

A SIMPLE FILTER-PAPER DISC METHOD FOR DETERMINING THE SENSITIVITY OF *MYCO. TUBERCULOSIS*

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Because of the success of modern chemotherapy in tuberculosis, it has become necessary to estimate the drug sensitivity of most strains of *Mycobacterium tuberculosis* isolated from patients. What has been until recently a specialist investigation is now a routine test performed in a general hospital laboratory.

With this transition from a research to a routine laboratory procedure, certain modifications of existing methods are worth considering, not only to obtain economy in time, media, glassware, and incubator space, but also to reduce the risks of infection of laboratory staff.

Drug sensitivity of *Mycobacterium tuberculosis* can be determined in fluid media (Medical Research Council, 1953) or by slide culture technique (Rubbo and Morris, 1951), but both methods are time consuming and hazardous to the operator, and the former uses much incubator space. More commonly, solid media impregnated with various concentrations of appropriate drugs are used, the aim being to determine the minimum drug concentration which inhibits growth of a test strain and of a sensitive control. This method has the disadvantages that the gaps between drug concentrations are wide, making interpretation of results sometimes difficult, and that several slopes of media are required for each drug investigated.

Hoyt (1951) reported the use of compressed tablets of streptomycin in performing sensitivity tests. Collins (1955, 1956) used filter paper discs impregnated with streptomycin or isoniazid, placed upon a solid medium, in parallel with tube dilution in Dubos liquid medium, and found close correlation between them. In the present investigation, we have performed sensitivity tests on organisms using impregnated paper discs, and have compared the results of this method with those obtained using impregnated solid media in parallel.

Materials

First, 94 cultures of *Mycobacterium tuberculosis*, isolated principally from sputum, were tested for sensitivity to streptomycin, then 205 cultures were tested for sensitivity to streptomycin, P.A.S., and isoniazid. Later 49 and 52 cultures were tested with "dipasic" and "viomycin," respectively.

Fifty-seven positive sputum concentrates were tested directly with streptomycin, P.A.S., and isoniazid.

Methods

Culture Media.—All primary cultures were grown on Löwenstein-Jensen medium (Mackie and McCartney, 1953) dispensed in 7 ml. amounts in 1 oz. (30 ml.) screw-capped bottles sloped at 10° to the horizontal during inspissation. The same medium was used in performing tests with paper discs.

The impregnated Löwenstein-Jensen slopes used were obtained from the Southern Group Laboratory in $\frac{1}{2}$ oz. vials.

Inoculum.—Several colonies from each primary culture were picked off with a "spade" of flattened nichrome wire, and emulsified on the dry inside of a sterile bijou bottle above 1 ml. of distilled water. The emulsion was mixed to a smooth suspension, and, by dilution with water, matched with No. 1 Brown's opacity tube (500×10^6 organisms per ml.). One drop of this suspension, approximately 0.015 ml., was delivered from a sterile pipette terminating in a No. 19 gauge hypodermic needle, and allowed to run down the centre of each culture slope, which was then capped and incubated upright without spreading the inoculum.

Concentrates.—Sputa, homogenized by 3% NaOH for 20 minutes and neutralized by 10% HCl, were centrifuged at 3,000 r.p.m. for 30 minutes. The inoculum was 0.015 ml. of the neutral deposit. Only concentrates containing at least one bacillus per microscopic field of the $\frac{1}{7}$ in. (3.6 mm.) oil-immersion lens ($500 \times$) were accepted for the direct test.

TABLE I
COMPARISON OF IMPREGNATED MEDIA AND DISC METHOD TESTS WITH VARIOUS STRAINS

Drug	Impregnated Media Maximum Concentration per ml. Permitting Growth				Disc Result	Zones of Inhibition (mm.)			Total Strains Tested
	< 1 µg.	1 µg.	3 µg.	10 µg.		Range	Range of Majority	Mean	
Streptomycin ..	229	16	8 13	33	S PR R	10-24 5-7	12-18 (84%)	14.5 6.5	299
P.A.S.	177	7 10	10 11		S PR R	10-50* 6-9	20-40 (75%)	29 7	205
Isoniazid	148	0.2 µg. 6 3	1 µg. 14	5 µg. 34	S PR R	10-50† 6-8	25-40 (85%)	33 7	205
Dipasic	32	0.2 µg. 2	1 µg. 4	5 µg. 11	S R	10-50		28	49
Viomycin	45	10 µg. 4	50 µg. 3		S PR R	10-24 6-9	10-16 (91%)	12 7	52

S=Sensitive. PR=Partially resistant. R=Resistant. * Twenty-four strains were completely inhibited. † Six strains were completely inhibited.

Discs.—Discs, each of 6 mm. diameter, were cut from Ford 428 mill coloured blotting-paper, sterilized by dry heat, impregnated with the appropriate drug solution, and dried *in vacuo* in a desiccator over phosphorus pentoxide. The prepared discs showed no loss of potency when stored at 4° C. for 12 months. A simple colour code distinguished the drugs.

The amount of drug used in each type of disc was: streptomycin 10 µg., P.A.S. 5 µg., isoniazid 1 µg., "dipasic" 1 µg., and viomycin 25 µg. The drug concentrations per disc were selected by experiment in order that (a) a zone of at least 10 mm. occurred with strains accepted as sensitive by impregnated media; (b) no zone was obtained with strains accepted as resistant by impregnated media; and (c) a zone of less than 10 mm. occurred with partially resistant strains.

In testing, a disc was placed on the Löwenstein slope, near the top of the medium, as soon as convenient after seeding the culture suspension.

Zones were obtained by measurement of the radius from the centre of the disc to the edge of the inhibition of growth. Measurements were made from both sides of the slope and their average accepted.

Period of Incubation.—With discs it was possible to record sensitivities in 10 to 14 days, and no encroachment on the zone of inhibition occurred within 21 days. Growth on impregnated media was recorded at 21 days, and an organism was considered sensitive when less than 20 colonies grew on a slope.

Results

Comparison of Discs with Impregnated Media.

—From Table I it can be seen that there were no discrepancies between the methods performed in parallel. With all the drugs used, the 10 mm. zone of inhibition was found to provide a yardstick for sensitive strains.

Reliability of Discs.—Tables II and III show that results using discs are reliable with fully sensitive strains (Table II) and with strains of diverse sensitivity (Table III). The three tests made on each strain, illustrated in Table III, were carried out on slopes from the same batch of

TABLE II
IMPREGNATED MEDIA AND DISC METHOD TESTS WITH SENSITIVE H37Rv STRAIN

Drug	Total No. of Tests	Impregnated Media Maximum Concentration Permitting Growth (µg./ml.)	Zones of Inhibition (mm.)	
			Range	Mean
Streptomycin	28	< 1	13-16	14.5
P.A.S. ..	28	< 2	15-45*	28
Isoniazid ..	28	< 0.2	25-45†	31
Dipasic ..	24	< 0.2	15-45	30
Viomycin ..	24	< 10	10-16	12

* In four tests growth was completely inhibited. † In one test growth was completely inhibited.

medium, and were seeded on the same day with equal inocula from the same suspension of each strain. Under these conditions zone variation between tests was almost eliminated.

Effect of Size of Inoculum.—Highly concentrated inocula produced results indicating false resistance, and a too dilute inoculum gave results of false sensitivity. Typical results are shown in Table IV. For these tests, the inoculum, which was always 0.015 ml., was altered from the standard (No. 1 Brown's opacity tube) to give $0.1\times$, $10\times$, and $100\times$ the concentration of bacteria.

Discussion

The two methods, impregnated media and impregnated discs, show close correlation and no advantageous features in the use of impregnated media. In fact, because of the wide spacing of concentrations in impregnated media, discs appeared to give clearer results with strains showing partial resistance. For example, nine strains resisting $0.2\ \mu\text{g./ml.}$ of isoniazid but sensitive to $1.0\ \mu\text{g./ml.}$ were separated by the disc technique into six strains partially resistant and three resistant.

Mixed strains of differing sensitivities in a culture can be detected more readily by discs than by impregnated media. In one test isoniazid inhibited most of the growth at 25 mm., but 50 colonies grew within the zone: the latter on sub-culture proved to be uniformly resistant. The readings with impregnated media suggested

doubtful sensitivity. Inocula from only one colony of the primary culture are therefore quite unreliable for sensitivity testing.

The mean zones of the H37Rv strain (Table II) and of other sensitive strains (Table I) compared closely, although zones of much greater range occurred with the latter, confirming that the degree of sensitivity does influence zone size. The repeated testing of H37Rv, however, showed variations which suggest that it would be unwise to construct standard graphs to convert zones into $\mu\text{g./ml.}$ as suggested by Maccabe and Gould (1954). It seems likely that zones were also influenced by batch-to-batch differences in media. Zone variations between tests were insignificant when tests were carried out under as nearly identical conditions as possible (Table III).

Maccabe and Gould (1954) deduced that zones were altered by not more than 1 mm. by variations some tenfold in the inoculum, and Middlebrook (1954) that a concentrated inoculum may cause a fully sensitive strain to simulate complete resistance. These observations are borne out in Table IV where it is shown that the inoculum should be no more dilute than the "standard" nor concentrated more than tenfold. This standard was found to be both reliable and reproducible.

Sensitivity Testing of Concentrates.—This was of little value and the best results were obtained from pre-treatment specimens. During a course of chemotherapy, heavily positive concentrates yielded insufficient growth for the interpretation

TABLE III
DISC METHOD TESTS IN TRIPPLICATE UNDER IDENTICAL CONDITIONS

Strain	Streptomycin			P.A.S.			Isoniazid					
	Result	Zone (mm.)			Result	Zone (mm.)			Result	Zone (mm.)		
		1	2	3		1	2	3		1	2	3
998	PR	7	8	8	R	—	—	—	S	19	16	20
1034	S	12	12	13	S	20	20	18	S	30	38	30
1062	R	—	—	—	S	22	22	20	R	—	—	—
1151	R	—	—	—	R	—	—	—	R	—	—	—
1182	S	12	12	13	S	36	+	+	S	38	35	41
1313	PR	6	8	7	S	15	15	17	S	27	30	33

+ Growth was completely inhibited. — Denotes absence of inhibition.

TABLE IV
DISC METHOD TESTS WITH VARYING INOCULA (ZONES IN MM.)

Strain	Streptomycin				P.A.S.				Isoniazid			
	0.1 ×	Standard*	10 ×	100 ×	0.1 ×	Standard	10 ×	100 ×	0.1 ×	Standard	10 ×	100 ×
1151	8	—	—	—	†	—	—	—	—	—	—	—
1182	13	12	13	—	†	36	35	—	—	38	30	—

* Standard equivalent to No. 1 Brown opacity tube. † Growth was completely inhibited. — Denotes absence of inhibition.

of sensitivity. Uneven distribution of viable bacilli is, however, easily detected by the disc technique since some growth is expected on each slope, whereas a chance distribution of negative slopes of impregnated media may lead to a false sensitivity report.

The disc method is readily adapted for testing strains of *Myco. tuberculosis* against other drugs, and a number of tests have been performed with terramycin (25 µg./disc) and cycloserine (50 µg./disc).

Since this material was collected, more than 700 strains of *Myco. tuberculosis* have been tested for sensitivity by the disc method, without any apparent difficulties. Promising results for combinations of drugs have been obtained by placing discs containing different drugs on top of each other, and this is being further investigated.

Summary

A simple and reliable method of testing the sensitivity of *Myco. tuberculosis* to various drugs

is described. Discs containing a single strength of each drug are placed at the upper ends of Löwenstein-Jensen slopes seeded with standard inocula of strains under test.

The method has many advantages over the use of impregnated media, not the least being a substantial reduction in the number of slopes. For doubtfully resistant strains interpretation has been more exact.

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