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STANDARDIZATION OF METHODS FOR CONDUCTING MICROBIC SENSITIVITY TESTS

Second Report of the Expert Committee on Antibiotics

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GENEVA

1961

EXPERT COMMITTEE ON ANTIBIOTICS

Geneva, 11-16 July 1960

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STANDARDIZATION OF METHODS FOR CONDUCTING MICROBIC SENSITIVITY TESTS

Second Report of the Expert Committee on Antibiotics

1. INTRODUCTION

An Expert Committee on Antibiotics met in Geneva from 11 to 16 July 1960. In an opening address, Dr P. Dorolle, Deputy Director-General of the World Health Organization, stressed the importance of the problems which the Committee was going to discuss, stating that, for obvious reasons, it would be of great public health value if reliable and comparable information could be obtained on bacterial sensitivity and resistance to antibiotics in different parts of the world.

Professor Maurice Welsch was elected Chairman, Dr William Wright Vice-Chairman, and Professor Lawrence P. Garrod Rapporteur. The draft agenda was discussed and adopted with minor alterations.

2. IMPORTANCE OF BACTERIAL RESISTANCE TO ANTIBIOTICS

Bacterial resistance to antibiotics is the principal obstacle to their successful therapeutic use. When resistance develops during a course of treatment, it may deprive an antibiotic of its proper therapeutic effect in the patient being treated. More important in the long run is the effect on the general community, since the elimination of sensitive strains and the dissemination of resistant ones leads to a situation in which many infections are resistant *ab initio* and alternative treatment must be adopted. For this reason, the estimation of bacterial sensitivity or resistance to antibiotics has assumed great importance. Such estimations are an essential prerequisite for the rational use of antibiotics and for preserving the efficacy of this important group of therapeutic substances.

2.1 Necessity for the adoption of reliable and standard methods

It is well known that for determining bacterial sensitivity to antibiotics several principal methods are used, each of which has innumerable variations. Some published results have been obtained by methods to which there are serious objections, and there are good reasons for believing that some laboratories that do not publish their findings use methods which are even less satisfactory. The universal adoption of reliable methods, standardized as far as possible, would have the following three advantages :

1. It would afford the best possible guidance to the clinician in the treatment of his patients.

2. It would enable comparative assessments to be made of the frequency, importance and epidemiology of resistant strains of bacteria in different institutions, areas and countries.

3. It would facilitate the interpretation of published findings, which often cannot be compared with those of other workers.

In connexion with the last point, it is important that published accounts of the effects of antibiotic treatment should include details of sensitivity determinations and the methods by which they were made.

2.2 The nature of microbic resistance to antibiotics

2.2.1 *Definition of terms*

To describe a micro-organism simply as " sensitive " or " resistant " to an antibiotic, although a common practice, is inexact. Resistance is never absolute and calls for quantitative expression. It is therefore preferable to say that a micro-organism is sensitive (or resistant) to a specified concentration. Since there is no internationally accepted standard method, this implies that the technique used to measure sensitivity be adequately described.

It should also be observed that the term " resistant " is used, without further definition, to describe two rather different properties :

1. A micro-organism is said to be resistant when the concentration of antibiotic which it is able to withstand is appreciably higher than the concentration obtainable *in vivo*.

2. A micro-organism is also said to be resistant when it tolerates a concentration of antibiotic appreciably higher than that which inhibits the growth of the majority of individuals of the same culture ; similarly, a microbial strain is said to be resistant when it tolerates a concentration of antibiotic appreciably higher than that which inhibits the growth of the majority of other strains of the same species.

The term " sensitive " can similarly be used in two senses. The property of being sensitive or resistant, whether quantitatively stated or not, is often ascribed to a microbial species or to a particular strain. In fact, the assay is always performed with a certain culture of which a very small sample only is used as an inoculum. The result thus obtained is extrapolated, for instance, to the whole microbial population infecting a patient, a procedure that is not always entirely justifiable.

Depending upon the technique adopted, the result of a sensitivity test sometimes tells only the degree of sensitivity of the most resistant individuals present in the inoculum, and sometimes shows a heterogeneity in this respect among members of the microbial population actually tested. It is important to underline that failure to detect heterogeneity despite the use of an adequate technique is not proof that it does not exist in the larger population from which the sample tested was obtained, since the sample may well be too small and not sufficiently representative.

In fact, heterogeneity is always present : wide in the case of some species and of some antibiotics ; narrow, and of no practical importance in others. Ideally, however, the sensitivity analysis of a species or of a culture should show the frequency distribution of individual strains or of micro-organisms, respectively, as a function of the maximal tolerated concentration.

2.2.2 *Biological heterogeneity and selection*

The consequences of heterogeneity within species, strains, cultures or even clones are of the utmost importance with respect to antibiotic sensitivity. Immediately suggesting the unavoidable effects of natural selection, heterogeneity will explain the emergence of resistant organisms. This heterogeneity, which varies in degree with species and still more in relation to different antibiotics, is the result either of phenotypical differences, which are unstable and of little practical importance, or of genotypical differences, which are more stable and possibly the origin of resistant clones.

That genotypically resistant individuals may arise by spontaneous mutation quite independently of the antibiotic has been conclusively shown by several ingenious techniques, in particular by indirect selection. It has been confirmed by genetical analysis that a high degree of resistance may be the result of either a single mutation or a series of additive mutations. Although it is not inconceivable that resistance might be acquired through a mechanism of enzymatic adaptation, the actual occurrence of such a process has, up to now, not been satisfactorily demonstrated.

In any sufficiently large microbial population the unavoidable selection of resistant mutants able to withstand the usual concentration of antibiotic reached *in vivo* will lead to the emergence of resistant clones, especially in chronic infections and during the indiscriminate prophylactic use of

certain antibiotics. These resistant clones progressively replace the sensitive micro-organisms, prevent the success of specific therapy and, when transmitted to new subjects, sometimes induce infections that, from the start, are not amenable to treatment with the supposedly active drug.

2.2.3 *Biochemical mechanisms responsible for resistance*

These are imperfectly understood, but three principal mechanisms can be recognized :

(1) non-penetration of the antibiotic into the bacterial cell, and its failure to reach the sensitive receptor by this or any other mechanism ;

(2) destruction of the antibiotic by an enzyme or inactivation by a specific inhibitor ;

(3) absence of the sensitive receptor from the resistant cell, or maintenance of a metabolic activity permitting growth, even though the receptor is attacked.

2.2.4 *Conditions influencing the acquisition of resistance*

Among these may be mentioned, because of its practical interest, the presence of a second antibiotic. Although simple *in vitro* observation may suggest that this strongly discourages the acquisition of resistance, it is necessary to remember that conditions *in vivo* are very much more complex, and it should not be too lightly assumed that bacteria subjected to the action of two antibiotics within a focus of infection will behave as they do *in vitro*. Selection *in vivo* involves many more variables than those operating *in vitro*, since many host and other ecological factors come into play.

3. INDICATIONS FOR PERFORMING SENSITIVITY TESTS

In relation to the individual patient, a test need not or should not be performed :

(1) When the infection is due to a micro-organism that is invariably sensitive to the antibiotic. The nature of the infection may be evident from the clinical diagnosis or may have been verified in the laboratory. An example is *Streptococcus pyogenes* of Group A, which is always sensitive to penicillin.

(2) When the nature of the infection cannot be determined. When a mixed growth is obtained containing bacteria of doubtful pathogenicity, the value of sensitivity tests is at least doubtful : at worst they may be misleading by encouraging treatment that has no sound basis.

On the other hand, tests of sensitivity are desirable (assuming that the infection is one meriting antibiotic treatment at all) when the causative organism can be identified as a species known to be capable of exhibiting resistance to antibiotics. Such tests are particularly necessary :

(1) in infections caused by staphylococci, *Mycobacterium tuberculosis*, or varieties of Gram-negative rods (*Escherichia*, *Klebsiella*, *Pseudomonas* and *Proteus* in particular) since these micro-organisms are peculiarly liable to be resistant to one or several antibiotics ;

(2) in certain infections in which bodily resistance is defective, and the antibiotic must consequently play a greater part in overcoming them ;

(3) in relation to particular antibiotics, such as streptomycin, against which resistance develops commonly and rapidly during treatment.

3.1 Relative importance of desirable features in a test

A method of test may have advantages of several kinds, some of which are mutually incompatible. Without anticipating detailed recommendations to be made later, it may be pointed out here that requirements differ according to the purpose of the test. If its object is to direct the treatment of a patient, rapidity and practicability in a busy laboratory are the foremost considerations, and accuracy within narrow limits must be secondary although reproducibility is important. On the other hand, for the requirements of a survey, rapidity is of no consequence, but accuracy and reproducibility are of first importance. It may therefore be doubted whether the same method is likely to be the most suitable for both purposes.

3.2 Choice of antibiotics for the performance of routine tests in clinical laboratories

The following discussion excludes antibiotics with highly specialized uses, such as those acting exclusively on fungi (nystatin, amphotericin, griseofulvin) or on tubercle bacilli (e.g., viomycin). It is concerned only with tests appropriate for common pathogenic species of bacteria other than *Myco. tuberculosis*.

3.2.1 *Groups of antibiotics*

In order to simplify routine tests, it is advisable to include only one respresentative of any group of antibiotics with closely similar actions between which there is cross-resistance. The results should however always be reported in relation to the antibiotic actually used.

Penicillins. Tests performed with penicillin G are adequate for most clinical purposes, but it should not be forgotten that the activity of penicillin V differs from that of penicillin G against some species, and other

penicillins are now being introduced into therapeutics which differ more widely in activity. In certain circumstances, it may therefore be advisable to perform a test with a penicillin other than G.

Streptomycin and dihydrostreptomycin are identical in antibacterial action and only one of them needs to be used.

Tetracyclines. Although there are some differences between members of this group in activity against individual bacterial species, cross-resistance between them is complete when resistance is considerably increased above the normal level, and only one need be used, usually tetracycline itself. Tests with chlortetracycline are unsatisfactory if allowance is not made for its instability *in vitro*.

Neomycin group. Neomycin, kanamycin, framycetin and paromomycin are closely related, and for most purposes not more than one of them need be used, but a test should preferably be performed with whichever member of the group it is proposed to use for treatment.

Erythromycin group. Organisms resistant to erythromycin are sometimes also resistant to oleandomycin and spiramycin and sometimes not : the two latter may therefore have to be included if their therapeutic use as alternatives to erythromycin is contemplated.

Polymyxin B and colistin are very closely related.

Vancomycin and ristocetin are related, but tests with both are desirable if the administration of either is seriously contemplated.

The choice of antibiotics within these groups for laboratory tests should be determined by consultation between the bacteriologist and the clinician.

3.2.2 *Nature of the infection*

It is presumed that either (*a*) the test is to be performed with a pure culture, or (*b*) the probable identity of the infecting organism is known from the examination of a stained film in cases where a test has to be performed on a primary culture. The kinds of test required depend on the nature of the infection in two senses : (*a*) on the bacterial species : for example, haemolytic streptococci of Group A and pneumococci are rarely or never resistant to antibiotics of first choice, and an elaborate series of tests is quite superfluous, whereas staphylococci are commonly resistant to the older antibiotics, and a series of tests may be necessary to identify one to which they are sensitive ; (*b*) on the severity of the disease : in serious infections such as septicaemia or meningitis it may be important to gain extensive and accurate information as speedily as possible, whereas in trivial infections the laboratory cannot be expected to exert itself to such an extent. The nature of the disease affects the choice in another way. If it involves a part of the body where higher concentrations of antibiotics

can be attained than in the blood and tissues generally, then it may be worth while to include tests with antibiotics to which the organism is likely to be relatively resistant.

3.2.3 *A general scheme for the choice of antibiotics*

An attempt is made in the accompanying table to suggest which antibiotics may be appropriate for tests of organisms within eight common bacterial genera. Only one antibiotic representative of each of the groups already referred to is included. The antibiotics are grouped from left to right into :

(1) those active almost exclusively against Gram-positive organisms ;

(2) those having activity against both Gram-positive and Gram-negative species (including penicillin, because it inhibits some strains of *Escherichia*, *Salmonella* and *Proteus* in concentrations easily attainable in some parts of the body, e.g., in the urinary tract) ;

(3) one antibiotic, polymyxin, acting exclusively on Gram-negative species.

With some possible additions from among the above-mentioned groups of antibiotics, this table represents the full range of tests likely to afford useful information. This full range need only be used for organisms such as staphylococci and *Pseudomonas* which commonly exhibit multiple resistance. A shorter series of tests will often suffice : much depends on the type of organism, and on the nature of the disease which it causes.

APPROPRIATE TESTS OF ANTIBIOTIC SENSITIVITY

	Erythromycin	Novobiocin	Vancomycin	Bacitracin	Penicillin	Tetracycline	Chloramphenicol	Neomycin	Streptomycin	Polymyxin
Staphylococcus	+	+	+	+	+	+	+	+	+	
Streptococcus	+	+	+	+	+	+	+		(+)	
Haemophilus	+	+			+	+	+	+	+	+
Escherichia					(+)	+	+	+	+	+
Salmonella					(+)	+	+	+	+	+
Proteus		(+)			(+)	(+)	+	+	+	
Shigella						+	+	+	+	+
Pseudomonas						(+)	(+)	+	+	+

\+ = organism normally sensitive to readily attainable concentrations
(+) = organism normally sensitive to high concentrations

4. METHODS IN PRESENT USE FOR DETERMINING MICROBIC SENSITIVITY

The methods at present in use are divisible into two main categories :

4.1 Dilution methods

In these methods different amounts of the antibiotic are added to a series of tubes or other containers of a culture medium, each of which is then inoculated with the micro-organism to be tested. The presence or absence of growth is recorded after a given period of incubation. The medium used in tubes is usually a liquid one : if plates of a solid medium are used it may be possible to test a number of different strains on each plate.

One advantage of the dilution methods is that they yield directly a value for the minimum inhibitory concentration of the antibiotic for the micro-organism under test. The liquid medium is best adapted for single determinations and the plate for multiple tests, as in surveys. One disadvantage of the method for clinical purposes is that, since it involves the preliminary isolation of a pure culture, the time required for it cannot be much shortened. To obtain consistent and significant results by this, as by any other method, it is naturally necessary to standardize every feature of the performance of the test, notably the composition of the culture medium, the size of the inoculum, and the period of incubation.

Single-tube or plate methods. A much simplified variation of this method is to inoculate a single tube or plate of medium containing a critical concentration of antibiotic, classifying bacteria as sensitive or resistant according to whether their growth is inhibited or not. This appears to be oversimplification, and a proceeding subject to grave errors which may be difficult to detect. An adequately staffed laboratory should be able to carry out tests providing more information than this about the degree of sensitivity or resistance.

4.2 Diffusion methods

In these the antibiotic diffuses in a solid medium, usually an agar medium in a plate. Of several variants of this method the gradient plate and the " ditch " plate appear more suitable for research purposes or for the comparison of different strains of bacteria than for the performance of tests for clinical purposes. In three other variations of the method, the antibiotic diffuses in every direction from a smaller " focus " and the presence of a zone within which growth is inhibited is then an indication

of the sensitivity of the micro-organism to the antibiotic. The cylinder method is better suited to assays of antibiotics than to determinations of sensitivity: for the latter purpose the focus may be a "hole" or "cup" filled with a solution of the antibiotic, or a paper disc or tablet containing the antibiotic and placed on the surface of the medium. The merits of different variants of this proceeding are considered later, but it may be stated here that, properly conducted, a test of this kind can be made to yield a quantitative result of the same order of accuracy as a dilution method.

4.3 Other methods

The dilution and diffusion methods described above are by no means the only forms of test that may be considered useful; others include:

4.3.1 *Rapid methods*

In a tube dilution test speed can only be increased by shortening the period of incubation; preliminary isolation of a pure culture cannot be dispensed with. A reduction in the period of incubation has been achieved by including a substance (whether pH indicator or a dye which is reduced) in the medium as an indication of early growth. A much greater reduction in the whole period required is obtainable by including a diffusion test in the primary plate culture.

4.3.2 *Methods of estimating bactericidal action*

Tests of bactericidal action are necessary only in exceptional cases. A simple method is to perform a broth dilution test of bacteriostatic action and to subcultivate those tubes showing no growth, either into further tubes of broth or on solid medium, in order to enumerate survivors. Similarly, areas on a plate where growth has been inhibited may be scraped with a wire loop or swab from which further cultures are prepared. A further alternative, applicable to any original plate culture in which the antibiotic has diffused, is replica plating, transfer being made by means of a velvet pad or other such device.

4.3.3 *Methods of studying combined action*

Tube dilution tests are applicable to the study of combined bacteriostatic action. Diffusion tests employing either a single disc containing two antibiotics or two discs superimposed are useless, since it has been shown that the effect obtained is only that produced by the antibiotic giving the wider zone of inhibition when acting alone. Diffusion tests in which two antibiotics diffuse from separate adjoining sources (discs or

strips), although not subject to the same criticism, afford results that are often difficult to interpret.

The following method of determining combined bactericidal action is valuable but laborious: from tubes of a liquid medium containing single antibiotics and antibiotics in different combinations but in a fixed concentration or, at most, in two different concentrations, sub-cultures are prepared in such a way as to determine what proportion, if any, of the original inoculum has survived. A transfer proceeding from a culture in which two antibiotics have diffused from adjoining sources is also suitable for this purpose.

5. RECOMMENDED STANDARD METHODS

5.1 The paper disc method for general clinical use

The Committee believed that a tube dilution method, properly conducted, is too laborious and time-consuming for general clinical use. As for the diffusion methods, the use of a hole or cup filled with solution has many advocates, but is somewhat more intricate and difficult than the use of a disc or tablet. Tablets, as hitherto produced, are often unsatisfactory for several reasons, including slow and incomplete liberation of the antibiotic from the tablet, and the possible effect on bacterial growth of the base of which the tablet is composed. For the present, it is therefore recommended that the method for general clinical use should be one in which filter-paper discs are applied to the surface of an inoculated culture medium in a plate. This method is the most economical in labour, and can be made to yield a quantitative result of sufficient accuracy for clinical purposes. The following conditions must be observed if such a test is to yield satisfactory results.

5.1.1 *The disc*

This should be made of an absorbent material, usually paper, which has no interfering effect either on bacterial growth or on the action of the antibiotic. It must be capable of absorbing moisture rapidly and the antibiotic should be evenly distributed in it. A diameter of 5-7 mm is preferable to a larger diameter. Thickness should be sufficient to ensure rigidity and to permit the complete absorption of an adequate volume, e.g., 0.02 ml of antibiotic solution. Exact definition is unnecessary if the conditions already mentioned are fulfilled. The identity of the antibiotic must be denoted either by a colour or by means of a letter or sign; the dye or ink used must not affect either bacterial growth or the antibiotic.

Except in large laboratories with highly skilled staffs it is better to use commercially prepared discs. These should be in sealed containers and bear an expiry date. Once the container has been opened, extreme caution must be exercised to avoid exposure to conditions of even moderate humidity such as those produced by removing the container from the refrigerator and opening it immediately at room temperature. In any case, the period during which the contents of a container that has been opened may be used should be restricted to a maximum of one month, or for discs containing penicillin to a maximum of two weeks. Laboratories using a small number of discs per day should give preference to containers holding only a few discs. The label should also give the generic name of the antibiotic, the batch number, the quantity of antibiotic, as estimated by a performance test such as is described below, and instructions for satisfactory storage conditions.

It is desirable that each government should ensure some system of control over the quality and particularly the antibiotic content of such commercially prepared discs. It is necessary that batches from each manufacturer should be tested from time to time, and the Committee recommended that a " performance test " be used for this purpose. In such a test the widths of the inhibition zones produced by a number of discs are compared with each other for uniformity, and in order to determine the content, with the widths of the inhibition zones produced by a range of standard discs, some containing less and others more of the antibiotic than the stated content of the discs being tested. A tolerance of $\pm$ 40% is permitted in the USA ; the view is sometimes taken that these limits could be narrower and should perhaps be different for different antibiotics.

Standard discs are at present being prepared in control laboratories and used within a short period of their preparation. The Committee recommended that the possibility be investigated of preparing standard discs which can be circulated internationally for the purpose of such tests. If research in this direction can be undertaken, attention might also be given to the composition of the disc itself. It appears by no means certain that absorbent paper, prepared in fact for entirely different purposes, is the ideal material. A material might be found which will regularly liberate a higher proportion of the antibiotic introduced.

If discs are prepared in the laboratory itself, the recommended method is the addition from a pipette or burette of a measured drop of antibiotic solution which is completely and uniformly absorbed by the paper. This must then be dried before use. Discs so prepared must be stored in the cold unless used immediately. The use of wet discs is unsatisfactory and still more so is the dropping of antibiotic solution on a disc already placed on the medium and consequently already saturated with moisture.

5.1.1.1 *Quantity of antibiotic in the disc*

It is preferable that an estimation of sensitivity by this method should be performed with a single disc. If so the quantity of antibiotic which it liberates should be such that :

(1) in tests of a fully sensitive organism the inhibition zone has a diameter of not more than 30 mm ;

(2) in tests of a rather more resistant organism, but one causing infection amenable at least to treatment with larger doses, an inhibition zone of at least an easily measurable diameter will be obtained.

If one disc will not meet these requirements, two may be used containing appropriate amounts of antibiotic.

The quantity of any antibiotic necessary to satisfy these requirements depends on three factors :

(*a*) its absolute activity ;

(*b*) the rate at which it diffuses into the medium ;

(*c*) the concentrations attainable *in vivo*, and the therapeutic activity of the antibiotic in different infections.

Because of these variables and of the considerable variations in disc content at present employed, it is difficult to recommend precise amounts for each antibiotic, and the Committee made no attempt to do so. On the other hand, it may be said that for most antibiotics the disc content should not be less than 1 microgram nor more than 50 micrograms. Penicillin, which is the most readily diffusible and against some species the most active of all known antibiotics, requires the lowest content ; one often adopted, with good reason, is of the order of 2 micrograms. On the other hand, streptomycin, with a generally lower activity and a much lower diffusibility, requires a disc content of 20 micrograms or more. For antibiotics of very low diffusibility, such as polymyxin, much higher disc contents are necessary, if this method is considered to be applicable at all.

The use of a much higher content than here prescribed, in either discs or tablets, obscures the existence of moderate degrees of resistance, and is to be condemned. On the other hand, a second disc of somewhat higher content may be used to assess the possible value of the treatment of urinary tract infections or of local application, since in both these cases higher concentrations are obtained at the site of infection.

5.1.2 *Culture medium*

It would naturally be futile, in proposing a method for international adoption, to define in precise terms the culture medium to be used. All that can usefully be said of it is :

(1) For common bacteria, the same medium should be used so far as possible, regardless of the species. The use of selective or other special media is quite inappropriate.

(2) As far as possible the medium should be one that is widely used, at least in the country in which the test is done.

(3) It should afford satisfactory growth of all common bacteria causing human diseases.

(4) It should not contain anything capable of interfering with the action of the antibiotic.

(5) It should permit satisfactory diffusion of the antibiotic.

(6) Its pH should be constant, preferably at 7.2-7.4. The adjustment of pH to encourage the action of a particular antibiotic is not advisable.

5.1.3 *Plates*

These must be flat-bottomed, both inside and out, and they must be laid on a flat surface for pouring to give a uniform depth of medium. This depth must be kept constant, a suitable depth being 4-5 mm.

5.1.4 *Inoculum*

This may be either a pure broth culture, or a suspension prepared in broth from colonies in a primary culture. It should be representative of the growth in the primary culture, and if there is a possibility that this may be heterogeneous despite apparent colonial identity (an instance of this is the known occurrence of two distinct strains of staphylococcus in primary culture) it may with advantage be prepared from 5-10 or more colonies, provided that these can be sampled without the danger of introducing a contaminant. If such a suspension is used, or alternatively a very young sub-culture in broth, the actual test can be carried out on the day after receipt of the specimen.

The size of the inoculum should be such as to produce a dense but not confluent growth. The quantity of culture required to produce such a growth, or the extent to which it must be diluted, depends on the volume of the inoculum, which is related, in turn, to the method by which the inoculum is spread. The most uniform distribution possible should be aimed at, and undoubtedly the best method of achieving this is by flooding the plate with a bacterial suspension, and immediately draining it by tilting and removing the excess with a pipette. The various streaking methods give much less regular results, although reasonably uniform distribution of a drop of bacterial suspension can be secured by thorough spreading with a bent glass rod.

5.1.5 *Ensuring a dry surface of the medium*

The medium should be dried before inoculation so that the inoculum is rapidly absorbed; if necessary, as is often the case after flooding, there should be a brief period of drying after inoculation.

5.1.6 *Application of the discs*

Discs should be firmly applied so that they are in uniform close contact with the medium. The number per plate should be such that the zones of maximum inhibition do not coalesce, at least not to the extent that it is difficult to read the diameters of the zones.

5.1.7 *Pre-diffusion of the antibiotic*

Pre-diffusion is highly desirable in order to obtain a practically stable diffusion gradient. As this cannot be achieved in less than three hours, a period of 3-4 hours is recommended. The temperature should not exceed 20°C, in order to ensure that bacterial growth does not begin during this period. An incubator maintained at 20°C is ideal for this purpose. Room temperature will serve if it approximates to this level. In tropical countries, if a temperature of 20°C cannot be maintained, refrigeration (4°C) is permissible, although diffusion may be somewhat slower under these conditions and troublesome condensation may occur.

5.1.8 *Incubation*

The plates, preferably inverted, are incubated at 37°C for 18 hours.

5.1.9 *Reading of results*

Inhibition zones should be measured. These measurements can be translated into minimum inhibitory concentrations in micrograms per ml if graphs are available relating these two values for each antibiotic under the exact conditions employed for the test. A warning is necessary against accepting manufacturers' instructions for translating zone size into minimum inhibitory concentration; such instructions are valid only if the precise conditions of the test are specified. The necessary data will not be available in many laboratories and, although the ideal method of interpretation and reporting is in terms of minimum inhibitory concentrations, a simple alternative must be permitted.

This is to interpret zone size as indicating full sensitivity, partial resistance or resistance, the assessment being made in accordance with the experience of the bacteriologist and by comparison with parallel tests on organisms of known sensitivity; such tests should be conducted from time to time, or even daily. In no case should zone size as such be reported.

5.1.10 *Multi discs*

Discs connected by a bridge either with a central sheet of paper or with a peripheral ring are inferior to individual discs as already described, because the zones of inhibition are incomplete and they are often not sufficiently widely separated.

5.2 Special methods

5.2.1 *Rapid method*

The only truly rapid method is one in which the test is performed by diffusion in primary plate culture. This is prepared in the usual way, and antibiotic-containing discs are placed on the surface of the inoculated medium. Conditions to be observed are :

1. At least half of one plate must be left free of discs to permit normal growth and enable all organisms present to be identified.

2. The spreading of the inoculum must be as uniform as possible : experience has shown that this is easily achieved with a bent glass rod.

3. There must be an adequate number of colonies to define the border of the inhibition zone exactly.

4. In the case of mixed cultures, it is important to ensure that, with the exception of the pathogenic species the sensitivity of which is being determined, no organism is present which destroys any of the antibiotics. The only clear instance of such interference occurs with organisms producing penicillinase : if the only infecting organism present is a staphylococcus, for example, the test will give a valid result, but if a streptococcus is also present it may appear to be penicillin-resistant when in fact it is not.

Results by this method can be read after overnight incubation. If necessary, they can be read in about 5 hours, since in this time on a good culture medium, most species form small colonies which are visible through a magnifying glass or to the naked eye as a haze. They may be seen a little sooner if looked for with a microscope, but this refinement seems unnecessary and possibly more subject to error. This ultra-rapid method is only reliable when the material will yield a pure culture of the infecting organism, since different types of colony cannot be identified at this early stage of growth.

It is to be observed that, in the interests of bacteriological diagnosis, growths in such plates must consist of fewer colonies than those obtained when the disc test is done with a pure culture as already recommended. It may also be found inconvenient, in cultures made in the latter part of the day, to allow the recommended period of pre-diffusion.

Results obtained by such a test should not be expressed in exact terms, and should if possible be verified by a subsequent test with a pure culture.

5.2.2 *Methods for* Mycobacterium tuberculosis

The Committee had before it two memoranda on this subject and heard some observations on it from the WHO Secretariat.

Tests for *Myco. tuberculosis* present problems quite distinct from those for any other bacteria. The slow growth of the micro-organism renders the usual diffusion tests inapplicable, and since a dilution method must therefore be employed, there is not the same scope for variation in the techniques used. It is most important to use a test affording information about the distribution of degrees of resistance in the population, and for this reason a solid medium on which colonies can be enumerated is to be preferred. However, the Secretariat informed the Committee that an approximate estimate of the number of colonies is possible in a newly-developed liquid medium which contains no wetting agents, can be fully standardized in advance, and has a long expiry date, thus making comparison between different laboratories possible. This subject is evidently receiving adequate attention, and the Committee agreed that the definition of all details of the methods to be used should be left to WHO.

5.2.3 *Methods for micro-organisms causing venereal diseases*

The Committee had before it a memorandum on this subject and heard some observations on it from the WHO Secretariat. It is evidently most desirable that a more practicable method than is at present available should be devised for testing the sensitivity of *Treponema pallidum* to antibiotics; this is only likely to be possible if cultivation of *T. pallidum* is achieved. In view of the already recognized increase of resistance of the gonococcus to penicillin and even to other antibiotics, extensive surveys to determine the distribution and degree of this change are very necessary, and for these to yield satisfactory results a standard method of test is required. The Committee suggested that these investigations should be undertaken by WHO, but the members of the Committee expressed their willingness to provide information whenever needed. The Committee also urged that stocks of lyophilized cultures should be kept, in order that future direct comparisons may be possible between strains of gonococcus isolated at different times. It would be advantageous to include tests with other antibiotics, such as streptomycin, tetracycline and erythromycin, which may be used for the treatment of gonorrhoea in the future.

5.2.4 *Methods for staphylococci and penicillin*

The staphylococcus is an exceptional micro-organism in that resistance to penicillin in strains isolated from clinical material almost always depends on the formation of an enzyme, penicillinase, which inactivates the antibiotic, and not on capacity to grow in the presence of the antibiotic. The inoculum for the test will contain some of this enzyme and produce

more during the early stage of incubation : hence the result of the test is influenced to a very high degree by the size of the inoculum. Various test methods have been proposed for use in these exceptional circumstances. Whatever the method used, the size of inoculum requires the strictest regulation, and it should be exactly measured in every test by whatever method the laboratory finds most convenient, so that results with different strains may be strictly comparable. In the Committee's view, the disc method is appropriate for this purpose, and a single disc should suffice, but the quantitative value of the test may be improved by using two plates, one heavily and the other more lightly inoculated, a suitable difference being of the order of 2 on a $\log_{10}$ scale. The heavy inoculum should give confluent growth and the light one separate colonies. According to the findings in the two plates, a resistant strain may be classed as producing much or little penicillinase and described accordingly in the report of the test. The view is widely held that infections due to strains in the former class are unamenable to treatment with penicillin, whereas those in the latter may be. It is not suggested that this test should be performed with all strains, but only those causing infections the treatment of which is a serious therapeutic problem.

5.2.5 *Methods for anaerobes*

Tests of anaerobes should be performed in the usual way, the cultures being placed in an oxygen-free atmosphere, as obtainable in a jar in which oxygen is removed by combustion with hydrogen.

The use of media containing reducing substances such as thioglycollate is inadvisable, because these substances inactivate some antibiotics, notably penicillin.

5.2.6 *The use of dilution methods for clinical purposes*

A clinically isolated strain may require to be submitted to a dilution test in the following circumstances :

1. When an exact and direct determination of the minimum inhibitory concentration is desired, as for determining the dosage of an antibiotic required for the treatment of bacterial endocarditis.

2. For organisms of exceptionally slow growth (other than *Myco. tuberculosis*). These include *Actinomyces israeli* and some species of *Bacteroides*.

3. For tests with antibiotics that are very poorly diffusible and thus give an inconveniently narrow zone, or no zone at all, in a disc test : examples are polymyxin and colistin.

4. For tests of filamentous fungi, which do not form colonies with a well-defined edge. It may be added that these fungi are sensitive only to

few antibiotics in clinical use, and that resistance to these is not yet known to occur to any degree of practical consequence.

5.2.7 *Tests of combined antibiotic action*

Methods for this purpose have already been referred to briefly, and the Committee does not feel able to propose the exclusive adoption of any one of them, or to define any method in detail. It nevertheless takes the view that in cases where it is advisable to assess bactericidal action for clinical purposes, as for instance in bacterial endocarditis and staphylococcal septicaemia, the most helpful method is likely to be that in which tubes of a liquid medium containing mixtures of antibiotics are subcultivated on a solid medium to estimate the proportion of survivors.

5.2.8 *Inclusion of tests for sensitivity to synthetic antibacterial drugs*

Discs containing a representative sulfonamide or one of the nitrofuran compounds may be included in any series of sensitivity tests performed by the disc method. If a sulfonamide is used, the medium must be sufficiently free from sulfonamide inhibitors to permit satisfactory inhibition of growth of a sensitive organism, and special care must be taken that the inoculum is not unduly heavy, as the results with sulfonamides are well known to be influenced by the density of the inoculum. These recommendations also apply to dilution methods.

5.3 Methods to be employed in surveys

The Committee agreed that a dilution method should be preferred in international surveys, and that every effort should be made to control all features of the method as closely as possible in order to ensure accuracy. Of the two variants of this method, the Committee recommended the plate dilution method whenever it is applicable, because many strains can be tested in a single plate, and a control of a standard organism of known susceptibility can be included. The following conditions require to be observed in such a test.

5.3.1 *Medium*

The medium is most important because of its growth-supporting properties. Variations in this respect can alter the response of the organism to the antibiotic. The question of the possible effect of the medium on the stability of the antibiotic is also important. No one medium will be satisfactory for testing all organisms. Some organisms, such as the pneumococci and streptococci, may require the addition of defibrinated blood. This addition in itself may constitute a dangerous variable. Other organisms

such as *Haemophilus influenzae* and *Neisseria gonorrhoeae* require a " chocolate " agar. Media available and reproducible in each laboratory should be specified and adhered to. Brands of peptone, meat extract and such other constituents should be rigidly specified. Ideally every laboratory engaged in a particular survey should use the same batch of medium. Since the antibiotic is uniformly distributed in the medium, the depth of the medium and uniformity of depth are unimportant within reasonable limits. The surface should be dried before inoculation.

5.3.2 *Antibiotics*

Antibiotic powders of known potency should be used. Methods of preparing stock solutions should be defined. Solutions should preferably be freshly prepared, although certain antibiotics may be kept in solution in the refrigerator or frozen for extended periods. The concentrations in the series should not be prepared by serial dilution, but rather in bulk by primary or secondary dilution of the stock solution.

5.3.3 *Antibiotic dilutions*

The maximum range covered by these should be from slightly below the minimum inhibitory concentration for the most sensitive strains of the species to somewhat above the highest concentration attainable in the body. The upper limit may be set higher if an aim of the study is to assess degrees of resistance outside the therapeutic range. It is not necessary to use the whole of any chosen range if the micro-organisms to be tested are known not to vary in sensitivity to this extent.

A preliminary test may be made with widely differing dilutions, but, in the test proper, these should differ by twofold steps. It is convenient, and enables direct comparison to be made between the results of different workers, if the baseline for the series of dilutions is a concentration of 1 microgram per ml and if concentrations are expressed on a $\log_2$ scale. Thus, 1 microgram per ml will be denoted by 0 ; 2, 4 and 8 micrograms per ml by 1, 2 and 3 respectively ; and 0.5, 0.25 and 0.125 micrograms per ml by -1, -2 and -3. This avoids the spurious accuracy of the usual method of reporting.

The greatest care must be taken that the antibiotic solution is uniformly mixed with the medium.

5.3.4 *Inoculation*

The plate is divided into areas, either horizontal strips or compartments, each of which is inoculated with one strain of the organism. Inoculation can be by means of a standard wire loop, a Heatley pipette with a fine capillary nozzle, or a multiple point mechanical inoculator. The dilution

of culture used should be so adjusted that the growth obtained consists of densely grouped colonies but not of a mat.

5.3.5 *Incubation*

A fixed period between 18 and 24 hours at 37°C is recommended.

5.3.6 *Reading of results*

Results should be recorded as :

\+ for growth as in the control
± for fewer or smaller colonies
— for no growth

The end-point may be taken as —. Alternatively, if a satisfactory method seems feasible for estimating the amount of growth denoted as ±, a 50% end-point reading has some advantages.

If the test is performed in tubes of a liquid medium, the same conditions apply to the medium, the antibiotic dilutions, incubation, and the reading of results. All the laboratories participating in a survey should use tubes of the same diameter and the volume of medium should be such that it is little affected by evaporation : 2-5 ml is recommended in preference to 1.0 ml or even 0.5 ml as sometimes used. In any case, if tubes of a different diameter must be used, the surface/volume ratio should be kept constant in order to control the gas exchange. The size of inoculum must be strictly standardized : one giving 10^5-10^6 bacteria per ml in the final culture is suitable.

6. RECOMMENDATIONS FOR FURTHER STUDY

In conclusion, the Committee made the following recommendations :

1. Surveys should be undertaken to determine the frequency of bacterial resistance in different parts of the world. The Committee attached the highest importance to the two surveys of this type already mentioned, namely, those concerned with *Myco. tuberculosis* and *N. gonorrhoeae*.

As regards other bacteria, some of these have already been examined extensively, although not by uniform methods in different countries. In the opinion of the Committee, the ecological factors have been neglected in much of this work ; the findings would be more valuable if they could be related to many circumstances concerning the individuals from whom strains of micro-organisms have been obtained and likely to affect the selection of resistant strains. The possibility of amplifying the information

available in such directions has influenced the Committee in proposing further subjects for study.

Another important consideration is that a survey conducted now will enable a collection to be made of present-day strains of micro-organisms which can then be preserved for purposes of comparison with a further series isolated, perhaps, in 5 or 10 years' time.

The Committee proposed that the following surveys be initiated :

(*a*) resistance of *Staphylococcus aureus* to the newer antibiotics ;

(*b*) resistance of pathogenic *Escherichia coli* to all antibiotics suitable for the treatment of infantile gastroenteritis ;

(*c*) resistance of *Salmonella typhi* to chloramphenicol ;

(*d*) resistance of *Neisseria meningitidis* to sulfonamides and penicillin.

These projects are listed in the order of importance which the Committee attached to them, except that no definite priority was allotted between (*b*) and (*c*).

Before the main survey is undertaken, the suitability of the methods used for these surveys should be verified by pilot trials on a small scale but in several countries.

2. A study should be undertaken of the possibility of producing standard antibiotic discs, and improving the composition of discs (see page 13 of this report).

3. Every effort should be made to have available strains of bacteria of known sensitivity.

A prerequisite for the adoption and maintenance of such strains is a highly accurate dilution test for verifying their sensitivity. The Committee recommended that WHO investigate the possibility of formulating such a test. It is advisable in this connexion that a standard medium be prepared and distributed in dried form to participating laboratories.

In selecting strains of bacteria, those already in use for similar purposes should be preferred, provided that they are otherwise suitable. The micro-organism should always be an easily growing one, not necessarily of a pathogenic species, and should have the lowest possible tendency to mutation to greater resistance or sensitivity.

It is advisable that at least three strains, having different degrees of sensitivity, should be available for each antibiotic. They need not be of the same species, and those of lower sensitivity should owe this to a natural property and not to a change artificially produced. The range of sensitivity represented should be between the maximum known for organisms causing human diseases and the minimum still consistent with the possibility of successful treatment by maximum dosage.

Once the strains have been chosen, they should be preserved by some suitable means such as lyophilization and kept available for distribution. It will be necessary to check their sensitivity periodically.