

# THE CONCENTRATION, MICROCULTURE, AND SENSITIVITY TESTING OF *M. TUBERCULOSIS*

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The Tuberculosis Chemotherapy Trials Committee, in the summary of their second report to the Medical Research Council (1953), state that "there is a great need for simple, reliable, and speedy techniques for the culture of tubercle bacilli and for the determination of their sensitivity to chemotherapeutic agents."

Since Pryce (1941) introduced the method of culturing *M. tuberculosis* in smears of sputum on glass slides, in a liquid medium, many workers have reported their success with a variety of technical modifications. Rubbo and Morris (1951) applied the microculture technique to streptomycin sensitivity tests.

The great advantage of microculture is the speed with which the growth of the organisms can be detected. Particularly in general hospital practice, the possibility of a diagnostic or sensitivity report in two to five days instead of in two to five weeks is a great advance. We set out to investigate the reliability of the method. The need is for a technique which is simple, reliable, and relatively inexpensive, equally good for concentrates of urine, pus, etc., as for direct smears of sputum. Most published reports on microculture are restricted to work on direct smears of sputum, which are notoriously easy to handle and which give a high proportion of successes.

After trying the trisodium phosphate, sulphuric acid, and Jungmann's methods of concentration we had, by 1951, gone back to Petroff's technique, which in our hands gave the best results (cf. Medical Research Council, 1952). But, especially in the early stages, we found difficulty with the Petroff concentrates in the micromethod, as the smears tend to wash off the cover-glasses on which, for economy and convenience, we make our preparations.

One of us had, some years ago, experimented with enzymes, and had found pancreatin to be a very useful concentrating agent, superior to pepsin, trypsin, and papain. A series of cultures using

pancreatin and Petroff concentrates in parallel was therefore made. This has yielded markedly better results with pancreatin. Growth on Loewenstein-Jensen medium has been both more rapid and more profuse—presumably largely because more viable organisms are inoculated (Tables I and II).

Pancreatin has also been found to give excellent results in the microculture method. Smears of concentrates from sputum, pus, urine, etc., readily adhere to the cover-slips.

Our practice has been to prepare smears for microculture and to make Loewenstein-Jensen cultures from the same concentrate. In this way we have been able to compare the efficiency of micro- and macro-culture.

## Concentration Technique with Pancreatin

**Solutions.**—*Pancreatin* (2.5%) in 1% aqueous sodium bicarbonate (A.R.). This must be freshly prepared and filtered. (Hopkin and Williams' brand pancreatin has been found satisfactory.)

*Hydrochloric acid* (8%).

*Sodium hydroxide* (4%).

*Indicator*, wide-range universal.

**Method.**—To 1 vol. of sputum (in universal container) 2 vol. of pancreatin solution is added, and shaken in a shaking-machine for five minutes. The suspension is incubated at 37° for 30 minutes (mucoid specimens require up to 60 minutes) and centrifuged at about 3,000 r.p.m. for 10 minutes. The supernatant is discarded, and the sediment resuspended in about 3 ml. 8% hydrochloric acid, mixing thoroughly. The suspension is allowed to stand for 10 minutes to kill contaminants, and spun as before. The supernatant is removed and the sediment resuspended in about 3 ml. of distilled water. The pH is adjusted to 6.8 using 4% sodium hydroxide. The solution is spun and the supernatant removed.

The pancreatin solution is unstable, even in the refrigerator, and must be freshly prepared each day. Homogenization is not so complete as with other methods, cells being relatively unaffected. With very mucoid sputa the addition of about 0.1 g. of dry

pancreatin will rapidly produce lysis in cases where the solution has not been effective. Pancreatin allows considerable latitude in timing. The longer it is permitted to act the better the concentrate will be, up to about three hours, when the multiplication of contaminants begins.

Contamination has caused some difficulty. Acid is used to combat this and exposure to 8% hydrochloric acid for six minutes is generally effective. *Candida* has been the commonest contaminant: after that, diphtheroids and micrococci.

### Microculture

Even smears are made on cover-slips with a large loop. (Cornet forceps may be used for holding the cover-slips.) They are placed film upwards on sticks in petri dishes and dried in the incubator at 37° C. for 10 to 15 minutes. The cover-slip is placed in 8% hydrochloric acid for six minutes. It is then removed, any excess acid drained off against the side of the tube, and transferred to a tube of distilled water for two minutes. The process is repeated into a second tube of distilled water for two minutes, and into a third tube of distilled water for the same time. The cover-slip then is placed edgewise in a tube containing about 10 mm. of medium, and plugged with cotton-wool. (Flat-bottomed tubes about 1 in. wide are convenient.) The tube is incubated at 37° C.

It is necessary to inspect all culture-tubes daily, and top up with distilled water, if necessary.

The smear is stained by the Ziehl-Neelsen method, holding it in "cornet" forceps, and it is mounted on a clean slide using "xam."

The lower-powered objectives are generally sufficient for examination, but oil-immersion objectives can be used if necessary.

### Sensitivity Tests

The full test requires 12 cover-slip preparations for each specimen: three controls, eight test-films, and one for reference.

The final concentrations of antibacterial substances in the medium are: streptomycin 1, 10, and 100  $\gamma$ /ml., P.A.S. 10 and 80  $\gamma$ /ml., and isoniazid 0.2, 1, and 10  $\gamma$ /ml. One test-film is incubated at each of these concentrations, and the controls in the plain medium.

The first control is examined after three days. If growth is abundant the test-cultures may be examined: if not, incubation is continued and the second control examined after five days. In the majority of cases growth is now obvious under the low power, and the tests may be read. Occasionally the test must run an extra one or two days, but no known positive specimen has so far required a longer period of incubation.

Organisms are considered sensitive if there is no evidence of fasciculate growth (cording) in the test-film concerned. A slight increase in numbers of organisms, compared with the reference film, is ignored.

### Media (Microculture)

Various media have been tried and discarded: the S.G. medium of Rubbo and Morris, plain and with the addition of 10% horse-serum, ox-serum, or lysed horse-blood; Dubos' medium, which in our hands has given variable results; 50% horse-serum; 50% ox-serum; and 50% lysed human blood.

Much the most satisfactory for speed, yield, reliability, and simplicity has been found to be 50% horse-blood, lysed with distilled water. Many batches have been made from commercial horse-blood over the past two years, with complete satisfaction. The addition of asparagin 0.5% was tried, but did not appear to affect the results. Penicillin, in a final concentration of 10 units per ml., is now used routinely to help to combat contaminating bacteria.

We have found that cotton-wool plugging for culture tubes gives better results than paraffin-wool or cork plugs.

### Results

**Comparison of Concentration Methods.**—Comparison of pancreatin and Petroff concentrates, prepared from the same material and cultured in parallel on Loewenstein-Jensen medium, has shown better results with the former method (Table I). One hundred and twenty-two sputa yielded 34 positives with pancreatin and 32 with Petroff's method. Eight specimens of pus gave four and two positives, and 19 urines five and four positives, respectively. Two specimens of pus from the same patient, which were positive on direct examination, failed to grow from concentrates by both methods. The organism was thought to be bovine in type. Contamination of both cultures occurred with two urine samples. One more urine and one sputum culture were contaminated following Petroff's method only. From a total of 149 parallel cultures five more positives were obtained with pancreatin than with Petroff concentrates, the totals being 43 and 38 respectively.

TABLE I  
PETROFF v. PANCREATIN CONCENTRATION

	No.	Positive		Contaminated		
		Petroff	Pancreatin	Petroff	Pancreatin	
Sputum	Direct positive	28	27	28	1	0
	Direct negative	94	5	6		
	Total	122	32	34		
Pus	Direct positive	4	2	2		
	Direct negative	4	0	2		
	Total	8	2	4		
Urine	Direct positive	4	4	4		
	Direct negative	15	0	1	3	2
	Total	19	4	5		

TABLE II  
CULTURES POSITIVE BY BOTH METHODS

	Total	Length of Incubation Required																												
		Days																												
		8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30						
Petroff .. ..	37	4	0	1	0	0	0	2	1	2	0	4	0	5	4	0	0	4	1	3	0	3	1	2						
Pancreatin ..	37	4	2	5	2	2	0	2	2	1	1	7	0	2	5	0	1	1	0	0	0	0	0	0						

A further advantage of the pancreatin method has been that growth on Loewenstein-Jensen medium has tended to be more rapid and more profuse. The incubation periods of 37 specimens positive in both cultures are compared in Table II.

Only 14 Petroff concentrates gave positive cultures in less than 20 days, against 28 by the pancreatin method. At 10 days the proportions were similar, five Petroff against 11 pancreatin positives. Conversely, only two pancreatin positives took more than 21 days (23 and 24 days), while 14 Petroff cultures took from 24 to 30 days.

Comparison of the incubation times of the individual specimens show that for 13 it was the same: in all the others the pancreatin method resulted in more rapid growth, the difference being 10 or more days in eight instances.

Other materials cultured in parallel have included stomach-washings and exudates, but no positives have been recorded. Owing to the large volume of deposit required, comparative results from tuberculous cerebrospinal fluid have not been obtainable.

**Microculture.**—Typical appearances at two, three, and five days are shown in Figs. 1–3 (all × 500).

The efficiency of microculture compared with culture on Loewenstein-Jensen medium (macro-culture) is shown in Table III.

With sputum 75 directly positive samples yielded 70 micro- and 74 macro-cultures, with four and one contaminated cultures respectively. Micro-culture failed in one case. Seventy-eight sputa negative to direct examination gave three positives on micro- and four on macro-culture. From three specimens of pus, all direct positives, only one positive culture was obtained, by both methods. The other two specimens, referred to above, were believed to be from a bovine infection. No positive microculture was obtained from five direct-negative pus specimens, but two were grown on macroculture. With urine, six direct positives yielded five positive cultures and one failure by both methods. Six direct negatives gave one positive by macroculture only.

Again, parallel results on cerebrospinal fluid have not been obtainable owing to scarcity of material. Relatively large amounts are required for the micromethod, which is, however, readily applied to cerebrospinal fluid.

TABLE III  
MACRO- v. MICRO-CULTURE

	No.	Both Positive	L.-J.* Positive Micro-negative	Contam-inated		Failure	
				L.-J.*	Micro	L.-J.*	Micro
Sputum:							
Direct positive ..	77	72	4	1	4	—	1
Direct negative ..	96	3	1	—	—		
Total ..	173	75	5				
Pus:							
Direct positive ..	3	1	0			2	2
Direct negative ..	5	0	2			(believed bovine)	
Total ..	8	1	2				
Urine:							
Direct positive ..	6	5	0			1	1
Direct negative ..	6	0	1				
Total ..	12	5	1				

\* Loewenstein-Jensen.

**Sensitivity Tests.**—The results of 21 sensitivity tests for streptomycin, nine of them in parallel with tests for isoniazid and P.A.S., are given below. All are expressed in terms of γ per ml. of medium. (A number of tests were made before the final concentrations of drugs had been decided upon.)

**Streptomycin.**—Eleven specimens were sensitive to 1 γ, six to 10, one to 20, one to 50, and one to 100 γ. One strain was resistant to 100 γ.

**Isoniazid.**—Five specimens were sensitive to 0.2 γ, two to 3, and one to 5 γ. One strain was resistant to 10 γ.

**P.A.S.**—Seven strains were sensitive to 10 γ, one to 20, and one resistant to 80 γ.

The same organism was resistant to isoniazid and P.A.S., but was sensitive to 50 γ of streptomycin.

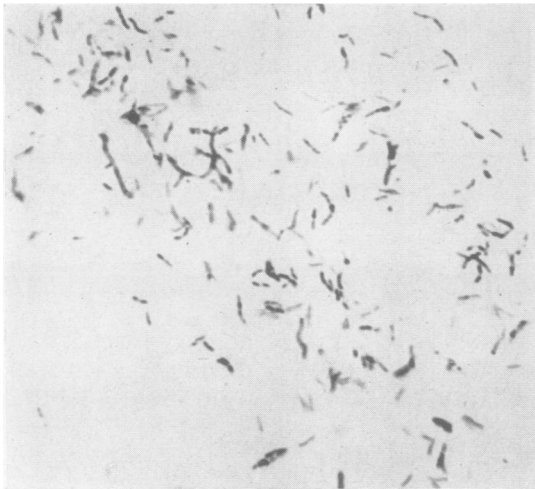


FIG. 1.

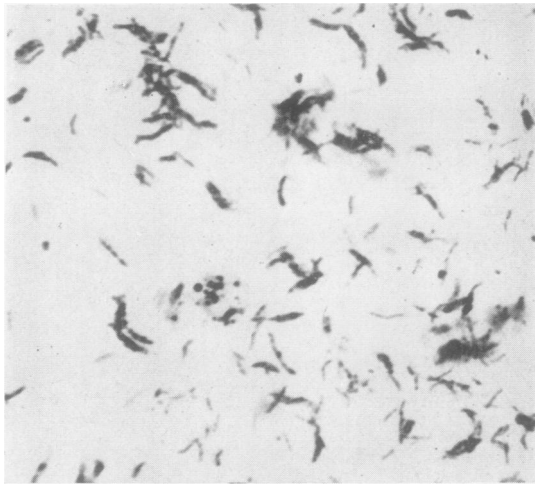


FIG. 2.

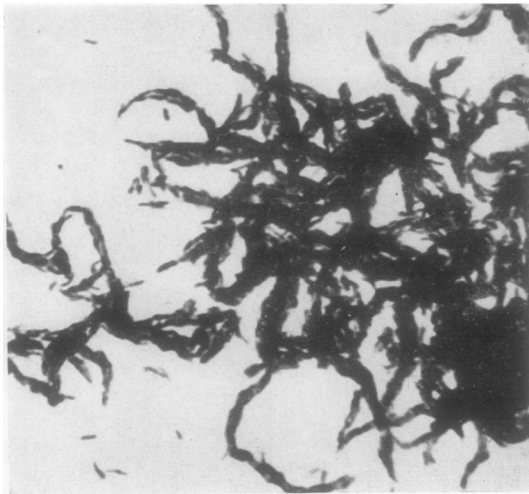


FIG. 3.

The times for these tests varied from three to seven days, most taking five, six, or seven days.

### Discussion

The work described in this paper was the result of the endeavour to speed up routine reports on the culture of *M. tuberculosis*. Since it was necessary, initially, to establish the reliability of microculture, the material used in the earlier part of the investigation was largely sputum which was microscopically positive ("direct positive"). Our results conformed with those of previous workers, and we therefore extended the use of the method to other materials.

Specimens examined before the final technique was evolved are not now considered, as parallel cultures were not always made. The results were similar to those now described.

Pancreatin was first used to overcome the difficulty of Petroff concentrates tending to float off the cover-slips. As soon as parallel macro- and micro-cultures were made on all specimens, its further advantages became apparent.

Our experiences may be considered under three headings.

(1) **Concentration Technique.**—We have found the use of pancreatin, as described, to give a number of positive cultures on Loewenstein-Jensen medium, when Petroff's technique, applied to the same material, gave negative results. The reverse has not occurred. A further advantage of the pancreatin method is that growth on Loewenstein-Jensen medium tends to be more profuse and to become visible sooner. Following its use, smear-preparations for microculture show no increased tendency to wash off the glass.

(2) **The Reliability of Microculture.**—The advantages of speed are so obvious that many workers would doubtless employ this method if they were satisfied with its reliability. For diagnostic purposes the procedure is very well worth while, if not quite so efficient, as culture on Loewenstein-Jensen medium. If, however, both macro- and micro-methods are applied to the same specimen, no risk of failure is run, while, in the great majority of cases, a positive microculture will give the desired early result. Especially in general hospital practice this is of very great value.

Success has been greatest with sputa and urines. As these constitute a very large proportion of tuberculous material in routine laboratories, the relative failure with pus (where the organism is often bovine) does not, in our opinion, detract

from the general usefulness of the method. The need for culture on solid media and for guinea-pig inoculation remains. Little extra labour is involved in the parallel use of micro- and macro-culture.

(3) **Sensitivity Tests.**—Microculture technique is so readily applied, and appears to be so reliable, that sensitivities to streptomycin, P.A.S., and isoniazid can regularly be reported in three to seven days. Most tests may be reported after five days.

Routine sensitivity tests on solid media generally take several weeks to perform, rendering the results largely of academic interest. From the clinical point of view the microculture method is of practical value.

We are aware that the results presented do not afford statistical proof of our opinions, but we regard them as sufficiently suggestive to merit an early report, which may lead to a more extended trial of the methods than can be made in a single department. Work on this subject continues as material becomes available.

### Summary

A technique is described for the use of pancreatin as a concentrating agent for tuberculous material. Culture on Loewenstein-Jensen medium has yielded more rapid and more profuse growth than Petroff's method applied to the same material.

Microculture in 50% lysed horse-blood, though not so reliable as culture on Loewenstein-Jensen medium, gives positive results in a high proportion of cases, enabling an early diagnosis to be made in some. Success has been greater with sputum and urine concentrates than with pus.

Routine sensitivity tests to streptomycin, P.A.S., and isoniazid can regularly be reported in three to seven days, rendering such tests of practical value from the clinical point of view.

No elaborate or expensive apparatus is required for the methods described.

### REFERENCES

- Medical Research Council (1952). *Monthly Bull. of Minist. Hlth Lond.* **11**, 187.  
— (1953). *Brit. med. J.*, **1**, 521.  
Pryce, D. M. (1941). *J. Path. Bact.*, **53**, 327.  
Rubbo, S. B., and Morris, D. M. (1951). *Journal of Clinical Pathology*, **4**, 173.