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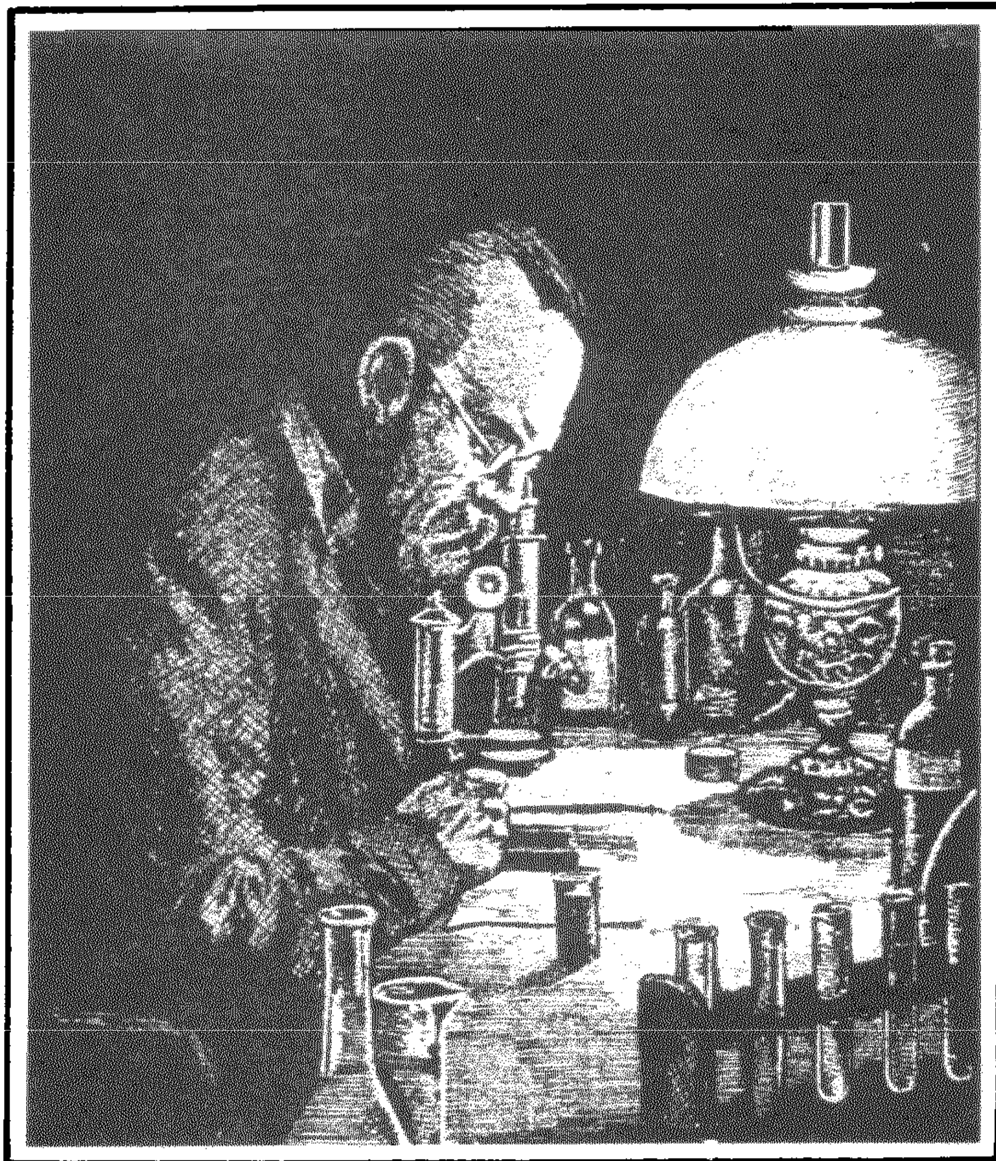

antibiotic sensitivity testing by the CDS method

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CLINICAL MICROBIOLOGY UPDATE PROGRAMME

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NO. 21: ANTIBIOTIC SENSITIVITY TESTING BY THE CDS METHOD

January, 1984

by

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Contents

	Page
Editorial	1
1. Introduction	2
2. Materials	3
2.1 Antibiotic discs	4
2.2 Media	4
2.3 Inoculating wire	5
3. Method	6
3.1 Preparation of sensitivity agar plates	6
3.2 Preparation of inoculum	6
3.3 Inoculation of sensitivity plates	6
3.4 Control organisms	7
3.5 Reading the test	7
3.6 Interpretation of results	8
4. Antibiotics used in testing	9
4.1 Range of antibiotics for which test has been calibrated	9
4.2 Use of a representative disc to indicate sensitivity to an antibiotic group	9
4.3 Suggested combinations of antibiotic discs	10
5. Tests of unusual or fastidious pathogens	12
5.1 *Streptococcus* species	12
5.2 *Haemophilus influenzae*	12
5.3 *Neisseria gonorrhoeae*	13
5.4 Anaerobic bacteria	13
6. Control measures in disc testing	14
6.1 Care and precision in technique	14
6.2 Media	14
6.3 Antibiotic discs	15
6.4 Inoculum	15
6.5 Incubation of sensitivity plates	16
6.6 Standard sensitive strains	16

	Page
7. Confirmation of resistance to penicillins	18
7.1 β-lactamase activity	18
7.2 Chemical tests for penicillinase production	18
7.3 Penicillin zone edge of *Staphylococcus aureus*	19
7.4 Confirmation of methicillin resistance in *Staphylococcus aureus*	20
8. Problems and pitfalls of disc sensitivity testing	21
8.1 Limitations of a disc test	21
8.2 Intermediate sensitivity	22
8.3 Common pitfalls of disc sensitivity testing	23
8.3.1 Errors in recording results	23
8.3.2 The mixed culture	24
8.3.3 Sulphonamide and trimethoprim with *Proteus* species	24
8.3.4 Fine growth within the sulphonamide and trimethoprim inhibitory zone	24
8.3.5 Mucoid *Klebsiella* or *Pseudomonas*	24
8.3.6 Defining the zone edge	25
8.3.7 Failure of the test organism to grow on the sensitivity plate	25
8.3.8 Direct antibiotic sensitivity testing	25
9. The clinical significance of tests of antibiotic sensitivity	27
9.1 Antibiotic sensitivity testing in perspective	27
9.2 Predictive value of antibiotic sensitivity tests	28
9.3 The implications of an antibiotic sensitivity report	29
References	30

Editorial

Antibiotic sensitivity testing is a frequently performed test in the routine microbiology laboratory and yields valuable information necessary for successful patient management. When laboratories report an organism as sensitive or resistant to an antibiotic it is important that they should be able to do so with confidence, therefore it is essential that they should know that their method of antibiotic sensitivity testing gives accurate and reproducible results.

Between 1968 and 1974 Dr. Sydney Bell at The Prince of Wales Hospital developed a method of disc sensitivity testing specifically for use in diagnostic laboratories. The method is simple, reproducible and economical and can be used by both large and small laboratories. In the calibration of the method, antibiotic content of the discs was selected to demonstrate clearly the division of sensitivity of organisms into either sensitive or resistant, i.e., a dichotomous division. More extensive grading of antibiotic sensitivity into categories such as "intermediate" or "moderately sensitive" is not used. The method is called the calibrated dichotomous sensitivity (CDS) test.

This monograph will be of value to the large number of laboratories already using the CDS method. It contains the results of calibration of many of the more recently available antibiotics and, also, several details of technique and interpretation are explained in greater depth. Other laboratories may not be satisfied completely with their present method of sensitivity testing. This may be because it is neither a calibrated technique nor a valid and reproducible quantitative method, or perhaps their methods are too difficult to perform or yield unreliable results. These laboratories may wish to consider using the CDS test.

The value of the CDS method in practice was demonstrated in the microbiology surveys conducted by the Royal College of Pathologists of Australasia. In the 1971 survey, 96 laboratories tested six strains of *Staph.aureus* to five antibiotics (i.e. 2,880 tests) and the following results were obtained:-

Method used	No. of errors
Laboratory's own method	296 (10.2%)
CDS method	41 (1.4%)

The results of the 1972 survey in which the sensitivities of six strains of *E.coli* to eight antibiotics were tested are as follows:-

Method used	Percentage obtaining correct results
86 laboratories using own method	19
24 laboratories using CDS method	100

In this issue Dr. Bell describes the details of the method and the interpretation of the results. Problems that have been encountered by laboratories are described and solutions suggested. Finally, the clinical significance of the antibiotic sensitivity tests and the implications of reporting sensitivities on laboratory reports are discussed.

1. Introduction

The Calibrated Dichotomous Sensitivity (CDS) test is a disc method of antibiotic sensitivity testing where the results of disc testing are related to quantitative antibiotic sensitivity (minimum inhibitory concentration) by prior calibration. The CDS test was developed to meet the needs of the vast majority of diagnostic laboratories in Australia and it was shown it could be applied successfully by laboratories irrespective of their size or availability of resources. Early in the development of the CDS test it was recognized that in practice no disc test could accurately and consistently grade antibiotic sensitivity of organisms more precisely than either sensitive or resistant. For this reason, and because when variation in antibiotic sensitivity of strains of common pathogens occurs it does so mainly in a bi-modal distribution, the method was designed to be dichotomous, that is it divides antibiotic sensitivities into two groups, "sensitive" and "resistant".

The CDS method was described first over eight years ago[1]. Since then a considerable number of antibiotics have been introduced and laboratories have found themselves under increasing pressure to test a much broader range of antibiotics than was necessary in 1975. Although it has not been possible, nor even necessary, to include every new antibiotic in the CDS test there are several antibiotics which have proven themselves as worthwhile additions to the range of antibiotics tested by diagnostic laboratories.

A prerequisite of the CDS method is that no antibiotic can be tested by this technique without prior calibration of the results of disc testing to quantitative sensitivity. Although this calibration has been achieved with a large proportion of newer antibiotics the results have been communicated to interested laboratories solely on a personal basis. The opportunity is taken here to disseminate the information accumulated more widely than that which could be achieved by private means.

The second reason for republishing the method is that experience has shown that in the course of time standard procedures are frequently modified to suit a laboratory's own requirement or to conform with the personal impressions of a particular operator. In many areas of microbiology such modifications cannot always be criticised in that they may be beneficial and lead to new and improved techniques. However, with any calibrated disc method of antibiotic sensitivity testing such as the CDS test or the Kirby-Bauer method, *even a minor modification of the method will invalidate the test.* Calibrated disc tests rely on a faithful reproduction of the original method using the calibration experiments for their accuracy in practice. Laboratories which make any change, no matter how subtle, to the materials or techniques or use the test for organisms or antibiotics not included in the description of the CDS method are not carrying out the CDS test. They are, in fact, performing their own test and unless they have carried out extensive calibration of this test under the new circumstances their method has no scientific basis and has little, if any, diagnostic value.

Finally our own and other people's experience has drawn attention to some common difficulties or pitfalls which may arise in testing antibiotic sensitivity by a disc method. I have taken the opportunity here to describe in more detail those aspects of technique where attention to detail may help to overcome at least some of these problems.

2. Materials

The materials required for carrying out the CDS test are summarized in Table 1 and described in more detail below.

Table I. Materials used in the CDS Test

Antibiotic discs

Multodisks - (Oxoid Limited)

or

Antibiotic discs - (Oxoid Limited)

or

Sensidiscs - (BBL)

Agar

Sensitest - (Oxoid Limited, CM.409)

Inoculating wire

Nichrome SWG.24 (0.56 mm diameter) - 10 cm length

Petri dishes

Plastic 90 mm

Sterile normal saline

2.5 mls in 10 x 1 cm test tubes

2.1 Antibiotic discs

Antibiotic discs supplied by either Oxoid Limited or Baltimore Biological Laboratories (BBL) are the only discs used in the CDS method. Antibiotic discs prepared by other manufacturers either yielded unsatisfactory results or were not available at the time of calibration of the test. Discs supplied by Oxoid Limited are available as single discs or as Multodisks® (see Figure 1 on page 7). Multodisks have a combination of six antibiotics attached by spokes to a central paper carrier. They are convenient and very easily applied to the sensitivity plate and the use of discs in this form is the method of choice at The Prince of Wales Hospital. A disadvantage of Multodisk found by some laboratories is that the combination of antibiotics is fixed and there is a limit to the number of different combinations available unless a particular combination is made to order. Single discs, which are supplied in cartridges, are available from both Oxoid Limited and BBL and each company also markets a six-disc dispenser (Oxoid Disc Dispenser Mark II, BBL Sensidisc Dispenser). Both types of disc dispensers perform satisfactorily but unfortunately the cartridges from the two manufacturers are not interchangeable between the different companies' dispensers. Ejectors which dispense a disc from an individual cartridge also are available from each source and these are convenient for dispensing discs in those situations where less than six antibiotics are required in a sensitivity test.

Care and Storage of Antibiotic Discs

Improvements in the packaging and the presentation of the two brands of antibiotic discs has led to a greater stability in the potency of the discs. Discs now have a stated shelf life of up to two years provided they are stored and maintained in accordance with instructions which are freely available from the supplier. Moisture particularly and/or heat cause a rapid deterioration in the potency of antibiotic discs and discs containing β-lactam antibiotics (penicillins and cephalosporins) in particular will deteriorate well before the stated shelf life if the discs are not handled correctly. Laboratories are advised not to over order discs and to store stock supplies of antibiotic discs at $-20^{o}C$. All antibiotics in daily use should be kept in a desiccator unless they are in a sealed dispenser (e.g. the Oxoid Mark II) which has its own desiccant pack *in situ*. The desiccant in the desiccator or the desiccant pack in the dispenser should be renewed or reactivated when necessary. If the desiccator is kept in a refrigerator the temperature of its contents will be below dew point and the container must be allowed to equilibrate to room temperature before it is opened. If this procedure is not followed carefully water will condense on the antibiotic discs and the desiccant, resulting in a rapid deterioration in the potency of the discs and a loss of activity of the desiccant.

2.2 Media

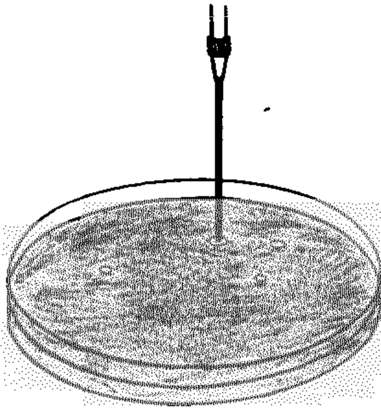
Only one basic medium is acceptable in the CDS Test and this is Sensitest (Oxoid Limited CM409). The calibration of zone sizes to MIC was carried out with this medium alone and the use of any other medium invalidates the

test. *Even minor modifications or improvements of the original Sensitest® agar may disturb the relationship between zone size and minimal inhibitory concentration (MIC)* and for this reason other types of sensitivity agar such as Isosensitest (Oxoid Limited) are unacceptable alternatives in the CDS method. The need for supplementary media for testing some of the more fastidious pathogens was recognised in the development of the CDS method and calibration of the technique was carried out with these media. Horse blood was added to Sensitest agar to enable streptococci and pneumococci to be tested and a chocolate horse blood agar was required for testing *Haemophilus influenzae*. *These supplementary media are intended for use only with the organisms specified and should not be substituted for Sensitest agar for testing other pathogens.*

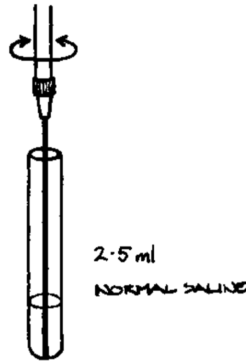
2.3 Inoculating wire

The inoculating wire specified in the CDS method is Nichrome SWG.24 (0.56 mm in diameter). The thickness of the wire is an important determinant of the size of the inoculum in the CDS test. As the inoculum size is a critical factor in determining the reproducibility of the test the use of the specified wire gauge is of the utmost importance. Nichrome wire is inexpensive and readily available from electrical wholesale suppliers. The wire should be cut so that the end is square and not chisel-shaped and this can be achieved best by cutting the lengths with a pair of large, sharp scissors. The inoculating wire should be 10 cm in length and the wire is held more firmly in a standard loop holder if 0.5 cms of the proximal (loop holder) end is doubled back on itself. Nichrome wire will corrode with use and its tip will eventually become tapered as oxidised material is burnt off by the Bunsen burner. The wire should be replaced before it reaches this stage which significantly reduces the size of the inoculum achieved.

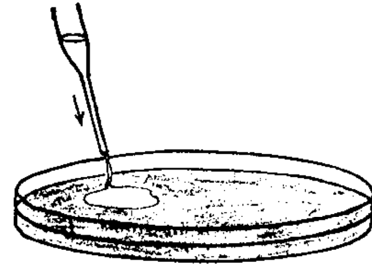
THE C.D.S. ANTIBIOTIC SENSITIVITY TEST



1. SAMPLE COLONY

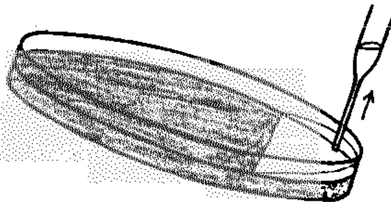


2. PREPARE SUSPENSION



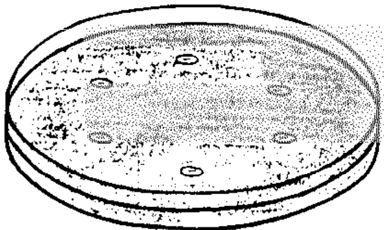
3. INOCULATE PRE-DRIED PLATE (2 hours, 37°C)

4. DISTRIBUTE INOCULUM BY ROCKING

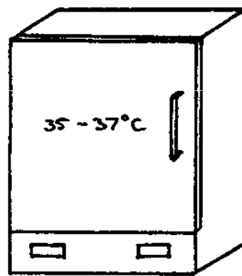


5. REMOVE EXCESS INOCULUM

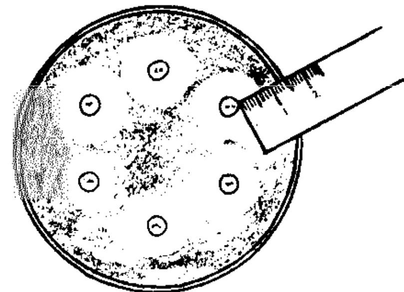
6. DRY, ROOM TEMPERATURE, 3/4 HOUR



7. LOAD PLATE WITH ANTIBIOTIC DISCS



8. INCUBATE FOR 18 HOURS



9. MEASURE ANNULAR RADII

ANNULAR RADIUS ≥ 6mm – SENSITIVE

ANNULAR RADIUS < 6mm – RESISTANT

(NOTE EXCEPTIONS)

10. INTERPRET ZONE SIZES

3. Method

The steps in performing the CDS test are represented diagrammatically on the opposite page. *The need to carry out each step precisely as described is emphasised* and the details of the method are set out below.

3.1 Preparation of sensitivity agar plates

The sensitivity test agar is reconstituted strictly according to the manufacturer's directions and 20 ml of agar is aseptically dispensed into each 90 mm petri dish so that the depth of agar in each plate is approximately 4 mm. For streptococci and pneumococci horse blood in a final concentration of 5% is added to the medium. The plates are stored in the refrigerator before use and are used within seven days of pouring. On the day of the test the plates are dried by inverting each plate with the lid removed in an incubator at 35-37°C for two hours.

3.2 Preparation of inoculum

The inoculum is prepared from the primary isolation plate or from a subculture of each strain grown preferably on a blood agar plate. The straight nichrome wire is used to prepare the inoculum. A typical colony of at least 2 mm in diameter is selected and the wire, after flaming, is cooled by touching several times the agar surface free of colonies. After cooling the wire is passed vertically *once* through the typical colony until the wire touches the agar surface but does not penetrate the surface of the agar. The wire is withdrawn from the colony and transferred to a labelled test tube containing 2.5 ml of sterile normal saline. The wire is rotated back and forth at least ten times with the tip of the wire in contact with the bottom of the test tube under the saline (this suspension contains 10^6-10^7 viable cells/ml). In the preparation of the inoculum for *Streptococcus pyogenes* and *Streptococcus pneumoniae* the method is modified so that the straight wire is passed through *three* colonies before it is transferred to the saline.

3.3 Inoculation of sensitivity plates

Each sensitivity plate is inoculated by flooding the plate with the saline suspension of the organism under test and rocking the plate several times to ensure that the suspension is evenly distributed over the plate. The plate is tilted to drain and the excess inoculum is drawn off with a Pasteur pipette. Each plate is allowed to dry with the lid off at room temperature for up to but not exceeding three quarters of an hour. The plate is not inverted or covered during drying. When the plate is dry no more than six discs are placed on each plate. If a dispenser or

Multodisk are not used the discs are placed with forceps in a circular fashion so that the disc centres are equidistant and each disc centre is approximately 15-20 mm from the edge of the plate. Contact with the agar of each antibiotic disc is ensured by gently pressing on each disc with the tips of a pair of sterile forceps. The plates are inverted and placed immediately in an incubator with a temperature strictly controlled at 35-37°C and incubated overnight (approximately 18 hours) in *air*.

3.4 Control organisms

The same sensitivity test is performed on a sensitive control organism with each batch of test strains. The Oxford strain of *Staphylococcus aureus* (NCTC.6571) is used for Gram-positive organisms and a sensitive strain of *E.coli* (NCTC.10418) for Gram-negative species. A control strain of *Pseudomonas aeruginosa* (NCTC.10490) is used in testing *Pseudomonas* species. (These organisms can be purchased from the Culture Curator, School of Microbiology, University of N.S.W., Kensington, 2033, telephone (02) 662-3835).

3.5 Reading the test

The annular radius of the zone of inhibition of each antibiotic is measured by either a metric rule or Vernier caliper with the plate inverted over a black surface and illuminated from above. The annular radius of the zone of inhibition is the distance between the edge of the antibiotic disc and the edge of confluent growth. Where a Multodisk is used the measurement is made where possible from the side of the disc opposite to the paper spoke.

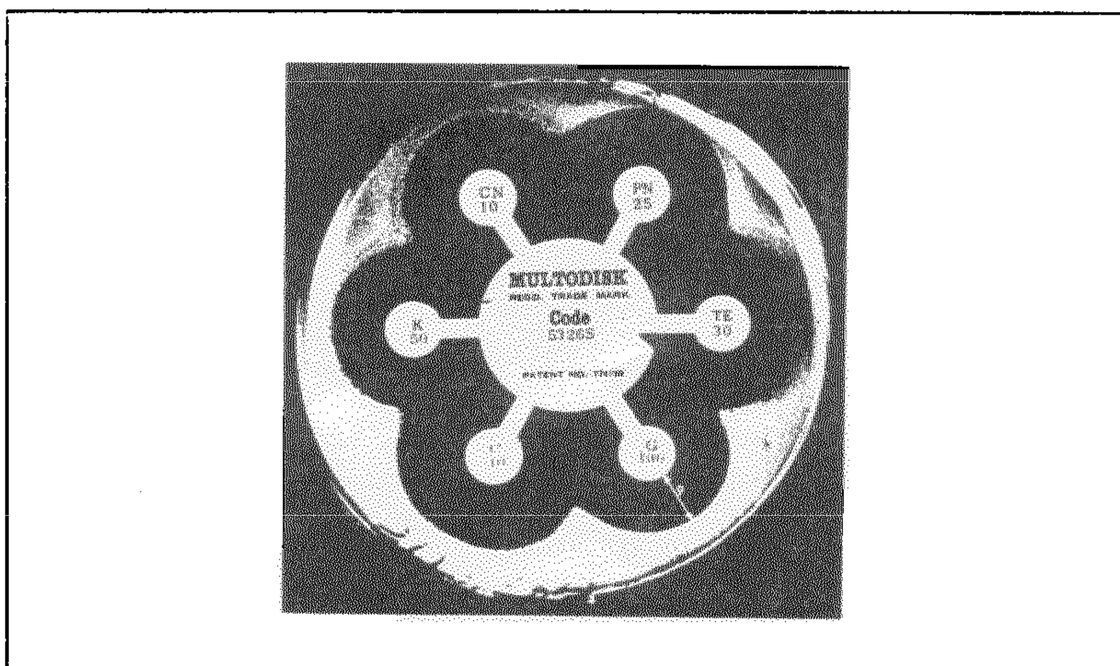


Figure I. Sensitivity test of the control strain of *E.coli* with an Oxoid Multodisk. The annular radius is measured as shown by the arrow marked "a" with the sulphonamide disc (G.300)

3.6 Interpretation of results

An annular radius of 6 mm or more indicates that the organism is sensitive. Where the annular radius is less than 6 mm or there is an absence of an inhibitory zone the organism is regarded as resistant.

Special Points of Interpretation

Pseudomonas species.

With gentamicin and polymyxin a zone size of 4 mm or more annular radius indicates a sensitive strain.

Staphylococcus aureus

Confirmatory tests of penicillin or methicillin sensitivity which are described on pages 18,20 should be carried out on strains of staphylococci where the zones of inhibition have radii between 5-7 mm with either antibiotic.

Vancomycin resistance in *Staphylococcus aureus*

This has not been encountered in our experience to date. However, despite the use of a 60 µg disc this antibiotic diffuses poorly in agar and sensitive strains may have an annular radius as small as 4 mm.

Enterobacter species and *Citrobacter freundii*

All strains of *Enterobacter* spp. and *Citrobacter freundii* so far tested against cefotaxime and moxalactam were demonstrated to produce an inducible β-lactamase which inactivated both antibiotics. In addition all strains yielded resistant variants at a frequency of 10^{-6} to 10^{-7}. The resistant variants produce large amounts of β-lactamase which inactivates the cephalosporins and this is reflected in a considerable increase in the MIC of this antibiotic group. For these reasons members of the *Enterobacter* spp. and *Citrobacter freundii* should be regarded as resistant to cephalosporins even though this is not always apparent on primary testing and *Enterobacter* spp. and *Citrobacter freundii* are not included in the CDS test of cephalosporin sensitivity.

Co-trimoxazole (Bactrim®, Septrin®) sensitivity

The sensitivity of Gram-negative organisms to sulphonamide and trimethoprim is tested with a separate disc for each agent. A disc containing a mixture of these agents *is not used* as the results with a combined disc are fundamentally uninterpretable and may be misleading. If laboratories wish to convey in the sensitivity report an indication of the organism's sensitivity to co-trimoxazole in addition to its sensitivity to sulphonamide and trimethoprim this can be done by using the results observed with the individual agents. Sensitivity to both drugs can be regarded as indicating sensitivity to co-trimoxazole and organisms resistant to either sulphonamide or trimethoprim should be reported as resistant to co-trimoxazole.

Table II. **Disc content, MIC of sensitive strains and the mean and standard deviation of annular radii of the reference strains of antibiotics used in the CDS Test**

Antibiotic and disc content (µg)		MIC of sensitive strains (mg/l)	Mean (± S.D.) of annular radii of reference strains (mm)
Gram positive			
Benzylpenicillin	(1.5 U)	≤ 0.05	10.0 ± 0.6
Tetracycline	(30)	≤ 1.0	11.2 ± 0.5
Erythromycin	(15)	≤ 0.25	11.1 ± 0.7
Chloramphenicol	(30)	≤ 6.25	9.4 ± 0.7
Kanamycin	(50)	≤ 3.1	7.6 ± 0.5
Methicillin	(10)	≤ 3.1	8.0 ± 0.6
Gentamicin	(10)	≤ 0.6	7.9 ± 0.7
Fusidic acid	(2.5)	≤ 0.5	9.5 ± 1.1
Rifampicin	(1)	≤ 0.3	9.8 ± 1.0
Vancomycin	(60)	≤ 2.0	6.9 ± 1.0
Gram negative			
Ampicillin	(25)	≤ 6.25	9.0 ± 0.6
Tetracycline	(30)	≤ 3.1	8.6 ± 0.7
Chloramphenicol	(30)	≤ 6.25	10.7 ± 1.1
Sulphonamide	(300)	≤50.0	9.7 ± 1.2
Trimethoprim	(2.5)	≤ 1.6	9.6 ± 0.7
Nitrofurantoin	(200)	≤25.0	8.7 ± 0.7
Nalidixic acid	(30)	≤ 3.1	9.1 ± 0.5
Kanamycin	(50)	≤ 6.25	8.6 ± 0.6
Gentamicin	(10)	≤ 0.6	8.6 ± 0.7
Tobramycin	(10)	≤ 0.6	7.6 ± 0.5
Netilmicin	(30)	≤ 1.25	9.8 ± 0.8
Amikacin	(30)	≤ 5.0	8.5 ± 0.5
Cefotaxime	(5)	≤ 0.8	12.5 ± 0.9
Moxalactam	(5)	≤ 0.8	11.4 ± 0.8
Pseudomonas			
Carbenicillin	(100)	≤50.0	15.3 ± 1.3
Ticarcillin	(75)	≤50.0	15.5 ± 1.6
Piperacillin	(50)	≤12.5	13.1 ± 1.0
Gentamicin	(10)	≤ 5.0	5.9 ± 0.2
Polymyxin	(300 U)	≤ 1.25	6.5 ± 0.4

4. Antibiotics used in testing

4.1 Range of antibiotics for which test has been calibrated

The antibiotics for which the CDS method has been calibrated are shown in Table II (on the opposite page). Included in this table is the potency of each of the antibiotic discs which was used in the initial calibration procedures and therefore is the only acceptable strength which can be used in practice. Also listed is the minimum inhibitory concentration of each antibiotic which equates to a "sensitive" zone size with Gram-positive, Gram-negative species and *Pseudomonas aeruginosa*. A series of measurements of inhibitory zones observed with the three control organisms (*Staph. aureus* NCTC.6571, *E.coli* NCTC. 10418 and *Ps.aeruginosa* NCTC.10490 were carried out in our laboratory and the mean annular radius (± standard deviation) with each appropriate antibiotic is included.

4.2 Use of a representative disc to indicate sensitivity to an antibiotic group

With many antibiotics it is necessary to test only one representative of a group of related antibiotics and the results with the representative disc can be taken to indicate sensitivity to other members of the group. The relationship of results with the representative antibiotic disc to other members of the group is shown in Table III.

Table III. Antibiotic sensitivity indicated by the results achieved with a representative antibiotic disc

Representative antibiotic (Disc content)	Antibiotic sensitivity indicated by representative
Gram positive	
Benzylpenicillin (1.5 U)	Benzylpenicillin Phenoxymethylpenicillin Ampicillin Amoxycillin Cephaloridine
Tetracycline (30 µg)	All tetracyclines including doxycycline and minocycline
Erythromycin (15 µg)	Erythromycin Lincomycin Clindamycin
Methicillin (10 µg)	Methicillin Cloxacillin Flucloxacillin Cephalothin
Gram negative	
Ampicillin (25 µg)	Ampicillin Amoxycillin
Sulphafurazole (300 µg)	All sulphonamides
Polymixin (300 U)	Polymixin Colistin
Tetracycline (30 µg)	All tetracyclines including doxycycline and minocycline

The representative disc specified in the method must be used and the substitution of this representative by antibiotic discs of other members of the group will yield invalid results in the CDS test, e.g. when testing *Staph. aureus* the results with a methicillin disc of 10 µg can be interpreted to indicate accurately in addition the sensitivity of cloxacillin and flucloxacillin. However, neither cloxacillin nor flucloxacillin discs have been used in the calibration of the CDS method and their use in the test is therefore unacceptable.

4.3 Suggested combinations of antibiotic discs

It is unnecessary and generally not feasible for laboratories to test every significant isolate against all the antibiotics listed in Table II. It is our practice to test isolates to no more than six antibiotics in the first instance. Tests of additional antibiotics are carried out only if the pathogen is resistant to all of the primary antibiotics or clinical circumstances warrant using an antibiotic other than those included in the primary set. The combinations used at The Prince of Wales Hospital are set out in Table IV.

Table IV. Suggested combinations of antibiotics for sensitivity testing by the CDS method

Primary set		Secondary set (for multiresistant organisms)
Gram positive species		
Benzylpenicillin (1.5 U)		*Staph.aureus*
Kanamycin (50 µg)		Rifampicin (1 µg)
Tetracycline (30 µg)		Fusidic acid (2.5 µg)
Erythromycin (15 µg)		Vancomycin (60 µg)
Chloramphenicol (30 µg)		Gentamicin (10 µg)
Methicillin (10 µg)		
Gram negative species (excluding Pseudomonas species)		
Urinary infection	*Systemic infection*	
Ampicillin (25 µg)	Ampicillin (25 µg)	Cefotaxime (5 µg)
Sulphafurazole (300 µg)	Sulphafurazole (300 µg)	Moxalactam (5 µg)
Gentamicin (10 µg)	Gentamicin (10 µg)	Kanamycin (50 µg)
Nalidixic acid (30 µg)	Tetracycline (30 µg)	Tobramycin (10 µg)
Nitrofurantoin (200 µg)	Chloramphenicol (30 µg)	Amikacin (30 µg)
Trimethoprim (2.5 µg)	Trimethoprim (2.5 µg)	Netilmicin (30 µg)
Pseudomonas species		
Carbenicillin (100 µg)		
Ticarcillin (75 µg)		
Piperacillin (50 µg)		
Gentamicin (10 µg)		
Polymixin (300 U)		

These combinations have been found useful not only for providing a sensitivity report but for epidemiological studies within the hospital. For example, although Gram-positive organisms are tested against kanamycin the result generally is not reported but this antibiotic is used to identify and trace aminoglycoside resistant *Staph.aureus* in the hospital environment. Laboratories may find that they have different requirements and wish to use a different combination to that shown in Table IV. This practice does not invalidate the test provided the

substitution is with an antibiotic which is listed in the relevant section in Table IV; that is, antibiotics listed for Gram-negative species only cannot be used to test Gram-positive species, and vice versa. For example, the ampicillin disc cannot be used to test Gram-positive species as its use will result in serious errors in interpretation of sensitivity. Similarly, if a tetracycline disc is included in a sensitivity test of *Ps.aeruginosa* it can, on occasions, give rise to an inhibitory zone but this zone has no clinical relevance and, as such, is not an indication of sensitivity.

5. Tests of unusual or fastidious pathogens

The CDS Antibiotic Disc Test is designed primarily to test the sensitivity of pathogens which are commonly isolated in a diagnostic laboratory. Organisms which have unusual nutritional requirements generally need to be tested by quantitative techniques such as the agar dilution method[2]. Nevertheless it is possible to derive some information in a limited sense by the use of simple techniques with a few fastidious pathogens and these techniques are described below.

5.1 *Streptococcus* species

All members of the genus can be tested by the CDS method using the modification given on page 6.

5.2 *Haemophilus influenzae*

Strains of *Haemophilus influenzae* may be unencapsulated (respiratory) or encapsulated, most commonly Type b. The respiratory strains may be associated with bronchitis and only rarely give rise to more serious systemic disease. On the other hand the encapsulated variety of *Haemophilus influenzae* is a common cause of meningitis and other serious systemic infections such as septic arthritis and epiglottitis. From the mid 1970's on, strains of *Haemophilus influenzae* Type b which were resistant to ampicillin were isolated from patients with serious systemic disease. The mechanism of resistance of the encapsulated strains is due almost exclusively to destruction of ampicillin by a β-lactamase. A similar β-lactamase can be found in some respiratory strains of *Haemophilus influenzae*. In addition we have found that some of these respiratory strains may have an intrinsic diminished sensitivity to ampicillin without production of the β-lactamase.[3]

It was found necessary to calibrate the CDS method to test mainly encapsulated strains of *Haemophilus influenzae*. The test is carried out on chocolate blood agar. This is prepared by heating at 80°C for ten minutes Columbia agar (Oxoid CM331) containing 8% horse blood. Only two antibiotic discs are used in the test and these are ampicillin *2 µg* and chloramphenicol *30 µg* otherwise the test is performed and interpreted precisely as described for the CDS test. A report of sensitivity to ampicillin is not issued until the results of disc testing have been confirmed by the performance of a chemical test for β-lactamase (see page 18). The CDS test can also be used to test respiratory strains of *Haemophilus influenzae* but the method will not demonstrate adequately those strains of *Haemophilus influenzae* which have a diminished sensitivity to ampicillin by an intrinsic mechanism.

Under no circumstances should a 25 μg disc of ampicillin be used for testing Haemophilus influenzae. Strains which produce β-lactamase and therefore are resistant to ampicillin can yield sizeable zones with a disc of this potency and this in turn can lead to a false report of sensitivity to the antibiotic with potentially disastrous consequences.

5.3 *Neisseria gonorrhoeae*

At present there is no satisfactory disc test for determining antibiotic sensitivity of gonococci. Resistance to penicillin in this species also may be intrinsic or due to the production of β-lactamase. It is probably sufficient for most diagnostic laboratories to test the isolates of gonococci simply for the presence or absence of β-lactamase by a chemical test (see page 18). Where more extensive testing of gonococci to either penicillin or other antibiotics such as tetracycline is required for epidemiological purposes a quantitative technique is necessary. Quantitative techniques for testing antibiotic sensitivity of gonococci are not without considerable technical problems and difficulties in interpretation. Laboratories which contemplate undertaking this method of testing the antibiotic sensitivity of gonococci are advised to contact the Reference Laboratory in their state. Further information also can be obtained from the coordinator of the Gonococcal Surveillance Programme, Dr. J. Tapsall, C/o the Microbiology Department of The Prince of Wales Hospital, Randwick, 2031, N.S.W.

5.4 Anaerobic bacteria

In our laboratory we have seldom found it necessary to test in detail sensitivity of anaerobic bacteria. We have relied more on the identification of the anaerobic species and the accepted susceptibility of the species to particular antibiotic agents, e.g. penicillin for *Clostridium perfringens*. Disc testing of anaerobes presents considerable technical problems and, in addition, patients with anaerobic infections are invariably treated with a broad combination of antibiotics prior to or irrespective of the laboratory's report. For example, in a patient with a pelvic abscess the laboratory not uncommonly receives its specimen as a result of drainage of the abscess (a procedure once considered to be adequate treatment in itself) and generally the patient, at the time of operation, is being treated with a mixture of antibiotics including ampicillin, aminoglycosides and metronidazole. In these circumstances it is doubtful that on a cost benefit basis many laboratories could justify the expense of setting up and maintaining anaerobic sensitivity testing on a routine basis. However, those laboratories which are interested in antibiotic disc testing of anaerobes are referred to the paper of Oitmaa and Benn[4].

6. Control measures in disc testing

6.1 Care and precision in technique

The most important control measure which can be adopted in the use of a calibrated disc test is an understanding that in practice the operator is in fact attempting to measure a minimum inhibitory concentration by maintaining a relationship which has been established previously between an inhibitory zone size and the MIC. The first consequence of this is that zone sizes observed with antibiotics or organisms for which the test has not been calibrated cannot be related to an MIC value and, therefore, have no meaning. Secondly, the relationship between MIC and zone size established by calibration can be maintained only by a faithful and precise reproduction of the conditions which were implemented at the time of the initial calibration. *Control of a calibrated disc test in practice therefore depends largely on an unquestioned use of the specified materials and care and precision in carrying out each of the steps described in the method.*

It would be unrealistic to assume that the technique of any disc test could not be improved, that better media would not become available or that cheaper or more convenient antibiotic discs could not be produced. However, the operator is locked in to the specified materials and methods by the basis of the test unless the test is recalibrated under the changed conditions. The author welcomes criticism and suggestions which could lead to improvement of the CDS test and, where necessary, the test would be recalibrated on the basis of these improvements. In the meantime users of the CDS test or any other calibrated disc test need to understand clearly that any modification is an invalidation unless accompanied by recalibration.

6.2 Media

The only medium which was used for all the calibration tests was Sensitest (Oxoid) and the validity of these tests will be disturbed with even minor variations to this medium. For example, Isosensitest (Oxoid), a similar medium to Sensitest, was not used in any of the calibration experiments and is not an acceptable substitute for Sensitest. Horse blood is added only for testing streptococci and pneumococci and horse blood Sensitest and Sensitest alone are not interchangeable.

The agar must be made up according to the manufacturer's specification and each 90 mm plate must contain 20 mls of agar. The agar depth should be approximately 4 mm and the gross weight of a freshly prepared sensitivity plate should be approximately 35 g. The prepared plates cannot be stored at $4^{o}C$ for longer than one week otherwise excessive drying out of the plates

occurs. Once the plates have been dried or pre-dried for two hours they must be used for the test and unused dried plates should be discarded. The storagê of previously dried plates in a refrigerator results in crazing and cracking of the agar and renders the plates unsuitable for use in the test.

6.3 Antibiotic discs

The improved technology and the stringent quality control measures used by reputable manufacturers of antibiotic discs today is associated with a consistently high performance of these products. Nevertheless there are still no regulatory control measures governing the sale of antibiotic discs in this country and discs which may not match the standard of those discs specified in the method (Oxoid and BBL) continue to be produced and sold. Laboratories attempting cost saving exercises should resist the temptation of substituting cheaper antibiotic discs for those specified in the CDS test.

Antibiotic discs should not be used after the expiry date stated and the manufacturer's recommendations for storage of the discs need to be followed closely to avoid the damaging effect of exposure of the discs to heat and humidity. The antibiotics which are the first to deteriorate are those of the β-lactam group, namely, benzylpenicillin, ampicillin and particularly methicillin. A careful watch on the zone sizes which are achieved each day with the discs of these antibiotics will give an early indication of any problem that the laboratory may have with the storage or maintenance of its antibiotic discs.

6.4 Inoculum

The straight wire technique is a simple and reproducible method of achieving an inoculum of a uniform size. The inoculum must contain from 10^6-10^7 viable organisms per ml and it should result in a uniform confluent growth on the sensitivity plate. If the operator is inexperienced in the technique or is in any doubt as to the size of the inoculum, this should be verified. Verification of the size of the inoculum is carried out as follows:

First the suspension prepared for the CDS inoculum has a barely discernible turbidity when compared carefully with an uninoculated tube of normal saline. This turbidity can be best seen when the test tube is viewed obliquely using transmitted light. The turbidity can also be confirmed using a spectrophotometer (Spectronic 20, Bausch and Lomb) on which the inoculum should have an O.D. of 0.02-0.04 when read at a wavelength of 640nm. Further verification of the inoculum size can be carried out by performing a viable count on the inoculum by the technique of Miles and Misra[9] as follows:-

> A pasteur pipette delivering 40 drops/ml is used to deliver one drop of the inoculum into 2.5 ml of normal saline. With a fresh Pasteur pipette this dilute suspension is thoroughly mixed and one drop is delivered into another tube containing 2.5 ml of normal saline. Again with a fresh pipette this 10^4 dilution of the original inoculum is thoroughly mixed and eight drops are delivered from a uniform height and evenly spaced on a dried sensitivity agar plate. After overnight incubation the colonies in each drop are counted. The size of the original inoculum is calculated by multiplying the average number of colonies/drop with $4x10^5$. An average of from 5 to 100 colonies/drop indicates a satisfactory conformity with the inoculum specified.

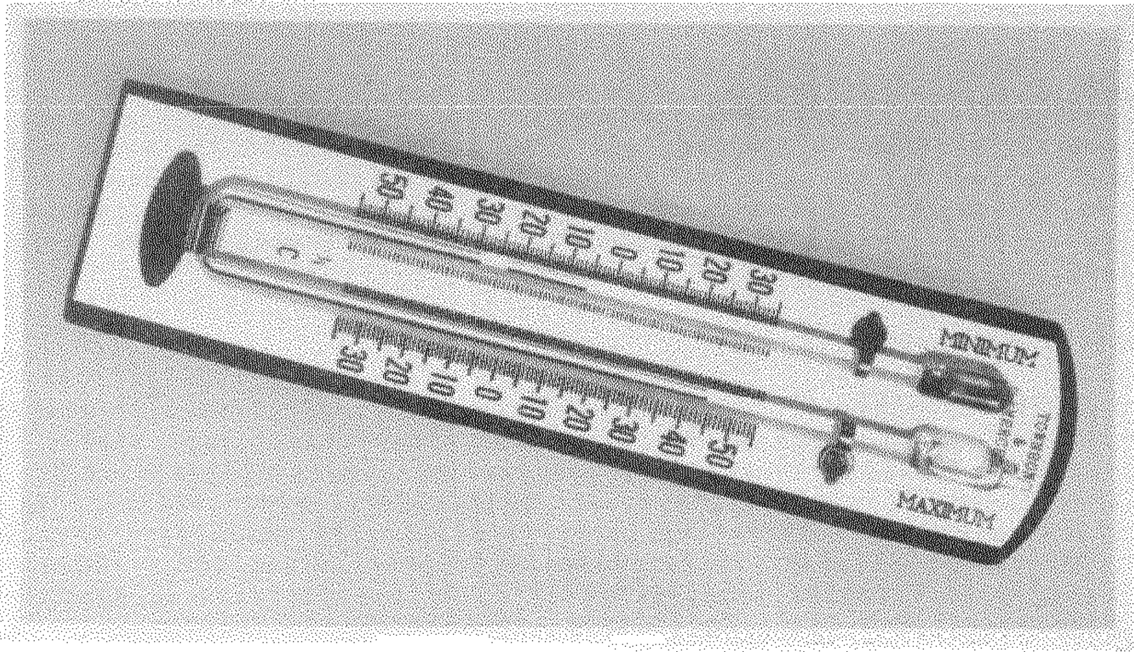
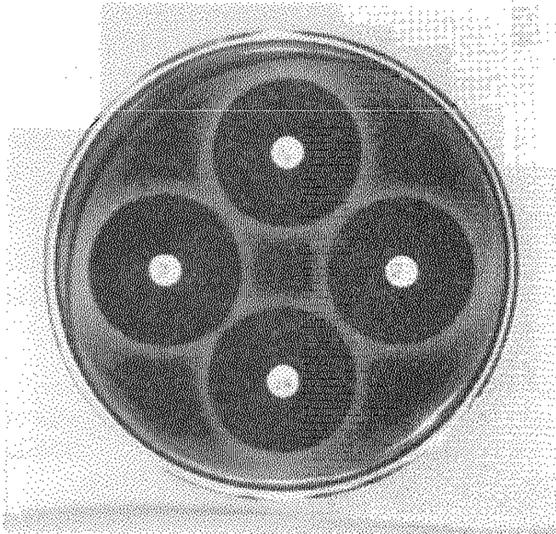
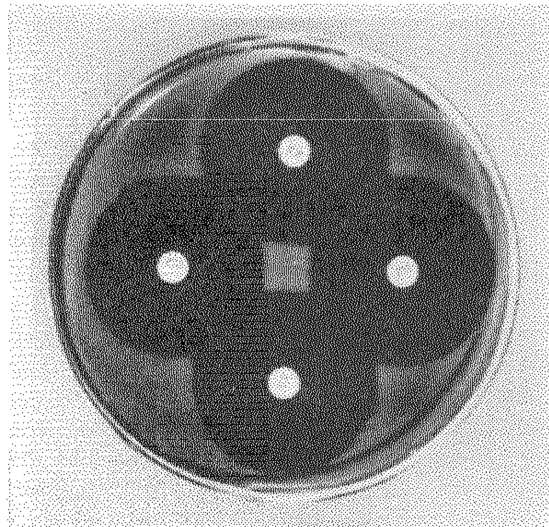


Figure II. A maximum/minimum thermometer. An inexpensive yet accurate means of monitoring the temperature of incubation. The thermometer should be placed on the shelf containing the sensitivity plates and it should indicate that the temperature is maintained at 35-37°C.



IIIa.



IIIb.

Figure III. An example of the effect of alteration of atmospheric conditions on the size of inhibitory zones.
In this example the zone size of the Oxford strain of *Staphylococcus* to tetracycline on Columbia blood agar in air (Plate IIIa), and in air + 5% CO_2 (Plate IIIb) was measured. The addition of 5% CO_2 to the environment increased the mean zone diameter from 27 mm to 33 mm.

6.5 Incubation of sensitivity plates

The temperature of incubation of the sensitivity plates must be carefully controlled to lie within the range of 35-37°C. The simplest and cheapest method of monitoring the incubator temperature is with a maximum/minimum thermometer (see Figure II on page opposite) which should be placed on the same shelf in the incubator as the sensitivity plates. The plates should be stacked in a way to allow free circulation of the air around them and the number of plates per stack should be limited to five. Crowding or overstacking the plates will result in a failure of the central plates to reach the required temperature for the test. The CDS test is carried out in *air*. A change in the gaseous environment, such as an anaerobic atmosphere or the addition of carbon dioxide, may have a profound effect on the size of the inhibitory zones (see Figure III on page opposite). The time of incubation is overnight (16-18 hours) and although a lawn of growth may be obvious within six hours there is often a considerable difference in the sizes of inhibitory zones with further incubation. Therefore, attempts to obtain a more rapid result than that which can be achieved with overnight incubation with a disc test can result in serious errors in the sensitivity report.

6.6 Standard sensitive strains

The sensitivity test on the Oxford *Staphylococcus* (NCTC.6571) for Gram-positive organisms and a sensitive *E.coli* (NCTC.10418) is generally carried out with each series of tests on the unknown strains. *Pseudomonas aeruginosa* (NCTC.10490) is used as a control strain for sensitivity tests of this species. Variation in zone sizes with the control strains are of value in indicating gross deterioration in the potency of batches of antibiotic discs, marked differences in media or generally poor technique of the operator. Table II (page 9) lists the mean annular radii and the standard deviations of measurements of inhibitory zones when the three control strains were tested against appropriate antibiotics. Laboratories could expect that their technique of carrying out the CDS test is satisfactory if their results with the control strains lie with + or - two standard deviations of the listed means. For example, the mean annular radius observed with benzylpenicillin is 10 mm (standard deviation ± 0.6) and the acceptable range of measurements would be from 8.8 mm to 11.2 mm.

It is important to recognise the limitations of the value of the use of control strains. These strains were selected originally because of their extreme sensitivity to the vast majority of antibiotics. As a result the sizes of the inhibitory zones with the reference strains is at the upper end of the sensitive zone range. In some cases the annular radii of the inhibitory zones with the control strains may be 12 mm or more, that is at least twice the radius which separates sensitive and resistant strains. Because of the mechanisms which govern diffusion tests variation in some factors may have considerably less effect on the size of larger zones than they do on zones of smaller radii. Changes in disc potency, for example, may be reflected by very little change in the zone size of the reference strain but can result in a gross and critical variation with the strains which have zones with radii close to the separation point of 6 mm. For

this reason we include a laboratory strain of a penicillin-resistant *Staph.aureus* as an additional control organism and we recommend this practice to other laboratories. Penicillin-resistant strains, although sensitive to methicillin, in general have a higher MIC than the Oxford *Staphylococcus* and consequently yield a smaller inhibitory zone around the methicillin disc than that observed with the Oxford strain. A change in the inhibitory zone around the methicillin disc with the penicillin-resistant strain is a much more sensitive indicator of variation in disc potency than is the change in zone size which is observed with the Oxford *Staphylococcus*. As stated previously, methicillin is generally the first antibiotic to deteriorate in a set of discs so that the results with this additional control strain and methicillin serve as a valuable guide in monitoring the conditions of storage and user handling of all the antibiotic discs in the laboratory.

7. Confirmation of resistance to penicillins

7.1 β-lactamase activity

Many organisms are resistant to the penicillins or cephalosporins because they elaborate an enzyme, β-lactamase (penicillinase), which destroys the antibiotic. Details of the various aspects of the role of β-lactamases in resistance to the penicillins and cephalosporins are so complex as to be outside the scope of this monograph. It is sufficient to observe here that there are a variety of β-lactamases, each with a different substrate profile, so that it is necessary to recognise that antibiotics which are regarded as β-lactamase stable in dealing with one particular species may not necessarily be stable to the β-lactamases elaborated by other species. For example, methicillin or cloxacillin are accepted as resistant to staphylococcal β-lactamase but these antibiotics may be destroyed by some of the β-lactamases elaborated by Gram negative species. In some species β-lactamase may be produced in large amounts at all times - "high constitutive production". With other species such as *Staphylococcus aureus* the organism needs to be growing in the presence of a penicillin or cephalosporin before high levels of β-lactamase are "induced". In addition, in some species the enzyme may be relatively easily detected because it is found in considerable amounts extracellularly, while in others the enzyme is cell bound but is produced in sufficient quantities to endow the organism with a high level of resistance to a particular penicillin or cephalosporin. The β-lactamase story is still more complicated, particularly in Gram negative species, by the demonstration with sensitive methods of β-lactamase detection of the presence of low levels of β-lactamase in organisms which appear to be particularly sensitive to susceptible β-lactam antibiotics. Despite the complexities of β-lactamase production, in practice tests of β-lactamase activity are simple and are worthwhile confirmatory tests of resistance to the penicillin provided that the tests are well controlled and the interpretation is restricted to confirmation of resistance in *Staph. aureus*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*.

7.2 Chemical tests for penicillinase production

Chemical tests for penicillinase production fall into three categories. The first are those which demonstrate a fall in pH of a strong penicillin solution as a result of its conversion to penicilloic acid by β-lactamase. pH change is generally detected by a simple indicator such as Andrade's indicator or phenyl red. Paper strips impregnated with penicillin and an indicator are available commercially and these are claimed to be a simple and cheap method of confirming β-lactamase activity. Although our

experience with these strips is limited there is some concern that under conditions of the test some strains of *Staph.aureus* may not produce sufficient penicilloic acid to yield a definite colour change in the indicator.

The second group of tests are based on the method of Foley and Perret[5] which is more specific in that it relies on decolourisation of a starch-iodine mixture by penicilloic acid. The test is more difficult to perform and the reagents need to be freshly prepared for each test.

The third and preferred test is the nitrocefin test[6]. Nitrocefin is a chromogenic cephalosporin which is highly susceptible to β-lactamase and which, when degraded, changes colour from yellow to red. Nitrocefin is now commercially available (Oxoid Ltd., SR.112) but unfortunately it is very expensive. Nitrocefin solution needs to be made up and stored according to the manufacturer's instructions. Our experience is that made up solutions of nitrocefin will keep for at least a month provided they are kept at less than 4°C in a bottle wrapped in aluminium foil. Various ways of conducting the nitrocefin test are described in the package insert but we have found the clearest method of demonstrating β-lactamase activity is to suspend a colony in one or two drops of nitrocefin solution contained in a microtitre well or Durham tube. The test is incubated for no more than 30 minutes at 37°C and a positive reaction is indicated by change in colour of the solution to deep red.

Whichever of the chemical methods of demonstrating β-lactamase activity is used it is always necessary to include a positive and negative control strain with each test and to follow the steps specified in the test carefully. Also, because the β-lactamase of *Staph.aureus* requires induction, chemical tests may yield equivocal results with some strains. However, a much stronger reaction is observed if the test is performed on the growth taken from the edge of the zone around the methicillin disc of the primary sensitivity plate. This improved reaction arises because methicillin is a highly effective inducer of β-lactamase activity with this species. Neither *Haemophilus influenzae* nor *Neisseria gonorrhoeae* need prior induction before carrying out chemical tests for β-lactamase activity.

7.3 Penicillin zone edge of *Staph. aureus*

The simplest demonstration of the production of β-lactamase which results in resistance to penicillin is observed with *Staph.aureus* in the disc sensitivity test itself. The effect of the destruction of penicillin with resistant strains is evidenced when the colonies on the edge of the inhibitory zone surrounding the penicillin disc are examined with a hand lens (x 10). The colonies are of normal size and shape and retain their opacity and the growth ends in complete inhibition. The zone edge, although it may be ragged, is sharp and well defined. Penicillin-sensitive strains on the other hand have inhibitory zones with a hazy edge due to the progressive reduction in size and substance of the colonies as the area of total inhibition is approached.

The effect of β-lactamase on the penicillin zone edge is specific to *Staph. aureus* and observation of this phenomenon cannot be applied to other species. In Gram negative species, particularly, there may be factors other than β-lactamase which influence the character of the zone edge around the ampicillin disc and the presence of a sharp edge does not indicate specifically resistance to the antibiotic mediated by β-lactamase production.

7.4 Confirmation of methicillin resistance in *Staph. aureus*

Several tests which confirm the resistance of staphylococci to methicillin are available and each relies on a change in conditions of the disc test which results in a more adequate demonstration of the resistance of the staphylococcus to this antibiotic. Under these conditions there is a marked reduction in the size of the zone of inhibition surrounding the methicillin disc with methicillin-resistant strains but not with methicillin-sensitive staphylococci. In Annear's method[7] the sensitivity test is carried out at 30°C and Barber[8] recommended the addition of 7% sodium chloride to the sensitivity test agar for the confirmatory sensitivity test. A practical modification of Barber's method which is used in our laboratory is to carry out a sensitivity test to methicillin in the usual way on mannitol salt agar. This medium is readily available from commercial sources (Oxoid Limited CM.85, BBL 11407) and on this medium, independent of the size of the inoculum or temperature of incubation, methicillin-resistant strains yield no zone around a methicillin disc. An additional advantage is that the yellow colour change of the indicator produced in the medium by this species confirms a positive coagulase test in identification of the organism as *Staph. aureus*.

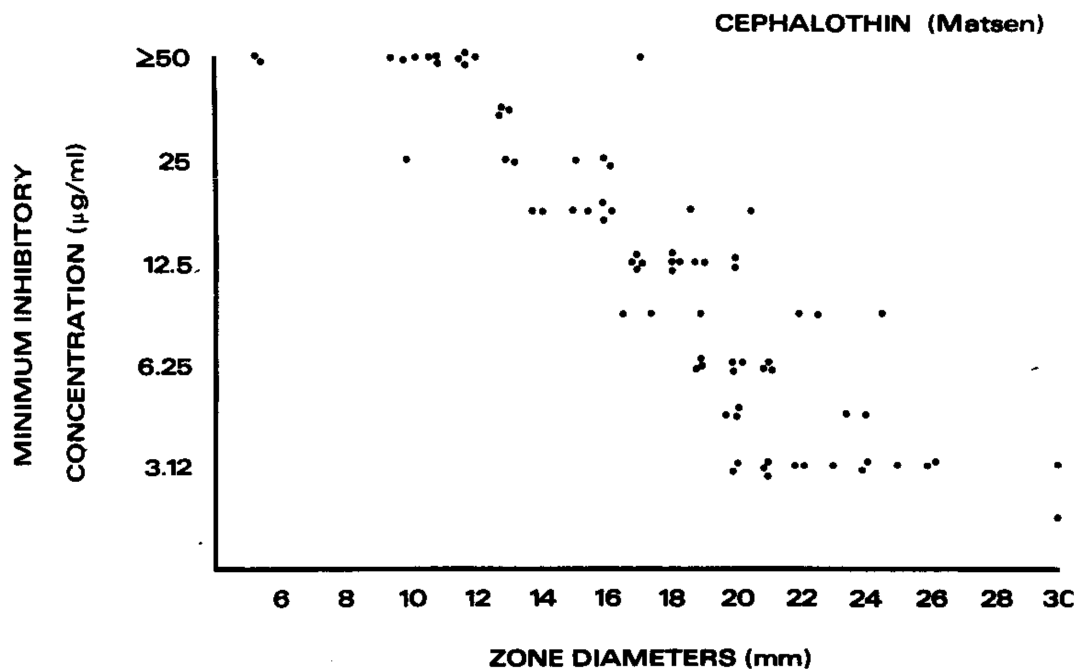


Figure IV. Results of calibration experiments with cephalothin tested by the Kirby-Bauer technique. There is considerable overlap of zones of similar size with strains of significantly different MICs. (Extracted from Matsen, J.M. *et al*, 1969. Antimicrob. Agents Chemother., 9: 445).

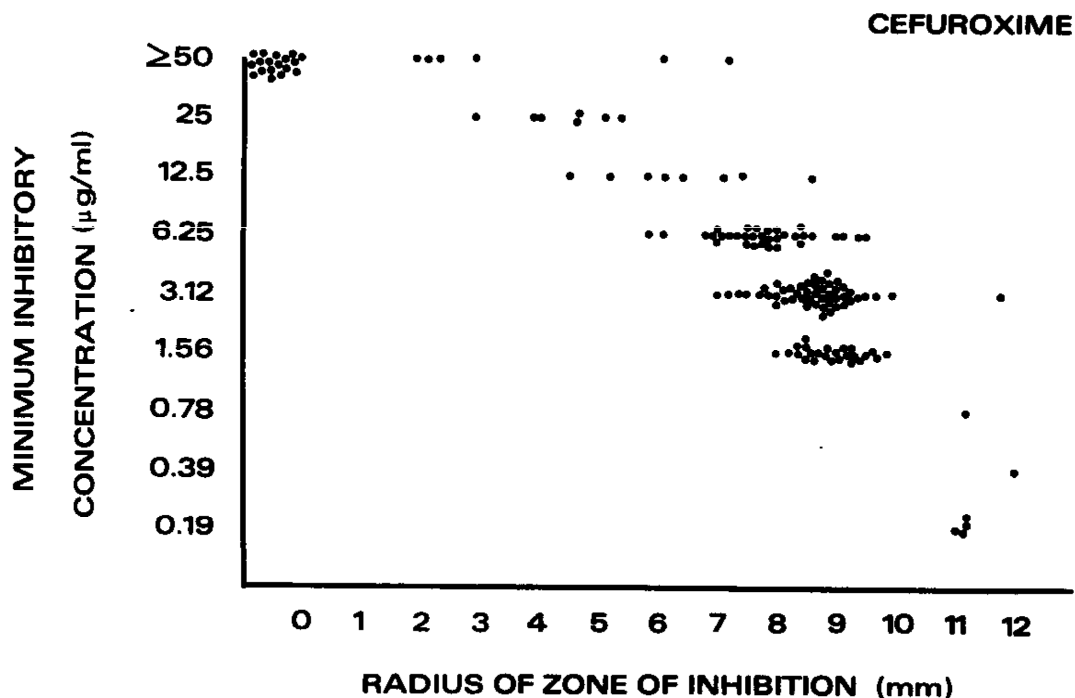


Figure V. An unacceptable result of attempted calibration of the CDS test with cefuroxime. There is a continuous distribution of MICs and similar zone sizes were observed with strains with up to a 32-fold difference in MIC.

8. Problems and pitfalls of disc sensitivity testing

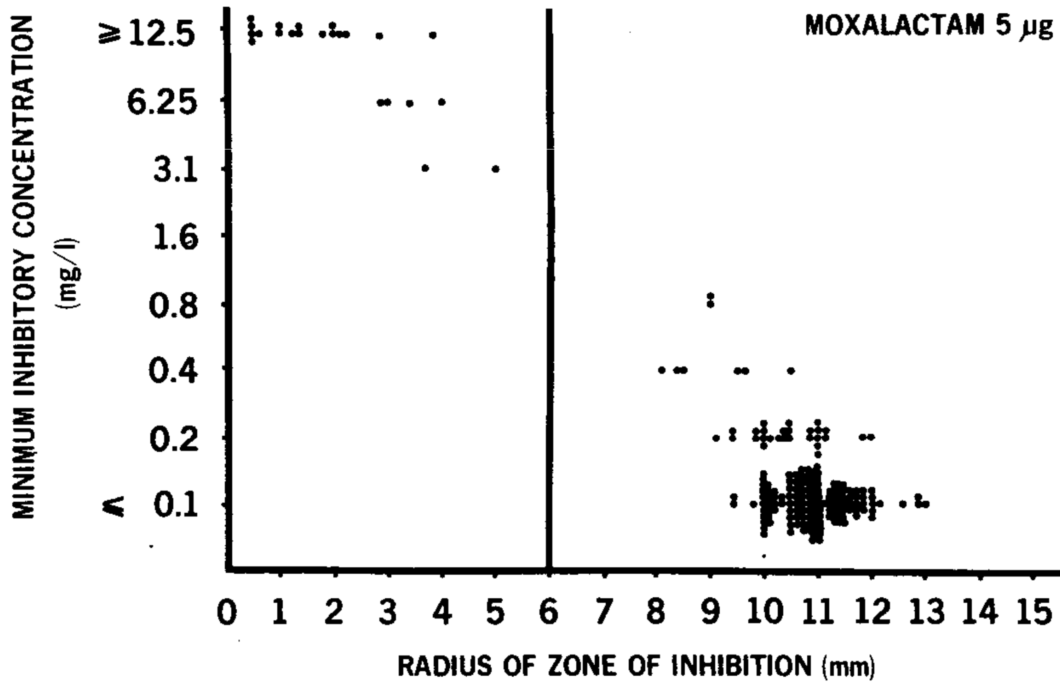
Calibrated disc tests have been shown to be the most inexpensive and the simplest methods for the determination of antibiotic sensitivity. The results of calibrated disc tests are highly reproducible in practice and, up to the present, they are the only methods which have been shown can be applied generally by diagnostic laboratories irrespective of the resources available to these laboratories. The materials used in the CDS test are now highly standardised and the test itself was designed to ensure that the results it yields can be related to those obtained by internationally accepted quantitative methods. However, disc methods do have their limitations and these are discussed below.

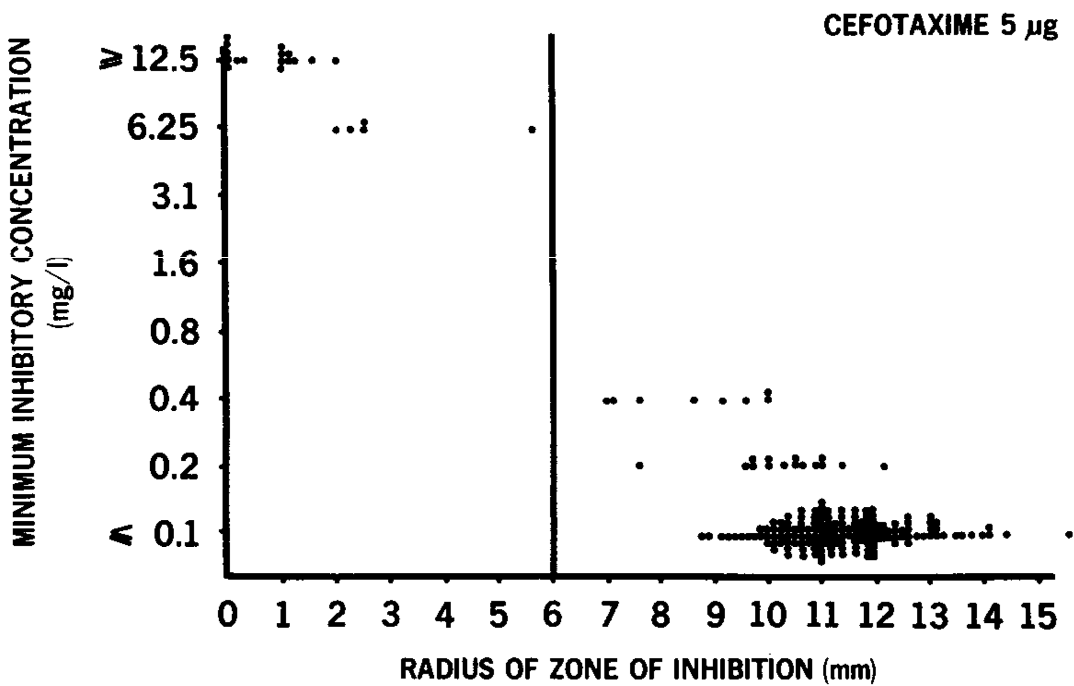
8.1 Limitations of a disc test

Problems with a calibrated disc test can arise in two ways; the first is the failure of an operator to follow the method precisely as described. This aspect has been discussed in detail in the description of the CDS test and can only be emphasised once more. The second problem arises from a failure to recognise the limitations of a calibrated disc test. Specific mention already has been made of the groups of organisms which can be tested by the CDS method. Disc methods, in general, are inappropriate for testing slow growing pathogens and attempts to test these organisms by any disc method usually yield inaccurate results.

Another limitation of disc tests is that not all antibiotics can be tested accurately by these methods. Although there was an appreciation of this limitation in the early development of calibrated disc tests it is now commonly ignored in the description of methods other than the CDS test. Originally the basis of all calibrated disc tests was that they could be applied only where the quantitative sensitivity of organisms to a particular antibiotic is distributed in a bimodal fashion. That is, within a species there are two groups of organisms, a sensitive group where the MIC of the antibiotic is low and a resistant group where the MIC is considerably higher. Fortunately in the development of sensitivity testing this was the usual mode of distribution with most species and practically all antibiotics. However, with more recently developed antibiotics, particularly the cephalosporin group, this bimodal distribution is less common and quantitative sensitivities can be distributed in a continuous fashion. When determination of quantitative sensitivity to these antibiotics is carried out by the usual method of twofold dilution of the antibiotic a proportion of the species is found to be inhibited at each dilution. As a result there is a more or less even distribution of MICs varying from very low to very high. An examination of graphic representations of the relationship of inhibitory zone sizes to the MICs reveals a considerable overlap of zones so at the very best adjacent MICs may yield similar sized inhibitory zones.

This overlapping of zone sizes is not restricted to any particular method of disc testing and Figures IV and V (on the page opposite) show





Figures VI and VII. Successful calibration of moxalactam and cefotaxime. With both cephalosporins the MICs essentially distribute in a bimodal fashion and with a 5 µg disc there is a clear distinction of the zones representing high and low MICs.

an extreme lack of discriminatory zones when calibration was carried out by the Kirby-Bauer method and the CDS test with two different cephalosporins.

It also can be shown that the overlap of zone sizes with different MICs would be aggravated by the variation introduced by different operators when the disc test is used in practice. Whilst others have chosen to ignore the inability of disc tests to accurately discriminate sensitivities with a continuous distribution of MICs and overlapping inhibitory zones the CDS test specifically excludes these antibiotics where this occurs. For example, of the several cephalosporins where calibration was attempted only two (cefotaxime and moxalactam) yielded a distribution of quantitative sensitivities which could accurately be separated by the disc technique. These are the only two cephalosporins included in the method and the results of calibration with moxalactam and cefotaxime are shown graphically in Figures VI and VII (on the page opposite).

The sensitivity patterns of *Enterobacteriaceae* to moxalactam and cefotaxime were very similar in the strains that have been tested so far. For this reason, at present, laboratories who do not wish to test both cephalosporins could interpret the results with one of the two to indicate sensitivity to the other cephalosporin. However, it is possible with use of these drugs differences in sensitivity may arise and it will be necessary to include both cephalosporins in the disc test.

Sensitivity to cefotaxime or moxalactam does not necessarily indicate sensitivity to other cephalosporins, so that neither drug can be regarded as a representative disc of the cephalosporin group of antibiotics in general. Those laboratories which find the need to test other cephalosporins such as cephamandole, cephalexin or cefoxitin must use a quantitative technique such as Buckle's Agar Dilution Method[2].

8.2 Intermediate sensitivity

In the CDS method the division of antibiotic sensitivity is dichotomous and no attempt is made to grade the categories of sensitivity into other than "sensitive" or "resistant". Whilst other disc methods further divide those strains which are not sensitive into such categories as "resistant" and "intermediate" or "resistant" and "moderately resistant" this concept was rejected for several reasons in the development of the CDS method. As stated earlier the distribution of sensitivities is bimodal with the majority of antibiotics and very few strains are found with an "intermediate" type of resistance. Secondly, separation to so-called intermediate resistance from full resistance is beyond a disc method's capacity to discriminate between strains with relatively close quantitative sensitivities. The reason for this was shown earlier in the discussion on the types of distribution of antibiotic sensitivity. The irrationality of the use of the intermediate category is obvious from an examination of the interpretative tables of those methods where this category is accepted. In the Kirby-Bauer method, for example, the acceptable day to day variation in zone sizes with all antibiotics is considerably larger than the range of zone sizes specified as indicating an intermediate sensitivity.

A further and important objection to the use of the intermediate category arises with those organisms where resistance is mediated by an enzymic destruction of the antibiotic. For example, although *Staph.aureus* is highly resistant to penicillin because of its destruction by β-lactamase, the dynamics of the disc test are such that the test may not demonstrate this resistance to its full extent. As a result inhibitory zones are produced which could be easily misinterpreted as indicating "intermediate" sensitivity.

In those circumstances where laboratories wish to grade an antibiotic sensitivity into categories more detailed than either sensitive or resistant it is necessary for them to adopt some method of quantitative estimation. However, the results obtained by these methods in itself create a problem in interpretation which relates to the ultimate use of the results of sensitivity tests. If the aim of carrying out the test is to make a contribution to the prediction of the likely outcome of an infection when the patient is treated with a particular antibiotic, this prediction should then be based hopefully on a well established correlation between clinical response and the MIC of the antibiotic against a specific pathogen. Admittedly this correlation has not been established directly with all antibiotics and in some cases is arrived at by various indirect methods and reasonable assumptions. The important point, however, is that such correlations have been achieved only between sensitive MICs and success on the one hand and resistant MICs and failure on the other. Correlations of clinical response to intermediate resistance are rare or non existent, thus from a clinical point of view this term has little relevance to treatment of a patient.

8.3 Common pitfalls of disc sensitivity testing

Disc methods of antibiotic testing are similar to any other tests performed in the laboratory in that despite careful adherence to the technique unexpected situations can arise which may lead to error or cause difficulty in interpretation of the result. The most common pitfalls encountered in the CDS test and, hopefully, the means of overcoming or avoiding them are listed below.

8.3.1 Errors in recording results

A small but significant number of errors occurs in recording the results of otherwise technically adequate sensitivity tests. These errors most commonly arise when the results for one antibiotic are substituted for another antibiotic tested on the same plate. The discs should always be loaded in the same order in the dispenser and the generic name checked on the side of the disc container. Laboratory staff should acquaint themselves with the manufacturer's code letters printed on the antibiotic disc as these symbols often refer to the proprietary name of the antibiotic and not the generic term which is more commonly used in laboratories. For example, Oxoid's code for ampicillin is P.N. (Penbritin ®) and sulphafurazole discs are labelled G (Gantrisin ®).

If sensitivities are reported by filling in the abbreviations "S" or "R" for sensitive or resistant in a preprinted format, the order of antibiotics on the form should be the same as that on the sensitivity plate. A clerical error is much more likely to arise when the forms are completed in a non-sequential fashion. The letters "S" and "R" have become acceptable abbreviations of sensitive and resistant and in cases where the abbreviations are used it is preferable that somewhere on the report is a key indicating what the letters mean. Plus or minus signs are ambiguous as an indication of antibiotic sensitivity and will only confuse the recipient of the report and should not be used.

8.3.2 The mixed culture

The use of a single colony in the CDS test reduces the risk of carrying out the test on a mixed culture. However, swarming organisms, particularly *Proteus* species, may contaminate the selected colony without this appearing obvious to the operator. A mixed culture is suspected if the organism under test appears to have a bizarre sensitivity pattern or when double zones are apparent when the plate is viewed through transmitted light. A Gram stain of the lawn of the sensitivity plate will demonstrate, for example, the contamination of *Staph.aureus* with *Proteus* species. When contamination of a Gram negative under test by an organism such as *Proteus* is suspected subculture of the lawn of growth on to MacConkey's agar will yield morphologically distinct colonies.

8.3.3 Sulphonamide and trimethoprim with *Proteus* species

Some strains of *Proteus* species will swarm into the inhibitory zones around sulphonamide and/or trimethoprim, especially if the plate is inadequately dried. This does not necessarily indicate resistance to these agents and the size of the zone in an area free of swarming should be measured or the edge of the lawn of confluent growth is sought and measured under the swarm by viewing the plate against a strong light.

8.3.4 Fine growth within the sulphonamide and trimethoprim inhibitory zone

The very fine growth in the inhibitory zones of sulphafurazole and/or trimethoprim should not be interpreted as indicating resistance. With the use of Sensitest agar it occurs rarely and is usually seen when the inoculum is too heavy or where there is a contamination of the plate by substances inhibitory to sulphonamide or trimethoprim such as when a direct sensitivity is performed. In these cases the test should be repeated using the correct inoculum obtained from a single colony.

8.3.5 Mucoid *Klebsiella* or *Pseudomonas*

An occasional strain of *Klebsiella* species or *Pseudomonas aeruginosa* may be so mucoid that the edge of the lawn on the sensitivity plate literally falls into the inhibitory zone around an antibiotic disc. Generally not all the zone is obliterated and it may be possible to achieve a measurement in at least some area of the inhibitory zone. If this is not possible the test should be repeated making sure the plate is dried according to the method and the plate should be incubated on a flat surface, base down (that is the reverse of the usual method of incubation of the plates inverted). If

successful measurement is not achieved in the repeat test it may be necessary, with a very rare extremely mucoid strain, to carry out testing by an agar dilution method. Although of little consolation to the disc test operator these rare strains present similar problems in interpretation of the result of quantitative testing by agar dilution methods.

8.3.6 Defining the zone edge

The CDS method stipulates that the annular radius is measured from the edge of the disc to the edge of *confluent growth*. A diminishing fine growth inside the edge of confluent growth is ignored and the edge of confluent growth is best demonstrated by viewing the plate in good light over a black background. When haemolytic streptococci are tested the edge of confluent growth and the edge of haemolysis do not necessarily coincide. The operator must be careful to measure the zone of inhibition of growth and not the zone of inhibition of haemolysis.

8.3.7 Failure of the test organism to grow on the sensitivity plate

Occasionally an organism may fail to grow, or grow poorly, on a sensitivity plate. This may be due to failure of the operator to cool the straight wire sufficiently before touching the selected colony or it can arise when Sensitest agar is deficient in a nutrient essential for the growth of that particular strain. This most commonly occurs with thymidine dependent strains of *E.coli*, particularly those isolated from urine. Where this dependency is demonstrated the disc test can be carried out by adding 5 drops (125 µl) of a stock solution of thymidine (5 mg/l) to the inoculating saline. This technique should be restricted to those strains where thymidine dependence is demonstrated before the test and it should not be added as a routine to the saline for other organisms.

8.3.8 Direct antibiotic sensitivity testing

It is appreciated that laboratories are often put under pressure to accelerate the production of results of sensitivity tests and as a result they may consider it necessary on occasions to carry out a direct antibiotic sensitivity test by preparing the inoculum from the specimen itself. Laboratories should understand clearly that in doing this they are jeopardising the accuracy of the disc test and in many cases the patient may be better off receiving an empirical combination of antibiotics whilst awaiting a delayed accurate report. Inaccuracies arise in direct antibiotic sensitivity testing for at least three reasons: first, the inoculum size contained in the specimen is difficult to control, secondly more than one organism may be present in the inoculum and, finally, the specimen itself may contain substances which interfere with the test.

In those situations where the performance of a direct sensitivity test is unavoidable the operator should attempt to assess the number of organisms in the inoculum on the basis of microscopy of the specimen, e.g. a specimen of heavily infected urine or an overnight growth of blood culture may need to be diluted up to one hundred-fold in normal saline to yield

a satisfactory inoculum. In these cases it is worthwhile to carry out the sensitivity test on both the undiluted and the diluted specimen. Unfortunately there is little that can be done about the specimen which contains fewer than the optimal number of organisms in the inoculum. It is the use of these latter specimens which most commonly results in the most serious error of sensitivity testing, i.e. the failure to detect an enzyme mediated resistance. Reports of direct antibiotic sensitivity testing should be guarded and issued as a provisional report only. Confirmation of the sensitivity determined conventionally on a pure culture should be regarded as mandatory.

9. The clinical significance of tests of antibiotic sensitivity

9.1 Antibiotic sensitivity testing in perspective

Irrespective of how complicated, or simple, methods of sensitivity testing are it is important to realise that all such tests are no more than *in vitro* examinations. The conditions under which sensitivity tests are conducted in no way resemble those which pertain *in vivo* and, in the past, attempts to simulate what goes on in the body when the patient is treated with an antibiotic by the use of complicated *in vitro* models have yielded little more information than can be derived from the simplest antibiotic sensitivity test.

Developments in sensitivity testing over the past 20 years have been mainly in the area of standardisation. It is now generally accepted that the reference to which the result of all antibiotic tests should relate is a minimum inhibitory concentration determined under standard conditions by an agar dilution technique at an inoculum of 10^4 colony forming units (International Collaborative Study Reference Technique[10]). It should be emphasised that the milieu and dynamics of the test and the size of the inoculum of this reference technique were not intended to bear any direct relationship to what occurs *in vivo* but the conditions were selected to achieve the most reproducible results of this *in vitro* test. What is achieved by this standardisation is a numerical result which can be reproduced universally which, in turn, enables comparisons to be made of correlations of sensitivity testing to the clinical response of antibiotic therapy. To place any more significance than this on the numerical values achieved by sensitivity testing is unscientific and, for example, any attempt to relate directly the antibiotic serum levels achievable with an antibiotic to its *in vitro* MIC as a criterion of sensitivity is viewing the test out of all perspective. *The only acceptable definition of a sensitive MIC of an antibiotic is that MIC which correlates with a successful clinical response.* Similarly the MIC of a resistant strain is that which is associated, in itself, with clinical failure of the antibiotic. This correlation of clinical response to laboratory results depends on an accumulation of world wide clinical experience with an antibiotic. The more established an antibiotic, therefore, the firmer will be the relationship between laboratory and clinical results. With the more recently developed antibiotics the difference between "sensitive" and "resistant" MICs is far less soundly based and this should be taken into consideration by laboratories in their interpretation of the results of antibiotic sensitivity testing.

9.2 Predictive value of antibiotic sensitivity tests

Studies of the value of carrying out sensitivity testing have shown that, overall, the response to antibiotic treatment is significantly better when the isolated pathogen is demonstrated to be sensitive to the antibiotic used compared to the response in those patients treated empirically or treated with an antibiotic which is demonstrated *in vitro* to be inappropriate. Despite this it is not unusual to see patients apparently respond to antibiotic therapy which seems inappropriate by laboratory testing. Similarly, failure of antibiotic therapy may occur despite an apparent *in vitro* sensitivity of the organism to the antibiotic used in therapy. Neither of these situations detracts from the overall value of antibiotic sensitivity testing as factors other than the organism's sensitivity to a particular antibiotic are commonly responsible for the apparent difference in what is observed *in vivo* and *in vitro*.

The high natural resolution rate which is observed with the majority of common bacterial infections ensures that most patients who receive an inappropriate antibiotic will get better despite "treatment". For example, streptococcal sore throat in children may resolve naturally and rapidly despite inappropriate antibiotic therapy. Similarly, it was shown that some patients with a "urinary tract infection" who failed to take the prescribed antibiotic or who took an inappropriate antibiotic responded as quickly as those patients who carefully took full doses of an appropriate antibiotic[11].

Failure of *adequate and appropriate* therapy, in our experience, is most commonly due to host factors. Patients who are compromised either because of their disease or as a result of therapy and who consequently respond poorly to antibiotic therapy are increasing in numbers, at least in specialised areas such as oncology and renal units. Quite apart from these patients, host factors in otherwise healthy patients largely determine the response to antibiotic therapy. The failure to recognise or to deal with sequestrated infection such as an abscess, the presence of dead tissue or a foreign body will result in a poor response to an antibiotic. Similarly, the neglect of well established supportive measures such as rest, dependent drainage and fluid replacement will impede the patient's response to appropriate therapy.

There are a few serious specific infections where the results of antibiotic sensitivity do not necessarily indicate the appropriate antibiotic to be used in treatment. For example, *Salmonella typhi* may appear *in vitro* to be sensitive to the aminoglycosides but these drugs have not been shown to be effective clinically in typhoid fever whereas chloramphenicol and ampicillin are, provided the organism is sensitive to these drugs *in vitro*. Similarly, patients with bacterial endocarditis due to viridans streptococci should be treated with penicillin and the apparent sensitivity to other antibiotics such as tetracycline is irrelevant to their management. When laboratories are confronted with an organism from a patient with an unusual or serious specific infection their advice on the choice of antibiotic should be based on reference to a text containing well documented accounts of established antibiotic therapy in addition to the results obtained by sensitivity testing.

9.3 The implications of an antibiotic sensitivity report

Whether justified or not, clinicians have come to accept that the issuing of a laboratory report detailing the antibiotic sensitivity of the organism carries with it a strong implication that the patient should be treated with one of the nominated antibiotics. This implied suggestion to treat places a responsibility on the laboratory to make an assessment of not only the pathogenic role of the organism but the need for antibiotic therapy in a particular circumstance. In some instances this judgement can be made with relative ease. For example, throat commensals are easily recognised and would not be tested for antibiotic sensitivity, whereas the antibiotic sensitivity of a *Staph.aureus* isolated from the blood culture of a patient with osteomyelitis is an essential and valuable piece of information. In other circumstances the differentiation between a commensal and a pathogenic role of a particular organism is extremely difficult, particularly in the compromised host or in patients who have undergone replacement surgery. A detailed discussion of the selection of isolates which should or should not be subjected to further examination such as sensitivity testing is outside the scope of this description of the CDS method. The importance of this aspect of laboratory medicine is recognised, however, and it is intended that it will be the subject of another issue of the Clinical Microbiology Update Programme series in 1984.

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