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Prevalence of Macrolides-Lincosamides-Streptogramin B Resistance and *erm* Gene Classes among Clinical Strains of Staphylococci and Streptococci

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A total of 332 staphylococcal and 263 streptococcal isolates from three hospital microbiology laboratories were tested with erythromycin, clindamycin, and vernamycin B_α to determine the prevalence of macrolides-lincosamides-streptogramin B resistance. Constitutive resistance was detected in 28 *Staphylococcus aureus* isolates (15.5%), 53 coagulase-negative staphylococci (35.1%), and 20 streptococci (7.6%). Inducible resistance was observed in 13 *S. aureus* isolates (7.2%), 25 coagulase-negative staphylococci (16.6%), and 2 streptococci (0.8%). Eleven coagulase-negative staphylococci (7.3%) exhibited a novel phenotype, namely inducible resistance to erythromycin and vernamycin B_α but not clindamycin. Among the staphylococci, two variants of the inducible phenotype detected with the agar disk diffusion assay correlated with the presence of classical *ermA* or *ermC* genes, respectively, by dot-blot analysis. The prevalences of the staphylococcal phenotypes were different in the hospitals surveyed, and there was an apparent inverse correlation between the resistance observed and the use of erythromycin in each hospital.

Simultaneous resistance to macrolides, lincosamides, and the type-B streptogramins (MLS resistance) in clinical isolates is a form of acquired resistance due to several evolutionary variants of the resistance-conferring (*erm*) gene, which encodes a 23S rRNA methylase (34). The methylase, which renders affected ribosomes incapable of binding the MLS antibiotics, can be produced constitutively or inducibly (38), low levels of erythromycin being the most effective inducer (36).

This pattern of resistance has been demonstrated in many bacterial species, and its prevalence has been monitored in Europe (10, 11, 15, 16, 29, 30, 39), Canada (7–9), and Japan (18, 20–22). In the United States, systematic studies of the epidemiology of MLS resistance are sparse. However, studies of bacterial susceptibilities to erythromycin and clindamycin suggest that MLS resistance is present in clinical isolates in the United States (2–4, 17, 28). To determine the prevalence of the MLS resistance phenomenon in our geographic area, we studied more than 800 clinical isolates, predominantly staphylococci and streptococci, from three hospitals affiliated with our institution. All isolates were tested for susceptibility to erythromycin, clindamycin, and vernamycin B_α. Resistant strains were tested further to determine whether resistance was constitutive or inducible. Finally, selected strains were characterized with respect to the evolutionary class (*ermA*, *B*, or *C*) of the resistance determinant.

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MATERIALS AND METHODS

Antimicrobial agents and media. Prepared and dehydrated media and antimicrobial disks were obtained commercially (BBL Microbiology Systems, Cockeysville, Md.). Erythromycin powder was purchased from the Sigma Chemical Co., St. Louis, Mo. Clindamycin and spectinomycin powders were gifts from The Upjohn Co., Kalamazoo, Mich. Vernamycin B_α was a gift from the Squibb Institute for Medical Research, Princeton, N.J. Data on the amount of erythromycin and clindamycin use at each hospital were provided by the pharmacy directors at each institution.

Microorganisms. Bacterial strains were obtained from three clinical microbiology laboratories (Robert Wood Johnson University Hospital, New Brunswick, N.J.; St. Peter's Medical Center, New Brunswick, N.J.; and Raritan Bay Medical Center, Perth Amboy, N.J.) from July 1984 to June 1985. Staphylococci were identified to the species level with the API Staph-Ident kit (Analytab Products, Plainview, N.Y.). Streptococci were identified by hemolytic reaction, colony morphology, susceptibility with bacitracin and optochin disks, the CAMP reaction (12), and using commercially available kits for grouping these organisms (Streptex, Wellcome Diagnostics, Research Triangle Park, N.C.; or Phadebact, Pharmacia, Inc., Piscataway, N.J.). After initial susceptibility testing, the isolates were stored at −55°C until further analyses could be performed. Reference microorganisms were obtained from several sources (Table 1).

Susceptibility testing. Antimicrobial susceptibilities for single-colony isolates were determined by disk diffusion and broth dilution using standard methods (24, 25). Inoculum size was confirmed using the surface drop count method of Miles and Misra (19). All microorganisms were screened for MLS resistance with disks containing erythromycin (15 μg), clindamycin (2 μg), and vernamycin B_α (25 μg). Blunting of the clindamycin or vernamycin B_α zones near an erythromycin disk was an indication of inducible resistance to one of the above-mentioned antimicrobial agents. Resistant isolates were also tested with disks containing spectinomycin (50 μg). The vernamycin B_α and spectinomycin disks were prepared freshly each week, and all disks were stored at 4 to 8°C until they were used.

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TABLE 1. Reference microorganisms

Microorganism	Source	Plasmid	Strain or plasmid description (reference)
Staphylococcus aureus 1206	B. Weisblum		Original chromosomal *ermA* isolate containing Tn*554* (34)
Staphylococcus aureus RN4932	E. Murphy	pEM715	Tn*554* in multicopy plasmid pT181; used as source of *ermA* probe
Staphylococcus aureus RN2466	E. Murphy	pRN3173	Derivative of *ermB*-containing plasmid pI258; used as source of *ermB* probe (26)
Staphylococcus aureus RN2442	E. Murphy	pE194	Multicopy plasmid containing *ermC*
Escherichia coli HB101	K. Hardy	pKH80	Chimeric plasmid used as source of *ermC* probe (14)

Turbidimetric studies. The inducibility of resistance to the MLS antibiotics was studied in selected strains using the Abbott Avantage instrument (Abbott Laboratories, Irving, Tex.). Duplicate overnight cultures of each isolate, one grown in tryptic soy broth (uninduced) and the other grown in tryptic soy broth containing 0.1 μg of erythromycin per ml (induced), were diluted 1:100 with fresh tryptic soy broth 1 h before the institution of turbidity recordings. The fresh tryptic soy broth added to the induced culture contained 0.1 μg of erythromycin per ml, whereas the tryptic soy broth added to the uninduced culture did not contain erythromycin. Challenge concentrations of each MLS antibiotic were 60 μg/ml. Turbidity readings were recorded for 24 h.

Determination of genotypes. To determine *erm* genotype, cell lysates were prepared using lysozyme, lysostaphin, alkali, and detergent (32, 33). Samples were spotted onto nitrocellulose membranes for hybridization to ^{32}P-labeled DNA containing one of the classical *ermA*, *B*, or *C* genes (32; Table 1).

RESULTS

Initial screening of isolates by disk diffusion revealed the five phenotypes shown in Fig. 1. Two phenotypes (1 and 2, respectively) were variants of the classical inducible pattern, with blunting of the clindamycin and vernamycin B_α inhibitory zones facing the erythromycin disk.

Type 1 strains (Fig. 1A) exhibited a pattern indistinguishable from that of the original MLS *Staphylococcus aureus* 1206, the *ermA* prototype (36). This pattern was characterized not only by blunted zones facing the erythromycin disk but also by a narrow (2- to 3-mm) zone of inhibition around the erythromycin disk. No late growth was observed within the zones of inhibition during 48 h of incubation and observation. Six *S. aureus* isolates, three coagulase-negative staphylococci, and one group B streptococcus exhibited this phenotype (Table 2). With the exception of two *S. aureus* strains, all staphylococcal isolates exhibiting this phenotype (Fig. 1A) were resistant to spectinomycin (MIC, >300 μg/ml).

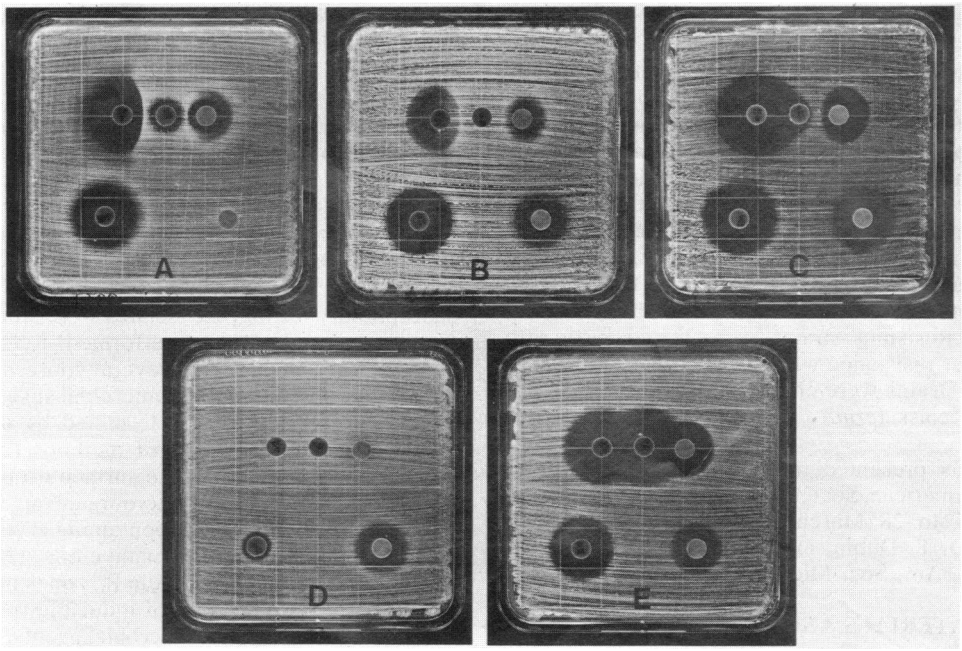

FIG. 1. MLS phenotypes detected by disk diffusion. Test disks were as follows. Top row, left to right: Clindamycin, 2 μg; erythromycin, 15 μg; vernamycin B_α, 25 μg. Bottom row, left to right: Chloramphenicol, 30 μg; spectinomycin, 50 μg. Growth within clindamycin and vernamycin B_α zones of inhibition for type 2 strains (panel B) required 36 to 48 h. See the text for description of each phenotype and correlation of panels A to E.

Type 2 strains (Fig. 1B) exhibited altered expression of inducible resistance with confluent growth around the erythromycin disk and delayed (36- to 48-h) growth within the clindamycin and vernamycin B_α zones, especially on the side nearest the erythromycin disk. This pattern was indistinguishable from that noted in a laboratory strain of *S. aureus* (RN2442) which contains *ermC* on plasmid pE194 (Table 1). Seven *S. aureus* isolates, eleven coagulase-negative staphylococci, and one group A streptococcus exhibited this phenotype (Table 2). All but one of the type 2 strains were inhibited by spectinomycin.

A third inducible phenotype, designated MS and shown in Fig. 1C, was noted in 11 coagulase-negative staphylococci (Table 2). This anomalous phenotype exhibited inducible resistance to erythromycin and vernamycin B_α but not clindamycin. All 11 strains were inhibited by spectinomycin.

The remaining two phenotypes, exhibiting either constitutive resistance or susceptibility to all three MLS antibiotics, are shown in Fig. 1D and 1E, respectively. The five phenotypes and the distribution of staphylococci and streptococci studied according to phenotype are shown in Table 2.

The inducibly resistant staphylococcal strains were examined for the nature of their *erm* determinants by hybridization using restriction fragments containing the classical *ermA*, *B*, or *C* genes (Table 3). Staphylococcal type 1 strains invariably had chromosomal genes of the *A* class. Of 11 staphylococcal type 2 strains subjected to dot-blot and Southern analysis (4 *S. aureus*, 5 *S. epidermidis*, 1 *S. haemolyticus*, and 1 *S. saprophyticus*), 10 had genes of the *C* class on a small plasmid resembling *S. epidermidis* plasmid pNE131 (17, 32); the exception was the *S. saprophyticus* isolate, which failed to hybridize significantly with our class *A*, *B*, or *C* probes. All of the MS coagulase-negative staphylococci and the two inducibly resistant streptococcal isolates also failed to yield positive dot blots with our probes.

TABLE 2. MLS and MS phenotypes of staphylococci and streptococci

Microorganism (no. of isolates)	No. of isolates with phenotype[a]:				
	MLS resistant			MS	Susceptible[b]
	Type 1	Type 2	Constitutive		
Staphylococci (332)					
S. aureus (181)	6	7	28	0	140
S. epidermidis (44)	0	7	34	3	
S. haemolyticus (15)	1	2	8	4	
S. hominis (9)	0	1	7	1	
S. saprophyticus (5)	0	1	2	2	
S. simulans (4)	2	0	2	0	
S. capitis (1)	0	0	0	1	
Other coagulase negative (73)	0	0	0	0	73[c]
Streptococci (263)					
Group A (105)	0	1	0	0	104
Group B (45)	1	0	1	0	43
Group C (2)	0	0	0	0	2
Group D					
Enterococci (13)	0	0	13	0	0
Nonenterococci (12)	0	0	2	0	10
Group G (6)	0	0	0	0	6
Other beta-hemolytic (16)	0	0	2	0	14
Viridans group (32)	0	0	2	0	30
S. pneumoniae (32)	0	0	0	0	32

[a] Phenotypes correspond to those shown in Fig. 1.
[b] Susceptible to erythromycin and clindamycin by National Committee for Clinical Laboratory Standards criteria.
[c] Susceptible coagulase-negative staphylococci were not identified to the species level.

TABLE 3. Correlation between disk diffusion phenotype and genotype for inducible MLS-resistant and MS staphylococci

Microorganism(s)	Phenotype	Genotype		
		A	*C*	Other[a]
Staphylococcus aureus	MLS type 1	6	0	0
	MLS type 2	0	7	0
Coagulase-negative staphylococci	MLS type 1	3	0	0
	MLS type 2	0	10	1
	MS	0	0	11

[a] No significant hybridization was noted with the *ermA*, *B*, or *C* probes used.

Timed turbidimetric measurements (Fig. 2) were used to confirm the inducibility of the MS strains as inferred from the disk diffusion results (Fig. 1). These strains consistently demonstrated inducible resistance to erythromycin and vernamycin B_α but not clindamycin using challenge concentrations of 60 μg/ml for each of the MLS antibiotics. These strains likewise demonstrated inducible resistance in repeated disk diffusion experiments (Fig. 1C), as well as significantly increased resistance to erythromycin and vernamycin B_α (MIC, ≥64 μg/ml) but not clindamycin in broth dilution experiments after an overnight exposure to an inducing level of erythromycin (0.1 μg/ml). Similar experiments conducted with the MLS-inducible strains confirmed their inducibility in most cases (data not shown). The exceptions were four type 1 strains that did not respond to induction in broth despite repeated attempts using different media (tryptic soy broth and Mueller-Hinton broth with and without divalent cation supplementation), variations in inducing concentrations of erythromycin (0.7, 0.07, and 0.007 μg/ml), and variations in challenge concentrations of erythromycin (2.4, 17.5, and 60 μg/ml).

Substantial differences were observed in the prevalence of the susceptible and constitutively resistant staphylococcal

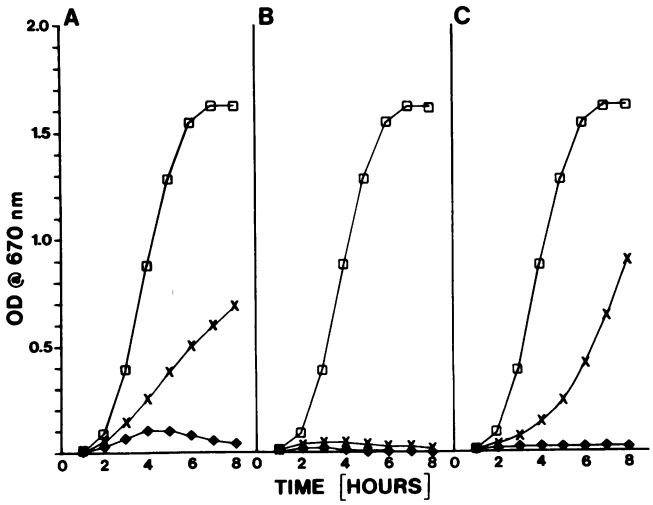

FIG. 2. Timed turbidimetric measurements of a representative strain, *S. haemolyticus*, exhibiting the MS phenotype. Challenge drugs (all at 60 μg/ml) were as follows. (A) Erythromycin, (B) clindamycin, (C) vernamycin B_α. Symbols: □, Control; ×, induced; ◆, uninduced.

phenotypes in the three institutions from which the microorganisms were obtained. There was an apparent inverse correlation between the rates of resistance found at the three hospitals and the pattern of erythromycin use. The hospital with the greatest erythromycin use yielded the lowest incidence of constitutively resistant strains ($P < 0.001$), whereas the hospital with the lowest erythromycin use yielded the highest incidence ($P < 0.001$). There also was an unequal distribution of the MS phenotype in the three hospitals, 9 of 11 strains with the MS phenotype coming from one hospital. There was no correlation between observed resistance patterns and clindamycin use.

DISCUSSION

The first reports in the English literature of MLS resistance in staphylococci appeared within 5 years of the introduction of erythromycin into medical practice (13). Since that time, several reports have monitored the prevalence of MLS resistance, particularly in *S. aureus*. Reports from Japan (21) and France (11) have shown a trend from predominantly inducible resistance to predominantly constitutive resistance, whereas in British (16) and German (39) reports the more common MLS phenotype continues to be inducibly resistant. In this study of three medical school-affiliated hospitals in New Jersey, 22.6% of *S. aureus* and 51.7% of coagulase-negative staphylococci (largely *S. epidermidis*) had MLS or MS phenotypes, with constitutive variants outnumbering inducible variants by at least 2:1. Similarly, Lampson and Parisi (17) recently reported a predominance of constitutivity among MLS strains of *S. epidermidis* in Missouri. It is possible that this change reflects selection by widely used noninducing MLS antimicrobial agents, in particular, clindamycin, although comparisons among our three hospitals, all of which experienced similar clindamycin usage patterns, do not support this supposition.

The epidemiology of MLS resistance has been less well studied in coagulase-negative staphylococci than in *S. aureus*, but MLS resistance has been demonstrated and characterized in *S. epidermidis* (27). Our results show that other coagulase-negative staphylococcal species (namely, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, and *S. simulans*) can acquire the MLS phenotype. Since coagulase-negative staphylococci are ubiquitous and may be reservoirs of resistance genes not only for other coagulase-negative staphylococci but also for *S. aureus* (5), the widespread nature of MLS resistance in these bacteria has potential clinical importance.

The epidemiology of MLS resistance in streptococci has been well studied, particularly in Japan (18, 22) and Canada (7–9), as well as in the United States (1, 3, 31). The proportion of non-group D streptococci in this study that exhibited MLS resistance, 7 of 238 isolates (2.9%), is similar to that reported in Canada (7–9) and elsewhere in the United States (1, 3) and much lower than that reported from Japan (18, 22). In this regard, it is interesting that the reported streptococcal *erm* genes are homologous to the staphylococcal *ermB* variant (37). The latter was described in Japan, and none of our staphylococcal or streptococcal isolates yielded a frankly positive *ermB* dot blot (Table 3). Perhaps there are characteristics of the *ermB* gene class (or its vectors) that facilitate spread between staphylococci and streptococci as well as among streptococci. A total of 15 of 25 group D streptococci studied were constitutively MLS resistant, a proportion similar to that found by Rollins et al. (31) in isolates from both animals and humans.

The gene of *S. aureus* 1206 (the *ermA* prototype) is part of Tn*554*, which also carries a spectinomycin resistance determinant (23). Thus, it was not surprising that of the nine inducible *ermA* staphylococcal strains examined, seven were resistant to spectinomycin. Tn*554* can harbor a defective spectinomycin resistance determinant (23), and this likely explains the susceptibility to spectinomycin in the two other strains. As noted, all but one of the inducible type 2 strains subjected to Southern analysis carried the *ermC* gene on plasmid pNE131. It is clear that pNE131 is not restricted to *S. epidermidis* (in which it was discovered) but occurs in other coagulase-negative staphylococci, as well as in *S. aureus*.

The correlation of the type 1 phenotype with *ermA* and type 2 with *ermC* is in agreement with models of induction based on comparative sequence analysis of the classical genes (23). According to these models, *ermA* would require sequential disruption of two leader hairpins by "ribosomal stall," rather than one, as for *ermC*. Murphy (23) predicted that this would result in more efficient induction of *ermC* strains by very low levels of erythromycin, a prediction borne out by the confluent growth of such strains around erythromycin disks. Further, the *ermC* leader has a potential metastable uninduced configuration that would permit synthesis of a basal level of *erm* methylase (13a, 35), whereas the *ermA* leader lacks such a configuration (23). This could result in slow induction of *ermC* strains in the presence of high and otherwise completely inhibitory levels of noninducing MLS antibiotics, thus explaining the extensive delayed growth around clindamycin disks. We note, however, that *ermC* was invariably plasmidborne in our isolates and *ermA* was chromosomal (32); thus, differences in gene copy number might have contributed to the phenotypic differences seen. Arguing against a major role of copy number is the fact that strain RN4932 (Table 1), which carries *ermA* on a plasmid, yielded a disk diffusion pattern more closely resembling that of type 1 than type 2: there was significant inhibition of growth around the erythromycin disk and only barely discernible delayed growth in the clindamycin inhibition zone with this strain (data not shown).

Eleven coagulase-negative staphylococci (7.3%) exhibited a novel phenotype; namely, erythromycin-inducible resistance to a streptogramin but not a lincosamide (MS phenotype). We are unaware of any previous study describing this phenotype in staphylococci. Because the strains are inducible by erythromycin, we doubt that they are explained on the basis of mutation causing an altered ribosomal protein. The potential clinical significance of this phenotype is not known. Since the usual studies of in vitro antibiotic activity tabulate clindamycin and erythromycin susceptibility separately and do not test vernamycin B_α, strains with this phenotype might be scored as inducible MLS isolates. Courvalin et al. described production of enzymes that inactivate one or another of the MLS antibiotic classes (6). Given the differing chemical structures of the macrolides and streptogramins, it seems unlikely that a single enzyme could inactivate both antibiotic classes. Thus, the molecular mechanism of the MS phenomenon is unclear.

Only two of our streptococcal isolates demonstrated inducible MLS resistance. We tested both (one group A, one group B) for the presence of *erm* genes using the classical staphylococcal *A*, *B*, and *C* probes with negative results, and we do not know the nature of the *erm* genes in these strains.

The relatively high proportion of constitutively resistant *S. aureus* in one hospital was noted despite lower erythromycin use in that institution than in the other hospitals

surveyed. However, this finding should be interpreted with caution, since we did not examine our isolates for resistance to multiple antibiotics at the time they were obtained and were unable in retrospect to retrieve this information from our laboratories. If, in fact, one hospital had more multiply resistant strains, there might have been more MLS resistance regardless of the amount of erythromycin used.

Finally, the results of this study suggest that, at least for staphylococci, inducible MLS phenotypes determined by disk diffusion methods correlate well with genotypes determined by hybridization techniques. The degree of correlation was so strong for inducible type 1 and type 2 strains that we believe disk diffusion results may be used to predict genotype in these isolates. This finding should be useful for epidemiologic studies in laboratories that do not have hybridization methodology available. Those wishing to use this technique should note, however, that the standard spacing of disks by commercial multiple-disk dispensers may not always be satisfactory; we found between-disk spacing of 1.0 to 1.5 cm to be optimal.

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