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Erythromycin-Resistant Group A Streptococcal Isolates Recovered in Sofia, Bulgaria, from 1995 to 2001

Antoaneta Detcheva,[1]* Richard R. Facklam,[2] and Bernard Beall[2]

National Center of Infectious and Parasitic Diseases, Sofia 1504, Bulgaria,[1] and World Health Organization Collaborating Center for Streptococci, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[2]

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The frequency of erythromycin resistance within group A streptococci in Sofia, Bulgaria, from 1995 to 2001 was 2.1% (26 isolates). Of this, 57.7% was macrolide-lincosamide-streptogramin (MLS) inducible, 7.7% was MLS constitutive, and 34.6% had the M phenotype. Eleven different *emm* sequence types were found among 25 erythromycin-resistant isolates tested. Nineteen of 26 erythromycin-resistant isolates were additionally resistant to tetracycline and/or chloramphenicol.

Group A streptococci (GAS) are common and important pathogens of humans, causing a wide variety of clinical manifestations that can involve virtually any tissue.

Penicillin has been used routinely for treating GAS infections for over 50 years, and yet GAS have remained penicillin susceptible. Macrolides are the first alternative for allergic patients and for cases where there is a poor response to penicillin. Unlike that of penicillin, the efficacy of clindamycin (a lincosamide) is not affected by GAS inoculum size or growth stage (13). Clindamycin also inhibits M-protein synthesis, thereby possibly promoting phagocytosis of GAS (13).

At present there are two known mechanisms of erythromycin resistance in GAS (9, 11, 12, 14). Methylation of 23S rRNA due to the *ermB*- or *ermTR*-encoded methylase results in the inability of all macrolides, lincosamides, and streptogramin B to bind to their common target site in the 50S ribosomal subunit (MLS type). The methylase can be expressed constitutively (MLS-cr phenotype) or inducibly (MLS-ir phenotype). The second mechanism, characteristic of the so-called M phenotype, involves energy-dependent efflux of 14- and 15-membered macrolides but not 16-membered macrolides, lincosamides, and streptogramin B. This efflux mechanism is encoded by the *mefA* gene (3, 11, 14).

Here we describe the overall incidence of macrolide resistance in GAS clinical isolates recovered in Sofia, Bulgaria. We also describe general features of these isolates, including resistance to other antibiotics, T-agglutination profiles, and *emm* gene sequence types. This work establishes the presence of 11 different strain types of GAS in Sofia exhibiting erythromycin resistance.

(Some of these results were presented at the XIV Lancefield International Symposium on Streptococci and Streptococcal Diseases, 1999, and were included in the proceedings of the symposium [4a]).

We tested 1,221 epidemiologically nonrelated GAS isolates recovered from 1995 to 2001 in five laboratories which process more than 50% of the microbiological samples in Sofia: 1,122 pharyngeal swabs (695 from patients with tonsillitis, tonsillopharyngitis, pharyngitis, or rhinopharyngitis; 339 from patients with scarlet fever or persons in contact with scarlet fever patients; and 88 from healthy carriers), 1 puncture of peritonsillar abscess, 55 nasal swabs (42 from patients with rhinopharyngitis, 8 from patients with scarlet fever, and 5 from healthy carriers), and 43 swabs from skin lesions (39 from patients with impetigo and 4 from patients with erysipelas). One thousand seventy-six of the isolates came from children in the age groups 0 to 4, 5 to 9, and 10 to 14 years.

Serologic group identification was performed by the capillary precipitin test of antigen extracts derived by Fuller's method and rabbit group-specific antisera (10).

Susceptibility testing. MICs of erythromycin and clindamycin (Sigma Chemical Co., St. Louis, Mo.) were measured by the agar dilution method for all isolates. Additionally, MICs of clarithromycin (Pfizer, Inc., New York, N.Y.), azithromycin (Pfizer, Inc.), and spiramycin (Rhone-Poulenc Rorer, Paris, France) were determined for all erythromycin-resistant and 96 random susceptible isolates. We followed procedures and breakpoints recommended by the NCCLS (6). Breakpoints for spiramycin were those proposed by the French Society for Microbiology (4).

Tetracycline and chloramphenicol resistance and susceptibility were determined by the Kirby-Bauer disk diffusion test (7) with commercial disks from Becton Dickinson Microbiology Systems (Cockeysville, Md.).

The double-disk diffusion test was performed as described by Seppälä et al. (11), except that we used different concentrations (15 μg of erythromycin and 2 μg of clindamycin [Becton Dickinson Microbiology Systems]) as described previously (16).

Induction experiments were carried out for the MLS-ir and M-phenotype isolates in order to confirm the phenotype as described by Seppälä et al. (11).

Subtyping. T-agglutination patterns and opacity factor reactions were determined as previously described (10). PCR of the *emm* gene and sequence analysis were performed as described on the website www.cdc.gov/ncidod/biotech/strep/protocols.htm. The *emm* gene sequences corresponding to the *emm* types and alleles listed for the isolates in this study are found on the website www.cdc.gov/ncidod/biotech/strep/emmtypes

* Corresponding author. Mailing address: National Center of Infectious and Parasitic Diseases, 26 Yanko Sakazov Blvd., Sofia 1504, Bulgaria. Phone: 359 2 9446999-314. Fax: 359 2 9433075. E-mail: Detcheva@hotmail.com.

TABLE 1. Levels of macrolide resistance found in GAS isolates in Sofia

Antibiotic	MIC range (μg/ml) for isolates with phenotype:			
	Erythromycin resistant			Susceptible
	MLS-cr	MLS-ir	M	
Erythromycin	1–8	1–4	0.5–4	0.03–0.125
Clarithromycin	0.5–4	1–2	0.5–4	0.03–0.125
Azithromycin	1–8	2–32	1–8	0.125–0.25
Spiramycin	2	0.5–2	0.5	0.03–0.5
Clindamycin	0.5	0.03–1→64–>256	0.015–0.125	0.03–0.125

.htm. One of these 26 isolates was not available for *emm* typing.

Among the 1,221 isolates, we found 26 that were erythromycin resistant: 20 from throat swabs (12 from patients with tonsillitis, tonsillopharyngitis, or pharyngitis; 6 from patients with scarlet fever; and 2 from healthy carriers), 1 from a puncture of a peritonsillar abscess, and 5 from swabs from skin lesions (4 from patients with impetigo and 1 from a patient with erysipelas). Twenty-four isolates were from children 1 to 12 years of age, one isolate was from a 23-year-old patient with pharyngitis, and one isolate was from a 40-year-old patient with erysipelas. These data indicated a low frequency of erythromycin resistance of GAS in Sofia, 2.1% for the whole period tested, varying from 4.1% in 1995 to 0.8% in 2001. These isolates represented each of the three different phenotypes of erythromycin resistance, with the MLS-ir phenotype being the most common (57.7% of the resistant isolates). The MLS-cr phenotype was least common (7.7%) and was not observed in isolates recovered after 1996. The M phenotype was found in 34.6% of the resistant isolates.

Table 1 presents the level of resistance of the erythromycin-resistant isolates to 14-, 15-, and 16-membered macrolides and to clindamycin. MLS-cr isolates were resistant to 14-, 15-, and 16-membered macrolides and clindamycin, but the level of resistance was low. MLS-ir isolates displayed a low level of resistance to the 14-membered macrolides erythromycin and clarithromycin, while MICs of the 15-membered macrolide azithromycin were higher (up to 32 μg/ml). Isolates exhibited either low-level resistance or sensitivity to clindamycin and the 16-membered macrolide spiramycin before the induction; however, after induction with a subinhibitory concentration of erythromycin, MICs of clindamycin increased to 64 to ≥256 μg/ml, indicating inducible resistance. Isolates with the M phenotype showed low-level resistance to 14- and 15-membered macrolides but were susceptible to spiramycin and clindamycin, with no inducible resistance evident.

Eleven different *emm* types were found among 25 erythromycin-resistant isolates (Table 2). Within the *emm* types found in multiple isolates, identical or closely similar T-agglutination patterns were found. The T-agglutination patterns associated with each of the 11 *emm* types were identical or closely similar to those of Centers for Disease Control and Prevention (CDC) reference strains for these specific *emm* sequence types.

The two MLS-cr isolates were T pattern 1 and type *emm1*. The 15 MLS-ir isolates comprised six different *emm* sequence types with *emm44/61* being most frequent, followed by *emm4* and *emm117*. Single isolates of types *emm43.4*, *emm77*, and *emm80* were also found. The M phenotype was found in nine isolates of types *emm12.10*, *emm78*, *emm75*, and *emm33*, with multiple isolates found within types *emm12.10* (five isolates) and *emm78* (two isolates).

The *emm12.10* allele differs by only a single deduced conservative substitution from type *emm12* (asparagine to serine at amino acid 36 of the mature M protein). We found this *emm12* allele in five independent erythromycin-resistant isolates recovered from individuals in Sofia, and we have not encountered this allele out of several hundred type *emm12* isolates recovered from patients with noninvasive and invasive infections in several other countries over the past 7 years. It was also interesting that no *emm* sequence types were found both in isolates with MLS phenotypes and in isolates with the M phenotype.

Nineteen erythromycin-resistant isolates were additionally resistant to tetracycline or both tetracycline and chloramphenicol (Table 3). The two MLS-cr isolates were susceptible to tetracycline and chloramphenicol. Within the MLS-ir and M-phenotype isolates, three resistance patterns were observed. Four of the six multiply represented *emm* types displayed more than one resistance pattern. For example, *emm117* was represented by all three resistance patterns.

Our results revealed that erythromycin resistance within GAS was rare in Sofia from 1995 to 2001, with an incidence of only 2.1%. In contrast, the incidence of erythromycin resis-

TABLE 2. Serotypes and *emm* sequence types of erythromycin-resistant GAS in Sofia

Erythromycin resistance phenotype	T pattern	*emm* sequence type	No. and source of isolates
MLS-cr	1	*emm1*	2 (1, tonsillitis; 1, scarlet fever)
MLS-ir	5/27/44	*emm44/61*	5 (1, healthy carrier; 1, tonsillitis; 1, scarlet fever; 2, impetigo)
	4/28	*emm4*	3 (1, healthy carrier; 2, pharyngitis)
	11/12/B3264	*emm117*	3 (2, impetigo; 1, erysipelas)
	3/13/B3264	*emm43.4*	1, tonsillitis
	3/13/B3264	Not tested	1, tonsillopharyngitis
	13	*emm77*	1, scarlet fever
	25	*emm80*	1, peritonsillar abscess
M	12	*emm12.10*	5 (2, scarlet fever; 2, tonsillitis; 1, tonsillopharyngitis)
	11	*emm78*	2, tonsillopharyngitis
	3/13/B3264	*emm33*	1, tonsillitis
	8/25	*emm75*	1, scarlet fever

TABLE 3. Resistance patterns of erythromycin-resistant GAS in Sofia

Erythromycin resistance phenotype	Resistance pattern	% (no. of isolates)	*emm* sequence type(s) (no. of isolates)
MLS-cr	Tet^s Chl^s	100.0 (2)	*emm1* (2)
MLS-ir	Tet^s Chl^s	13.3 (2)	*emm117* (1), not tested (1)
	Tet^r Chl^s	53.3 (8)	*emm80* (1), *emm4* (3), *emm43.4* (1), *emm117* (1), *emm44/61* (1), *emm77* (1)
	Tet^r Chl^r	33.3 (5)	*emm117* (1), *emm44/61* (4)
M	Tet^s Chl^s	33.3 (3)	*emm78* (1), *emm33* (1), *emm75* (1)
	Tet^r Chl^s	22.2 (2)	*emm78* (1), *emm12.10* (1)
	Tet^r Chl^r	44.4 (4)	*emm12.10* (4)

tance among invasive GAS isolates recovered in the United States in 1999 was 7.1% (C. Van Beneden, R. Facklam, R. Lynfield, et al., poster presented at the International Conference on Emerging Infectious Diseases, Atlanta, Ga., 24 to 27 March 2002.). In a recent survey of 109 random GAS isolates recovered in Italy, 28% were found to be erythromycin resistant (G. Dicuonzo and G. Gherardi, unpublished data). In Bulgaria macrolides are rarely prescribed for GAS infections and are prescribed mainly for allergic patients and patients who respond poorly to penicillin. We feel that it is likely that the low number of macrolide prescriptions given in Bulgaria is a primary reason for the low rate of erythromycin resistance of GAS observed in Sofia. Moreover, all resistant isolates had low-level resistance to erythromycin (MICs not exceeding 8 μg/ml), clarithromycin (MICs of ≤4 μg/ml), spiramycin (MIC of 2 μg/ml), clindamycin (MIC of ≤1 μg/ml), and azithromycin (MIC of ≤32 μg/ml) relative to isolates from countries with a higher incidence of erythromycin resistance.

Within 25 isolates, 11 *emm* sequence types could be distinguished, indicating that there are at least 11 different GAS clonal types in Sofia associated with erythromycin resistance. Seven *emm* sequence types were associated with MLS phenotypes, and four *emm* types were associated with the M phenotype. As has been seen elsewhere (Dicuonzo and Gherardi, unpublished), the majority of erythromycin-resistant isolates were of *emm* types that are also commonly associated with the presence of the serum opacity factor-fibronectin binding gene (*sof*). Previous data have shown the presence of the *sof* gene in types *emm44/61*, *emm4*, *emm117*, *emm12*, *emm78*, and *emm75*, which comprised 20 of the 25 isolates (80%) in this study of known *emm* type (1). Possibly *sof* contributes to differing tissue specificities exhibited by *sof*-positive strains relative to *sof*-negative strains, effecting physical separation and reducing the frequency of horizontal transfer events between these two groups.

We found the *emm12.10* allele in five independent isolates. Since we have not encountered *emm12.10* elsewhere, it is possible that the population from which we obtained these GAS isolates is relatively segregated. However, except for types *emm117*, *emm33*, and *emm43* (subtype *emm43.4*), all of the sequences described in this study have been associated with erythromycin resistance in other countries (2, 5, 8, 15; B. Beall, unpublished data), indicating that the majority of erythromycin-resistant GAS strains could have originated in other countries prior to their introduction into Sofia.

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