streptococcus pyogenes* isolated in Germany, 2002-2003. Int. J. Antimicrob. Agents, 24: 43-47.

Rodriguez, C., P. Rojas, A. Wozniak, A.M. Kalergis and I. Ceron *et al.*, 2011. [Resistance phenotypes and genotypes of *Streptococcus pyogenes* clinical isolates in Chile over a 10-year period]. Revista Medica Chile, 139: 1143-1149, (In Spanish).

Rowe, R.A., R.M. Stephenson, D.L. East and S. Wright, 2009. Mechanisms of resistance for *Streptococcus pyogenes* in Northern Utah. Clin. Lab. Sci., 22: 39-44.

Rubio-Lopez, V., S. Valdezate, D. Alvarez, P. Villalon, M.J. Medina, C. Salcedo and J.A. Saez-Nieto, 2012. Molecular epidemiology, antimicrobial susceptibilities and resistance mechanisms of *Streptococcus pyogenes* isolates resistant to erythromycin and tetracycline in Spain (1994-2006). BMC Microbiol., Vol. 12. 10.1186/1471-2180-12-215

Sabuncu, E., J. David, C. Bernede-Bauduin, S. Pepin and M. Leroy *et al.*, 2009. Significant reduction of antibiotic use in the community after a nationwide campaign in France, 2002-2007. PLoS Med., Vol. 6. 10.1371/journal.pmed.1000084

Seppala, H., A. Nissinen and P. Huovinen, 1993. Three different phenotypes of erythromyein-resistance in group *A Streptococci* in Finland. J. Antimicrob. Chemother., 32: 885-891.

Seppala, H., T. Klaukka, J. Vuopio-Varkila, A. Muotiala, H. Helenius, K. Lager and P. Huovinen, 1997. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. N. Engl. J. Med., 337: 441-446.

Stein, G.E., S. Christensen and N. Mummaw, 1991. Comparative study of clarithromycin and penicillin V in the treatment of streptococcal pharyngitis. Eur. J. Clin. Microbiol. Infect. Dis., 10: 949-953.

Yoon, E.J., A.R. Kwon, Y.H. Min and E.C. Choi, 2008. Foggy D-shaped zone of inhibition in *Staphylococcus aureus* owing to a dual character of both inducible and constitutive resistance to macrolide-lincosamide-streptogramin B. J. Antimicrob. Chemother., 61: 533-540.