European Review for Medical and Pharmacological Sciences 2014; 18: 2960-2965

Typing of macrolide resistant group A streptococci by random amplified polymorphic DNA analysis

I. GAJIC, V. MIJAC, M. STANOJEVIC, L. RANIN, A. SMITRAN[1], N. OPAVSKI

Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Belgrade, Serbia
[1]Department of Microbiology and Immunology, Faculty of Medicine, University of Banjaluka, Banjaluka, Republic of Serbia

Abstract. – OBJECTIVE: Several studies of group A streptococci (GAS) have revealed that a small number of dominant resistant clones might be responsible for the spread of *Streptococcus (S.) pyogenes* resistance to macrolides. We aimed to determine the genetic diversity of macrolide resistant group A streptococci (MRGAS), isolated from patients with pharyngitis in Serbia.

MATERIALS AND METHODS: The clonal relationships among 76 MRGAS isolates collected during 2008 were studied using two molecular typing methods: *emm* typing and random amplified polymorphic DNA (RAPD) analysis. Isolates that share the same *emm* type and RAPD pattern were considered to belong to the same clone.

RESULTS: Out of 7 distinct *emm* types identified, the 3 most frequently occurring overall were *emm*12, *emm*75 and *emm*77 (> 90% of isolates). Although as many as 26 different RAPD patterns were found among the isolates studied, two clones with *emm*12 and *emm*77 accounted 32 out of 76 (42%) isolates.

CONCLUSIONS: The results indicate a polyclonal spread of erythromycin-resistant *Streptococcus pyogenes* in our country. Furthermore, predominance of two clones, particularly among *emm*12 and *emm*77 strains indicates that erythromycin-resistant GAS of the same clonal origin are widely distributed in Serbia.

Key Words:
Emm typing, Group A streptococci, Macrolide resistance, RAPD fingerprinting.

Introduction

Group A streptococcus (GAS), also known as *Streptococcus pyogenes* is a highly prevalent human pathogen with a worldwide distribution. GAS causes a variety of disease, including pharyngitis and invasive infections. GAS is responsible for more than 600 million cases of pharyngitis each year worldwide[1]. Penicillin is a first choice therapy for infections caused by GAS, since penicillin resistance in streptococci has not yet emerged. Macrolides are preferred for treatment of GAS infections in patients with beta-lactam hypersensitivity, in chronic, recurrent pharyngitis due to prior treatment failure and lack of oral forms of penicillin[2]. Significant increase in macrolide resistance has been reported from many countries, in the last years[3-6]. Particularly high level of GAS macrolide resistance was reported in southern European countries[7].

Numerous typing schemes have been used to characterize and measure the genetic diversity among macrolide resistant isolates of *S. pyogenes*. The most common tool used today is *emm* typing[8,9], which is based on sequence at the 5' end of *emm* gene that encode M protein, one of the major virulence factor in GAS. So far, more than 150 *emm* types have been described. An association between certain *emm* types and macrolide resistance has already been documented[10,11]. Compare to *emm* typing, several methods, such as multilocus sequence typing (MLST) and pulsed filled gel electrophoresis (PFGE) better suited towards characterizing the genetic relationships among the organisms of a bacterial species. Therefore, for the precise identification of genetic lineages, *emm* typing should be complemented with other typing methods[12]. Nevertheless, in case of GAS, MLST requires 14 sequencing reactions per isolate. On the other hand, PFGE is time consuming and technically highly demanding method. In order to overcome these difficulties, alternative approaches to characterizing the genetic relationships between isolates were developed. Randomly amplification of polymorphic DNA (RAPD) analysis is genotyping gel based method, with relatively high dis-

Corresponding Author: Ina Gajic, MD; e-mail: ina.gajic@med.bg.ac.rs

criminatory index[13]. Comparing to MLST and PFGE, RAPD profiling is faster and more economical. In RAPD the genomic DNA is amplified with a primer of an arbitrarily selected nucleotide sequence. The resulting RAPD fingerprints of different isolates can be compared according to number of PCR products and their size.

Although macrolide resistance rates among GAS were shown to be associated with consumption of macrolides, the prevalence of antimicrobial resistance is also due to several circulating clones associated with certain *emm* types. Several clinical and epidemiological studies of group A streptococci have revealed that a small number of dominant resistant clones might be responsible for the spread of *S. pyogenes* resistance to macrolides. However, limited information about the genetic relatedness of macrolide resistant group A streptococci circulating in Serbia is available.

The aim of this study was to determine the genetic diversity of macrolide resistant group A streptococci (MRGAS), isolated from patients with pharyngitis in Serbia.

Materials and Methods

Bacterial Isolates

Eleven laboratories from Serbia collected 3893 GAS isolates from outpatients with pharingitis through a nationwide surveillance study. Among those strains we identified isolates resistant to macrolide antibiotics. Seventy six MRGAS were randomly chosen and further investigated in the National Reference Laboratory for Streptococci.

GAS isolates were identified on the basis of Gram staining, typical colony morphology, bacitracin (0.04U) sensitivity test (BioRad, Hercules, CA, USA) and latex agglutination with group A-specific antisera (bioMerieux, Marcy L'Etoile, France). The isolates were then stored at –80°C in Todd Hewitt Infusion Broth (Biomedics, Barcelona, Spain), containing 10% glycerol.

Antimicrobial Susceptibility

Susceptibilities to penicillin, erythromycin, clindamycin, tetracycline, norfloxacin and chloramphenicol were tested by disk diffusion method, according to the CLSI recommendations[14]. The minimum inhibitory concentrations (MICs) of erythromycin, clindamycin and tetracycline were determined by E-test (bioMerieux, France). *Streptococcus pneumoniae* ATCC 49619 was used as control strain.

Emm Type Assignment

Briefly, the 5' end of *emm* genes were amplified by PCR method, following a previously published protocol[15]. N-terminal hypervariable portions of the *emm* genes were sequenced, as described previously[8]. DNA sequence alignment and *emm* type assignment were performed using CDC reference strains, available at http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm. Isolates which expressed > 95 percent homology with reference strain, were designated the particular *emm* type/subtype.

RAPD Fingerprinting

RAPD fingerprinting was carried out to determine the clonal characteristics of the isolates. RAPD amplifications were performed with a single primer H2 by previously described protocols of Seppala et al[16]. Amplified products were resolved in 2 percent agarose gel, stained with ethidium bromide and visualized by UV transilluminator. Fingerprints were compared by assessment of the number and distribution of the bands but not their intensity. Generated banding patterns were scored as presence (1) and absence (0) of band of a particular molecular size. Distance matrix based on RAPD patterns was created and then subjected to cluster analysis, using Restdist and Neighbor programs from the Phylip package.

Statistical Analysis

SPSS software, version 13.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. To quantify diversity (D) among the GAS isolates, statistics were used to incorporate the number of clones and their frequency of occurrence[17], whereby a clone is defined in this report as a unique *emm* type/RAPD fingerprint. Discrimination index (DI) of 100% (1.0) indicates that the clonal typing method is able to discriminate among all isolates and a DI value of 0% (0.0) indicates that all isolates are the identical clone. An estimation of confidence intervals (CI) was also done following the methods of Grundmann et al[17].

Results

Antimicrobial Susceptibility

We identified 484 out of 3893 (12.4%) macrolide resistant GAS. Co-resistance to erythromycin and tetracycline was detected in 17 out of 76 MRGAS isolates (22.4%). All MRGAS strains were fully susceptible to penicillin, chloramphenicol and norfloxacin.

Distribution of emm Types in Serbia

Emm genotyping revealed the presence of 7 different *emm* types: *emm*1 (1.3%), *emm*11 (1.3%), *emm*12 (32.8%), *emm*28 (2.6%), *emm*75 (39.5%), *emm*77 (18.5%) and *emm*118 (1.3%). Number of isolates with specific *emm* type are presented in Table I. Three and 2 different *emm* subtypes are identified within *emm*12 (*emm*12.0; *emm*12,7; *emm*12.31) and *emm*75 (*emm*75.0; *emm*75.1), respectively. Two isolates were nontypable (2.6%). Out of 7 distinct *emm* types identified, the 3 most frequently occurring overall were *emm*12, *emm*75 and *emm*77, accounted for 69 isolates (> 90%).

Specific geographical distribution of *emm* types was observed. Although *emm*75 dominated in Serbian Capitol, Belgrade, 4 more types (*emm*11, *emm*12, *emm*28, *emm*118) were detected out there. The highest *emm* diversity was found in Belgrade. Emm12, *emm*77 and *emm*75 predominated in other parts of the country.

In the erythromycin and tetracycline-resistant population (N=17), 3 *emm* types were observed (*emm*1, *emm*12 and *emm*77), the majority being *emm*77 (82.4%).

RAPD Fingerprinting

Amplification of genomic DNAs from the GAS isolates with a primer H2 resulted in RAPD patterns consisting of three to nine distinct DNA fragments, generally ranging from approximately 150 to 2,000 bp. A total of 26 distinct RAPD profiles were found among the 76 GAS isolates studied. Most of the patterns were observed in a small number of strains. Out of 26 different RAPD fingerprints, 12 (46%) were shared and 14 (54%) were unique, representing polymorphic nature of the tested isolates. However, 2 RAPD profiles that were the most prevalent patterns, accounted for 32 (42%) out of 76 isolates. RAPD profile B predominated in *emm*12 (92%), and profile E was associated only with the *emm*75 (67%). To visualize the genetic relationships among the different strains, a dendrogram of the cluster analysis based on RAPD fingerprinting was generated. Four RAPD clusters were associated with tested strains (cluster A through D) (Figure 1). Strains belonging to cluster D are different but genetically related, while most strains within clusters A, B and C have the same clonal origin.

Great RAPD diversity was observed in *emm*75 strains (N=30), whereas 19 distinct patterns were distinguished and most of them (13) were unique. Two and three different RAPD patterns were found in *emm*12 (25 strains) and *emm*77 (14 strains), respectively.

Discrimination index (DI) of the RAPD fingerprinting was 84% (0.84) with CI 75.8-92.2, while DI of the *emm* typing was 69% (0.69) with CI 58.5-79.5.

Discussion

Association between most of the MRGAS *emm* types found in Serbia (*emm*75, *emm*28, *emm*12 and *emm*11) and macrolide resistance was previously described in several reports[11,17].

Three dominant *emm* types of MRGAS in Serbia (*emm*12, *emm*75 and *emm*77) are among a few *emm* types that have been highly associated with macrolide resistance[5,17-19,20]. Emm12 is the major resistant *emm* type in Germany, Greece, Italy, Portugal, Israel[11,19] and the second one in the United States, being surpassed only by *emm*75[17]. In Europe the most common macrolide resistant *emm* type during 2003 and 2004 was *emm*28[21]. The highest *emm* diversity in Serbian Capitol was expected, since almost a third of entire population live in Belgrade. On the contrary, *emm* homogeneity was observed in regions with lower population density and in smaller cities.

In the present study *emm*77 was the most prevalent *emm* type co-resistant to tetracycline and erythromycin. Emm77 has been previously associated with resistance to tetracycline in Israel, Norway as well as in Italy[11]. On the contrary, the heterogeneity of *emm* types among macrolide and tetracycline co-resistant isolates was reported in USA[22]. It is believed that associ-

Table I. Numbers of *emm* types and RAPD fingerprints of MRGAS isolates.

	emm1	*emm11*	*emm12*	*emm28*	*emm75*	*emm77*	*emm119*
N. of isolates (%)	1 (1.3)	1 (1.3)	25 (32.8)	2 (2.6)	30 (39.5)	14 (18.5)	1 (1.3)
N. of RAPD fingerprints	1	1	2	1	19	3	1

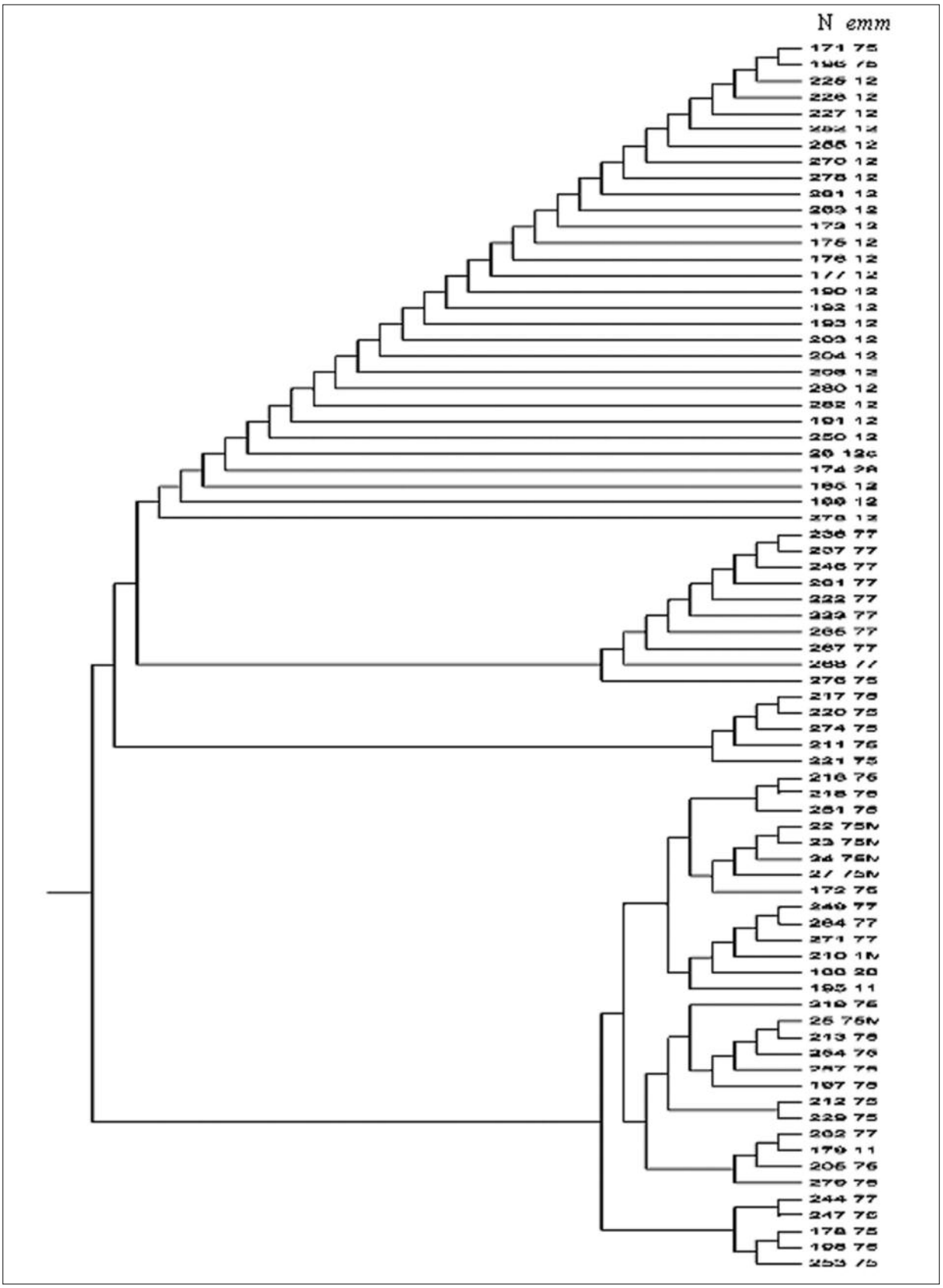

Figure 1. Dendrogram of the cluster analysis based on RAPD fingerprinting.

ation between erythromycin and tetracycline resistance contributes in the selection of erythromycin resistance. Relatively high frequency of macrolide-tetracycline co-resistance, found in our country, indicates that tetracycline resistance can be considered as a cofactor in selection of macrolide-resistant strains in Serbia.

Our results related to RAPD fingerprinting indicate a high degree of genetic variability among MRGAS isolates in Serbia, suggesting a polyclonal spread of erythromycin-resistant *S. pyogenes*. However, a striking finding of this study is the degree to which multiple isolates of a given *emm* type share identical or highly similar RAPD profiles. Although our results show that macrolide resistant isolates of *S. pyogenes* in Serbia are genetically diverse, some widely spread clones exist, particularly among *emm*12 and *emm*77 strains. Furthermore, these association imply strong clonal relationship among strains within these *emm* types. The finding that macrolide resistance rate of GAS isolates increased in Central Serbia, where *emm*12 and *emm*77 are dominant, suggests that it is mainly consequence of clonal spread of particular macrolide resistant clones of *S. pyogenes*.

Discrimination index (DI) of the RAPD fingerprinting was 84% (0.8) with CI 75.8-92.2, while DI of the *emm* typing was 69% (0.69) with CI 58.5-79.5. That finding confirm that the RAPD profiling is very good discriminatory method for GAS genotyping. Although it has been shown to be possible to increase the discriminatory power of RAPD analysis by using two primers together[21,23,24], in the present study we achieved good discrimination index by using only one primer. Regional heterogeneity or homogeneity of RAPD profiles was in accordance with *emm* types distribution through Serbia, although RAPD profiling reflected more discriminatory power than sophisticated *emm* technique.

Conclusions

Erythromycin resistance is mainly consequence of clonal spread of *emm*12 and *emm*77 in our country, whereas co-resistance to tetracycline and erythromycin is due to clonal spread of exclusively *emm*77. Use of PCR based RAPD method for typing of macrolide resistant GAS was found to be a high-resolution genetic typing approach.

Acknowledgements

This study was funded in part by Serbian Ministry of Education and Science, Project No 175039. We gratefully acknowledge all colleagues for providing group A streptococcal isolates.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. Lancet Infect Dis 2005; 5: 685-694.
2) Kaplan EL, Johnson DR. Unexplained reduced microbiological efficacy of intramuscular benzathine penicillin G and of oral penicillin V in eradication of group A streptococci from children with acute pharyngitis. Pediatrics 2001; 108: 1180-1186.
3) Martin JM, Green M, Barbadora KA, Wald ER. Erythromycin-resistant group A streptococci in schoolchildren in Pittsburgh. N Engl J Med 2002; 346: 1200-1206.
4) Perez-Trallero E, Marimón JM, Montes M, Orden B, de Pablos M. Clonal differences among erythromycin-resistant *Streptococcus pyogenes* in Spain. Emerg Infect Dis 1999; 5: 235-240.
5) Scott RJ, Naidoo DJ, Lightfoot NF, George RC. A community outbreak of group A beta-haemolytic streptococci with transferable resistance to erythromycin. Epidemiol Infect 1989; 102: 85-91.
6) Malli E, Tatsidou E, Damani A, Pantelidi K, Petinaki E. Macrolide-resistant *Streptococcus pyogenes* in Central Greece: prevalence; mechanism and molecular identification. Int J Antimicrob Agents 2010; 35: 614-615.
7) Seppala H, Nissinen A, Jarvinen H, Houvinen S, Henriksson T, Herva E, Holm SE, Jahkola M, Katila ML, Klaukka T. Resistance to erythromycin in group A streptococci. N Engl J Med 1992; 326: 292-297.
8) Beall B, Facklam R, Thompson T. Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. J Clin Microbiol 1996; 34: 953-958.
9) Facklam RF, Martin DR, Lovgren M, Johnson DR, Efstratiou A, Thompson TA, Gowan S, Kriz P, Tyrrell GJ, Kaplan E, Beall B. Extension of the Lancefield classification for group A streptococci by addition of 22 new M protein gene sequence types from clinical isolates: *emm*103 to *emm*124. Clin Infect Dis 2002; 34: 28-38.
10) Michos AG, Bakoula CG, Braoudaki M, Koutouzi FI, Roma ES, Panoalis A, Nikolopoulou G, Kirikou E, Syriopoulou VP. Macrolide resistance in *Streptococcus pyogenes*: prevalence, resistance determinants, and *emm* types. Diagn Microbiol Infect Dis 2009; 64: 295-299.

11) Nir-Paz R, Block C, Shasha D, Korenman Z, Gorodnitzky Z, Jaffe J, Ron M, Michael-Gayego A, Cohen-Poradosu R, Shapiro M, Moses AE. Macrolide, lincosamide and tetracycline susceptibility and *emm* characterisation of invasive *Streptococcus pyogenes* isolates in Israel. Int J Antimicrob Agents 2006; 28: 313-319.

12) Carriço JA, Silva-Costa C, Melo-Cristino J, Pinto FR, de Lencastre H, Almeida JS, Ramirez M. Illustration of a common framework for relating multiple typing methods by application to macrolide-resistant *Streptococcus pyogenes*. J Clin Microbiol 2006; 44: 2524-2532.

13) Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 1988; 26: 2465-2466.

14) Clinical Laboratory Standards Institute. Wayne IN. Performance Standards for Antimicrobial Susceptibility Testing: 19th Informational Supplement, M100-S19, 2009.

15) Podbielski A, Melzer B, Lütticken R. Application of the polymerase chain reaction to study the M protein(-like) gene family in beta-hemolytic streptococci. Med Microbiol Immunol 1991; 180: 213-227.

16) Seppala H, He Q, Osterblad M, Huovinen P. Typing of group A streptococci by random amplified polymorphic DNA analysis. J Clin Microbiol 1994; 32: 1945-1948.

17) Grundmann H, Hori S, Tanner G. Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganims. J Clin Microbiol 2001; 39: 4190-4192.

18) Green MD, Beall B, Marcon MJ, Allen CH, Bradley JS, Dashefsky B, Gilsdorf JR, Schutze GE, Smith C, Walter EB, Martin JM, Edwards KM, Barbadora KA, Wald ER. Multicentre surveillance of the prevalence and molecular epidemiology of macrolide resistance among pharyngeal isolates of group A streptococci in the USA. J Antimicrob Chemother 2006; 57: 1240-1243.

19) Malhotra-Kumar S, Mazzariol A, Van Heirstraeten L, Lammens C, de Rijk P, Cornaglia G, Goossens H. Unusual resistance patterns in macrolide-resistant *Streptococcus pyogenes* harbouring erm(A). J Antimicrob Chemother 2009; 63: 42-46.

20) Reinert RR, Lutticken R, Sutcliffe JA, Tait-Kamradt A, Cil MY, Schorn HM, Bryskier A, Al-Lahham A. Clonal relatedness of erythromycin-resistant *Streptococcus pyogenes* isolates in Germany. Antimicrob Agents Chemother 2004; 48: 1369-1373.

21) Luca B, Ekelund K, Darenberg J. Genetic determinants and epidemiology of antibiotic resistance among invasive isolates of *Streptococcus pyogenes* in Europe. Porto Heli, Greece, 2008. Federation European Microbiological Societies; p. 164. (Abstract of the XVII lancefield international symposium on streptococci and streptococcal diseases).

22) Tanz RR, Shulman ST, Shortridge VD, Kabat W, Kabat K, Cederlund E, Rippe J, Beyer J, Doktor S, Baell BW; and the North American Streptococcal Pharyngitis Surveillance Group. The Community-based surveillance in the United States of macrolide-resistant pediatric pharyngeal group A streptococci during 3 respiratory disease seasons. Clin Infect Dis 2004; 39: 1794-1801.

23) Micheli MR, Bova R, Calissano P, D'Ambrosio E. Randomly amplified polymorphic DNA fingerprinting using combinations of oligonucleotide primers. BioTechniques 1993; 15: 388-390.

24) Welsh J, McClelland M. Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. Nucleic Acids Res 1991; 19: 5275-5279.