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Molecular characterization of macrolide resistant *Streptococcus pyogenes* isolates from pharyngitis patients in Serbia

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ABSTRACT

A steady increase in macrolide resistance in *Streptococcus pyogenes*, group A streptococci (GAS) was reported in Serbia during 2004–2009 (9.9%). However, there are no data on the molecular epidemiology of pharyngeal macrolide resistance GAS (MRGAS) isolates. Therefore, the aims of this first nationwide study were to examine the prevalence of macrolide resistance in Serbian GAS and to determine their resistance phenotypes, genotypes and clonal relationships. Overall 3893 non-duplicate pharyngeal *S. pyogenes* isolates from outpatients with GAS infection were collected throughout country during 2008 and 2009. Among 486 macrolide resistant pharyngeal isolates collected, 103 were further characterized. Macrolide resistance phenotypes and genotypes were determined by double-disk diffusion test and PCR, respectively. Strain relatedness was determined by *emm* typing, multilocus sequence typing (MLST), multilocus variable tandem repeat analysis (MLVA), phage profiling (PP) and virulence factor profiling (VFP). Overall, macrolide resistance among GAS isolates in Serbia was 12.5%. M phenotype was the most common (71.8%), followed by iMLS (18.4%) and cMLS (9.7%). Three clonal complexes – *emm75*/*mefA*/ST49, *emm12*/*mefA*/ST36 and *emm77*/*ermA*/*tetO*/ST63 comprised over 90% of the tested strains. Although MLVA, PP and VFP distinguished 10, 20 and 12 different patterns, respectively, cluster analysis disclosed only small differences between strains which belonged to the same *emm*/ST type. Our data indicate dominance of three major internationally widely disseminated macrolide resistant clones and a high genetic homogeneity among the Serbian MRGAS population. Continued surveillance of macrolide resistance and clonal composition in MRGAS in Serbia in future is necessary to determine stability of MRGAS clones and to guide therapy strategies.

1. Introduction

Streptococcus pyogenes (group A Streptococcus, GAS) is an important human pathogen that causes a wide variety of diseases (Walker et al., 2014). Penicillin is still the drug of choice for GAS infections, whereas macrolides, such as erythromycin, are recommended as a first alternative therapy for patients who are allergic to penicillin. Since the 1990s a significant increase of erythromycin resistance was observed among *S. pyogenes* isolates worldwide (Ardanuy et al., 2010; Cornaglia et al., 1996). However, in recent years a steady decline in macrolide resistance was recognized (Silva-Costa et al., 2008; Van Heirstraeten et al., 2012).

There are two major mechanisms of macrolide resistance in *S. pyogenes*: (i) target modification due to methylation of the ribosomal RNA (encoded by *ermA* or *ermB* genes) which results in co-resistance to macrolides, lincosamides and streptogramins (MLS phenotype) and (ii) efflux of antibiotics (encoded by *mefA* gene) that affects 14- and 15-membered macrolides only and gives rise to the so-called M phenotype of resistance (Leclercq, 2002). The MLS phenotype could be further subdivided into inducible (iMLS) and constitutive (cMLS).

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M protein gene (*emm*) sequence typing is the most widely used method for epidemiological classification of GAS strains (Beall et al., 1996). The correlation between macrolide resistance and emm types has been noted worldwide (Michos et al., 2009; Silva-Costa et al., 2008). However, for the precise detection of the clonal relationships between the macrolide-resistant strains, additional methods, such as pulse field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) should be used (Carrico et al., 2006). Beside these techniques there are several other approaches for GAS typing – multiple-locus variable number tandem repeat analysis (MLVA) (Obszanska et al., 2011) and recently designed phage profiling (PP) and virulence factor profiling (VFP) (Borek et al., 2011).

The incidence of macrolide resistance and the distribution of resistance phenotypes and genotypes are significantly variable in different parts of the world (Green et al., 2006; Silva-Costa et al., 2008; Van Heirstraeten et al., 2012). In Serbia macrolide consumption has substantially increased during 2000s and a significant rise in macrolide resistance was observed in *S. pyogenes* pediatric isolates (9.9%) (Mijac et al., 2014). However, there are no data on the molecular epidemiology of pharyngeal macrolide resistant GAS (MRGAS).

The aims of this study were to examine the prevalence of macrolide resistance in Serbian pharyngeal isolates of *S. pyogenes* and to investigate phenotypic and genotypic characteristics and genetic similarity of MRGAS isolates.

2. Materials and methods

2.1. Strain collection and identification

Serbia is Southeastern European country with roughly 7 million inhabitants. It could be arbitrary divided into three regions: Belgrade (the capital, 2 million), Central Serbia (3 million) and Vojvodina (2 million). During 2008 and 2009, 5 laboratories from Belgrade and 6 laboratories located throughout Vojvodina and Central Serbia collected 3893 non-duplicate pharyngeal *S. pyogenes* isolates from outpatients with GAS infection. Among those strains, MRGAS isolates were identified and sent into the National Reference Laboratory. One hundred and three (21.2% of all MRGAS) were randomly chosen for further characterization: 25 out of 123 (20.3%) from Belgrade; 29 out of 139 (20.9%) from Vojvodina and 49 out of 224 (21.9%) from the Central Serbia. The majority of these isolates (89%) originated from school age children.

Isolates were identified by slide agglutination with group A antisera (Slidex Streptokit; bioMerieux, France), susceptibility to bacitracin and positive PYR test (Rosco, Denmark).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested using a disk diffusion test for penicillin (10 IU), erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg), ofloxacin (5 μg) and chloramphenicol (30 μg) (BioRad, USA), in accordance with the Clinical and Laboratory Standard Institute guidelines (CLSI) guidelines (2012). Macrolide resistance phenotypes were determined by the double-disk diffusion test (CLSI, 2012). MICs of erythromycin and clindamycin were determined by *E* test (bioMerieux, France). *Streptococcus pneumoniae* ATCC 49619 was used as a control strain.

2.3. Antibiotic resistance gene detection, emm typing, MLST, MLVA, VFP and PP

Detection of the macrolide resistance genes *mefA*, *ermA*, *ermB*, and tetracycline resistance genes *tetM*, *tetO* was performed by PCR as described previously (Perez-Trallero et al., 2007).

The *emm* types of MRGAS isolates were determined according to the recommendations of Centers for Disease Control and Prevention http://www.cdc.gov/streplab/protocol-emm-type.html.

MLST was carried out according to Enright et al. (2001). Allele numbers and the sequence type were assigned using the MLST website http://spyogenes.mlst.net.

Multilocus variable tandem repeat analysis (MLVA) was performed using the procedure described by Obszanska et al. (2011).

Virulence factor profiling (VFP) and phage profiling (PP) was done using the set of multiplex PCR reactions which detect simultaneously 20 GAS virulence factors (*spd3*, *sdc*, *sdaB*, *sdaD*, *speB*, *spyCEP*, *scpA*, *mac*, *sic*, *speL*, *speK*, *speM*, *speC*, *speI*, *speA*, *speH*, *speG*, *speJ*, *smeZ* and *ssa*) and 21 phage end integrative and conjugative element (ICE) integration sites as previously described (Borek et al., 2011).

The results of MLVA, VFP and PP were analyzed using BioNumerics software (Applied Maths). Similarity coefficient between strains was calculated using ranked Pearson correlation. MLVA based clusters were assessed using UPGMA method.

2.4. Cluster typing

Cluster designation was done by a typing system recently developed by Sanderson-Smith et al. (2014). It is based on *emm* typing results and distinguishes 48 GAS clusters containing closely related M proteins.

2.5. Statistics

The χ^2 test was used for statistical analysis when appropriate. A $p < 0.05$ was considered significant.

3. Results

3.1. Antibiotic resistance and detection of resistance determinants

In this study 486 (12.5%) macrolide resistant strains were detected among 3893 GAS pharyngeal isolates identified over a two-year (2008 and 2009) period. The erythromycin resistance rates were 15% in Belgrade, 14% in Central Serbia and 10% in Vojvodina. Among 103 analyzed MRGAS strains, the M phenotype, moderately resistant to erythromycin, was detected in 74 strains, iMLS pattern in 19 strains, while the cMLS phenotype, characterized by high level of erythromycin and clindamycin resistance was represented by only 10 isolates (Table 1).

The mefA gene was found in three-quarters of isolates among which 97.4% expressed M phenotype. The *ermA* and *ermB* genes were present in a minority of strains (Table 1). Resistance genes were not found in one MLS-phenotype strain. All *ermA* positive isolates expressed iMLS, while all *ermB* positive strains showed cMLS phenotype. The *mefA*/*ermA* and *mefA*/*ermB* genotypes were observed in two isolates, displaying an iMLS and cMLS phenotype, respectively.

Strains with M phenotype were predominant in Belgrade (68%) and in the Central Serbia (89.8%), whereas MLS was the most prevalent phenotype in Vojvodina (55.2%). Strains with iMLS were more frequent than cMLS isolates.

The overall frequency of tetracycline resistance (TR) among the 103 studied MRGAS strains was 19.4%. TR was detected in 20 isolates with MLS phenotype. All *tetO* genes were found in 15 *ermA* positive iMLS strains, while a total of 5 *tetM* genes were identified among *ermB* positive cMLS isolates (Table 1).

All 103 analyzed MRGAS strains were uniformly susceptible to penicillin, vancomycin, ofloxacin and chloramphenicol.