Changes in Macrolide Resistance Among Group A Streptococci in Serbia and Clonal Evolution of Resistant Isolates

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In Serbia, the frequency of macrolide-resistant group A streptococci (MRGASs) increased significantly from 2006 to 2009. MRGAS analysis in 2008 revealed the presence of three major clonal lineages: *emm75/mefA*, *emm12/mefA*, and *emm77/ermTR*. The aim of the present study was to determine the prevalence of macrolide resistance and to evaluate variations in the clonal composition of MRGASs. The study included 1,040 pharyngeal group A streptococci collected throughout Serbia, which were tested for antimicrobial susceptibility. MRGAS isolates were further characterized by the presence of resistance determinants, *emm* typing, and pulsed-field gel electrophoresis analysis. The prevalence of macrolide resistance was 9.6%, showing a slight decrease compared with the rate of 12.5% (2008). Tetracycline resistance was present in 6% of isolates, while norfloxacin nonsusceptibility detected for the first time in Serbia was 9.8%. The M phenotype dominated (84%), followed by the constitutive macrolides, lincosamides, and streptogramin B phenotype (12%). Five *emm* types were detected: *emm75*, *emm12*, *emm1*, *emm28*, and *emm89*. The *emm75/mefA* (62%), *emm12/mefA* (14%), and *emm12/ermB/tetM* (6%) were predominant clones and were found in both the present and the previous study periods at different frequencies. The major change was the loss of *emm77/ermTR/tetO*, which contributed to 15% of MRGASs in 2008.

Keywords: antibiotics, bacteria, epidemiology, genotyping, resistance

Introduction

STREPTOCOCCUS PYOGENES (group A *Streptococcus* [GAS]) is considered to be one of the leading human pathogens responsible for a broad spectrum of infections, ranging from relatively mild sore throat to more severe forms, such as streptococcal toxic shock syndrome, necrotizing fasciitis, bacteremia, and nonsuppurative sequelae. In the Western Hemisphere, pharyngitis is the most common clinical manifestation of GAS disease and it is estimated to affect about 616 million people per year worldwide.¹

Although penicillin remains the antibiotic of choice in the treatment of GAS infections, macrolides and lincosamides are recommended as suitable alternatives for patients with β -lactam allergy.² Furthermore, macrolides such as azithromycin are being increasingly used for convenience due to the once-daily dosing and shorter course of therapy.

Two main mechanisms of macrolide resistance in GAS are macrolide efflux and target site modification. The *mefA* gene encodes the efflux pump, which selectively removes 14- and 15-membered macrolides from the bacterial cell, and thus confers resistance to these macrolides only (M phenotype). The *erm* genes encode 23S rRNA methylases that mediate target site modification, resulting in coresistance to macrolides, lincosamides, and streptogramin B (MLS phenotype). The expression of *erm* genes may be either constitutive (c) related to the cMLS phenotype, or inducible (i) related to the iMLS phenotype.³ Studies on clonal characteristics of GAS strains carrying resistance determinants have shown that resistance is associated with a limited number of *emm* types.⁴⁻⁶

Moreover, molecular characterization employing techniques such as pulsed-field gel electrophoresis (PFGE) revealed that there is a significant genetic homogeneity of resistant strains sharing the same *emm* type.⁴

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The overall rate of macrolide resistance among *S. pyogenes* isolates and distribution of resistance phenotypes varies throughout the world and over time. In Europe, after an initial increase in macrolide resistance among GASs observed in several countries in the late 1990s, continual levels of resistance of up to 30% have persisted in the Mediterranean area.^{7–11} From the mid-2000s, an overall decreasing incidence of macrolide resistance has been noted in different regions of Europe, including Southern countries such as Spain, Portugal, France, and Italy.^{5,12–14}

The variation in frequency of macrolide resistance was accompanied by changes and fluctuation in the composition of the macrolide-resistant GAS (MRGAS) population. An initial dominance of the M phenotype across Europe was followed by an increase in the frequency of the MLS phenotype.^{12,13,15} Apart from Europe, high levels of macrolide and tetracycline resistance in *S. pyogenes* were recorded in the Far East, particularly in China (>95%).^{16,17} Selective pressure of antibiotics is viewed as a major factor in favoring the spread of macrolide resistance. However, there are many regions where high macrolide consumption is not accompanied by an equivalent level of resistance, underlining the influence of other factors on the population structure of MRGAS isolates.¹⁸

In Serbia, macrolide resistance has been rather low (<2%) until 2005.¹⁹ However, since 2006, a significant rise in macrolide resistance has been noted, reaching 12.5% at a national level in 2008–2009,²⁰ with even higher rates in some regions, as has been shown for the pediatric population in the capital city, Belgrade (19%).¹⁹ The observed increase in resistance correlated with a large increase in macrolide consumption in the same period.¹⁹ The main characteristic of Serbian MRGAS strains was the predominance of M phenotype (~70%) over MLS (~30%). Three major clones were found: *emm75/mefA*, *emm12/mefA*, and *emm77/ermTR*, representing more than 90% of the resistant isolates.²⁰

Knowing that MRGAS is a dynamic population and that surveillance of macrolide resistance is of particular importance, our main goal was to examine the evolution of Serbian MRGAS. We determined susceptibility patterns, phenotypes, genotypes, *emm* types, and PFGE profile distribution and demonstrate changes in the macrolide resistance rate and variation in the clonal structure of MRGAS isolates.

Materials and Methods

Bacterial isolates

A total of 1,040 epidemiologically unrelated *S. pyogenes* isolates recovered from patients with acute tonsillopharyngitis were included in this study. The strains were collected in 23 microbiology laboratories located in different regions of Serbia over two study periods: May 2011–July 2011 and October 2011–January 2012. All available isolates (except for 24 that were lost during transport) were included in the study.

Isolation and identification of *S. pyogenes*

The isolates were identified as GASs by colony morphology, β -hemolysis, catalase test, bacitracin susceptibility (BioRad), pyrrolidonyl arylamidase test (Pyrase Strips; Sigma-Aldrich, Germany), and a commercial latex aggluti-

nation technique (SlidexStrepto A; bioMerieux, France). All strains and relevant patient information were submitted to the National Reference Laboratory for streptococci.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed according to recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), Clinical breakpoints–bacteria, version 3.1.2013. Susceptibilities to penicillin (1 U), erythromycin (15 μ g), clindamycin (2 μ g), tetracycline (30 μ g), chloramphenicol (30 μ g), vancomycin (5 μ g), and norfloxacin (10 μ g) (BioRad) were tested by the disk diffusion test. Minimum inhibitory concentrations (MICs) of erythromycin and clindamycin were determined for MRGAS isolates (Etest; bioMerieux). Erythromycin-resistant strains were assigned a cMLS, iMLS, or M phenotype on the basis of the double-disk diffusion test by placing a 2- μ g clindamycin disk 12 mm apart from a 15- μ g erythromycin disk on blood agar plates, as described previously.²¹

Multidrug resistance was defined as resistance to three or more antibiotics. Quality control testing was performed with *Streptococcus pneumoniae* ATCC 49619.

Erythromycin and tetracycline resistance gene detection

All GAS isolates showing resistance to erythromycin were screened for the presence of *ermTR*, *ermB*, *mefA*, *tetM*, and *tetO*, as previously reported.^{6,22,23}

Molecular typing

All erythromycin-resistant isolates were further characterized by *emm* typing performed according to the Centers for Disease Control and Prevention protocol.²⁴

PFGE was performed for 50% of the randomly selected MRGAS isolates from each *emm* type. The PFGE procedure followed the previously published protocol with slight modifications.²⁵ Briefly, chromosomal DNAs of MRGAS isolates expressing the MLS phenotype were digested with the *Sma*I (10 U) restriction enzyme (Thermo Scientific), (run time: 18 hr, switching intervals: 5–35 sec, angle: 120°, voltage: 6 V, and cooling temperature: 14°C), while DNAs of strains with an M phenotype were treated with the *Cfr*9I (30 U) restriction enzyme (Thermo Scientific), (run time: 18 hr, switching intervals: 5–35 sec, angle: 120°, voltage: 6 V, and cooling temperature: 14°C). DNA fragments were separated on a 1% agarose gel (SeaKem Gold Agarose; Lonza, Rockland, ME). The separation was done using a CHEF DR-III system (Bio-Rad).

The BioNumerics 7.5 software (Applied Maths, Belgium) was used to create an unweighted pair-group method with arithmetic mean (UPGMA) dendrogram of the *Sma*I and *Cfr*9I fragment patterns. The Dice similarity coefficient between strains was calculated using ranked Pearson correlation, with optimization and position tolerance settings of 1 and 1.2, respectively. PFGE clusters were defined as isolates with a similarity of 80% or higher on the dendrogram. The genomic DNA of *Salmonella enterica* subspecies *enterica* serovar Braenderup strain H9812 (ATCC BAA-664) digested with *Xba*I was used as the molecular marker for PFGE analysis.²⁵

Statistical analysis

For statistical analysis, χ^2 or Fisher exact test was used when appropriate. A value of $p < 0.05$ was considered statistically significant. Data were analyzed using SPSS, version 14.0 (Chicago, Illinois).

Results

Of 1,040 pharyngeal GAS isolates, 91.9% ($n=956$) were obtained from children (1 to ≤ 15 years of age) and 8.1% ($n=84$) from adults (>15 years). The mean age of children with streptococcal pharyngitis was $8.5 (\pm 3.9)$ and there were 440 boys (46%) and 516 girls. Distribution of GAS isolates among different age groups is summarized in Table 1.

Antimicrobial susceptibility testing and macrolide resistance phenotypes and genotypes

All *S. pyogenes* isolates were susceptible to penicillin, chloramphenicol, and vancomycin. Macrolide resistance was detected in 100 GAS isolates and its overall incidence was 9.6%. Resistance to tetracycline and norfloxacin was present in 6% and 9.8% of isolates, respectively. Overall, the incidence of macrolide and tetracycline resistance was higher in isolates from adults compared with isolates from children (Table 1). On the other hand, norfloxacin non-susceptibility was almost twice as high in children compared with adults. Obtained results were not statistically significant ($p > 0.05$). Among MRGAS isolates, six strains (0.6%) were both clindamycin and tetracycline resistant. Norfloxacin resistance was detected in 10 MRGAS strains. Antimicrobial susceptibility of GAS isolates according to the patient groups is summarized in Table 1.

The double-disk diffusion test revealed that the M phenotype of macrolide resistance dominated (84%), followed by the MLS phenotype, which was present in the remaining 16% of MRGAS isolates. The cMLS phenotype was more frequent compared with iMLS, and they were detected in 12 and 4 isolates, respectively. A strong statistically significant phenotype/genotype association was noted between the M phenotype and presence of the *mefA* gene ($p < 0.001$). The majority of isolates with a cMLS/iMLS phenotype harbored the *ermB*/*ermTR* gene. Interestingly, in two iMLS phenotype isolates, *ermB* was detected. There were two isolates with a cMLS phenotype that harbored both the *ermB* and *mefA* genes and two isolates with a cMLS phenotype that lacked resistance genes.

Tetracycline resistance was present in six of 100 MRGAS isolates, and they all harbored the *tetM* gene. Neither *tetO* nor the silent form of *tetM* was found. Among tetracycline-

resistant MRGAS isolates, *tetM* was exclusively associated with the presence of the *ermB* gene and the cMLS phenotype. The distribution of macrolide resistance genotypes and phenotypes is summarized in Table 2. As expected, MIC₅₀ and MIC₉₀ of erythromycin for isolates with the M phenotype of resistance were lower (8 $\mu\text{g/ml}$), while isolates with iMLS and cMLS phenotypes showed a high level of resistance to erythromycin with MIC₅₀ and MIC₉₀ $\geq 256 \mu\text{g/ml}$. Among both M phenotype and iMLS phenotype isolates, clindamycin MIC₅₀ and MIC₉₀ were 0.25 and 0.5 $\mu\text{g/ml}$, respectively. In cMLS strains, clindamycin MIC₅₀ and MIC₉₀ were $\geq 256 \mu\text{g/ml}$.

Characterization of MRGAS clones

emm genotyping. Among 100 MRGAS strains, five different *emm* types were identified, with *emm75* (62%, $n=62$) and *emm12* (24%, $n=24$) being the most frequent. The three remaining *emm* types, *emm1*, *emm28*, and *emm89*, were found in 14 isolates (14%). *emm75* was the dominant genotype, both in MRGAS isolates from children (42.9%) and in MRGAS isolates from adults (65.1%). All isolates of *emm75* harbored the *mefA* gene. Among *emm12* isolates, the distribution of macrolide resistance determinants was heterogeneous: *mefA* was most frequent, detected in 14 (58.3%) isolates, followed by *ermB* found in 6 (25%) and *ermTR*, which was rarely encountered.

Tetracycline resistance was detected only among isolates of *emm12* that harbored the *ermB* gene. On the other hand, distribution of norfloxacin nonsusceptibility among MRGASs was more heterogeneous: eight resistant isolates belonged to *emm12* and two were of *emm75*. The distribution of *emm* types and resistance determinants is presented in Table 2.

PFGE patterns and correlation with *emm* types

PFGE macrorestriction profiling generated 20 pulsotypes with a similarity range of 34% to 97% (Fig. 1). Nevertheless, the majority of strains with the M phenotype ($n=42$) were grouped in one predominant PFGE cluster E ($n=28$). Twelve MRGAS isolates with the M phenotype were clustered into four minor PFGE clusters (A, B, F, and G) containing ≤ 5 isolates each. Two remaining isolates expressing the M phenotype had a unique PFGE pattern and did not belong to any of the described clusters. Clusters A and E–G included isolates of a single *emm* type each: *emm12* for cluster A ($n=2$), *emm75* for cluster E ($n=28$), *emm1* for cluster F ($n=3$), and *emm12* for cluster G ($n=5$).

Among MRGAS strains with MLS phenotypes ($n=8$), five distinct PFGE pulsotypes with a similarity range of 66%

TABLE 1. ANTIMICROBIAL RESISTANCE OF PHARYNGEAL GROUP A *STREPTOCOCCUS* ISOLATES BY PATIENT GROUPS

Patient group	Age range (years)	Total, n (%)	Nonsusceptibility rates, n (%)			
			Erythromycin	Clindamycin	Tetracycline	Norfloxacin
Children	≤ 5	362 (34.8)	36 (9.9)	6 (1.7)	16 (4.4)	36 (9.9)
	$>5-9$	468 (45)	48 (10.3)	2 (0.4)	24 (5.1)	52 (11.1)
	10–15	126 (12.1)	2 (1.6)	—	14 (11.1)	10 (7.9)
Total		956 (91.9)	86 (9)	8 (0.8)	54 (5.6)	98 (10.3)
Adults	>15	84 (8.1)	14 (16.7)	6 (7.14)	8 (9.5)	4 (4.8)
Total		1,040 (100)	100 (9.6)	14 (1.3)	62 (6)	102 (9.8)

TABLE 2. PHENOTYPES AND GENOTYPES OF MACROLIDE RESISTANCE AND *emm* TYPE DISTRIBUTION OF MACROLIDE-RESISTANT GROUP A STREPTOCOCCI

emm type	n	Phenotype (n)	Genotypes (no. of isolates)						Nonsusceptibility (n)		
			mefA	ermTR	ermB	mefA+ermB	tetM	tetO	CL	TET	NOR
1	6	M (6)	6	0	0	0	0	0	0	0	
12	24	M (14)	14	0	0	0	0	0	0	6	
		iMLS (2)	0	2	0	0	0	0	0	2	
		cMLS (8)	2	0	6	2	6	0	8	6	
28	4	M (2)	2	0	0	0	0	0	0	0	
		cMLS (2)	0	0	2	0	0	0	2	0	
75	62	M (62)	62	0	0	0	0	0	0	2	
89	4	iMLS (2)	0	0	2	0	0	0	0	0	
		cMLS (2)	0	0	2	0	0	0	2	0	
Total	100		86	2	12	2	6	0	12	6	10

CL, clindamycin; cMLS, constitutive macrolides, lincosamides, streptogramin B; iMLS, inducible macrolides, lincosamides, streptogramin B; M, macrolides; NOR, norfloxacin; TET, tetracycline.

FIG. 1. Dendrogram showing the genetic relationship of 50 macrolide-resistant *Streptococcus pyogenes* established from PFGE patterns obtained after *Sma*I and *Cfr*9I digestion and their antibiotic resistance gene content. The dendrogram, to the left, compares the percent of genetic relatedness among resistant isolates. The level at which the vertical line transects the horizontal line from the PFGE of each isolate determines its similarity based on the percent scale above the dendrogram. PFGE clusters were defined as isolates sharing at least 80% similarity. Each major lineage is designated by a capital letter. PFGE, pulsed-field gel electrophoresis.

to 98% were found. They were classified into two PFGE clusters: C and D (Fig. 1). Cluster C comprised four MRGAS isolates with *emm12*, while cluster D included isolates with two distinctive *emm* types: *emm89* ($n=2$) and *emm12* ($n=1$). A single isolate of *emm28* had a unique PFGE pattern. Three of four isolates of cluster C were multidrug resistant and belonged to the same clone *emm12/ermB/tetM*.

Overall, PFGE analysis revealed the presence of four major MRGAS clones: *emm75/mefA*/PFGE profile E ($n=28$), *emm12/mefA*/PFGE profile G ($n=5$), *emm1/mefA*/PFGE profile F ($n=3$), and *emm12/ermB/tetM*/PFGE profile C ($n=3$).

Discussion

In this study, the prevalence of macrolide resistance among pharyngeal GASs in 2011–2012 in Serbia was 9.6%, indicating a slight but statistically nonsignificant decrease compared with the rate of 12.5% found in 2008.²⁰

The decline in macrolide resistance was accompanied by changes in the prevailing phenotypes of resistance and in the structural composition of the MRGAS population. The main difference compared with our previous study was the complete disappearance of the *emm77/ermTR* clone that accounted for 15% of macrolide-resistant isolates in the period 2008–2009.^{20,26} As a consequence, the frequency of the iMLS phenotype primarily related to this clone substantially decreased (from 18% to 4%). This major change was accompanied by a noticeable increase in the prevalence of the M phenotype (72% in 2008–2009 vs. 84% in 2011–2012) and, to a lesser extent, of the cMLS phenotype (10% vs. 12%).

emm type distribution among Serbian MRGAS isolates revealed that two *emm* types, *emm75* and *emm12*, continually predominate. These types accounted for over 80% of macrolide resistance in both current and previous study periods and they still contribute to the continuing dominance of the *mefA*-related M phenotype of resistance, as revealed by the finding that over 90% of M phenotype strains belong to these *emm* types.

In other studies of macrolide-resistant GASs, both *emm75* and *emm12* have been identified: *emm12* is widely distributed and commonly found at a high frequency in many European countries as well as in other regions in the world.^{5,9,16,18,27–29} Interestingly, *emm75* was the most frequent type in Serbia in the period 2008–2009 (~45%), with its incidence having risen to 62% in 2011–2012. Based on the high frequency of a single *emm* type in two periods, we might speculate that in Serbia, *emm75* has continually dominated during the 4-year period.

Compared with our previous survey, of the five minor MRGAS clones identified, two remained: *emm1* and *emm28*. Both types are well recognized among the MRGAS population and are widely distributed across Europe.¹⁰ In spite of their low prevalence in Serbia, the continuous presence and broad distribution of these *emm* types probably reflect their good fitness and ability to persist. The only newly encountered type *emm89* was previously identified among the population of both macrolide-susceptible and macrolide-resistant GASs in other regions and was mainly related to *erm* gene-associated resistance,^{4,30} as found in our work.

In the present study, macrolide resistance was more frequent among adults (16.7%) than in children (9%), and these findings are consistent with data from other regions.¹²

The overall tetracycline resistance rate of 6% in Serbian GAS isolates is rather low, indicating its decreasing tendency. Among MRGASs, the significant reduction of tetracycline coresistance (from 19.4% to 6%) was due to the loss of the *emm77/ermTR/tetO* clone, which contributed to a great deal of resistance in 2008–2009. The main clone related to erythromycin and tetracycline coresistance (6% of MRGASs) in the present study was the *emm12/ermB/tetM*.

Fluoroquinolone nonsusceptibility emerged for the first time in Serbia at a surprisingly high frequency of 9.8%. Among MRGASs, fluoroquinolone nonsusceptibility was detected among isolates of *emm12/mefA*, *emm12/ermTR*, and *emm75/mefA*. Unfortunately, we were not able to further characterize these isolates or to explore the genetic background of resistance, but it is intended to be included in our future studies.

Data from the literature show that fluoroquinolone nonsusceptibility among GASs is increasing not only across Europe, especially in Spain and Belgium, but also in other countries and is frequently related to the spread of *emm6*.^{31–33} This type was not found in the present study probably due to its rare association with macrolide resistance.^{32,34} On the other hand, the presence of fluoroquinolone resistance among *emm75* and *emm12* as observed here was also documented in other countries.^{31–33}

PFGE characterization of MRGAS isolates revealed the strong association between *emm* type, resistance determinants, and particular PFGE clusters, indicating high genetic homogeneity of strains belonging to a certain *emm* type and sharing the same resistance determinant. The predominant MRGAS clone circulating in Serbia was *emm75/mefA* with PFGE cluster E ($n=28$). Additional frequently encountered genotypes were *emm12/mefA*/PFGE cluster G ($n=5$), *emm12/ermB*/PFGE cluster C ($n=3$), and *emm1/mefA*/PFGE cluster F ($n=3$).

Even though in our previous study typing methods other than PFGE were used and therefore the results could not be compared with data in the present survey, we assume that the predominant clones remained the same in both study periods with slight variations in their overall frequencies. This assumption is based on the finding of two highly dominant *emm* types with genetically homogeneous isolates within the particular *emm* type in both study periods.

During the present study, the level of consumption of macrolides in Serbia remained high: a total macrolide consumption of 5 DDD/1000 inhabitants/day was the second highest in Europe in 2011.³⁵ Particularly worrisome was the high level of consumption of long-acting macrolides (azithromycin): 2.75 DDD/1000 inhabitants/day in 2011. Although the overall macrolide expenditure decreased from 2008 (5.8 DDD/1000 inhabitants/day), consumption of long-acting macrolides remained unchanged.

This might explain the small and insignificant variation in the rate of macrolide resistance observed. A high-level of consumption of long-acting macrolides is postulated to be the major promoting factor for escalation of high-level macrolide resistance related to the MLS phenotype.³⁶ Interestingly, the prevalence of cMLS phenotype in the present study, although higher than in the previous period, was still low compared with the noticeable dominance of M phenotype of resistance.

Although the present study was conducted in two relatively short study periods and a restricted number of MRGAS isolates were genotyped, all *S. pyogenes* isolates detected in routine clinical care of patients of all ages

throughout the country were included. Since, to the best of our knowledge, data on GASs and MRGASs in the Western Balkan region are limited, we believe our results contribute to a better understanding of MRGAS dynamics in this area.

In conclusion, the prevalence of macrolide resistance in Serbia is moderate (~10%) despite a very high level of macrolide consumption. A small decline of resistance compared with the previous study period (2008–2009) has been recorded that did not reach statistical significance.

Persisting dominance of the *emm75/mefA* clone comprising highly genetically related strains and hence the overrepresentation of the M phenotype of resistance characterize the Serbian MRGAS population. Changes in the clonal structure of MRGASs have been noted, in particular, the loss of the *emm77/ermTR/tetO* clone, while other major genetic lineages were still present. Overall, a decreasing trend of tetracycline resistance was noted and its relationship with macrolide resistance was due to the *emm12/ermB/tetM* clone. Fluoroquinolone nonsusceptibility was detected for the first time with a significant rate that warrants further thorough characterization. These results underscore the importance of active surveillance of GAS resistance and investigation of its clonal composition.

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Disclosure Statement

No competing financial interests exist.

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