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Use of Clindamycin Disks To Detect Macrolide Resistance Mediated by *ermB* and *mefE* in *Streptococcus pneumoniae* Isolates from Adults and Children

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We studied 198 macrolide-resistant *S. pneumoniae* isolates obtained from adults and children to evaluate whether 2-μg clindamycin disks can distinguish between isolates manifesting *ermB*- versus *mefE*-mediated resistance to clarithromycin and to determine the relative frequency with which each resistance mechanism occurred in these populations. The *mefE* gene was predominant among 109 isolates from children, occurring in 73.4% versus 50.6% of 89 isolates from adults. Three isolates (1.5%) did not amplify either gene. Among 125 *mefE*$^+$ isolates, the MIC of clarithromycin at which 90% of the isolates tested were inhibited, determined by Etest, was 32 μg/ml versus >256 μg/ml in 70 *ermB*$^+$ isolates. All *ermB*$^+$ isolates were highly resistant to clindamycin (MICs >256 μg/ml), whereas all *mefE*$^+$ isolates were susceptible to clindamycin using the 2-μg disk. Testing *S. pneumoniae* from the respiratory tract for susceptibility to clindamycin by agar disk diffusion is an easy and inexpensive method to estimate the frequency of resistance mediated by *ermB* in specific patient populations. Macrolide resistance mediated by *ermB* is usually of greater magnitude than that due to *mefE*. Clinical studies are needed to determine the significance of high- versus low-level macrolide resistance in *S. pneumoniae*.

Macrolide resistance in *Streptococcus pneumoniae* has increased during the 1990s to the extent that over 30% of clinical isolates are now resistant in some communities (16–18). Formerly, it was believed that all macrolide resistance in *S. pneumoniae* was due to target modification by *ermB* methylase carried on the conjugative transposon Tn*1545*, which dimethylates a specific adenine residue in the peptidyl transferase center of 23s rRNA, simultaneously conferring high-level resistance to lincosamides and streptogramin B (MLS_B phenotype) (1, 3, 4, 7, 8, 10, 16). More recently, it has been observed that many isolates of *S. pneumoniae* are susceptible to clindamycin but are resistant to macrolides, including erythromycin and clarithromycin (M phenotype). These isolates have been shown to exhibit a noninducible, macrolide-specific efflux mechanism, encoded by *mefE* (5, 10–16; K. Y. Gay, D. Miller, M. Farlay, F. Tenover, and D. Stephens, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemotherapy, abstr. C-33, 1998).

We studied 198 macrolide-resistant *S. pneumoniae* isolates obtained from clinical specimens from adults and children to evaluate whether 2-μg clindamycin disks can accurately distinguish between isolates manifesting *ermB*- versus *mefE*-mediated resistance and to determine the relative frequency with which each resistance mechanism occurred in these populations.

MATERIALS AND METHODS

Microorganisms. One hundred ninety-eight nonduplicate clinical isolates of *S. pneumoniae* obtained primarily from upper and lower respiratory tract specimens from 109 children in Birmingham, Ala., Greenville-Spartanburg, S.C., and Nashville, Tenn. between 1990 and 1999 were evaluated. Eighty-nine additional isolates obtained from adults in Birmingham, Ala. between 1995 and 1999 were studied for comparative purposes. Isolates were chosen from the culture collections of the investigators based solely on resistance to erythromycin as determined by prior screening on blood agar plates containing erythromycin (1 μg/ml). Isolates were stored frozen at −70°C and then thawed and passaged twice on Trypticase soy agar with 5% sheep blood (Remel, Inc., Lenexa, Kans.). A single colony of each isolate was cloned for susceptibility testing and PCR analysis.

Susceptibility tests. Clindamycin disks (2 μg; Remel) and Etest strips (AB BIODISK, Piscataway, N.J.) for clindamycin and clarithromycin were used to determine susceptibilities for lincosamide and macrolide antibiotic classes. Testing was performed by the standard agar disk diffusion methodology published by the National Committee for Clinical Laboratory Standards (NCCLS) (9) and according to the manufacturer's instructions for Etest strips, using Mueller-Hinton agar with 5% sheep blood (Remel). Agar plates were incubated for 24 h in an atmosphere supplemented with 5% CO_2. NCCLS criteria (9) were used for interpretation of disk diffusion inhibitory zones and Etest MICs. *S. pneumoniae* ATCC 49619 was used as a control.

Genetic analysis by PCR. The PCR used in this study, performed with primers complementary to conserved regions in the *erm* genes, allows detection of MLS_B and M phenotype resistance in *S. pneumoniae* (1, 12–16; Gay et al., 38th ICAAC). Genomic DNA from all clinical isolates was obtained by removing a 1-cm^2 area of bacteria grown overnight on Trypticase soy agar with 5% sheep blood (Remel), resuspending it in 50 μl of Millipore water, and boiling it for 5 min. Samples were placed on ice and centrifuged to remove cellular debris. PCRs were prepared using 5 μl of this lysate plus oligonucleotide primers designed to amplify *ermB* (0.6 kb) and *mefE* (1.7 kb) at a concentration of 4 μM for each primer (15, 17). Reactions were carried out using Ready-to-Go PCR Beads (Pharmacia Biotech, Piscataway, N.J.) and were subjected to 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min in a Perkin-Elmer 480 DNA Thermal Cycler (Perkin-Elmer, Norwalk, Conn.). Ten microliters of each reaction mixture was subjected to 1% agarose gel electrophoresis for 1.5 h, and the gel was then stained with ethidium bromide. Positive controls included primers for RR142, a conserved *S. pneumoniae* sequence of 0.4 kb, to demonstrate the presence of amplifiable DNA and the absence of inhibitors. Negative controls were carried out using primers for both genes with an erythromycin-susceptible isolate.

RESULTS

Table 1 summarizes clarithromycin and clindamycin MICs and clindamycin susceptibility according to agar disk diffusion in relation to the presence of *ermB* or *mefE*. The distribution of individual MICs is shown graphically in Fig. 1. Clindamycin

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TABLE 1. Susceptibilities of *S. pneumoniae* by disk diffusion and Etest according to mechanism of macrolide resistance

Resistance mechanism	Etest MICs (μg/ml) of:						Clindamycin 2-μg disk inhibitory zone range (mm)
	Clarithromycin			Clindamycin			
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	
mefE$^+$ (n = 125)	2–128	4	32	≤0.25	≤0.25	≤0.25	21–31
ermB$^+$ (n = 70)	2–>256[a]	>256	>256	>256	>256	>256	6[b]
Neither (n = 3)	4–>256	—	—	0.25–64	—	—	6[b]–22

[a] Two *ermB*$^+$ isolates had high-level resistance to clindamycin (>256 μg/ml) but clarithromycin MICs were 2 μg/ml.
[b] The width of the disk is 6 mm. There was no evidence of inhibition surrounding it.

susceptibility and resistance results determined by Etest and disk diffusion were 100% concordant, using NCCLS criteria for interpretation (9).

High-level clindamycin resistance (MICs >256 μg/ml) was observed in all 70 isolates in which *ermB* was amplified. There were two isolates that expressed the MLS_B phenotype (clindamycin MICs, 8 and 64 μg/ml; clarithromycin MICs, >256 μg/ml) that could not be shown to contain either *mefE* or *ermB* despite repeated attempts by PCR or by DNA-DNA hybridization. A third isolate that expressed the M phenotype (clindamycin MIC, ≤0.25 μg/ml; clarithromycin MIC, 4 μg/ml) also could not be shown to possess either genetic marker. Among 125 clarithromycin-resistant isolates in which *mefE* was amplified, all were clindamycin susceptible (M phenotype), and clarithromycin MICs were generally lower than those for the *ermB*$^+$ isolates. The clarithromycin MIC at which 90% of the isolates tested were inhibited (MIC_{90}) for *mefE*$^+$ isolates was at least fourfold less than that for the *ermB*$^+$ isolates (32 and >256 μg/ml, respectively). There were only 2 of 70 (2.9%) *ermB*$^+$ isolates for which high-level clarithromycin resistance (>256 μg/ml) was not present. In both cases the clindamycin MIC was >256 μg/ml, whereas the clarithromycin MIC was only 2 μg/ml. MIC tests were repeated a second time by Etest and again by broth microdilution, yielding the same results, within 1 dilution.

Table 2 shows the relative frequencies of *ermB* and *mefE* in the three pediatric populations and the single adult population studied. Among pediatric isolates, *mefE* was clearly predominant, accounting for 63.5 to 87% of all macrolide-resistant *S. pneumoniae* isolates studied, with an average of 73.4%. However, in adult isolates, the frequencies of *ermB* and *mefE* were very similar, at 49.9 and 50.6%, respectively. There were no isolates shown to possess both genetic markers.

DISCUSSION

Disk diffusion has been recommended for determining susceptibilities of *S. pneumoniae* to erythromycin and clindamycin (3). Erythromycin disks (15 μg) also predict clarithromycin susceptibilities (9), even though actual MICs of the latter are likely to be somewhat lower. We have shown that the 2-μg clindamycin disk can be used to distinguish macrolide resistance mediated by *ermB* versus *mefE* in clinical isolates of *S. pneumoniae*. Isolates containing *ermB* were highly resistant to clindamycin in every instance and had no detectable inhibitory zones surrounding the disks. In contrast, isolates containing *mefE* were uniformly susceptible to clindamycin using either MICs or disk inhibitory zone data (9). Although clindamycin resistance was present in every instance in which the *ermB* gene could be demonstrated in macrolide-resistant *S. pneumoniae*, a small percentage of macrolide-resistant isolates, 1.5%, do not amplify *ermB* or *mefE* genes. Thus, a different mechanism of

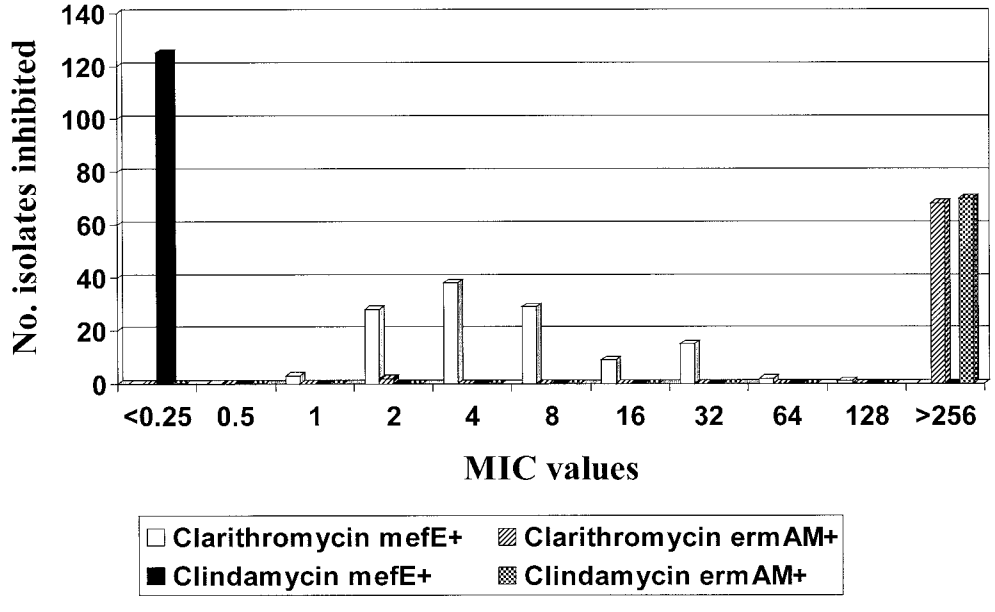

FIG. 1. Clindamycin and clarithromycin MIC distribution for 195 *S. pneumoniae* isolates in which either *mefE* or *ermB* was amplified.

TABLE 2. Frequency of *mefE* and *ermB* in macrolide-resistant *S. pneumoniae* isolates from children versus those from adults

Group	No. (%) of isolates that were:		
	mefE$^+$	*ermB*$^+$	Neither
Alabama adults (n = 89)	45 (50.6)	44 (49.4)	0
Alabama children (n = 52)	33 (63.5)	17 (32.7)	2 (3.8)
South Carolina children (n = 46)	40 (87)	5 (10.9)	1 (2.2)
Tennessee children (n = 11)	7 (63.6)	4 (36.4)	0
Total pediatric isolates (n = 109)	80 (73.4)	26 (23.9)	3 (2.2)
Total isolates (n = 198)	125 (63.1)	70 (35.4)	3 (1.5)

resistance is operative, possibly related to a mutation in ribosomal RNA, and clindamycin activity can be variable (4, 14).

Susceptibility or resistance to clindamycin in macrolide-resistant *S. pneumoniae* also gives clues to the magnitude of macrolide resistance in most instances. Among 125 *mefE*$^+$ isolates, clarithromycin MICs were ≤32 μg/ml in 122 (97.6%) isolates versus >256 μg/ml in 68 of 70 (97.1%) *ermAM*$^+$ isolates. The MIC_{90} for *mefE*$^+$ isolates (32 μg/ml) was identical to that reported by Johnston et al. (4) and eightfold higher than the findings of Shortridge et al. (13). However, both of these studies were performed using broth microdilution cultures incubated in ambient air as opposed to agar-gradient diffusion with Etests incubated in 5% CO_2 where MICs of macrolides for *S. pneumoniae* macrolides may be elevated by 1 to 2 dilutions. Despite increasing macrolide resistance in *S. pneumoniae* amidst widespread usage of these drugs as empiric therapy for respiratory infections, reports of macrolide treatment failures are relatively uncommon. This observation may be related to the predominance of low-level resistance mediated by *mefE* in some populations and to the favorable bronchopulmonary pharmacokinetics of drugs such as clarithromycin. Concentrations of clarithromycin in epithelial lining fluid are substantially higher than the MICs for many *mefE*$^+$ isolates (11–13). Outcome-based clinical studies with microbiologic data will be necessary to fully understand the clinical significance of low-level macrolide resistance in *mefE*$^+$ isolates of *S. pneumoniae*.

Clindamycin MIC breakpoints and inhibitory zone diameters for *S. pneumoniae* have been included in NCCLS documents (9), but no recommendations have been made for routine testing of clindamycin against this organism, apparently because of the belief that macrolide resistance is predominantly of the MLS_B type, and possibly because macrolide resistance has become more widespread in the United States only within the past few years. However, clindamycin is often the only oral agent available for treating children with pneumococcal infections caused by beta-lactam- and/or macrolide-resistant organisms since fluoroquinolones are not approved for general use in this population, and it may become more widely used in pediatrics when a pneumococcal etiology of an infection is known or strongly suspected. Clinical laboratories should routinely test and report the drug, and the NCCLS should consider adding it to their list of suggested agents for testing against this organism.

The final observations from this study relate to epidemiologic data for various patient populations. Although there have been limited reports of the frequency of *mefE* in macrolide-resistant *S. pneumoniae* isolates, varying from 54 to 85% (4, 13, 16; Gay et al., 38th ICAAC), this is the first study to describe the frequency of these genes specifically in pneumococcal isolates from pediatric patients from different geographic locations and to compare the frequencies of these genes in isolates from children and adults in the same community. Based on our preliminary findings, *mefE* accounted for the majority of macrolide resistance in pneumococci from pediatric populations in three southeastern states, ranging from 63.5 to 87%, in contrast to isolates from adults from Alabama in whom rates of *mefE* and *ermB* occurrence were similar, each accounting for approximately 50% of the macrolide-resistant isolates. *S. pneumoniae* isolates obtained from children are often different from those found in adults. Therefore, it is not surprising that we observed different frequencies of *mefE* in children and adults from the same geographic area.

In summary, we suggest that testing clindamycin susceptibility routinely in respiratory isolates of *S. pneumoniae* is desirable in view of the fact that this drug is often the most active oral agent, other than quinolones, against this organism. Testing *S. pneumoniae* with clindamycin and erythromycin disks is an easy and inexpensive method to estimate the frequency of MLS_B resistance. Since treatment of pneumococcal respiratory infections is usually empiric and bacterial isolates are often unavailable for testing, this information can be valuable for patient care.

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