

## DISTRIBUTION OF MACROLIDE-RESISTANT GENES AMONG ISOLATES OF MACROLIDE-RESISTANT *STREPTOCOCCUS PYOGENES* AND *STREPTOCOCCUS PNEUMONIAE* IN SERBIA

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**Abstract** – Macrolide resistance in *Streptococcus pneumoniae* and in group A streptococci (GAS) is a significant problem worldwide. In Serbia, data on the mechanisms of resistance and the corresponding resistance genes in streptococci are largely lacking. Therefore, we analyzed the distribution of macrolide resistance phenotypes and genotypes in 44 macrolide-resistant GAS (MRGAS) and 50 macrolide-resistant *S. pneumoniae* (MRSP) isolates collected in the same period. The double disk diffusion test and PCR were used to analyze resistance phenotypes and resistance genes, respectively. Among MRSP, the MLS<sub>B</sub> phenotype dominated, whereas the M phenotype was the most prevalent among MRGAS isolates. Consequently, in MRSP, the *ermB* gene was the most common (n=40, 80%), followed by the *mefA* gene (n=7, 14%). In MRGAS strains, *mefA* dominated (n=27, 61%), followed by *ermA* (n=15, 33%) and *ermB* (n=3, 7%). In 3 MRSP isolates no resistance genes were detected, while one MRGAS strain with iMLS<sub>B</sub> phenotype harbored both *ermA* and *mefA* genes.

**Key words:** *Streptococcus pyogenes*, *Streptococcus pneumoniae*, macrolide resistance

### INTRODUCTION

*Streptococcus pyogenes* (Lancefield group A *Streptococcus*, GAS) causes a broad spectrum of illnesses, ranging from pharyngitis to severe life-threatening invasive diseases (Carapetis, 2005). It is the most common cause of bacterial pharyngitis (15-30%). *Streptococcus pneumoniae* is a significant cause of morbidity and mortality. Pneumococci remain the most common causative agents of community-acquired pneumonia (CAP), bacterial meningitis, bacteremia and otitis media, worldwide (Mitchell et al., 1995).

Resistance of *S. pneumoniae* and *S. pyogenes* to macrolides has become an increasing problem

worldwide. In two world surveillance studies carried out throughout the period from 1997 to 2000 (Gordon, 2002; Jacobs, 2003), the regional rates of erythromycin resistance ranged from 7 to 67.3% among *Streptococcus pneumoniae* strains, and from 2.7 to 18.6% among beta-hemolytic streptococci.

Genes conferring macrolide resistance are usually located in mobile elements such as transposons, suggesting a putative transmission of macrolide-resistant genes between different bacteria, particularly in *S. pneumoniae* and *S. pyogenes* (Gordon, 2002).

Two main well-described molecular mechanisms are responsible for macrolide resistance among

streptococci (Leclercq, 2002): target site modification and antibiotic efflux. Target site modification due to methylase activity has been linked to the presence of the *erm* gene. This mechanism confers cross-resistance to macrolides, lincosamides and streptogramin B (MLS<sub>B</sub> resistance). It can be constitutive (cMLS<sub>B</sub>), usually mediated by the *ermB* gene, or inducible (iMLS<sub>B</sub>) and mediated by the *ermA* gene. The other mechanism of resistance, macrolide efflux, is encoded by the *mefA* gene and confers low-level resistance to 14 and 15-membered macrolides, but not to 16-membered macrolides, lincosamides, or streptogramin B (Sabharwal et al., 2006; Sutcliffe et al., 1996).

Surveillance studies from European countries indicate that the distribution of MLS<sub>B</sub> phenotype (due to *erm* genes) and M phenotype (due to the *mefA* gene) among clinical isolates of GAS and pneumococci greatly varies in different regions in Europe (Richter, 2008, Gordon, 2002; Linares, 2000; Roberts, 1999).

Little data are available on the mechanisms of macrolide resistance in GAS and *S. pneumoniae* in Serbia. Therefore, the aim of the present study was to examine the distribution of macrolide-resistant phenotypes and the related resistance genotypes among macrolide-resistant GAS (MRGAS) and macrolide-resistant *S. pneumoniae* (MRSP) isolates obtained from upper respiratory tract specimens of patients with pharyngitis and other upper respiratory tract infections.

## MATERIALS AND METHODS

### *Bacterial isolates*

A total of 44 isolates of MRGAS and 50 isolates of MRSP originating from patients with pharyngitis and other upper respiratory tract infections were collected from several regional laboratories between June 2008 and December 2009. Strains were sent to the National Reference Laboratory for Streptococci and Pneumococci for further testing. GAS identification was confirmed on the basis of Gram stain mor-

phology, typical colony morphology, beta hemolysis, bacitracin (0.04U) sensitivity test (BioRad, USA) and latex agglutination with group A-specific antisera (bioMerieux, France). Pneumococci were identified according to typical colony morphology, presence of alpha hemolysis on blood agar plates, Gram stain morphology, optochin (BioRad, USA) sensitivity, bile solubility test and slide agglutination test (bioMerieux, France). GAS and pneumococcal isolates were preserved in Todd Hewitt Infusion Broth (Biomedics, Spain) and Brain Heart Infusion Broth (Biolife, Italy), respectively, containing 10% glycerol, and were stored at -80°C.

### *Macrolide resistance phenotypes*

A double disk diffusion test was used to determine the macrolide resistance phenotype, using erythromycin (15µg) and clindamycin (2µg) disks (BioRad, USA) (Seppala et al., 1993; Montanari et al., 2001). The M phenotype was scored when the isolate was non-susceptible to erythromycin only. Resistance to both erythromycin and clindamycin was designated as MLS<sub>B</sub> phenotype. Inducible MLS<sub>B</sub> (iMLS<sub>B</sub>) phenotype was assigned if a D-shaped inhibition zone around the clindamycin disk was observed, while the absence of an inhibition zone around the two disks characterized constitutive MLS<sub>B</sub> (cMLS<sub>B</sub>) phenotype.

MICs of erythromycin and clindamycin were determined using the E test (BioMerieux, France). Results were interpreted according to the EUCAST guidelines (EUCAST, 2013).

### *Genes of macrolide resistance*

MRGAS isolates were tested for the presence of *ermA*, *ermB* and *mefA* genes, using PCR, according to the previously published protocols (Brandt et al., 2001; Weber et al., 2001; Perez-Trallero et al., 2007.). MRSP strains were screened for the *ermB* and *mefA* genes. After initial denaturation at 95°C for 10 min, 35 cycles were repeated under the following conditions: denaturation at 95°C for 30 s, annealing at 59°C for 30 s and elongation at 72°C for 30 s. Final

elongation was done at 72°C for 10 min. The primers used were previously described by Farrell et al. (2001).

Statistical analysis was performed using SPSS software, version 13.0.

## RESULTS

### *Macrolide resistance phenotypes*

On the basis of the double-disk test, MRGAS isolates (n=44) were assigned to the following resistance phenotypes: M phenotype – 26 isolates, (59%), iMLS phenotype – 15 isolates, (34%), and cMLS phenotype – 3 isolates, (7%). Among the 50 MRSP isolates, 8 (16%) expressed M phenotype and 42 (84%) were assigned to the cMLS phenotype (Table 1).

The overall MIC range of erythromycin for both MRGAS and MRSP isolates was 0.5- $\geq$ 256  $\mu$ g/ml, while the range of clindamycin was 0.032- $\geq$ 256  $\mu$ g/ml. The MIC ranges of erythromycin and clindamycin of strains with particular phenotypes are shown in Table 2.

All MRGAS and MRSP strains with M phenotype expressed low-level macrolide resistance (MIC<sub>50</sub> – 4  $\mu$ g/ml), while isolates with iMLS<sub>B</sub> phenotype showed moderate erythromycin resistance (MIC<sub>50</sub> – 128  $\mu$ g/ml) and were susceptible to clindamycin (MIC<sub>50</sub> –  $\leq$ 0,032  $\mu$ g/ml) without induction. The cMLS<sub>B</sub> isolates showed a high level of both erythromycin and clindamycin resistance (MIC<sub>50</sub>  $\geq$ 256  $\mu$ g/ml).

The MLS<sub>B</sub> phenotype was significantly more common among MRSP isolates than among MRGAS isolates ( $p < 0.05$ ), whereas M phenotype dominated among MRGAS in comparison to MRSP isolates ( $p < 0.05$ ).

### *Macrolide resistance genes*

In this study, the prevalent erythromycin resistance gene among MRGAS isolates was *mefA* (n=27, 61%), followed by *ermA* (n=15, 33%) and *ermB* (n=3, 7%).

The *mefA* gene was detected in all strains expressing M phenotype, while *ermA* and *ermB* genes were found in all strains with iMLS and cMLS phenotypes, respectively. One MRGAS strain with iMLS<sub>B</sub> phenotype harbored both *ermA* and *mefA* genes (Table 1).

In MRSP, 7 out of 8 isolates showing M phenotype carried the *mefA* gene (14%), while among isolates with cMLS phenotype, 40 out of 42 carried the *ermB* gene (80%). In three isolates (one with M phenotype and two with cMLS phenotype, respectively), no resistance genes were detected. These findings are summarized in Table 1.

## DISCUSSION

An increase in macrolide resistance in streptococcal species is becoming a serious problem in recent decades (Van Bambeke, 2007; Richter, 2008). In Serbia, macrolide resistance in GAS is mainly due to the presence of the *mefA* gene. Our data are in agreement with the findings obtained in other countries, including Germany (Bley, 2011), Spain (Tamayo, 2005), USA (Green, 2006) and Canada (Wierzbowski, 2005). However, the epidemiology of GAS resistance is not uniform in Europe. Likewise, in Norway (1993-2002) and in Bulgaria (1993-2002), iMLS<sub>B</sub> was the prevalent phenotype in GAS isolates (Littauer et al., 2006), and a recent study from France demonstrated predominance of the cMLS<sub>B</sub> phenotype (d'Humieres, 2012). Richter et al. (2008) in a study of macrolide resistance that included a significant number of GAS isolates from many European countries concluded that there is a continual increase in the incidence of MLS<sub>B</sub> phenotype over M phenotype in Europe. Therefore, the dominance of the M phenotype in Serbia and the low prevalence of clindamycin resistance among our GAS isolates are encouraging, since high rates of clindamycin resistance, associated with *erm* genes, may have a significant impact on the treatment of invasive GAS diseases.

Unlike *S. pyogenes*, in *S. pneumoniae* in Serbia, the MLS<sub>B</sub> phenotype due to the *ermB* gene was overrepresented compared to the M phenotype. Similar results were found in other Mediterranean

**Table 1.** Distribution of macrolide-resistant genes.

| Isolates | Phenotypes | Macrolide-resistant genes |             |             | No gene |
|----------|------------|---------------------------|-------------|-------------|---------|
|          |            | <i>mefA</i>               | <i>ermA</i> | <i>ermB</i> |         |
| MRGAS    | M          | 26 (58%)                  | /           | /           | /       |
|          | iMLS       | 1 (2%)                    | 15 (33%)    | /           | /       |
|          | cMLS       | /                         | /           | 3 (7%)      |         |
| MRSP     | M          | 7 (14%)                   | /           | /           | 1 (2%)  |
|          | cMLS       | /                         | /           | 40 (80%)    | 2 (4%)  |

**Table 2.** MIC values of erythromycin and clindamycin.

| Phenotype | Antibacterial agent | MIC (µg/ml) |       |             |
|-----------|---------------------|-------------|-------|-------------|
|           |                     | 50%         | 90%   | Range       |
| M         | erythromycin        | 4           | 16    | 2-16        |
|           | clindamycin         | 0.047       | 0.094 | 0.032-0.094 |
| iMLS      | erythromycin        | 128         | 128   | 12->256     |
|           | clindamycin         | 0.25        | 0.25  | 0.25        |
| cMLS      | erythromycin        | > 256       | >256  | >256        |
|           | clindamycin         | >256        | >256  | >256        |

an countries like Spain (Perez-Trallero, 2001) and France (Fitoussi, 2001), where the MLS<sub>B</sub> phenotype was dominant in pneumococci. Contrary to this, in the USA, the M phenotype is considerably represented among MRSP isolates, although an increase in MLS<sub>B</sub> phenotype in recent years has been noted (Jenkins, 2009). In Germany as well, efflux-mediated macrolide resistance is the predominant mechanism found in both *S. pneumoniae* and GAS, as shown by several studies (Bley, 2011). On the other hand, the dominance of the MLS<sub>B</sub> phenotype over the M phenotype in pneumococci, and the opposite distribution of macrolide resistance phenotypes in GAS, as in Serbia, was also noted in several reports from Spain (Perez-Trallero, 2001). The explanation for the discrepancy of resistance mechanisms in related streptococcal species found in our study could be in the different clinical presentations and differences in the age of the patients: GAS isolates were obtained from patients with pharyngitis that were mostly children, while pneumococci were collected from patients with upper respiratory tract diseases (mostly adults).

Our results confirmed a strong correlation between the M phenotype and the *mefA* gene and between the MLS<sub>B</sub> phenotype and *erm* genes in GAS, as well as in pneumococcal isolates. These results are also in accordance with the susceptibility pattern of the tested isolates. A high level of resistance to macrolides was characteristic of the MLS<sub>B</sub> phenotype/*erm* positive isolates, while low-level resistance was related to the M phenotype/*mefA*-positive strains. An association between certain macrolide resistance phenotypes and erythromycin resistance genes has already been documented (Giovanetti et al., 1999; Sutcliffe, 1996). Of note is that dual resistance mechanisms (*ermA* and *mefA* in GAS and *ermB* and *mefA* genes in pneumococci) were uncommon in our study. However, in three pneumococcal isolates none of resistance genes were found, indicating the presence of other resistance genes/mechanisms.

Since the prevalence of the recognized phenotypes of erythromycin-resistant *S. pyogenes* and *S. pneumoniae* may vary from area to area and over

time, continued monitoring is important for a better understanding of the epidemiology of macrolide resistance in related streptococcal species. Indeed, further studies are needed to evaluate potential changes of macrolide resistance phenotypes and genotypes in streptococci in Serbia.

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