Role of β-Lactamase and Different Testing Conditions in Oxacillin- 
Borderline-Susceptible Staphylococci

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A group of staphylococcal isolates for which oxacillin MICs were intermediate (1 to 4 μg/ml) were studied to establish the role of β-lactamase in this phenomenon. MICs and MBCs of oxacillin and penicillin with and without clavulanic acid or sulbactam (4 or 16 μg/ml, respectively) were determined for 11 Staphylococcus aureus and 2 coagulase-negative Staphylococcus isolates for which oxacillin MICs were 1 to 4 μg/ml. The susceptibility studies were done with incubation at 35 and 30°C, and the MICs were read at 24 and 48 h. Of the 13 isolates, 4 became resistant when longer incubation or 30°C incubation was used, and the MICs for 9 remained in the intermediate range. Only three of these strains were susceptible to penicillin, and β-lactamase was not detected. For 6 of 10 β-lactamase-positive strains, there was a greater-than-twofold-dilution reduction in oxacillin MICs with the addition of clavulanic acid or sulbactam. Of the four strains that became resistant with incubation at the lower temperature, a clavulanic acid effect was observed in three but only at 35°C. The oxacillin MIC for one of the β-lactamase-negative strains was also reduced with clavulanic acid; however, this strain was inhibited by 1 μg of clavulanic acid per ml alone. Bactericidal activity was observed with two or four times the oxacillin MIC in eight strains tested at both temperatures, and the combination with clavulanic acid was bactericidal at higher than four times the MIC in five of the strains at 30°C. Our results suggest that oxacillin intermediate MICs for staphylococcal isolates are due not only to β-lactamase hyperproduction but also to some other unidentified factor. The reduction in oxacillin MIC observed when clavulanic acid was added to one strain was probably due to the intrinsic inhibitory activity of clavulanic acid.

Strains of Staphylococcus aureus for which oxacillin MICs are 1 to 2 μg/ml have been called acquired resistant, borderline, or partial borderline susceptible by McDougal and Thornsberry (8). In their studies, MICs for oxacillin-susceptible strains of S. aureus were ≤0.5 μg/ml. This phenomenon is thought to be mediated by production of β-lactamase in sufficient high quantity to hydrolyze the penicillinase-resistant penicillins (8). In contrast, the resistance in intrinsically resistant staphylococci is not caused by β-lactamase production but is due to the presence of a penicillin-binding protein, called 2’ or 2a, which has very low affinity for oxacillin (3–5). β-Lactam antibiotics are generally ineffective in treating infections caused by intrinsically resistant S. aureus (1, 6), and vancomycin remains the drug of choice in such cases (15). Not enough information is available yet on whether borderline-susceptible staphylococci should be considered similar to intrinsically resistant staphylococci for purposes of treatment or whether β-lactam antibiotics can be used; most authors, however, agree at this time that these organisms should be considered oxacillin resistant and treated with vancomycin (8). We have isolated not only staphylococcal strains with characteristics similar to those described by McDougal and Thornsberry (8) but also some strains for which oxacillin MICs are intermediate, that produce β-lactamase, and in which clavulanic acid does not modify the oxacillin MIC and others that are susceptible to penicillin and do not produce β-lactamase and are, therefore, different from the borderline-susceptible strains described by McDougal and Thornsberry (8).

We conducted this study first to investigate the role of β-lactamase production in a group of strains for which oxacillin MICs range from 1 to 4 μg/ml and, second, to determine whether this group of strains is acquired resistant, as described by McDougal and Thornsberry (8), or intrinsically resistant with a very small subgroup of resistant cells. Finally, we wanted to characterize the effects of different testing conditions and different susceptibility testing methods on these strains.


MATERIALS AND METHODS

Bacterial strains. Thirteen clinical isolates of staphylococci (eleven S. aureus and two coagulase-negative staphylococci) for which oxacillin MICs were intermediate (1 to 4 μg/ml), as determined by broth microdilution with a direct inoculum of 5 × 10⁵ CFU/ml, incubation temperature of 35°C, and reading at 24 h at the Cleveland Clinic Foundation, made up our initial study population. When further testing was done under different conditions (see below), for only nine of these strains were MICs still in the intermediate range.

Antibiotics. Sterile standard powders of oxacillin, penicillin, clavulanic acid, and sulbactam were used as supplied by the manufacturers. Clavulanic acid and sulbactam were used in fixed concentrations of 4 and 16 μg/ml, respectively, when combined with oxacillin and penicillin. Trays containing the antibiotics were prepared in advance and stored at −70°C until use, within 1 month of preparation.

Antibiotic susceptibility testing. MICs were determined by broth microdilution as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (10). For purposes of this study, we considered oxacillin MICs from 1 to 4 μg/ml as intermediate, ≤0.5 μg/ml as susceptible, and

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>4 \mu g/ml as resistant. A direct inoculum from an overnight blood agar plate and a mid-log-phase inoculum at a final concentration of 5 \times 10^7 or 5 \times 10^8 CFU/ml were used. Antibiotics were diluted in cation-supplemented Mueller-Hinton broth with 2% NaCl, the trays were inoculated at 30 and 35°C, and readings were done at 24 and 48 h. MBCs were determined from the trays inoculated at mid-log phase by transferring 100 \mu l of broth from the wells with no visible growth to sheep blood agar and counting the colonies after 48 h of incubation (14). The MBC was defined as the lowest concentration of antibiotic yielding >99.9% of killing (12).

An agar screen test was performed on all strains by spot inoculating approximately 10^6 CFU/ml on a Mueller-Hinton agar plate containing 6 \mu g of oxacillin per ml and 4% NaCl. If no growth was observed at 24 h, plates were incubated for an additional 24 h. Strains that grew on the agar were considered resistant. Disk diffusion was carried out as described in the NCCLS disk diffusion standard (9), using a 1-\mu g oxacillin disk. The inoculum was prepared from an overnight blood agar plate to yield the equivalent of a 0.5 McFarland standard, and 4% NaCl was added to the agar.

\textbf{\beta-Lactamase determination.} \textit{\beta}-Lactamase production was determined by using nitrocephin disks (BBL Microbiology Systems, Cockeysville, Md.) and iodometric methods (11, 13) with colonies growing at the zone of inhibition surrounding cephalothin (30 \mu g) and oxacillin (1 \mu g) disks.

\textbf{RESULTS}

For four of the strains for which oxacillin MICs originally were 1 to 4 \mu g/ml, MICs were found to be >4 \mu g/ml when the strains were incubated at 30°C with 2% NaCl (Table 1). Another eight strains remained in the intermediate range with low incubation temperature, salt supplementation, and 48 h of incubation. For two of these strains, however, oxacillin MICs did increase to >8 \mu g/ml when the high inoculum was used. For one strain (no. 2) for which the initial oxacillin MIC was 1 \mu g/ml, the MIC was 0.5 \mu g/ml upon retesting at 35°C; however, since the oxacillin MIC for this strain was 1 \mu g/ml with the direct inoculum, the strain was retained in the study. Oxacillin MICs derived from an inoculum prepared directly from overnight growth on agar were the same for four strains, 1 dilution higher for seven strains, and 1 dilution lower for one strain compared with MICs derived from mid-logarithmic-phase inocula (Table 2).

\textit{\beta}-Lactamase was detected in 10 of the 13 strains. Both the nitrocephin and iodometric methods failed to detect \textit{\beta}-lactamase in the other three strains for which penicillin MICs were between 0.06 and 0.125 \mu g/ml.

MICs for the 10 \textit{\beta}-lactamase-producing strains are shown in Table 1. The four strains for which oxacillin MICs were >4 \mu g/ml at 30°C were not tested with the high inoculum. Oxacillin MICs for the other six strains ranged from 0.5 to 4 \mu g/ml with an inoculum of 5 \times 10^6 CFU/ml and remained in the intermediate range when the strains were incubated at 30°C and MICs were read at 48 h. Of the 10 \textit{\beta}-lactamase-positive strains, 6 (including 3 that became resistant with lower-temperature incubation) there was a greater-than-twofold reduction in oxacillin MICs in the presence of clavulanic acid. This reduction in oxacillin MICs with clavulanic acid was seen after incubation at either 35 or 30°C in four of the six strains and at both temperatures in the other two. The effect of clavulanic acid was best detected when readings were done at 48 h and at 35°C. Oxacillin MICs for the same six strains were also reduced by sulbactam. Penicillin MICs for all 10 \textit{\beta}-lactamase-positive strains decreased substantially in the presence of clavulanic acid (Table 1).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Strain no.} & \textbf{Inoculum (CFU/ml)} & \textbf{Temp (°C)} & \textbf{Oxacillin} (\mu g/ml) & \textbf{Oxacillin-clavulanic acid} & \textbf{Penicillin} (\mu g/ml) & \textbf{Penicillin-clavulanic acid} \\
\hline
1 & 10^5 & 35 & 4 & 2 (4)\textsuperscript{a} & 0.5 (0.5) & >4 & 0.5 \\
2 & 10^5 & 35 & 0.5 (0.5) & >4 & >4 & 0.5 \\
3 & 10^7 & 30 & 1 (0.25) & 2 (2) & 2 (2) & >4 & 0.5 \\
4 & 10^5 & 35 & 1 (2) & 1 (0.5) & >4 & 1 \\
5 & 10^5 & 35 & 4 (8) & >4 & >4 & 0.5 \\
6 & 10^5 & 35 & 1 (2) & 1 (0.5) & >4 & 2 \\
7 & 10^5 & 35 & 2 (2) & 2 (2) & 2 (2) & >4 & 1 \\
8 & 10^5 & 35 & 1 (4) & 1 (0.5) & >4 & 1 \\
9 & 10^5 & 35 & 4 (4) & 4 (4) & >4 & 0.5 \\
10 & 10^5 & 35 & 8 (8) & >4 & >4 & 0.5 \\
11 & 10^5 & 35 & 8 (8) & >4 & >4 & 0.5 \\
12 & 10^5 & 35 & 2 (2) & 2 (2) & >4 & 0.5 \\
13 & 10^5 & 35 & 2 (2) & 2 (2) & >4 & 0.5 \\
14 & 10^5 & 35 & 2 (2) & 2 (2) & >4 & 0.5 \\
15 & 10^5 & 35 & 2 (2) & 2 (2) & >4 & 0.5 \\
\hline
\end{tabular}
\caption{Oxacillin and penicillin MICs for \textit{\beta}-lactamase-positive, borderline-oxacillin-susceptible staphylococci and effect of clavulanic acid}
\end{table}

\textsuperscript{a} Numbers in parentheses are MICs read at 48 h.

**\textit{\beta}-Lactamase-negative staphylococcus.**

MICs for the three \textit{\beta}-lactamase-negative strains are shown in Table 3. All were susceptible to penicillin (MICs of 0.06 to 0.125 \mu g/ml), and the oxacillin MICs were between 1 and 4 \mu g/ml at both temperatures with the standard inoculum. For one strain of this group, the oxacillin MIC increased to 16 \mu g/ml when the high inoculum was used. One of these strains showed an unexpected substantial decrease in oxacillin MICs when clavulanic acid was added; however, when this strain was tested against clavulanic acid alone, the MIC was 1 \mu g/ml.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Strain no.} & \textbf{Broth microdilution} \textsuperscript{b} oxacillin MIC (\mu g/ml) & \textbf{Disk diffusion zone diam (mm)} & & \\
\hline
1 & 1 & 2 & 13 & \\
2 & 1 & 0.5 & 12 & \\
3 & 1 & 0.5 & 12 & \\
4 & 1 & 0.5 & 12 & \\
5 & 1 & 0.5 & 12 & \\
6 & 1 & 0.5 & 12 & \\
7 & 1 & 0.5 & 12 & \\
8 & 1 & 0.5 & 12 & \\
9 & 1 & 0.5 & 12 & \\
10 & 1 & 0.5 & 12 & \\
11 & 1 & 0.5 & 12 & \\
12 & 1 & 0.5 & 12 & \\
13 & 1 & 0.5 & 12 & \\
14 & 1 & 0.5 & 12 & \\
15 & 1 & 0.5 & 12 & \\
\hline
\end{tabular}
\caption{Comparison of disk diffusion and broth microdilution tests in 12 isolates for which oxacillin MICs were intermediate}
\end{table}

\textsuperscript{b} One-microgram oxacillin disk.

Incubation at 35°C for 24 h.

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\textit{Incubation at 35°C for 24 h.}

\textit{One-microgram oxacillin disk.}
TABLE 3. Oxacillin and penicillin MICs for β-lactamase-negative, borderline-oxacillin-susceptible staphylococci and effect of clavulanic acid

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Inoculum (CFU/ml)</th>
<th>Temp (°C)</th>
<th>MIC (μg/ml)</th>
<th>Oxacillin</th>
<th>Oxacillin-clavulanate</th>
<th>Penicillin</th>
<th>Penicillin-clavulanate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10⁶</td>
<td>35</td>
<td>1 (1)†</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>10⁷</td>
<td>30</td>
<td>1 (2)</td>
<td>≤0.06</td>
<td>0.25</td>
<td>2</td>
<td>0.125</td>
</tr>
<tr>
<td>9</td>
<td>10⁶</td>
<td>35</td>
<td>2 (4)</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>10⁷</td>
<td>30</td>
<td>2 (4)</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>15</td>
<td>10⁵</td>
<td>35</td>
<td>2 (4)</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>10⁷</td>
<td>30</td>
<td>4 (ND)‡</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

† Coagulase-negative staphylococcus.
‡ Numbers in parentheses are MICs read at 48 h.
§ Clavulanic acid MIC = 1 μg/ml.
†‡ ND, Not done.

Bactericidal activity was observed with two or four times the oxacillin MIC in eight strains at both 30 and 35°C. MBC/MIC ratios for these strains are shown in Table 4. The combination of oxacillin and clavulanic acid was bactericidal at higher than four times the MIC for five of the strains at 30°C.

By the agar screen test, all strains were susceptible. The results of the disk diffusion tests are shown in Table 2. The zone diameter was 12 mm (intermediate) in three of the strains (no. 2 through 4), ≤10 (resistant) in four strains (no. 5, 7, 10, and 12), and ≥13 (susceptible) in five strains (no. 1, 6, 8, 14, and 15). One strain (no. 9) was not tested by this method.

**DISCUSSION**

*S. aureus* isolates for which oxacillin MICs are intermediate were first recognized by Thornsberry and McDougal (16). These authors postulated that, although rare, such strains might actually be resistant ones that did not grow well enough to give an MIC indicating resistance (7) and suggested that such strains be incubated for 24 h longer. If the MIC did not increase with additional incubation, McDougal and Thornsberry considered such strains truly intermediate. In a later study, McDougal and Thornsberry attributed the intermediate MICs for these organisms to excessive β-lactamase production (8) and suggested the term acquired resistant to differentiate them from the intrinsically resistant staphylococci, in which resistance is chromosomally medi-

TABLE 4. MBC/MIC ratio in eight borderline-oxacillin-susceptible staphylococci cultured with oxacillin alone and with clavulanic acid

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>No. of strains with MBC/MIC ratio of:</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>&gt;4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin–2% NaCl</td>
<td>30°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxacillin–2% NaCl-clavulanate</td>
<td>30°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

We studied staphylococcal strains for which oxacillin MICs are intermediate (1 to 4 μg/ml), some of which, according to NCCLS, would be considered susceptible (≤2 μg/ml) and others resistant (≥4 μg/ml), with the purpose of determining whether excess β-lactamase production was responsible for this phenomenon, as has been previously described by McDougal and Thornsberry (8). We found that 4 of the 13 staphylococcal strains for which oxacillin MICs were initially intermediate became resistant when a lower temperature (i.e., 30°C) or longer incubation time was used and that MICs for the other 9 remained in the intermediate range. The effect of lower temperature or prolonged incubation on increasing detection of resistance in four strains could be due to one of two factors: larger amounts of β-lactamase produced under these conditions or growth of a small number of resistant subpopulations of bacteria. The latter factor is more likely, because an effect of clavulanic acid on oxacillin MICs was observed only at 35°C in three of the strains. When those three strains were incubated at 30°C and the oxacillin MICs increased to resistant ranges, no effect of clavulanic acid on oxacillin MICs was seen, which suggests that β-lactamase is responsible for the intermediate oxacillin MICs observed at 35°C but not for the increased MICs observed at 30°C. That β-lactamase inhibitors are inactivated at the lower temperature is unlikely, because the effect of β-lactamase inhibitors on penicillin activity was still seen under these conditions (Table 1).

In the group of nine strains for which oxacillin MICs were intermediate despite all conditions used, β-lactamase was not detected by using nitrocephin disks or iodometric methods in three strains. These three strains were susceptible to penicillin, which confirmed that no or very little β-lactamase was produced by them. Clavulanic acid did not affect the oxacillin MICs for these strains except for one (no. 8) that was susceptible to clavulanic acid alone.

A significant effect of clavulanic acid on oxacillin MICs (greater-than-twofold reduction) was seen in only 6 of the 10 β-lactamase-positive strains (3 that remained intermediate and 3 that became resistant with lower-temperature incubation). In the other four β-lactamase-positive strains, addition of β-lactamase inhibitors did not affect the oxacillin MICs at either temperature, which suggests that mechanisms other than β-lactamase production may be responsible for this phenomenon.

A poor correlation was found among results obtained by the disk diffusion, microdilution, and agar screen tests. Whereas by agar screen all strains were susceptible, by disk diffusion four were resistant and three were intermediate. On the basis of NCCLS breakpoints, the microdilution method yielded 7 susceptible and 6 resistant strains with the direct inoculum and 12 susceptible and 1 resistant strain with the mid-log-phase inoculum. The discrepancies observed when testing these strains are probably responsible for reported differences among different methods (P. J. Taylor, P. C. Appelbaum, B. Bixler, K. Todd, and S. L. Hansen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, C319, p. 385).

Our findings suggest that staphylococci for which oxacillin MICs are intermediate (1 to 4 μg/ml) can have different mechanisms of resistance. Some of those tested in this study became resistant upon exposure to lower temperature or longer incubation. These strains (nos. 1, 6 and 10) would be called susceptible if incubated at 35°C but resistant if incubated at 30°C. As mentioned above, they are probably heteroresistant organisms; however, the clinical significance...
of detecting resistance in these strains is unknown. Susceptibility testing of staphylococci against oxacillin at either 30 or 35°C is correct according to NCCLS recommendations (10). Among the strains that remain intermediate, there are some that produce no or very little β-lactamases. These strains are susceptible to penicillin, and β-lactamase inhibitors do not affect the oxacillin MICs unless the inhibitor itself is inhibitory to the bacteria at the concentration used. Of the β-lactamase-positive strains, there is one group in which the enzyme is responsible for the intermediate oxacillin MICs. For these strains, penicillin MICs are very high, β-lactamase is detected easily, and penicillin and oxacillin MICs decrease substantially with addition of clavulanic acid. For another group of β-lactamase-positive strains, penicillin MICs may or may not be so high, β-lactamase is detected easily, and penicillin but not oxacillin MICs decrease with addition of clavulanic acid. These observations suggest that inactivation of penicillinase-resistant penicillins by β-lactamase is probably not the only mechanism that explains oxacillin intermediate MICs.

Although our data suggest that mechanisms other than β-lactamase production are involved in the phenomenon of oxacillin intermediate MICs, the nature of these mechanisms remains to be clarified. It is possible that penicillin-binding proteins with low affinity to oxacillin, like those found in oxacillin-resistant S. aureus (3–5), are present in some of these strains, especially those that are β-lactamase negative and those for which oxacillin MICs do not change in the presence of β-lactamase inhibitors. However, this hypothesis remains to be tested by appropriate studies. Moreover, the reason why they do not express fully the resistant phenotype is unclear. Even if penicillin-binding protein 2a could be detected in some of these organisms, the organisms would still differ from the typical oxacillin-resistant staphylococci in important characteristics, such as their susceptibilities to clindamycin, erythromycin, and imipenem (data not shown).

Until experimental studies address the question of whether infections caused by these organisms can be treated with penicillinase-resistant penicillins or alternative antibiotics in vitro, we believe that these strains should be considered resistant by clinical laboratories. It is possible that eventually a new category will need to be created because these strains are not typical oxacillin-resistant staphylococci, yet they do not exhibit full in vitro susceptibility to oxacillin. It is therefore likely that in clinical situations in which high bactericidal titers of antibiotics are needed, oxacillin will be less effective against these groups of strains than are other antistaphylococcal antibiotics.

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LITERATURE CITED


