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# Resistance to Macrolides in *Streptococcus pyogenes* in France in Pediatric Patients

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**A total of 1,500 recent throat isolates of *Streptococcus pyogenes* collected between 1996 and 1999 from children throughout France were tested for their susceptibility to erythromycin, azithromycin, josamycin, clindamycin, and streptogramin B. The erythromycin-resistant isolates were further studied for their genetic mechanism of resistance, by means of PCR. The clonality of these strains was also investigated by means of serotyping and ribotyping. In all, 6.2% of the strains were erythromycin resistant, and 3.4 and 2.8% expressed the constitutive $MLS_B$ and M resistance phenotypes and harbored the *ermB* and *mefA* genes, respectively; *ermTR* was recovered from one isolate which also harbored the *ermB* gene. Ten serotypes and 8 ribotypes were identified, but we identified 17 strains by combining serotyping with ribotyping. Among the eight ribotypes, the *mefA* gene was recovered from six clusters, one being predominant, while the *ermB* gene was recovered from four clusters, of which two were predominant.**

Penicillin is the drug of choice for the treatment of *Streptococcus pyogenes* infection. However, for patients sensitive to β-lactam antibiotics, and when these drugs fail, macrolides are often the recommended substitute. Penicillin resistance has not yet been described in *S. pyogenes*, but resistance to erythromycin and related antibiotics has been widely reported (2, 3, 5, 8, 10, 11, 17, 20, 29, 33, 43, 46). The mechanism of acquired resistance to erythromycin involves a target site modification mediated by a methylase which modifies the 50S ribosomal subunit, leading to the $MLS_B$ resistance phenotype encoded by *erm* genes (25, 36, 47). Erythromycin resistance due to an efflux mechanism (M phenotype), encoded by *mef* genes, has recently been described (6, 45). While the prevalence of the *S. pyogenes* resistance to macrolides has been reported worldwide, very few recent data deal with the French situation (1, 8). The aims of this study were to assess the macrolide sensitivity of recent throat isolates of *S. pyogenes* collected from French children, to determine the genetic mechanisms of resistance, and to explore clonality by means of serotyping and molecular methods.

## MATERIALS AND METHODS

**Bacterial isolates.** A total of 1,500 consecutive *S. pyogenes* isolates were collected between 1996 and 1999 throughout France. They were isolated by swabbing the throats of children, 4 to 17 years old (mean age, 11 years), with pharyngitis. The isolates were identified as *S. pyogenes* by colony morphology, beta-hemolysis on blood agar, and a commercial agglutination technique (Murex Diagnostics UK).

**Susceptibility testing.** The procedures for susceptibility testing were those recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (30).

**(i) Detection of erythromycin resistance and determination of resistance phenotypes.** Erythromycin-resistant strains were initially identified by the disk diffusion method on Mueller-Hinton agar supplemented with 5% defibrinated horse blood (Diagnostic Pasteur, Marnes la Coquette, France) using 15-μg erythromycin disks (Diagnostic Pasteur) according to NCCLS guidelines (30). Simultaneously, the resistance phenotypes of erythromycin-resistant *S. pyogenes* isolates were determined by the double-disk test with erythromycin and clindamycin disks, as previously described (42). Blunting of the clindamycin inhibition zone proximal to the erythromycin disk indicated an inducible type of $MLS_B$ resistance, and resistance to both erythromycin and clindamycin indicated a constitutive type of $MLS_B$ resistance. Susceptibility to clindamycin with no blunting indicated the new erythromycin resistance phenotype (M phenotype).

**(ii) Determination of MICs.** The MICs of erythromycin (Roussel Uclaf, Paris, France), azithromycin (Pfizer, Orsay, France), josamycin (Rhône-Poulenc-Rorer, Vitry-sur-Seine, France), clindamycin (Pharmacia Upjohn, Saint-Quentin-en-Yvelines, France), and streptogramin B (Rhône-Poulenc-Rorer) were determined for all the isolates that had an erythromycin inhibition zone diameter of less than 21 mm (30). MICs were determined by the agar dilution method with Mueller-Hinton medium supplemented with 5% defibrinated sheep blood. The plates were incubated overnight at 35°C in ambient air. The NCCLS breakpoints for resistance (30) were as follows: susceptible (MIC ≤ 0.25 μg/ml) or resistant (MIC ≥ 1 μg/ml) to erythromycin and clindamycin and susceptible (MIC ≤ 0.5 μg/ml) or resistant to azithromycin (MIC ≥ 2 μg/ml). Given the lack of NCCLS recommendations on josamycin, we used the breakpoints recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (9), i.e., a susceptibility MIC of ≤1 μg/ml and a resistance of MIC ≥4 μg/ml.

**Detection of erythromycin resistance genes.** All erythromycin-resistant isolates were screened for erythromycin resistance genes. Isolates were grown in 20 ml of TGY broth (Diagnostic Pasteur) for 18 h. After centrifugation, bacterial DNA was prepared as previously described (4). The *mef*, *erm*, and *ermTR* genes were detected by PCR amplification, using previously published primers (24, 36). We used 5′-AGT ATC ATT AAT CAC TAG TGC-3′ and 5′-TTC TTC TGG TAC TAA AAG TGG-3′ to detect *mefA*, 5′-CGA GTG AAA AAG TAC TCA ACC-3′ and 5′-GGC GTG TTT CAT TGC TTG ATG-3′ to detect *ermB*, and 5′-GCA TGA CAT AAA CCT TCA-3′ and 5′-AGG TTA TAA TGA AAC AGA-3′ to detect *ermTR*. Amplification was performed in a DNA thermal cycler (no. 9600; Perkin-Elmer Cetus, Norwalk, Conn.) programmed for one cycle of denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 2 min, and extension at 72°C for 10 min (24). Amplification products were run through 1% agarose gels. Gels were stained with ethidium bromide, and the DNA bands were visualized with a UV transilluminator. *S. pyogenes* 02C 1061, *S. pyogenes* 02 61110 and *S. pyogenes* 02C 1064 were used as positive PCR controls for the *ermB*, *ermTR*, and *mefA* genes, respectively (6, 7, 12, 44). Five erythromycin-susceptible *S. pyogenes* isolates were used as negative controls. Amplification of DNA from the positive controls with the corresponding primers produced PCR products of expected sizes (616, 348, and 206 bp for *erm*, *mef*, and *ermTR*, respectively) (Fig. 1). These PCR products were used for direct sequencing with an Applied Biosystems model 373 sequencer, using a modification of the method of Sanger et al. (35). The analysis showed that the amplimers were identical to *mefA*, *ermB*, and *ermTR* genes (6, 36, 47).

After amplification, hybridization with DNA probes was performed for the *mef* and *erm* PCR products. The PCR products were transferred, by using a standard method, to Hybond-N+ membranes (Amersham Bioproducts). The 616 and 348-bp PCR product from *S. pyogenes* 02C 1061 and 02C 1064, respectively, were

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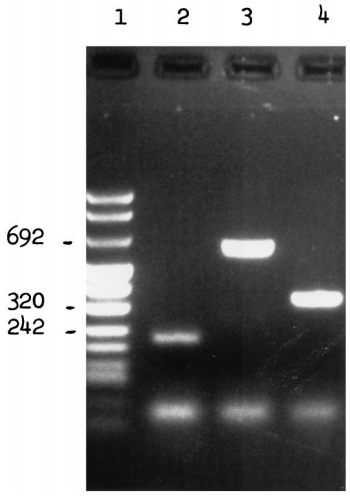


FIG. 1. PCR analysis of the *S. pyogenes* erythromycin-resistant control strains using specific primers for detection of *ermTR* (lane 2), *ermB* (lane 3), and *mefA* (lane 4). Lane 1, DNA molecular weight marker.

chemiluminescence labeled according to the manufacturer's instructions and hybridized to the PCR products from each of the erythromycin-resistant isolates.

No hybridization was observed with the five erythromycin-susceptible control isolates.

**Restriction fragment length polymorphism analysis of the *mef* and *erm* PCR products.** The PCR products of the erythromycin resistance genes *mef* and *erm* from 15 randomly selected isolates were digested by *Mse*I and *Tsp*509I (*mefA*) or *Fok*I and *Sau*III (*ermB*), and electrophoresis on 6% polyacrylamide gel was performed as previously described (13). Gels were stained with ethidium bromide, and DNA bands were visualized with a UV transilluminator.

**Serotyping.** Erythromycin-resistant *S. pyogenes* isolates were studied by T and M serotyping, and the presence of serum opacity factor was also determined as previously described (23), in the Colindale laboratory (Central Public Health Laboratory, London, United Kingdom).

**Genotyping.** Genotyping based on restriction fragment length polymorphism of the rRNA gene (ribotyping) was performed on all the erythromycin-resistant isolates. DNA was digested with *Hin*dIII and *Hha*I (Boehringer, Mannheim, Germany) according to the manufacturer's instructions and analyzed by Southern blotting with a chemiluminescent ribosomal probe, as previously described (4). Initial experiments on a few strains showed that *Hin*dIII produced a better distribution of restriction length fragments than did *Hha*I. *Hin*dIII was thus used to ribotype all the strains.

## RESULTS

**Antimicrobial susceptibility patterns of and MICs for the *S. pyogenes* isolates with different erythromycin resistance phenotypes.** The screening plate method showed that 6.2% (93 of 1,500) of the *S. pyogenes* isolates had decreased susceptibility to erythromycin. Among those isolates, 3.4% (51 of 1,500) and 2.8% (42 of 1,500) expressed the constitutive $MLS_B$ and M resistance phenotypes, respectively. None of the isolates had the inducible $MLS_B$ resistance phenotype.

Table 1 shows the MICs for *S. pyogenes* according to the erythromycin resistance phenotype. All 93 strains were resistant to erythromycin (MIC breakpoint $\geq$1 μg/ml).

Isolates with the $MLS_B$ phenotype were resistant to the 14-, 15- and 16-membered-ring macrolides (azithromycin and josamycin, respectively) and showed cross-resistance to clindamycin and streptogramin B. Isolates with the M phenotype were resistant to erythromycin and azithromycin, but the 16-membered-ring macrolides josamycin and clindamycin showed good activity against these isolates.

**Erythromycin resistance genes of *S. pyogenes* isolates with different erythromycin resistance phenotypes.** PCR amplification followed by Southern hybridization showed that all the resistant isolates with the $MLS_B$ and M phenotypes harbored the *ermB* and *mefA* genes, respectively. Digestion of *mef* PCR products by *Mse*I and *Tsp*5091 produced a single restriction pattern. We also observed a single restriction pattern when *erm* PCR products were digested by *Fok*I and *Sau*III. PCR amplification with primers corresponding to *ermTR* identified only one isolate harboring this gene. Interestingly, this isolate also harbored the *ermB* gene.

TABLE 1. MICs of macrolides and related agents against 93 erythromycin-resistant *S. pyogenes* isolates

| Erythromycin resistance genotype ($n^b$) | Antimicrobial agent | MIC (μg/ml)[a] | | |
|---|---|---|---|---|
| | | 50% | 90% | Range |
| *ermB* (51)[c] | Erythromycin | >128 | >128 | >128 |
| | Josamycin | >128 | >128 | >128 |
| | Azithromycin | >128 | >128 | >128 |
| | Clindamycin | >128 | >128 | >128 |
| | Streptogramin B | 64 | 64 | 32–128 |
| *mefA* (42) | Erythromycin | 8 | 8 | 4–16 |
| | Josamycin | 0.25 | 0.25 | 0.25–0.5 |
| | Azithromycin | 8 | 8 | 8–16 |
| | Clindamycin | 0.064 | 0.064 | 0.064–0.125 |
| | Streptogramin B | 1 | 1 | 0.5–2 |

[a] 50 and 90%, $MIC_{50}$ and $MIC_{90}$, respectively.
[b] Number of isolates.
[c] Including one strain harboring both *ermB* and *ermTR*.

**Genotyping.** After digestion by *Hin*dIII, a total of 8 ribotypes (A to H) were observed among the 93 erythromycin-resistant isolates. Twenty-nine (31%), 20 (22%), 12 (13%), and 28 (30%) of the 93 erythromycin-resistant strains belonged to ribotypes A, G, E, and C, respectively. Four isolates (4%) had unique individual patterns. The ribotype distribution according to the genetic mechanism of resistance is reported in Table 2. The *mefA* gene was recovered from six clusters, with one being predominant, while the *ermB* gene was recovered from four clusters, with two being predominant.

**Serotyping.** Serotyping identified 10 serotypes (Table 3). Thirty-two isolates (34%) were of serotype T4M4, 18 (19%) were of serotype T12M22, 12 (13%) were of serotype T12M12, 12 (13%) were of serotype T28R28, 5 (5%) were of serotype T2M2, 8 (9%) were of serotype T11M11, 3 (3%) were of serotype T1M1, and one each was of serotype T6M6, T22M22, and T13M77. No apparent geographic clustering of isolates of any serotype was observed.

The serum opacity factor typing was positive for all the isolates of serotypes T2M2, T4M4, T11M11, T12M22, and T28R28.

A strong correlation of some serotypes with particular erythromycin-resistance genotypes was found, as the gene *mefA* was present in all but one of the serotype T4M4 isolates and all the serotype T1M1 isolates. The *ermB* gene was found in all the isolates of serotypes T11M11, T12M22, and T28R28 and in all but one of the serotype T2M2 isolates.

TABLE 2. Distribution of ribotypes according to the genetic mechanism of resistance

| Genetic mechanism of resistance ($n^a$) | No. of isolates with ribotyping profile | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H |
| *ermB* (51) | 0 | 0 | 26 | 0 | 4 | 0 | 20 | 1 |
| *mefA* (42) | 29 | 1 | 2 | 1 | 8 | 1 | 0 | 0 |
| *ermTR* (1) | | | 1 | | | | | |

[a] Number of isolates.

TABLE 3. Ribotypes of 93 erythromycin-resistant *S. pyogenes* isolates with various serotypes and genetic resistance mechanisms

| Serotype | *n* | Ribotypes | | | | | | | | Genes | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F | G | H | *ermB* | *mefA* | *ermTR* |
| T1M1 | 3 | | 1 | 1 | | 1 | | | | | 3 | |
| T2M2 | 5 | | | 5 | | | | | | 4 | 1 | |
| T4M4 | 32 | 29 | | 1 | 1 | 1 | | | | 1 | 31 | |
| T6M6 | 1 | | | | | | 1 | | | | 1 | |
| T11M11 | 8 | | | 8 | | | | | | 8 | | |
| T12M12 | 12 | | | | | 10 | | 1 | 1 | 6 | 6 | |
| T12M22 | 18 | | | | | | | 18 | | 18 | | |
| T22M22 | 1 | | | | | | | 1 | | 1 | | |
| T13M77 | 1 | | | 1 | | | | | | 1 | | |
| T28R28 | 12 | | | 12 | | | | | | 12 | | 1 |

The number of ribotypes within each serotype varied between one and four. The combination of ribotyping and serotyping allowed us to identify 17 strains (Table 3). Seventy percent of macrolide resistance due to *mefA* was due to T4M4 ribotype A, and 60% of *ermB* resistance was found in two clones.

## DISCUSSION

Although *S. pyogenes* is consistently susceptible to penicillin, resistance to macrolides, first described in the United Kingdom in 1958 (26) and in the United States in 1968 (34), has been reported worldwide. The incidence of *S. pyogenes* erythromycin resistance remains low in most parts of the world but recently reached 17% in Finland (23), 27 to 34% in Spain (2, 32, 33), and 30 to 35% in Italy (5, 11). In our study, the prevalence of erythromycin resistance in *S. pyogenes* isolates from French children was only 6.2%. Similar percentages (4 to 10%) have been reported in Germany (G. Cornaglia, P. Huovinen, and The European GAS Study Group, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-149, 1998), the United Kingdom (Cornaglia et al., 38th ICAAC), Portugal (Cornaglia et al., 38th ICAAC), Greece (46), Canada (K. Weiss, M. Laverdière, C. Restieri, N. Persico, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-153, p. 213, 1998), and the United States (samples collected throughout continental United States) (3). Our low percentage of erythromycin-resistant *S. pyogenes* isolates contrasts with the high prevalence of macrolide resistance associated with penicillin resistance in French *S. pneumoniae* isolates.

Until recently, the only known mechanism of resistance to erythromycin was target modification mediated by methylase, a phenomenon encoded by *erm* genes. At least 12 classes of *erm* genes have been identified by nucleic acid hybridization analysis and nucleotide sequence comparison (25, 47; K. Weiss, C. Restieri, L. A. Galarneau, M. Gourdeau, P. Harvey, J. F. Paradis, J. de Azavedo, K. Salim, and D. Low, Program Abstr. 5th Int. Conf. Macrolides, Azalides, Streptogramins, Ketolides, Oxazolidinones, abstr. 7.23, 2000). The latest gene in that class is designated *ermTR* (36). An efflux mechanism of resistance encoded by the *mefA* gene has also been reported (6). The gene encodes a hydrophobic 44.2-kDa protein sharing homology with membrane-associated pump proteins (6). The *ermB* and *mefA* genes were harbored by 3.4 and 2.8% of our isolates, respectively, conferring the $MLS_B$ and M phenotypes. Contrary to a previous report, none of our strains harbored both the *ermB* and *mefA* genes (18). However, the isolate which carried *ermTR* also harbored *ermB*, as previously observed (E. Di Modugno, A. Felici, M. Guerrini, H. Mottl, P. Piccoli, and D. Sabatini, Program Abstr. 5th Int. Conf. Macrolides, Azalides, Streptogramins, Ketolides, Oxazolidinones, abstr. 7.14, 2000). The M phenotype was found to be predominant among erythromycin-resistant *S. pyogenes* isolates in Finland (23), Sweden (20), Spain (32), and some areas in Italy (5), while an equal distribution of the M and inducible $MLS_B$ phenotypes was reported in Greece (46). Genetic investigations were made in Finland and Spain and showed the predominance of the *mefA* gene (23, 32). In agreement with previous studies (22, 33), the *ermB* gene conferred coresistance to 14-, 15-, and 16-membered-ring macrolides and lincosamides (MICs at which 90% of the isolates tested are inhibited [$MIC_{90}$s] exceeding 128 μg/ml for both drugs), and to streptogramin B ($MIC_{90}$ = 64 μg/ml); the *mefA* gene conferred resistance only to 14- and 15-membered-ring macrolides ($MIC_{90}$ = 8 μg/ml).

Previous reports have suggested that erythromycin resistance is often associated with specific serotypes (21). Indeed, the increase in erythromycin resistance among *S. pyogenes* isolates has been linked to the spread of serotype T12 in Japan (27, 28, 29) and serotype T4M4 in Finland (23). Eight T and 9 M agglutination patterns were observed in our study among the 93 erythromycin-resistant isolates.

Although serotyping has provided useful epidemiologic information, its discriminatory power is considered poor because of genetic heterogeneity among isolates of the same serotype on the one hand and the existence of the same genotype among different serotypes on the other hand. Genomic typing methods such as restriction endonuclease analysis (REA), random amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis have been applied to *S. pyogenes*, together with ribotyping (15, 32, 40, 41). Ribotyping was found to be less discriminatory than REA (4). However, the complexity of the restriction patterns makes it difficult to analyze a large number of isolates by REA. We used ribotyping for strain differentiation, identifying eight ribotypes among the 93 erythromycin-resistant *S. pyogenes* isolates. We combined ribotyping and serotyping. In 3 out of 10 cases, ribotyping revealed genetic differences among isolates of the same serotype. Although ribotyping identified many different clones within a single serotype, identical ribotypes were detected among isolates of different serotypes. Analysis of the M protein sequences has shown that some serotypes are phylogenetically closer than others (14). Ribotyping adds to the discriminatory power of serotyping, especially when epidemiologically unrelated isolates are compared (41); we identified 17 different strains in this way.

Six of the eight ribotypes carried either the *mefA* or the *ermB* gene. The *mefA* gene was recovered from six clusters. One cluster was predominant, and most of the strains were serotype T4M4. In Finland, the increase in erythromycin-resistant *S. pyogenes* with the M phenotype was also due to strains of serotype T4M4 (23). The *ermB* gene was recovered from four clusters, two being predominant. Thus, most isolates carrying the *mefA* gene were unrelated to those harboring the *ermB* gene. The location of *erm* and possibly *mef* on a chromosomal conjugative transposon may explain the spread of erythromycin resistance to different clones of *S. pyogenes* (25). The isolate harboring *ermTR* was of serotype M28T28. Interestingly, the resistance of *S. pyogenes* to macrolides in Quebec, Canada, is due to a single M28T28 clone possessing the *ermTR* gene (K. Weiss et al., 5th ICMASKO).

Macrolide resistance is closely related to the extent to which these agents are used, and national surveys have shown that decreases in macrolide use lead to decreases in the incidence of macrolide resistance (16, 37, 39). Moreover, a significant negative correlation has been found between the age of pa-

tients and the occurrence of erythromycin-resistant organisms (38). This seems to be the result of prescription of more antibiotics for children together with a greater risk of cross-colonization among children than among adults. The fact that the level of erythromycin resistance among our *S. pyogenes* isolates was below 7% may be related to the stable consumption of macrolides in France since 1980 (19, 31) and to the absence of clonal spread of erythromycin-resistant strains. The pharyngeal origin of all our isolates may also explain the relatively low incidence of erythromycin resistance, as a significantly higher rate of resistance is observed with invasive strains (43, 48). However, it has been suggested that strain virulence is not related to antimicrobial resistance (21).

In conclusion, we found a low level of erythromycin resistance in *S. pyogenes* in France. Moreover, as only 2.8% of the isolates had the M phenotype, clindamycin and josamycin would be the drugs of choice for treating children with clinical failure after phenoxymethyl penicillin, and for penicillin-allergic patients. Further careful surveillance of erythromycin resistance with other macrolides-lincosamides-streptogramin B is required to follow changes in *S. pyogenes* resistance patterns in France.

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