Susceptibility of the 'penicillinase-resistant' penicillins and cephalosporins to penicillinase of *Staphylococcus aureus*

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SUMMARY The activities of some semisynthetic penicillins and cephalosporins have been tested against clinical strains of *Staphylococcus aureus*. The apparent activity *in vitro* varies with the method of testing used. Determination of MICs using light inocula fails to detect the destructive effect of penicillinase on the antibiotic. This was, however, demonstrated reproducibly by the use of a technique in which a heavy inoculum was pre-incubated for two hours before application of antibiotic to wells. This method of testing probably represents most of the clinical situations in which the drugs are used since both *in vitro* and *in vivo* a growing culture is exposed to an antibiotic gradient.

Flucloxacillin was inactivated by penicillinase considerably more than either methicillin, cloxacillin, or nafcillin. Cephaloridine was the most vulnerable of the cephalosporins. Cephalozin, cephalothin, and cephalexin were intermediate. Cephradine was the least hydrolysed by staphylococcal penicillinase.

It is recommended that the activities of all penicillins and cephalosporins against staphylococci should be tested by diffusion at 37°C with pre-incubation of the culture for two hours at this temperature.

Determination of minimum inhibitory concentrations (MIC) has become accepted as the ideal method by which laboratories express antibiotic sensitivity and resistance. This method has the advantage that most of the conditions of the test can be controlled accurately and reproduced easily, so that different laboratories can, by reference to standard organisms and antibiotics, obtain confidence in the accuracy of their sensitivity testing.

In most clinical situations the MIC level obtained *in vitro* is thought to correlate well with the outcome of antibacterial therapy *in vivo*. That laboratories do not express routinely many results as MICs is an indication of the length of time that this method takes compared to diffusion methods.

There is probably, however, one serious drawback in the use of MIC methods to report antibiotic sensitivity. Under certain conditions (particularly when a dilute inoculum is used) the assessment of sensitivity obtained by MIC methods will fail to take into account the production of certain enzymes.

These enzymes are released into the media with little residual protein adherent to the cell. In this category the enzyme that is of paramount clinical importance is the β -lactamase which, to varying degrees, hydrolyses all the penicillins and cephalosporins (see below). Although a large proportion of the β -lactamase synthesised by Gram-negative bacteria is still cell-associated after subculture of the organism to fresh media, the amount of lactamase retained by the staphylococcal cell is small. Thus, MIC methodology may fail to detect much of the effect of staphylococcal lactamase on penicillins and cephalosporins.

This phenomenon was well recognised in the 1940s and 1950s when resistance to benzyl penicillin *in vitro* was usually identified by diffusion testing, since determination of MIC was thought to give a falsely optimistic view of the activity of this drug against penicillin-resistant strains of *Staphylococcus aureus*.

However, with the arrival of the 'penicillinase-resistant' penicillins and cephalosporins throughout the 1960s and 1970s, this shortcoming of MIC assessment has apparently been forgotten. The activity of each new penicillin or cephalosporin has

been assessed primarily by MICs. The possibility that the drug may be significantly hydrolysed by staphylococcal penicillinase has usually been discounted, at least in the promotional literature.

There is, however, mounting evidence, derived both from the chemotherapy of experimentally induced infections in animals and from clinical experience in man, that certain of these new 'penicillinase-resistant' penicillins are indeed vulnerable to staphylococcal penicillinase *in vivo*.

Thus, cephaloridine fares poorly in the treatment of induced staphylococcal osteomyelitis in rabbits, probably because of its destruction by staphylococcal penicillinase (Norden and Dickens, 1973). Burgess and Evans (1966) found that cephaloridine failed in the treatment of staphylococcal endocarditis in seemingly adequate dose, again due to the production of staphylococcal penicillinase.

We have found that flucloxacillin was unable to eliminate penicillinase-producing staphylococci in two patients (Lacey and Lewis, 1975, 1976). In each instance detailed evidence was presented which indicated that the therapeutic failure was due to destruction of flucloxacillin *in vivo* by staphylococcal penicillinase.

A large number of authors have described an 'inoculum effect' with cephaloridine, ie, the MIC for a particular strain rises with increasing numbers of bacteria in the inoculum. We have found that such an effect occurs only with penicillinase-producing strains.

These observations suggest that both cephaloridine and flucloxacillin are significantly inactivated *in vivo* by staphylococcal penicillinase.

The purpose of this paper is to identify the conditions *in vitro* under which this inactivation occurs, and to assess which of the penicillins and cephalosporins are likely to be least vulnerable to staphylococcal penicillinase *in vivo*.

Material and methods

STRAINS

Strains of *Staph. aureus* were isolated consecutively from lesions of inpatients and outpatients at Wisbech and King's Lynn District Hospitals during 1975. In addition, three pairs of strains isolated in Bristol in 1971 were investigated; the latter were constructed so that they were isogenic except for variation in their ability to produce penicillinase (see results).

SENSITIVITY TESTING

MICs were determined by inoculating dilutions of overnight peptone water (Oxoid) cultures on to the surface of agar (Oxoid blood base No. 2) containing appropriate levels of antibiotic. The plates were

incubated aerobically for 18 hours at 37°C and the end point was taken as no visible growth.

DIFFUSION TESTING

These experiments were performed using a standard surface seeding technique with a neat overnight culture in peptone water that was applied to the surface of plates containing DST agar (Oxoid). These were drained for 10 minutes and then allowed to dry for a further 10 minutes. Solutions of antibiotics were applied in wells, 8 mm diameter, either immediately or after incubation of the seeded plates at 37°C for various intervals, usually two hours. The diameters of the inhibition zones were measured after incubation at 37°C for a further 18 hours. In general, eight replicas of each antibiotic solution for each test organism were allocated randomly to the plates. Relative antibiotic activities for each strain were calculated by reference to a standard curve. This was obtained by measuring the inhibition zones resulting from various concentrations of each antibiotic and a 'standard' penicillinase-negative culture. This organism was used throughout. The reproducibility of the technique was established as follows.

Six antibiotics were assessed for activity against four different strains. The experiments were then repeated 'blind' after randomising of strains, and the absolute activities were calculated on each occasion. The standard error between the two sets of results was 30%.

Controls included the checking of the variation in the surface seeding, examination for possible differences in the growth rate of the inoculum once applied to the plate, and measurement of inhibition zones obtained with other antibiotics, including tetracycline and gentamicin. It was concluded that variations in zone size attained were unlikely to be due to factors other than variation in the ability of the culture to produce penicillinase. Penicillinase was demonstrated both chemically (starch-iodine method) and by the effect on benzyl penicillin in disc testing.

Results

The MICs of 'single cell' inocula (ie, an inoculum of about 50-200 discrete colony-forming units when applied to the surface of antibiotic-free medium) are shown in Table 1. For each antibiotic the range of MIC was narrow among the 60 strains and was not affected by the ability of the culture to produce penicillinase. As many other workers have found, methicillin was the least potent of the penicillins. Of the cephalosporins, cephaloridine was the most active and cephalixin and cephradine the least.

Since several reports have indicated that the MIC

Table 1 MICs ($\mu\text{g/ml}$) of penicillins and cephalosporin for penicillinase-positive and penicillinase-negative strains of *Staph. aureus*

Antibiotic	Penicillinase-positive strains (44): mean (range)	Penicillinase negative strains (14): mean (range)
Methicillin	2.38 (1.0-5.0)	1.75 (1.0-2.5)
Cloxacillin	0.28 (0.25-0.5)	0.24 (0.1-0.25)
Flucloxacillin	0.25 (0.1-0.5)	0.23 (0.1-0.25)
Nafcillin	0.38 ¹ (0.25-0.5)	0.25 ¹ (0.25-0.25)
Cephaloridine	0.071 (0.05-0.1)	0.039 (0.025-0.1)
Cephalexin	1.84 (1.0-5.0)	1.64 (1.0-5.0)
Cephadrine	1.45 (1.0-2.0)	1.11 (0.5-2.0)
Cephalothin	0.27 (0.25-0.5)	0.23 (0.1-0.5)
Cephazolin	0.34 (0.25-0.5)	0.26 (0.1-0.5)

¹5 only tested

of cephaloridine may be increased if a heavy inoculum is used, this was investigated for 10 penicillinase-positive and six penicillinase-negative cultures by placing drops of neat and serially diluted (10-fold dilutions) cultures onto the surface of plates containing varying levels of cephaloridine. With each of the penicillinase-producing cultures a marked inoculum effect was demonstrated. Indeed, five of the cultures that produced penicillinase became 50 times more resistant when a neat culture was applied to the agar rather than a single cell inoculum. In contrast, no inoculum effect was demonstrated with penicillinase-negative cultures.

SENSITIVITY OBTAINED BY DIFFUSION TESTING

Preliminary experiments indicated that the zones of inhibition obtained in diffusion testing were variable, and many factors contributed to this. Of particular importance was the temporal relationship of the application of antibiotics to the growth phase of the culture. Thus, cultures in exponential phase when first exposed to the drug gave smaller zones of inhibition than those in stationary phase, particularly if that culture produced penicillinase. The following experiments were performed with a standardised pre-incubation time of two hours at 37°C before application of antibiotic to the wells in the agar. Two hours' incubation was selected because the difference between the activity of the antibiotics under test was markedly and reproducibly different between penicillinase-positive and -negative cultures. It was felt that two hours was sufficient time for the culture to be in early exponential phase and would resemble the

clinical situation in which an antibiotic is administered to a patient with a developing bacterial infection. Pre-incubation beyond two hours gave more marked differences but the zones were less clearly defined and difficult to read.

ACTIVITIES OF METHICILLIN, CLOXACILLIN, NAFICILLIN, AND FLUCLOXACILLIN AGAINST 50 PENICILLINASE-PRODUCING STAPHYLOCOCCI IN DIFFUSION TESTING

Initially, 50 consecutively isolated strains were examined; many of these produced low levels of penicillinase. The activities of each strain were expressed as relative to the activities of that of a constant standard penicillinase-negative culture (Table 2). There was considerable variation in the

Table 2 Relative activities of some semi-synthetic penicillins to 50 consecutively isolated strains of *Staph. aureus*: activities expressed as % of the standard in diffusion testing

Antibiotic	Penicillinase-positive strains (38)		Penicillinase-negative strains (12)	
	Mean	Range	Mean	Range
Methicillin	78.1	38.0-200.0	76.2	21.0-122.0
Nafcillin	58.4	2.5-123.0	49.5	9.0-90.0
Cloxacillin	39.2	4.8-141.0	71.6	12.0-141.0
Flucloxacillin	30.0	3.3-76.0	88.6	5.4-330.0

sensitivity of these isolates to each of these antibiotics, although, in general, those strains that were particularly active against one drug were also active against the others. The relative resistance of the drugs to penicillinase was in the following order: methicillin (most resistant), nafcillin, cloxacillin, and flucloxacillin (most vulnerable).

ACTIVITIES OF CEPHALOSPORINS AGAINST PENICILLINASE-POSITIVE CULTURES

In preliminary experiments, the activities of cephaloridine, cephazolin, cephalothin, cephalexin, and cephadrine were tested in a way similar to that of the penicillins. Cephadrine was the least vulnerable to penicillinase and cephaloridine the most.

One surprising finding from these experiments was that the antibiotics cephalexin and cephadrine, although structurally almost identical, had apparently significant differences in their resistance to penicillinase. This was confirmed as follows:

The activities of 60 strains, for which the MIC data had been obtained, were each examined against these antibiotics by diffusion testing. The activities of the two drugs against 13 penicillinase-negative strains from these cultures were, as expected, almost identical; the mean relative activity of cephalexin

was 122% and that of cephadrine 120% of the standard ($t = 0.13$; $P > 0.50$). In contrast, there were marked differences between the activities of the 47 penicillinase-positive cultures for cephadrine (89%) and cephalixin (41%) relative to that of the standard. Differences even greater than this could be detected if the antibiotics were added to a culture in mid-exponential phase.

ACTIVITIES OF PENICILLINS AND CEPHALOSPORINS AGAINST 15 STRAINS OF *Staph. aureus* PRODUCING HIGH LEVELS OF PENICILLINASE
Fifteen strains that produced high levels of penicillinase were then selected from another 50 cultures. When tested against these strains, flucloxacillin was found to be inactivated to a greater extent than the other semisynthetic penicillins, and of the cephalosporins cephaloridine was the most vulnerable, and cephradine the least (Table 3). Combined data for

Table 3 *Relative activities (as % of standard sensitive strain) of cephalosporins and penicillins for 15 penicillinase-positive strains of Staph. aureus*

Antibiotic	Mean	Range
Cephaloridine	0.12	0.01- 0.2
Cephalexin	6.6	1.5 - 20.0
Cephadrine	12.2	2.4 - 44.2
Cephalothin	2.6	0.6 - 6.9
Cephazolin	4.4	2.7 - 8.9
Methicillin	48.4	20.9 - 118.0
Nafcillin	42.5	17.0 - 87.0
Cloxacillin	122.1	28.8 - 398.0
Flucloxacillin	12.9	4.2 - 29.5

the 38 strains previously examined, and the selected 15, indicated that flucloxacillin (mean activity 25%) was much more vulnerable to penicillinase than either methicillin (69%), nafcillin (54%), or cloxacillin (63%).

EVIDENCE THAT VARIATION IN ACTIVITY IS DUE TO PRODUCTION OF PENICILLINASE
Three cultures of *Staph. aureus* that produced either A, C, or D-type penicillinase (Rosdahl, 1973) were treated (Lacey, 1971) with a mutagen, nitrosoguanidine, and then plated on media containing 10^{-4} M cadmium acetate. (This step was essential to select plasmid-positive cells. Nitrosoguanidine treatment produces loss of the entire penicillinase plasmid at a frequency much greater than point mutations in that plasmid; since each of the plasmids employed carried the genes for cadmium-ion resistance, this plating ensured the retention of each plasmid.) The resultant colonies were replica-plated onto media containing starch, and penicillinase-negative variants were identified. These were examined for metal-ion resistance; those derivatives that showed metal-ion

resistance identical with the wild strain were considered to possess plasmids intact except for their inability to cause the cells to synthesise penicillinase. Each of these plasmids was then transduced into fresh clone of the corresponding original host cell. Thus, three cultures were prepared that were apparently identical except for variation in their ability to produce penicillinase. These six cultures were then tested for their resistance to penicillins and cephalosporins. As expected, no variation in MIC was seen between members of each pair, but in diffusion testing greatly reduced zones were seen around wells of most of the cephalosporins and also flucloxacillin compared to their penicillinase-negative counterparts.

The zones of both penicillinase-positive and -negative cultures were similar to corresponding types of strains described above. Thus, it appears highly likely that it was the production of penicillinase itself that caused the reduction in zone sizes in diffusion testing.

The reason for this reduction in zone size could be due either to destruction of the antibiotic or to the presence of the antibiotic acting as an inducer of enzyme production during diffusion. Attempts to demonstrate the latter have been unsuccessful. Cultures were pre-incubated in subinhibitory concentrations of each drug singly before the diffusion experiment was performed as before. No differences were observed.

Discussion

These results indicate that, while the activity of any one of the penicillinase-resistant penicillins or cephalosporins is almost uniform against staphylococci if tested by MIC methods, it is extremely variable in diffusion testing. This variation has been demonstrated (most emphatically) by pre-incubation (for two hours) of the seeded plate before application of the drug. Evidence has been presented that this variation is due to two main factors: (1) the production of penicillinase by some cultures, and (2) variation in the ability of the various antibiotics to resist penicillinase.

The reason why MIC methodology gives uniform results for any one of the drugs against a variety of test cultures is that it fails to detect the production of penicillinase, since that enzyme is readily lost from the dormant cell.

We believe that, in general, the diffusion/pre-incubation method of sensitivity testing relates more closely to the clinical situation than MIC methodology. An exception to this may be the use of antibiotic prophylactically. It is of extreme practical importance to assess the correctness of laboratory

methods in sensitivity testing, since consistently different results are obtained, depending on which method is used. Perhaps under ideal conditions the method should be selected that represents most closely the context of each individual prescription. Thus, if an antibiotic is used prophylactically it should be selected on the basis of expected MIC of the predicted pathogens. In the treatment of established infections, diffusion methods would be preferable. Such refinement is obviously difficult to achieve in a routine laboratory.

We believe, therefore, that the correct general policy for testing the activity of these penicillins and cephalosporins against staphylococci should be by diffusion against actively growing inocula at 37°C.

Assuming that this method is valid, how do the various drugs fare against penicillinase-producing staphylococci? Of the penicillins tested, methicillin, cloxacillin, and nafcillin were relatively resistant to penicillinase, methicillin and cloxacillin being most resistant, whereas flucloxacillin was much less resistant (approximately 10 times more susceptible than cloxacillin against some strains). While flucloxacillin has an advantage over cloxacillin in that higher blood levels are obtained after oral administration, it seems to us unlikely that this would counteract the increased susceptibility to penicillinase. It is, however, hazardous to predict the clinical outcome from such considerations, and more clinical data than the two cases quoted by us would be desirable. Meanwhile, in the absence of other considerations (eg, cost) and of other clinical data, we recommend the use of cloxacillin in these circumstances.

Of the cephalosporins, the following order of

resistance to staphylococcal penicillinase has been found: cephaloridine (the most vulnerable), cephazolin, cephalothin, cephalixin, and cephadrine (the most stable). It is impossible to draw many specific conclusions from this as these drugs differ in many properties other than their resistance to penicillinase. However, it would seem unwise to use cephaloridine where a staphylococcal infection is suspected, and cephadrine is preferable to cephalixin and probably also cephazolin and cephalothin where a staphylococcal infection is a possibility.

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References

- Burgess, H. A. and Evans, R. J. (1966). Failure of cephaloridine in a case of staphylococcal endocarditis. *British Medical Journal*, **2**, 1244.
- Lacey, R. W. (1971). Transfer of tetracycline-resistance between strains of *Staphylococcus aureus* in mixed cultures. *Journal of General Microbiology*, **69**, 229-237.
- Lacey, R. W. and Lewis, E. L. (1975). Further evolution of a strain of *Staphylococcus aureus* in vivo: evidence for significant inactivation of flucloxacillin by penicillinase. *Journal of Medical Microbiology*, **8**, 337-347.
- Lacey, R. W. and Lewis, E. L. (1976). Inactivation of 'penicillinase-resistant' penicillins and cephalosporins by staphylococcal penicillinase; clinical and laboratory studies. *III International Symposium on Staphylococci and Staphylococcal Infections*. Springer, Berlin. (In press.)
- Norden, C. W. and Dickens, D. R. (1973). Experimental osteomyelitis. III. Treatment with cephaloridine. *Journal of Infectious Diseases*, **127**, 525-528.
- Rosdahl, V. T. (1973). Naturally occurring constitutive β -lactamase of novel serotype in *Staphylococcus aureus*. *Journal of General Microbiology*, **77**, 229-231.