

Macrolide and Tetracycline Resistance and Molecular Relationships of Clinical Strains of *Streptococcus agalactiae*

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Mechanisms for tetracycline and macrolide resistance in 54 isolates of erythromycin-resistant *Streptococcus agalactiae* were analyzed by PCR. The *erm(B)*, *erm(A)*, and *mef(A)* genes, either alone or in combination, were detected in all the erythromycin-resistant isolates. The *tet(M)* and *tet(O)* genes were responsible for tetracycline resistance. Random amplification of polymorphic DNA indicated different clonal origins of the isolates.

Group B streptococcal infections are a leading cause of neonatal mortality and also affect pregnant women and the elderly. β -Lactam agents are the treatment of choice for these infections, but macrolides and related drugs provide useful alternative therapy for allergic patients. *Streptococcus agalactiae* is considered to be susceptible to β -lactam antimicrobial agents, but the emergence of strains resistant to macrolides and tetracycline has been increasingly reported (15, 17).

The mechanisms of erythromycin resistance in *S. agalactiae* include target site modification and active drug efflux. Target modification is conveyed by the action of a family of methyltransferase enzymes encoded by the *erm* genes. The *erm* genes found in *S. agalactiae* are *erm(B)* and *erm(A)*. Both genes may be inducibly or constitutively expressed (14). Active drug efflux is mediated by the *mef(A)* gene and causes resistance to 14- and 15-membered macrolide compounds (4).

Tetracycline resistance genes are often found on the same mobile unit as erythromycin resistance genes (22). In a variety of gram-positive and gram-negative species, *erm(B)* is frequently found linked with *tet(M)* (23). Seventeen different tetracycline resistance determinants have been characterized to date. Most of these determinants code either for a protein which pumps tetracycline out of the cell or for a ribosomal protection protein which protects the ribosomes from the action of tetracycline (2).

The purpose of this study was to investigate the phenotypic and genotypic distribution of erythromycin-resistant *S. agalactiae* isolates and to explore the clonality of these isolates by molecular methods. Rates of tetracycline resistance and the genes responsible were also investigated.

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From 1992 through 2000 a total of 109 *S. agalactiae* (55 erythromycin-susceptible and 54 erythromycin-resistant) isolates were collected in the Microbiology Department of the Hospital Clínico San Carlos. In order to avoid duplication, only one isolate per patient was studied. The clinical sources were as follows: skin and soft tissues (57 isolates), urine (29 isolates),

vagina (12 isolates), blood (5 isolates), upper respiratory tract (4 isolates), and abdomen (2 isolates). Isolates were identified by a commercial latex agglutination technique (Slidex Strepto B; bioMérieux, Marcy L'Etoile, France).

All *S. agalactiae* isolates were tested for antibiotic resistance. MICs of erythromycin (Abbott, Madrid, Spain), clindamycin (Pharmacia & Upjohn, Barcelona, Spain), and tetracycline (Sigma Chemical Co., St. Louis, Mo.) for all the erythromycin-resistant strains were determined by the agar dilution method in accordance with NCCLS guidelines (18, 19). Plates were incubated overnight at 37°C under 5% CO₂. The resistance phenotypes of all erythromycin-resistant isolates were determined by the double-disk diffusion method using erythromycin (15 μ g) and clindamycin (2 μ g) disks as described elsewhere (24).

For the detection of different resistance genes, DNA of *S. agalactiae* was extracted as previously described (3). DNAs of resistant isolates were amplified by using primers specific for the *erm(A)*, *erm(B)*, *erm(C)*, and *mef(A)* genes. The sequences of the primer sets and PCR conditions were as previously described (14, 26). All isolates were also tested for the presence of the *tet(K)*, *tet(L)*, *tet(M)*, and *tet(O)* tetracycline resistance determinants (27).

Epidemiologic typing of *S. agalactiae* isolates was performed with two different primers (M13 and H2) and Ready-to-Go Analysis Beads (Pharmacia Biotech) as described by Seppälä et al. (25), and the same criteria were used to interpret and compare the patterns.

The activities of the three antimicrobial agents tested against erythromycin-resistant and erythromycin-susceptible *S. agalactiae* strains are summarized in Table 1. As expected, MICs of clindamycin were lower for erythromycin-susceptible (range, 0.03 to 0.06 μ g/ml) than for erythromycin-resistant (range, 0.03 to >64 μ g/ml) *S. agalactiae* isolates. It should be stressed that for 13 erythromycin-resistant *S. agalactiae* isolates, clindamycin MICs were higher than erythromycin MICs. Rates of tetracycline resistance in erythromycin-resistant and -susceptible isolates (87 and 72%, respectively) were very similar, as were the MICs at which 50 and 90% of isolates were inhibited (MIC₅₀ and MIC₉₀, respectively) (Table 1).

The numbers of strains with various macrolide-resistant phenotypes, and the genes associated, are given in Table 2. Most (53.7%) of the erythromycin-resistant *S. agalactiae* isolates showed macrolide-lincosamide-streptogramin B cross-resis-

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TABLE 1. Activities of erythromycin, clindamycin, and tetracycline against 54 erythromycin-resistant and 55 erythromycin-susceptible *S. agalactiae* isolates

Organisms (no. of isolates tested) and antimicrobial agent	MIC ^a (μg/ml)		
	50%	90%	Range
Erythromycin-resistant isolates (54)			
Erythromycin	4	>64	1->64
Clindamycin	16	>64	0.03->64
Tetracycline	32	64	0.05-64
Erythromycin-susceptible isolates (55)			
Erythromycin	0.03	0.03	0.01-0.06
Clindamycin	0.03	0.06	0.03-0.06
Tetracycline	16	32	0.01-64

^a Breakpoints from the NCCLS for resistant isolates are as follows: erythromycin and clindamycin, ≥1 μg/ml; tetracycline, ≥8 μg/ml.

tance, whereas 37.0 and 9.3% of the strains had the inducible and M phenotypes, respectively.

The *mef(A)* gene was detected by PCR in all isolates with the M resistance phenotype, either alone (two strains) or associated with the *erm(A)* gene (three strains). The *erm(B)* gene was detected either alone (16 isolates) or associated with either *erm(A)* (18 isolates) or *mef(A)* (2 strains).

Genes responsible for tetracycline resistance were determined in erythromycin-resistant isolates. Forty-seven erythromycin-resistant *S. agalactiae* isolates were also resistant to tetracycline; 31 of these had *tet(M)*, and 8 had *tet(O)*. The *tet(L)* and *tet(K)* genes did not appear in any of the strains. In 8 isolates, we were not able to find any of the tetracycline-resistant genes tested for. It is possible that these isolates carried another *tet* determinant.

The most widely distributed tetracycline resistance determinant in gram-positive bacteria is *tet(M)* (21). The *tet(O)*, *tet(K)*, and *tet(L)* genes also appear in gram-positive microorganisms but are much less common. As expected, the majority of our isolates harbored the resistance determinant *tet(M)*. We found *tet(M)* associated with all macrolide-resistant genes.

TABLE 2. Distribution of erythromycin resistance genes among 54 erythromycin-resistant *S. agalactiae* isolates categorized according to erythromycin resistance phenotype and erythromycin and clindamycin MIC ranges

Gene(s)	No. of strains	Phenotype ^a	MIC range	
			Erythromycin	Clindamycin
<i>erm(B)</i>	13	Cr	4->64	4->64
	3	I	2->64	0.03-0.2
<i>erm(A)</i>	7	Cr	2->64	64->64
	5	I	1-4	0.06-0.5
<i>erm(B)</i> + <i>erm(A)</i>	9	Cr	2->64	32->64
	9	I	2->64	0.06-0.2
<i>mef(A)</i> + <i>erm(B)</i>	2	I	4->64	0.03-0.1
<i>mef(A)</i> + <i>erm(A)</i>	3	M	1-4	0.06-0.1
	1	I	1	0.06
<i>mef(A)</i>	2	M	4	0.06

^a Cr, cross-resistance; I, inducible; M, efflux.

Resistance to macrolides and tetracyclines in *Streptococcus pneumoniae* has been assumed to be mainly due to the presence of a conjugative transposon that encodes *erm(B)* in addition to *tet(M)* (5). However, in group C and group G streptococci this association between erythromycin and tetracycline resistance determinants was not found (14). From this study, it seems that tetracycline resistance in *S. agalactiae* is not linked to erythromycin resistance.

Mechanisms of erythromycin and tetracycline resistance in *S. pneumoniae* and *Streptococcus pyogenes* have been widely investigated (7, 9, 10, 12, 13, 16), but there are few studies of *S. agalactiae*, and those are focused only on erythromycin resistance (1, 6, 8, 11, 20). In agreement with the findings of these previous studies, we observed that erythromycin resistance in *S. agalactiae* was mainly associated with the presence of the *erm(B)* gene. *erm(A)* was the second most common gene found. A high percentage of strains in our collection (44.5%) carried more than one macrolide resistance gene. These data agree with some of the previous studies (6, 20). Nevertheless, the association of *mef(A)* with *erm(A)* in *S. agalactiae* was first detected in our study. Novel subphenotypes of macrolide-lincosamide-streptogramin B resistance due to the concomitant presence of *erm* and *mef* genes have recently been described for *S. pyogenes* (12, 13) and *S. agalactiae* (6). However, the correspondence with our findings appears to be only partial. The erythromycin MIC range for our strains was 1 to 4 μg/ml, and three of these strains were fully susceptible and did not show inducible resistance to clindamycin.

Our collection of *S. agalactiae* strains was very heterogeneous with regard to macrolide MICs, the presence of erythromycin resistance determinants, and association between different tetracycline- and macrolide-resistant genes. A similar heterogeneity was found when we analyzed the clonal relationships of isolates.

A total of 35 different randomly amplified polymorphic DNA (RAPD) patterns were found among the 54 erythromycin-resistant *S. agalactiae* strains studied. RAPD-PCR typing with both the M13 and H2 primers resulted in 27 unique fingerprint patterns. Of the remaining 27 isolates, 2 different profiles appeared in 4 isolates each, 2 appeared in 3 isolates each, and 3 different profiles appeared in 2 isolates each. One DNA profile was demonstrated in 7 isolates.

We conclude that, among *S. agalactiae* strains, resistance to erythromycin and tetracycline in our area is due to multiclonal dissemination of resistance within the streptococcal population rather than to the epidemic spread of single clones.

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