Effect of Temperature on the In Vitro Susceptibility of *Staphylococcus aureus* to Penicillinase-Resistant Penicillins

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Heteroresistant (methicillin-resistant) and nonheteroresistant strains of *Staphylococcus aureus* were tested for their susceptibility to penicillinase-resistant penicillins at incubation temperatures of 37, 35, and 30 C. Susceptibilities were determined by agar dilution and by the standard Kirby-Bauer agar diffusion tests. Minimal inhibitory concentrations were higher at 35 and 30 C than at 37 C. Heteroresistance could be detected with the Kirby-Bauer test if the incubation temperature was 30 or 35 C instead of 37 C, when tests were performed against methicillin, oxacillin, or nafcillin, because the resistant organisms grew up to the disks even though the susceptible organisms were inhibited. At 37 C, the resistance was detectable with some strains but not with others. When cloxacillin disks were used, the temperature effect was not seen. The incubation temperature did not affect results with nonheteroresistant strains. Therefore, it is recommended that all Kirby-Bauer tests be incubated at a temperature of 35 C to insure detection of methicillin-resistant *S. aureus* strains. Detection of these strains is of increasing importance because the incidence of infections with these organisms is increasing, particularly in hospitalized patients.

Strains of *Staphylococcus aureus* that are resistant to the penicillinase-resistant penicillins have been isolated principally from hospitalized patients, particularly in Europe (1, 3, 6, 11, 12, 15, 16, 19, 22, 24, 25) but also in this country (4, 7–9, 21, 23, 26–28). These strains have been called “methicillin-resistant” or “heteroresistant” *S. aureus*. They are characterized by the marked heterogeneity of their resistance. A clone of these staphylococci will contain both methicillin-susceptible cells and resistant cells (31). The resistant variants usually produce small colonies on isotonic media but larger colonies on hypertonic media (3, 12, 14, 16). They also grow more quickly and luxuriantly at lower temperatures (2, 16). It is not surprising, then, that clinical laboratories have had difficulty in recognizing these heteroresistant staphylococci.

Methods that have been proposed for laboratory detection of heteroresistance are (i) testing on media containing 5% NaCl (3, 12), (ii) the use of a larger inoculum than is normally used for susceptibility tests (14), (iii) incubation of the tests for 48 h (7, 8, 14), and (iv) incubation of the tests at 30 C (2, 16). None of these four methods can be used with a standardized agar diffusion test such as the method of Bauer and associates (5). However, in this report we present data to show that the resistance of *S. aureus* to methicillin can be detected by the method of Bauer et al. (5) when cultures are incubated at 35 C, but not when they are incubated at 37 C.

**MATERIALS AND METHODS**

The strains of *S. aureus* used in these studies were selected from cultures submitted for bacteriophage typing to the Staphylococcus Laboratory, Center for Disease Control. These strains had been isolated from a variety of infections and came from several different parts of this country. Forty-five methicillin-resistant strains and 11 methicillin-susceptible strains were selected by screening cultures with methicillin disks at 30 C.

The methicillin, oxacillin, and cloxacillin used in the study were obtained from Bristol Laboratories, and the nafcillin was from Wyeth Laboratories.

The *S. aureus* strains were studied for their susceptibility to methicillin, oxacillin, nafcillin, and cloxacillin by agar diffusion and agar dilution methods. The agar diffusion method was the standardized method of Bauer and associates (5) as modified by the NCCLS Subcommittee for Antimicrobial Susceptibility Testing (Performance standards for antimicrobial disk susceptibility tests, submitted for publication). The agar dilution method was described in the 1971
International Collaborative Study Report by Ericsson and Sherris (14), and with this method a Steer's replicator was used (30).

In both the agar diffusion and the agar dilution tests, Mueller-Hinton agar, pH 7.2 to 7.4, was used. For the agar diffusion test, 150-mm plates were filled with approximately 60 ml of agar. The cultures used to inoculate the agar plates were adjusted to a density equivalent to a 0.5 McFarland standard. For the agar dilution tests, working dilutions of the antibiotics were made in sterile distilled water so that they could be combined with the Mueller-Hinton agar in a ratio of nine parts of agar to one part of antibiotic solution to achieve the desired concentration. The antibiotic solution was added to agar that had been cooled to 50 C. Approximately 25 ml of the agar-antibiotic solution was added to each 100-mm square petri plate. The inocula for the agar dilution studies were prepared by diluting a 4- to 5-h broth culture to a density equivalent to a 0.5 McFarland standard and then making another 10-fold dilution. When these cultures were applied to the agar with a Steer's replicator, approximately 10^6 bacteria from each culture were deposited on the agar surface.

Three identical sets of plates were prepared for each culture, so that each culture could be incubated at 30, 35, and 37 C. The tests were read after 18 to 24 h of incubation. In the agar dilution tests, the minimal inhibitory concentration (MIC) was read as that concentration which allowed the growth of no more than one colony.

The incubators used in the study contained fans which maintained constant circulation of the air in the incubator. During the early parts of these studies, the temperature was monitored continuously by a recorder that was placed inside the incubator. During the rest of the study, temperatures were read four to five times during the working day. The temperatures did not vary more than 1 degree, and when this variation occurred it was always to the lower temperature.

RESULTS

Agar diffusion tests. An example of the diameter of the zones of inhibition seen with methicillin-susceptible and methicillin-resistant strains is shown in Table 1. By the interpretive standards of the Kirby-Bauer test, the susceptible strain was interpreted as being susceptible to all four antibiotics regardless of the temperature of incubation.

With incubation at 37 C, the resistant strain was also interpreted as being susceptible to all four antibiotics, as judged by the diameter of inhibition zones. However, at 30 and 35 C, the zone sizes indicated that it was susceptible only to cloxacillin. With the other three antibiotics, at these temperatures, there was a zone where some of the growth had been inhibited, but inside this zone there was growth up to the disk. Therefore, these zones were read as 6 mm, which is the diameter of the disk. In Table 1, these zones are recorded as 6i, to indicate that there was some inhibition. Therefore, this strain would be erroneously interpreted as being susceptible to these four antibiotics if the cultures were incubated at 37 C. However, at 35 and 30 C, it would be properly interpreted as being resistant to methicillin, oxacillin, and nafcillin, but not to cloxacillin.

Although the results shown in Table 1 are for only one strain, similar results were obtained for all strains tested. Resistance was detected in all of the 45 heteroresistant strains when they were tested at 35 and 30 C with methicillin, oxacillin, and nafcillin. Some resistant strains could also be detected at 37 C. At all three temperatures, the susceptible strains were susceptible to cloxacillin as well as to the other three antibiotics.

Examples of the effect of incubation temperatures on agar diffusion tests for susceptibility of S. aureus to penicillinase-stable penicillins are shown in Fig. 1–6. Figures 1–3 show results of tests with a methicillin-susceptible strain and Fig. 4–6 show results with a methicillin-resistant strain. Cephalothin disks were included in these tests because the methicillin-resistant strains often show cross-resistance to the cephalosporins. Although resistance to cephalothin was not detected in the strains shown in these figures, a few methicillin-resistant strains have been detected when the incubation temperature was 35 or 30 C. Further studies have shown that the agar diffusion test is not reliable in the detection of cephalothin resistance in heteroresistant S. aureus.

Agar dilution tests. The effect of incubation temperature was also seen with the agar dilution tests. The MICs of methicillin, oxacillin, nafcillin, and cloxacillin for the 45 methicillin-resistant strains are shown in Table 2. With
Fig. 1. Methicillin-susceptible strain of S. aureus tested at 37°C. Clockwise, the disks are oxacillin, nafcillin, cloxacillin, cephalothin, and methicillin.

Fig. 2. Methicillin-susceptible strain of S. aureus tested at 35°C. Clockwise, the disks are oxacillin, nafcillin, cloxacillin, cephalothin, and methicillin.

Fig. 3. Closer view of the zone of inhibition around the methicillin disk in the test with the methicillin-susceptible strain at 35°C shown in Fig. 2.

Fig. 4. Methicillin-resistant strain of S. aureus tested at 37°C. Clockwise, the disks are oxacillin, nafcillin, cloxacillin, cephalothin, and methicillin.

Fig. 5. Methicillin-resistant strain of S. aureus tested at 35°C. Clockwise, the disks are oxacillin, nafcillin, cloxacillin, cephalothin, and methicillin.

Fig. 6. Closer view of the zone of inhibition around the methicillin disk in the test with the methicillin-resistant strain at 35°C shown in Fig. 5.
most of the strains, the MIC at 37 C was lower than that at 35 or 30 C. For example, when tests were performed with methicillin at 37 C, only 2 of 45 strains showed an MIC of 50 μg/ml or greater. At 35 and 30 C, 42 of 45 strains showed an MIC of 50 μg/ml or greater. A similar effect was seen with the other three drugs. One strain was unusual in that it showed the temperature effect with oxacillin, nafcillin, and cloxacillin, but not with methicillin. This strain was still classified as being heteroresistant.

The MIC for the nonheteroresistant strains was the same regardless of the temperature at which they were tested. These MICs were generally much lower than those of the resistant strains as shown in Table 3. With all of the susceptible strains, the MIC was 3.12 μg or less/ml except with one strain for which the MIC of methicillin was 6.25 μg/ml. This strain did not show the temperature effect and was, therefore, considered to be nonheteroresistant.

Although the MICs of cloxacillin for the resistant strains were similar to those of methicillin, oxacillin, and nafcillin, the amount of growth at particular concentrations was much less. This can be seen in Fig. 7.

**DISCUSSION**

Infections with methicillin-resistant *S. aureus* strains are apparently occurring with increasing frequency. This seems to be particularly true in Europe. Parker (24) and Parker and Hewitt (25) reported that in England the number of infections increased moderately between 1960 and 1963; then, after a stationary period, another increase began in 1968 and continued at least through 1969. More recently, Kayser and Mak (19) reported that the frequency of infections due to methicillin-resistant *S. aureus* strains was increased in the Zurich, Switzerland area and that sometimes up to 50% of the staphylococcal infections in a hospital in that area were caused by these strains of *S. aureus*.

The incidence of infections due to these strains appears to be lower in the United States. Most reports have been of isolated cases (7-9, 21, 29). However, Barrett and associates (4) and O'Toole and associates (23) reported hospital outbreaks involving 18 and 6 patients in their respective hospitals, one of which was on the East Coast and the other on the West Coast of this country. R. B. Lindberg et al. (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 113, 1972) recently reported an epidemic of methicillin-resistant *S. aureus* infections in patients in a burns institute located in another section of the country.

Therefore, it appears that methicillin-resistant *S. aureus* infections are, at present, principally nosocomial infections. Although these organisms have been a part of the bacterial flora of many hospitals for several years, they are becoming more widely disseminated among hospitalized patients (25). The spread of methicillin-resistant staphylococci often cannot be correlated with increased use of methicillin but can be correlated with increased use of “conventional” penicillins (24). This has led Parker (24) to suggest that other penicillins and related antibiotics may act as selective agents for the methicillin-resistant strains and thus encourage their spread among hospital patients.

These organisms are virulent, and they produce serious disease. However, according to Parker (24), the significance of methicillin resistance to the infected patient is less clear. Although little information is available on whether these infections can be treated success-

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of cultures with MIC (μg/ml) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤7.8</td>
</tr>
<tr>
<td>Methicillin</td>
<td>0</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>9</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>3</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>10</td>
</tr>
</tbody>
</table>

*The MICs were identical at all three temperatures.*

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**TABLE 2. Minimal inhibitory concentrations (MIC) of 45 methicillin-resistant *S. aureus* strains**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Temp (C)</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>&gt;50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methicillin 37</td>
<td>1</td>
<td>15</td>
<td>27</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>9</td>
<td>33</td>
</tr>
<tr>
<td>Oxacillin 37</td>
<td>1</td>
<td>10</td>
<td>17</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Nafcillin 37</td>
<td>13</td>
<td>27</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td>Cloxacillin 37</td>
<td>30</td>
<td>12</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>3</td>
<td>12</td>
<td>10</td>
<td>20</td>
</tr>
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<td>30</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>15</td>
<td>27</td>
</tr>
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</table>
fully with the penicillinase-resistant penicillins, most treatment regimens have been with other antibiotics. Cephalothin has been used with varied degrees of success to treat a number of infections due to methicillin-resistant *S. aureus* strains (1, 6, 8, 21, 23). It has also been used in combination with kanamycin or gentamicin (1, 8, 23), but there is some doubt about the efficacy of these combinations against all strains (1), even though there is in vitro evidence that they might be successful (17, 18, 20). Vancomycin (7), lincomycin (21), and fusidic acid (12) have been used successfully in these infections.

In a study of the treatment of rats with renal infections due to a methicillin-resistant strain of *S. aureus*, Bulger and associates (10) reported that either vancomycin or a combination of cephalothin and kanamycin was more effective than cephalothin, kanamycin, or methicillin alone. Cephalothin and kanamycin were found to be equally effective and were more effective than methicillin. Methicillin was no more effective than saline.

Therefore, it is imperative that methicillin-resistant *S. aureus* strains be recognized by clinical bacteriology laboratories. Since recommended methods are intolerable variations of the Kirby-Bauer agar diffusion test (5, 32), it is of practical importance that these organisms can be recognized by the Kirby-Bauer test if it is performed at 35 C. All other organisms that can be tested by the Kirby-Bauer test can also be tested at 35 C. However, incubation temperature must not be allowed to rise above 35 C, since some methicillin-resistant *S. aureus* strains cannot be recognized at 37 C by the agar diffusion test. This observation was independently confirmed by Drew and associates (13).

Routine incubation of agar diffusion tests at 30 C has not been recommended because we do not know whether incubation at this temperature would affect the results obtained with other bacteria. On the other hand, we and others (13) have shown that incubation at 35 C does not adversely affect the results obtained with the Kirby-Bauer test. Furthermore, as far as we know with our present knowledge, no additional information could be obtained if incubation was at 30 C.

For the determination of resistance of *S. aureus* to penicillinase-resistant penicillins by agar diffusion, methicillin, oxacillin, or nafcillin disks may be used. However, the resistance may be missed if cloxacillin disks are used, possibly because there are fewer cloxacillin-resistant organisms present in a population of these *S. aureus* strains than there are organisms resistant to the other three penicillins, as shown by the population studies of Drew and associates (13). In our agar dilution studies, many more organisms were inhibited with cloxacillin at 30
and 35°C than with methicillin, oxacillin, or nafcillin, as can be seen in Fig. 7. However, there were some resistant colonies, and the MICs of cloxacillin were therefore higher at the lower temperatures.

We agree with the conclusions of Drew and associates (13) that oxacillin disks are more stable than methicillin disks. However, the U.S. Food and Drug Administration has recently ruled that only methicillin disks may be manufactured (Federal Register, 37:20525–20529, 30 September 1972) for commercial use. In view of this decision, most, if not all, of the laboratories will be using methicillin disks. Since these disks are more susceptible to deterioration, laboratories must have rigid quality control programs which will detect disks that have lower concentrations of active antibiotic. This program should include the use of standard control cultures, preferably the “Seattle strains” of Escherichia coli (ATCC 25922) and S. aureus (ATCC 25923). As long as the methicillin disks maintain their potency, they are well-suited for detecting susceptibility or resistance to the penicillinase-resistant penicillins.

S. epidermidis strains may also be resistant to the penicillinase-resistant penicillins, and in this country at least there are more resistant strains of S. epidermidis than of S. aureus (27). Before reporting that an organism is a methicillin-resistant S. aureus strain, a microbiologist should make sure that it is not S. epidermidis.

ACKNOWLEDGMENT

We express our appreciation to Gary Hancock, Staphylococcus Laboratory, Center for Disease Control, for furnishing most of the cultures used in this study.

LITERATURE CITED

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