

## CHARACTERISTICS OF ERYTHROMYCIN-RESISTANT CLINICAL ISOLATES OF *STREPTOCOCCUS PYOGENES*

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### ABSTRACT

Erythromycin resistance of clinical isolates *Streptococcus pyogenes* recovered in Bulgaria during 1995-2005 was studied by phenotypic and genetic methods. The frequency of erythromycin resistance was low during the whole period tested (0.5 to 4.1%). Fifty percent of the resistant isolates belonged to the MLS-inducible resistance phenotype, 38.9% were from the M phenotype and 11.1% were from the MLS-constitutive resistance phenotype. Two thirds (63.9%) of the erythromycin-resistant isolates were additionally resistant to tetracycline and / or chloramphenicol. Our data suggest that the resistance itself is not associated with increased pathogenicity of the clinical isolates *S. pyogenes*. The statistical analysis revealed that there was no difference between resistant and susceptible isolates regarding the following factors tested: production of protease, possession of *speA* (encoding streptococcal pyrogenic exotoxin A), *speF* (encoding streptococcal pyrogenic exotoxin F) and *prtF1* (encoding protein F1). The MLS-inducibly resistant isolates were more frequently serum-opacity factor positive (81.3%) than susceptible isolates (32.4%) ( $p < 0.05$ ). *Emm* sequence typing revealed that the erythromycin resistant isolates were a heterogenous group. Overlapping of sequence types between isolates of different resistance phenotypes was very rare. For isolates with the target site modification mechanism of resistance, *emm* 44/61 was the most frequent sequence type. For isolates with efflux mechanism, *emm* 12.10 was the most common one. The group of erythromycin-susceptible isolates was also heterogenous as a whole with *emm* 1, *emm* 3 and *emm* 12 being most common types. Overlapping of sequence types between susceptible and resistant isolates was observed for *emm* 1, *emm* 12, *emm* 75 and *emm* 77.

### INTRODUCTION

*Streptococcus pyogenes* is one of the common and important etiological agents of bacterial infections in humans, especially for children. It causes wide spectrum of clinical forms ranging from tonsillopharyngitis, scarlatina, impetigo, erysipelas to necrotizing fasciitis and life-threatening streptococcal toxic shock syndrome with rapid clinical

course within a few hours and mortality rate 30-70% (1).

All isolates of *S. pyogenes* which have been recovered until now have been found in vitro susceptible to penicillin and this remains the antibiotic of choice. However, about 25% of the patients fail to respond to penicillin in vivo. In such cases (and also for allergic patients) the alternatives are macrolide antibiotics (erythromycin, clarithromycin, azithromycin, spiramycin, etc.) and lincosamides (clindamycin).

There are two known mechanisms of resistance to erythromycin for *S. pyogenes*: target site modification and efflux mechanism (2, 3). The target site modification is due to two genes – *ermB* or *ermTR* (4). These are transferred by transposones and encode production of methylases which specifically methylate a certain aminoacid in the ribosomal RNA of the 23S ribosomal subunit. Macrolides, lincosamides and streptogramins group B share a common target site so the modification results in resistance to all these antibiotics. This mechanism issues two phenotypes - constitutive (MLS-cr) and inducible (MLS-ir). The efflux mechanism is due to a *mefA* gene (5), which is also transferred by transposones and encodes production of a pump which thrusts out specifically 14- and 15-membered macrolides but not 16-membered macrolides and lincosamides. This mechanism is presented by a third – M phenotype. Isolates of different phenotypes need different treatment so the differentiation is important (2): isolates of the MLS-constitutive phenotype are resistant to all macrolides, lincosamides and streptogramins group B so none of them can be used; for isolates from the MLS-inducible phenotype only 16-membered macrolides can be used (spiramycin, josamycin, midecamycin), yet it is possible that resistance develops during the treatment course; isolates of the M phenotype can be treated by lincosamides and 16-membered but not 14- (erythromycin, clarithromycin) and 15-membered (azithromycin) macrolides.

The Bulgarian National referent laboratory for streptococci and diphtheria has been monitoring the erythromycin resistance of clinical isolates *S. pyogenes* since 1995. Our data reveal that the frequency is low – about 2% in con-

trast to other countries (20-30%) (3, 6).

The aim of the present study is to characterize erythromycin-resistant clinical isolates of *Streptococcus pyogenes* in the following aspects: phenotype of resistance, multiresistance, clonality and pathogenicity. Clonality was studied by sequence typing of the *emm* gene which encodes the main pathogenic factor of *S. pyogenes* – the antiphagocytic M protein. More than 120 *emm* sequence types (with subtypes) have been identified until now in isolates of various parts of the world. Pathogenicity of the erythromycin-resistant isolates was studied with respect to the following pathogenic factors: streptococcal pyrogenic exotoxin A which is associated with the pathogenesis of scarlatina and toxic shock; streptococcal pyrogenic exotoxin F which probably plays role in the pathogenesis of toxic shock; protein F1 which helps the bacteria to enter the epithelial cells; pyrogenic exotoxin B (proteinase) which also acts as an invasin and is associated with tissue necrosis; serum-opacity factor – an adhesin whose production correlates with certain streptococcal M serotypes (*emm* sequence types).

## MATERIALS AND METHODS

We tested **1862 clinical isolates of *S. pyogenes*** which were recovered during the period 1995-2005 from patients with throat and skin infections. The isolates were identified by standard methods recommended by the World Health Organization (7).

**Antibiotic susceptibility testing** was performed by the disk diffusion method following the criteria of NCCLS for erythromycin, clindamycin, tetracycline and chloramphenicol (8).

**Phenotypes of erythromycin resistance** were defined by the double-disk diffusion test as described by Sepala et al. (3).

We performed **polymerase-chain reaction (PCR)** for detection of the following genes: *speA* – encoding production of streptococcal pyrogenic exotoxin A; *speF* – encoding production of streptococcal pyrogenic exotoxin F; *prtF1* – encoding production of protein F1. Primers and cycling parameters of the PCR have already been described by Bianco et al. (9).

We tested the strains for **proteinase** production by a proteinase agar method described by Hynes and Tagg (10).

Production of **serum-opacity factor** was tested by a microtechnique with horse serum, described by Johnson and Kaplan (11).

***emm* sequence typing** was performed on ABI 310 sequencing machine using the technique described on the following webpage of Centers of Disease Control and Prevention, Atlanta, GA, USA: [www.cdc.gov/ncidod/biotech/strep/emmtypes](http://www.cdc.gov/ncidod/biotech/strep/emmtypes). Minimal modifications were applied in quantities of reaction mixtures and amplification cycling due to the different class of the machinery used.

**Statistical analysis** was performed by  $\chi^2$  test with

Yates' correction for small numbers and with Fischer's  $\phi$  transformation.

## RESULTS

Table 1 presents the frequency of erythromycin resistance of *S. pyogenes* by years during 1995-2005. It has been low during the whole period tested – from 0.5 to 4.1% in the earlier years. The MLS-inducible phenotype was the most frequent and the MLS-constitutive phenotype was the rarest one.

Table 2 presents the resistotypes (towards tetracycline and chloramphenicol) within the different erythromycin-resistance phenotypes. From the whole number of thirty-six erythromycin-resistant strains, thirteen were susceptible to tetracycline and chloramphenicol (36.1%), twenty-two were resistant to tetracycline (61.1%), ten were resistant to chloramphenicol (28.1%), nine were resistant to both tetracycline and chloramphenicol (25%). At first glance it seemed that multiresistance was a more frequent phenomenon in those strains which had acquired erythromycin resistance through target site modification than those with efflux mechanism but the statistical analysis of the data proved that there was no significant difference in the number of multiresistant strains between the different phenotypes ( $p > 0.05$ ).

The distribution of pathogenic factors within erythromycin-resistant and susceptible isolates of *S. pyogenes* is presented in table 3. Proteinase production and possession of the *speF* gene were quite frequent in all isolates. The genes *speA* and *prtF1* were equally distributed in both groups of resistant and susceptible isolates. The statistical analysis of the data by  $\div 2$  with Yates' correction for small numbers and by Fischer's  $\phi$  transformation, revealed that there was no difference between erythromycin-resistant and susceptible isolates with respect to the three genes tested *speA*, *speF*, *prtF1* and proteinase production. However, there was statistically significant difference in the production of serum-opacity factor between the MLS-inducibly resistant isolates and the susceptible isolates ( $p < 0.05$ ).

Table 4 presents the *emm* sequence types which we identified in thirty erythromycin-resistant and thirty-five susceptible isolates. The group of erythromycin-resistant isolates was heterogeneous as a whole. When comparing the *emm* sequence types of isolates of different phenotypes, it could be seen that except one case of *emm* 4, there was no overlapping – the different phenotypes were presented by different genotypes. For isolates with the target site modification mechanism of resistance, *emm* 44/61 was the most frequent sequence type. For the isolates with efflux mechanism, *emm* 12.10 was the most common one. This allele variation of type 12 has for the first time been found in Bulgarian isolates in 2001 and has been already described (12). The group of erythromycin-susceptible isolates was heterogeneous as a whole with *emm* 1, *emm* 3 and *emm* 12 being

most common. For the majority of isolates tested, the sequence types found in sensitive strains were different from those in resistant ones but *emm* 1, *emm* 12, *emm* 75 and *emm* 77 were found in both groups.

## DISCUSSION

Erythromycin resistance of *Streptococcus pyogenes* was a rare phenomenon in Bulgaria for the period tested 1995-2005 (about 2%). At present, no trend for increase could be seen. This means that macrolides and lincosamides remain proper alternatives for penicillin treatment failure cases and for allergic patients. The MLS-inducible phenotype is the dominant one for Bulgaria. It presents the phylogenetically older mechanism of resistance - target site modification. In regions where high resistance rate has been registered, the phenomenon usually has been due to expansion of clones with the efflux mechanism of resistance which is the newer mechanism for *S. pyogenes*, widely investigated in the last decade of the XXth century (3, 5, 6).

Two thirds (63.9%) of the erythromycin-resistant isolates were additionally resistant to tetracycline and / or chloramphenicol. Tetracycline resistance in erythromycin-susceptible isolates varied from 9 to 38% for the same period but for most of the years it was about 20%. It seems that tetracycline resistance is more common among erythromycin-resistant isolates in comparison to susceptible ones. It is probable that transposones which transfer erythromycin-resistance genes, also transfer genes for tetracycline re-

sistance.

Our data show that the resistance itself is not associated with increased pathogenicity of the clinical isolates of *S. pyogenes*. The statistical analysis revealed that there was no difference between resistant and susceptible isolates regarding the factors tested: production of protease, possession of *speA*, *speF*, *prtF1*.

The serum-opacity factor (SOF) divides the species *Streptococcus pyogenes* in two main groups of isolates: producers (SOF+) and nonproducers (SOF-). It is well known that each of the two groups is associated with certain M serotypes (*emm* sequence types, respectively) (7). The statistically significant difference ( $p < 0.05$ ) which we found for the production of SOF between susceptible isolates (32.4%) and MLS-inducibly resistant isolates (81.3%) suggests that isolates that belong to SOF+ serotypes more frequently are infected by transposones carrying *erm B* and / or *ermTR* in comparison to isolates that belong to SOF- serotypes.

*Emm* sequence typing revealed that the erythromycin-resistant isolates of *S. pyogenes*, recovered in Bulgaria during the last decade are a heterogenous group and this correlates with the low frequency of erythromycin resistance. Although *emm* 44/61 was the most frequent sequence type for isolates with target site modification and *emm* 12.10 was the most common one for isolates with efflux mechanism of resistance, at present there is no evidence for domination of a certain clone.

**Table 1.** Frequency and phenotypes of erythromycin-resistant isolates of *Streptococcus pyogenes*

period	Total number of isolates tested	Erythromycin-resistant isolates % (number)	Phenotype of erythromycin resistance		
			MLS-cr	MLS-ir	M
1995	98	4.1% (4)	25% (1)	75% (3)	0% (0)
1996	61	3.3% (2)	50% (1)	0% (0)	50% (1)
1997	235	3.8% (9)	0% (0)	77.8% (7)	22.2% (2)
1998	320	1.9% (6)	0% (0)	16.7% (1)	83.3% (5)
1999	121	1.7% (2)	0% (0)	100% (2)	0% (0)
2000	118	0.9% (1)	0% (0)	0% (0)	100% (1)
2001	373	0.5% (2)	0% (0)	100% (2)	0% (0)
2002	207	0.97% (2)	0% (0)	50% (1)	50% (1)
2003	126	2.4% (3)	33.3% (1)	33.3% (1)	33.3% (1)
2004	110	2.7% (3)	0% (0)	33.3% (1)	66.7% (2)
2005	93	2.2% (2)	50% (1)	0% (0)	50% (1)
<b>1995-2005</b>	<b>1862</b>	<b>1.93% (36)</b>	<b>11.1% (4)</b>	<b>50.0% (18)</b>	<b>38.9% (14)</b>

**Table 2.** Resistotypes of erythromycin-resistant isolates of *Streptococcus pyogenes*

Phenotype of erythromycin resistance	resistotype	Number of isolates
4 isolates MLS-cr	Tet <sup>S</sup> Chl <sup>S</sup>	3
	Tet <sup>R</sup> Chl <sup>S</sup>	1
18 isolates MLS-ir	Tet <sup>S</sup> Chl <sup>S</sup>	3
	Tet <sup>R</sup> Chl <sup>S</sup>	10
	Tet <sup>R</sup> Chl <sup>R</sup>	5
14 isolates M	Tet <sup>S</sup> Chl <sup>S</sup>	7
	Tet <sup>R</sup> Chl <sup>S</sup>	2
	Tet <sup>R</sup> Chl <sup>R</sup>	4
	Tet <sup>S</sup> Chl <sup>R</sup>	1

Tet – tetracycline; Chl - chlorampheni

**Table 3.** Distribution of SOF, *prtF1*, *speA*, *speF*, proteinase according to erythromycin resistance / susceptibility of *Streptococcus pyogenes*

factor	Susceptible isolates 37	Resistant isolates 29				
		All resistant isolates 29	MLS-cr 3	MLS-ir 16	MLS cr + ir 19	M 10
SOF +	12 (32.4%)	16 (55.2%)	0	13 (81.3%)	13 (68.4%)	3 (30.0%)
<i>PrtF1</i> +	20 (54.1%)	11 (37.9%)	0	7 (43.8%)	7 (36.8%)	4 (40.0%)
<i>SpeA</i> +	24 (64.9%)	20 (69.0%)	3 (100%)	10 (62.5%)	13 (68.4%)	7 (70.0%)
<i>SpeF</i> +	37 (100%)	25 (86.2%)	3 (100%)	13 (81.2%)	16 (84.2%)	9 (90.0%)
Protease +	34 (91.9%)	28 (96.6%)	3 (100%)	15 (93.8%)	18 (94.7%)	10 (100%)

**Table 4.** *Emm* sequence types (genotypes) in erythromycin-resistant and susceptible isolates of *Streptococcus pyogenes*

Phenotype	<i>emm</i> sequence type (number of isolates)
MLS-cr 4 isolates	<i>Emm</i> 1 (2)
	Not tested (2)
MLS-ir 18 isolates	<i>Emm</i> 4 (3)
	<i>Emm</i> 27 L (1)
	<i>Emm</i> 43.4 (1)
	<i>Emm</i> 44/61 (5)
	<i>Emm</i> 77 (3)
	<i>Emm</i> 80 (1)
	<i>Emm</i> 117 (3)
	Not tested (1)
M 14 isolates	<i>Emm</i> 4 (1)
	<i>Emm</i> 12 (1)
	<i>Emm</i> 12.10 (5)
	<i>Emm</i> 33 (1)
	<i>Emm</i> 75 (1)
	<i>Emm</i> 78 (2)
	Not tested (3)

S (susceptible) 35 isolates	<i>Emm</i> 1	(8)
	<i>Emm</i> 2	(1)
	<i>Emm</i> 3	(5)
	<i>Emm</i> 6	(3)
	<i>Emm</i> 12	(4)
	<i>Emm</i> 18	(1)
	<i>Emm</i> 28	(2)
	<i>Emm</i> 49	(1)
	<i>Emm</i> 59	(1)
	<i>Emm</i> 65	(2)
	<i>Emm</i> 74	(1)
	<i>Emm</i> 75	(1)
	<i>Emm</i> 77	(1)
	<i>Emm</i> 78	(1)
	<i>Emm</i> 123	(1)
ST-2974	(1)	
ST-4935	(1)	

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