Successful Use of Broth Microdilution in Susceptibility Tests for Methicillin-Resistant (Heteroresistant) Staphylococci

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We studied the broth microdilution antimicrobial susceptibility testing procedure to see whether it could be made reliable for determining resistance of staphylococci to methicillin, oxacillin, nafcillin, and cephalothin. With 45 selected strains of *Staphylococcus aureus* and 12 selected strains of *Staphylococcus epidermidis* we found that the addition of 2% NaCl to cation-supplemented Mueller-Hinton broth permitted us to discriminate reliably between resistant and susceptible organisms. A screening test in which resistant staphylococci grew on agar containing 4% NaCl and methicillin (10 µg/ml), oxacillin (6 µg/ml), or nafcillin (6 µg/ml) incubated at 35°C for 24 h (additional 24 h if no growth) was also reliable. In vitro cephalothin resistance occurred in heteroresistant *S. aureus* but usually did not occur in heteroresistant *S. epidermidis*.

Methicillin-resistant staphylococci were reported in Great Britain in 1961, shortly after methicillin was introduced for use in therapy (4, 15). They were later found in hospitals in other parts of Great Britain and Europe (5, 23) but were not a significant problem in the United States, where they tended to be found more in large hospitals (13) until the mid-1970s. These strains of *Staphylococcus aureus* have been called methicillin resistant and also heteroresistant because the resistant cultures are heterogeneous in resistance to these penicillinase-resistant penicillins. Both terms are used interchangeably in this paper.

The recognition of methicillin resistance in these heteroresistant *S. aureus* strains has always been a problem because of differences in growth characteristics of the organisms that make up the populations (23). Each heteroresistant population contains not only methicillin-susceptible organisms that exhibit the usual growth characteristics of non-heteroresistant *S. aureus* but also methicillin-resistant organisms that grow more slowly, prefer temperatures below 37°C, and prefer a higher salt content (23, 24). Thus, in a disk diffusion test, the susceptible organisms will grow normally and a zone will be formed around the methicillin disk, but the slower-growing resistant strains will later grow within that zone (11, 23, 26). The size of the initial zone and the amount of later growth within that zone will vary with the strain, environmental conditions, inoculum, and time of incubation.

Recommendations to improve detection of methicillin-resistant strains have been to use a

temperature of 30°C, incubate for 48 h, use a larger inoculum, or increase the salt content (2, 5, 12, 14). These procedures are a marked departure from the standard disk diffusion test (7, 21), and in the past we have not found it necessary to use them if we incubated the plates at 35°C, a temperature amenable to routine use for all organisms and drugs (26).

The heteroresistant *S. aureus* organisms have also posed problems when tested by the dilution susceptibility tests. In the agar dilution test, there has been an endpoint at which there was a marked diminution of growth, followed by less-er, often spotty, growth to a final endpoint. In the broth microdilution tests, the minimal inhibitory concentrations (MICs) have often been lower, often too low for the organisms to be called methicillin resistant (6, 14, 16).

Until recently, it was our opinion that the accuracy of these tests, in descending order, was disk diffusion, agar dilution, and broth microdilution.

Although these heteroresistant *S. aureus* are more often called methicillin-resistant *S. aureus*, they generally exhibit cross-resistance to oxacillin, nafcillin, cloxacillin, and dicloxacillin. In earlier studies we determined that, in addition to methicillin, oxacillin and nafcillin could also be used for disk diffusion testing, but cloxacillin or dicloxacillin could not (26), and Parker also reported problems with cloxacillin (23).

More recently, we have seen the problems associated with testing for methicillin resistance increase so much that we were often unsure of the accuracy and sensitivity of any susceptibility

test. We have also seen an apparent increase, although slight, in strains that do not show complete cross-resistance to methicillin, oxacillin, and nafcillin.

Since there has been an increase in the incidence of heteroresistant *S. aureus* in the United States (13), we reexamined the susceptibility methods used for these organisms to determine whether changes could be made to make the tests reliable, accurate, and reproducible. We believed that our initial efforts should be directed toward improving the microdilution MIC test since further studies could be better related to these results. In particular, we wanted to devise a procedure that would yield MICs for resistant strains significantly higher than the susceptibility breakpoints for methicillin, oxacillin, and nafcillin to make it unlikely that lower values owing to methodological variation would be interpreted as indications of susceptibility. This is a report of those studies.

MATERIALS AND METHODS

Bacteria. The strains used in the study were 57 selected strains of *S. aureus* (45 strains) and *Staphylococcus epidermidis* (12 strains) that had been referred to us for antibiotic studies. They were selected to contain both heteroresistant and methicillin-susceptible strains. Three *S. aureus* strains (112, 819, and 577), selected because of their discrepant results in previous tests (i.e., different results with different methods), were used in the preliminary studies to evaluate the different media and media supplements in obtaining a more definitive MIC. Standard reference strains were derived from *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853.

Antimicrobial agents. Antimicrobial powders suitable for susceptibility tests were obtained directly from the appropriate pharmaceutical companies.

Culture media. Media and supplements were chosen because they might support better growth of staphylococci or make the media more hypertonic. Most of the studies were done with Mueller-Hinton (MH) broth (Difco Laboratories) used alone or supplemented with various compounds, e.g., horse blood (5%), thiamine (0.005%, 0.01%, or 0.025% plus 0.25% NaCl), Tween 80 (0.01% plus 0.025% NaCl), KCl (various concentrations), or NaCl (various concentrations). Some MH broth also contained calcium (50 mg/liter) and magnesium (25 mg/liter) (called CSMH) as described in the National Committee for Clinical Laboratory Standards (NCCLS) dilution standard (22). In early tests, Tryptic soy broth (Difco), Schaedler broth (BBL Microbiology Systems), and Sceptor broth (BBL) were also used. The bacteria were subcultured on Trypticase soy agar (BBL) with 5% sheep blood, and the screening tests were done on MH agar (BBL) supplemented with 4% NaCl.

MIC tests. The MIC tests were done by the microdilution method, as described in NCCLS standard M7-T (22), except for variations in media, incubation, and inoculum preparation. The trays were incubated at 34

to 35°C, and the endpoints were determined at 24 and 48 h, although a few were read at 18 h also. The inoculum was prepared from growth on a 24-h culture on agar; enough organisms from the agar culture were added to MH broth to yield a turbidity equal to an 0.5 McFarland standard, approximately 10^8 CFU/ml. The final inoculum was approximately 10^5 CFU/ml.

Screening test. Methicillin (10 µg/ml), oxacillin (6 µg/ml), or nafcillin (6 µg/ml) was incorporated in molten MH agar containing 4% NaCl at 50°C, mixed, and dispensed into 100-mm petri plates. By using the MIC 2000 inoculator, an inoculum of approximately 10^4 organisms was placed on the surface of the agar. These plates were incubated at 35°C for 24 h, but if there was no growth, they were reincubated for 24 h more. If the organism grew, it was defined as resistant, and if it failed to grow, it was defined as susceptible.

Interpretive breakpoints. The susceptibility and resistance breakpoints were: for methicillin, ≤ 4 and ≥ 16 µg/ml; for oxacillin and nafcillin, ≤ 2 and ≥ 8 µg/ml; and for cephalothin, ≤ 8 and ≥ 32 µg/ml.

RESULTS

In the initial phase of this study, several broth media other than MH broth were tested to see whether they would yield more definitive MICs for methicillin, oxacillin, and nafcillin. Tryptic soy broth was more discriminatory between resistant and susceptible strains than other broths were. The addition of 5% horse blood, thiamine, or Tween 80 to MH broth with 0.25% NaCl did not enhance its ability to detect methicillin-resistant staphylococci. The addition of NaCl or KCl did, however, make the MH broth yield higher MICs with methicillin-resistant strains, and there was no detectable difference in the effects of the two salts. Since MH is the medium recommended for the NCCLS standard dilution susceptibility tests (22), we concentrated on the use of MH, and for supplementation we arbitrarily chose NaCl. The results of the studies with NaCl supplementation of MH broth are shown in Table 1. These data show that the methicillin-resistant strains (112 and 819) had higher MICs with increased salt concentrations, with modal MICs being four- to eightfold higher in MH broth plus 2% NaCl than in unsupplemented MH broth. Corresponding increases for oxacillin were 32- to 128-fold higher. For nafcillin, they were 8- to 32-fold higher. Strain 577, which we consider to be a true intermediate strain, was affected very little by the increase in salt concentration with an increase of only 1 log₂ dilution. MICs for a methicillin-susceptible strain (ATCC 29213) were also not affected by more than 1 log₂ dilution with the increase in NaCl. For reference, medium B in Table 1 (CSMH) is the medium that we routinely use for microdilution susceptibility tests.

In a separate study, we examined the effect of the addition of 5 and 10% NaCl to CSMH broth. A total of 27 selected strains (15 were heterore-

TABLE 1. MICs of methicillin, nafcillin, oxacillin, and cephalothin obtained with selected strains of *S. aureus* in different concentrations of salts, incubated for 24 h at 35°C

Antibiotic	Strain	MIC (μ g/ml) obtained with medium ^a :					
		A	B	C	D	E	F
Methicillin	112	4	8	8	8	16	16
	819	4	8	16	16	32	32
	577	4	4	4	4	4	8
	29213 ^b	ND ^c	2	ND	ND	ND	2
Nafcillin	112	0.5	2	4	8	16	16
	819	2	4	8	16	16	16
	577	2	4	4	2	2	2
	29213	ND	0.25	ND	ND	ND	0.5
Oxacillin	112	0.5	4	16	16	32	32
	819	0.25	4	16	32	32	32
	577	2	4	4	4	4	4
	29213	ND	0.25	ND	ND	ND	0.5
Cephalothin	112	ND	1	2	8	8	16
	819	ND	2	2	2	8	16
	577	ND	2	2	4	2	2
	29213	ND	0.25	ND	ND	ND	0.5

^a Media: A, unsupplemented MH broth; B, MH plus cation supplement (CSMH); C, CSMH plus 0.25% NaCl; D, CSMH plus 0.5% NaCl; E, CSMH plus 1.0% NaCl; F, CSMH plus 2.0% NaCl.

^b Standard reference strain, *S. aureus* ATCC 29213.

^c ND, Not done.

sistant and 12 were methicillin susceptible) were tested in CSMH broth plus 0, 2, 5, or 10% NaCl with methicillin, oxacillin, nafcillin, and cephalothin. Although the use of 10% NaCl will often yield the appropriate resistant result, this concentration is too inhibitory to be recommended. The use of 5% NaCl has both advantages and disadvantages when compared with 2% NaCl. Growth in 5% NaCl is better than in 2% NaCl,

and this can be an advantage occasionally in detecting methicillin resistance at 24 h. Of the 15 methicillin-resistant strains in this part of the study, one strain was resistant to methicillin at 24 h in 5% NaCl but not in 2% NaCl (it was resistant at 48 h in 2% NaCl). There were no differences between 2 and 5% NaCl in the qualitative results for nafcillin and oxacillin. For cephalothin, the results obtained with the two media were similar, with MICs approximately 1 dilution higher in the 5% NaCl. The disadvantage of using 5% NaCl occurs with the methicillin-susceptible strains. The use of 5% NaCl, in contrast to 2% NaCl, created a trailing effect with the methicillin-susceptible strains, which often yields MICs of 8 μ g/ml with methicillin and 4 μ g/ml with oxacillin and nafcillin, i.e., intermediate results. Because of these results with the methicillin-susceptible strains, we decided to use the 2% NaCl supplement, even though growth was quantitatively better with the 5% supplement.

The other staphylococci were tested in CSMH plus 2% NaCl for susceptibility to methicillin and seven other antibiotics. These data are shown in Table 2. The methicillin-resistant strains were more multiply resistant than the other strains, but even the methicillin-susceptible strains were resistant to two or three other drugs. Only 1 of the 45 *S. aureus* strains (577 mentioned above) and only 1 of 12 *S. epidermidis* strains had an intermediate susceptibility to methicillin. Only methicillin-resistant *S. aureus* strains were also resistant to gentamicin, chloramphenicol, sulfamethoxazole-trimethoprim, and rifampin. Only methicillin-resistant *S. epidermidis* was resistant to gentamicin and sulfamethoxazole-trimethoprim.

These MICs were read at 24 h rather than after 18 h or overnight incubation for the following reason. In the early part of the study, the strains were read at both 18 and 24 h. For a majority of strains, the results were essentially identical, but

TABLE 2. Classification of antibiotic-resistant strains of staphylococci according to category of susceptibility to methicillin

Organism	Susceptibility to methicillin ^a	% of total strains resistant to ^b :						
		AMP	GTM	TET	ERY	CLP	SMX-TMP	RIF
<i>S. aureus</i> (45 strains)	Resistant (60)	60	11	22	58	2	2	2
	Susceptible (38)	31	0	2	11	0	0	0
	Intermediate (2)	2	0	0	2	0	0	0
<i>S. epidermidis</i> (12 strains)	Resistant (42)	42	25	0	25	8	8	0
	Susceptible (50)	25	0	17	25	0	0	0
	Intermediate (8)	8	0	8	8	8	0	0

^a Numbers in parentheses show percentages of strains for each species.

^b AMP, Ampicillin; GTM, gentamicin; TET, tetracycline; ERY, erythromycin; CLP, chloramphenicol; SMX-TMP, sulfamethoxazole-trimethoprim; RIF, rifampin.

for other strains the results were significantly different. For these latter strains, the MICs read at 24 h were more often than not four times the MICs read at 18 h.

The results obtained with the screening plates containing either 10 µg of methicillin or 6 µg of oxacillin or nafcillin per ml are shown in Table 3, in which the screen results are compared with categories of resistance as determined by MIC testing. With each of the three penicillins, the numbers of resistant strains as determined by the screen procedure were comparable. The lone discrepancy with *S. aureus* was a strain (577) that grew lightly on all three screen plates; this strain was classified as intermediate towards methicillin and oxacillin (MICs, 8 and 4 µg/ml) but susceptible to nafcillin and cephalothin (MICs, 2 and 0.5 µg/ml). Except for this same strain and three strains that were judged intermediate (MICs, 16 µg/ml), the categorization of MICs of cephalothin for *S. aureus* was comparable to that of the penicillins. Similar results were

obtained with the *S. epidermidis* strains and the three penicillins. One strain was intermediate to methicillin and susceptible to oxacillin and nafcillin but failed to grow on any of the three screen plates. In addition, one strain was susceptible to all three penicillins at 24 h but became resistant to methicillin at 48 h (MIC, 16 µg/ml); this strain had very light growth on the methicillin and oxacillin screening plates but none on the nafcillin screening plate. In contrast to the *S. aureus* strains, in which the cephalothin category results were essentially the same as the three penicillins, the *S. epidermidis* strains were very different in that only one strain was resistant to cephalothin. These results were also obtained at 24 h for the reasons listed previously.

These data show, then, that when staphylococci were tested in CSMH broth supplemented with 2% NaCl, MICs of methicillin, oxacillin, and nafcillin for heteroresistant strains were ≥ 16 , ≥ 8 , and ≥ 8 µg/ml, respectively. The MICs of the same drugs for methicillin-susceptible

TABLE 3. Susceptibility of *S. aureus* and *S. epidermidis* to four antibiotics as determined by microdilution (with 2% NaCl in CSMH broth and 24 h of incubation) and an agar screening technique

Antibiotic	Test ^a			No. of strains	
	Method	Breakpoints		<i>S. aureus</i> (total, 45)	<i>S. epidermidis</i> (total, 12)
		Concn (µg/ml)	Category		
Methicillin	MIC	≤ 4	Susceptible	17	6 ^b
		≥ 16	Resistant	27 ^c	5
	Screen	10	Resistant	28 ^d	6 ^e
Oxacillin	MIC	≤ 2	Susceptible	16	7
		≥ 8	Resistant	28	5
	Screen	6	Resistant	29	6 ^e
Nafcillin	MIC	≤ 2	Susceptible	18	8
		≥ 8	Resistant	27	4 ^f
	Screen	6	Resistant	28	5
Cephalothin	MIC	≤ 8	Susceptible	18	11
		≥ 32	Resistant	24	1

^a For MIC tests, data indicate susceptibility or resistance at the breakpoints shown. For the screen test, data indicate resistance of growth on medium containing the given concentration of each drug. No screening test was used for cephalothin.

^b Susceptible and resistant strains total 11 for methicillin and 12 for the other drugs because 1 strain of *S. epidermidis* was intermediate for methicillin but susceptible to oxacillin and nafcillin. This strain did not grow on any of the three screen plates.

^c Numbers of resistant strains as determined by the two tests differ because at 24 h one strain of *S. aureus* was resistant to oxacillin but susceptible to the other three drugs. At 48 h, however, results with all four drugs indicated resistance.

^d Numbers of resistant strains as determined by the two tests differ because at 24 h with the MIC test, one strain of *S. aureus* was intermediate for methicillin and oxacillin but susceptible to nafcillin and cephalothin. On the methicillin and oxacillin screen plates, there was growth at 24 h. (A very light growth occurred on the nafcillin plate at 48 h; there was no screen test for cephalothin). The MICs did not change at 48 h. With cephalothin, three *S. aureus* strains were intermediate (MIC, 16 µg/ml).

^e There was one more resistant strain by the screen test than by the MIC test because one strain of *S. epidermidis* was susceptible to methicillin and oxacillin by the MIC test at 24 h but was resistant by the screen. This strain was susceptible to nafcillin by both methods.

^f One strain was susceptible to nafcillin by the MIC test but was resistant by the screen test.

strains were ≤ 4 , ≤ 2 , and ≤ 2 $\mu\text{g/ml}$, respectively. The MICs of cephalothin were ≥ 16 $\mu\text{g/ml}$ for the heteroresistant strains of *S. aureus* and ≤ 8 $\mu\text{g/ml}$ for the methicillin-susceptible strains. The majority of *S. epidermidis* strains were susceptible to cephalothin (MIC, ≤ 8 $\mu\text{g/ml}$), and there appeared to be no correlation with resistance to methicillin.

DISCUSSION

The difficulties that are being experienced in determining the resistance of staphylococci to methicillin, oxacillin, and nafcillin are cause for alarm since recent studies have shown that methicillin-resistant (or heteroresistant) *S. aureus* strains are significant causes of infections in many institutions (13). Haley et al. have reported that the increase has occurred primarily in large, tertiary referral centers but that these infections do occur sporadically in smaller institutions (13). They also indicate that the problem is unlikely to disappear (13). Failure to detect these resistant organisms by susceptibility tests would certainly lead to a false sense of security and could lead to erroneous and inadequate therapy for infections. On the other hand, false resistance results could lead to the use of inappropriate, and possibly toxic, antimicrobial agents. The need for a sensitive, reproducible antimicrobial susceptibility method is obvious.

The problems of susceptibility testing of heteroresistant *S. aureus* are caused by the innate genetic characteristics of these strains, particularly the occurrence of both methicillin-susceptible and methicillin-resistant bacteria in each population and the slower growth of the resistant strains (23, 24, 26). To overcome some of these problems, microbiologists soon after the discovery of these strains and several times since recommended using lower temperatures for incubation (30 or 35°C rather than 37°C), larger inocula, longer incubation times, or additional salt in the medium (5, 6, 11, 14, 26). In our earlier studies of these organisms, our data indicated that the disk diffusion test was very reliable if the incubation temperature did not exceed 35°C (26). We recognized then that there were problems with MIC testing because of slower growth, and this problem has been magnified with the increased use of microdilution MIC testing in clinical laboratories (6, 17, 19). Problems of determining methicillin resistance in staphylococci have also been reported with some newer automated systems (8–10). Until recently, we believed that the disk diffusion procedure was consistently reliable and recommended it as the ultimate in vitro arbitrator of methicillin resistance in staphylococci. Recent problems have forced us to reassess this recommendation.

The cause of the recent problems may be related to changes in the media that affect the rate of growth of the heteroresistant strains of staphylococci. Even in the past, when we believed that the test was reliable, the growth of some of the resistant strains was quite slow, and only subtle changes in media or environmental conditions could result in slowing the growth to the point at which it could not be detected and thus would not be recognized. Although tryptic soy broth (Difco) worked well in this test, we believed that we would rather not adopt this medium but would stay with the standard CSMH broth and supplement it with 2% NaCl. In this medium, heteroresistant strains could be easily determined because the window between susceptible and resistant MICs was wide enough to overcome the technical problems experienced previously.

Our recommendations for performing MIC tests are as follows: (i) use CSMH broth as the basic medium for performing these tests, as recommended by the NCCLS (22) and add 2% NaCl to the broth that will be used for testing methicillin, oxacillin, nafcillin, and cephalothin; (ii) do the test as described in NCCLS standard M7-T (22) but prepare the inoculum from growth on an overnight agar plate and incubate the trays or tubes at 35°C for a full 24 h; (iii) record the MIC as the lowest concentration that inhibits macroscopic growth.

Doing the test this way has the advantage of varying very little from the routine method already in use in many laboratories. It was for this reason that we preferred not to use a lower incubation temperature such as 30°C, not to routinely incubate for 48 h, and not to use a larger than standard inoculum. Similarly, we do not recommend adding the 2% NaCl to all the broth because it would interfere with testing some drug-organism combinations, e.g., aminoglycosides and *P. aeruginosa*, and because it would interfere with the growth of some organisms. We do not advocate it for all the gram-positive set of antimicrobial agents for the latter reason. Although some gram-positive organisms other than staphylococci might fail to grow in broth containing 2% NaCl, that would be of little consequence since these drugs in the gram-positive set are there only for antistaphylococcal purposes.

The use of additional salt in the test medium has been suggested by several investigators. Barber used 5% NaCl or 7% $(\text{NH}_4)_2\text{SO}_4$, and Hewitt et al. used 5% NaCl, and both reported better growth in the salt-supplemented media than in unsupplemented media (5, 14). Barry and Badal reported that the addition of 5% NaCl to MH broth increased detection of methicillin resistance (6). As indicated above, we found that

the addition of 2% NaCl to the MH broth was adequate for discriminating between methicillin-susceptible and heteroresistant strains whether methicillin, oxacillin, nafcillin, or cephalothin was being tested and yet did not cause problems with the non-heteroresistant strains. We do not object to the use of the 5% NaCl supplement since it does permit better growth, but we do caution the user that it may make occasional strains appear to be intermediate to the β -lactam antibiotics used in this study.

We believe that the use of an inoculum prepared from an 18- to 24-h agar culture, rather than from a 2- to 4-h MH broth culture generally described for this method, is a definite advantage. This inoculum will contain more of the resistant cells because they grow slower, and a 24-h culture will contain still more of them. The use of inocula prepared from growth on an overnight agar culture for the microdilution test has been shown to be an accurate and reproducible alternative to the standard method (3). Preparing and using the inoculum in this manner probably yields the same results as using a large inoculum such as that suggested by Barber (5) and recommended by Ericsson and Sherris (12).

The third minor change that we have made in the standard procedure is to recommend that the incubation time be 24 h rather than overnight or 18 to 24 h, as usually recommended. We think that this change, although minor, is a significant one for obtaining accuracy in the test. On the other hand, we think that 48 h of incubation is not usually needed except in certain cases that we will mention later, e.g., reincubation of a strain that has been categorized as intermediate.

Although the MIC method we have described here will detect most strains of heteroresistant staphylococci, certain points should be emphasized because they will aid in detecting even the most difficult strains. Certain results can serve as "flags" to alert the tester to the possibility of heteroresistant staphylococci. The first of these flags is an intermediate result. Although we have described an intermediate strain here, they are rare, and such a result may indicate that the strain is actually a resistant one that did not grow well enough to give an MIC indicating resistance. In other words, almost every strain should fall within the susceptible or resistant categories listed previously; e.g., the MIC of methicillin should be ≤ 4 or ≥ 16 $\mu\text{g/ml}$. We recommend that strains that yield intermediate results be reincubated for an additional 24 h; if it is resistant, the MIC will be higher, but a true intermediate strain will remain intermediate. A second flag concerns cross-resistance between cephalothin and the three penicillins. Methicillin-resistant *S. aureus* strains should also be resistant to cephalothin, and methicillin-suscep-

tible strains should also be susceptible to cephalothin. An MIC of cephalothin of 2, 4, or 8 $\mu\text{g/ml}$ should be a clue that the strain is probably methicillin and cephalothin resistant (MICs of cephalothin for methicillin-susceptible strains are usually ≤ 0.25 to 1.0 $\mu\text{g/ml}$). These strains should be incubated an additional 24 h. One should expect that there will be cross-resistance to all three penicillins in the vast majority of strains if the test is being done properly. If there is any indication that there is not cross-resistance, tests should be repeated with an incubation time of 48 h. Even if complete resistance cannot be demonstrated in vitro, it may be prudent for therapeutic purposes, in our present state of knowledge, to assume that total cross-resistance occurs anyway. Finally, the third flag is the occurrence of multiple resistance in a strain that may include resistance to erythromycin, clindamycin, chloramphenicol, aminoglycosides, and penicillin (β -lactamase positive).

We add one other note of caution. In some recent studies, we have had occasional strains (all from the same location) that showed a paradoxical result in that they were inhibited at lower concentrations of methicillin (e.g., 2 to 8 $\mu\text{g/ml}$) but were not inhibited at higher concentrations (e.g., 16 and 32 $\mu\text{g/ml}$). This is a problem because the range of methicillin concentrations tested often does not exceed 8 $\mu\text{g/ml}$, and the strain would be read as susceptible. The concentrations tested, therefore, should cover these ranges. For example, a test in which the highest concentration of methicillin tested was 4 or 8 $\mu\text{g/ml}$ might miss a strain that shows this kind of paradoxical result. With nafcillin, this phenomenon tended to be a diminution of the amount of growth rather than absence of growth (skips), but it occurred at concentrations low enough (0.5 to 1.0 $\mu\text{g/ml}$) not to create a problem generally. This skipping phenomenon did not occur with oxacillin.

If a screening method is needed, we recommend the one that we used in this study, i.e., the use of MH agar containing 4% NaCl and either 10 μg of methicillin, 6 μg of oxacillin, or 6 μg of nafcillin per ml. With the inoculum as described in the methods, growth of a *Staphylococcus* strain on one of these plates would reliably indicate resistance to that drug. This screening test is similar to those used by Barber (5) and Hewitt et al. (14). We have not used screening plates containing cephalothin.

The results that we obtained with cephalothin deserve further comment. In the past, most tests with cephalothin and heteroresistant *S. aureus* would yield results indicating susceptibility to cephalothin, particularly with the disk diffusion test. Infections caused by these strains have, however, been shown to be generally clinically

resistant to cephalothin (1, 19), resulting in a recommendation that any heteroresistant *S. aureus* should be reported resistant to cephalothin (21). This recommendation is usually extended to other cephalosporins also, even though there are few data on which to make such a decision. Nevertheless, many clinicians are reluctant to use any cephalosporin in infections due to these heteroresistant *S. aureus*. The issue is much less clear with methicillin-resistant *S. epidermidis* (20), but some clinicians have taken the conservative approach and have not used cephalosporins to treat infections caused by heteroresistant *S. epidermidis*. Karchmer et al. recommended the use of vancomycin plus rifampin or an aminoglycoside to treat prosthetic valve endocarditis caused by methicillin-resistant *S. epidermidis* (18). Our MIC data with cephalothin support the recommendation for cephalothin resistance in *S. aureus* and shows that with the test reported here results with cephalothin should be correct and reproducible, i.e., a heteroresistant *S. aureus* should be cephalothin-resistant. Thus, it should no longer be necessary to assume resistance based solely on clinical results obtained in the past. On the other hand, our results indicate that most methicillin-resistant *S. epidermidis* may or may not be susceptible to cephalothin as determined by this susceptibility test. Studies are needed to ascertain whether *S. epidermidis* strains that are susceptible to cephalosporins by this in vitro test are clinically susceptible to cephalosporins. Only then can the in vitro results be accepted as the basis on which to devise a therapeutic strategy that includes a cephalosporin.

These data also show that apparently rare strains of *S. aureus* and *S. epidermidis* are truly intermediate in their resistance to some of these penicillins. Three strains for which the MICs of cephalothin were 16 µg/ml were, by definition, intermediate. For therapy, it would be no problem and may be wise to consider the intermediate *S. aureus* to be resistant and use an alternative drug. It may be possible to treat infection by these organisms with increased doses, but this must be proved by clinical studies. Although not shown here, all these strains were susceptible to vancomycin.

These studies have only partially solved the problems associated with susceptibility tests of heteroresistant staphylococci since most clinical laboratories do not do MIC testing but rather use the disk diffusion procedure. In a recent survey by the College of American Pathologists, only 25% of the responding laboratories used MIC testing (16). Preliminary studies in our laboratory indicate that adding extra salt to the MH agar will not solve the problems with disk diffusion and that other approaches will have to be

used. Those studies are in progress in our laboratory. Even though the MIC is not the predominant testing procedure, we believe that studying the MIC method first was necessary because the disk procedure is based on MIC results and correct disk diffusion methods cannot be developed without correct MICs.

Some laboratories use the newer automated methods, and as indicated previously, there are reports of problems in detecting heteroresistant staphylococci (8–10). In a collaborative study of the Autobac I system, we did not test heteroresistant staphylococci because we tested only clinical isolates (27). In a collaborative study of the MS-2 system (Abbott Diagnostics), we were fairly successful in detecting these strains (25), but reports of some recent investigations have indicated less success (8, 9). We had thought, in retrospect, that the success we had may have been because the broth used in the MS-2 system contained more salts than MH broth, but that does not explain the failures in other studies. One could postulate that the addition of more salt, i.e., NaCl, to the appropriate broth in the gram-positive cuvette of the MS-2 or Autobac I system might eliminate these problems, but that has not proven true in one recent study (10).

It is reasonable to suggest, however, that organizations that sell susceptibility testing systems, including microdilution, should investigate the use of increased NaCl content in their tests with these penicillins and with cephalothin and staphylococci.

In conclusion, the standard microdilution broth MIC test will reliably detect heteroresistant staphylococci if 2% NaCl is added to the CSMH broth containing methicillin, oxacillin, nafcillin, and cephalothin. These strains can also be detected by a screening procedure in which methicillin (10 µg/ml) or oxacillin (6 µg/ml) or nafcillin (6 µg/ml) is incorporated into MH agar containing 4% NaCl: resistant strains grow, and susceptible strains do not. Until further studies are done on the problems associated with other susceptibility testing systems, we recommend that one of these two tests be used as the arbitration test for heteroresistance in staphylococci when questionable results arise.

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