Reliability of the Kirby-Bauer Disc Diffusion Method for Detecting Methicillin-Resistant Strains of *Staphylococcus aureus*

W. LAWRENCE DREW, A. L. BARRY, RICHARD O'TOOLE, AND JOHN C. SHERRIS

Department of Pathology and Laboratory Medicine, Mount Zion Hospital and Medical Center, San Francisco, California 94120; Hospital Infection Research Laboratory, Departments of Medical Microbiology and of Medicine, College of Medicine, University of California, Irvine, California 92650; and Departments of Medicine and Microbiology, University of Washington and Harborview Medical Center, Seattle, Washington 98104

The resistance of *Staphylococcus aureus* to methicillin and related drugs can be reliably determined by using the Kirby-Bauer method of susceptibility testing if the incubation temperature is 35 C or below, but resistance may be missed at 37 C. The 1-μg discs of oxacillin and nafcillin or the 5-μg discs of methicillin may be used for this purpose but not the 1-μg discs of cloxacillin. The latter fail to discriminate between sensitive and resistant staphylococci by zone measurement; some resistant strains of staphylococci may show larger zones of inhibition than sensitive strains. Stability of these antibiotic-containing discs was studied under conditions of temperature and humidity variation that might be encountered in a clinical laboratory refrigerator. Oxacillin discs were the most stable and are to be preferred for susceptibility testing. Nafcillin discs were less stable, and methicillin discs lose their potency rapidly unless carefully stored in a refrigerator with a desiccant.

Strains of *Staphylococcus aureus* which are resistant to methicillin and other penicillinase-stable penicillins have been isolated regularly from infected patients in the hospitals of Europe and the United Kingdom. In the United States, there have been only a few reported outbreaks of infections due to these organisms (4, 8, 15).

Disc diffusion methods for susceptibility testing may fail to detect all methicillin-resistant strains of *S. aureus*, largely because such strains are remarkably heterogenous in their resistance (3, 9, 10, 12, 16, 20, 21). That is to say, the majority of cells within a clone may be fully susceptible, and only a small proportion may be capable of growing in the presence of an increased concentration of methicillin. On ordinary media, the resistant portion of the population may grow rather slowly and tend to form small colonies (17). An incubation period of 48 hr has been recommended for detecting the resistant portion of the population, but this can be decreased by using a hypertonic medium (2, 10) or by incubating the tests at a lower temperature (1, 12, 13). The present study was undertaken to determine whether the disc diffusion method of Bauer et al. (16), without modification, would suffice for detecting methicillin resistance among isolates of *S. aureus* or whether an additional type of screening test must be utilized. Because cloxacillin, nafcillin, and oxacillin are structurally and pharmacologically related to methicillin, detection of resistance to these agents was also studied.

MATERIALS AND METHODS

Tests were carried out with 52 isolates of methicillin-resistant *S. aureus* obtained from several sources and 25 isolates of methicillin-susceptible *S. aureus* obtained from the clinical laboratories of the University Hospital, Seattle, Wash.

Strains of methicillin-resistant *S. aureus* were kindly supplied by E. J. Benner, University of California, Davis (24 strains); R. J. Bulger, University of Washington, Seattle (6 strains); S. Cohen, University of California, San Francisco (3 strains); and R. Sutherland, Beecham Research Laboratories, Brockham

Park, Batchwork, Surrey, England (17 strains). All filter-paper discs were obtained from Difco Laboratories. For agar dilution studies methicillin, cloxacillin, and oxacillin were obtained from Bristol Laboratories, Syracuse, N.Y., and nafcillin was obtained from Wyeth Laboratories, Philadelphia, Pa.

Susceptibility tests were performed by the disc diffusion method of Bauer et al. (6) with Mueller-Hinton agar (Difco). Zones of inhibition were measured after 18 hr and again after 48 hr of incubation at 35 C. Studies were also carried out by incubating the plates at 35 and 37 C. Simultaneous agar dilution susceptibility tests were performed with the same suspension of bacteria as prepared for the disc test but diluted so that a Steers-Foltz (19) inoculum replicator would deposit approximately 10^5 viable cells of each strain onto each plate. Doubling dilutions of antibiotic were incorporated into Mueller-Hinton agar (Difco) for these tests. The minimal inhibitory concentration (MIC) was recorded as the lowest concentration of drug permitting the growth of no more than one colony after 48 hr of incubation at 35 C. Population analyses were carried out in a similar manner but by inoculating the plates with varying dilutions of the bacterial suspension. The number of colony-forming units capable of growing in the presence of each concentration of drug was then determined.

The comparative stability of discs containing methicillin, oxacillin, cloxacillin, and nafcillin was determined by storing cartridges in open containers and in a closed storage rack with a desiccant which was replaced when needed. Both types of containers were stored at room temperature (12 to 28 C, 10 to 50% relative humidity) and in a household refrigerator located in the center of a clinical microbiology laboratory. During the working day, the door to this refrigerator was frequently opened and closed and, consequently, the temperature varied from 3 to 18 C and the relative humidity from 56 to 80%. Once a week, for 12 weeks, the discs were removed from storage and tested in triplicate against a fully susceptible clinical isolate of *S. aureus* by using the agar overlay modification of the Kirby-Bauer method; (5) the diameters of the inhibitory zones were recorded after overnight incubation. Because chloramphenicol is very stable under a variety of conditions of temperature and humidity, discs containing this drug were also included as a methodology control. The zone diameters around chloramphenicol discs varied no more than 1 mm during the 12-week study, regardless of storage conditions, thus confirming the reproducibility of the test method.

RESULTS

The inactivation of antibiotic-containing discs during 12 weeks of storage under four different conditions is depicted in Fig. 1. When exposed to the temperature and humidity of the laboratory, methicillin discs lost all ac-
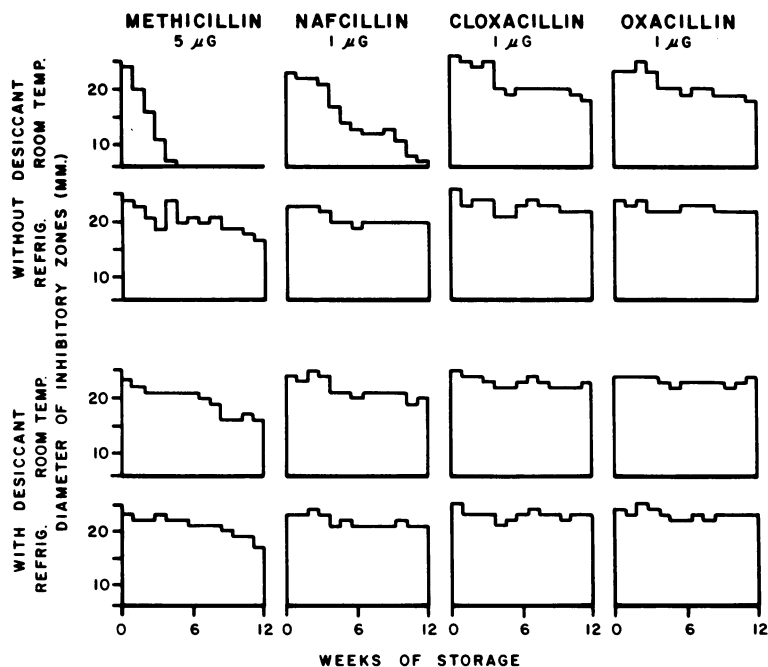

FIG. 1. *Stability of methicillin, nafcillin, cloxacillin, and oxacillin discs at refrigerator or room temperature, with and without desiccant.*

tivity after 6 weeks, and nafcillin was completely inactivated after 12 weeks. Inactivation of the drugs was not as pronounced when the discs were refrigerated with a desiccant. Even under these conditions, methicillin lost a significant amount of activity during the 12-week study period, but the other three drugs were not markedly affected. Of the four drugs tested, oxacillin and cloxacillin were somewhat more stable than nafcillin, and all three were much more stable than methicillin.

These four drugs were then tested for their ability to separate methicillin-resistant *S. aureus* from susceptible strains by the disc diffusion technique of Bauer et al. (6) The relationship between the sizes of inhibitory zones read after 18 hr and the 48-hr MIC is described in Fig. 2 and 3. Oxacillin and nafcillin discs (1 μg) both readily detected resistant strains (MIC > 1.25 μg/ml) all of which gave no zone of inhibition, whereas susceptible strains (MIC $\leq$ 0.62 μg/ml) gave zones of 11 mm or greater. Methicillin discs (5 μg) were also capable of separating resistant strains from susceptible strains; with one exception, resistant strains (MIC > 5.0 μg/ml) grew up to the edge of the disc, and all the susceptible strains (MIC $\leq$ 2.5 μg/ml) gave zones 15 mm in diameter or greater.

Some resistant strains showed reduced density of growth in the immediate vicinity of the discs; occasionally an "outer" zone of inhibition could actually be measured. In these instances bacterial growth in the "inner" zone could be seen with careful inspection. This appearance was more frequent around nafcillin discs than around oxacillin or methicillin discs.

With 1-μg cloxacillin discs, zone measurements provided no clear separation of resistant and sensitive staphylococci. Resistant strains, with MIC ranging from 1.5 to 100 μg/ml, gave inhibitory zones 13 to 19 mm in diameter, whereas susceptible strains (MIC $\leq$ 1.25 μg/ml) had zones measuring 14 to 23 mm. Plates seeded with resistant organisms often revealed tiny colonies within the zone of inhibition, but these colonies were not always visible to the naked eye and did not always appear even after 48 hr of incubation. Figure 4 demonstrates the type of zones observed with an isolate of *S. aureus* which is actually resistant to all four drugs by agar dilution tests. Limited experience with dicloxacillin discs revealed results similar to those observed with cloxacillin.

Ten isolates were retested by the disc diffusion technique and incubated at two different temperatures (Table 1). The ten resistant isolates gave very small zones, if any, around oxacillin, nafcillin, and methicillin discs at 35 C, but at 37 C there was a marked increase in the zone sizes. With cloxacillin discs the sizes of the zones of inhibition were not affected by the temperature of incubation.

Population survival studies were carried out to determine whether the percentage of cells resistant to high concentrations of cloxacillin is smaller than that with comparable antibiotics and accounts for the apparent susceptibility of these bacteria in disc diffusion susceptibility tests. Figure 5 represents the re-
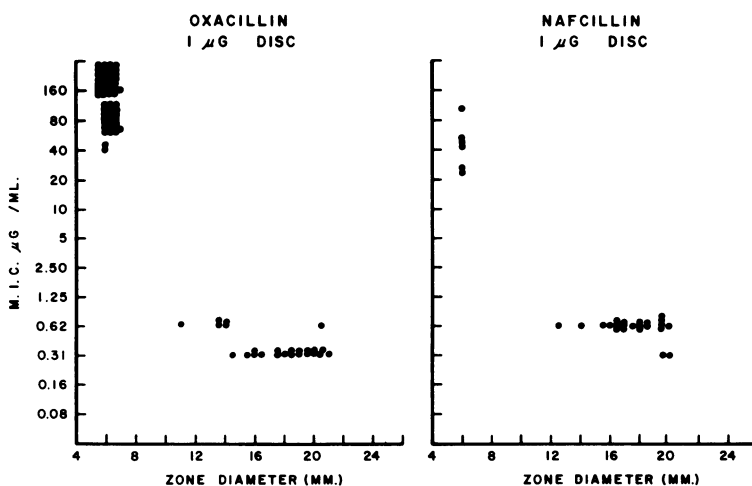

FIG. 2. *Relationship of zone sizes to minimal inhibitory concentration (MIC).*
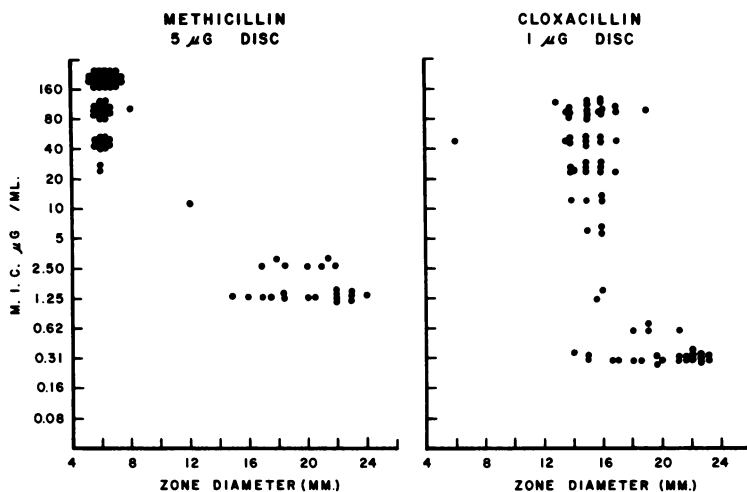
FIG. 3. *Relationship of zone sizes to minimal inhibitory concentrations (MIC).*
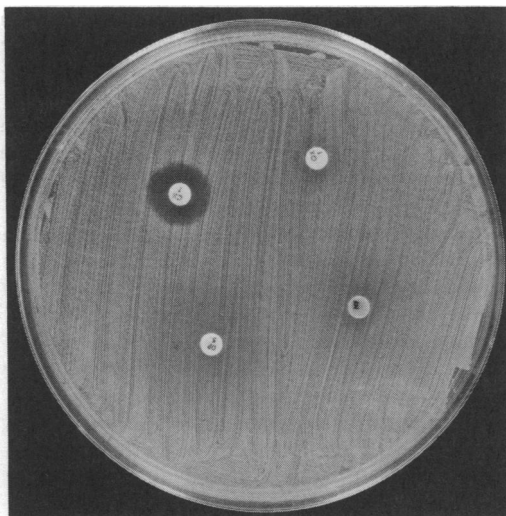

FIG. 4. *Appearances of inhibitory zones with methicillin-resistant S. aureus and the following discs: oxacillin (OX, upper right); cloxacillin (CX, upper left); nafcillin (NF, lower right); and methicillin (DP, lower left).*

sults of such an experiment in which the number of viable cells capable of growing in the presence of increasing concentrations of cloxacillin was compared with the same capability in nafcillin. The resistant isolate tested in this experiment was capable of growing on agar plates containing 100 µg of either drug per ml, but cloxacillin was much more active in that the percentage of resistant cells surviving intermediate concentrations was much lower. Indeed, 99.99% of the viable cells in this culture was inhibited by 1.6 µg of cloxacillin/ml

whereas 50 µg of nafcillin/ml was required for comparable results.

A similar population survival study was carried out with two other isolates of *S. aureus*, both of which had a cloxacillin MIC of 50 µg/ml. One isolate showed confluent growth right up to the edge of a 1-µg cloxacillin disc, whereas the other had the more typical pattern of a 16-mm zone with several inner colonies. The latter organism was more susceptible to intermediate concentrations of cloxacillin than the former (Fig. 6). In this instance, 50 µg of cloxacillin/ml was required to inhibit 99.99% of the cells in the more resistant culture, whereas only 1.6 µg/ml was required for comparable inhibition of the culture which gave large zones of inhibition with the disc method.

DISCUSSION

The data reported here indicate that the disc diffusion technique of Bauer et al. (6) is reliable for the detection of *S. aureus* resistant to methicillin and related to antimicrobials when incubated at 35 C. However, several precautions must be taken to avoid erroneous results. In practice, many "methicillin-resistant *S. aureus*" are actually coagulase-negative *S. epidermidis* which have been misidentified for one reason or another. Methicillin resistance is not uncommon among strains of *S. epidermidis*, and mixtures of resistant *S. epidermidis* and susceptible *S. aureus* are often found in clinical specimens. The identification of resistant colonies within a zone of inhibition should be confirmed before being reported.

Inactivation of the susceptibility test discs is

TABLE 1. *Effect of temperature of incubation on zone-size measurements*

Strain	Temp	Zone diameters (mm)[a]			
		Oxa-cillin	Naf-cillin	Methi-cillin	Cloxa-cillin
CCAU	35	6[b]	6[b]	6	17
	37	13	16	14	17
N37	35	6	6	6	16
	37	6 (11)	14	11.5	16
387	35	6	6[b]	6	17
	37	14	17	15	18
UC2	35	6 (11)	6 (13)	6[b]	16.5
	37	13	16	10	17
68/8585	35	6	6	6	16
	37	6	6[b]	6[b]	15
1484	35	6	6	6	16
	37	12	17	15	16
7913	35	6	6	6	16
	37	6 (11)	14	12	16
996	35	6	6	6	19
	37	13	14	12	17
968	35	6 (14)	6 (15)	6 (10)	19
	37	15	18	6 (13)	19
E164	35	6 (12)	12	6 (11)	16
	37	13	17	14	16

[a] A diameter of 6 mm equals no inhibition because this is the diameter of the disc. Figures in parentheses equal the "outer" zone in those tests where there was marked reduction of the density of growth in the immediate vicinity of the disc.

[b] These tests showed only a suggestion of an outer zone.

another significant source of error in the clinical laboratory. Griffith and Mullins (11) studied a number of storage conditions and documented inactivation of penicillinase-stable penicillins under conditions of high humidity and temperature. They recommended that the stock supply of discs should be stored in a vacuum desiccator at −20 C to provide maximum stability. The desiccator should be removed from the freezer at least 1 hr before the discs are used to prevent condensation on the discs with subsequent inactivation of the drug. The present study documents the extremes of temperature and humidity to which working supplies of discs may be exposed within one laboratory. In other parts of the country, greater or lesser extremes of tem-

perature and humidity certainly could be encountered. The conditions of storage during transit from the manufacturer to the laboratory is always an unknown factor. Once in the laboratory, the discs can be brought in and out of the refrigerator several times before the working supply is consumed. For this reason it is difficult to determine how long the discs can be used before the drug is inactivated. Daily quality control tests should be carried out to check the performance of the discs being used with each batch of tests. Obviously, fewer technical problems will be encountered if only the more stable penicillinase-resistant penicillins are used for routine testing. Our studies indicate that oxacillin is satisfactorily stable under the storage conditions tested. Nafcillin would be the next best choice on the basis of stability alone, with methicillin the most likely to become inactivated during storage. Cloxacillin discs failed to discriminate between strains which were sensitive and resistant by the dilution test; thus, although stable, they are not suitable for use.

Because oxacillin discs are the most stable and provide clearcut separation of susceptible and resistant populations when tested with the disc method of Bauer et al. (6), they are preferred for routine testing purposes. Nafcillin discs also discriminate between susceptible and resistant *S. aureus* but are somewhat less stable than those of oxacillin. Also, some resistant strains may show only a fine film of bacterial growth around these discs suggesting a zone of inhibition. Methicillin discs are suitably discriminatory, but their instability can provide a serious potential source of technical error unless they are stored under optimal conditions (18).

Zone size criteria for these discs are shown in Table 2. Interpretive zone standards for cloxacillin could not be established because some resistant strains gave large, clear zones of inhibition which were as large or larger than those seen with susceptible strains.

Other reports have indicated that methicillin resistance can be detected with filter-paper discs only when the agar medium is stabilized with 5% NaCl (2, 10, 12) or by incubating the test plates at 30 C rather than 35 to 37 C (1, 12, 13). However, most of these studies were done with lighter inocula and more potent discs which could cause resistant staphylococci to show large zones of inhibition under the usual conditions of temperature and tonicity. With the Kirby-Bauer disc diffusion technique, using the recommended disc contents and an incubation temperature of 35 C,
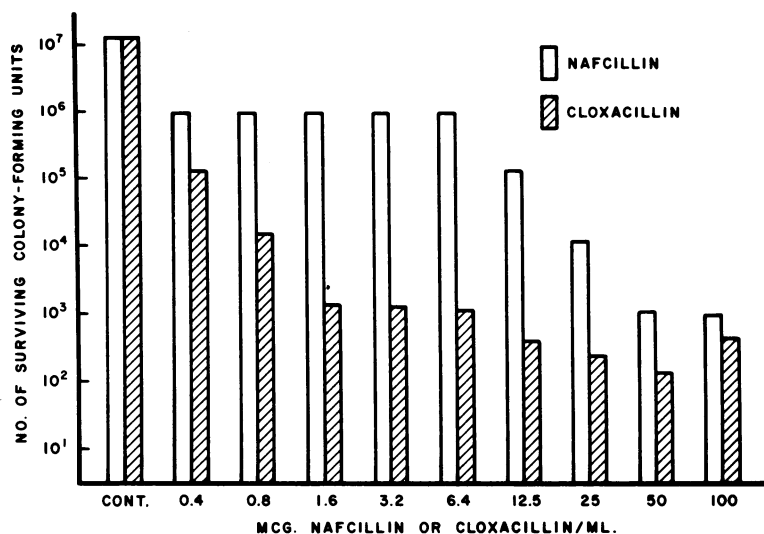
FIG. 5. Comparison of the survival of one strain of methicillin-resistant S. aureus when exposed to increasing concentrations of nafcillin and cloxacillin.
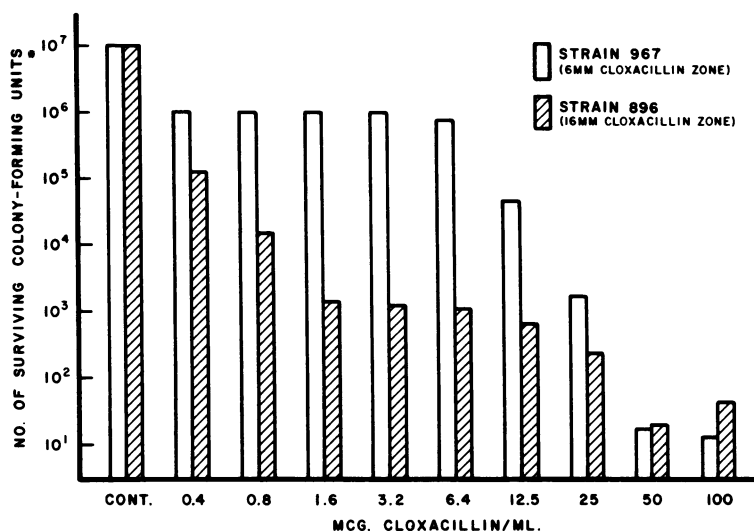

FIG. 6. Comparison of the survival of two strains of methicillin-resistant staphylococci when exposed to increasing concentrations of cloxacillin. Strain 967 had shown a 6-mm zone of inhibition around the 1-ug cloxacillin disc when tested by the Kirby-Bauer method; strain 896 had shown a 16-mm zone of inhibition.

these additional procedures appear to be unnecessary.

The success of the Kirby-Bauer method in detecting methicillin-resistant staphylococci depends on close control of incubator temperature. Increasing the temperature of incubation from 35 to 37 C may cause resistant strains to appear sensitive. Another laboratory has had similar findings (Thornsberry et al., *personal communication*). Regulation of incubation temperature in a clinical laboratory can be quite difficult due to frequent opening and closing of the incubator doors. For accurate susceptibility testing of staphylococci, incubation temperature must not exceed 35 C throughout incubation. To ensure this, incubator temperature should be checked after overnight use, before the doors are opened in the morning.

The failure of cloxacillin discs to detect strains which show high cloxacillin MIC and are resistant to other penicillinase-stable peni-

TABLE 2. *Disc zone sizes and standards*

Drug	Disc potency (μg)	Inhibitory zone diameter (mm)			MIC (μg/ml)
		Resist-ant (mm)	Inter-medi-ate	Suscep-tible	Suscep-tible
Methicillin	5	9 or less	10–13	14 or more	2.5 or less
Nafcillin	1	10 or less	11–12	13 or more	0.6 or less
Oxacillin	1	10 or less	11–12	13 or more	0.6 or less

cillins has been reported previously. Kayser (14) found discrepancies between MIC and zone sizes. Hewitt et al. (13) also documented that cloxacillin discs failed to discriminate between sensitive and resistant staphylococci, even when a hypertonic medium was used.

The inability of cloxacillin discs to detect all resistant staphylococci is due, in part at least, to the low proportion of resistant cells within the population. Intermediate concentrations of cloxacillin inhibit a greater proportion of the individual cells than do other related drugs. Therefore, few, if any, colonies are able to grow on the agar in the immediate vicinity of the cloxacillin disc where the drug concentration is relatively high. With methicillin, oxacillin, or nafcillin growth around the discs may show reduced density but, nonetheless, there is usually confluent growth right up to the edge of the disc. We have not yet found a strain of *S. aureus* with a sufficiently low proportion of resistant cells to yield a "false-sensitive" disc test with these agents under the specified testing conditions.

Whether the extreme heterogeneity of staphylococci resistant to cloxacillin provides an advantage in the clinical use of the antibiotic is uncertain, but any potential advantage of cloxacillin is probably offset by its high degree of protein binding. Clinical studies adequately answering this question are not available. It may be that more quantitative approaches to susceptibility testing, such as population survival studies, are necessary for determining the relative efficacy of these agents.

The current data confirm that isolates of *S. aureus* can be separated into two distinct groups (resistant and susceptible to methicillin and related antimicrobials) and indicate that the Kirby-Bauer disc test will distinguish between these groups provided the incubator temperature is 35 C or below. Cloxacillin and

dicloxacillin discs should not be used. Oxacillin discs are preferred; if methicillin discs are used, careful attention must be paid to the conditions of storage. Use of nafcillin discs requires more careful inspection of the "inner" zone of inhibition to detect the growth of resistant staphylococci.

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