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False Susceptibility of Enterococci to Aminoglycosides with Blood-Enriched Mueller-Hinton Agar for Disk Susceptibility Testing

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Disk diffusion susceptibility tests for enterococci are frequently modified by adding 5% sheep blood (SB) to Mueller-Hinton agar; the performance standards from the National Committee for Clinical Laboratory Standards sanction this addition. Susceptibility testing of aminoglycoside antibiotics is not recommended for enterococci; in actual practice, however, some laboratories do include aminoglycoside antibiotics routinely, and others may test upon request or in selected situations. In examining 50 clinical isolates of enterococci, SB-enriched Mueller-Hinton agar frequently gave enlarged zone sizes that falsely indicated susceptibility (72% for gentamicin and tobramycin), with the average increase in zone size being 6.3 and 7.6 mm, respectively. Comparison agar dilution MICs demonstrated uniform resistance, with or without added SB. The effect was shown to be caused by heme in concentrations as low as 0.03 μg/ml, which, when combined with aminoglycoside antibiotics, caused a synergistic growth inhibition of the enterococci, resulting in larger aminoglycoside antibiotic zones. We postulate that the heme effect is related to a catalytic cleavage of intracellular H_2O_2 and resultant lipid peroxidation. No other organism or antimicrobial agent tested demonstrated a similar effect, although other investigators have shown a similar phenomenon with the broad-spectrum cephalosporins. Because enterococci grow well and give accurate susceptibility results on Mueller-Hinton agar without SB supplementation and because of the spectrum of definable problems with a number of antimicrobial agents, we recommend that enterococci routinely be tested without SB.

Enterococci are known to be clinically resistant to the aminoglycoside antibiotics and almost always demonstrate MICs in the resistant range; however, disk diffusion antimicrobial susceptibility testing may yield susceptible results, depending on testing circumstances. For example, *Cumitech 6* (24) states, "Many, if not most, strains of enterococci have been shown to give zone diameters with gentamicin above the present breakpoint for susceptibility despite MICs in the range of 8-16 μg/ml." Although the data involved in arriving at this statement were not discussed, our own experience demonstrates that this is indeed true, specifically when enterococci are tested on Mueller-Hinton agar (MHA) supplemented with 5% sheep blood (SB). In contrast, disk diffusion testing with nonsupplemented MHA only rarely yields susceptible patterns.

Supplementation of MHA with 5% SB for disk diffusion susceptibility testing of enterococci is a common practice in many laboratories and is in accordance with the publication *Performance Standards for Antimicrobic Disc Susceptibility Tests* (17), published by the National Committee for Clinical Laboratory Standards in 1979, which states in section 3.1.2, "For enterococci, defibrinated blood can be added to the cooled medium to give a final concentration of 5% (v/v)." The updated 1983 National Committee for Clinical Laboratory Standards tentative standard also states that 5% defibrinated SB may be added to test strains that fail to grow satisfactorily on unsupplemented MHA, although it does not specifically address supplementation for enterococci (20).

The study reported here evaluates this problem, and the results demonstrate that the blood component heme is responsible for the discrepancy which, in synergy or combination with the aminoglycosides, results in the growth inhibition of enterococci. Although routine susceptibility testing of enterococci for the aminoglycosides is not recommended, in actual laboratory practice enterococci may be tested with a battery of antimicrobial agents that includes the aminoglycosides. This is most likely to occur in laboratories that do not have separate disk dispensers for enterococci and that test enterococci with either gram-positive organism or urinary tract isolate antimicrobial disk batteries that include an aminoglycoside, as suggested recently (9).

MATERIALS AND METHODS

Organisms. Fifty consecutive clinical isolates of enterococci were collected from the Clinical Microbiology Laboratory at the University of Utah Medical Center over a 3-week period. All were identified by esculin hydrolysis and 6.5% NaCl tolerance testing (11).

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains for the antimicrobial disk susceptibility testing. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as control strains for the agar dilution susceptibility testing.

Disk diffusion susceptibility testing. Disk diffusion antimicrobial susceptibilities were performed as described by the standard Bauer-Kirby technique (3, 4) with single-lot, noncation-adjusted MHA (BBL Microbiology Systems, Cockeysville, Md.). These tests with the above controls were performed in duplicate with standard-content diffusion disks. The following zone diameters were interpreted as being intermediate in susceptibility: gentamicin, 13 to 14 mm; kanamycin, 14 to 17 mm; tobramycin, 13 to 14 mm; and amikacin, 15 to 16 mm (19). Fifty isolates of enterococci

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were tested for susceptibility to kanamycin, gentamicin, tobramycin, and amikacin by the disk diffusion technique to compare media made with and without 5% defibrinated SB and to document the extent of the false susceptibility.

In looking for other antimicrobial agents that might demonstrate similar false susceptibility, seven enterococcal strains were tested by the disk diffusion technique for susceptibility to a wide range of antimicrobial agents to compare media prepared with and without 5% SB. The antimicrobial agents tested included cephalothin, cefoxitin, cefamandole, carbenicillin, ampicillin, penicillin, clindamycin, erythromycin, chloramphenicol, polymyxin B, sulfisoxazole, and nafcillin. In an attempt to find other organisms demonstrating false susceptibility to aminoglycosides, 14 nonenterococcus isolates were tested by disk diffusion for susceptibility to gentamicin, tobramycin, kanamycin, and amikacin to compare media made with and without 5% SB. The isolates tested included the following: *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus bovis*, *Streptococcus equinus* (group D streptococci, nonenterococcus), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhi*, *Providencia stuartii*, *Morganella morganii*, *Enterobacter cloacae*, and *Citrobacter freundii*.

To search for the component of SB responsible for the false susceptibility to aminoglycosides, susceptibilities of 10 isolates of enterococci to gentamicin, tobramycin, kanamycin, and amikacin were tested by the disk diffusion technique to compare MHA media alone with that containing several different additive agents. These agents included the following: 5% SB with a hematocrit of 38%; pooled sheep serum (62% of the volume of the whole SB employed); 5% saline-washed and pooled sheep erythrocytes with a hematocrit of 36% (sheep blood, erythrocytes, and serum obtained from Colorado Serum Co., Denver, Colo.); hemoglobin, 650 mg/dl (BBL); hemin, 26 and 13 mg/dl (Eastman Kodak Co., Rochester, N.Y.); protoporphyrin IX, 22 and 11 mg/dl (Porphyrin Products, Logan, Utah); ferrous sulfate, 12 mg/dl (J. T. Baker Chemical Co., Phillipsburg, N.J.); and delta-aminolevulinic acid, 10 mg/dl (Porphyrin Products). The concentrations chosen were generally the amounts present in 5% SB.

Agar dilution. Agar dilution MICs were used rather than broth dilution, because the addition of blood made interpretation difficult by the broth dilution method. Agar dilution MICs were performed by a standard technique that employs serial twofold dilutions of antibiotic and gives final plate concentrations ranging from 256 to 0.0155 μg/ml in MHA. The replicator device of Steers, Foltz, and Graves provided standardized inoculation of test and control strains (27). For comparison with the disk dilution method, agar dilution MICs were performed in duplicate on 50 isolates of enterococci for tobramycin, gentamicin, kanamycin, and amikacin to compare media made with and without 5% SB. MICs were interpreted as resistant when in the following ranges: gentamicin, ≥8 μg/ml; kanamycin, ≥25 μg/ml; tobramycin, ≥8 μg/ml; and amikacin, ≥32 μg/ml. MICs were interpreted as susceptible when in the following ranges: gentamicin, ≤4 μg/ml; kanamycin, ≤6 μg/ml; tobramycin, ≤4 μg/ml; and amikacin, ≤16 μg/ml (19).

Growth curves. Growth studies were performed by one of two methods. Quantitative growth studies were performed with Mueller-Hinton broth (BBL). Eighteen milliliters of broth was inoculated with 2 ml of an enterococcal suspension standardized to contain 10^7 to 10^8 CFU/ml with the Autobac 1 photometer (General Diagnostics Division, Warner-Lambert Co., Morris Plains, N.J.). Samples were obtained hourly for colony counts while incubating under continuous agitation for 6 h at 37°C. Samples were serially diluted and plated on SB agar by the calibrated-loop technique for quantitation. Growth curves were plotted for two enterococcal strains and one *S. aureus* control strain to compare growth rates in the presence of broth alone, 5% SB, and hemin (3 μg/ml). Each broth was tested with and without tobramycin at 4 μg/ml (MIC = 32 μg/ml).

Growth curves were also performed with the Autobac 1 system, with Mueller-Hinton broth rather than the customary eugonic broth. Hemin was added to three of the four broth lots in concentrations of 3.0, 0.3, and 0.03 μg/ml; 18 ml of each broth was inoculated with 2 ml of enterococcal suspension standardized to contain 10^7 to 10^8 CFU/ml and dispensed into each cuvette. Cuvettes divided the broth into 12 test chambers of ca. 1.4 ml per chamber, to which various concentrations of aminoglycosides were added. Four different concentrations of tobramycin, gentamicin, kanamycin, and amikacin were individually added in 0.1-ml samples to chambers representing each of the four broth lots. The concentrations chosen were generally the second through the fifth twofold dilution below the MIC of each of the five strains of enterococci tested. The cuvettes were incubated at 37°C for 6 h while being rotated continuously at 220 rpm. Hourly nephelometric recordings were performed with the photometer in the calibrate mode, providing raw voltage readings in proportion to turbidity.

Statistical analyses. The *t* test was used to assess the probability of the blood components causing the increased zone diameters in disk diffusion antimicrobial susceptibility testing and the decreased MICs in agar dilution MIC testing.

RESULTS

The addition of SB to MHA for disk diffusion antimicrobial susceptibilities of enterococci resulted in increased zone diameters for tobramycin (mean, 7.6 mm), gentamicin (6.3 mm), amikacin (4.8 mm), and kanamycin (4.0 mm). In all cases, the change was statistically significant ($P < 0.0001$). For tobramycin, the maximum change was 12 mm. Only one organism showed no change, and only three had discrepancies of less than 3 mm. The maximum change for gentamicin was 13 mm, with eight organisms having less than 3 mm of change. The maximum change for amikacin was 11 mm, and six strains showed no change. The maximum change for kanamycin was 8.5 mm, with 17 isolates exhibiting no change.

These changes in zone diameters caused significant changes in the interpretations of susceptibility to the specific antimicrobial agents, as outlined in Table 1. For tobramycin, 36 organisms (72%) changed from resistant to susceptible when 5% SB was present, whereas only 10% had unchanged susceptibility interpretations. False susceptible results with gentamicin occurred with 35 organisms (70%). This was less of a problem with amikacin and kanamycin, as changes to false susceptibility were present with only 4 and 2% of the isolates, respectively. Changes to false intermediate susceptibility were present with 6 and 16%, respectively. Many organisms showed no change in zone diameters when tested with kanamycin and amikacin; uniformly, these were the organisms with zone diameters of 6 mm (i.e., confluent growth to the disk), indicating high-level resistance. The vast majority had MICs in excess of 256 μg/ml.

The agar dilution MIC control organisms gave reliable results with no significant change as a result of the addition

of SB. Both *S. aureus* and *E. coli* showed very sharp growth endpoints, with luxuriant growth at sub-MIC levels of the aminoglycoside, and no growth whatsoever at or above the MIC level of the aminoglycoside. The enterococci, on the other hand, did not show sharp growth endpoints, and the MIC became a somewhat subjective observation. The test was read as positive when any obvious growth was noted by the naked eye; fine or barely visible haze was ignored. Unlike the controls, the enterococcal growth did not become luxuriant until as many as five or more dilutions below the MIC; microscopic growth was often evident at one or more dilutions above the MIC. This was evident for the MHA plates, both with and without 5% SB. Agar dilution MICs did not show the false aminoglycoside susceptibility nearly to the extent that disk diffusion did. In fact, there were no changes that resulted in false susceptibility for amikacin or kanamycin in the SB agar dilution plates and only three such false susceptible results for both gentamicin and tobramycin (Table 2). The only consistent change was a pervasive decrease in the growth on the plates containing SB supplementation for several dilutions below the MIC, indicating slower or poorer growth. Although many MICs decreased by one and rarely two dilutions (mean of 0.98 for gentamicin, 0.46 for amikacin, 0.40 for tobramycin, and 0.04 for kanamycin), it was not enough to change many of the MIC interpretations.

We noted no other significant changes of zone disk diffusion diameters attributed to SB in the presence of any of the nonaminoglycoside antimicrobial agents tested on the enterococcal isolates. In addition, no other organisms demonstrated SB-induced increases in zone diameters with the exception of *Streptococcus bovis* (group D streptococcus, nonenterococci). The *Streptococcus equinus* strain grew so poorly that an interpretation of the disk diffusion results was impossible. Group A and B hemolytic streptococci grew so poorly on media without SB that zone sizes decreased with the addition of SB, as a result of the nutritive effect of blood.

The blood component responsible for the zone diameter discrepancy appeared to be present in the erythrocyte fraction and specifically appeared to be hemin, the oxidized, ferric form of heme (Table 3). Heme-containing blood components demonstrated the discrepancy, whereas the products of heme degradation did not. Growth kinetic studies demonstrated that the addition of 5% SB did not affect the growth pattern of enterococci when compared with Mueller-Hinton broth alone. Although hemin was toxic to enterococci and *Staphylococcus aureus* control organisms in amounts down to 30 μg/ml, concentrations of 3 μg/ml or less had absolutely no effect on the growth of enterococci. However, the combination of sub-MIC amounts of an aminoglycoside with either 3.0, 0.3, or 0.03 μg of hemin per ml caused a striking decline in growth rates when compared with the effect of the aminoglycoside alone. The growth kinetic studies of hemin at 3 μg/ml follow closely the curves of 5% SB, as both clearly show synergy with tobramycin at 4 μg/ml in inhibiting enterococci. A quantitative growth curve exhibiting this phenomenon is shown in Fig. 1. This synergy was documented with all four aminoglycosides on the five enterococci tested. An example of the effect of synergy between hemin and multiple sub-MIC levels of tobramycin is shown in Fig. 2. These studies showed that

TABLE 1. Differences in interpretation of enterococcal disk diffusion susceptibility testing of aminoglycosides with 5% SB supplementation of MHA

Antimicrobial agent and result on MHA (*n*)	Result on MHA–5% SB (*n*)		
	Resistant	Intermediate	Susceptible
Tobramycin			
Resistant (50)	5	9	36
Intermediate (0)	0	0	0
Susceptible (0)	0	0	0
Gentamicin			
Resistant (45)	4	6	35
Intermediate (2)	0	0	2
Susceptible (3)	0	0	3
Amikacin			
Resistant (50)	45	3	2
Intermediate (0)	0	0	0
Susceptible (0)	0	0	0
Kanamycin			
Resistant (50)	41	8	1
Intermediate (0)	0	0	0
Susceptible (0)	0	0	0

TABLE 2. Differences in interpretation of agar dilution susceptibility testing of aminoglycosides with 5% SB supplementation of MHA

Antimicrobial agent and result on MHA (*n*)	Result on MHA–5% SB (*n*)		
	Resistant	Intermediate	Susceptible
Tobramycin			
Resistant (50)	47	0	3
Intermediate (0)	0	0	0
Susceptible (0)	0	0	0
Gentamicin			
Resistant (49)	46	0	3
Intermediate (0)	0	0	0
Susceptible (1)	0	0	1
Amikacin			
Resistant (49)	49	0	0
Intermediate (0)	0	0	0
Susceptible (1)	0	0	1
Kanamycin			
Resistant (49)	49	0	0
Intermediate (1)	0	1	0
Susceptible (0)	0	0	0

TABLE 3. Enterococcal zone size enlargement from blood components[a]

Blood component	Range (%)
Washed sheep cells	87–100
Pooled sheep serum	19–35
Hemolyzed sheep serum	49
Bovine hemoglobin	77
Hemin	80–100
Protoporphyrin IX	36–44
Delta-aminolevulinate	0
Ferrous sulfate	0

[a] The values represent the zone size increases caused by the supplementation of MHA with the blood components listed in disk diffusion testing of 10 enterococci and are percentages as compared with the increase caused by 5% SB supplementation. Ranges represent values for four aminoglycosides. Single values represent testing of tobramycin.

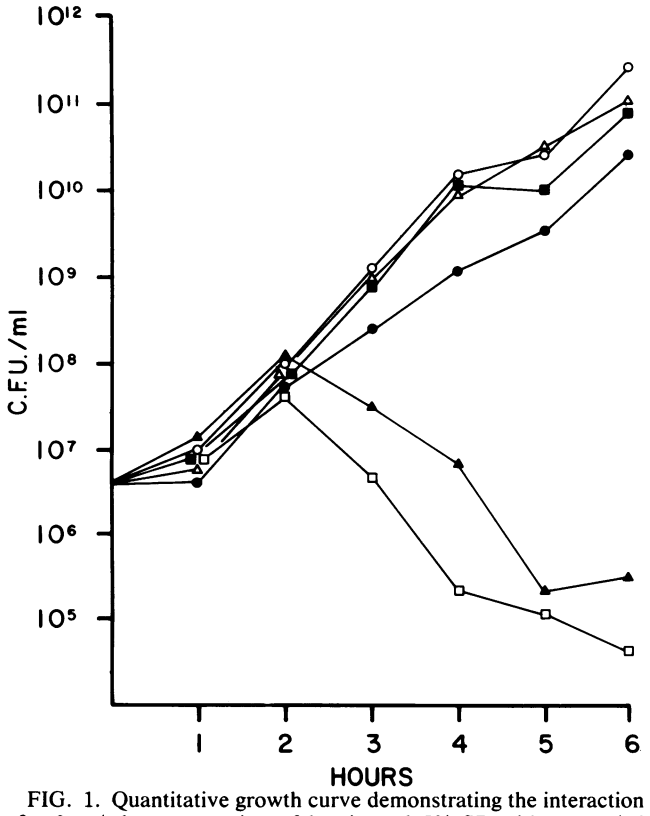

FIG. 1. Quantitative growth curve demonstrating the interaction of a 3-μg/ml concentration of hemin and 5% SB with a 4-μg/ml concentration of tobramycin with enterococcal growth rate. The MIC of the strain shown is 32 μg/ml. Symbols: ○, Mueller-Hinton broth plus 5% SB; △, Mueller-Hinton broth plus hemin; ■, Mueller-Hinton broth alone; ●, Mueller-Hinton broth plus tobramycin; ▲, Mueller-Hinton broth plus 5% SB plus tobramycin; □, Mueller-Hinton broth plus tobramycin plus hemin.

synergy was documented best with hemin levels of 3 μg/ml and most prominently with gentamicin and tobramycin. The degree of synergy, measured in the number of dilutions below the MIC for each aminoglycoside showing synergy with hemin, closely paralleled the order of zone size increases with disk diffusion testing. The number of dilutions showing synergy were 5, 4, 3, and 2.5 for tobramycin, gentamicin, amikacin, and kanamycin, respectively, when combined with hemin at 3 μg/ml.

DISCUSSION

These results demonstrate the high incidence of false susceptibility of enterococci to aminoglycosides, particularly to tobramycin and gentamicin, when SB is used to supplement MHA in disk diffusion antimicrobial susceptibility testing. The interaction appeared to be limited to the group D streptococci when tested with the aminoglycosides. In that light, the effect is not simply an antimicrobial agent-blood or organism-blood interaction but actually involves a synergistic inhibitory effect of blood and aminoglycosides specific for enterococci. It is important to understand that such an effect occurs and can be obviated by avoiding test circumstances which could lead to erroneous results.

Other investigators have shown a similar interaction between enterococci and several broad-spectrum cephalosporins, with enhanced susceptibility from blood enrichment of the test medium (16, 22, 23, 25). The enhancement was found to be both medium dependent (commercial brand and blood content) (22) and inoculum dependent (16). Moellering et al. found variable enhancement with several blood products, however, and designated the α_2-globulin fraction as the best candidate for the etiological agent. They also suggested that the activity involved binding of the globulin to the enterococcus (16).

In our studies, the blood component responsible for the false susceptibility was determined to be heme, the iron-containing porphyrin ring structure. The mechanics of the heme interaction was not readily apparent. A clue that heme could actually have antimicrobial effects came with the observation that agar dilution MICs showed particularly poor growth of enterococci on media that contained 5% SB at several dilutions below the MIC when compared with media without SB. This caused us to look for synergy between heme and sub-MIC levels of aminoglycosides. Alone, neither blood nor heme has an effect on the growth of enterococci; however, when combined with an aminoglycoside, they each have a powerful inhibitory effect on enterococci.

Because the inhibition is manifested as a decline in the rate of growth and is not a cidal effect, the agar dilution MICs theoretically should not be, and in fact were not, altered

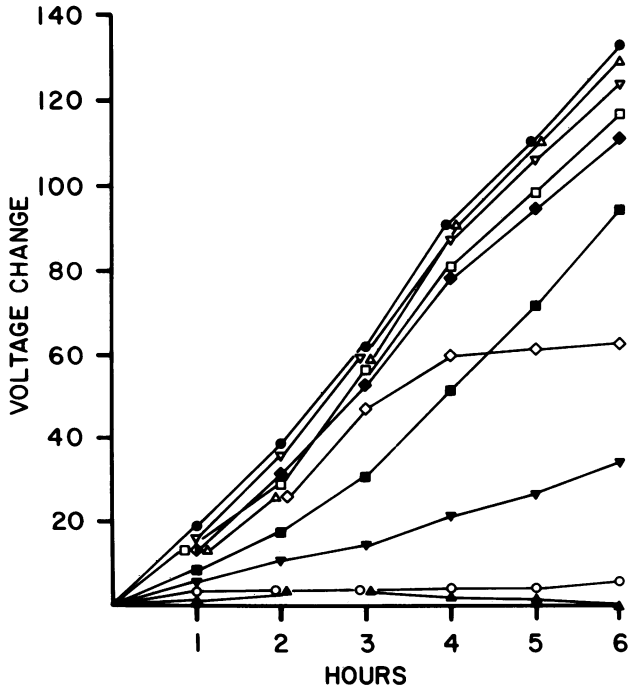

FIG. 2. Autobac growth kinetic studies demonstrating synergy between sub-MIC levels of tobramycin and a 3-μg/ml concentration of hemin in inhibiting enterococcal growth. The MIC of the strain shown is 32 μg/ml. Symbols: ●, Mueller-Hinton broth plus hemin; △, Mueller-Hinton broth alone; ▽, Mueller-Hinton broth plus 1 μg of tobramycin per ml; □, Mueller-Hinton broth plus 2 μg of tobramycin per ml; ◆, Mueller-Hinton broth plus 4 μg of tobramycin per ml; ■, Mueller-Hinton broth plus 1 μg of tobramycin per ml plus hemin; ◇, Mueller-Hinton broth plus 8 μg of tobramycin per ml; ▼, Mueller-Hinton broth plus 2 μg of tobramycin per ml plus hemin; ○, Mueller-Hinton broth plus 4 μg of tobramycin per ml plus hemin; ▲, Mueller-Hinton broth plus 8 μg of tobramycin per ml plus hemin.

significantly by the addition of blood. As the amount of growth in the first dilution below the MIC was marginal in many cases, it is understandable why one, and rarely two, twofold dilution result changes were possible in MICs done with SB.

While Sahm et al. questioned whether the blood-enhanced susceptibility of the enterococci to the broad-spectrum cephalosporins was a better reflection of the in vivo susceptibility, our studies with the aminoglycosides show that the disk diffusion susceptibility test can be falsely sensitive (22). The reduction in the MIC of one or two dilutions is generally insufficient to cause a susceptible test result, indicating the probable true resistance of the enterococci to the aminoglycosides.

The synergistic effect of heme and aminoglycosides on growth rate is much more important with disk diffusion testing. A zone of inhibition is determined at a critical growth time during incubation. This is a result of the balance between the constantly diffusing antimicrobial agent against a logarithmically multiplying microorganism (3). One can then conceptualize how the addition of heme effectively expands the concentric ring of aminoglycoside in a disk diffusion test as the peripheral sub-MIC levels of aminoglycoside interact with heme, slowing the growth of enterococci at the margins of the ring. This allows increased time for aminoglycoside diffusion and alters the critical time of zone formation with the resultant larger zones.

The exact mechanism of heme-aminoglycoside interaction on enterococci is perhaps beyond the scope of this paper and probably only of academic interest; however, from what we now know, there are a few plausible explanations. It is likely that a heme enzyme or hemoprotein is involved. Recent research has shown that heme and heme products demonstrate damaging effects on lipid membranes. This has been specifically seen in mouse erythrocytes (8), *Trypanosoma brucei* (15), and *Plasmodium berghei* (21) in causing hemolysis and trypanocidal and antimalarial activity. Meshnick et al. postulated that the trypanocidal activity is mediated through hydroxyl radicals formed from the cleavage of intracellular hydrogen peroxide by heme (15). The effectiveness of heme in catalyzing the cleavage of peroxide with free-radical formation is documented in the literature (26).

Trypanosomes are known to be free of catalase, as are enterococci. This lack of catalase is associated with significant amounts of intracellular hydrogen peroxide, making catalase-negative organisms vulnerable to compounds that form free radicals from peroxide cleavage. The addition of riboflavin, a free-radical scavenger, decreased the hemin trypanocidal activity, whereas the addition of diamide, a chemical that depletes reduced glutathione, increased the trypanocidal activity. Albumin was noted to neutralize the trypanocidal activity by tightly binding free heme (15).

In malarial parasites, hemoglobin is taken up, and heme is produced and sequestered in malarial pigment in an innocuous form (12). In the presence of chloroquine, however, a chloroquine-hemin complex is formed, which has antimalarial effects. This is felt to be the basis of the chloroquine antimalarial effect, as chloroquine alone has no harmful effects on malarial parasites (7). Other studies document that indeed hemin is the chloroquine receptor, mediating antimalarial activity by forming aggregates between hemin and chloroquine. Chou et al. demonstrated that chloroquine binding to hemin enhanced the hemolysis of mouse erythrocytes (8).

With this background, it appears likely that enterococcus, a catalase-negative organism, would be susceptible to hemin-caused peroxide cleavage and lysis from free-radical damage. Hemin is potentially made available by enterococcal proteases so that it may bind with aminoglycosides in the way it binds to chloroquine. By this mechanism hemin-aminoglycoside aggregates may be activated and cause membrane damage analogous to the chloroquine potentiation of hemin in the malaria model. In addition, the effect of hemin on the membrane may be additive to the antibacterial effect of sub-MIC levels of aminoglycoside. Evidence of aggregation between hemin and aminoglycosides was provided in an experiment combining a 30-μg/ml concentration of hemin with an 8-μg/ml concentration of gentamicin. By nephelometry, the turbidity increased and plateaued by 1 h, although this was not related to bacterial growth. We postulate that the cephalosporin interaction may work on a similar mechanism. If this theory is correct, then we would expect other catalase-negative organisms to be similarly affected. We would also expect that the effect of hemin on zone diameters would be enhanced by diamide and decreased by riboflavin.

Preliminary testing of this hypothesis has been suggestive of this mechanism but is not diagnostic and is particularly hard to test because of the rapid reversal of the diamide depletion of reduced glutathione upon incubation (13). More definitive test results could be obtained in testing for malondialdehyde, a product of lipid peroxidation; however, this is beyond the means of our laboratory (14).

Where the idea of blood supplementation for enterococcal susceptibilities began is difficult to determine. Several studies contained in the international collaborative study on susceptibility testing advise blood supplementation of fastidious organisms and uniformly claim no consistent differences noted between zone diameters with and without blood. Although multiple organisms were tested, none of the studies included an enterococcus (10). A study by Brenner et al. also specifically looked at the use of blood supplementation in disk diffusion testing but again failed to test enterococci (5). More recently, a text, *Antibiotics in Laboratory Medicine*, also states that defibrinated blood may be used to supplement MHA for fastidious organisms in disk diffusion susceptibility testing (1, 2). It is unclear from this just how the use of blood for enterococcal disk diffusion testing was sanctioned.

Before the discovery of the heme-induced false susceptibility of enterococci to aminoglycosides, our laboratory routinely used 5% SB enrichment for enterococcal disk diffusion testing, although we did not test aminoglycoside antibiotics against these strains. We expect that the practice of using SB for enterococcal testing remains fairly widespread, as it was specifically sanctioned in the National Committee for Clinical Laboratory Standards performance standards (17). Although two updated supplements and a newer tentative standard have been published, they do not specifically address the problem (18–20). However, they do state that antimicrobial agents not suggested for routine testing against enterococci, such as the cephalosporins, clindamycin, and aminoglycosides, should not be tested, as the reporting of their results can be dangerously misleading. We realize that aminoglycosides are not routinely necessary for the testing of streptococci because of their clinical resistance to aminoglycosides. The exception is bacterial endocarditis in which an awareness of the possibility of high-level resistance is useful when considering combination antimicrobial therapy (6, 24). Because of the clinical resistance, it is imperative that those laboratories using blood-

supplemented MHA for enterococcal disk diffusion testing be aware that a falsely reported aminoglycoside "sensitive" report may well be used as a basis of treatment by those not experienced with using aminoglycosides for enterococcal infections. Based on the studies of others, the same holds true for the broad-spectrum cephalosporins (16, 22, 23, 25). Blood enrichment of MHA should therefore be abandoned in this situation, particularly since we have shown that susceptibilities of enterococci are accurate without SB supplementation of MHA. Further studies need to be done to evaluate the effect of individual commercial brands of MHA on this phenomenon.

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