

## PHENOTYPES AND GENOTYPES OF MACROLIDE-RESISTANT *STREPTOCOCCUS PNEUMONIAE* IN SERBIA

MIRJANA HADNAĐEV<sup>1</sup>, INA GAJIĆ<sup>2</sup>, VERA MIJAC<sup>2</sup>, TATJANA KURUCIN<sup>1</sup>, ANIKA POVAŽAN<sup>1</sup>,  
ALEKSANDRA VULIN<sup>3</sup> and NATAŠA OPAVSKI<sup>2</sup>

<sup>1</sup>Institute for Pulmonary Diseases of Vojvodina, 21208 Sremska Kamenica, Serbia

<sup>2</sup>Institute for Microbiology and Immunology, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia

<sup>3</sup>Institute for Cardiovascular Diseases of Vojvodina, 21208 Sremska Kamenica, Serbia

**Abstract** - Although macrolides are widely used for treating pneumococcal infections, an increase in macrolide resistance might compromise their use. The objective of this study was to determine the prevalence of macrolide-resistant phenotypes and genotypes in macrolide-resistant *S. pneumoniae* isolates in Serbia. A total of 228 macrolide-resistant strains isolated during the period of 2009-2012, were analyzed. Macrolide resistance phenotypes were determined by a double disk diffusion test. The presence of macrolide resistance genes was detected by PCR. Antibiotics susceptibilities were tested using the VITEK2 system and E test. Among the examined isolates, the MLS<sub>B</sub> phenotype which is linked to the presence of the *erm(B)* gene dominated (83.3%), while the *mef(A)* gene which is associated with the M phenotype, was identified in 16.7% isolates. Over 40% of isolates expressed co-resistance to penicillin. A multiple-resistant pattern was found in 36.4% strains, more frequently in children. However, all strains were susceptible to telithromycin, vancomycin, linezolid, fluoroquinolones and rifampicin.

**Key words:** *Streptococcus pneumoniae*, macrolides, resistance, phenotype, genotype

### INTRODUCTION

*Streptococcus pneumoniae* is a leading cause of diseases, inducing invasive (meningitis, septicemia, bacteremic pneumonia) and mucosal infections (otitis media, sinusitis) in both children and adults worldwide (Centers for Disease Control and Prevention, 2000; Robinson et al., 2001; Farha, 2005). In addition, pneumococcus is the most common causes of community-acquired pneumonia (CAP) (Ortqvist et al., 2005; Marrie, 2001).

Beta lactam and macrolide antibiotics have remained a first choice for the empirical treatment of pneumococcal infections (Whitney et al., 2000).

However, in recent decades, the treatment of pneumococcal infections is becoming more difficult due to a global increase and expansion of pneumococcal resistance to these antibiotics. This is of particular importance, since strains exhibiting penicillin and macrolide are frequently resistant to other classes of antibiotics. The prevalence of resistance varies greatly among countries (Lynch et al., 2005).

Macrolide resistance in *Streptococcus pneumoniae* is mediated by two major mechanisms – ribosomal methylation encoded by the *erm(B)* gene and the drug efflux, encoded by the *mef(A)* gene. Modification of ribosomal targets leads to cross-resistance to macrolides, lincosamides and streptogramin B

(MLS<sub>B</sub> phenotype) (Douthwaite et al, 2000; Hansen et al., 2002; Kresken et al., 2004). This phenotype can be either inducibly (iMLS<sub>B</sub>) or constitutively expressed (cMLS<sub>B</sub>). Drug efflux confers resistance to 14- and 15-membered macrolides (M phenotype).

There are few data concerning the pneumococcal resistance to macrolides in Serbia. The aim of this study was to examine the prevalence of macrolide-resistant phenotypes and genotypes among invasive and noninvasive macrolide-resistant *S. pneumoniae* isolates in Serbia.

## MATERIALS AND METHODS

A total of 228 invasive and non-invasive macrolide-resistant pneumococcal strains (one strain per patient) were analyzed. The strains were isolated in 16 laboratories throughout Serbia, during 2009-2012, and sent for further investigation to the National Reference Laboratory for Streptococci and Pneumococci, Institute for Microbiology and Immunology, Medical Faculty, Belgrade. Out of 228 isolates, 34 (14.9%) were derived from invasive infections, while 194 (85.1%) came from noninvasive infections. Invasive isolates were obtained from blood (n=19), cerebrospinal fluid (n=13), and pleural fluid (n=2). Non-invasive isolates were cultured from nose swab (n=173), sputum (n=8), ear swabs (n=6), conjunctival swabs (n=5), and bronchial and tracheal aspirates (n=2). Isolates were obtained from children ≤16 years old (n=157) and adults >16 years of age (n=71).

Pneumococcal isolates were identified by alpha hemolysis and colony morphology on 5% sheep blood agar, Gram stain characteristics, optochin sensitivity (BioRad, USA), slide agglutination test (bioMerieux, France) and bile solubility. The strains were stored at -80°C.

Macrolide resistance phenotypes were determined by a double disk diffusion test using erythromycin (15μ) and clindamycin (2μg) disks (bioRad, USA), as described elsewhere (Montanari et al., 2003). The absence of an inhibition zone around both disks suggested cross-resistance to macrolides-

lincosamides-streptogramin B (cMLS phenotype). Blunting of the clindamycin inhibition zone proximal to the erythromycin disk indicated inducible resistance (iMLS<sub>B</sub>). Susceptibility to clindamycin with resistance to erythromycin suggested the M phenotype (Calatayud et al., 2007; Van der Linden et al., 2007).

Macrolide resistance genes – *erm*(B) and *mef*(A) – were determined by PCR. Previously published primers for *mefA* and *ermB* were used (Farell et al., 2001).

Amplification was performed using the following conditions: initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 59°C for 30 s and 72°C for 30 s. Final elongation was performed at 72°C for 10 min.

The antibiotic susceptibilities against 19 antibiotics were tested by VITEK2 (bioMerieux, France) automated system (bio Merieux, France, card AST-P576). Minimal inhibitory concentrations (MICs) of erythromycin, clindamycin, penicillin and ceftriaxone were determined by the E test (bioMerieux, France). *Streptococcus pneumoniae* ATCC 49619 was used as a quality control strain. The obtained results were interpreted according to CLSI guidelines (CLSI, 2013).

The statistical analysis was performed using SPSS software, version 13.0. The chi-square test was applied when appropriate. P values of 0.05 were considered statistically significant.

## RESULTS

Among 228 macrolide-resistant isolates, 190 (83.3%) exhibited the MLS<sub>B</sub> phenotype: 182 (79.8%) belonged to the cMLS, and 8 (3.5%) to the iMLS phenotype. The remaining 38 isolates (3.5%) were confirmed as M phenotype.

The prevalence of MLS resistance was 82.3% (28/34) in invasive isolates and 83.5% (162/194) in noninvasive-site isolates (Table 1). The MLS pheno-

**Table 1.** Distribution of resistance phenotypes in erythromycin-resistant *S. pneumoniae* invasive (n=34 ) and noninvasive (n=194) strains collected from children and adults.

Phenotypes	Children (n=157)		Adults (n=71)		Total
	invasive (n)	noninvasive (n)	invasive (n)	noninvasive (n)	
cMLS	15	118	11	38	182
iMLS	0	5	2	1	8
M	3	16	3	16	38
Total	18	139	16	55	228

**Table 2.** Distribution of resistance related genes in 216 tested macrolide-resistant pneumococci.

Phenotypes	<i>erm</i> (B)	<i>mef</i> (A)
cMLS	170	1
iMLS	8	0
M	0	38

**Table 3.** Distribution of macrolide resistance phenotypes and MIC ranges for erythromycin, clindamycin, penicillin and ceftriaxone.

Phenotype	n (%)	Antibiotics	MIC ( $\mu$ g/ml)		
			Range	50%	90%
cMLS	182 (79.8)	Erythromycin	4->256	>256	>256
		Clindamycin	4->256	>256	>256
		Penicillin	0.06-8	1	3
		Ceftriaxone	0.06-4	1	2
iMLS	8 (3.5)	Erythromycin	3->256	>256	>256
		Clindamycin	0.064- $\geq$ 256	2	8
		Penicillin	0.06-3	0.5	2
		Ceftriaxone	0.06-2	0.5	1
M	38 (16.7)	Erythromycin	1.5->256	3	4
		Clindamycin	0.016-0.25	0.25	0.25
		Penicillin	0.06-2	0.25	1
		Ceftriaxone	0.06-2	0.25	1

type was predominant in both age groups; 87.9% in children and 73.2% in adults.

The distribution of *erm*(B) and *mef*(A) resistance genes is presented in Table 2. In 178 (82.4%) of 216 tested strains, the *erm*(B) gene was identified, while

the *mef*(A) gene was found in 39 (18.1%). All the strains assigned to the MLS<sub>B</sub> phenotype harbored the *erm*(B) gene, while all the strains with M phenotype had the *mef*(A) gene. The presence of both resistance genes was confirmed in one strain with MLS<sub>B</sub> phenotype.

**Table 4.** The most common patterns of co-resistance and multiresistance in macrolide-resistant pneumococci.

Resistance profile	Children (n= 157)	Adults (n=71 )	<i>p</i>	$\chi^2$
	%	%		
Macrolide and penicillin co-resistance (I+R)	54.1	21.1	0.001	21.64
Macrolide and cefotaxime (I+R) co-resistance	29.9	8.5	0.001	12.65
Multiresistance (ERY+PEN+TET+SXT)	45.9	15.5	0.001	19.47

\*I-intermediate, R-resistant, PEN-penicillin, TET-tetracycline, SXT-trimethoprim-sulfamethoxazole.

The MLS<sub>B</sub>/*erm*(B) positive strains demonstrated a high level of resistance to erythromycin (MIC<sub>50</sub>>256µg/ml), while M/*mefA*-positive isolates expressed moderate resistance (MIC<sub>50</sub>=3µg/ml) (Table 3). Almost all (98.9%) *erm*(B)-positive isolates were resistant to clindamycin (MIC<sub>50</sub>>256µg/ml). A low percentage of clindamycin-susceptible strains (1.1%) among the *erm*(B)-positive isolates belonged to the iMLS<sub>B</sub> phenotype. All M/*mefA* strains were susceptible to clindamycin (MIC<sub>50</sub>=0.25µg/ml).

Among macrolide-resistant strains, penicillin and third generation cephalosporin (cefotaxime, ceftriaxone) nonsusceptibility was observed in 43.9% and 23.2%, respectively. Significantly, higher resistance rates to both antibiotics were observed in children than in adults (21.1%) (Table 4). The non-invasive strains proved to be more resistant than the invasive ones, but the difference was not statistically significant.

More than one-third (36.4%) of macrolide-resistant strains showed co-resistance to at least one of the following agents: penicillin, tetracycline and trimethoprim-sulfamethoxazole. Multiresistant isolates were significantly more prevalent among children (45.9%) than adults (15.5%) (Table 4).

The rates of resistance to trimethoprim-sulfamethoxazole and tetracycline were high – 71% and 82%, respectively. On the other hand, all strains were fully susceptible to telithromycin, vancomycin, linezolid, fluoroquinolones and rifampicin.

## DISCUSSION

The results of our study showed that a constitutive MLS<sub>B</sub> phenotype predominates among macrolide-resistant *S. pneumoniae* isolates in Serbia. Although a high rate of macrolide resistance in pneumococci in Serbia (41%) was previously demonstrated (Gajić et al., 2012), it is of particular concern, since the isolates with cMLS<sub>B</sub> phenotype are highly resistant to all macrolide-lincosamide-streptogramin antibiotics (Leclercq et al., 2002). These strains exhibit very high MICs to erythromycin (MIC<sub>50</sub>>256µg/ml), which was also demonstrated in our study.

The analysis of resistance genes has shown that the *ermB* gene, which encodes methylation of 23S rRNA, is present in the majority (82.4%) of our strains. Globally, the *erm*(B) gene is the most common macrolide resistance determinant in pneumococci. A high prevalence of the *erm*(B) gene is registered in Belgium (91%), France (90%), Spain (88%), Hungary (82%). Poland (80%), China (77%), Italy (56%) and Japan (58%) (Felmingham et al., 2007). Conversely, the *mefA* gene is the prevailing macrolide resistance mechanism in USA (66%), Australia, Germany, Greece, Finland, Ireland and UK (Farrell et al., 2008).

In recent years, the number of pneumococcal strains with a dual resistance mechanism related to the presence of both *erm*(B) and *mefA* genes has increased globally and the prevalence of these strains reaches up to 12% (Farrell et al., 2008). The countries

with a high prevalence of the dual resistance mechanism in pneumococci are South Korea, South Africa, USA and Australia. The majority of these strains are multiresistant and clonally related (Jenkins et al., 2008; McGee et al., 2001) and their spreading is worrying. In our study, the dual resistance mechanism was detected in only 1 out of 216 macrolide-resistant strains, suggesting it is uncommon in Serbia.

Of note is our finding that pneumococcal macrolide resistance in Serbia is frequently associated with resistance to other antibiotics. We observed a high rate of penicillin nonsusceptible pneumococci (PNSP) among MRSP (43.9%) and a significant difference in the prevalence of PNSP in children (54.1%) compared to adults (21.1%). Similar findings were reported from other regions (Brown et al., 2004; Dias et al., 2006; Farrell et al., 2008). The explanation for these results could be the overuse of antibiotics in children since localized pneumococcal infections are more common in this age group. Most pediatric invasive strains were isolated from meningitis cases and criteria that are more stringent were used in defining penicillin nonsusceptibility (CLSI, 2012). Therefore, pediatric patients revealed significantly higher penicillin resistance rates.

The prevalence of pneumococcal penicillin and macrolide dual nonsusceptibility varies greatly among countries (Felmingham et al., 2007). Percentages ranged from zero (Estonia, Germany and Latvia) to 44.4% (Romania) (EARS-Net, 2011).

In our study, resistance to 3<sup>rd</sup> generation cephalosporins was present in 23.2% of the isolates, and it was more common in children (29.9%) than in adults (8.5%). The resistance of our strains to tetracycline and trimethoprim-sulfamethoxazole was exceptionally high – 82% and 71%, respectively. As tetracycline resistance determinants are located on the same transposons as the *erm(B)* gene, pneumococcal resistance to tetracyclines is commonly associated with their macrolide resistance. It is therefore evident that high levels of tetracycline resistance are often reported in the countries with a high level of macrolide resistance (Cochetti et al., 2008; Seral et

al., 2001). Multiresistant phenotype – co-resistance to penicillin, macrolides, tetracycline and trimethoprim-sulfamethoxazole, was registered in up to 30% of our MRSP strains and again more often in children.

It should be stressed out that our strains were fully susceptible to telithromycin, vancomycin, linezolid, fluoroquinolones and rifampicin. These antibiotics are used for empiric treatment of community-acquired pneumonia (telithromycin, levofloxacin) or infections caused by resistant pneumococcal strains.

The introduction of PCV7 into the childhood immunization schedule in European countries and the USA resulted in a significant reduction in the incidence of invasive pneumococcal diseases in children aged  $\leq 5$  years due to vaccine serotypes (Pilishvili et al., 2010). The use of PCV7 also reduced the pneumococcal resistance in the vaccinated, as well as non-vaccinated groups. There is strong evidence that a significant decline in overall penicillin nonsusceptibility, particularly in children, was registered in Spain, the USA and other countries (Fenoll et al., 2009) in the recent period, which was probably due to a heptavalent conjugate vaccine (PCV7) implementation and decrease in antibiotic consumption (EARSS 2010). Pneumococcal conjugate vaccines are recommended in Serbia, but not reimbursed, and vaccine coverage is low. We assume that only after its introduction into the national immunization programs could some reduction in pneumococcal resistance in Serbia be expected.

It could be concluded that the constitutive *MLS<sub>B</sub>/erm(B)* phenotype/genotype is predominant among macrolide-resistant pneumococcal strains in Serbia. The frequency of co-resistance to penicillin, as well as the frequency of the multiple resistance, is high. Future studies on macrolide resistance and its relation to specific serotypes are necessary and foreseen.

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