A SLIDE-CULTURE TECHNIQUE FOR DETERMINING STREPTOMYCIN SENSITIVITY OF *M. TUBERCULOSIS* IN SPUTUM

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It is now widely recognized that a rapid method for determining the streptomycin sensitivity of tubercle bacilli is highly advantageous in the management of tuberculosis undergoing, or about to undergo, treatment with this drug. Up to the present three different types of streptomycin sensitivity tests have been described. The first depends on preliminary isolation of the organism by culture on Lowenstein or similar media and the subsequent assay of the isolate in a fluid medium or by slide-culture (M.R.C. Report, *Lancet*, 1948; Giammalvo, Natsios, and Elton, 1949). This method is so slow (six to eight weeks) that its clinical usefulness is doubtful. In the second method the sputum is directly cultured after digestion on agar egg yolk media (Pyle, 1947; Karlson and Needham, 1948; Youmans, Ibrahim, Sweany, and Sweany, 1950), Lowenstein (Holt and Cruickshank, 1949; Hauduroy and Rosset, 1948) or potato-egg media (Anderson, 1949) containing appropriate dilutions of streptomycin. Even under the most advantageous conditions results are not obtained before the fourteenth day by this direct plating method. The third procedure is to use a slide-culture technique (Rubbo, Woodruff, and Morris, 1949; Sievers, 1949; Cummings and Drummond, 1949; Bernard and Kreis, 1949). The present communication is an extension of our previous work on slide-cultures and describes the development of a technique now being used as a routine test in our laboratories.

Methods

The essential problem in the direct micro-culture of tubercle bacilli is to achieve a simple method of treating the sputum without destroying its fixative properties or interfering with subsequent micro-growth. The choice of a fluid culture medium not affecting the activity of streptomycin but at the same time promoting rapid growth is also an obvious requirement. These points will be discussed.

Treatment of Sputum.—Sputa from four cases of pulmonary tuberculosis, two of which were receiving streptomycin therapy, were cultured on slides before and after treatment with 1% trypsin for 30 minutes, 5% oxalic acid for 30 minutes, 4% sodium hydroxide for 30 minutes, 6% (1.2 N) sulphuric acid for 20 minutes, and 23% trisodium phosphate for 24 hours at 37° C. After digestion the sputa were centrifuged at 3,000 r.p.m. for 30 minutes. The sediments were smeared on slides and allowed to dry in an incubator under cover of sterile petri dishes. The slides prepared from the tryptic digestion and the untreated sputa were sterilized by immersion in 6% sulphuric acid. All

slides were then immersed in a fluid serum synthetic medium and incubated for six days at 37° C. After incubation the slides were stained and examined microscopically for typical fasciculate growth (Fig. 1). In addition, all treated specimens were cultured on Lowenstein media for 30 days at 37° C.

FIG. 1.—Typical fasciculate. Micro-colonies of *Mycobacterium tuberculosis*. Medium: serum synthetic medium pH 7.0–7.2. Incubation: six days at 37° C.

From the results shown in Table I the following conclusions were reached: (1) fasciculate growth was more pronounced in smears prepared from the untreated specimens; (2) digestion by NaOH and H₂SO₄ was unsatisfactory, as the smears tended to float off the slides during culture; (3) oxalic acid treatment inhibited growth on slides, but not on Lowenstein medium; (4) after trypsin or trisodium phosphate digestion growth was slight and variable although good growth was obtained on Lowenstein medium.

These preliminary results were confirmed by more extensive tests, particularly with trypsin and trisodium phosphate. In regard to tryptic digestion a fairly consistent pattern of results was obtained in which slide-culture was usually successful before streptomycin therapy was begun, but was seldom successful after eight days' treatment. This inhibi-

TABLE I
THE EFFECT OF DIGESTION OF SPUTA ON GROWTH BY SLIDE-CULTURE

Patient	Strepto- mycin Therapy	No Digestion Slide- culture	NaOH Digestion		H ₂ SO ₄ Digestion		Na ₂ PO ₄ H ₂ O Digestion		(COOH) ₂ Digestion		Trypsin Digestion	
			Slide- culture	Lowen- stein	Slide- culture	Lowen- stein	Slide- culture	Lowen- stein	Slide- culture	Lowen- stein	Slide- culture	Lowen- stein
H.H. . .	0	++	±	++	+	++	±	++	—	++	±	++
M.M. . .	15 g.	++	±	++	±	++	±	++	—	++	±	++
P.P. . .	38.5 "	++	±	++	±	++	±	++	—	++	±	++
B.F. . .	60 "	++	+	++	±	++	±	++	—	++	—	++

TABLE II
THE EFFECT OF TREATMENT DESIGNED TO ELIMINATE CONTAMINANT ORGANISMS ON GROWTH BY SLIDE-CULTURE

Patient	Strepto- mycin Therapy	Degree of Growth Following											
		No Treatment			6% H ₂ SO ₄ Treatment			CH ₃ OH Treatment			Penicillin and Sulphadiazine in Medium		
		Contami- nants	T.B. Bacilli	Unsatisfactory	Contami- nants	T.B. Bacilli	Satisfactory	Contami- nants	T.B. Bacilli	Unsatisfactory	Contami- nants	T.B. Bacilli	Unsatisfactory
L.W.	Nil	+	—	Unsatisfactory	—	+	Satisfactory	±	+	Unsatisfactory	—	+	Unsatisfactory
M.W.	"	±	—	Unsatisfactory	±	+	Satisfactory	±	±	Unsatisfactory	—	±	Unsatisfactory
B.C.	"	+	—	Unsatisfactory	—	+	Satisfactory	+	—	Unsatisfactory	+	+	Unsatisfactory
M.M.	"	+	±	Unsatisfactory	—	+	Satisfactory	+	±	Unsatisfactory	—	+	Unsatisfactory
R.T.	"	+	—	Unsatisfactory	—	+	Satisfactory	+	+	Unsatisfactory	—	+	Unsatisfactory
H.H.	"	+	—	Unsatisfactory	—	+	Satisfactory	+	+	Unsatisfactory	—	+	Unsatisfactory
K.T.	"	+	—	Unsatisfactory	—	+	Satisfactory	+	+	Unsatisfactory	—	+	Unsatisfactory
F.D.	"	+	—	Unsatisfactory	—	+	Satisfactory	+	+	Unsatisfactory	—	+	Unsatisfactory
T.N.	"	+	—	Unsatisfactory	—	+	Satisfactory	+	+	Unsatisfactory	—	+	Unsatisfactory
P.P.	"	+	—	Unsatisfactory	—	+	Satisfactory	±	+	Unsatisfactory	—	+	Unsatisfactory
	29 g.												
	30 "												
Comment on result		Unsatisfactory			Satisfactory			Unsatisfactory			Unsatisfactory		

+++ signifies luxuriant growth. ++ signifies good growth. + signifies moderate growth. ± signifies slight growth. — signifies no growth. Serum synthetic medium pH 7.0-7.2. Incubation for six days at 37° C.

tion of growth occurred only with the slide-cultures, since the organisms were easily cultured from the same material on Lowenstein medium.

It was clear that the usual methods of treating sputa were of little value for the preparation of slide-cultures. From the practical point of view it is unlikely that this affects the sensitivity of the test, since Spendlove, Cummings, and Patnode (1949) have shown that concentration methods do not increase the number of detectable bacilli in a direct smear.

The problem of determining the simplest technique of destroying the varied population of non-acid-fast bacilli and yeasts present in sputum was studied. Four procedures were compared: (1) immersion of the smeared slide in 6% sulphuric acid for two minutes before culture in serum synthetic medium; (2) immersion of the smear in 70% methyl alcohol for five minutes; (3) immersion of the untreated smear in the medium to which 5 units per ml. of penicillin and 50 μ g. per ml. of sulphadiazine had been added; (4) combination of (2) and (3). The treated slides in methods (1), (2), and (4) were washed in distilled water before immersion in the serum synthetic medium. The results for 10 sputa are listed in Table II.

It will be seen that the acid treatment gave the best results, in that fasciculate growth was observed in every specimen with slight contamination occurring only in one instance. Methyl alcohol treatment was invariably unsuccessful in controlling contaminant organisms whose growth tended to inhibit that of the tubercle bacilli. The penicillin-sulphadiazine combination was equally disappointing.

Further investigations of the acid treatment method showed that the number of contaminants decreased as the exposure time to acid increased but that this was accompanied by a decreasing size of fascicle formation. It was found that the optimal time of acid treatment was approximately four minutes, which is shorter than that recommended by other authors (Hauduroy and Rosset, 1948; Sievers, 1949; Cummings and Drummond, 1949; and Bernard and Kreis, 1949).

Choice of Fluid Medium.—Up to the present stage of the investigation we had used a serum synthetic medium described by Williston and Youmans (1949) for all cultural work. However, since Giammalvo *et al.* (1949) had used a saponated blood medium, Sievers (1949) a lysed defibrinated blood, and Bloch (1948) had indicated that chicken embryo extract was of value, we decided to modify the serum synthetic medium along the following lines.

Using the basic medium (SG) containing asparagin 0.5%, KH_2PO_4 0.5%, K_2SO_4 0.5%, glycerol 2.0%, magnesium citrate 0.15% in distilled water (pH 7), modifications were prepared by adding (a) 10% ox serum (SGS, serum synthetic medium as above), (b) 10% defibrinated blood (SGB), (c) 10% yeast extract (SGY), (d) 1% chick embryo extract (SGC), (e) 10% serum and 1% chick embryo extract (SGSC), and (f) 10% egg yolk (SGE). Also included in the series was the saponated blood medium.

Nine sputa from tuberculous patients were cultured on slides (after acid treatment) by immersion in these media for six days at 37° C. The results are summarized in Table III.

It will be seen that the improvement in the degree of growth obtained by supplementing the basic medium (SG) with serum (SGS) was equal to that effected by the various extracts. The addition of egg yolk and yeast extract caused films to deposit on the surface of the slides, making the microscopic observation of fasciculate growth difficult. Saponated blood medium prepared by lysing citrated blood with an equal volume of 1% saponin supported growth equally as well as did serum synthetic medium (SGS).

Examination of Slide-cultures.—Two methods of demonstrating tubercle bacilli and their fasciculate micro-colonies were open to us—either the Ziehl-Neelsen stain, using the

TABLE III
THE EFFECT OF MODIFICATION OF SERUM SYNTHETIC MEDIUM† ON GROWTH BY SLIDE-CULTURE

Medium	Code	Degree of Growth									Average Result	Comment
		Sputum										
		1	2	3	4	5	6	7	8	9		
Basic medium: (a) plus 10% serum ..	SGS	++	+++	+	++	+	++	++	++	++	++	Satis- factory
Basic medium: (b) plus 10% defibrin- ated blood	SGB	++	+++	±	+++	+	++	++	++	++	++	Satis- factory
Basic medium: (c) plus 10% yeast extract	SGY	++	—*	—*	++*	+	±*	++	++	+	+	Unsatis- factory
Basic medium: (d) plus 1% chick em- bryo extract	SGC	++	—	—	—	—	—	—	—	—	—	Unsatis- factory
Basic medium: (e) plus 10% serum and 1% chick embryo extract	SGSC	+	+++	+	++	+	++	++	++	++	++	Satis- factory
Basic medium: (f) plus 10% egg yolk	SGE	++	±*	+	++*	±*	++	++	++	++	+	Unsatis- factory
(g) Saponated blood medium		++	+++	+	++	+	++	++	++	++	++	Satis- factory

signifies luxuriant growth. — signifies good growth. ± signifies moderate growth. + signifies slight growth. — signifies no growth. * Film over slide making reading difficult. † See text for composition. Incubation for six days at 37° C.

light microscope, or the auramine stain (Hughes, 1943), using the fluorescent microscope. Since the latter is used as a routine method in these laboratories we adopted this procedure from the outset. Comparison, however, was made between two techniques with identical results, but the tediousness of the Ziehl-Neelsen method is so great, when compared to the fluorescent technique, that it is easy to appreciate the reluctance in accepting the slide-culture assay as a routine method. In view of the improvements and simplification of apparatus recently described by our colleague (Matthaei, 1950) for fluorescent microscopy we consider the classical method of Ziehl-Neelsen or its modifications (Hallberg, 1946) unnecessarily time-consuming and fatiguing, and do not recommend their use in assessing the results of slide-cultures.

Method.—The following method is now being used in our diagnostic laboratories for the rapid measurement of streptomycin or P.A.S. sensitivity of tubercle bacilli in sputum. Specially cut glass slides, 1 cm. by 6 cm., are prepared and sterilized by heat. Eight marked slides are mounted on metal slide carriers (Fig. 2) smeared liberally with sputum, choosing, where possible, caseous-like particles. The slides are dried in the incubator at 37° C. for 15 minutes and are then immersed in a petri dish containing 6% (1.2 N) sulphuric acid for four minutes. After this they are washed twice by immersion for two minutes in two petri dishes containing sterile distilled water. All these operations are carried out on a bench covered with brown paper, and, as a further precaution, it is useful to place the lid of the dish, which holds the acid or water, inside its cover as shown in Fig. 2.

FIG. 2.—Slide-culture technique showing acid treatment of slides.

The treated slides are then transferred to eight small test-tubes, 10 cm. by 1.5 cm., holding 5 ml. of serum synthetic medium (SGS) or saponated blood medium. In preparing the former it is important to remove traces of soap from all glassware by washing first in dilute chromic acid cleaning mixture and then in water (six rinses). Each pair of tubes contains 0, 2, 10, and 100 μ g. (microgram) per ml. of streptomycin. Although it has been our practice to add streptomycin immediately before a test, Youmans and Karlson (1947) have shown that the drug is quite stable at 10° C. in SGS medium

for at least 35 days. The tubes are sealed with paraffined plugs and incubated for six days at 37° C.

A slide from one of the control tubes is removed with forceps, fixed and sterilized by heating on a hot plate (90° C. for 15 to 30 minutes), and stained with warmed auramine-rhodamine stain for 10 minutes. It has been found that excessive heating of the slides before staining (viz., by passing the slide directly through the bunsen flame) results in a marked reduction in the number of stainable bacilli, and hence should be avoided. The stained smear is examined by fluorescent microscopy, using either ultra-violet light as a radiant or a 100-watt globe as described by Matthaei (1950).

Growth of the tubercle bacilli in the preparation can be readily detected by the presence of cord-like masses of golden acid-fast bacilli, best described as fasciculate micro-colonies (Fig. 1). If growth is observed on this slide the remainder are then examined. If, however, no growth is observed in the control, the cultures are incubated for a further four days. If fascicles do not form after 10 days' incubation it is unlikely that any growth will occur subsequently. On some occasions slight contamination of the medium has been noted, due particularly to yeasts, but this does not affect the titration except when the contamination is very heavy.

The end-point is the lowest concentration of streptomycin inhibiting fasciculate growth of the majority of acid-fast bacilli seen in the preparation. In some tests small fascicles (when compared with the controls) and single cells are seen on the same slide indicating a mixture of partially resistant and sensitive (or non-viable) organisms. Such observations are of value in forecasting the development of resistance.

In view of the fact that the sensitivity of tubercle bacilli to streptomycin may be greatly influenced by the technique employed (Williston and Youmans, 1949) it was decided to investigate further the method just described. To this end the following tests were performed. Streptomycin-sensitive and resistant cultures of *Mycobacterium tuberculosis* H37Rv were prepared in Dubos and Davis liquid albumin medium (Dubos and Davis, 1946) containing 0.05% of Tween 80 to ensure a dispersed growth. The cultures were standardized to the same turbidity, then mixed in different proportions as shown in Table IV. To these mixed culture suspensions sterile ox serum was added to give a

TABLE IV
COMPARISON OF SLIDE-CULTURE ASSAY WITH TUBE DILUTION METHOD

Inoculum	Mixture	Slide-culture Assay (μ g. streptomycin/ml.)			Tube Dilution Method (μ g. streptomycin/ml.)		
		0	10	100	0	10	100
H37Rv sensitive H37Rv resistant	100% 0%	+++	—	—	+++	—	—
H37Rv sensitive H37Rv resistant	90% 10%	+++	+	±	+++	+	±
H37Rv sensitive H37Rv resistant	50% 50%	+++	++	++	+++	++	++
H37Rv sensitive H37Rv resistant	0% 100%	++	++	++	++	++	++

+++ signifies luxuriant growth. ++ signifies good growth. + signifies moderate growth. ± signifies slight growth. — signifies no growth. Serum synthetic medium pH 7.0–7.2. Incubation for six days at 37° C.

final concentration of 80% serum. This provided a bacterial suspension capable of adhering to the slides when cultured by immersion in fluid medium. The tests were made in duplicate from each suspension in serum synthetic medium containing 0, 10, and 100 μ g. of streptomycin per ml. At the same time the suspensions were used to sow heavily 0.05 ml. inoculum and 5-ml. quantities of the same medium containing the same streptomycin dilutions. The slide-cultures and tube cultures were incubated at 37° C. and the results noted after six days' incubation.

It will be seen that the results obtained by the two methods were identical and that it was possible to detect resistant forms in a predominantly streptomycin-sensitive population (90%) by slide-culture. It was evident that slide-culture was as sensitive and gave the same results as the tube dilution method.

A second series of experiments was made with the object of discovering whether this direct method of assay yielded the same results as the indirect method. Tests were done with sputa of six patients by direct slide-culture and later with the same organisms isolated on Lowenstein medium. The latter were subcultured in liquid Tween albumin medium for six days to provide an inoculum for assay by the tube dilution method in serum synthetic medium. The results are shown in Table V, in which it will be noted that

TABLE V
COMPARISON OF SENSITIVITY DETERMINED BY DIRECT SLIDE-CULTURE AND TUBE DILUTION
USING CULTURE ISOLATED FROM SPUTUM

Patient	Streptomycin Therapy	Lowest Concentration of Streptomycin Inhibiting Growth	
		Direct Slide-culture using Sputum	Tube Dilution using Isolated Pure Cultures
E.T.	Nil	2 μ g. per ml.	2 μ g. per ml.
L.M.	"	2 " " "	2 " " "
N.P.	"	2 " " "	2 " " "
E.D.	"	2 " " "	2 " " "
B.F.	30 g.	>800 " " "	>800 " " "
P.P.	45 g.	>1,000 " " "	>1,000 " " "

Serum synthetic medium pH 7.0-7.2. Incubation for six days at 37° C.

no difference in sensitivity (or resistance) was observed between the organisms tested directly and those assayed indirectly from pure cultures.

It may be added that in both series the results were read after 6, 14, and 21 days' incubation without detecting any change in sensitivity. It appears that the short incubation time is equally as effective as longer periods of incubation.

Discussion

The emergence of streptomycin-resistant strains of tubercle bacilli *in vivo* is now well established. While the statistical evidence obtained by the Streptomycin Committee of the Veterans Administration (1948) indicated that resistant forms may develop in 40% of cases after 60 days' treatment the management of the individual case must be based on the clinical, radiological, and bacteriological evidence available. One important aspect of the latter is the determination of streptomycin resistance before and during therapy. As already pointed out, a number of direct and indirect methods have been described, but, in our opinion, slide-culture assay is the method of choice for routine purposes. Of the various techniques dealing with

slide-culture the method described here appears to satisfy the important clinical and laboratory criteria demanded of such a test. The procedure is technically simple, rapid in performance, and yields an accurate result six days after receipt of the specimen. Furthermore, extremely small quantities of sputa can be handled, and the medium is easily prepared and stored. Slide-cultures possess the advantages of direct plating, but without such disadvantages as homogenization of the sputa, the tedious preparation and storage of solid media, the delay in reaching a result (viz., not less than 14 days), and the confusion in readings due to contamination.

The present method differs from those previously described in the following details. (a) Direct smears are made from selected particles as recommended by Spendlove *et al.* (1949). (b) Slides are mounted on a slide carrier to reduce manipulation to a minimum (Fig. 2). (c) Sputum smears are sterilized by immersion in 6% H_2SO_4 for four minutes and then washed twice in distilled water, using a double-sided petri dish (Fig. 2). (d) Serum synthetic medium is used containing 0, 2, 10, and 100 μg . per ml. of streptomycin. Saponated blood medium would be equally satisfactory, but the Dubos and Davis Tween albumin medium is not recommended on the grounds that fasciculate or cord-like growth is inhibited in the presence of Tween 80 (Middlebrook, Dubos, and Pierce, 1947). (e) Growth is determined microscopically with the fluorescent microscope, using Hughes's stain (Hughes, 1943) and Matthaei's optical system (1920). These requirements simplify yet increase the reliability and safety of the test.

It is obvious that the test can only be applied to sputa in which organisms are visible by direct smear. This limitation is not so serious as it appears, since the most important cases requiring investigation are those in which the sputum is positive. The early exudative lesion found radiologically in a symptomless individual without sputum would not provide a suitable specimen for a streptomycin sensitivity either by slide or direct plate methods, but, should the lesion progress, then in all probability the sputum would become positive and hence suitable for slide-culture assay. Other pathological fluids containing visible tubercle bacilli can be treated by slide-culture, provided a suitable fixative, such as serum or albumin, is used.

So far over 100 sputa have been examined in these laboratories with consistently good results. Only on a few occasions has it been necessary to repeat the test because of technical imperfection of the method. As a result of this experience we arbitrarily designate grades of resistance as follows:

Sensitive strain	..	..	growth inhibited by 2 μg . per ml. of streptomycin.
Slightly resistant	..	..	growth in 2 μg . per ml. but inhibited in 10 μg . per ml.
Moderately resistant	..	..	growth in 10 μg . per ml. but inhibited in 100 μg . per ml.
Highly resistant	..	..	growth in 100 μg . per ml.

Summary

A description of a slide-culture method for determining streptomycin-sensitivity of tubercle bacilli in sputum has been presented. The method involves culture of the organisms as micro-colonies on the surface of glass slides immersed in serum synthetic medium containing three concentrations of streptomycin. As the method does not involve preliminary isolation of the tubercle bacilli a result can be given six days after receipt of the specimen. The method possesses the advantages of the direct plate without its practical disadvantages.

A good deal of comparative testing was undertaken with a view to assessing the reliability and accuracy of the slide-culture assay. It was found that the results were identical with those obtained in liquid medium and the method was capable of detecting resistant mutants in a predominantly sensitive population of cells.

Arguments have been advanced on the grounds of simplicity and rapidity that slide-culture is the method of choice for determining streptomycin-sensitivity of tubercle bacilli in sputum, provided the organisms are visible in the direct smear from selected particles.

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