Resistance of Coagulase-Positive Staphylococci to Methicillin and Oxacillin

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Abstract
GRAVENKEMPER, CHARLES F. (University of Washington School of Medicine, Seattle), JEAN L. BRODIE, AND WILLIAM M. M. KIRBY. Resistance of coagulase-positive staphylococci to methicillin and oxacillin. J. Bacteriol. 89:1005-1010. 1965.—Two strains resistant to methicillin were discovered among 541 strains of Staphylococcus aureus isolated in a clinical laboratory during a 1-yr period, and their properties were compared with those of strains isolated in Europe. The two strains were very active producers of penicillinase, and exhibited cross-resistance with other antistaphylococcal antibiotics. Like the European strains, our resistant cultures showed resistance to methicillin only with large inocula, and consisted of a mixture of cells. The great majority were sensitive and underwent early swelling and lysis, and only a small minority of the bacteria were able to grow in the presence of methicillin. The methicillin-resistant strains caused destruction of methicillin and oxacillin in vitro, but the rate of hydrolysis was slow. Antibiotic destruction was probably due to high concentrations of staphylococcal penicillinase, and not to another specific enzyme. These observations are helpful in explaining why resistance of staphylococci to the synthetic penicillins has not become a significant clinical problem.

After more than 5 years of clinical usage, relatively few infections due to methicillin-resistant staphylococci (coagulase-positive) have been reported (Dowling, 1961; Chabbert and Baudens, 1962; Stewart and Holt, 1963a). Coagulase-negative staphylococci have often been found to be resistant to methicillin (Kjellander, Klein, and Finland, 1963), but these organisms are much less virulent than coagulase-positive strains. Jevons (1961) and Jevons, Coe, and Parker (1963), in Great Britain, noted an increase of coagulase-positive, methicillin-resistant staphylococcal isolates from 0.55% in 1960 to 0.8% in 1962, but the clinical significance of the resistant strains was not known in detail. Barber and Waterworth (1962) found a higher incidence (2.2%) at the Hammersmith Hospital, London, but these strains caused few significant infections. However, Stewart and Holt (1963a) described an endemic methicillin-resistant strain, which was isolated from 37 persons and caused one death in a British hospital. All of the above resistant strains have been thoroughly studied, and they all demonstrate penicillinase production, similar phage types (group III), cross-resistance with the other semisynthetic antistaphylococcal penicillins, and a marked alteration in susceptibility to the new penicillins with a change in inoculum size (Jevons, 1961; Chabbert and Baudens, 1962; Jevons et al., 1963; Barber, 1962; Barber and Waterworth, 1962; Ayliffe and Barber, 1963; Stewart and Holt, 1963a; Eriksen and Ericksen, 1963).

Other properties of these resistant staphylococci have been the subject of controversy. Stewart and Holt (1963a) stated that their strains consisted of uniformly resistant organisms which could destroy the isoxazolyl penicillins (i.e., oxacillin and cloxacillin), but could not inactivate methicillin. Knox and Smith (1963a) and Ayliffe, Barber, and Waterworth (1963) were unable to confirm a difference in the destruction of the isoxazolyl penicillins by the Stewart strains as compared with methicillin-sensitive staphylococci. Most workers have found that cultures of the resistant staphylococci are composed of a mixture of cells, the large majority showing sensitivity to methicillin, and a much smaller percentage representing highly resistant variants (Rolinson, 1963; Knox and Smith, 1961). Methicillin destruction by these resistant strains was later demonstrated by Eriksen and Ericksen (1963) with cultures actively growing in broth in the presence of methicillin. Ayliffe and Barber (1963) also related methicillin destruction to growth, and showed that a methicillin-sensitive
strain of Staphylococcus aureus could also inactivate methicillin, but only if the culture were able to grow in the presence of antibiotic after first being treated with a subinhibitory concentration of methicillin. This latter study was confirmed by Knox and Smith (1963b), who concluded that antibiotic destruction was not due to a specific "methicillinase," but to markedly increased penicillinase production when either the sensitive or resistant strains were able to grow in the presence of methicillin.

A study was undertaken at the University Hospital, Seattle, to determine the incidence of methicillin-resistant staphylococci during a 1-year period (March 1963 to March 1964). Of 541 strains of coagulase-positive staphylococci isolated, only 2 were resistant to methicillin. This report describes the specific methods that were used to find these strains, and also compares the characteristics of these bacteria with those isolated in Europe.

**Materials and Methods**

**Screening test.** From disc sensitivity plates (Bauer, Perry, and Kirby, 1959), heavy inocula (10⁵ cells) of overnight cultures of staphylococci growing outside the inhibitory zone of the methicillin disc, and also of small colonies growing within the inhibitory zone, were transferred to tubes containing 10 µg/ml of oxacillin, and the presence or absence of growth was noted after 48 hr of incubation at 37 C. Oxacillin was chosen as the screening antibiotic because of its greater susceptibility to inactivation by methicillin-resistant staphylococci (Stewart and Holt, 1963a). A total of 73 strains (13.5% of the 541 tested) yielded positive results in screening tests, but only 2 proved to be methicillin-resistant (growth in >10 µg/ml) when minimal inhibitory concentrations (MIC) were tested in the usual manner (Sidell et al., 1963). Inactivation of oxacillin by large amounts of penicillinase (Naylor et al., 1962) was probably responsible for the high percentage of positive results in the screening tests.

**Other studies.** The effect of varying the inoculum size, and the presence of cross-resistance, were determined for the two methicillin-resistant strains by performing MIC tests with penicillin G, methicillin, oxacillin, cloxacillin, and cephalothin. Population characteristics were studied by comparing the number of colonies that would grow in pour plates containing 10 µg/ml of methicillin with the number growing in pour plates without antibiotic. Penicillinase production was measured quantitatively by a modification of the cylinder-plate method of Wallmark (1964); cultures were exposed to penicillin G for 15 min, and penicillinase activity was calculated as the amount of penicillin destroyed per minute. To test the effect of nonmultiplying cultures, cup-plate assays of oxacillin and methicillin were performed after 6 and 24 hr of contact with fully grown overnight cultures.

**Correlation of growth curves and antibiotic destruction.** These studies were made with actively growing cultures of the two methicillin-resistant strains and two methicillin-sensitive strains, Kelly (penicillin-resistant) and WS (penicillin-sensitive). Overnight cultures were diluted 10-fold and agitated in a shaker for 2 hr, and appropriate mixtures of cultures and antibiotics were then prepared in quadruplicate. Turbidity (optical density) was recorded with a Coleman spectrophotometer, growth curves were measured by performing plate counts, and residual antibiotic was assayed at various intervals by the cup-plate method (Sarcina lutea), after samples were removed for flash heating and Seitz filtration.

**Results**

**Description of the two resistant strains.** One strain (Russell) originated from a large, single colony adjacent to the methicillin disc, and organisms picked from outside the inhibitory zone were sensitive by the screening test. The other strain (Villaluz) was isolated both from colonies outside and inside the methicillin inhibitory zone. Neither strain came from an infected patient. The Russell strain was cultured from the throat of a 20-month-old girl who had previously been treated with penicillin V for otitis media but had never received methicillin. The anterior nares of an infant girl who had not received previous antibiotic therapy was the site of origin for the Villaluz strain. MIC values of methicillin for the Russell and Villaluz strains were 20 and 50 µg/ml, respectively. After several subcultures in broth, absent or small zones were noted around methicillin and oxacillin discs with both strains. Both strains were susceptible to chloramphenicol, kanamycin, and vancomycin, and the Russell strain was resistant to tetracycline and erythromycin. The phage types for the Russell and Villaluz strains were 55/77 and 53/77/83A, respectively, very similar to the phage types of the European strains.

**Cross-resistance and inoculum effect.** Table 1 demonstrates cross-resistance and a marked inoculum effect with one of the two methicillin-resistant strains for four antistaphylococcal antibiotics and penicillin G; the other strain (Russell) was slightly less resistant with the larger inocula. Both strains were in the susceptible range with 10⁶ organisms per milliliter or less, but antibiotic resistance was present with the higher inocula. Cephalothin and cloxacillin were somewhat more active than methicillin and oxacillin with inocula of 10⁷ to 10⁸ organisms per milliliter. In contrast, the MIC of a methicillin-sensitive penicillin-re-
antibiotic destruction of various penicillins with a changing inoculum of the Villaluz strain of Staphylococcus aureus

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>10^3</th>
<th>10^4</th>
<th>10^5</th>
<th>10^6</th>
<th>10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>6.0</td>
<td>6.0</td>
<td>20</td>
<td>100</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Methicillin</td>
<td>2.0</td>
<td>4.0</td>
<td>4.0</td>
<td>8.0</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0.4</td>
<td>1.0</td>
<td>2.0</td>
<td>1.0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>2.0</td>
<td>10</td>
</tr>
</tbody>
</table>

* Results expressed as minimal inhibitory concentrations (micrograms per milliliter).

Resistant strain (Kelly) remained at a level of 3.12 for inocula of 10^4 to 10^6 organisms per milliliter.

Population characteristics. Only 0.02% (Russell) and 1.6% (Villaluz) of the organisms were able to grow in the presence of methicillin (10 μg/ml), compared with the number that would grow in pour plates without antibiotic. The resistant cells formed very small colonies, indicating that methicillin probably had an inhibitory effect upon their growth. This mixed population of organisms was also observed by most of the British workers. The larger percentage of resistant organisms noted with the Villaluz strain was correlated with a somewhat greater degree of resistance to the penicillins (Table 1) than was present with the Russell strain.

Penicillinase production. The two methicillin-resistant strains destroyed penicillin G at a rate approximately three times that of a highly active penicillinase-producing methicillin-sensitive staphylococcus (Kelly strain, MIC of penicillin G >1,000 μg/ml). After each overnight culture was incubated with an equal volume of antibiotic-broth solution (2,000 units of penicillin G/ml), the calculated rates of penicillin destruction were 300, 263, and 96 units per min per 10^6 bacteria per ml for the Russell, Villaluz, and Kelly strains, respectively. In contrast, Ayliffe and Barber (1963), with a different method for measuring penicillinase activity, found no difference in the rate of penicillinase production between methicillin-resistant and -sensitive staphylococci.

Antibiotic destruction by inactive cultures. Methicillin and oxacillin destruction was demonstrated when very large inocula (10^6 organisms per milliliter) of three fully grown overnight cultures, consisting of essentially nonmultiplying staphylococci, were incubated in the presence of antibiotic. Approximately 75% of the methicillin (initial concentration, 10 μg/ml) was destroyed after 24 hr of incubation by both methicillin-resistant strains and the Kelly strain. All of the oxacillin (initial concentration, 5 μg/ml) was inactivated by the three strains after only 6 hr of incubation. Thus, there was no difference in the rate of antibiotic destruction between the methicillin-resistant strains and the susceptible (Kelly) strain when fully grown cultures were added to the antibiotic solutions. This was in marked contrast with the difference in destruction rates for actively growing cultures (see below).

Growth and methicillin destruction. Figures 1 and 2, representing an average of two separate studies, illustrate the optical density (turbidity), colony counts, and residual antibiotic activity...
Table 2. Residual methicillin concentrations (micrograms per milliliter) after incubation of four active staphylococcal cultures in the presence of methicillin (initial concentration, 50 μg/ml)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Methicillin sensitivity</th>
<th>Growth at 72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hours of incubation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Russell</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Villaluz</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>WS</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Kelly</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

* R, resistant; S, sensitive.

strains showed lysis, with almost a 10-fold decrease in number of organisms after 4 hr of incubation, but bacterial growth occurred between 4 and 24 hr in each instance. The initial lysis of the resistant cultures was probably due to killing of susceptible bacteria, which were shown previously to outnumber greatly the resistant organisms in each resistant culture. Methicillin was completely inactivated by the two resistant strains at the end of 24 hr of incubation, whereas there was very little or no destruction of this antibiotic by the sensitive strains. The rate of oxacillin destruction was somewhat faster (Fig. 2); even the Kelly strain (a strong producer of penicillinase) was able to inactivate over 90% of this antibiotic, but enough oxacillin remained to inhibit growth. The faster destruction of oxacillin by the Kelly strain is in accord with the observations of Nayler et al. (1962), who found that oxacillin is more susceptible than methicillin to staphylococcal penicillinase. Growth of the resistant cultures probably represented initial multiplication of the small minority of inherently resistant organisms, together with multiplication of the remaining sensitive cells after destruction of the antibiotic was complete.

When this same experiment was repeated with 50 rather than 10 μg of methicillin per ml, growth of the methicillin-resistant strains did not occur until after 48 hr of incubation. Table 2 illustrates the residual antibiotic activity after 6, 24, and 72 hr of incubation. Methicillin destruction by the two resistant strains was incomplete, but was much greater than that caused by the two sensitive strains. MIC determinations for cultures showing growth after 72 hr of incubation were compared with the MIC values of the same strains prior to the study. The MIC of methicillin for the Russell strain increased from 10 to 400 μg/ml, and the Villaluz MIC rose from 20 to 400 μg/ml. These increased MIC values were probably due to selection of the resistant cells.
from the mixed population of sensitive and resistant organisms present in the original culture.

The pH determinations on the filtrates in the lysis studies were determined with nitrazine paper, and showed very little change. The pH of the Russell and Villaluz filtrates dropped from 7.5 to just under 7.0, whereas the filtrates of the sensitive cultures showed a lesser fall in pH. Since it has been demonstrated that methicillin can be hydrolyzed in solutions with a pH less than 6.0 (Simon and Rantz, 1962), acidity was not present to a sufficient degree in these studies to account for the inactivation of methicillin.

**DISCUSSION**

In a hospital where methicillin and oxacillin are widely used, the isolation of only 2 methicillin-resistant strains among 541 cultures of penicillinase-producing *S. aureus* makes it apparent that resistance to methicillin has not yet become a problem. No other studies of this kind have been reported in the United States, but extensive surveys in Great Britain have similarly yielded a very low incidence of methicillin-resistant staphylococci (Jevons, 1961; Barber, 1962; Jevons et al., 1963; Stewart and Holt, 1963a). Our two strains were detected by the use of a special oxacillin tube sensitivity test upon very large inocula of staphylococcal isolates, and also upon cultures of colonies that appeared within the methicillin inhibitory zone from the single-disc sensitivity plates. If the colonies appearing within the inhibitory zone of the methicillin disc had not been studied, one of the resistant strains (Russell) would not have been found. Our testing method has recently been proposed independently as a reliable approach by Sutherland and Rolinson (1964), i.e., "a serial dilution test, with a heavy inoculum and incubation for 48 hr."

The clinical significance of methicillin-resistant staphylococci is minor in comparison with the large number of infections caused by penicillinase-producing staphylococci which began appearing soon after penicillin G first became available. Our two strains were not pathogens, but methicillin-resistant staphylococci have caused a few infections in Europe (Chabbert and Baudens, 1962; Stewart and Holt, 1963a), and only one well-documented infection in the United States (Dowling, 1961). Cross-infections have not been described. Several factors probably account for the relative rarity of clinical infections, one being the fact that cultures are actually sensitive to the new antistaphylococcal penicillins with inocula of $10^4$ organisms per milliliter or less. In addition, the resistant strains consist of a mixed population of cells, the great majority being sensitive to methicillin, and the small minority of resistant cells growing only slowly in the presence of methicillin (10 µg/ml). The initial swelling and lysis observed with our strains was undoubtedly due to the presence of a great preponderance of methicillin-sensitive cells.

The mechanism of resistance for methicillin-resistant staphylococci has not been completely explained. Steinman (1961) showed that methicillin can be hydrolyzed by staphylococcal penicillinase, but the rate of destruction was very slow when compared with that of penicillin G by the same enzyme. Knox (1962) attributed methicillin resistance to inherently resistant cells, and presented indirect evidence indicating that the slow methicillin inactivation by the resistant strains was due to penicillinase, and not due to a specific "methicillinase" (Knox and Smith, 1963b; Knox, 1962). Sutherland and Rolinson (1964) were unable to confirm the methicillin destruction shown by Eriksen and Eriksen (1963), Ayliffe and Barber (1963), Knox and Smith (1961), and our present studies. With our strains, actively growing cultures completely inactivated methicillin and oxacillin in concentrations of 10 µg/ml within 24 hr. Since the rate of destruction was very slow, we doubt the presence of a specific enzyme that would inactivate the antibiotic. These cultures were able to grow in the presence of methicillin, allowing production of large amounts of penicillinase by induction (Knox and Smith, 1963b). Thus, the slow inactivation of methicillin or oxacillin was probably due to staphylococcal penicillinase, although the possibility of a specific enzyme has not been entirely excluded.

**LITERATURE CITED**


