

## Prevalence and Mechanisms of Macrolide Resistance in Clinical Isolates of Group A Streptococci from Ontario, Canada

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Received 4 February 1999/Returned for modification 19 May 1999/Accepted 24 June 1999

**A total of 3,205 group A streptococcal isolates were collected in 1997 through a private laboratory which serves community physicians in southern Ontario and which represents a population base of 6 million people. Nonsusceptibility to erythromycin was detected for 67 (2.1%) isolates both by disk diffusion and by broth microdilution. Of these, 47 (70%) were susceptible to clindamycin and were found by PCR to possess the *mef* gene. Of the other 20 strains, 18 and 2 showed inducible and constitutive resistance, respectively, to clindamycin. Nineteen of these strains were shown by PCR to possess the *ermTR* gene, and a single constitutively resistant strain harbored an *ermB* gene. Sixteen (24%) erythromycin-resistant strains were also resistant to tetracycline. All were susceptible to penicillin and chloramphenicol.**

Although there have been no confirmed reports of decreased susceptibility to penicillin in group A streptococci (GAS), resistance to erythromycin has emerged in some countries. Rates of resistance in Europe in the last decade have ranged from 10% in Sweden (13) up to 17% in Finland (15), 22% in the United Kingdom (23), and 47% in parts of Italy (3). Rates of resistance in excess of 50% have been reported in Taiwan and Japan (12, 19). Prevalence rates in some countries are still low, as in Argentina, where the current rate of erythromycin resistance is less than 1% (18).

Macrolides exert their action by binding to the bacterial ribosome and inhibiting protein synthesis. Resistance in streptococci has been shown to arise either through the presence of *erm* gene-encoded methyltransferases which induce ribosomal modification or through efflux. Macrolide efflux in GAS is effected by a membrane protein encoded by the *mefA* gene and is specific for 14- and 15-member macrolides such as erythromycin, clarithromycin, and azithromycin; the lincosamides and streptogramin antibiotics remain unaffected (M phenotype) (2). Erm methylases methylate 23S rRNA and induce ribosomal modification, which results in loss of binding not only to macrolides but also to lincosamides and the streptogramin B class of antimicrobials (MLS<sub>B</sub> phenotype) (29). Several methylases have been described for gram-positive bacteria (6); the ErmB (ErmAM) group is that most commonly found in streptococci (17). The *ermTR* gene in GAS has more homology to the *ermA* gene (83%) found in staphylococci than to the *ermB* gene (58%) from GAS (25). In a recent report, it was noted that in Finland, 60% of erythromycin-resistant strains had the M phenotype and all but one of the remaining strains with the MLS<sub>B</sub> phenotype had the *ermTR* gene (16).

Strains with a particular mechanism of resistance may have different prevalence rates depending on the country of origin. For example, with erythromycin-resistant *Streptococcus pneumoniae*, the M phenotype predominates in strains isolated in Canada and the United States (14, 28), whereas in South Africa, the MLS<sub>B</sub> phenotype is more prevalent (30). Although

GAS strains with the M phenotype have been reported in the United States (4), there is little information about prevalence rates of *mef* and *erm* genes.

In this study we looked at the prevalence of erythromycin resistance in GAS clinical isolates collected in southern Ontario and examined their mechanisms of resistance.

### MATERIALS AND METHODS

**Strains.** From August to November 1997, all consecutive clinical isolates of GAS, 3,205 strains in total, were collected by a private laboratory serving community physicians throughout southern Ontario, Canada, representing a population base of 6 million people. Strains were identified by PathoDx (Diagnostic Products Corp., Los Angeles, Calif.).

**Susceptibility testing.** Erythromycin-resistant strains were initially identified by disk diffusion on Mueller-Hinton agar supplemented with 5% sheep blood using 15- $\mu$ g erythromycin disks (Becton Dickinson, Cockeysville, Md.) according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (21). MICs of penicillin, erythromycin, clindamycin, tetracycline, chloramphenicol (Sigma-Aldrich, Canada, Oakville, Ontario), and quinopristin-dalfopristin (Rhône-Poulenc Rorer, Collegeville, Pa.) were determined for erythromycin-resistant strains by using cation-supplemented Mueller-Hinton broth with 2% lysed horse blood in accordance with NCCLS guidelines (21). Incubation was performed at 37°C in O<sub>2</sub>, except for two strains which required CO<sub>2</sub> for growth. *S. pneumoniae* ATCC 49619 and ATCC 6303 and *Enterococcus faecalis* ATCC 29212 were used as controls. In order to distinguish between M and MLS<sub>B</sub> phenotypes, erythromycin (15  $\mu$ g) and clindamycin (2  $\mu$ g) disks were placed on plates approximately 12 mm apart, and the plates were incubated overnight at 37°C in 5% CO<sub>2</sub>. Blunting of the growth inhibition zone around clindamycin in the area between the two disks was considered to indicate inducible MLS<sub>B</sub> resistance (MLS<sub>B</sub> phenotype); constitutive resistance was defined as zones of  $\leq 15$  mm around both the clindamycin and the erythromycin disk (21). The M phenotype was characterized by resistance to erythromycin and susceptibility to clindamycin.

**PCR.** For the identification of *ermABC* and *mef* genes, multiplex PCR was performed with published primer sequences (27). Template DNA was prepared by mixing a loopful of bacteria grown overnight on blood agar with 100  $\mu$ l of lysis buffer (100 mM NaCl, 10 mM Tris HCl [pH 8.3], 1 mM EDTA [pH 8.0], 1% Triton X-100) and boiling in a water bath for 10 min. After cooling, the suspensions were spun, and 5  $\mu$ l of the supernatant was used as a template. Reactions were performed in a Perkin-Elmer 9600 thermocycler with a final reaction volume of 25  $\mu$ l by using 4 mM MgCl<sub>2</sub> and the following cycling conditions: denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. A final elongation step was performed at 72°C for 5 min. The following primer sequences based on the sequence of the *ermTR* gene (GenBank accession no. AF002716) were used for detection of *ermTR*: GAAGTTTAGCTTTCCTAA (*ermTRA*, sense, 5' to 3') and GCTTCAGCACCTGCTTAATTGAT (*ermTRB*, antisense, 5' to 3'). Control strains were GAS 0261110 (*ermTR*) and *S. pneumoniae* 02J1175 (*mefE*), both kindly provided by J. Sutcliffe (Pfizer, Groton, Conn.). Reaction conditions were as described above except that 1.5 mM MgCl<sub>2</sub> was used and amplification was carried out for 35 cycles with

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TABLE 1. In vitro susceptibility of 67 GAS strains to select antimicrobial agents

Antimicrobial agent	MIC ( $\mu\text{g/ml}$ )		Range
	50%	90%	
Erythromycin	8.0	16.0	1.0->16.0
Clindamycin	0.25	0.25	0.25->16.0
Quinupristin-dalfopristin	<0.12	0.12	<0.12-1.0
Tetracycline	1.0	16.0	$\leq$ 1.0-32.0

denaturation at 94°C for 30 s and annealing at 42°C for 30 s. PCR products were resolved by electrophoresis on 1% agarose gels. The expected sizes were 640 bp for *ermA*, *ermB*, or *ermC*; 348 bp for *mef*; and 400 bp for *ermTR*.

**Southern hybridization.** Genomic DNA was isolated by lysis of a cell pellet derived from 1.5 ml of an overnight culture. Cells were washed with 1.0 ml of lysis buffer (50 mM glucose, 25 mM Tris [pH 8.0], 10 mM EDTA [pH 8.0], 150 mM NaCl) and incubated in 0.5 ml of lysis mixture consisting of lysis buffer plus 10  $\mu\text{l}$  of DNase-free RNase (10 mg/ml), 50  $\mu\text{l}$  of mutanolysin (1 mg/ml), and 50  $\mu\text{l}$  of lysozyme (10 mg/ml) (all components were purchased from Sigma-Aldrich, Canada). Following lysis, DNA was extracted as described by Smith et al. (26) and resolved by electrophoresis on 1% agarose gels. Southern blotting was performed according to standard procedures (1). Hybridization was carried out with *mefAE*, *ermB*, and *ermTR* probes, derived by PCR as described above, from the following control strains. *S. pneumoniae* 02J1175 was used for *mefE*, and GAS 0261110 was used for *ermTR*. The *ermB* probe was amplified from *S. pneumoniae* BSP3585, which contains Tn1545 harboring *ermB*. *Staphylococcus aureus* RN4220, harboring plasmid pE194, was used for *ermC*, and *ermA* was amplified from *Escherichia coli* RN7951, containing an *ermA* gene from Tn554 cloned into pUC18 (both kindly provided by B. Kreiswirth, Public Health Research Institute, New York, N.Y.). Labeling and detection were carried out by using the ECL direct labeling enhanced chemiluminescence kit (Amersham Life Science, Oakville, Ontario, Canada) as outlined by the manufacturer.

**PFGE.** DNA to be used for pulsed-field gel electrophoretic (PFGE) analysis was extracted essentially as described by Murray et al. (20) with modifications. A sample of  $10^8$  CFU from an overnight culture was washed in PIV buffer (1 M NaCl-10 mM Tris-HCl [pH 7.6]) and resuspended in 750  $\mu\text{l}$  of the same buffer. An equal volume of 1.6% low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) at 65°C was added to the PIV-bacterial suspension; approximately 260  $\mu\text{l}$  was aliquoted into PFGE plug molds (Bio-Rad Laboratories) and cooled to 4°C for 30 min. Plugs were incubated at 37°C in 1.5 ml of lysis mixture (100 mM Tris-HCl at pH 8.0-50 mM EDTA-1 M NaCl) containing 67  $\mu\text{g}$  of RNase (Boehringer-Mannheim Canada, Laval, Quebec)/ml, 33  $\mu\text{g}$  of mutanolysin/ml, and 300  $\mu\text{g}$  of lysozyme (Sigma-Aldrich, Canada)/ml. After 5 h, 50  $\mu\text{l}$  of 10-mg/ml proteinase K (Boehringer-Mannheim Canada) was added to the lysis mixture, and incubation was continued for 18 h. Plugs were washed three times with TE buffer (10 mM Tris-HCl-0.1 mM EDTA [pH 7.5]) and incubated with 10 U of *SmaI* (Boehringer-Mannheim Canada) at 25°C. DNA was resolved by using a contour-clamped homogeneous electric field (CHEF)-DRII apparatus (Bio-Rad Laboratories) in a 1% agarose gel at 175 V with an initial pulse time of 5 s and a final pulse time of 60 s for 24 h at 12°C in 0.5 $\times$  Tris-borate-EDTA.

**M typing.** Serotyping was carried out by the National Reference Center for Streptococci, Edmonton, Alberta, Canada, according to standard protocols (10).

## RESULTS

**Phenotypic characterization.** Of 3,205 isolates tested, 67 (2.1%) were found to be erythromycin resistant by disk diffusion. These isolates were also found to be erythromycin resistant by broth microdilution tests. Disk susceptibility results demonstrated that 47 (70%) were of the M phenotype and 20 (30%) were of the MLS<sub>B</sub> phenotype. Of the 20 MLS<sub>B</sub> resistant strains, 18 demonstrated blunting of the clindamycin inhibition zone when placed 12 mm from an erythromycin disk, indicating inducible resistance. Only two strains showed constitutive resistance to clindamycin.

Erythromycin MICs ranged from 1 to >16  $\mu\text{g/ml}$  (Table 1). The MIC at which 90% of strains were inhibited (MIC<sub>90</sub>) of erythromycin for MLS<sub>B</sub> strains was 4  $\mu\text{g/ml}$ , whereas that for strains demonstrating the M phenotype was 16  $\mu\text{g/ml}$ . For all the erythromycin-resistant strains, including MLS<sub>B</sub> strains, the MIC of quinupristin-dalfopristin was 0.25  $\mu\text{g/ml}$  or lower. Methylation of 23S rRNA results in resistance to streptogramin B-type compounds such as dalfopristin but not to strep-

togramin A-type compounds such as quinupristin, and synergy between the two compounds is conserved. All strains were susceptible to chloramphenicol and penicillin, but 16 (0.5%) were not susceptible to tetracycline; of these, 14 were of the MLS<sub>B</sub> phenotype.

**Genotypic characterization.** As predicted, all of the 47 (70%) strains bearing an M phenotype yielded an amplification product of 348 bp, which was consistent with the presence of the *mef* gene. The primers chosen in this study were based on conserved regions of the *mefA* gene from GAS and the *mefE* gene from *S. pneumoniae*, which have 90% homology. None of the strains with an M phenotype contained an *erm* determinant. Surprisingly, in the multiplex PCR assay using *ermABC* primers, only a single (constitutively resistant) MLS<sub>B</sub> strain yielded a product consistent with the presence of *ermA*, *ermB*, or *ermC*. This strain, for which the MIC was >16  $\mu\text{g/ml}$ , was shown by Southern blotting to possess an *ermB* gene. However, when primers based on the *ermTR* gene were used, 19 strains were found to harbor *ermTR* (Table 2). Southern blotting of 12 representative strains confirmed these results. No hybridization with any of the probes was observed for five randomly selected erythromycin-sensitive strains. MICs for strains harboring *ermTR* genes generally were lower than those for strains possessing *mef* genes (Fig. 1).

**PFGE.** Of the 20 strains harboring *ermTR*, 12 showed different patterns, indicating that they were not clonal in origin. Despite several attempts, it was possible to resolve only 11 of 46 *mef*<sup>+</sup> strains by PFGE, and among these, nine patterns were observed. No reason can be given for the lack of success in resolving M phenotype strains by PFGE, but this phenomenon has been noted by other groups (9).

TABLE 2. Correlation of phenotype, M type, and genotype

No. of strains with the indicated phenotype <sup>a</sup>	M type	T type	<i>mef</i>	<i>ermA</i>	<i>ermB</i>	<i>ermC</i>	<i>ermTR</i>
<b>M (<i>n</i> = 46)</b>							
18	4	4	+	-	-	-	-
1	11	11/12	+	-	-	-	-
2	12	12	+	-	-	-	-
1	28	28	+	-	-	-	-
21	NT <sup>b</sup>	4	+	-	-	-	-
2	NT	2/25	+	-	-	-	-
1	NT	NT	+	-	-	-	-
<b>MLS<sub>B</sub> (IR) (<i>n</i> = 19)</b>							
1	2	2/28	-	-	-	-	+
1	4	4	-	-	-	-	+
1	12	12	-	-	-	-	+
1	22	NT	-	-	-	-	+
1	28	28	-	-	-	-	+
2	58	58	-	-	-	-	+
1	77	13	-	-	-	-	+
1	77	9/13	-	-	-	-	+
1	77	9/13/B3264	-	-	-	-	+
3	NT	12	-	-	-	-	+
1	NT	58	-	-	-	-	+
3	NT	3/13/B3264	-	-	-	-	+
1	NT	8/25/Imp19	-	-	-	-	+
1	NT	NT	-	-	-	-	+
<b>MLS<sub>B</sub> (CR) (<i>n</i> = 2)</b>							
1	58	58	-	-	-	-	+
1	22	12/B3264	-	+	-	-	-

<sup>a</sup> IR, inducible resistance; CR, constitutive resistance.

<sup>b</sup> NT, nontypeable.

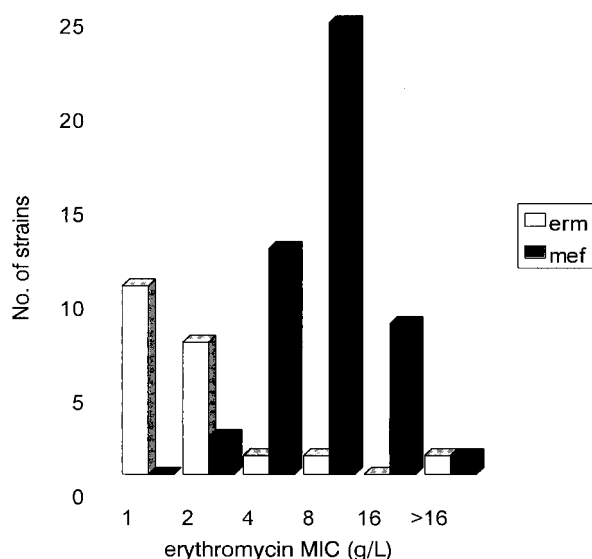


FIG. 1. Correlation of the erythromycin MIC with the presence of *mef* and *erm* genes. For a single MLS<sub>B</sub> phenotype strain, the erythromycin MIC was >16 g/liter. This strain was constitutively resistant and was the only strain found to possess an *ermB* gene.

**Strain characteristics.** Of the 67 erythromycin-resistant strains, 64 were throat isolates and 3 were isolated from wounds. As shown in Table 2, 19 strains (28%) were M serotype, type 4; however, a large proportion of strains were non-typeable (34 of 67; 51%). The presence of *mef* or *ermTR* genes did not correlate with particular serotypes.

## DISCUSSION

In this study, the overall rate of erythromycin resistance in GAS strains was low, at 2.1%, compared with the overall rates of 10 to 47% reported in European countries (3, 13, 15, 23). In an earlier study of Canadian strains, resistant strains were detected at a rate of 0.24% in 1971, increasing to 1.4% in 1972 (7). It is encouraging that the prevalence has remained low in Canada. In some countries, such as Spain, it increased steadily from 1.2% before 1990 to 35% in 1995, falling to 18% in 1996, possibly as a result of decreased macrolide use (22). The prevalence of erythromycin-resistant strains with the M phenotype (70%) in this study is lower than that reported in Spain and Sweden (90%) but is similar to that recently reported in Finland (13, 16, 22). In Sweden, the M phenotype has been highly prevalent since the early 1980s (13), and it was also present in Spanish isolates collected in the late 1980s (22). In the study of Canadian isolates collected in 1971 to 1972 in which 145 erythromycin-resistant strains were examined, no M phenotype isolates were identified (7). It is interesting that this phenotype has become so prevalent in Canada in a relatively short time, not only in GAS but also in *S. pneumoniae* (14). Kataja et al. (16) have shown that the *mefA* gene can be transferred from GAS to *E. faecalis* by conjugation, which may explain the spread.

All but 1 of the 20 strains showing the MLS<sub>B</sub> phenotype possessed the *ermTR* gene. The MICs of erythromycin for these strains, in general, were lower than those for strains harboring *mef* genes. This is unusual, since it has been noted previously in GAS, and also in *S. pneumoniae*, that MICs for MLS<sub>B</sub> (*ermB*<sup>+</sup>) strains tend to be higher than those for strains with the M phenotype (14, 22). It was also interesting that 14

of 16 tetracycline-resistant strains were of the MLS<sub>B</sub> phenotype. In *S. pneumoniae*, Tn1545, which confers MLS<sub>B</sub> resistance through the presence of the *ermB* gene, also confers tetracycline resistance through the *tetM* gene (5). Future studies should examine whether MLS<sub>B</sub> resistance in GAS might also be transposon mediated. The fact that the *ermTR* strains in this study had 12 different PFGE patterns suggests that the gene may have spread by horizontal transfer to strains of different genetic backgrounds.

It is encouraging that the GAS strains in this study were uniformly susceptible to penicillin and indeed that resistance to most antimicrobials was nonexistent or extremely low. Resistance to penicillin has not emerged in GAS despite extensive use of  $\beta$ -lactam drugs in many countries, and the reason for this is unknown (11). On the other hand, erythromycin resistance has correlated with antibiotic usage both in Finland and in Japan, and resistance was shown to decline when the use of macrolides was restricted (8, 24). It is, therefore, important to continue to monitor macrolide resistance rates on a national level so that if prevalence rates begin to increase significantly, attempts to control their use can be made.

## ACKNOWLEDGMENTS

This work was funded in part by the Canadian Bacterial Diseases Network.

We thank the MDS Laboratory Service for providing the clinical isolates.

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