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MACROLIDE-RESISTANT PHENOTYPES OF INVASIVE *STREPTOCOCCUS PNEUMONIAE* ISOLATES IN SERBIA

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Abstract – Macrolide resistance in *Streptococcus pneumoniae* has emerged as an important worldwide problem over the past decade. The aim of this study was to investigate macrolide-resistant phenotypes and the antimicrobial susceptibility patterns of invasive pneumococci in Serbia. A total of 68 invasive pneumococcal strains, collected from 2009 to 2011, were sent from regional laboratories to the National Reference Laboratory. Susceptibility testing was performed using the VITEK2 system and phenotypes were determined by triple-test. Overall penicillin and erythromycin nonsusceptibility rates were 26% and 43%, respectively. Resistance rates were higher in children than in adults. Co-resistance to penicillin and erythromycin was detected in 18% strains. Resistance rates to the third generation of cephalosporins, TMP-SXT and tetracycline were 16%, 37% and 29%, respectively. All isolates were fully susceptible to vancomycin, linezolid, fluoroquinolones, telithromycin and rifampicin. Twenty-two isolates (79%) an expressed macrolide-lincosamide-streptogramin B (MLS_B) resistance phenotype and M phenotype was found in 21% of macrolide resistant strains.

Key words: *Streptococcus pneumoniae*, invasion, macrolide resistance, phenotype

INTRODUCTION

Worldwide, *S. pneumoniae* remains the most common cause of community-acquired pneumonia (CAP), bacterial meningitis, bacteremia, and otitis media. (Mitchell et al., 1995). In recent decades, the increase in the prevalence of antimicrobial resistant pneumococci has been observed, with considerable geographical variation among the genotypes and phenotypes involved. Therefore, the emerging high-level antimicrobial resistance of pneumococci is a major challenge worldwide and may lead to treatment failures (Iannini et al., 2007; Neuman et al., 2007).

Beta-lactam antibiotics and macrolides are extensively used for the treatment of respiratory tract infections due to their broad spectrum of activity and safety profile. Penicillin-nonsusceptibility rates increased worldwide during the 1990s and 2000s (Sogstad et al., 2006). Macrolide resistance among *S. pneumoniae* isolates has risen to prominence during the last decade. These antibiotics are being used as initial empiric therapy for community-acquired respiratory tract infections. Over the 1990s, an increase in the proportion of pneumococcal isolates with combined nonsusceptibility to penicillin and erythromycin was observed (Felmingham et al., 2005b). Selection and dissemination of penicillin and/or erythromycin nonsusceptibility in *Streptococcus pneumoniae* were associated with antibiotic consumption, mainly of oral cephalosporins (among β-lactams) and long-half-life macrolides (Whitney et al., 2005; Felmingham et al., 2007a; ESAC, 2010).

Macrolide resistance (MR) in *S. pneumoniae* is primarily due to two mechanisms: target site modification, encoded by the *erm* gene and efflux pump expulsion, mediated by the *mef* gene. Target site modi-

fication leads to reduction in the binding affinity of all macrolides, lincosamides, and streptogramin B to the 23S rRNA (MLS_B phenotype) and it is defined by a high-level cross-resistance to all MLS_B antibiotics. Expression of MLS_B resistance can be either constitutive ($cMLS_B$) or inducible ($iMLS_B$). Efflux-mediated erythromycin resistance is associated with a particular resistance pattern (M phenotype) characterized by resistance only to 14- and 15-membered macrolides, usually at a low level (Lambert et al., 2007; Ambrose et al., 2005).

The aim of this study was to analyze the antimicrobial susceptibility patterns of invasive pneumococcal isolates received at the National Reference Laboratory (NRL) from 2009 to 2011, and to determine the prevalence of macrolide-resistant phenotypes of invasive *S. pneumoniae* isolates in Serbia.

MATERIALS AND METHODS

A total of 68 invasive pneumococcal strains were received from twelve regional microbiological laboratories throughout the entire country at the NRL for streptococci and pneumococci, Institute of Microbiology and Immunology, Medical Faculty, Belgrade, between July 2009 and July 2011. Isolates were obtained from blood (n=31), cerebrospinal fluid (n=22) and pleural fluid (n=15). Twenty-three out of 68 strains were from children (14 among ≤5 years old) and 45 from adults (21 from persons ≥65 years).

Pneumococci were identified on the basis of typical colony morphology, alpha hemolysis, Gram-stain morphology, optochin sensitivity (BioRad, USA), and a bile solubility and slide agglutination test (bioMerieux, France). The isolates were stored at -80°C in Brain Heart Infusion Broth (Biolife, Italy) containing 10% glycerol.

Susceptibility testing against 17 antibiotics was done by the VITEK2 (bioMerieux, France) automated system. Minimal inhibitory concentrations (MICs) of erythromycin and clindamycin were determined by E test (bioMerieux, France). *Streptococcus pneumoniae* ATCC 49619 was used as a quality control strain. Results were interpreted according to the EUCAST guidelines (EUCAST, 2011). Isolates with intermediate or high-level resistance were classified as nonsusceptible.

Macrolide resistance phenotypes were determined by a triple-disk diffusion test, using erythromycin (15μg), clindamycin (2μg) and spiramycin (100μg) disks, (bioRad, USA), as described elsewhere (Montanari et al., 2001). The M phenotype was scored when the isolate was nonsusceptible only to erythromycin. Resistance to both erythromycin and clindamycin was considered a MLS_B phenotype. An inducible MLS ($iMLS_B$) phenotype is characterized by a D-shaped zone around clindamycin and the constitutive MLS ($cMLS_B$) phenotype, without blunting of the zone.

Seven out of 28 randomly selected erythromycin resistant strains were serotyped by Quelling reaction, using antisera (Statens Serum Institute, Copenhagen, Denmark).

SPSS software, version 13.0, was used for statistical analysis. The chi-square test was used when appropriate. Two-sided P values of 0.05 were considered to be statistically significant.

RESULTS

Overall, the macrolide nonsusceptibility rate was 41% (28 out of 68 strains). The proportion was higher in children [57%, (13/23)] than in adults [33% (15/45)] (Table 2). However, the difference was not statistically significant ($\chi2=2.49$; $p=0.06$). The lowest macrolide nonsusceptibility rate was observed in adults ≥65 years [24% (5/21)].

The proportion of penicillin nonsusceptible *S. pneumoniae* among all strains studied was 28% (19 out of 68 strains). A significantly higher percentage of penicillin resistance rates ($\chi2=3.08$; $p=0.04$) were observed in children [44% (10/23)] than in adults [20% (9/45)] (Table 2). Penicillin resistance in adults ≥65 was 29% (6/21).

Table1. Macrolide-resistant phenotypes and corresponding MICs values of invasive *S. pneumoniae* isolates.

Phenotype	n (%)	Antibiotic	MIC (μg/ml)	
			90%	range
M	6(21%)	Erythromycin	6	4-6
		Clindamycin	0,094	0,032-0,094
iMLS	3(11%)	Erythromycin	>256	0,5->256
		Clindamycin	0,25	0,25->256
cMLS	19(68%)	Erythromycin	>256	16->256
		Clindamycin	>256	>256

Table 2. Susceptibility to antibiotics of child and adult invasive *S. pneumoniae* isolates.

Antibiotic		Penicillin n (%)	Ceftriaxone n (%)	Erythromycin n (%)	Co-resistance to penicillin and erythromycin n (%)
Children n=23	S	13 (56%)	16(70%)	10 (43%)	7 (30%)
	I	2 (9%)	3(13%)	0 (0%)	
	R	8 (35%)	4(17%)	13 (57%)	
Adults n=45	S	36 (80%)	42(94%)	30 (67%)	5 (11%)
	I	1 (2%)	2(4%)	2 (4%)	
	R	8 (18%)	1(2%)	13 (29%)	

Co-resistance to penicillin and erythromycin was expressed in 11 out of 68 isolates (16%).

Ceftriaxone and cefotaxime nonsusceptible isolates became less frequent [15% (10/68)]. The rate was higher in children [30% (7/23)] than in adults [7% (3/45)] (Table 2). Nonsusceptibility rates to trimethoprim-sulfamethoxazole and tetracycline were 40% and 29%, respectively, with no statistical significant difference between the two age groups.

Among 28 MR *S. pneumoniae* examined in this study, 43% (12/28) and 36% (10/28) of the isolates displayed nonsusceptibility to penicillin and cefotaxime, respectively. A total of 20 isolates (29%) revealed multiresistant profiles exhibited resistance to penicillin, erythromycin, tetracycline and trimethoprim-sulfamethoxazole. Multiresistant strains were most frequently isolated in children.

However, all isolates were fully susceptible to vancomycin, linezolid, ofloxacin and newer fluoroquinolones, telithromycin and rifampicin. In addition, 25 strains (37%) were susceptible to all tested antibiotics.

Among MR isolates, the MIC range of erythromycin was 0.5 (≥256 μg/ml), while the range of clindamycin was 0.032 (≥256 μg/ml).

On the basis of the erythromycin-clindamycin-spiramycin triple-disk test, 19 out of 28 (68%), and 3 out of 28 (11%) strains were assigned to the cMLS and iMLS phenotype, respectively, while 6 out of 28 (21%) isolates were referred to the M phenotype. These findings, together with the ranges of MICs of erythromycin and clindamycin, are summarized in Table 1. Isolates displaying the MLS_B phenotype showed higher MICs to erythromycin than isolates with the M phenotype and were associated with a high percentage of resistance to ceftriaxone, tetracycline and trimethoprim-sulfamethoxazole.

Serotypes 19F and 14 were found in five of seven MR isolates. Two strains belonged to serotypes 23A and 11A.

DISCUSSION

Our study highlights a high level of macrolide resistance among invasive pneumococcal strains in Serbia

(43%), especially in children. The finding that the majority of strains displayed the MLS_B phenotype, i. e. a high-level macrolide resistance, is of particular concern. There is considerable geographic variation in the prevalence of erythromycin-resistant pneumococci, from 30-55% in France, Spain, Greece, South Africa, United States of America, and Asia to as low as 4-7% in parts of northern and western Europe (Czech Republic, Netherlands, and Sweden) (Whitney et al., 2005; Felmingham et al., 2007). Our finding of a high level of macrolide resistance among invasive pneumococcal strains in Serbia is consistent with similar reports from southern European countries in the Alexander project (Felmingham et al., 2005). Differences in erythromycin resistance are believed to reflect variations in macrolide consumption (ESAC, 2010) and the spread of multidrug-resistant clones.

Among the 28 erythromycin-resistant clinical strains of *S. pneumoniae* investigated in this study, we found that the MLS_B resistance phenotype was present in 22 out of 68 (79%) strains. Therefore, the MLS_B phenotype, which demonstrates high-level resistance to MLS antibiotics, was significantly dominant. This phenotype is predominant in southern European countries, Italy, Spain and the entire Mediterranean region (Reinert et al., 2005), whereas the M phenotype predominates in North America, England, and Germany (McEllistrem et al., 2005).

We also found that the prevalence of penicillin nonsusceptible pneumococcal (PNSP) strains is high (26%), with a significant difference between pediatric (44%) and adult (20%) isolates. Most pediatric strains were isolated from patients with meningitis, while the majority of adult strains were obtained from patients with pneumonia. Since current CLSI (Clinical Laboratory Standard Institute, USA) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines use the different penicillin breakpoints for meningeal (resistant, MIC≥0,012μg/ml) and non-meningeal strains (nonsusceptible, MIC≥4μg/ml) (CLSI, 2011), we interpret our results in accordance with revised cut-off values. Therefore, pediatric patients revealed significantly higher penicillin resistance rates. The frequency of penicillin nonsusceptible pneumococcal isolates in our collection is similar to those observed among invasive isolates in other countries, such as Spain (30% in 2010) and France (28% in 2010) (EARSS, 2010). It was, however, higher than those in countries of central and northern Europe, and lower than those in Cyprus (41.7%), South Africa (74%), the Far East (63%), and the Middle East (54%) (EARSS, 2010; Jenkins et al., 2008).

Sixteen percent of our invasive strains expressed co-resistance to penicillin and erythromycin. The last EARSS report noted that the overall rate of dual nonsusceptibility to these antibiotics remained below 6.2% in 2010 in Europe. Proportions of simultaneous nonsusceptibility to penicillin and macrolides ranged from 0.0% (Estonia, Lithuania) to 36.4% (Cyprus). The rate of pneumococcal co-resistance in Serbia is similar to that reported in Spain (17%) and Belgium (22%) (EARSS, 2010).

We found that erythromycin nonsusceptibility was also associated with resistance to tetracycline and trimethoprim-sulfamethoxazole. The high prevalence of such strains can be explained by co-resistance, mainly between tetracycline and macrolides. This association is commonly associated with the presence of several transposons that carry the genetic determinants encoding resistance to both antibiotics (Brenciani et al., 2007). Up to 30% of the isolates expressed multiresistant pattern. These strains were most frequently isolated in children.

Differences in rates of pneumococcal antibiotic resistance between countries have been shown to be associated with levels of antimicrobial consumption and the introduction of pneumococcal conjugate vaccine. It has been established that the proportion of resistant *S. pneumoniae* isolates has remained generally stable in Europe due to the introduction of the pneumococcal conjugate vaccine at the beginning of the 2000s. The widespread use of this vaccine is an important factor that may have influenced the decrease in antibiotic resistance levels, eliminating the infections (and more important, the children's car-

riage) of frequent 'classic' resistant serotypes. This vaccine was licensed in Serbia, but it is not included in the national vaccination program.

In spite of the scarce number of serotyped isolates, we found that almost all of them belong to the most common macrolide-resistant serotypes (19F and 14). Our results are in line with the results reported from other countries, conducted over a similar period (Imohl et al., 2009).

This is the first study assessing macrolide-resistant phenotypes and antimicrobial susceptibility of invasive *S. pneumoniae* isolates from Serbia. Its limitation was the relatively small number of isolates, which should be considered in the interpretation of the results.

Nevertheless, the high level of macrolide resistance, with the predominance of the MLS_B phenotype among invasive pneumococcal isolates in Serbia, should be stressed. Further studies are needed to investigate comprehensively the serotype distribution and molecular epidemiology of these isolates.

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