

Reevaluation of the Ability of the Standardized Disk Diffusion Test to Detect Methicillin-Resistant Strains of *Staphylococcus aureus*

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To reevaluate the ability of the disk diffusion method to detect methicillin-resistant *Staphylococcus aureus*, 73 such isolates from 13 cities were tested for antimicrobial susceptibility with the standardized disk diffusion test. Duplicate plates were incubated at 30 and 35°C and read after 18, 24, and 48 h. After incubation at 35°C for 24 h, 97% of isolates appeared resistant to methicillin, and 99% appeared resistant to oxacillin. A significantly smaller proportion of isolates appeared resistant to cephalothin ($P < 0.001$) and cefamandole ($P < 0.001$). Isolates from some cities had no zones of inhibition around methicillin and oxacillin disks, whereas those from other cities had measurable zones of inhibition, with light growth inside the zones. Patterns of growth around cephalothin and cefamandole disks also varied among isolates from different cities. Incubation at 30°C for 24 h did not result in better detection of methicillin or oxacillin resistance. All study isolates appeared resistant to methicillin and oxacillin after 48 h of incubation at 35°C. The results suggest that methicillin-resistant *S. aureus* strains from many areas will be detected if standardized disk diffusion tests are incubated at 35°C for 24 h.

The Kirby-Bauer disk agar diffusion test and its modifications (6, 7, 32, 33) are among the most widely used antimicrobial susceptibility testing systems employed in American hospitals (25). This technique was developed at the University of Washington in the early 1950s, and in 1959 Bauer et al. (8) reported on use of the method for testing staphylococci. These early evaluations demonstrated that the system was well suited for performing susceptibility tests on *Staphylococcus aureus* isolates. Methicillin was not included in these early studies because the drug did not become available until about 1960.

Between 1960 and 1966, when the Kirby-Bauer test was undergoing further evaluation, methicillin-resistant (MR) *S. aureus* strains were discovered in Britain (24) and became an important cause of nosocomial infections. However, such organisms were rarely recovered from patients in this country in the early 1960s. As a result few, if any, MR *S. aureus* strains were included in later studies during which the Kirby-Bauer method was standardized. A description of the standardized disk diffusion test was published in 1966 (7).

The first reported outbreak of MR *S. aureus* infections in this country occurred in 1967 (4). In the years after the 1967 outbreak, few other institutions in this country reported epidemics of MR *S. aureus* infection (34). Most isolates from these early American outbreaks and from Britain were multidrug resistant and showed a heterogeneous resistance to methicillin. That is, only a fraction of the daughter cells derived from a single colony were phenotypically resistant to methicillin at 37°C (17, 29, 38).

Researchers in Britain and other countries, using other disk diffusion tests (not the Kirby-Bauer method), determined that heterogeneous resistance to methicillin was not detected reliably when susceptibility tests were performed at 37°C (3, 23, 41). Additional studies revealed that the resistance could be detected more easily if one or more of the following procedures was used: incubation of plates at 30°C for 18 h, or at 37°C for 48 h, use of a heavy inoculum, or use of agar containing 5% NaCl (3, 23, 40, 43).

In 1968 Benner and Kayser (9) reported that the Kirby-Bauer method did not detect MR *S. aureus* strains reliably. Although the authors did not state the incubation tempera-

ture used, earlier descriptions of the method had recommended that plates be incubated at 37°C. In the early 1970s, two other groups of investigators studied the ability of the standardized Kirby-Bauer method to detect MR *S. aureus* strains (18, 42). These groups determined that incubation of plates at 35°C for 18 h (18) or overnight (42) resulted in adequate detection of MR *S. aureus* strains available at that time.

Since these two studies were conducted, MR *S. aureus* strains have become much more prevalent in this country (11, 21). In addition, it has become apparent that strains from some medical centers are homogeneously resistant to methicillin (35, 39) and that the level of heteroresistance demonstrated by more typical strains varies among isolates from different geographic areas. Interestingly, several studies have shown that a number of other antimicrobial susceptibility testing systems developed in the last decade have not detected MR *S. aureus* isolates reliably (5, 12, 15, 16). In light of these developments, a reevaluation of the Kirby-Bauer method appeared warranted. This report describes a study in which 73 MR *S. aureus* isolates from hospitals in 12 states were tested for antimicrobial susceptibility by using the standardized disk diffusion technique.

MATERIALS AND METHODS

Study strains. A total of 73 MR *S. aureus* isolates recovered from patients in university-affiliated hospitals in 13 cities were included in the study. Isolates were from Ann Arbor (5 isolates), Atlanta (3), Charleston (6), Charlottesville (5), Detroit (5), Houston (11), Jackson (11), Miami (6), New Orleans (2), New York (7), Seattle (5), St. Louis (5), and St. Paul (2). All isolates were coagulase positive and DNase positive. All isolates had methicillin MICs of 16 µg/ml or greater on two occasions when tested by standard agar dilution techniques performed at 35°C (44). A quality control strain of known antimicrobial susceptibility (*S. aureus* ATCC 25923) was included in all experiments. Study strains and the control strain were stored in brain heart infusion broth at -70°C.

Antimicrobial susceptibility testing methods. Isolates were thawed and streaked onto blood agar plates which were

incubated at 35°C for 18 to 24 h. Isolated colonies were suspended in tryptic soy broth, and the suspension was adjusted to the turbidity of a 0.5 McFarland standard by using an API-IR nephelometer (Analytab Products, Plainview, N.Y.). Duplicate Mueller-Hinton plates were inoculated with cotton swabs, commercially available disks were applied as recommended, and the plates were incubated at 30 and 35°C. The temperature in the 35°C incubator was monitored with a minimum-maximum thermometer (Brannan Thermometers, Cumberland, England). Plates were held over a black, nonreflecting background, and zones of inhibition were measured with calipers after 18, 24, and 48 h of incubation. Standard zone-size criteria were used to define isolates as susceptible, intermediate, or resistant (33). Zones of inhibition were examined carefully for colonies or faint growth inside the zone margins.

Phage typing. All study isolates were phage typed at the Centers for Disease Control by Gary Hancock, using standard methods (10). The following phages were used to type study isolates: group 1—29, 52, 52A, 79, and 80; group 2—3A, 3C, 55, and 71; groups 3 through 6, 42E, 47, 53, 54, 77, 83A, 84, and 85; miscellaneous—81, 94, 95, and 96.

Statistical analysis. Differences in unpaired data were analyzed with the Mann-Whitney U test, whereas paired data were compared with the McNemar test (30, 46).

RESULTS

The temperature in the 35°C incubator never varied by more than $\pm 1^\circ\text{C}$, whereas the temperature in the 30°C incubator varied by $\pm 2^\circ\text{C}$.

Results at 35°C for 18 h. The methicillin, oxacillin, cephalothin, and cefamandole inhibitory zones for *S. aureus* ATCC 25923 were within the acceptable range for individual daily test control zone diameter limits (33) in all runs when incubation was at 35°C for 18 h, with one exception. On 1 of 10 runs, the zone around cefamandole was 1 mm less than the lower individual daily test control limit for ATCC 25923. Individual colonies or faint growth was never observed inside the zones around methicillin, oxacillin, cephalothin, or cefamandole disks with the control strain.

Of the 73 MR *S. aureus* isolates, 96% (70) were categorized as methicillin resistant, 3% as intermediate, and 1% as susceptible (Table 1). A total of 97% appeared oxacillin resistant, and 3% appeared susceptible to oxacillin. Compared with oxacillin, significantly fewer isolates appeared resistant to cephalothin and cefamandole ($P < 0.0001$ and $P < 0.0001$, respectively) (Table 2).

Results at 35°C for 24 h. For the control strain incubated at

35°C for 24 h, all inhibitory zones around methicillin, oxacillin, cephalothin, and cefamandole disks were within the acceptable individual daily test control zone diameter limits, with one exception. On one run, the zone around the cephalothin disk was 1 mm outside the acceptable limits.

Some MR *S. aureus* isolates which appeared to be intermediate or susceptible to methicillin or oxacillin at 18 h had detectable growth inside the zone margin after 24 h of incubation. As a result, all isolates appeared to be intermediate or resistant to methicillin, and only one appeared to be susceptible to oxacillin. Compared with oxacillin, a significantly smaller proportion of isolates appeared resistant to cephalothin ($P < 0.001$) and cefamandole ($P < 0.001$).

Results at 35°C for 48 h. Inhibitory zone diameters for the control strain when incubation was at 35°C for 48 h were within acceptable limits for cephalothin and cefamandole in all runs. Inhibitory zone diameters were less than the lower acceptable limits (as published for 18-h cultures) for methicillin in two runs and for oxacillin in three runs. However, *S. aureus* ATCC 25923 always had methicillin inhibitory zones of 15 mm or more and oxacillin inhibitory zones of 16 mm or more in diameter.

All 73 study isolates were categorized as resistant to methicillin and oxacillin, 90% appeared to be intermediate or resistant to cephalothin, and 93% were intermediate or resistant to cefamandole.

Effect of incubation at 30°C. Examination of duplicate Mueller-Hinton plates which were incubated for 24 h at 30°C revealed that zone diameters for the control strain were all within acceptable limits (as published for 35°C cultures). At 48 h, zone diameters for the control strain were out of control on several occasions: methicillin (one run), cephalothin (one run), and cefamandole (two runs).

The results obtained when MR *S. aureus* isolates were tested on duplicate Mueller-Hinton plates incubated at 30°C are shown in Tables 1 and 2. Inhibitory zone diameters and interpretive results obtained at 30°C were not significantly different from those obtained at 35°C for methicillin and oxacillin. Significantly fewer study isolates appeared intermediate or resistant to cephalothin at 30 than at 35°C, after incubation for 24 h ($P < 0.001$) and 48 h ($P < 0.025$). In contrast, a greater number of isolates appeared to be resistant to cefamandole when incubated at 30°C for 24 h ($P < 0.025$). At 48 h, there was no significant difference between the 30 and 35°C readings for cefamandole.

Reproducibility of disk diffusion results. To determine the reproducibility of the disk diffusion results, all MR *S. aureus* isolates were tested a second time at 35°C, and readings were taken at 18, 24, and 48 h. Analysis of the results obtained with the control strain revealed that all zone diameters were within acceptable limits for methicillin, oxacillin, cephalothin, and cefamandole after incubation at 35°C for 18 and 24 h.

Table 3 shows the results of duplicate determinations for the 73 MR *S. aureus* isolates when tested for susceptibility to methicillin, oxacillin, cephalothin, and cefamandole. Of the 73 study isolates, 69 appeared to be resistant to methicillin on both occasions after 18 h of incubation at 35°C. After 24 h of incubation, 71 isolates appeared to be resistant to methicillin on both occasions. Similar results were obtained for oxacillin. Disparities between the interpretive results obtained on the first and second runs were significantly more common with cephalothin ($P < 0.01$) and cefamandole ($P < 0.001$) than with oxacillin.

Effect of strain variation on disk diffusion results. MR *S. aureus* strains from different geographic areas yielded a

TABLE 1. Susceptibility of 73 MR *S. aureus* isolates to methicillin and oxacillin^a

Drug	Time (h)	Susceptibility ^b (no. of isolates) at:					
		30°C			35°C		
		S	I	R	S	I	R
Methicillin	18	— ^c	—	—	1	2	70
	24	1	1	71	0	2	71
	48	0	0	73	0	0	73
Oxacillin	18	—	—	—	2	0	71
	24	2	1	70	1	0	72
	48	1	0	72	0	0	73

^a Determined by the disk diffusion method.

^b S, Susceptible; I, intermediate; R, resistant (33).

^c —, Not determined.

TABLE 2. Susceptibility of 73 MR *S. aureus* isolates to cephalothin and cefamandole^a

Drug	Time (h)	Susceptibility ^b (no. of isolates) at:					
		30°C			35°C		
		S	I	R	S	I	R
Cephalothin	18	— ^c	—	—	30	5	38
	24	33	2	38	21	4	48
	48	14	2	57	7	3	63
Cefamandole	18	—	—	—	30	18	25
	24	28	10	35	19	20	34
	48	8	19	46	5	23	45

^a Determined by the disk diffusion method.

^b S, Susceptible; I, intermediate; R, resistant (33).

^c —, Not determined.

variety of patterns of growth inhibition and differing zone diameters. After incubation at 35°C for 18 h, MR *S. aureus* isolates from most cities had little or no inhibitory zones around methicillin disks. In contrast, isolates from Atlanta, Miami, and New Orleans had relatively large zones of partial growth inhibition, with a light growth within the zone. Growth inside the zone was easily detected without the use of special maneuvers such as the use of a hand lens. Isolates from these same three cities plus those from Detroit and Ann Arbor had zones of partial growth inhibition around oxacillin disks, whereas a majority of strains from other cities had virtually no inhibitory zones around oxacillin disks.

Three patterns of growth were noted around cephalothin disks. Strains from Virginia and South Carolina showed confluent growth up to the edge of cephalothin disks, with no apparent inhibition of growth. The zone diameters for isolates from these two areas were significantly less than for MR *S. aureus* isolates from other areas for both cephalothin ($P < 0.0002$) and cefamandole ($P < 0.0002$).

In contrast, MR *S. aureus* isolates from Atlanta, Miami, and Detroit had large zones of complete growth inhibition around cephalothin disks after 18 h of incubation at 35°C. Organisms from four cities had large inhibitory zones with light growth inside the zone, whereas groups of MR *S. aureus* isolates from the remaining areas had variable patterns.

Results of phage typing. MR *S. aureus* isolates from Detroit fell into phage group 1, whereas a majority of isolates from Atlanta, Miami, St. Louis, St. Paul, Charlottesville, Seattle, and Charleston had phage group 3 reactions. Organisms from Jackson, Ann Arbor, and New Orleans had mixed reactions, and most of those from New York and Houston were nontypable. Analysis of the disk diffusion results for methicillin, oxacillin, cephalothin, and cefamandole, by phage type, revealed no apparent pattern. Susceptibility patterns seemed to be more related to geographic region than to phage type.

Susceptibility to other agents. Examination of plates after 18 h of incubation at 35°C revealed resistance to erythromycin in 100% of isolates, clindamycin (90%), tetracycline (64%), and gentamicin (67%). All isolates were resistant to erythromycin and clindamycin, or to erythromycin and tetracycline. All MR *S. aureus* isolates were susceptible to vancomycin.

The results obtained at 30°C are compared with those obtained at 35°C in Table 4. The interpretive results obtained at 30°C were not significantly different than those obtained at 35°C for erythromycin, clindamycin, tetracycline, gentamicin, or vancomycin.

DISCUSSION

When new techniques for performing antimicrobial susceptibility tests are developed, or modifications of existing systems are being evaluated, strains of bacteria that are known to be susceptible and others that are moderately resistant to representative antimicrobial agents should be included in premarketing evaluations (20). Strains used in such studies should possess genetically stable antimicrobial resistance patterns (20) and should presumably come from different geographic areas (including areas where the new system is likely to be used after licensure).

Interestingly, the early evaluations of the single-disk agar diffusion tests described by Ericsson (19) and by Bauer et al. (8) were conducted before methicillin was commercially available. Hence, MR *S. aureus* strains were not included in the preliminary studies of the disk diffusion test.

As part of the International Collaborative Study on Antibiotic Sensitivity Testing, 45 strains of MR *S. aureus* were tested by a disk diffusion method that employed tryptic soy agar plates inoculated by the flooding technique (20). The study revealed that results were not reliable when penicillinase-resistant penicillins and cephalosporins were tested. The International Collaborative Study report noted that incubation of susceptibility tests for 48 h, or at 30°C, might improve detection of MR *S. aureus* strains (20). The authors concluded that further studies were needed to determine the ability of the various proposed reference methods to detect hetero-resistant staphylococci.

In 1972, Drew et al. (18) used MR *S. aureus* strains from stock collections at three American medical centers and a British pharmaceutical firm to study the ability of the Kirby-Bauer method to detect MR *S. aureus* strains. Disk diffusion tests were incubated at 35 and 37°C for 18 and 48 h. The authors found that all isolates were correctly categorized as methicillin resistant if plates were incubated for 18 h at 35°C. Results were not accurate at 37°C.

Forty-five MR *S. aureus* isolates from an unspecified number of hospitals were included in an evaluation of the standardized disk diffusion test performed by Thornsberry et al. (42). Plates were incubated overnight (18 to 24 h) at 30, 35, and 37°C. All isolates appeared to be resistant to methicillin when plates were incubated overnight at 35°C. Although resistance to methicillin was detected reliably at 30°C, routine incubation of plates at 30°C was not recommended because too little information was available about the effects of 30°C incubation on susceptibility results for other bacteria.

Recently, Aldridge et al. (2) reported that the disk diffusion method failed to detect oxacillin resistance in 64% of isolates and cephalothin resistance in 100% of isolates when

TABLE 3. Reproducibility of disk diffusion results for 73 MR *S. aureus* isolates tested for susceptibility at 35°C

Result of two determinations ^a	No. of isolates with susceptibility pattern at time indicated							
	Methicillin		Oxacillin		Cephalothin		Cefamandole	
	18 h	24 h	18 h	24 h	18 h	24 h	18 h	24 h
S-S	1	0	1	1	29	19	26	18
S-I	0	0	0	0	3	3	13	11
S-R	0	0	2	0	5	6	0	2
I-I	0	1	0	0	2	3	8	8
I-R	3	1	0	0	5	5	6	10
R-R	69	71	70	72	29	37	20	24

^a S, Susceptible; I, intermediate; and R, resistant (33).

TABLE 4. Susceptibility of 73 MR *S. aureus* isolates tested at 30 and 35°C

Result at 30°C ^a	Correlation with results at 35°C after 24 h (no. of isolates)														
	Erythromycin			Clindamycin ^b			Tetracycline			Gentamicin			Vancomycin		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
R	0	0	73	0	0	62	0	0	47	0	0	47	0	0	0
I	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
S	0	0	0	7	0	3	26	0	0	24	0	0	73	0	0

^a S, Susceptible; I, intermediate; and R, resistant (33).

^b Results of duplicate tests not available for one isolate.

plates were incubated at 35°C for 24 h. The origins of the 25 MR *S. aureus* isolates studied were not listed. Presumably, the disparity between this recent survey and earlier evaluations is due to differences in the MR *S. aureus* strains used or to differences in study design.

Seventy-three isolates recovered in hospitals in 13 cities located throughout the United States were included in the present evaluation. Although bacterial population studies were not performed on study isolates during this investigation, the patterns of growth observed around the methicillin, oxacillin, and cephalothin disks suggest that strains from Charlottesville and Charleston are probably homogeneously resistant to methicillin and cephalothin, whereas strains from most other cities appeared to be heteroresistant. The epidemic strain recovered from patients in Charlottesville has been shown to be homogeneously resistant (35).

After incubation of plates for 18 h at 35°C, as currently recommended (33), 96% of isolates included in the present study were categorized as resistant to methicillin and 97% as resistant to oxacillin. Strains from some cities had little or no zones of inhibition, whereas those from other cities had measurable zones, with growth inside the zones. A few appeared to be susceptible to one or both agents. Repeat determinations performed at 35°C suggest that the results are reproducible. The high detection rate noted in this study is similar to the rate obtained during a recent survey by the College of American Pathologists (26). In that survey, 96.8% of participating laboratories that used disk diffusion methods detected the MR *S. aureus* isolate that was included in the survey.

In the present study, incubation of plates for a full 24 h resulted in detection of resistance of 97% of isolates by methicillin disks and 99% by oxacillin disks. After prolonged incubation (48 h) at 35°C, all MR *S. aureus* isolates included in this study appeared to be resistant to methicillin and oxacillin. On several occasions, the control strain had 48-h methicillin and oxacillin inhibitory zones that were smaller than the accepted limits, as published for 18-h cultures. This finding suggests that it may be useful to establish individual daily test control zone diameter limits for disk diffusion susceptibility tests that are incubated for 48 h.

Incubation of plates at 30°C for 24 h did not improve detection of methicillin or oxacillin resistance among MR *S. aureus* isolates included in this study. Although these conditions did not adversely affect the results when the 73 MR *S. aureus* isolates were tested for susceptibility to erythromycin, clindamycin, tetracycline, gentamicin, or vancomycin, there seems to be little reason to recommend use of a 30°C incubator when testing *S. aureus*, since it does not appear to result in better detection of MR *S. aureus* isolates.

Only 52% of MR *S. aureus* isolates in this study appeared to be resistant to cephalothin after 18 h of incubation at 35°C.

The percentage categorized as resistant increased to 66 and 86% after 24 and 48 h of incubation, respectively. A number of other studies have also shown that the standard Kirby-Bauer test does not detect cephalosporin resistance reliably (5, 13, 18, 36, 37, 42). Unlike some previous reports (13, 14, 22), use of a 30°C incubator did not improve detection of cephalothin resistance among MR *S. aureus* strains included in the present investigation, but it did result in a greater number of isolates being categorized as resistant to cefamandole.

In view of the problems associated with detecting cephalosporin resistance among MR *S. aureus* isolates, it may be prudent to report MR *S. aureus* isolates as resistant to cephalothin, regardless of the results obtained in vitro (33). Administration of cephalosporins to patients infected with MR *S. aureus* has been associated with treatment failures (1, 28, 31, 37), and as a result, these drugs are not recommended for treatment of serious MR *S. aureus* infections (31, 45). Further studies in appropriate animal models are needed to help establish whether or not these agents can eradicate such organisms in vivo.

The results of the present study suggest that the standardized disk diffusion test will detect a majority of MR *S. aureus* strains if the following precautions are taken. (i) Inoculated plates should be incubated at 35°C, and the temperature of the incubator should be monitored carefully. (ii) *S. aureus* isolates that are multidrug resistant should be suspected of being MR *S. aureus*, and zones of inhibition around oxacillin or methicillin disks should be examined carefully for evidence of growth inside the zone. (iii) Plates should be incubated for a full 24 h if an *S. aureus* isolate appears to be multidrug resistant. If such organisms appear to be intermediate in susceptibility to oxacillin or methicillin at 24 h, it may be helpful to hold the plate an extra 24 h at 35°C. (iv) Use of both oxacillin and methicillin disks on the same plate may increase the likelihood of detecting a few strains that show differing degrees of heteroresistance to these agents. If a single class disk is used, previous studies suggest that oxacillin disks are preferable because of their greater stability (18).

Finally, McDougal and Thornsberry (L. K. McDougal and C. Thornsberry, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, Las Vegas, Nev., abstr. no. 535, 1983) have just completed an investigation of the ability of the disk diffusion test to detect MR *S. aureus*. They have recommended that the antibiotic content of methicillin and oxacillin disks be changed, and that the zone diameter cutoff points used when testing *S. aureus* isolates for susceptibility to these agents be revised. Approval of these proposed changes by the Food and Drug Administration and commercial production of the new disks may take some time. Until the new disks are commercially available, the present study suggests that the current method will detect MR *S. aureus* strains in many geographic areas.

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