A study of antibiotic sensitivity testing with proposals for simple uniform methods

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SUMMARY The findings in the preceding paper on pages 773-778 are reviewed and the possible causes of error revealed by them are analysed.

The disc test has been studied with a view to defining suitable culture media, and disc contents such that six can usually be placed on a 9 cm plate. Emphasis is placed on controlling the weight of the inoculum and on ensuring its uniform distribution. It is suggested that such cultures should be interpreted by comparison of inhibition zone sizes with those of a suitable control organism on the same medium.

Indications and methods for tests in primary culture are discussed.

Proposals are made for eliminating superfluous or inappropriate tests, thus limiting the number of antibiotics or other drugs with which tests need initially be done.

There are now on record at least four studies in which the results obtained by numerous different laboratories in testing the antibiotic sensitivity of given cultures have been compared. Either pure cultures (*Journal of Medical Laboratory Technology*, 1960; College of Pathologists of Australia, 1968) or clinical specimens (Association of Clinical Pathologists, 1965) have been sent for testing by whatever method each laboratory favoured; in another study (Beaney, Goodwin, Jones, Winter, and Sippe, 1970) cultures were circulated for testing by a prescribed method. Comparison of the findings showed numerous discrepancies. When, for instance, 34 laboratories report an organism to be sensitive to a certain antibiotic and 26 find it resistant, one of these sets of reports must be wrong.

The reason for an erroneous finding can only be either in the method used, in its execution, or in its interpretation. An inquiry into methods should therefore be of interest, and the most extensive yet in this country—and probably indeed anywhere—is reported in the preceding paper (Castle and Elstob, 1971). Analysis of replies to a questionnaire shows a consistent lack of uniformity in every detail of technique in performing the disc test. Various culture media are used: the inoculum varies from light to

heavy and is not always regulated at all; methods of spreading it vary, and that most favoured is the one least capable of ensuring uniform distribution. Disc contents employed vary widely, and an extraordinary variety of antibiotics and other antibacterial drugs is tested. Perhaps the most disturbing feature is the frequent absence of controls. Unless every factor affecting the result of a test is rigidly standardized, in the way attempted in two methods about to be described, it should be interpreted by comparison with a culture of known normal sensitivity.

Towards a Uniform Method

It has been recognized for years that the general adoption of a method so standardized as to minimize the influence of variables would be a great advance. Two moves in this direction have been in progress for some time, in which the methods proposed are very similar.

One is the Kirby-Bauer method (Bauer, Perry, and Kirby, 1959; Bauer, Kirby, Sherris, and Turck, 1966; Anderson, 1970), which is widely and increasingly practised in the United States, and is understood to be undergoing consideration for official adoption in that country. This employs the Mueller-Hinton medium, and the bacterial suspension used for inoculation is adjusted to an opacity standard, although since inoculation is with a swab, the size and mode of

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use of this can evidently affect the weight of inoculum considerably. The resulting growth is confluent. A single disc only is used for each antibiotic, and most of their contents are high, giving zones of inhibition of a size necessitating the use of plates 15 cm in diameter containing 80 ml of medium. Inhibition zones are measured and interpreted from tables stating limits of diameters for full and intermediate sensitivity and resistance to each antibiotic.

The second method, based largely on that originally described by Ericsson (1960), is proposed by a working group of WHO which has been engaged in an international collaborative study for nearly 10 years (Ericsson and Sherris, 1971). This report defines two dilution methods as well as a disc method. The latter is proposed as either a routine method or a reference method whereby the validity of an existing routine method can be tested. It uses large plates of Mueller-Hinton medium with 5% sheep's blood, an inoculum yielding 'dense but not completely confluent growth' and high content discs (only those for five groups of antibiotics and for chloramphenicol are actually specified). Zones are measured, and can be translated into the minimum inhibitory concentration (MIC) by reference to regression lines (plots of zone diameters against MIC for organisms of differing degrees of sensitivity). This enables the organism to be placed in a category of sensitivity by reference to tables of 'break-points' (in terms of MIC) separating these categories. This process is simplified in instructions for this test issued in Sweden kindly supplied to us by Dr Hans Ericsson. Scales are provided in this for each antibiotic on which a caliper reading of zone diameter can be directly read as indicating 'sensitive', 'fairly sensitive', 'slightly sensitive', and 'resistant'. The report also recommends that national reference laboratories should issue full instructions on methods and their interpretation in each country, and it is understood that France, Germany, and the United States as well as Sweden are engaged in implementing this proposal. In view of these developments it appears necessary that the pros and cons of adopting a test of this type should be officially considered in Great Britain.

It should be clearly understood that the preparation of satisfactory regression lines is an enormous task, and that they are only valid for tests on precisely the same medium as that used in constructing them. Those who are now asking disc manufacturers to provide them with regression lines are presumably unaware of this.

The general adoption of either of these methods would greatly improve the reliability of reporting, but it may be questioned whether they provide the best solution of the difficulty. Their validity depends

on rigid standardization of every feature of the test, including particularly the composition of the medium, and it will be shown later in this paper that the composition of the medium recommended for both tests varies considerably. It is also difficult to standardize the inoculum, and it may be questioned whether some laboratories whose methods are most in need of reform will always trouble to regulate it by comparison with an opacity standard. Another serious objection is cost. At present prices in this country the cost in materials (medium and dishes) for a test on large plates is almost exactly three times that for one using a Petri dish of ordinary size. General adoption of such a method would thus involve a considerable outlay to add to the present soaring costs of the National Health Service. In present circumstances this would be justifiable only if no more economical improvement could be devised.

A further objection has less force, since it only applies if such a test is misinterpreted, as it certainly sometimes will be. High content discs produce large zones of inhibition even of resistant organisms: in the 'zone size interpretative chart' for the Kirby-Bauer method (Anderson, 1970) 20 mm or less denotes resistance to penicillin in a staphylococcus, and 14 mm or less resistance to cephalothin or tetracycline in any organism. With this plain evidence of the activity of the antibiotic before their eyes, some workers will be reluctant simply to class the organisms as resistant.

It is our aim in this paper to suggest a method for routine performance of the disc test to which these objections do not apply. No originality is claimed for this: most of its features are already common practice in Great Britain, although it will be evident from the paper on pages 773-778 that many laboratories adopt only some of them. A comparison has been made of different culture media and their effect on the growth and inhibition zone size of various bacterial species. Disc contents are defined such that inhibition zones will usually not overlap if six discs are placed on the medium in a 9 cm plate, and a scheme is proposed whereby the number of drugs which can usefully be tested against each principal species can usually be reduced to six or less. Secondly, we believe it to be too difficult for many laboratories so to control the conditions of the test that degrees of sensitivity can safely be read from a table of zone diameters or a regression line. We suggest that every daily batch of tests should be controlled by, and results read by comparison with, an identically prepared culture on the same medium (with the same addition of blood if this is necessary) of an organism of known normal sensitivity. This should be a strain of *Staph. aureus* if the infection is systemic, and of *E. coli*, a generally more resistant organism if

the infection is in the urinary tract, where higher antibiotic concentrations are attained.

Materials and Methods

MEDIA

The principal culture media studied, with the abbreviations used to denote them, were: 'LLP' Lab-Lemco peptone agar (Oxoid nutrient broth no. 2 solidified with 1% Oxoid agar no. 3); 'DST' Oxoid diagnostic sensitivity test agar; 'MH' Mueller-Hinton agar (Oxoid); 'WST' Wellcotest sensitivity test agar (Wellcome Reagents Ltd); Mueller-Hinton agars from two other sources, Difco and Baltimore Biological Laboratories, were also used in some experiments.

Horse blood (4%) was added for tests with *Strept. pyogenes*, *Strept. pneumoniae*, and *H. influenzae*, the medium being heated for the last-named. Lysed horse blood (4%) was added to all media except WST for tests with trimethoprim and sulphonamide. Plastic 9 cm dishes containing 16 ml medium were used throughout.

DISCS

These were supplied by Oxoid Limited and Mast Laboratories. Some containing quantities not commercially available were made in this laboratory.

TEST ORGANISMS

Those principally used were *Staphylococcus aureus*, Oxford (NCTC 6571), *Escherichia coli* NCTC 10418, and *Pseudomonas aeruginosa* NCTC 10662. Others were recent clinical isolates.

DIFFUSION TESTS

With the exception of tests by the Stokes method, when a swab was used for inoculation, all plates were inoculated by flooding with a culture so diluted as to give dense but not confluent growth. Discs were applied and the culture incubated forthwith. Unless otherwise stated incubation was in air at 37° for 18 to 20 hours.

MINIMUM INHIBITORY CONCENTRATIONS

These were determined by the plate dilution method with two-fold differences based on 1 µg/ml. Inoculation was by multiple replicator with cultures diluted to contain about 10⁶ organisms/ml.

Results

GROWTH-SUPPORTING PROPERTIES

Strept. pyogenes and *Strept. pneumoniae* grow well on LLP but poorly on all the sensitivity test media even with the addition of blood. Growth is improved

by CO₂, but is still inferior to that on ordinary nutrient media.

Staph. aureus grew very poorly on DST and hardly at all when the culture was incubated at 30°. The addition of aneurin, made at our suggestion, removed this defect, and the further observations to be described were made with the currently available aneurin-containing product.

The addition of heated blood enabled *H. influenzae* to grow profusely on all four media. Other common bacteria grew satisfactorily on all four.

EFFECT OF MEDIUM ON INHIBITION ZONES

Diameters of inhibition zones of two standard organisms produced by discs of different drugs are stated in Table I. Differences in zone size related to medium composition are considerable with only two drugs.

TETRACYCLINE

Zones are much smaller on Lab-Lemco peptone agar. The fact that some media reduce the apparent activity of tetracycline, possibly in part because of their Ca and Mg content, is now well known. In our hands an increase in the peptone content of a medium reduces the size of the tetracycline inhibition zones, and the agar used, because of its varying mineral content, may also have an effect.

SULPHONAMIDE

Even with the addition of lysed horse blood, the zones on LLP are smaller than on the other three media and their edges are indistinct. Oxoid diagnostic sensitivity test agar has a low inhibitor content, and the addition of as little as 2% lysed blood is enough to neutralize it. Wellcotest sensitivity test agar is inhibitor-free, and no addition of blood is necessary. We have examined MH from three different manufacturers, and found considerable differences in their inhibitor content, one product not being cleared even by the addition of 4% lysed blood, small colonies of some species occupying the entire inhibition zone. These observations confirm those of S. R. M. Bushby (personal communication) who found similar variations not only between supplies of MH from different manufacturers, but between batches prepared in his own laboratory. Media suitable for tests of sulphonamides are equally so for trimethoprim.

Gentamicin zone sizes differ little in Table I, but in tests of *Ps. aeruginosa* the composition of medium affects the result very considerably, the difference being probably attributable to the magnesium content of the agar used (Garrod and Waterworth, 1969). Table II states the MIC of gentamicin for a strain of *Ps. aeruginosa* on six different media, three

Antibiotic	Disc Content (μg)	Oxford Staph. aureus				E. Coli (NCTC 10418)			
		LLP	DST	MH	WST	LLP	DST	MH	WST
Ampicillin	2	36	36	38	38	19	19	20	21
	10	43	44	43	44	23	24	24	25
	30	46	46	46	46	27	28	27	28
Carbenicillin	100	50	48	48	50	30	33	32	33
Cephaloridine	2	38	38	40	40	14	11	13	11
	10	43	44	43	43	22	21	23	22
	30	48	48	48	49	27	25	26	26
Cephalexin	30	38	36	37	38	23	24	23	24
Chloramphenicol	10	25	24	25	25	26	26	27	27
Clindamycin	2	32	33	31	31	—	—	—	—
Colistin	50	—	—	—	—	17	17	17	16
Erythromycin	2	25	25	25	27	tr	tr	tr	tr
Fucidin [†]	5	30	28	29	30	—	—	—	—
Gentamicin	10	26	27	25	25	26	25	24	24
Kanamycin	30	26	26	26	25	25	24	24	26
Lincomycin	2	26	25	26	25	—	—	—	—
Nalidixic acid	30	15	17	16	20	26	30	30	31
Nitrofurantoin	200	27	27	26	30	23	26	25	26
Novobiocin	5	26	27	26	28	—	—	—	—
Penicillin	1	37	38	39	39	—	—	—	—
Polymyxin B	+300 [‡]	tr	tr	tr	tr	16	16	16	16
Streptomycin	30	25	25	25	26	23	23	23	21
Sulphafurazole	100	23	30	31	32	22	29	29	28
Sulphamethoxazole/ trimethoprim	23.5/ 1.25	24	32	32	33	28	35	34	35
Tetracycline	30	24	33	30	35	17	26	26	28
	5	20	28	26	30	13	21	20	20
Trimethoprim	1.25	17	22	21	21	22	30	29	31

Table I Comparison of zones of inhibition (diameter mm) of the Oxford Staph. aureus and E. coli (NCTC 10418) produced by discs of recommended content on four culture media

[†]tr = trace of inhibition

[‡] = zones substantially smaller when the medium contains blood

[‡]+ = units

	LLP	DST	Mueller-Hinton Medium (as Supplied by Three Manufacturers)			WST
			Oxoid	BBL	Difco	
E. coli (NCTC 10812)	1.0	1.0	0.5	1.0	1.0	1.0
Ps. aeruginosa (NCTC 10662)	8.0	1.0	0.5	2.0	16.0	1.0

Table II Effect of medium on MIC ($\mu\text{g/ml}$) of gentamicin

of which are Mueller-Hinton media from different sources, giving values of 0.5, 2.0, and 16 $\mu\text{g/ml}$. The first of these, Oxoid MH, is solidified with agar no. 1, which has a very low Mg content. The contrasting effects of these three media are also illustrated in Figure 1.

THE EFFECT OF STARCH

It was accidentally observed that the addition of 0.15 % starch to LLP substantially increased the zone of inhibition of *Strept. faecalis* by penicillin and ampicillin, which was otherwise smaller than on the other three media. This enhancement of effect increased with storage of the medium, which suggested that breakdown products were responsible. LLP contains no glucose: when 150 mg/100 ml starch was added its glucose content was found

to be 25 mg/100 ml immediately after preparation, and this was found to increase to 35 mg/100 ml in one week. The addition of glucose itself caused a similar but somewhat lesser increase in zone size. The MICs on these media were unaltered. The larger zones on the three sensitivity test media may therefore be explained by the presence of glucose in DST and of starch in MH and Wellcotest. Some hydrolysis of starch must occur during the preparation of the two latter media, and a sample of MH was found on reconstitution to contain 75 mg glucose per 100 ml. The degree of this change must affect the performance of the medium, and constitutes a most unsatisfactory variable.

TESTS OF H. INFLUENZAE

Inhibition zones produced by cephaloridine in

Fig. 1 Effect of medium on the sensitivity of *Ps. aeruginosa* to gentamicin. The top half of each plate is inoculated with *Ps. aeruginosa* and the lower half with *E. coli*. The discs contain 10 µg gentamicin. Each plate contains Mueller-Hinton medium.

a = Oxoid b = Difco c = BBL

Fig. 2 Effect of medium on clarity of zone edges. All plates are inoculated with *E. coli* and discs contain (upper left) ampicillin 25 µg. The plates contain: a) WST, b) DST, and c) MH.

cultures of this organism were much larger on Wellcotest (differences of 10-14 mm) than on LLP or DST, diameters on MH being intermediate. Starch also had an effect on zone size with this species, its addition to LLP increasing the diameter of the zones, and with some but not all strains reducing the minimum inhibitory concentration. Similar effects were seen with cephalothin and cephalixin.

APPEARANCE AT ZONE EDGES

A clearly defined zone edge is necessary for accurate measurement. Tetracycline produces indefinite edges, particularly on LLP and to a lesser extent on WST and DST, but a fairly well defined edge on MH (Fig. 2). In contrast, zones produced by aminoglycosides in cultures of *E. coli* on MH are either indefinite, or as seen in Fig. 2 are double, there being an inner zone of diminished growth. These zones are clearest on Wellcotest.

TESTS WITH METHICILLIN

No values for methicillin are given in Table I, because it is now recognized that resistance to this drug cannot satisfactorily be demonstrated by the usual method. We fully accept the contention of Hewitt, Coe, and Parker (1969) that it should be tested for with a 10 µg disc on a plate incubated at 30°C. (An alternative is a test on medium containing 5% NaCl.) It is also fairly widely recognized that a test for methicillin resistance should be taken to apply to other penicillinase-resistant penicillins and cephalosporins, and that tests with these drugs themselves give misleading results.

A test of a methicillin-resistant strain of *Staph. aureus* with discs of methicillin, cloxacillin, and flucloxacillin is shown in Figure 3. In a plate incubated at 30° there is growth almost up to the disc of methicillin, whereas there are large clear zones around discs of clox- and flucloxacillin, strongly suggesting full sensitivity. That this organism nevertheless is resistant to these two drugs is shown by the MIC determinations in Table III, in which

Fig. 3 Disc sensitivity test on a methicillin-resistant strain of *Staph. aureus* incubated at 30°C. Discs contain: (top) methicillin 10 µg, (lower left) flucloxacillin 5 µg, and (lower right) cloxacillin 5 µg.

the resistant strain is the same as that used in the test illustrated in Figure 3.

This difficulty is not overcome by reducing the amount of cloxacillin in the disc so as to bring the contents of this and the methicillin disc into a similar relation to the minimum inhibitory concentration. Hewitt, Coe, and Parker found that the use of 2 µg cloxacillin discs 'gave little improvement'. The reason for the different behaviour of these drugs in diffusion tests remains unexplained, but whatever differences may be seen *in vitro* it is agreed that clinically they show cross resistance.

DISC CONTENTS

The object of the experiments in Table I, which included trial of disc contents other than those given in the table, was to define contents such that zones of control organisms should preferably be about 25 to 30 mm in diameter, thus not occupying too large an area, yet permitting the detection of moderate degrees of resistance. Those given are considered suitable. So far as possible, those are chosen which have been recommended for use in other methods.

There has been much discussion whether discs with a single content will serve all purposes. The use of a single disc is required in the Kirby-Bauer method, but Ericsson and Sherris (1971) acknowledge that two contents may be advisable for penicillin, ampicillin, and carbenicillin. We agree that in testing drugs, of which the dosage is elastic, and the concentrations attainable, particularly in the urine, are

		Methicillin	Cloxacillin	Flucloxacillin
Light inoculum (10 ⁴)	37°C	0	2	2
	30°C	32	16	8
Heavy inoculum (10 ⁷)	37°C	16	>16	>16
	30°C	>32	>16	>16

Table III Fold¹ increase in MIC of methicillin, cloxacillin, and flucloxacillin for the methicillin-resistant *Staph. aureus* shown in Fig. 3 when tested with a light and a heavy inoculum and incubated at 37°C and 30°C

¹MIC of methicillin-sensitive, penicillin-resistant control: methicillin 4 µg/ml, cloxacillin and flucloxacillin 0.5 µg/ml.

high, a high-content disc is required for detecting lower degrees of sensitivity. The range of sensitivity to penicillins compatible with successful treatment is also so wide, and the inhibition zone of a fully sensitive organism produced by a disc of only moderate content is so large, that discs of at least two contents are clearly required. Penicillin, ampicillin, and cephaloridine give very large zones with highly sensitive cocci even when the disc content is only 1 or 2 μg , but it seems inadvisable to reduce the content further.

We question the need for the regular use of a higher content disc of penicillin; the only common Gram-positive organism requiring this is *Strept. faecalis* and sensitivity in this organism differs little from that to ampicillin. Such Gram-negative organisms as *E. coli*, *Proteus*, and *H. influenzae* are more sensitive to ampicillin, and only their sensitivity to this antibiotic is usually of interest. We also doubt the need for more than one disc content of carbenicillin. The position of this antibiotic is exceptional. There is cross-resistance between it and ampicillin, and ampicillin is the more active, and hence therapeutically preferable, against all organisms except *Ps. aeruginosa* and indole-positive *Proteus*. *Ps. aeruginosa*, much the most important of these species, is relatively resistant, and hence only a high-content carbenicillin disc is required.

On the other hand, we suggest that for ampicillin and cephaloridine discs of three contents are advisable to be used as follows: 2 μg for tests of Gram-positive organisms from systemic infections; 10 μg for tests of more resistant organisms (eg, coliform) from systemic infections; 30 μg for tests of organisms from urine.

The choice of a correct control organism in these three tests is important. For any test of organisms from specimens other than urine, the more sensitive *Staph. aureus* is required, whether a 2 or 10 μg disc is used. The inhibition zones of more resistant organisms will be smaller than in the control cultures, and the organism will be reported as moderately sensitive or sensitive only to high doses. Infections due to more resistant organisms in the urinary tract may respond to normal doses, and the more resistant *E. coli* control is therefore employed here.

Discussion

The variety of methods in current use in this country, as revealed in the foregoing paper (Castle and Elstub, 1971), now calls for further comment.

CULTURE MEDIUM

Most laboratories use a medium formulated for sensitivity tests, but 21 % do not. Various other media

may differ in their suitability, but any ordinary nutrient medium is likely to be excessively inhibitory of the action of certain drugs, and is consequently unsuitable. On the other hand, sensitivity test media are unsuitable for primary cultures of material containing fastidious organisms, eg, *Strept. pyogenes* and *Strept. pneumoniae*. It seems unlikely that medium depth is an important source of error, provided that it is kept fairly constant. Additions of whole or lysed blood greatly reduce the activity of highly protein-bound antibiotics, and comparison with a culture of a control organism on the same medium is essential for interpretation.

INOCULUM

The majority test only pure cultures, but 17 % employ direct plating and 30 % either method according to the nature of the specimen. The dependability of direct plating depends very much on how it is done: growth must be of the required density and uniformly distributed, and certainly not spread in the usual way so as to produce areas with progressively diminishing numbers of colonies.

When pure cultures are used, a surprising number of laboratories state that they make no attempts to control the size of the inoculum. It is even more surprising that neglect of this factor is commonest in teaching hospitals (40 %). The resulting growth varies from confluent (a much more frequent type of growth in teaching hospitals than elsewhere) to well separated colonies, although 59 % of all hospitals usually obtain dense but not confluent growth, which is widely accepted as the optimum.

This is clearly a factor of major importance as a source of error. A heavy inoculum can abolish sulphonamide activity completely, and much reduce the apparent activity of some antibiotics. Steps to regulate the inoculum, preferably so as to ensure dense but not confluent growth, are essential.

SPREADING THE INOCULUM

Another important requirement is uniform distribution. Flooding, which best achieves this, is exclusively used by only 16 %, a swab by 14 %, and a glass rod by 3 %. The most popular implement is the wire loop, used exclusively by 46 % and along with other methods in 15 % more. Uniform distribution is very rarely achieved if whole plates are spread with a loop, and inhibition zones on such plates cannot be accurately measured. This can be seen from published illustrations of the results (eg, Schwarz and Brown, 1954). The abolition of loop spreading would alone be an important step forward.

CONTROL CULTURES

Unless all the conditions of a test are rigidly standard-

ized, it must be interpreted by comparison with a control culture of an organism of known normal sensitivity. Only 46% of laboratories prepare such cultures at all, and only a third of these control each test, the remainder using them only for such purposes as checking new batches of discs or medium. This is clearly another important defect which needs to be rectified.

DISC CONTENT

Discs of a wide range of drug contents are used. Although 78% of laboratories use higher contents for testing organisms from the urinary tract, this can account for only some of the differences. Many of the amounts are very high, and could produce fairly wide zones of inhibition even of a clinically resistant organism. Nearly all laboratories were in favour of international standards for disc contents, and everyone must agree that far greater uniformity is desirable.

CHOICE OF DRUGS TO TEST

An extraordinary variety of practice is also shown in the tables stating the frequency with which various drugs are tested in urinary, staphylococcal, and 'other' infections. Detailed analysis of these data is prohibited by considerations of space, but it may be pointed out that some of the choices are of drugs inapplicable to their purpose (nitrofurantoin and nalidixic acid for organisms not from urine, framycetin for organisms from urine, and polymyxin or nystatin for staphylococci), and many others are superfluous since they should be covered by a single test with another drug of the same group. Thus there is no point in testing a staphylococcus with ampicillin as well as penicillin, since resistance if any will be to both. As shown earlier in this paper, a disc test with cloxacillin may not reveal resistance in a staphylococcus, and resistance to this drug should be deduced from a special test with methicillin. Nevertheless, only 84 laboratories used methicillin and 162 used cloxacillin in first-line testing. Likewise only one tetracycline need be tested.

Even allowing for special types of use and for any reasonable personal predilections, these lists could evidently be cut severely.

In the light of the results of this inquiry, and on the basis of studies reported in this paper, we venture to suggest the performance of sensitivity tests by the following methods.

Recommended Proceedings

MEDIUM

The medium used should be free so far as possible of inhibitors (particularly of tetracycline, sulphonamide,

and trimethoprim activity). Of the three tested WST alone is entirely free of sulphonamide and trimethoprim inhibitors. On the other hand the inhibitors in DST can be neutralized by the addition of only a small amount of horse blood. Wellcotest has the advantage of enabling tests of sulphonamide and trimethoprim sensitivity to be performed in countries where horse blood is unobtainable, but further experience of its practical use may be desirable. This medium and DST are products of single manufacturers and can be relied on to be of constant composition. Mueller-Hinton agar is a more variable product, as the present observations show, and has another serious drawback in the indefinite zones produced by aminoglycosides in cultures of *E. coli*. We regard it as the least satisfactory of the three.

Blood should be added to promote adequate growth of *Strept. pyogenes* and pneumococci, heated blood for *H. influenzae* and lysed blood for tests including sulphonamides (except to Wellcotest). Whenever such an addition is made, there should naturally be a control culture of a standard sensitive organism on the same medium.

TESTS IN PRIMARY CULTURE

These are only advisable when the organisms present are such as will grow well on a sensitivity

Fig. 4 Primary sensitivity test on a swab from a boil. The top and bottom areas are inoculated with the Oxford staphylococcus and the centre area with the swab, which yielded a pure growth of *Staph. aureus*.

Discs: Top left streptomycin 25 µg
Top right tetracycline 10 µg
Lower left penicillin 1.5 unit
Lower right erythromycin 5 µg

test medium. All organisms commonly found in urine will do so, and if microscopy indicates the presence of infection, tests in primary culture of urine are to be commended. A simple diagnostic culture should be made in addition. Most specimens of pus can satisfactorily be treated in the same way.

The most reliable method is the Stokes plate (Stokes, 1968; Garrod and O'Grady, 1971) on which a wide area across the centre is inoculated with the specimen and areas on either side with the control culture, discs (two on each side) being placed on the line separating the two inocula. A test so performed is illustrated in Figure 4. This method is unique in controlling the performance of each disc, as well as producing test and control inhibition zones in immediate juxtaposition, thus facilitating comparison. The difficulty of ensuring growth of the required density has been much exaggerated. Uniform distribution is best achieved with a swab. Any unsatisfactory test should be repeated with a pure culture.

This method can also be used for tests of pure cultures, and is preferable to any other when few drugs are applicable and an unusual control organism is required. Thus in tests of *Ps. aeruginosa* not more than four drugs are worth testing (Table IV) and the control should be not *Esch. coli*, but a standard sensitive strain of *Ps. aeruginosa* itself (NCTC 10662) (Waterworth, 1969a).

TESTS OF PURE CULTURES

Inoculum

A suspension is prepared from parts of at least six or eight colonies, since different but colonially indistinguishable strains, particularly of a staphylococcus, may be present. How dense this should be depends very greatly on the method to be used for

inoculating the plate. In our view, its density should be such that growth is dense but not confluent. When growth is confluent, there is no indication of how heavy the inoculum was: any number from 10^3 to 10^6 organisms may have been deposited per sq cm of medium. Too heavy an inoculum invalidates comparison with a properly inoculated control culture, and is liable to abolish sulphonamide activity altogether.

Inoculation

The only method of inoculation which ensures really uniform distribution of colonies, which is essential if inhibition zone sizes are to be measured or indeed to be accorded any exact significance, is flooding and draining. If for any bacterial species this method is considered too risky, assiduous spreading with a swab or a bent glass rod gives acceptable results. A method now in use at University College Hospital, which is described in a forthcoming publication (D. Felmingham and E. J. Stokes, *Med. Lab. Technol.*, in press), ensures good distribution with a dry swab and at the same time regulates the density of the growth without requiring dilution of the culture.

CHOICE OF DRUGS

The number of drugs used in routine tests, according to Table IX in the preceding paper, varies from one to 17, and we know of a laboratory in which the number is even higher. Any such figure must include superfluous tests, including several members of groups acting alike: there is no need for more than one sulphonamide¹ or tetracycline. A further

¹Sulphafurazole is commonly used. There are few instances of superior activity in individual sulphonamides against particular species, but that of sulphadiazine against *Ps. aeruginosa* may be mentioned, since a test with this or with sulphatriad may indicate sensitivity when one with another sulphonamide does not. It is doubtful whether the degree of this sensitivity is of therapeutic significance.

Pathogen	Pen	Meth	Amp	Car	Cep ¹	Ery	Lin ¹	Fuc	Tet	Chl	Str	Kan	Gen	Pol	Sul	Tri
<i>Staph. aureus</i>	+	+				+	+	±	(+)	(+)	(+)	(+)	(+)		(+)	(+)
<i>Str. pyogenes</i>	+				+	+	+									
<i>Str. faecalis</i>			±			+			+		+					
<i>H. influenzae</i>			±						+	+					+	+
<i>Bacteroides</i>			±			+	±		±	+						
<i>Escherichia</i>			±		+				(+)	(+)	+	(+)	+	(+)	+	+
<i>Klebsiella</i>			±		+				(+)		+	(+)	+	(+)	+	+
<i>Proteus</i>				±	+											
<i>Salmonella</i>			±							+					+	+
<i>Shigella</i>			±							+					+	+
<i>Pseudomonas</i>				+							+		±	±		

Table IV Choice of drugs for tests of principal pathogens

± = drugs of first choice.

(+) = drugs with which further tests may sometimes be indicated.

¹ = Cephalothin may be used instead of cephaloridine, or clindamycin instead of lincomycin.

In addition to the 16 drugs named, nalidixic acid and nitrofurantoin may be used in tests of organisms from urine, but only for this purpose. Cloxacillin and flucloxacillin are excluded, because resistance to these drugs and to cephaloridine in staphylococci should be determined by a special test with methicillin only (see text).

reduction of numbers commonly used must follow acceptance of a conclusion reached earlier in the paper, namely, that a test of the sensitivity of a staphylococcus to methicillin gives a result not only applicable to all penicillinase-resistant penicillins and cephalosporins, but more indicative of resistance than a test with any of these other antibiotics themselves.

Excluding drugs used for special purposes (mycoses, tuberculosis, etc) this leaves a list of 16, given in Table IV, together with suggestions with regard to those indicated for first-line testing against common bacterial species or genera. These are chosen on a basis of superior activity *in vitro* and known therapeutic applicability, but individual workers may prefer to vary these lists. We are mainly concerned to point out that intelligent choice can limit the number of drugs in a primary test to six, or at the most eight, and it will not often be necessary to follow this with more.

Table IV is based on one given by Garrod and O'Grady (1971) (Table LI, p. 466), which also includes suggestions for tests in primary culture of three kinds of specimen. That most likely to be examined in this way is urine, and if the Stokes method is used only four drugs can be tested in a single plate. It may be of interest that a ballot of clinical staff in this hospital chose sulphonamide, trimethoprim, ampicillin, and nalidixic acid for this primary test, and others are made only when the culture proves resistant to two or more of these drugs. Alternatively, the initial use of two plates would enable a full range of eight tests to be performed. Recently a square plastic Petri dish (96 mm × 96 mm) has become available which accommodates six discs.

DISC CONTENTS AND CONTROL ORGANISMS

These are fully discussed in a preceding section. It may be added that in addition to the two usual control cultures, a normally sensitive strain of *Ps. aeruginosa* (NCTC 10662) is required for testing the sensitivities of other strains of this species.

INTERPRETATION

This is based on comparing inhibition zones of test and control organisms. When the Stokes method is used, and direct comparison is therefore possible, zones need not be measured if that of the test organism is as large or larger than that of the control. When the test zone is smaller, and in all tests in which the control is on a separate plate, zones should be measured with calipers. We admit that a scheme for interpreting differences in zone size might with advantage be based on more extensive studies than we have been able to carry out, but few mistakes are

likely to be made if the following definitions are accepted:

Sensitive

Zone diameter equal to or larger than that of control.

Moderately sensitive

Zone diameter smaller than the control by at least 4 mm.

Resistant

Zone diameter <10 mm (diameter of disc being 6 mm).

The inocula of test and control organisms must be comparable and both measurements made by the same operator.

These instructions are also applicable to tests with high content discs and the more resistant control for examining organisms from urine.

Special considerations apply to the following drugs and organisms:

Polymyxins

These antibiotics diffuse so slowly that zones are small and the dimensions above do not apply. Only infections by Gram-negative bacilli are likely to respond to treatment and the *E. coli* or *Ps. aeruginosa* controls should always be used.

Trimethoprim

This drug is efficacious in infections caused by organisms having a wide range of MIC, and *E. coli*, proposed here as a more resistant control, is one of the most sensitive to it. It also diffuses rapidly, hence inhibition zones exceeding the optimum diameter chosen may be obtained if a disc of more than 1.25 µg is used. Results with a disc containing both trimethoprim and sulphamethoxazole are given in Table I, but it is preferable to test each component separately (Waterworth, 1969b).

Ps. aeruginosa

This organism is less sensitive than *E. coli* to either carbenicillin or gentamicin, and its apparent sensitivity to gentamicin is much affected by the composition of the medium. Both difficulties can be overcome by using a normally sensitive strain of *Ps. aeruginosa* itself (NCTC 10662) as the control, preferably in parallel culture by the Stokes method. Interpretation is on the same basis, and when test and control zones are of similar size the strain can be reported as showing normal sensitivity.

Penicillin-resistant staphylococci

The amount of penicillinase formed by resistant strains varies between very wide limits, and those

forming smaller amounts may give quite large inhibition zones round a penicillin disc, although they can be recognized both by a somewhat smaller measured diameter than in the staphylococcus control and by the large colonies forming the heaped-up edge of the zone. The scheme of interpretation proposed would class these as moderately sensitive, but the now common practice of reporting all penicillinase-forming staphylococci simply as resistant appears preferable.

References

- Anderson, T. G. (1970). Testing of susceptibility to antimicrobial agents and assay of antimicrobial agents in body fluids. *Man. Clin. Microbiol.*, edited by J. E. Blair, E. H. Lenette, and J. P. Truant, p. 299-310. Bethesda, Md. American Society for Microbiology.
- Association of Clinical Pathologists (1965). Report on antibiotic sensitivity test trial organized by the Bacteriology Committee of the Association of Clinical Pathologists. *J. clin. Path.*, **18**, 1-5.
- Bauer, A. W., Kirby, W. M. M., Sherris, J. C., and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Amer. J. clin. Path.*, **45**, 493.
- Bauer, A. W., Perry, D. M., and Kirby, W. M. M. (1959). Single disk antibiotic sensitivity testing of staphylococci. *Arch. intern. Med.*, **104**, 208-216.
- Beaney, N., Goodwin, N. G., Jones, R. V., Winter, R., and Sippe, G. R. (1970). Antibiotic sensitivity testing. *Med. J. Aust.*, **1**, 483-484.
- Castle, A. R., and Elstob, J. (1971). Antibiotic sensitivity testing—a United Kingdom survey undertaken in September 1970. *J. clin. Path.*, **24**.
- College of Pathologists of Australia (1968). A survey of antibiotic sensitivity testing. *Med. J. Aust.*, **2**, 171-172.
- Ericsson, H. (1960). Rational use of antibiotics in hospital: studies on laboratory methods and discussion of the biological basis for their clinical application. *Scand. J. clin. Lab. Invest.*, **12**, Suppl. 50.
- Ericsson, H. M., and Sherris, J. C. (1971). Antibiotic sensitivity testing: report of an international collaborative study. *Acta path. microbiol. scand.* [B], Suppl. 217.
- Garrod, L. P., and O'Grady, F. (1971). *Antibiotic and Chemotherapy*, 3rd ed., p. 463. Livingstone, Edinburgh.
- Garrod, L. P., and Waterworth, P. M. (1969). Effect of medium composition on the apparent sensitivity of *Pseudomonas aeruginosa* to gentamicin. *J. clin. Path.*, **22**, 534-538.
- Hewitt, J. H., Coe, A. W., and Parker, M. T. (1969). The detection of methicillin resistance in *Staphylococcus aureus*. *J. med. Microbiol.*, **2**, 443-456.
- Institute of Medical Laboratory Technology. (1960). A survey of antibiotic sensitivity tests. *J. med. Lab. Technol.*, **17**, 133-143.
- Schwarz, L. H., and Brown, B. A. (1954). An improved rapid method of tube dilution antibiotic sensitivity testing. *Antibiot. and Chemother.*, **4**, 333-337.
- Stokes, E. J. (1968). *Clinical Bacteriology*, 3rd ed., p. 179. Arnold, London.
- Waterworth, P. M. (1969a). Interpretation of disc sensitivity tests on organisms of intermediate sensitivity. *J. med. Lab. Technol.*, **26**, 106-110.
- Waterworth, P. M. (1969b). Practical aspects of testing sensitivity to trimethoprim & sulphonamide. *Postgrad. med. J.*, **45**, Suppl. (Nov.), 21-29.

Addendum

When we performed the experiments with WST we were uninformed about its composition. We now know that it contains 0.38% of glucose and 0.2% starch. This is considerably more carbohydrate than either DST (0.25% glucose) or MH (0.15% starch) and it seems probable that this is the cause of the misleadingly large zones seen on WST with *H. influenzae* and cephalosporin discs.

As it has previously been reported (Association of Clinical Pathologists, 1965) that the presence of larger quantities of glucose in the medium made *Pr. mirabilis* appear falsely sensitive to nitrofurantoin, we have now tested a number of strains of this species against this drug. We found that zones on WST were 7-10 mm larger than those on DST though there was only 1-3 mm difference between the control zones.

These findings have been discussed with the manufacturers of WST who have agreed to review the carbohydrate content of the medium.

The publishers are also to be congratulated on the high quality of the printing, binding, and photographic reproduction, and even at the price of £15.00 it represents extremely good value for money, even at today's inflated prices.

J. A. MILNE

Pathology Annual Volume 5, 1970 Edited by Sheldon C. Sommers. (Pp. x + 436; illustrated. £7.00. \$16.50.) New York: Meredith Corporation. London: The Butterworth Group. 1970.

This fifth volume of the *Pathology Annual* maintains its high standard of selection of material and presentation. Naturally enough electronmicroscopic studies are prominent and include the ultrastructure of the normal and neoplastic prostate, primary carcinoma of the liver, adrenal medullary tumours, and acute inflammation. Microdissections of the nephron in a number of diseases are beautifully demonstrated, including some of Darmady's work in this country. More classical histopathological study of diagnostic problems involving nodal lymphomata, although not novel, does indicate commonly encountered pitfalls. There is also an excellent chapter on lupus nephritis in which the EM studies are shown to confirm the present view of the changes in the nephron. The EM dense deposits correspond to the areas which by immunofluorescent examination are found to contain immune globules.

Further studies on the pathogenicity of antigen-antibody complexes by fluorescent and EM techniques demonstrate the presence of such complexes in many parts of the body, but the author suggests that the factors predisposing certain parts of certain organs to injury by the complexes is apparently a non-immunological process.

The final chapter is a very practical demonstration of the use of polarized light in pathology.

A. G. SIGNY

Human Tumors, Histology, Diagnosis, and Technique. 2nd ed Edited by Pierre Masson. (Pp. 1359; illustrated. \$45.00.) Detroit: Wayne State University Press. 1971.

The second (French language) edition of Professor Pierre Masson's book 'Tumeurs humaines' was published only a few years before the author's death in 1959. The majority of Masson's numerous scientific

articles, written between 1908 and 1957, were published in French language journals. The appearance of this English translation of Professor Masson's treatise on the pathology of human tumours is therefore to be welcomed, and Dr Sidney Kobernick should be thanked and congratulated on the production of a volume which will commemorate for us the life and work of a great pathologist. The short biography of Pierre Masson by Professor L. C. Simard is also a warm personal tribute.

In scope the book aims at a survey of the whole range of human neoplasms arising from all organs and tissues of the body, and there is a final section dealing with histological techniques, including various special staining procedures. No translation can ever capture all the flavour and nuances of the original text, and some of the phraseology may appear quaint and unfamiliar to English readers. If anything, this adds to the charm of the book and makes it stimulating reading. A large proportion of the illustrations of microscopic appearances are drawings; too large a proportion by modern standards, Dr Kobernick's skilful advocacy for their use notwithstanding. The photomicrographs vary considerably in quality, some being frankly poor.

There is no doubt that Dr Kobernick's translation will be read with great interest and profit by many professional pathologists, and especially by those whose particular concern is the histopathology of human neoplasms. However, in view of the excellent modern treatises and numerous monographs already available, it is unlikely to become a widely used reference work amongst English-speaking pathologists.

N. F. C. GOWING

Correction

We regret that in the legend for Fig. 2 in the paper by L. P. Garrod and Pamela M. Waterworth (*J. clin. Path.*, **24**, 779-789), a line of explanation is missing. It should read as follows:

Fig. 2 *Effect of medium on clarity of zone edges. All plates are inoculated with E. coli and discs contain (upper left) ampicillin 25 µg, (upper right) gentamicin 10 µg, (lower left) streptomycin 25 µg, (lower right) tetracycline 25 µg. The plates contain: a WST, b DST, and c MH.*

This correction applies also to the cover where there is a printing error in this legend.

Notices

Study of Ovarian Tumours

A panel of pathologists for the study of ovarian tumours has been formed under the auspices of the Royal College of Obstetricians and Gynaecologists with the support of the Cancer Research Campaign. The members of the panel are Professor A. R. Currie, Dr A. D. T. Govan (convener), Dr Magnus Haines, Dr F. A. Langley, Dr A. M. Neville, Dr C. W. Taylor, and Dr A. S. Woodcock.

At present the panel members are interested in gonadal tumours in females and apparent females under the age of 25 years. Dysgerminomas and gonadoblastomas are particular examples. They are also interested in related gonadal abnormalities in this age group such as streak gonads.

Material in any form would be welcome—sections stained or preferably unstained—paraffin blocks or fixed material. If fresh unfixed material for electron microscopy, tissue culture, and endocrine studies were available, arrangements could be made for its collection by phoning the convener of the panel.

All communications should be addressed to: Dr A. D. T. Govan at the Royal Maternity Hospital, Glasgow, C4.

Telephone numbers: 041-552-4513, 041-552-1942.

XX Annual Colloquium on Protides of the Biological Fluids

The XXth Annual Colloquium on 'Protides of the biological fluids' will be held from 3 to 7 May 1972, at Bruges, Belgium.

All information can be obtained from the Simon Stevin Instituut, Jerusalem Straat 34, B-8000 Bruges, Belgium.