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Patterns of Macrolide Resistance Determinants among Community-Acquired *Streptococcus pneumoniae* Isolates over a 5-Year Period of Decreased Macrolide Susceptibility Rates

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Erythromycin resistance rates were found to be increased, from 7.1 in 1993 to 32.8% in 1997, among community-acquired *Streptococcus pneumoniae* isolates from the Siena area of central Italy. Most of the erythromycin-resistant isolates carried *ermAM* determinants and were also resistant to josamycin and clindamycin, whereas a minority (5.8%) carried *mefA* determinants and remained susceptible to the latter drugs.

Macrolide and lincosamide antibiotics exhibit strong antimicrobial activity against streptococci and are among the drugs that can be used for chemotherapy of infections caused by *Streptococcus pneumoniae*. Macrolide resistance in pneumococci has been detected at variable rates in different epidemiological settings (see, for instance, references 1, 2, 4, 7, 15, 18, and 22), with a trend toward increasing resistance being reported by several investigators (4, 6, 9, 21). This is a matter of major concern, since macrolides are largely prescribed for the empiric chemotherapy of community-acquired respiratory tract infections and may be useful in case of intolerance to β-lactams as well as pneumococcal resistance to other antimicrobial agents.

In *S. pneumoniae*, macrolide resistance can be mediated by ribosomal modification (8) or active drug efflux (16). The former mechanism is associated with high-level resistance to macrolides, lincosamides, and streptogramin B (MLS_B-type resistance pattern) (8), while the latter mechanism is associated with low-level resistance to 14- and 15-membered-ring macrolides only (M-type resistance pattern) (16, 19). Both resistance effectors are encoded by acquired determinants: the *ermAM* gene encoding the ribosome-modifying enzyme (8, 20) and the *mefE* gene for the efflux system (19). The contributions of these mechanisms to macrolide resistance in pneumococci appears to be variable in different epidemiological settings (1, 2, 7, 15, 18, 22), although a molecular analysis of the resistance determinants was carried out only in a minority of cases (7, 22).

In the work described here we determined the macrolide and lincosamide susceptibilities of 302 *S. pneumoniae* isolates isolated from an area of central Italy over a 5-year period (1993 to 1997) and investigated the resistance determinants carried by macrolide-resistant isolates.

Pneumococci were randomly selected from among those that were classified as community acquired and that were cultured at the Laboratory of Clinical Bacteriology of the Institute of Infectious Diseases, University of Siena, during the period 1 January 1993 to 31 December 1997 from samples from patients who were residents of the Siena area. Repeated isolates from the same individual were not included unless the isolations were separated by a period of at least 1 year. Identification of pneumococcal isolates was performed by standard procedures (12). In vitro susceptibility testing was performed by a broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (10). Erythromycin and clindamycin were from Sigma Chemical Co. (St. Louis, Mo.); josamycin was from ICN (Costa Mesa, Calif.). The breakpoints for susceptibility classification were those specified by NCCLS (11). *S. pneumoniae* ATCC 49619 was used for quality control of susceptibility testing.

Of the 302 pneumococcal isolates, 233 (77.2%) were found to be susceptible to erythromycin (MICs, ≤0.015 to 0.06 μg/ml), josamycin (MICs, 0.06 to 0.25 μg/ml), and clindamycin (MICs, ≤0.06 to 0.25 μg/ml), while the remaining 69 (22.8%) were resistant to erythromycin. Of these, 65 (94.2%) exhibited high-level resistance to erythromycin (MICs, 128 to >1,024 μg/ml) and also to josamycin (MICs, 16 to >1,024 μg/ml) and clindamycin (MICs, 512 to >1,024 μg/ml), which is typical of a constitutive MLS_B-type resistance pattern, while the remaining 4 (5.8%) exhibited low-level resistance (MICs, 1 to 8 μg/ml) to erythromycin only, which is typical of an M-type resistance pattern. A double-disk diffusion assay (8) confirmed that susceptibility to josamycin and clindamycin was not influenced by the presence of erythromycin in the isolates with M-type resistance.

The prevalence of erythromycin-resistant isolates showed a steady increase during the study period, from 7.1% in 1993 to 32.8% in 1997 (Fig. 1).

The occurrence of *ermAM*- and *mef*-related genomic sequences was investigated in all the erythromycin-resistant isolates and in 54 (23.2%) randomly selected susceptible isolates by means of colony blot hybridization with ^{32}P-labeled DNA probes. Colony blot hybridization was performed as described previously (13) after an initial exposure (twice for 10 min at room temperature) of pneumococcal colonies to 1% (wt/vol) Na-Sarkosyl–1% (wt/vol) sodium deoxycholate–1.5 M NaCl. With *Streptococcus pyogenes* and *Streptococcus mitis*, colony blotting was performed as described previously (5). The *ermAM*-specific probe was a 764-bp PCR amplicon that contains the entire *ermAM*-coding sequence (20). The *mef*-specific probes were 346-bp PCR amplicons which contained part of *mefA* or *mefE*, and the amplicons were obtained as described previously (17). *S. pneumoniae* 4C1 (*ermAM*$^+$), *S. pyogenes*

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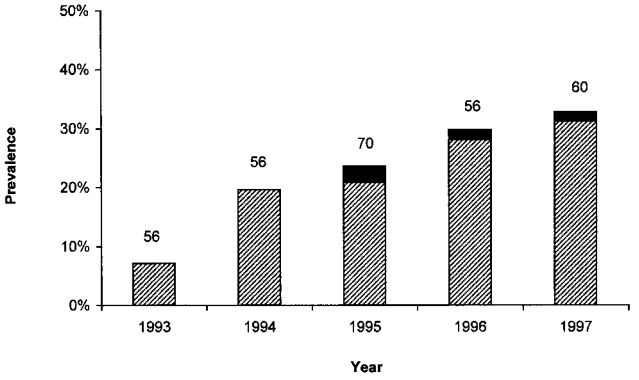

FIG. 1. Prevalences of MLS_B-type resistance (▨) and M-type resistance (■) among *S. pneumoniae* isolates in different years. The numbers reported at the top of each column indicate the total number of isolates analyzed in that year.

1A77 (*mefA*$^+$), and *S. mitis* 21A29 (*mefE*$^+$) were included as hybridization controls.

The *ermAM* probe yielded a strong hybridization signal for all isolates with MLS_B-type resistance, while it did not recognize any of the susceptible isolates or isolates with M-type resistance. The intensity of the hybridization signal was overall comparable to that obtained with a positive hybridization control for *ermAM* (data not shown). The presence of *mef*-related sequences was initially investigated with a *mefE* probe. This probe yielded a hybridization signal for the four isolates with M-type resistance, while it did not recognize any of the susceptible isolates or isolates with MLS_B-type resistance. The hybridization signal, however, was weaker than that obtained with a positive hybridization control for *mefE*. By using a *mefA* probe, a stronger hybridization signal was obtained for the four isolates with M-type resistance, and the signal was comparable to that obtained with a positive hybridization control for *mefA* and stronger than that obtained with a positive hybridization control for *mefE* (data not shown).

To verify the identities of the *mef* alleles carried by the four isolates with M-type resistance, PCR was carried out with primers MEFA-up (5′-GACCAAAAGCCACATTGTGGA) and MEFA-dn (5′-CCTCCTGTCTATAATCGCATG), which were designed on the basis of sequences that flank the *mefA* gene from *S. pyogenes* 02C1064 (3), by using the following cycling conditions: 94°C for 30 s, 58°C for 60 s, and 70°C for 80 s, which were repeated for 40 cycles. In all cases an amplimer of the expected size (1,431 bp) was obtained. Restriction analysis of the amplimers with *Ava*II, *Bam*HI, *Cla*I, *Hin*dIII, and *Nhe*I yielded in all cases a profile consistent with the sequence of *mefA* (with *Ava*II, products of 283, 419, and 729 bp; with *Bam*HI, products of 223 and 1,208 bp; with *Cla*I, products of 620 and 811 bp; with *Hin*dIII, products of 437 and 994 bp; with *Nhe*I, a product of 1,431 bp) (3) but not with that of *mefE* (with *Ava*II, products of 283, 372, and 776 bp; with *Bam*HI, a product of 1,431 bp; with *Cla*I, a product of 1,431 bp; with *Hin*dIII, a product of 1,431 bp; with *Nhe*I, products of 525 and 906 bp) (19).

Concluding remarks. Similar to what has been observed elsewhere (4, 6, 9, 21), the prevalence of macrolide-resistant pneumococci has also recently undergone a substantial increase in the Siena area of central Italy, with macrolide-resistant pneumococci making up nearly one-third of the community-acquired isolates in 1997. A similar situation is probably consequent to an increased selective pressure generated by increased prescriptions of macrolides in community medicine, as has previously been demonstrated for *S. pyogenes* (14).

The results of this study revealed that, in our region, the most prevalent macrolide resistance phenotype among pneumococcal isolates is the MLS_B type and that the relevant increase in the number of macrolide-resistant pneumococci observed during the study period was virtually completely a result of isolates that carry *ermAM* determinants. Isolates that have M-type resistance and that carry *mef* determinants have appeared only since 1995 and remain uncommon. A similar pattern of macrolide resistance determinants in pneumococci has also been observed in Spain (1), although it is different from those encountered in other countries where lower relative rates of isolates with MLS_B-type resistance have been observed (7, 22). The relative prevalence of the two macrolide resistance mechanisms in pneumococci therefore exhibits remarkable geographical heterogeneity. This essentially results from large variations in the absolute rates of occurrence of isolates with MLS_B-type resistance, whereas the prevalence of pneumococci with M-type resistance showed an overall low variability (0.45 to 1.6%) in different epidemiological settings (1, 7, 22; this study), suggesting that the *erm* determinants play a major role in the geographical and temporal variability in macrolide resistance rates among pneumococci. Molecular analysis also showed that all the pneumococci with M-type resistance encountered in this study apparently carried a *mefA* determinant, revealing that not only *mefE* but also *mefA* can be acquired by *S. pneumoniae*, resulting in an M-type resistance phenotype.

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