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Early Detection of Oxacillin-Resistant Staphylococcal Strains with Hypertonic Broth Diluent for Microdilution Panels

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A total of 292 coagulase-positive and 111 coagulase-negative staphylococcal strains were tested in microdilution MIC panels containing 16 to 0.13 μg of oxacillin per ml diluted in cation-supplemented Mueller-Hinton broth with and without an additional 2% NaCl. All strains were tested using the stationary-phase inoculum procedure with an incubation temperature of 35°C. Test results were recorded after 16 to 20 h of incubation; staphylococcal strains susceptible to oxacillin (≤2 μg/ml) were reincubated for 20 to 24 h, and endpoints were determined again. Oxacillin resistance was found in 27 (9%) of the 292 coagulase-positive strains and 39 (35%) of the 111 coagulase-negative strains. Of these resistant strains, 5 (19%) of the 27 coagulase-positive strains and 13 (33%) of the 39 coagulase-negative strains were detected 24 h earlier in cation-supplemented Mueller-Hinton broth with 2% NaCl than in cation-supplemented Mueller-Hinton broth without the additional NaCl. However, 9 (33%) of the 27 resistant coagulase-positive strains and 10 (26%) of the 39 resistant coagulase-negative strains were detected only after an additional 24 h of incubation. Oxacillin MICs for the 265 coagulase-positive susceptible strains and 72 coagulase-negative susceptible strains were not affected by the additional 2% NaCl. These results support the utility of adding 2% NaCl to the broth diluent for the early detection of oxacillin-resistant staphylococcal strains and the necessity of extended incubation for those strains which initially appear to be susceptible to oxacillin after only 16 to 20 h of incubation.

Susceptibility testing of methicillin- or oxacillin-resistant staphylococcal strains is complicated by their genetic heterogeneity. Broth cultures of resistant staphylococcal strains consist of various proportions of susceptible and resistant organisms (2, 8, 13, 14). In clinical specimens, resistant organisms may represent only a small proportion of the total number of organisms in the suspension (2, 8, 13, 14). Moreover, resistant staphylococcal strains may grow poorly in a broth medium (2, 8). For these reasons, resistant staphylococcal strains may not be detected in the standard overnight broth microdilution technique used in determining MICs.

To enhance the growth of the slower-growing resistant staphylococcal strains and thus to improve the detection of these strains in the microdilution MIC panel system, it has been recommended that test panels be incubated at 30 to 35°C (2, 15) and that the incubation time be increased to 48 h for those staphylococcal strains which appear to be susceptible after overnight incubation (2). Although these recommendations are relatively easy to implement, a method that would improve detection of resistant staphylococcal strains at 16 to 18 h with the microdilution MIC system would be an advantage.

Other methods of enhancing the growth of resistant strains include increasing the NaCl concentration of the broth (1, 2, 16), increasing the organism concentration of the inoculum (2), and a combination of these methods. The addition of NaCl to the broth diluent has not been extensively used in routine testing, since few laboratories presently prepare MIC panels for their own use, and most commercial MIC panel manufacturers have not incorporated additional NaCl in their oxacillin concentrations. When prepared MIC panels are used, the addition of NaCl to the wells can be accomplished through the inoculum diluent, but when NaCl is inoculated to all wells of the panel, it is likely to inhibit the activity of some aminoglycosides and the growth of some organisms (6, 16). An increase in the organism concentration in the inoculum could be useful in the oxacillin wells, but it is not recommended for use with other antimicrobial agents in the MIC panel (12).

Because of these difficulties, it has not been possible for many laboratories to readily improve the detection of resistant staphylococcal strains in the microdilution MIC method. Hence, many laboratories have chosen to perform the disk diffusion susceptibility technique (4) or the agar overlay method (3), both of which have been considered reliable methods for detecting methicillin- or oxacillin-resistant staphylococcal strains. For those laboratories that perform MIC determinations routinely, these variations from routine MIC susceptibility testing are disadvantageous.

This study was performed to determine whether oxacillin-resistant staphylococcal strains could be more reliably detected with a hypertonic broth diluent (2% NaCl) for oxacillin. Instead of being added to the inoculum, NaCl was added only to the oxacillin concentrations during preparation of the MIC panels. The effects of this modification on both susceptible and resistant staphylococcal strains at 16 to 24 h and 40 to 48 h of incubation were determined. Staphylococcal strains for which endpoints were in the range of 2 to 8 μg/ml by the standard MIC microdilution method (12) were confirmed by the standard disk diffusion method (11).

MATERIALS AND METHODS

Bacterial strains. A total of 292 coagulase-positive and 111 coagulase-negative staphylococcal isolates were identified by standard methodology (10). The isolation, identification, and subsequent MIC susceptibility testing was performed at four institutions (see Acknowledgments). These staphylo-

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TABLE 1. Detection of resistant staphylococcal strains[a]

Staphylococcal strains	Total no., (%) of resistant isolates detected after:			
	16 to 18 h at 35°C		40 to 48 h at 35°C	
	MHB[b] without NaCl	MHB[b] with NaCl[c]	MHB[b] without NaCl	MHB[b] with NaCl[c]
Coagulase positive	13 (48)	18 (67)	21 (78)	27 (100)
Coagulase negative	16 (41)	29 (74)	36 (92)	39 (100)

[a] Resistance = MIC of >2 μg/ml.
[b] MHB, Cation-supplemented Mueller-Hinton broth (50 mg of Ca^{2+} per liter, 25 mg of Mg^{2+} per liter).
[c] Two percent NaCl (20 g/liter).

coccal strains were all isolates from clinically significant infections for which antimicrobial susceptibility testing had been requested. Standard strains (derived from *Staphylococcus aureus* ATCC 29213 and ATCC 25923) were also included for quality control purposes and stability studies.

Inoculum standardization procedures. The inoculum was prepared and standardized by the stationary-growth-phase technique (12). Four to five isolated colonies from overnight growth on solid medium were transferred to 0.5 ml of prepared brain heart infusion broth (Pasco Laboratories, Inc., a subsidiary of Difco Laboratories, Wheat Ridge, Colo.). All media were obtained from Pasco Laboratories unless otherwise specified. This broth was incubated at 35°C for 4 to 6 h. Twenty-five microliters of this suspension, containing ca. 10^9 CFU/ml, was transferred to 12.5 ml of sterile distilled water with 0.02% Tween 80. This bacterial suspension was inoculated (Anderson Technical Inoculator, Lakewood, Colo.) into routine MIC panels as well as into the oxacillin test panels with and without additional NaCl. The final inoculum of 10^5 CFU/ml was checked for purity and organism concentration by the transfer of 0.01 ml to tryptic soy agar with 5% sheep blood.

Microdilution test panels. Because it is more stable than methicillin in this test system (2), oxacillin (Bristol Laboratories, Syracuse, N.Y.) was used in the preparation of the MIC microdilution panels (Pasco Laboratories). Oxacillin was serially diluted to concentrations of 16 to 0.13 μg/ml in Mueller-Hinton broth and supplemented to final concentrations of 50 mg of calcium per liter and 25 mg of magnesium per liter (12), with and without additional 2% NaCl. One hundred microliters of each of these dilutions was dispensed in duplicate into a total of 32 wells of a microtiter plate. The panels were immediately frozen and stored at −20°C for up to 6 months.

After inoculation, MIC microdilution panels were incubated for 16 to 20 h at 35°C, and MICs were determined with a viewbox (Sienco, Morrison, Colo.). MICs were recorded as the lowest concentration of antimicrobial agent at which there was no visible growth of the organisms. Susceptible strains for which oxacillin MICs were ≤2 μg/ml (12) were incubated for an additional 20 to 24 h, at which time the endpoints were again determined and recorded. This additional incubation time resulted in a total incubation of 40 to 48 h at 35°C. Test results in the MIC range of 2 to 8 μg/ml were confirmed with standard disk diffusion susceptibility methods (11) at an incubation temperature of 35°C.

RESULTS

Susceptible and resistant categories were interpreted according to the recommendations of the National Committee for Clinical Laboratory Standards (12). Staphylococcal organisms for which oxacillin MICs were >2 μg/ml were considered to be resistant. Endpoints were determined at 16 to 18 h and again at 40 to 48 h. Clearly susceptible (≤1 μg/ml) or resistant (≥8 μg/ml) strains were not confirmed by the disk diffusion method because of previously demonstrated correlations in these ranges (2). Strains for which oxacillin MICs were in the range of 2 to 8 μg/ml were confirmed by the standard disk diffusion method and showed complete correlation with the MIC microdilution susceptibility results when 2% NaCl was present in the oxacillin wells.

Of the 292 coagulase-positive and 111 coagulase-negative staphylococcal strains tested, 27 (9%) and 39 (35%), respectively, were determined to be resistant (>2 μg/ml) after extended incubation. Of these resistant organisms, 18 (67%) of the coagulase-positive and 29 (74%) of the coagulase-negative strains were detected at 16 h in cation-supplemented Mueller-Hinton broth with 2% NaCl, whereas only 13 (48%) of the coagulase-positive and 16 (41%) of the coagulase-negative strains were detected in cation-supplemented Mueller-Hinton broth without 2% NaCl. That is, 5 (19%) of the resistant coagulase-positive strains and 13 (33%) of the resistant coagulase-negative strains were detected 20 to 24 h earlier in the cation-supplemented Mueller-Hinton broth with 2% NaCl than in the broth without 2% NaCl (Table 1). The addition of 2% NaCl to the cation-supplemented Mueller-Hinton broth not only resulted in the earlier detection of resistant strains, but also improved the ease of detection at 24 h by increasing oxacillin MICs by fourfold or more for 33% of the resistant strains.

After 48 h of incubation, an additional 9 (33%) of the 27 resistant coagulase-positive and 10 (26%) of the 39 resistant coagulase-negative staphylococcal strains were detected (Table 1). Of these additional resistant strains, six coagulase-positive and three coagulase-negative strains (14%) were detected in the cation-supplemented Mueller-Hinton broth with NaCl and not in the cation-supplemented Mueller-Hinton broth without NaCl. These nine strains were tested with the standard disk diffusion method and were confirmed to be resistant.

The MICs determined in cation-supplemented Mueller-Hinton broth with and without 2% NaCl were essentially identical (within one dilution) for the 265 coagulase-positive and 72 coagulase-negative oxacillin-susceptible strains. There was no evidence of inhibition of staphylococcal growth by NaCl in any of the susceptible strains studied when 2% NaCl was added to the broth diluent.

Quality control included the determination of colony counts of the inoculum for each test panel. The expected colony count of the inoculum was 100,000 CFU/ml. The mean colony counts for coagulase-positive and coagulase-negative organisms were 84,000 CFU/ml (standard deviation = 28,000) and 60,000 CFU/ml (standard deviation = 28,000), respectively. Quality control of the MIC panel included the determination of MICs for reference strains. There was no variation from expected endpoint ranges over a six-month period, confirming the stability for up to 6 months of oxacillin diluted in hypertonic broth (2% NaCl) and stored at −20°C.

DISCUSSION

Difficulties in the detection of oxacillin-resistant staphylococci by microdilution MIC methods have been attributed to the slow growth of resistant strains in the Mueller-Hinton broth diluent (2, 8). This problem is particularly apparent in

those antimicrobial systems which rely upon shorter incubation times (4 to 6 h) (6, 7, 9). If the growth of these resistant strains could be enhanced, earlier and more reliable detection would result. Tolerance to high concentrations (6.5 to 10%) of NaCl has long been used as an identifying characteristic of staphylococci (10), and previous reports indicate that lower concentrations (2 to 5%) of NaCl actually enhance the growth of some oxacillin-resistant strains (1, 2, 16).

Although others have reported enhanced detection of methicillin- or oxacillin-resistant staphylococci in the MIC microdilution system with hypertonic broth diluent, they have relied on fewer clinical isolates, a predominance of stock organisms, and different NaCl concentrations (1, 2, 16). In this study, susceptibility testing was performed on 292 coagulase-positive and 111 coagulase-negative staphylococcal clinical isolates. Microdilution MIC panels were used to make a comparison of the susceptibility of these staphylococcal isolates to oxacillin diluted in cation-supplemented Mueller-Hinton broth with and without an additional 2% NaCl at 16 to 20 and 40 to 48 h of incubation at 35°C. These isolates were selected from clinically significant infections for which MIC determinations had been requested, and thus they did not represent a sample of all staphylococci isolated at the four institutions taking part in this study. This selection process may explain the high incidence of oxacillin-resistant strains in this study as compared with that in a previous report (5).

The addition of 2% NaCl to the oxacillin concentrations was made during preparation of the panels. If added to the panel as part of the inoculum, the NaCl could have deleterious effects on the activity of other antimicrobial agents in the panel and could inhibit the growth of some organisms (6, 16). Preliminary studies with a limited number of oxacillin-resistant isolates were done to determine the optimum NaCl concentration to enhance growth. There appeared to be no difference in oxacillin endpoints when they were determined in hypertonic broth at concentrations of 2, 3, and 5% NaCl. The 2% NaCl concentration was chosen to allow for possible minor increases in the NaCl concentration resulting from the dehydration of the MIC panels during freezer storage and incubation.

This study demonstrates that hypertonic broth enhances the growth of some resistant staphylococcal strains, thus allowing earlier detection. It was found that 18 (27%) of 66 resistant strains of staphylococcal clinical isolates could be detected up to 20 to 24 h earlier with cation-supplemented hypertonic Mueller-Hinton broth. Moreover, when 2% NaCl was omitted from the broth diluent, 9 (14%) of 66 resistant staphylococcal strains were not detected even after 40 to 48 h of incubation.

When a breakpoint of 2 μg/ml was used, as recommended by the National Committee for Clinical Laboratory Standards (12), there was a complete correlation between oxacillin resistance as demonstrated by disk diffusion and oxacillin resistance as demonstrated by microdilution MIC determinations when cation-supplemented Mueller-Hinton broth with 2% NaCl was used as the diluent. Oxacillin MIC endpoints for 83% of the susceptible strains were <1 μg/ml at 24 and 48 h, oxacillin MIC endpoints for 54% of the resistant strains were >8 μg/ml at 24 h, and oxacillin MIC endpoints for 89% of the strains were >8 μg/ml at 48 h.

The addition of 2% NaCl did not affect the MIC test results of susceptible staphylococcal strains. The MICs determined for the 337 susceptible clinical strains and control organisms tested in Mueller-Hinton broth with and without NaCl were identical or within the acceptable range of plus or minus one dilution, even after extended incubation for up to 48 h. These results would indicate that 2% NaCl could be added to the broth diluent for oxacillin with no effect upon the growth of susceptible organisms. Moreover, the stability of oxacillin was essentially the same in cation-supplemented Mueller-Hinton broth with or without 2% NaCl and was determined to be a minimum of 6 months when stored at −20°C or less.

A precaution should be observed by those working with gram-negative organisms, some of which are inhibited by the NaCl. Although oxacillin is not intended for use with these organisms, some MIC panels are used to test both gram-positive and gram-negative organisms. If experimenters are unaware of this inhibition phenomenon, oxacillin MICs reported for gram-negative organisms could be misinterpreted as indications of susceptibility.

This study demonstrates that a significant number (18 [27%] of 66) of oxacillin-resistant staphylococcal strains can be detected up to 24 h earlier in the microdilution MIC system when 2% NaCl is added to the broth diluent. It also demonstrates that there are a number of staphylococcal strains which are detected only after extended incubation. In this study, 9 (33%) of the 27 resistant coagulase-positive strains and 10 (26%) of the 39 resistant coagulase-negative strains were detected only after further incubation for 40 to 48 h. Since endpoints were not determined at intermittent incubation periods, we cannot rule out the possibility that some of these oxacillin-resistant strains could have been detected earlier if endpoints had been determined at 24 h, as recommended by Thornsberry and McDougal (16).

Colony counts of the inoculum for those resistant strains detected only after 40 to 48 h of incubation were consistently less than the expected 100,000 CFU/ml (mean = 53,000, standard deviation = 28,000). These data suggest that if further improvements are to be made in the earlier detection of this group of resistant strains, alternative methods for standardization of the inoculum may be required. The stationary-phase procedure utilizes the growth of the organism in broth culture before inoculation, and many resistant staphylococcal strains grow poorly in broth; therefore, preparation of the inoculum from growth on an overnight agar plate, as recommended by Thornsberry and McDougal (16), may be preferable. Until further improvements are made, it is prudent for those laboratories that use the stationary-phase inoculum procedure to incubate all staphylococcal strains for which the oxacillin MICs are ≤2 μg/ml for a total of 40 to 48 h to adequately exclude oxacillin resistance.

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