

MLS_B GENOTYPE IS PREDOMINANT MOLECULAR GENETIC MECHANISM AMONG ERYTHROMYCIN - RESISTANT *STREPTOCOCCUS PNEUMONIAE* FOR THE PERIOD: 2006-2008.

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SUMMARY

In total, 413 clinical isolates of *Streptococcus pneumoniae* were collected from four University laboratories in Sofia, Pleven, Plovdiv and Varna for the period of 2006-2008.

150 strains (36, 3%) of all 413 tested *S. pneumoniae* were erythromycin-resistant (ERSP). The most prevalence of ERSP were observed in children under 5 years (80.0%).

The results of conventional PCR showed presence of *erm(B)* gene among 64.7% of the strains, *mef(E)* gene among 29.3%, and 9 strains (6%) carried both *erm(B)+mef(E)*. Serotypes 6B, 19F and 19A were the most prevalent among ERSP with *erm(B)* gene, and serotype 14 was predominant among strains harboring *mef(E)* gene.

Phenotypic characterization of ERSP with the triple-disc test (4) completely confirmed the genotype: 106 strains were with MLS_B phenotype (70.7%, including the 9-th strains harboring both *erm(B)+mef(E)* genes) and 44 were with M phenotype (29.3%).

Macrolides are widespread used antibiotics in therapy of pneumococcus infections in our country and ERSP increased with accelerated rates: from 20% in 2000, to 25% in 2005 (5), till 36% in the present research.

The efflux mechanism of macrolide resistance, which were predominant before in Bulgaria (5), now give place to widely spread MLS_B phenotype and genotype referring with target modification. The dissemination of macrolide resistance encoded by *erm(B)* gene were 30% in 2000, increased to 43% in 2005 and 70% in 2008.

Keywords: *Streptococcus pneumoniae*, macrolide resistance, MLS_B genotype, Bulgaria

INTRODUCTION

Streptococcus pneumoniae is an important pathogen in many community-acquired respiratory infections such as

bacterial sinusitis, otitis media, community acquired pneumonia and in more invasive infections like meningitis and bacteremia.

The pneumococcus is becoming increasingly resistant to a variety of antibiotics (7, 8). Nowadays multiply resistant pneumococci that are resistant to penicillin, erythromycin, clindamycin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazol have been reported very often (2, 3). The present study examined the in-vitro susceptibility to macrolides of *S. pneumoniae* isolates from four main University hospitals in Bulgaria. The main aims were to determine the frequency of resistance to macrolides in our country, and to investigate the mechanisms of macrolide resistance. Resistance seems to be associated mainly with efflux mechanisms, but nowadays target site modification by the *erm(B)* gene is more common.

Pneumococcal macrolide resistance can be caused by *erm(B)* (erythromycin ribosome methylase)-encoded methylation of a residue in the 23S rRNA (1). Phenotypically, this results in the MLS_B resistance phenotype, with resistance to all macrolides plus lincosamides and streptogramin B antibiotics. An alternative resistance mechanism involves *mef(E)* (macrolide efflux), resulting in M-type resistance to 14- and 15-membered macrolides, but not to 16-membered macrolides, lincosamides or streptogramin B antibiotics. The simultaneous presence of *mef(E)* and *erm(B)* has been reported also.

MATERIALS and METHODS

Bacterial isolates

For the period of 2006 to 2008 year a total number of 413 pneumococcus were isolated of all ages **patient specimens:** cerebrospinal fluids (20 strains), blood (16), ear fluids (31), sputums and exudates (74), sinus pus (8), eye fluids (8) and nasopharynx (256).

Initial identification of isolates was done by test for

optochin susceptibility as a primary identification method and if it was necessary, by another test such as bile solubility.

Susceptibility testing

All macrolide resistant strains *S.pneumoniae* were tested both by Agar Dilution method and MIC testing for penicillin G, erythromycin and clindamycin on Mueller-Hinton agar (Oxoid, England) containing 5% sheep blood. Incubation (5% CO₂) was for 24 h at 37°C.

Identification of resistance phenotypes

Resistance phenotypes were determined by triple disk test with erythromycin, clindamycin and rokitamycin (4) for all macrolide-resistance strains *S.pneumoniae* which have erythromycin susceptibility $d \geq 20$ mm. All isolates (0.5x McFarland standard) were tested on Mueller-Hinton agar (Oxoid, England) containing 5% sheep blood.

Serotyping. The pneumococci were serotyped by slide agglutination with factor-specific antisera (Statens Serum Institute, Copenhagen, Denmark) and observed by phase contrast microscopy (Binocular, Carl Zeiss). Pneumococcal diagnostic antisera are intended for identification and typing of pneumococci by means of the capsular reaction test. A positive reaction is the result of an in situ immunoprecipitation, leading to a change in the refractive index. In addition the bacteria agglutinate.

Molecular identification methods

The presence of erythromycin resistance genes *erm(B)* and *mef(E)* were identified by conventional PCR according to the method of Sutcliffe *et al.* (6)

Following growth overnight on blood agar in 5 % CO₂, single colonies were grown in 5 ml of Todd Hewitt Broth with Yeast extract (BBL, Becton, Dickinson and Company,) for 6 h, and this was followed by extraction of DNA. All erythromycin-resistant isolates were analysed further by PCR.

PCR buffers and DNA polymerase were supplied by Abgene, UK and all DNA primers were purchased from Alpha DNA, Canada.

The PCR mix was with a magnesium concentration of 2 mM being preferred for the *erm (B)* primer set and 4 mM for the *mef (E)* primer set. Electrophoresis on 1% agarose gels in 40 mM Tris acetate-2 mM EDTA buffer was used to distinguish PCR products. The size of each PCR product was

estimated using standard molecular weight markers (Super DNA Ladder - Low 100-bp, Abgene, UK).

RESULTS

Macrolide resistance was found in 150 strains (36.3%) out of all 413 tested *S. pneumoniae*. The invasive isolates of all ERSP from blood, cerebrospinal fluids, ear fluids and exudates were 25 (16.67%).

We observed 87.34% penicillin resistance (Pen-R) and intermediate penicillin resistance (Pen-I) among erythromycin-resistant pneumococcus. Of these Pen-R and -I isolates 71.0 % were with MLS_B type. Resistant to clindamycin were more than a half (62%) or 93 strains of all ERSP isolates.

The most prevalence of macrolide resistant pneumococci we observed in children under 5 years old -120 patients (80.0%). In this age group 32.5% showed M phenotype and 67.5% of erythromycin-resistant *S.pneumoniae* were with MLS_B phenotype. The patients over 65 years old with infections, caused by ERSP were 17 (11.33%) and these between 18-65 years old were 13 (8.67%).

Macrolide resistance phenotypes

Resistance phenotypes that were determined by triple disk susceptibility method with erythromycin, clindamycin and rokitamycin, showed presence of iMcLS phenotype - 86 (57.33%) of all macrolide-resistant isolates, cMLS phenotype showed 16 (10.67%), four were with iMLS phenotype (2.67%) and 44 (29.33%) with M phenotype. (Tabl.1)

Macrolide resistance genotypes

Two different molecular genetic mechanisms of macrolide resistance have been described among pneumococci: targeted alteration in a result of methylase expression and erythromycin active cell efflux, encoded by *erm (B)* and *mef (E)* genes.

PCR analysis of the 150 macrolide-resistant pneumococcal isolates showed that 64.7% isolates harboured only the *erm (B)* gene and 29.3% only the *mef (E)* gene. Nine (6.0%) isolates carried both *erm (B)* and *mef (E)* gene. *Erm (B)*-positive isolates generally had higher MICs for erythromycin and clindamycin, while *mef (E)*-positive isolates had lower erythromycin MICs and remained susceptible to clindamycin. (Tabl.1)

Tabl.1. Genotypic and phenotypic characterization of 150 macrolide resistant *S.pneumoniae* isolates

Phenotype		Genotype		
		<i>ermB</i>	<i>mefE</i>	<i>ermB+mefE</i>
M		-	29.33%	-
MLS _B	iMcLS	57.33%	-	-
	cMLS	7.34%	-	3.33%
	iMLS	-	-	2.67%
Total number (%)		97 (64.67%)	44 (29.33%)	9 (6%)

According to specimen type, erythromycin resistance was found in 22.97% of isolates from sputum or the lower

respiratory tract (of which 52.93% were *erm* (B)-positive, 29.41% were *mef* (E) positive, and 17.66 % carried both *erm* (B) + *mef* (E)).

Macrolide resistance was found in 6.25% of isolates from blood (all *mef* (E) -positive), in 15 % of isolates from cerebrospinal fluid (all *erm* (B)-positive), in 25 % from sinus pus (of which 50% were *erm* (B)-positive and 50% carried both *erm* (B) + *mef* (E)). Resistant to erythromycin were 25% from eye fluids isolates (all *erm* (B)-positive). We observe prevalence again for *erm* (B) gene in 41.4% of isolates from nose or throat samples.

Of *S. pneumoniae* isolates from middle ear fluid samples, 61.3% were erythromycin-resistant and the distribution rates were: *erm*(B)-positive were 84.21%, *mef*(E) positive were 10.53% and only 5.26% were positive both to *erm* (B) and *mef* (E).

Although 80% of erythromycin-resistant isolates were from children aged < 5 years, only 32.5% were *mef*(E)-positive isolates from this age group. *Erm* (B)-positive isolates were 60.83% of erythromycin-resistant isolates in this group and only 8 (6.67%) carried both *erm* (B) and *mef*(E) genes.

These data suggest a link between high macrolide consumption and the selection of *erm* (B)-positive rather than *mef*(E)-positive strains.

Serotyping. A total of 109 strains of all 150 ERSP strains were tested up to now. ERSP serotypes/serogroups that we observed were: Serogroup 6- 31.19%, Serogroup 19 – 49.54%, Serotype 14 – 19.27%. Further analysis of serotyping were with factor specific antisera. Serotypes 6B, 19F and 19A were the most prevalent among ERSP with *erm*

(B) gene, and serotype 14 was predominant among strains harboring *mef* (E) gene.

CONCLUSION

Analysis of the genetic basis of macrolide resistance revealed two main genotypes, namely *erm* (B) and *mef*(E).

The study shows that resistance was associated predominantly with MLS_B cross-resistance to macrolides and lincosamides respectively with *erm* (B) and rare *mef* (E)-positive isolates. The efflux mechanism of macrolide resistance, which were predominant before in our country (5), now give place to widely spread MLS_B phenotype and genotype referring with target modification. This indicate that *erm* (B) remains the major macrolide-resistance mechanism among pneumococci in Bulgaria.

The dissemination of macrolide resistance encoded by *erm* (B) gene were 30% in 2000, increased to 43% in 2005 and 70% in 2008.

Serotypes 6B, 19F and 19A were the most prevalent among ERSP with *erm* (B) gene, and serotype 14 was predominant among strains harboring *mef* (E) gene.

Macrolides are widespread used antibiotics in therapy of pneumococcus infections in our country and ERSP increased with accelerated rates: from 20% in 2000, to 25% in 2005 (5), till 36% in the present research. Controlling the spread of resistant pneumococci through appropriate and judicious prescribing to reduce selective pressure is necessary. Efforts should be made to prevent pneumococcal infections in high-risk patients through vaccination.

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