Acta Microbiologica et Immunologica Hungarica 65 (4), pp. 477–488 (2018)
DOI: 10.1556/030.65.2018.035
First published online July 14, 2018

CHARACTERIZATION OF MACROLIDE-RESISTANT NON-INVASIVE PNEUMOCOCCI IN THE PRE-VACCINE ERA IN SERBIA

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(Received: 9 April 2017; accepted: 20 June 2018)

Numerous reports have confirmed that increased macrolide use in the treatment of respiratory tract infection has contributed to the emergence of antibiotic resistance worldwide. Studies have also shown that pneumococcal vaccine can reduce pneumococcal resistance. The aim of this study was to determine the prevalence of co-resistance to penicillin and other antibiotics in macrolide-resistant (MR) non-invasive pneumococcal isolates and to evaluate serotype distribution in resistant strains in the pre-vaccine era in Serbia. About 80% of MR isolates expressed the MLS phenotype with very high resistance to both erythromycin and clindamycin. A total of 132 (84.1%) MR isolates were multiresistant, i.e., they were resistant to erythromycin, penicillin, tetracycline, and trimethoprim–sulfamethoxazole. Among 157 MR pneumococci, 11 different serotypes were found. Four serotypes, 19F, 14, 6B, and 23F, accounted for 77.7% of all MR pneumococcal isolates. Among isolates with the cMLS phenotype, serotypes 19F and 14 were predominant, whereas serotype 6A was the most common among those with the M phenotype, followed by 14. In conclusion, co-resistance to macrolides and penicillin in our non-invasive pneumococcal isolates is high. The majority of tested strains (~80%) belonged to the four serotypes (19F, 14, 6B, and 23F) that are included in all conjugate vaccine formulations.

Keywords: macrolide resistance, pneumococci, serotype, vaccine

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Introduction

Streptococcus pneumoniae (pneumococcus) is one of the most important human pathogens. It is the most common cause of community-acquired pneumonia [1–3], sinusitis, and otitis media, as well as invasive diseases, such as bacteremia, sepsis, and meningitis. Parenteral antibiotics, such as high doses of penicillin, third-generation cephalosporins, meropenem, vancomycin, and linezolid, are used for the treatment of pneumococcal invasive diseases, while treatment for non-invasive infections typically includes oral beta-lactam antibiotics, macrolides, and newer generations of fluoroquinolones.

In the past, beta-lactam antibiotics were widely used as initial empirical treatment of community-acquired respiratory tract infections (CARTI), which led to an increase in the resistance of common respiratory pathogens. Global surveillance of resistance in bacteria causing CARTI has shown that beta-lactam non-susceptibility rates increased worldwide during the 1990s and 2000s and reaching the alarming level. The Alexander Project monitored resistance in *S. pneumoniae* in 9-year period during 1992–2001 and reported increase of pneumococcal non-susceptibility rate to penicillin. In some countries, such as Spain and France, pneumococcal resistance to penicillin raised from 25% to 7%, respectively, at the beginning of the 1990s to 30% and 35% in 2001 [4]. Consequently, macrolides were increasingly used for the treatment of respiratory tract infections. Thereafter, a rapid worldwide increase in the prevalence of macrolide resistance associated with an increase in macrolide consumption was observed.

The report of European Antimicrobial Resistance Surveillance System for 2014 indicated that there is a large variation in pneumococcal macrolide resistance rates across Europe. In northern European countries, resistance was low (5% to <10%), whereas in Mediterranean countries, Italy, Turkey, and France, resistance was 10% to <25% and levels reached up to 35%–40% in Malta and Romania [5].

Resistance to macrolides in *S. pneumoniae* is mediated by two major mechanisms. First is target modification caused by a ribosomal methylase encoded by the *ermB* gene and associated with high level of resistance to all macrolides, lincosamides, and streptogramin (MLS phenotype) [6, 7]. MLS resistance can be expressed either constitutively (cMLS) or inducibly (iMLS). The second mechanism is drug efflux, encoded by the *mefA* gene, which results in lower level resistance (M phenotype) to 14- and 15-membered macrolides [8–10].

A nationwide surveillance study performed during 2009–2011 in Serbia indicated that non-susceptibility of invasive pneumococcal strains to penicillin and erythromycin was 34% and 36%, respectively [11]. Pneumococcal conjugate vaccine (PCV) was approved for introduction in Serbia's national immunization program, and it will be implemented during 2018. It is documented that PCV has profound impact on the incidence of both pneumococcal invasive and non-invasive diseases. Also, vaccines can reduce pneumococcal resistance in vaccinated and unvaccinated populations by reducing the carriage of antibiotic-resistant serotypes [12]. Assessment of changes in serotype distribution and resistance potentially related to vaccine introduction requires valid baseline values of both serotype distribution and antimicrobial resistance.

The aim of this study was to determine the prevalence of co-resistance to penicillin and other antibiotics in macrolide-resistant (MR) non-invasive pneumococcal isolates and to evaluate serotype distribution in resistant strains in the pre-vaccine era in Serbia.

Materials and Methods

Bacterial isolates

A total of 157 non-invasive MR isolates of *S. pneumoniae* from all over Serbia were collected in 2014. The pneumococci were isolated from nasal discharge and nasopharyngeal swabs (103), sputum, tracheal, and bronchial aspirates (50), and middle ear fluid (4) obtained from patients with symptoms of an acute respiratory infection presumably of bacterial etiology (sinusitis, otitis media, and pneumonia). Out of 157 strains, 111 were isolated from children (75 among ≤5 years old) and 46 from adults (23 from persons ≥65 years old). After isolation and identification by conventional microbial techniques in regional microbiology laboratories, strains were sent into the National Reference Laboratory for further characterization. Strain identification was confirmed in our laboratory by optochin test, latex agglutination with the Slidex pneumo-kit (bioMérieux, France), bile solubility test, and PCR for the *lytA* gene. Serotyping was done by the capsular swelling test (Quellung reaction) using pool antisera and factor/type antisera (Statens Serum Institut, Copenhagen, Denmark).

Antibiotic susceptibility testing

Antibiotic susceptibility testing for oxacillin, erythromycin, chloramphenicol, trimethoprim-sulfamethoxazole, and tetracyclines was carried out by the disk diffusion method using disks (Bio-Rad, USA) on Mueller–Hinton agar with 5% defibrinated horse blood and 20 mg/L β-nicotinamide adenine dinucleotide (bioMérieux) according to the recommendations of European Committee on Antimicrobial Susceptibility Testing (EUCAST) [13]. Oxacillin susceptibility for screening benzylpenicillin resistance was interpreted using the recommended current EUCAST clinical breakpoints (≥20 mm) [13]. Oxacillin-resistant isolates were further tested for penicillin susceptibility using E-test strips (bioMérieux). The minimum inhibitory concentrations (MICs) of penicillin were interpreted according to the EUCAST guidelines for infections other than meningitis ($S \leq 0.06$ μg/ml; $R > 2$ μg/ml). Intermediate and resistant isolates were collectively grouped as non-susceptible.

Macrolide resistance phenotypes

Macrolide resistance phenotypes were determined by a double disk diffusion test using erythromycin (15 μg) and clindamycin (2 μg) disks (Bio-Rad), as described previously [14]. 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). Susceptibility to clindamycin with resistance to erythromycin suggested the M phenotype [15]. MICs of erythromycin and clindamycin were determined using E-test strips (bioMérieux).

Statistical analysis

Data were analyzed by descriptive statistic methods and the insignificance of the prevalence of MLS and M phenotype between children and adults was assessed using χ^2 test.

Results

The great majority (80.9%) of MR strains of *S. pneumoniae* tested in this study expressed MLS phenotype (Table I). Overall, 123 of 157 isolates had a cMLS phenotype with very high resistance to both erythromycin and clindamycin (MICs ≥256 μg/ml). Only four strains had iMLS phenotype with heterogeneous MICs values of erythromycin and clindamycin. Therefore, MIC_{50} and MIC_{90} values in iMLS group of isolates could not be calculated. Approximately, one fifth of isolates showed the M phenotype with MIC values of erythromycin that did not exceed 12 μg/ml. These strains were completely susceptible to clindamycin (Table I). There was no significant difference in the prevalence of MLS phenotype

Table I. Macrolide resistance phenotypes among pneumococcal isolates

				MIC (μg/ml)		
Phenotype	Number	Percentage (%)	Antibiotics	MIC_{50}	MIC_{90}	Range
M	30	19.12	Erythromycin	3	8	1.5–12
			Clindamycin	0.047	0.094	0.032–0.094
iMLS	4	2.54	Erythromycin	–	–	4 to ≥256
			Clindamycin	–	–	0.125 to ≥256
cMLS	123	78.34	Erythromycin	≥256	≥256	≥256
			Clindamycin	≥256	≥256	≥256

Note: MIC: minimum inhibitory concentration; MLS: macrolides, lincosamides, and streptogramin; iMLS: inducible MLS; cMLS: constitutive MLS.

in children and adults, but M phenotype was significantly more common in adults than in children ($\chi^2 = 19.08$, $p < 0.01$).

Oxacillin resistance was observed in 130 (82.8%) of MR pneumococcal strains. However, according to MIC values determined by E-test, 100 of oxacillin-resistant isolates were non-susceptible to penicillin, having MIC > 0.06 μg/ml. Therefore, penicillin non-susceptibility was found in 63.7% of MR pneumococcal isolates. Among them, 10 strains were resistant to penicillin, having MIC > 2 μg/ml, whereas 90 (57.3%) strains showed intermediate resistance. In total, 57 (36.3%) of MR pneumococcal strains were penicillin susceptible. The MIC range of penicillin for tested strains was 0.032–16 μg/ml.

About 80% and 70% of MR non-invasive pneumococci were resistant to tetracyclines and trimethoprim–sulfamethoxazole, respectively. Chloramphenicol remains relatively active with susceptibility rate of 77% (Table II).

A total of 132 (84.1%) MR isolates were multiresistant, i.e., they were resistant to three or more different antimicrobial classes: erythromycin, penicillin, tetracycline, and trimethoprim–sulfamethoxazole. The most frequently occurring phenotype included resistance to erythromycin, tetracyclines, and trimethoprim–sulfamethoxazole was found in 96 (61.1%) strains.

Among 157 MR pneumococci, 11 different serotypes were found, while four (2.5%) strains were non-typable (Table III). Four serotypes, 19F ($n = 51$),

Table II. Susceptibility of macrolide-resistant *S. pneumoniae* strains to other antibiotics

Antibiotics	Resistant (%)	Intermediate (%)	Susceptible (%)	Total (%)
Penicillin	10 (6.4)	90 (57.3)	57 (36.3)	157 (100)
Tetracycline	124 (79)	0 (0)	33 (21)	157 (100)
Chloramphenicol	36 (23)	0 (0)	121 (77)	157 (100)
Trimethoprim–sulfamethoxazole	105 (67)	8 (5)	44 (28)	157 (100)

Table III. Distribution of serotypes among different macrolide resistance phenotypic categories of 157 macrolide-resistant *S. pneumoniae* isolates

Serotypes	cMLS [*n* (%)]	iMLS [*n* (%)]	M [*n* (%)]	Children [*n* (%)]	Adults [*n* (%)]	Total [*n* (%)]
19F	48 (39.0)	0	3 (10.0)	43 (84.3)	8 (15.7)	51 (32.5)
14	33 (26.8)	0	6 (20.0)	33 (84.6)	6 (15.4)	39 (24.8)
6B	15 (12.2)	0	2 (6.7)	12 (70.6)	5 (12.4)	17 (10.8)
23F	12 (9.8)	0	3 (10.0)	10 (66.7)	5 (33.3)	15 (9.6)
6A	2 (1.6)	0	9 (30.0)	5 (45.5)	6 (54.5)	11 (7.0)
19A	5 (4.1)	4 (100)	0	5 (55.5)	4 (44.5)	9 (5.7)
3	1 (0.8)	0	3 (10.0)	1 (25.0)	3 (75.0)	4 (2.5)
11A	2 (1.6)	0	1 (3.3)	1 (33.3)	2 (66.7)	3 (1.9)
15B	1 (0.8)	0	1 (3.3)	0 (0)	2 (100)	2 (1.3)
8	1 (0.8)	0	0	0 (0)	1 (100)	1 (0.7)
23A	1 (0.8)	0	0	0 (0)	1 (100)	1 (0.7)
Non-typable	2 (1.6)	0	2 (6.7)	1 (25.0)	3 (75.0)	4 (2.5)
Total	123 (100)	4 (100)	30 (100)	111 (100)	46 (100)	157 (100)

Note: MLS: macrolides, lincosamides, and streptogramin; iMLS: inducible MLS; cMLS: constitutive MLS.

14 ($n = 39$), 6B ($n = 17$), and 23F ($n = 15$) accounted for 77.7% of all MR pneumococcal isolates. Among isolates with the cMLS phenotype, serotypes 19F [48/123 (39.0%)] and 14 [33/123 (26.8%)] were predominant, whereas serotype 6A was the most common among those with the M phenotype [9/30 (30.0%)], followed by 14 [6/30 (20.0%)]. Interestingly, all iMLS ($n = 4$) belonged to 6A serotype. The leading serotypes 19F, 14, and 6B were significantly more common in children than in adults ($p < 0.05$). Serotypes 8, 15B, and 23A were recovered only from adults.

Co-resistance to penicillin and erythromycin was predominantly found in MR isolates with 19F serotype.

According to European Medical Agency, there is sufficient evidence that PCV10 provides protection against cross-reactive serotype 19A [16]. Therefore, we calculated the PCV10 coverage with and without serotype 19A. Coverage with PCV10 for all MR pneumococci was 77.7% (increasing to 83.4% if 19A is included), whereas coverage with PCV13 was 92.9%. For pediatric isolates only, coverage for PCV10 was 88.3% (92.8% if 19A is included) and 98.2% for PVC13.

Discussion

In this study, we characterized the MR population of non-invasive respiratory tract pneumococcal isolates in Serbia. Among MR pneumococcal population, constitutive MLS phenotype predominated. More than 80% of isolates belonged to this phenotype, which is characterized by cross-resistance to all three classes of antibiotics – macrolides, lincosamides, and streptogramins – with extremely high MIC values (≥256 μg/ml). Only four isolates expressed inducible MLS phenotype, which is associated with susceptibility to clindamycin without induction, turning to high-level resistance after induction. M phenotype characterized by low-level erythromycin resistance and susceptibility to clindamycin was detected in almost 20% of our isolates. This phenotype was significantly more common in adults, whereas highly resistant MLS phenotype was equally distributed among isolates from children and adults.

Globally, cMLS is the most prevalent resistance phenotype among MR *S. pneumoniae* and it was more common than M phenotype. The high prevalence of *ermB* genotype, which mostly correlated with the cMLS phenotype, was registered in Belgium (91%), France (90%), Spain (88%), Hungary (82%), Poland (88%), Italy (56%), and Japan (58%) [17–20]. By contrast, the M phenotype predominates in USA [21], Canada [22], Greece [23], Ireland, and UK [19].

A high rate of macrolide resistance among invasive pneumococci in Serbia (41%) was previously described by Gajic et al. [11] with domination of highly resistant cMLS. It is well documented that the total macrolide use, especially long-lasting antibiotics, such as azithromycin, is associated with increased macrolide resistance in *S. pneumoniae* [24]. In Eastern Europe, in 2011, the highest macrolide consumption was noted in Montenegro and Serbia [25]. Therefore, it is not surprising that resistance to these drugs in *S. pneumoniae* in Serbia reaches alarmingly high rates.

The prevalence of dual penicillin and macrolide non-susceptibility in Serbian non-invasive isolates was very high (63.7%). However, a great majority of penicillin non-susceptible pneumococci were in the intermediate category, while 6.4% were highly resistant. The rate of penicillin and macrolide co-resistance of non-invasive pneumococcal isolates observed in this study was significantly higher compared to reports from Norway (1.7%) [26], the Netherlands (2.7%) [27], Czech Republic (3%) [28], and Estonia (6%) [29], even before the widespread use of PCV vaccination. Countries with high co-resistance rates were Greece (34.7%) [30], Poland (39.2%) [31], and Romania (83%) [32]. As the decrease of resistance to both penicillin and macrolides was reported due to the PCV vaccination [33, 34], we might anticipate these changes in Serbia as well in the future.

High percentage of our erythromycin non-susceptible isolates expressed concomitant resistance to tetracycline (~80%) and/or trimethoprim–sulfamethoxazole (~70%). These patterns of resistance are widespread not only in other countries (UK and Far East) [35, 36], but also in our close proximity (Bulgaria) [37]. Typically, *erm* and tetracycline resistance genes are carried on the same transposon [38]. Therefore, we can assume that the high prevalence (~80%) of macrolide and tetracycline resistance in our isolates might be due to the insertion of transposons of the Tn916 family, but future studies should address this issue.

Eight different serotypes (19F, 14, 6B, 23F, 6A, 19A, 3, and 11A) were found among MR pneumococci in both populations and three more (15B, 8, and 23A) only in adult group. Serotypes 19F, 14, 6B, and 23F, which are included in both PCV10 and PCV13, accounted for almost 80% of all MR pneumococcal isolates. The leading serotypes 19F, 14, and 6B were significantly more common in children than in adults. It is well described that antibiotic resistance, which may provide a survival advantage, is associated with specific pneumococcal serotypes – 6B, 6A, 9V, 14, 19F, and 23F in the pre-vaccine era. These are predominant carriage serotypes that colonize nasopharynx of children [5]. The serotype epidemiology of MR non-invasive pneumococcal isolates in our country corresponds to distribution of pediatric serotypes in European countries in the pre-vaccination period [5].

The PCV has been shown to exert a profound influence on the incidence of invasive pneumococcal diseases, epidemiology of *S. pneumoniae* serotypes, as well as on the resistance. In countries with universal PCVs vaccination, vaccine serotypes have virtually disappeared [39, 40]. Serotype coverage of both PCV10 and PCV13 of our resistant *S. pneumoniae* isolated from non-invasive diseases is high (≥78%). Until 2018, both PCV10 and PCV13 vaccines were licensed in Serbia, but vaccination was not compulsory and coverage is low. However, PCV is introduced in the new Law on Protection of Population against Communicable Diseases and is mandatory from March 2018. Therefore, we expect to achieve an overall reduction in pneumococcal resistance after introduction of PCVs.

In conclusion, macrolide–penicillin co-resistance in our non-invasive pneumococcal isolates is high. Four serotypes (19F, 14, 6B, and 23F) accounted for ~80% of all strains. The prevailing pneumococcal serotypes in our country are included in both PCV10 and PCV13 vaccine formulations indicating good coverage. However, continuous monitoring of antimicrobial resistance and serotype distribution of *S. pneumoniae* are essential for surveillance of changes in pneumococcal population.

Acknowledgements

The authors gratefully acknowledge all colleagues for providing isolates of pneumococci. The study was partially funded by the Serbian Ministry of Education and Science (project no. 175039).

Conflict of Interest

None.

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