TESTS OF BACTERIAL SENSITIVITY TO ANTIBIOTICS

BY

Captain H. J. WOODLIFF, M.B., D.C.P., M.R.C.P.(Edin.)

Royal Army Medical Corps

AND

Corporal J. M. GOODWIN

Royal Army Medical Corps

From The District Laboratory, Woolwich

INTRODUCTION

FOR the rational antibiotic treatment of patients suffering from bacterial infections a knowledge of the sensitivity of the causative micro-organism to the available drugs is required. This knowledge may come from previous experience or be determined in the laboratory. As examples of the former, pneumococcal pneumonia and hæmolytic streptococcal sore throat may be quoted. The organisms associated with these conditions are always penicillin sensitive. Laboratory tests are required when there is doubt as to the identity or the sensitivity of the infecting organism. If an organism whose sensitivity pattern is well known is isolated, sensitivity tests are not necessary but may be of interest. If the sensitivity of the organism is not known tests are performed. Whilst the *in vitro* tests of the reaction between the organism and the drug are not strictly comparable with their reaction *in vivo*, where other factors such as the natural resistance of the host play a part, they have been widely accepted, with a few exceptions, as a useful guide to treatment. In this paper we review the existing tests and recommend one suitable for use in a routine clinical laboratory. The sensitivity of *Mycobacterium tuberculosis* to various antibiotic and chemotherapeutic substances is not considered.

TYPES OF TEST

The aim of these tests is to observe the effect on the organism of differing concentrations of the antibiotic. Numerous methods have been devised with this aim in view and are briefly described below.

Tube dilution method

The antibiotic is diluted with a suitable fluid culture medium to form a series of known concentrations. The organism to be tested is inoculated into each tube, and into a control tube. After incubation the concentration of antibiotic which either inhibits or kills the organism is recorded. This method is, generally speaking, the most accurate, but as it is very laborious only a limited number of tests can be performed. It is not suitable for testing the penicillin sensitivity of penicillinase-producing staphylococci (Cruickshank, 1955).

Solid media incorporating antibiotics

One or more plates of solid media incorporating a known concentration of antibiotic are used. If an organism is inhibited, but grows on a control plate containing no antibiotic, it is considered sensitive. This method is wasteful, both of time and materials, for if only one organism is to be tested against five antibiotics, at least five carefully prepared plates will be required in addition to a control.

Agar diffusion methods

Many methods incorporating an agar diffusion technique are in use. The antibiotic is applied in a concentrated form to one part of a solid agar medium from which it diffuses into the surrounding medium. The concentration of drug gradually diminishes from the point of application outwards.

(*a*) *Ditch plate method.* This was the test originally used by Fleming (1929). A central ditch is removed from an agar plate and replaced by agar containing antibiotic. The organism to be tested is inoculated at right angles to the ditch. If, after incubation, the organism grows up to and over the ditch it is resistant, and if growth is inhibited near the ditch it is sensitive. A control organism is usually inoculated on to the same plate. A modification of this method uses a filter paper strip which, after being soaked in antibiotic solution, is laid on the plate. These methods have the disadvantage of requiring a separate plate for each antibiotic, which is wasteful if only a few organisms are to be tested.

(*b*) *Tablet method.* Small tablets containing antibiotics are obtainable commercially. These are placed on freshly inoculated plates and zones of inhibition noted after incubation. They are relatively expensive but are satisfactory for a qualitative test.

(*c*) *Cylinder plate method.* A plate is sown with the test organism and small sterile cylinders made of porcelain, glass or steel are pressed on to the surface of the medium. The cylinders are then filled with antibiotic solution and the plate is incubated. Sensitivity is shown by the failure of the organism to grow up to the cylinder. Holes bored in the medium can be used as an alternative. This method is more time consuming and exacting than the paper disc techniques which employ the same principle.

(*d*) *Paper disc method.* Paper discs, which have been impregnated with antibiotic solutions, are placed on an agar plate after it has been sown with the test organisms. After incubation the sensitivity of the organism is judged by the zone, if any, of inhibition of growth surrounding the disc. As this method is both economical and easy to perform, we consider it is the most suitable type of test for use in a routine clinical laboratory (see Plate I, facing page 38).

ANTIBIOTIC PAPER DISCS

As recommendations for the preparation of the discs vary they will be considered in more detail.

Choice of paper and size of disc

An absorbent paper is required and both filter paper (Gould & Bowie,

1952) and blotting paper (Ungar, 1951) can be used. Discs may be cut out with a paper hole puncher or a cork borer. The cork borer method is tedious. We recommend an ordinary office paper punch (Stationery Office two-hole punch No. 803) which stamps out discs 6 mm. in diameter. Ungar (1951) recommends 9 mm. discs which will absorb 0.02 ml. of fluid. 0.01 ml. of fluid is absorbed by the 7 mm. filter paper discs recommended by Gould and Bowie (1952) and by the 6 mm. blotting paper discs which we have used. The use of a standard dropper (Ungar, 1951) which adds a constant volume of antibiotic solution to each disc, which is then dried, is time consuming and is unnecessary as 1 ml. of antibiotic solution is completely and evenly absorbed by 100 (6 mm.) blotting paper discs. This is demonstrated by Experiment 1.

Experiment 1.   One ml. of a penicillin solution containing 200 units was added to 100 sterile discs. Plates were inoculated with a standard organism (*Staphylococcus aureus*, Oxford strain), and the discs (five to each plate) applied. After incubation for 18 hours the diameters of the zones of inhibition were measured. It was found that the diameters were 31.0 mm. ± 1 mm. (standard deviation 0.355).

Identification of the discs

Bowie & Gould (1952) recommend the use of Ford dyes which they incorporated into their filter paper discs. Coloured paper discs are available commercially. The dyes have no anti-bacterial action. We have found discs prepared from Ford blotting paper, already coloured, satisfactory. Different colours are reserved for the various antibiotic solutions. As the dyes are precipitated by streptomycin (Bowie & Gould, 1952) white discs are used for this antibiotic.

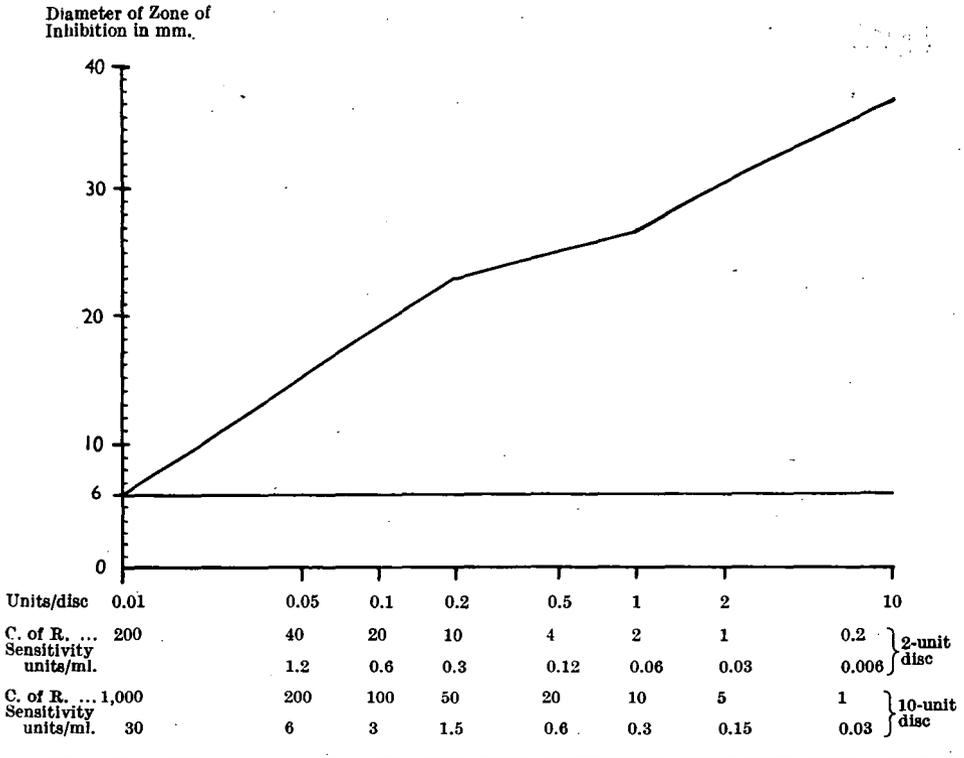### Concentration of antibiotic per disc

By using a standard technique and a standard organism (*Staph. aureus*, Oxford strain) the effect of using discs impregnated with varying concentrations of antibiotic can be measured and a graph constructed. From this a suitable concentration of antibiotic per disc can be chosen for routine work.
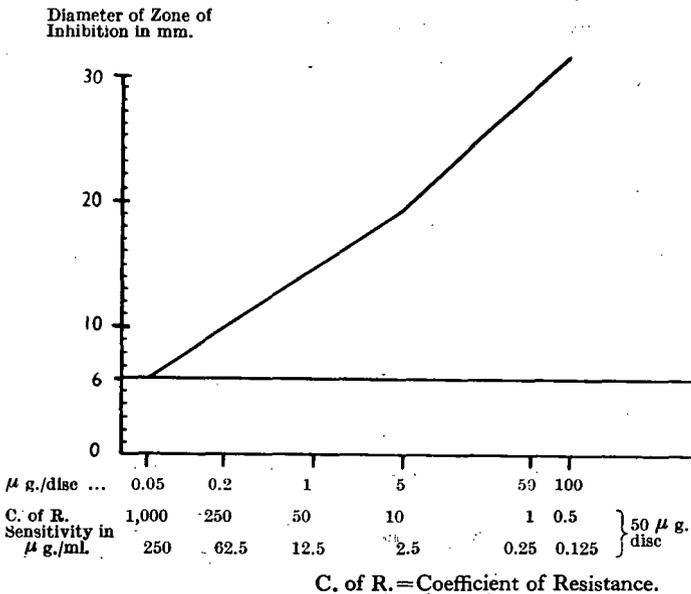
Construction of the graphs

(a) *Benzylpenicillin*.   Penicillin, obtained from the hospital dispensary, in the form of crystalline sodium penicillin G, was diluted with sterile distilled water to varying concentrations. 1 ml. of each concentration was added to 100 sterile discs and ten of each put up on plates inoculated with the standard organism. After 19 hours' incubation at 37° C. the diameters of the zones of inhibition were measured and plotted against the logarithm of the concentration of antibiotic per disc (Graph 1).

A two-unit disc giving a zone of 31 mm. with the standard organism was chosen for routine work. Using this disc, as described below, the antibiotic sensitivity of any test organism can be compared with the standard staphylococcus or recorded in units per ml. by measuring the zone of inhibition in millimetres and correlating it with the *abscissa* line indicating the Coefficient of Resistance
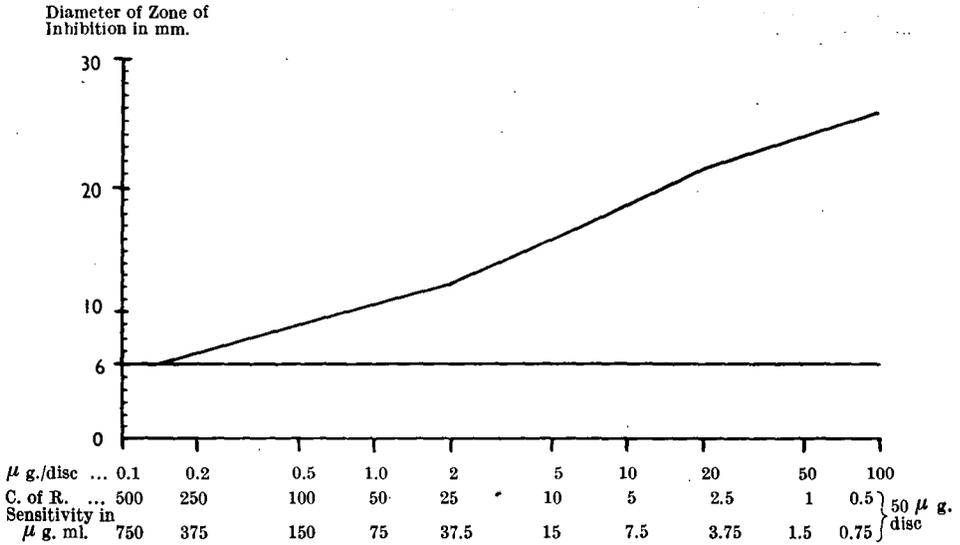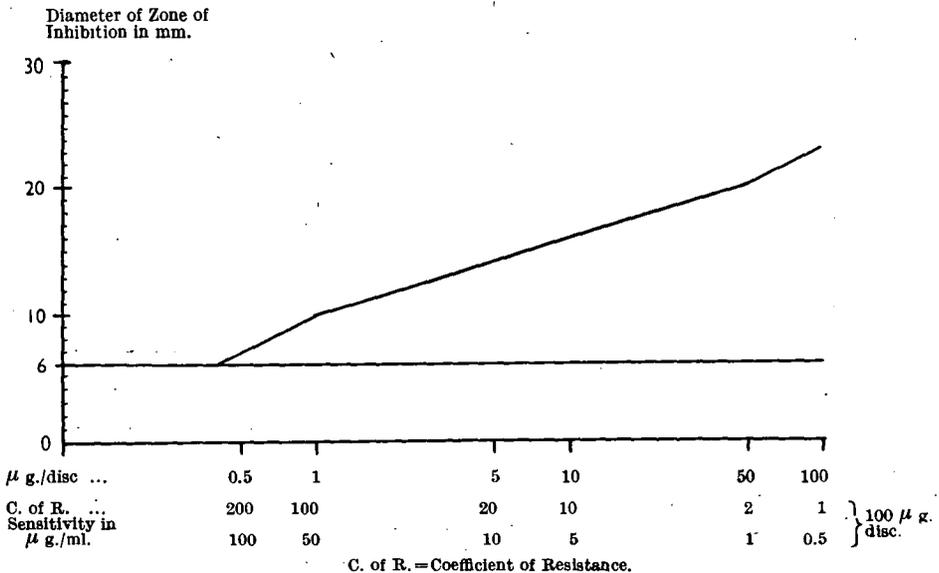
GRAPH No. 1. Penicillin.

Diameter of Zone of
Inhibition in mm.

Units/disc	0.01		0.05	0.1	0.2	0.5	1	2	10	
C. of R. ... Sensitivity units/ml.	200		40 1.2	20 0.6	10 0.3	4 0.12	2 0.06	1 0.03	0.2 0.006	} 2-unit disc
C. of R. ...1,000 Sensitivity units/ml.	30		200 6	100 3	50 1.5	20 0.6	10 0.3	5 0.15	1 0.03	} 10-unit disc

GRAPH No. 2. Tetracycline.

Diameter of Zone of
Inhibition in mm.

μ g./disc ...	0.05	0.2	1	5	50	100	
C. of R. Sensitivity in μ g./ml.	1,000 250	250 62.5	50 12.5	10 2.5	1 0.25	0.5 0.125	} 50 μ g. disc

C. of R. = Coefficient of Resistance.

GRAPH No. 3.   Chloramphenicol.

Diameter of Zone of
Inhibition in mm.

μ g./disc ...	0.1	0.2	0.5	1.0	2	5	10	20	50	100	
C. of R. ...	500	250	100	50	25	10	5	2.5	1	0.5	50 μ g.
Sensitivity in μ g. ml.	750	375	150	75	37.5	15	7.5	3.75	1.5	0.75	disc

GRAPH No. 4.   Streptomycin.

Diameter of Zone of
Inhibition in mm.

μ g./disc ...		0.5	1		5	10		50	100	
C. of R. ...		200	100		20	10		2	1	100 μ g.
Sensitivity in μ g./ml.		100	50		10	5		1	0.5	disc.

C. of R. = Coefficient of Resistance.

or Sensitivity in units per ml. The use of the graph in most cases, however, is not necessary as the relevant information can be obtained from Table 3.

The abscissæ—Coefficient of Resistance and Sensitivity in units per ml.—have been derived as follows (Gould, 1955): The standard organism gives a zone of inhibition of 31 mm. with a two-unit disc, and if the test organism also gives a zone of 31 mm. the sensitivities of the two organisms to penicillin are the same. The Coefficient of Resistance (*i.e.*, concentration required to inhibit test organism/concentration required to inhibit the standard organism) is 1. If, however, the diameter of the zone of inhibition of the test organism is 23 mm., reference to the graph shows that the standard organism requires only a 0.2 unit disc to give the same zone of inhibition, so we have C. of R.=2/0.2=10. The sensitivity or minimum inhibitory concentration of the standard organism, as measured by the tube dilution technique, is 0.03 units per ml., and so the C. of R. can be converted into absolute sensitivity values by using the factor 0.03. Thus sensitivity equals C. of R.×0.03, in this case 10×0.03=0.3 units per ml. The other figures are derived similarly.

(*b*) *Tetracycline.* The tetracycline was obtained from the manufacturers as the pure substance. After suitable dilution, discs of varying concentrations were prepared and used to obtain the data for the preparation of Graph 2. The sensitivity of the standard organism was found by the tube dilution method to be $0.25\mu g$. per ml. and a $50\mu g$. disc was chosen for routine use.

(*c*) *Chloramphenicol.* The chloramphenicol was obtained from the hospital dispensary in the form of oral capsules containing 250 mg. After suitable dilution, discs of varying concentrations were prepared and used to obtain the data for the preparation of Graph 3. The sensitivity of the standard organism was found by the tube dilution method to be $1.5\mu g$. per ml. and a $50\mu g$. disc was chosen for routine use.

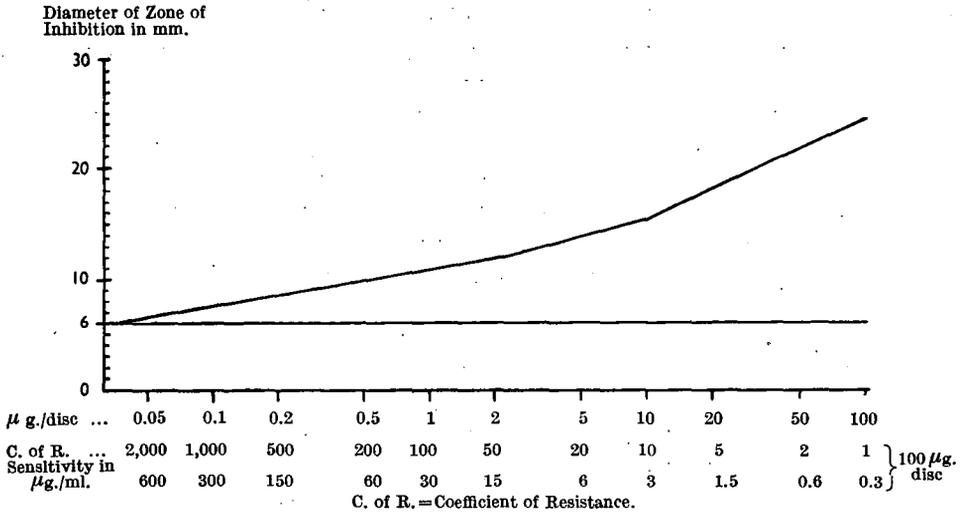(*d*) *Streptomycin.* The streptomycin was obtained from the hospital dispensary in the form of "Streptoquaine" Solution Stabilized Injection of Streptomycin Sulphate. After suitable dilution, discs of varying concentrations were prepared and used to obtain the data for the preparation of Graph 4. The sensitivity of the standard organism was found by the tube dilution method to be $0.5\mu g$. per ml. and a $100\mu g$. disc was chosen for routine use.

(*e*) *Erythromycin.* The erythromycin was obtained from the manufacturers as the pure substance. After suitable dilution, discs of varying concentrations were prepared and used to obtain the data for the preparation of Graph 5. The sensitivity of the standard organism was found by the tube dilution method to be $0.3\mu g$. per ml. and a $100\mu g$. disc was chosen for routine use.

Factors affecting the zone of inhibition

Various factors, apart from the actual sensitivity of the organism, may affect the diameter of the zone of inhibition and must be standardized if the test is to be used quantitatively. The size of the disc and the amount of antibiotic absorbed have already been mentioned and no errors should arise from these factors.

GRAPH No. 5.   Erythromycin.

Diameter of Zone of
Inhibition in mm.

μ g./disc ...	0.05	0.1	0.2	0.5	1	2	5	10	20	50	100	
C. of R. ... Sensitivity in	2,000	1,000	500	200	100	50	20	10	5	2	1	⎫ 100 μg. ⎬ disc
μg./ml.	600	300	150	60	30	15	6	3	1.5	0.6	0.3	⎭

C. of R. = Coefficient of Resistance.

Storage of the discs at 4° C. does not result in any appreciable loss of potency as is illustrated in Experiment 2.

Experiment 2.   Fifty discs of each antibiotic of routine concentration were made up and stored at 4° C. Each week the discs were placed on plates inoculated with the standard organism and after incubation for 18 hours the diameters of the zones of inhibition were recorded. During the three months the penicillin, streptomycin and erythromycin discs showed no deterioration, the zones of inhibition remaining constant. The tetracycline and chloramphenicol discs showed a slow deterioration, the zone of inhibition after three months being 2 mm. less than when the discs were freshly prepared.

The size of the inoculum is important, but by the methods described below a confluent growth is obtained and no appreciable variation in the size of the zone of inhibition occurs. If necessary the number of organisms in the inoculum can be standardized to 100 million per ml. The tests should be read after 18 hours' incubation so that the time of incubation is constant. Variations within the *p*H range 7.2-7.6 have no appreciable effect. The medium should contain 2 per cent. agar, and 5 per cent. blood agar plates are recommended for routine sensitivity tests. The depth of the medium is important and the graphs have been constructed using plates about 2 mm. deep. If the plates are prepared by adding 10 ml. of medium to each four-inch petri dish, this gives a depth of approximately 2 mm. and so the factor remains constant. Primary plates used for the isolation of the organism are often deeper; if antibiotic discs are used at this stage a qualitative answer only is possible and further tests on a subculture should be carried out. Useful information can often be obtained by using a penicillin disc in the well of all primary plates.   .

TECHNIQUE OF RECOMMENDED TEST

Preparation and storage of the discs

Discs are punched out of Ford blotting paper, of the appropriate colour, obtainable from any stationers. One hundred are carefully counted out, placed in a universal container and sterilized in the hot air oven at 160° C. for one hour. When the discs are cool 1.0 ml. of the appropriate concentration of antibiotic solution is added and the bottle vigorously shaken to ensure equal distribution of solution. The discs are then ready for use. The colours and concentrations used are given in Table 1. Provided the other factors affecting the zone of inhibition remain constant, the discs can be used with the graphs we have prepared. However, we have found that erythromycin supplied for laboratory use varies in potency and so it is advisable to prepare a graph for each new batch received. The discs are stored at 4° C. and will retain their potency for at least three months (Experiment 2).

Table 1.   *The colour and strength of antibiotic discs*

Antibiotic	Colour of disc	Amount of antibiotic	
		Per ml. (stock)	Per disc
Benzyl-penicillin ...	Purple	200 units (133 μg.)	2 units (1.3 μg.)
Tetracycline ...	Yellow	5 mg.	50 μg.
Chloramphenicol ...	Green	5 mg.	50 μg.
Streptomycin ...	White	10 mg.	100 μg.
Erythromycin ...	Red	10 mg.	100 μg.

Use of the discs

The tests are best carried out on a young pure subculture of the test organism, although in certain cases the discs can be used to give an early indication of the sensitivity on the primary culture.

(*a*) A sweep of several colonies of the test organism is subcultured into a nutrient broth and incubated for several hours. The culture is then poured on to the surface of a blood agar plate (nutrient agar and McConkey agar can also be used) and the excess removed with a sterile pasteur pipette into lysol. The plates are then dried in the incubator for one hour and the discs applied, using forceps with fine points which are kept in 70 per cent. alcohol and flamed before use. The discs should be placed around the periphery of the plate, 20 mm. from the edge and spaced equidistantly. The plates are then incubated for 18 hours before reading.

(*b*) The test can also be carried out by taking a sweep of several colonies of the test organism and making a heavy, even inoculum over the whole surface of a blood agar plate. The discs are then applied as before and the plates are incubated for 18 hours before reading.

Interpretation of results

Simple qualitative results only are generally required by the clinician. It is necessary, however, to have some idea of the meaning of "sensitive" and

Table 2. *Antibiotic serum concentrations*
After Valentine (1955) *and McCorry and Weaver* (1955)

Antibiotic	Loading dose	Maintenance dose	Peak blood levels
Streptomycin	1 g.	0.5 g. 6-hourly	20-40 μg./ml.
Chloramphenicol ...	2-3 g.	0.5 g. 6-hourly	16-33 μg./ml.
Chlortetracycline ...	1-2 g.	0.5 g. 12-hourly	1-3 μg./ml.
Oxytetracycline ...	1-2 g.	0.5 g. 12-hourly	2-4 μg./ml.
Tetracycline ... · ...	1 g.	0.5 g. 6-hourly	2.15-5.5 μg./ml.
Erythromycin	1 g.	0.5 g. 6-hourly	1-8 μg./ml.

Table 3. *Interpretation of results of sensitivity tests*

Antibiotic	Zone in mm.	Sensitivity in units or μg. per ml.	Description	Abbreviation
Penicillin (2-unit disc)	Greater than 23	Less than 0.3 units	Penicillin very sensitive	P.V.S.
	16-23	0.3-1.0 units	Penicillin sensitive	P.S.
	7-16	1.0-5 units	Penicillin slightly sensitive	P.S.S.
	Less than 7	More than 5 units	Penicillin resistant (to five units)	P.R. 5
Penicillin (10 unit disc)	Greater than 16	Less than 5 units	Penicillin slightly sensitive, sensitive or very sensitive. Use 2 unit disc	
	10-16	5-15 units	Penicillin resistant (to five units)	P.R. 5
	Less than 10	More than 15 units	Penicillin resistant (to fifteen units)	P.R. 15
Tetracycline (50 μg. disc)	Greater than 20	Less than 2.5 μg.	Tetracycline sensitive	T.S.
	15-20	2.5-12.5 μg.	Tetracycline relatively resistant	T.R.R.
	Less than 15	More than 12.5 μg.	Tetracycline resistant	T.R.
Chloramphenicol (50 μg. disc)	Greater than 17	Less than 10 μg.	Chloramphenicol sensitive	C.S.
	14-17	10-25 μg.	Chloramphenicol relatively resistant	C.R.R.
	Less than 14	More than 25 μg.	Chloramphenicol resistant	C.R.
Streptomycin (100 μg. disc)	Greater than 14	Less than 10 μg.	Streptomycin sensitive	S.S.
	10-14	10-50 μg.	Streptomycin relatively resistant	S.R.R.
	Less than 10	More than 50 μg.	Streptomycin resistant	S.R.
Erythromycin (100 μg. disc)	Greater than 20	Less than 1.0 μg.	Erythromycin sensitive	E.S.
	13-20	1.0-8 μg.	Erythromycin relatively resistant	E.R.R.
	Less than 13	More than 8 μg.	Erythromycin resistant	E.R.

"resistant" in terms of the concentration of antibiotic required to inhibit the growth of the organism (abscissa of graphs), and to correlate this with the concentration of antibiotic obtained in the serum during treatment (Table 2). If the organism grows right up to the disc it is resistant to the drug ; if there is a wide zone of inhibition the organism is sensitive or very sensitive. If a small zone of inhibition is seen the organism is regarded as "relatively resistant" or "slightly sensitive." Measuring the actual diameter of the zone of inhibition with a pair of dividers allows a quantitative result to be given. The exact con-

centration of antibiotic required to inhibit the organism can be read off the graphs, but this is not generally necessary. Table 3, which has been derived from the graphs, correlates the diameter of the zones of inhibition with the inhibitory concentrations of the antibiotic and the terminology used. By using this table the clinician will know more exactly what "sensitive" and "resistant," which are purely relative terms, actually mean. In the case of penicillin four grades of sensitivity are given. The "penicillin very sensitive" organisms are inhibited or killed by 0.3 units of penicillin per ml., a concentration readily obtained in the serum with doses as low as 100,000 units four-hourly or 100,000 units of crystalline penicillin with 300,000 units of procaine penicillin twelve-hourly. The "penicillin sensitive" organisms are inhibited by the usual doses of penicillin, but higher doses, repeated more frequently, are required for the "penicillin slightly sensitive" organisms. The organisms labelled "penicillin resistant 5" are not usually treated with penicillin as very large doses are required to provide a serum concentration greater than 5 units/ml. If necessary, however, the organism can be tested with a stronger penicillin disc containing 10 units, which will allow sensitivities equivalent to 15 units per ml. to be read. With the other drugs the usual clinical doses give rise to serum levels which will inhibit the organisms found "sensitive." Higher than usual doses may be required to inhibit the organisms found "relatively resistant" and are given only if the organism is not sensitive to any other drug.

OXYTETRACYCLINE, CHLORTETRACYCLINE, BACITRACIN, POLYMIXIN B AND NEOMYCIN

The Tetracyclines

Tetracycline itself is used to prepare the discs, and the sensitivities derived from their use apply, in the vast majority of cases, also to oxytetracycline (terramycin) and chlortetracycline (aureomycin). Discs can, if necessary, be made from these substances, using the same technique as for tetracycline. Discs containing chlortetracycline, which is less stable than tetracycline, do not keep so long.

Bacitracin, Polymixin B and Neomycin

Bacitracin, polymixin B and neomycin sensitivity tests are seldom requested and graphs have not been prepared. A qualitative test can be done by dipping one sterile disc into a solution containing bacitracin (1,000 units/ml.) or polymixin B (1,000 μg./ml.) and using the disc as above. A suitable concentration for neomycin sulphate is 1,000 μg. per disc which gives a zone of 27 mm. with the standard organism.

EXAMPLES OF RESULTS OBTAINED WITH THE METHOD DESCRIBED

The sensitivity pattern of fifty strains of *Staph. aureus* (coagulase positive) isolated from the patients and staff of the Royal Herbert Hospital was tested by the method described. The following results were obtained:

    (a) Benzylpenicillin—Very sensitive, 38 per cent. ; sensitive, 18 per cent. ; slightly sensitive, 22 per cent. ; resistant, 22 per cent.

(b) Tetracycline—Sensitive, 60 per cent.; relatively resistant, 28 per cent.; resistant, 12 per cent.

(c) Chloramphenicol—Sensitive, 100 per cent.

(d) Streptomycin—Sensitive, 88 per cent.; relatively resistant, 10 per cent.; resistant, 2 per cent.

(e) Erythromycin—Sensitive, 100 per cent.

SUMMARY

An antibiotic sensitivity test using a paper disc technique is described. The discs are easy to prepare and the test is simple to perform. The results which are recorded qualitatively are correlated with inhibitory concentrations of the antibiotic. The antibiotic sensitivity pattern of fifty strains of *Staph. aureus* is recorded.

ACKNOWLEDGMENTS

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Fig. 1. Standard *Staph. aureus* : T.S., C.S., S.S., E.S., P.V.S.

Fig. 2. *Staph. aureus* isolated from the fæces of a patient after treatment with chlorotetracycline : T.R., C.S., S.R., E.S., P.R.

The discs in both photographs, reading clockwise, contain tetracycline (12 o'clock), chloramphenicol, streptomycin, erythromycin and penicillin.

[*Photograph kindly taken by Photographer, R.A.M. College*
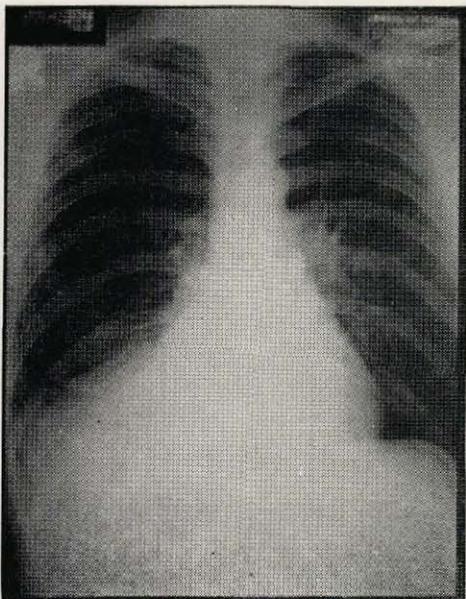
PLATE I
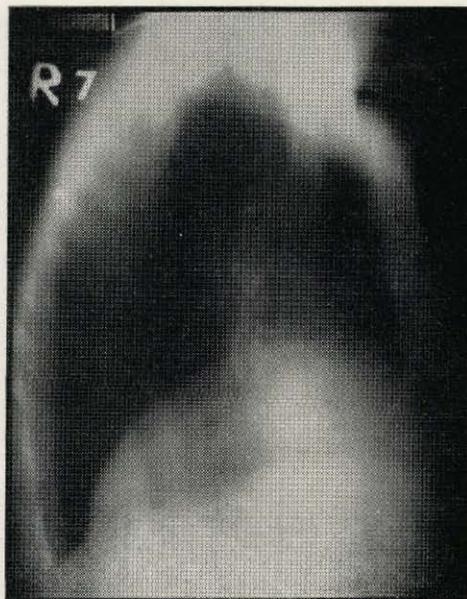
FIG. 1.   P.A. radiograph of the chest.

FIG. 2.   Right lateral tomogram 7 cm. cut suggestive of raised right cupola of diaphragm.

FIG. 3.   Right lateral radiograph where diagnostic pneumoperitoneum reveals the mass to be supradiaphragmatic.
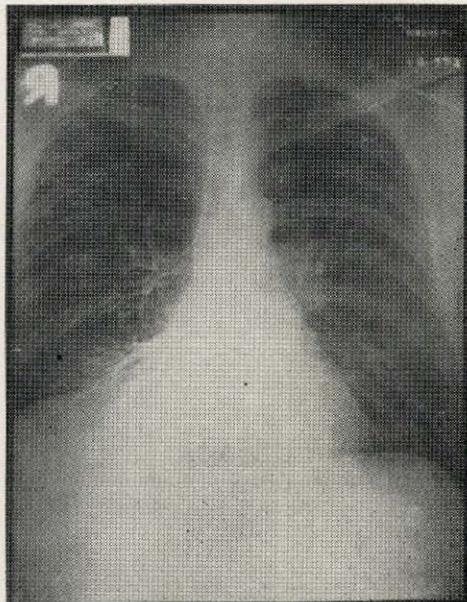

FIG. 4.   Right lateral bronchogram showing the distortion of the bronchial tree.

PLATE I