

Distribution of levels of penicillin resistance among freshly isolated strains of *N. gonorrhoeae*

Application of a novel sensitivity assay

R. SHANNON, A. J. HEDGES*, AND R. J. EDWARDS

From the Public Health Laboratory, Bristol BS2 8EL, and the *Department of Bacteriology, University of Bristol, Bristol BS8 1TD

Investigations into the distribution of levels of drug resistance within bacterial populations require sensitivity assay methods that are not only accurate and precise but also easy to carry out on large numbers of isolates. Certain organisms present special problems; for example, *Neisseria gonorrhoeae* is difficult to cultivate and shows very low levels—in absolute terms—of penicillin resistance. Such features put extra demands on the reproducibility and sensitivity of the assay method.

This paper describes the successful application of a novel diffusion zone method to surveying the distribution of levels of penicillin resistance among isolates of *N. gonorrhoeae* obtained from the Bristol area. It is suggested that the method may have wide application to such studies.

Material and methods

Source of gonococci

The strains used in this investigation were routine isolations from three clinics in the Bristol area. Details are given in the results section.

Cultivation of gonococci

Primary isolation was made on the special medium routinely used in our laboratories (Cooper, Mayr-Harting, and McLachlan, 1950), with and without the addition of 3 µg. per ml. vancomycin (Vancocin, Burroughs Wellcome), 3 µg. per ml. trimethoprim lactate (Lilly), and 12.5 µg. per ml. nystatin (Mycostatin, Squibb). Subcultures were maintained on the same medium without additions. All cultures were incubated at 37°C. in a candle-extinction jar (Cooper and others, 1950).

Penicillin

The sodium salt of benzyl penicillin (Crystapen, Glaxo) was used throughout. One unit was equivalent to 0.6 µg. of the dry salt. Solutions were made in sterile de-ionized water.

Determination of minimum inhibitory concentration (MIC)

Penicillin was incorporated in the cultivation medium at concentrations of 0.01, 0.03, 0.05, and 0.08 units per ml. Higher levels were used for certain more resistant strains.

Each of four test strains were applied to each plate by a standard procedure, which consisted of streaking out the inoculum so as to obtain growth ranging from confluent to isolated colonies on each plate. They were incubated overnight in a candle-extinction jar at 37°C. The MIC was taken to be midway between adjacent concentrations showing the transition from 'growth' to 'no growth'.

Determination of 'critical concentration' (m')

THEORETICAL BASIS OF THE METHOD

In their studies on the formation of inhibition zones due to antibiotics diffusing in agar gels, Cooper, Linton, and Sehgal (1958) showed that the time of formation, and therefore the size, of such zones was a definable function of two groups of factors:

- (a) Those influencing the rate of diffusion of the antibiotic;
- (b) Those influencing the time taken for the inoculum of the test organism to grow to some 'critical population' density.

Not only did their results reveal the reasons for the extreme variability of diffusion zones when such factors as temperature, inoculum size, and growth rate were insufficiently controlled, but the authors were also able to demonstrate a very important consequence.

It was well established that, if the square of the length of a linear diffusion zone was plotted against the logarithm of the antibiotic concentration from which diffusion took place, a linear graph resulted. Cooper and others (1958) showed that, for a specified organism and growth medium, the point at which the extrapolated linear graph cut the concentration axis at zero zone size was independent of the factors influencing growth, *i.e.* group (b) above. It was therefore a measure of the intrinsic sensitivity of the organism to that antibiotic and was called the 'critical concentration, (m')'. Although the linear relation holds strictly only for linear zones, it is well approximated by the radius of a radial diffusion zone provided that the diameter of the reservoir is sufficiently large (Cooper, 1963).

Thus, in the diagram (Fig. 1), organisms *A* and *B* have the same growth rate (and inoculum size) but *B* is ten times more resistant than *A* to the antibiotic. In contrast, *A* and *C* share the same intrinsic sensitivity, but *C* achieved the critical population faster than *A*; *C* may have had a faster growth rate or a larger inoculum.

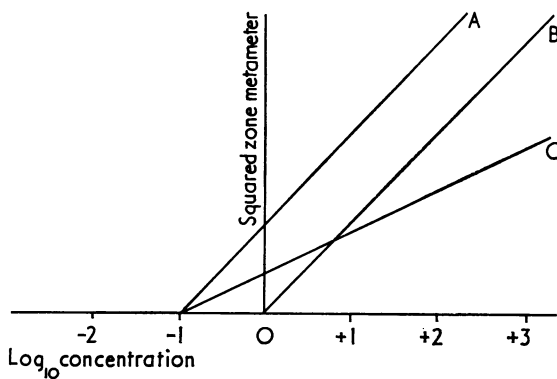


FIG. 1 Relation of zone size to concentration of a diffusing antibiotic for three hypothetical strains, A, B, C, of the same organism. A and C are equally sensitive, B is 10-fold more resistant (Simplified from Linton, 1961)

By using a few (3 or 4) logarithmically spaced antibiotic concentrations and plotting the linear graph formed by the squared radius, or diameter, of their corresponding inhibition zones—corrected for the diameter of the reservoir, it is possible to estimate m' on a specified medium from the results of a single plate and free from the common sources of inaccuracy. The critical concentration (m') is always larger than the minimum inhibitory concentration (MIC) determined by incorporation methods, for reasons discussed by Cooper (1963, 1972), but the two measures vary in constant ratio for specified conditions.

TECHNIQUE

Lysed blood agar medium was prepared by adding saponized horse blood (10 per cent. v/v) to Blood Agar Base No. 2 (Oxoid), and dispensing it in 65-ml. amounts in 14-cm. Petri dishes. The plates were well dried at 50°C. for 30 min. Growth from an overnight culture of a test strain on maintenance medium was removed by means of a sterile throat swab and inoculated into 5 ml. of nutrient broth (Lab lemco, Oxoid). An even suspension was obtained by adding a sterile nail and agitating on a magnetic stirrer. The density of the inoculum was not rigorously controlled because of the independence of the method of the inoculum effect, but ranged from approximately 3 to 6×10^8 organisms per ml. The suspension was flooded onto the surface of a lysed blood agar plate, the excess fluid was removed, and the plate left for a short time until dry. Four equally-spaced wells were then cut in the medium by means of a sterile No. 8 cork borer (1.4 cm. diameter). These were completely filled with solutions containing 0.1, 1.0, 10, and 100 units of penicillin per ml. and the plate was immediately incubated at 37°C. in a candle-extinction jar. After overnight incubation, the overall diameters of the inhibition zones were measured to the nearest 0.1 mm. by means of Vernier callipers fitted with needle points.

The estimation of m' was routinely made by the 'graphical method'. First, the square of the true radius of each diffusion zone was obtained from its overall

diameter by subtracting the well diameter (1.4 cm.) and halving and then squaring the result. It is not strictly necessary to use the radius, instead the corrected diameter may be used throughout; we used the radius because a simple Table had been constructed from which the squared radius could be obtained directly from the overall diameter. The values of (radius)² were then plotted against penicillin concentration on 4-cycle semi-logarithmic graph paper and a straight line was drawn through the points 'by eye'. The point at which the extrapolated line cut the concentration axis was the estimate of m' . The number of points plotted for each strain depended on the number of readable zones: fully sensitive strains yielded readable zones with all four concentrations, some resistant strains gave only the minimum usable number, *i.e.* two. It was found necessary to check that the graph paper had not become distorted in storage if erroneous estimates were to be avoided.

In some investigations we required 'best' estimates of m' and these were obtained algebraically by the 'least squares' method. If the relation of the squared zone metameter (y) to the logarithm of the antibiotic concentration (x) is

$$y = a + bx$$

then, for N zones, the least squares solution is

$$\log m' = -a/b = \frac{1}{N} \left\{ \frac{\sum x \sum y (\sum x^2 - (\sum x)^2/N)}{\sum xy - \sum x \sum y/N} \right\}.$$

Results

Relation of m' to MIC

Forty consecutive freshly isolated samples of gonococci were chosen for direct comparison of MIC, determined as described above, with the m' values obtained by the graphical method. In spite of the difficulty of relating the stepwise variation in MIC to the smoothly continuous m' values, a good degree of correlation was obtained. The correlation coefficient was calculated as $r = 0.74$ —a value that was statistically highly significant ($P < 0.001$). Assuming a linear relation over the 'useful' range (0.01 to 4.0 units per ml.), we estimated that

$$\text{MIC} = 0.276 m' \text{ approximately} = 0.3 m'.$$

This relation was very similar to that found by Cooper (1972) for *Staphylococcus aureus* and streptomycin.

Precision of the method

Four strains of gonococci were selected to span the levels of penicillin resistance observed. These were strains 21604 and 254 (MIC *c.* 0.03 unit per ml.), strain 20618 (MIC *c.* 0.3 unit per ml.), and strain 20637 (MIC *c.* 1.2 units per ml.). Ten replicate determinations of m' were made on each by the same technician, using the graphical method of estimation. His data were also subjected to the least squares treatment to yield 'best' estimates of m' . The precision of the values obtained by both methods was

TABLE Precision of graphical and least squares estimates of m'

| Strain no. | No. of readable zones per plate | Mean m' (units/ml.) | | Coefficient of variation* (per cent.) | | Worst result (per cent. deviation from best estimate) | Calculated MIC (units/ml.) ^b |
|------------|---------------------------------|-----------------------|----------------------|---------------------------------------|----------------------|---|---|
| | | Graphical method | Least squares method | Graphical method | Least squares method | | |
| 21604 | 4 | 0.082 | 0.096 | 11.3 | 9.9 | 0.070 Graphical (- 27.1) | 0.028 |
| 254 | 4 | 0.071 | 0.074 | 10.3 | 16.4 | 0.096 Least squares (+ 29.7) | 0.022 |
| 20618 | 3 | 1.003 | 0.960 | 6.4 | 4.6 | 0.863 Least squares (- 10.1) | 0.288 |
| 20637 | 2 | 3.880 | 4.035 | 6.7 | 12.0 | 4.986 Least squares (+ 23.6) | 1.211 |

*Coefficient of variation = $100 \times (\text{standard deviation} \div \text{least squares estimate of } m')$

^a Least squares estimate of m' taken to be the 'best estimate'

^b MIC = $0.3 \times (\text{least squares estimate of } m')$

judged by calculation of coefficients of variation and by noting the 'worst' result, *i.e.* that most deviant from the least squares mean m' , for each set. A comparison of the results of the two methods of estimation is shown in the Table.

The assay showed a satisfactorily high precision, the coefficient of variation being *c.* 10 per cent. Moreover, the precision of the graphical method was comparable with that of the least squares treatment. Both methods also yielded very similar mean values for m' ; the maximum difference was obtained for strain 21604 (- 14.6 per cent.), the other strains showed differences of only *c.* 4 per cent.

The 'worst' result was always less than 30 per cent. different from the best estimate, and it is interesting to note that the graphical method yielded the worst result for only one of the four strains examined.

Levels of penicillin resistance among local strains of gonococci

A total of 816 isolates of *N. gonorrhoeae* was obtained over a 6-month period (July to December, 1973) from three well separated clinics within the Bristol area, *viz.* Bristol, Bath (*c.* 12 miles from Bristol), and Cheltenham (*c.* 42 miles from Bristol). All isolations were made in our laboratory and each isolate was identified by means of Gram reaction, oxidase reaction, and sugar fermentation pattern. Of the 816 isolates, 661 were from the Bristol clinic, 107 from Bath, and 48 from Cheltenham. No selection of these strains was attempted and we recorded only whether they were isolated from male or female patients.

Estimates of m' were obtained by the graphical method for each isolate and converted to its corresponding MIC. We present the results as histograms showing the relative frequency of such MIC values rounded off according to scale. Figs 2 and 3 show the distribution for Bristol males and females, respectively. Fig. 4 shows the relative frequencies for

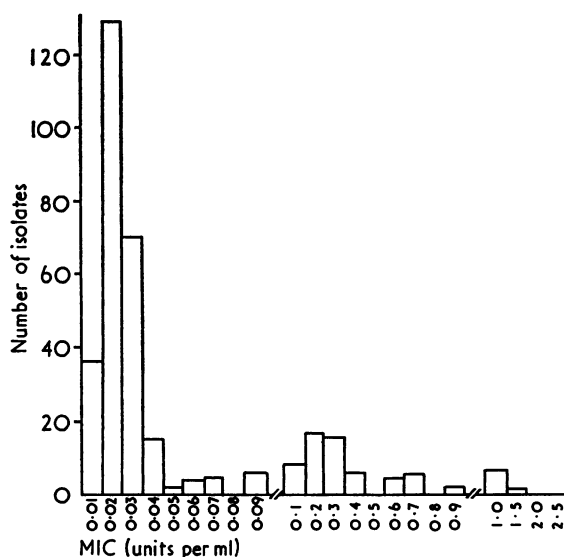


FIG. 2 Distribution of levels of penicillin resistance among 327 gonococcal isolates obtained from male patients in Bristol. Changes of scale on MIC axis indicated by double lines

the total 816 isolates as well as the relative frequency for each local clinic separately.

Discussion

In seeking to understand the characteristics and nature of penicillin resistance in strains of *N. gonorrhoeae*, it is obviously important to be able to define the distribution of levels of resistance as sharply as possible. For instance, an infinitely graded 'spectrum' of resistance should be readily distinguishable from a pattern of stepwise increases with phenotypic scatter at each level. The method of sensitivity assay commonly employed (*e.g.* Lynn, Nicol, Ridley, Rimmer, Symonds, and Warren,

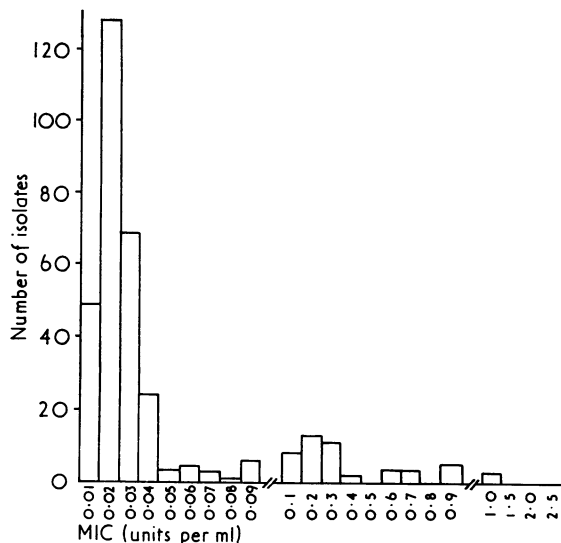


FIG. 3 Distribution of levels of penicillin resistance among 334 gonococcal isolates obtained from female patients in Bristol. MIC axis as for Fig. 2

1970; Stolz, Zwart, and Michel, 1974) has been to use graded concentrations of penicillin incorporated in a nutrient medium to which standard inocula of the test strains are applied (incorporation method). This has a number of inherent snags. First, the end-point (MIC = minimum inhibitory concentration) has to be guessed as either coinciding exactly with one of the steps of the series or as falling somewhere between two adjacent, but often logarithmically spaced, steps. Secondly, variation among plates in inoculum size or growth conditions may introduce inaccuracies in the estimates of MIC. Thirdly, the method requires a series of penicillin plates to be set up for each test strain—a tedious and space-consuming procedure.

Although methods that rely on the relation of degree of resistance to the size of inhibition zones produced by diffusion from a reservoir of known concentration of antibiotic (diffusion methods) can avoid these difficulties, such methods have their own inbuilt pitfalls. In spite of the basic theoretical studies of Cooper and his colleagues (summarized in Cooper, 1963, 1972) and a discussion of their technical implications by Linton (1961), we feel that the dangers are still not fully appreciated. All too often one encounters methods which rely on the notion that if two strains of the same organism produce unequal inhibition zones in response to the same reservoir concentration of antibiotic then the organism producing the larger zone *must* be more sensitive. This is simply not true: it is impossible to make such a statement if armed only with the evidence from a single concentration (see Fig. 1).

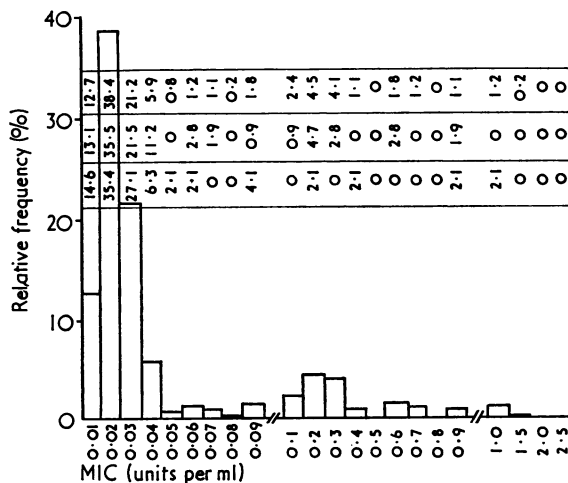


FIG. 4 Relative frequency distribution of penicillin resistance among 816 gonococcal isolates from Bristol area. Superimposed numbers give relative frequencies as percentages for each local clinic separately
Top row—Bristol
Middle row—Bath
Bottom row—Cheltenham
MIC axis as for Fig. 2.

Linton (1961) showed how the theoretical studies pointed the way to a method of estimating degrees of resistance that overcame the difficulties of the diffusion methods at the expense of very little extra manipulation. It is this approach that we have developed and applied to the definition of the distribution of levels of penicillin resistance among local strains of gonococci.

The estimate of resistance obtained by this method is the so-called 'critical concentration' (m'), which represents the concentration of antibiotic that has diffused to the zone edge at the time of its formation. Cooper's group (Cooper, 1963, 1972) found that m' was always greater than MIC, determined by the incorporation method, and discussed the likely reasons for this difference. Their important practical conclusion was that, for a specified sort of bacterium on a specified medium, the ratio m' : MIC remained constant within the experimental variation. The results presented above for the gonococcus and penicillin support this conclusion and, on the medium employed, MIC equals approximately $0.3 m'$.

From the clinician's viewpoint, what is most often needed is some measure of the intrinsic sensitivity of the organism that can be related to the therapeutic dose of antibiotic. There is no reason why m' should not, itself, serve this purpose but, for comparability with published results, we have converted all estimates to their corresponding MIC values by means of the equation given above.

Before applying the method to the central problem, it was first necessary to examine its precision when used in a 'routine' manner. From the results, we concluded that the precision obtainable 'by eye', *i.e.* by a graphical estimation where both slope and intercept of the linear graph were judged subjectively, was comparable with the precision obtainable from a least squares algebraic solution for the value of m' . For the representative strains tested the worst estimate was less than 30 per cent. different from the corresponding best estimate. On the basis of these observations, we suggest that, if a technician finds little difficulty in subjectively fitting the linear graph to his results, then a reasonable estimate of m' will be obtained. If difficulty is experienced, it probably indicates that the technician is not yet sufficiently familiar with the technique or that some other factor needs attention. In other words, we see no practical advantage to be gained from using a more sophisticated analysis—provided that the data are good enough.

The method was then applied to 816 isolates of gonococci obtained from three widely separated clinics in the Bristol area. It revealed a well-defined distribution of penicillin resistance among these strains. The strains clustered in four reasonably distinct groups, having median MIC values of *c.* 0.02, 0.20, 0.60, and 1.00 units per ml. The majority of isolates (82.3 per cent.) fell in the 'sensitive' cluster with MIC values ranging from 0.01 to 0.07 unit per ml. The next cluster comprised 13 per cent. (MIC: 0.09 to 0.40), and only 2.2 per cent. and 2.4 per cent. formed the clusters showing highest resistance (MIC 0.6 to 0.7 and 0.9 to 1.9 units per ml., respectively).

Each cluster was sharply demarcated from its neighbours, and a remarkable degree of similarity was evident in the distributions from the different centres; although it is possible that the two most resistant clusters will merge when more data are collected. It is obvious that the 'points of rarity' indicated by the histograms, *i.e.* MIC values which never or rarely occurred, would probably not have been detected if our survey had employed an incorporation method with logarithmically spaced concentrations; the four clusters could have merged into one.

We forbear from speculation, here, on the underlying mechanism(s) of penicillin resistance in gonococci, nor do we discuss the clinical implications;

these topics will be the subjects of future studies. We would merely point out that the method described in this paper appears to have the qualities required to facilitate such studies. Finally, we suggest that this same approach could be profitably applied to similar investigations with other organisms and other antibiotics.

Summary

A novel diffusion zone method for quantitative assay of the antibiotic sensitivity of bacterial strains was tested on freshly isolated gonococci. Smoothly variable estimates of the minimum inhibitory concentration of penicillin for these strains was obtained with sufficient accuracy and precision (coefficient of variation *c.* 10 per cent.) by means of a simple graphical analysis and without replication. Such estimates were free from the chief sources of error associated with the commonly applied 'incorporation' and 'diffusion' methods. The method revealed that 816 isolates of gonococci obtained in the Bristol area during a 6-month period fell into a large 'sensitive' group (MIC *c.* 0.02 unit per ml.) and three smaller more resistant groups, and that this pattern occurred in three widely spaced centres within the area. It is suggested that the method is capable of revealing details of distribution that may be masked by the usual techniques and that it is of wide applicability.

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