CULTIVATION OF MYCOBACTERIUM TUBERCULOSIS

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A REPORT by a working party of the Public Health Laboratory Service on the laboratory diagnosis of tuberculosis, reported in the Monthly Bulletin of the Ministry of Health and Public Health Laboratory Service (August, 1952, Vol. 11, p. 187 onwards) and referred to in the Bulletin of Hygiene (September, 1953, 28, 748), lays out the best methods of homogenization of sputa prior to culture on Lowenstein-Jensen medium and places in the following order four methods:

(1) 3 per cent. Sulphuric acid,
(2) 4 per cent. Sodium hydroxide,
(3) Jungmann's,
(4) Trisodium phosphate.

The second part of this report suggested that the heat evolved by thirty minutes' centrifuging exerted a deleterious effect on \textit{M. tuberculosis} over that which could be exerted over a period of fifteen minutes and recommended the latter period for all such procedures. Anderson, Hughes and Knox, writing in the \textit{J. Clin. Path.} (1953, vol. 6, 2, 124-7), suggested that the low grading of Jungmann's method was due to a departure from the original method of Jungmann by the workers embodied in the working party's report. They stated that the "3\% hydrogen peroxide" given in the original paper had been misinterpreted and that in effect one-tenth of the amount of $\text{H}_2\text{O}_2$ required had been used. They recommended 3 ml. of 10 vol. $\text{H}_2\text{O}_2$ for good results. They also recommended considerable other alterations to the method laid down by the working party.

We therefore decided to try out these conflicting statements both at the Royal Army Medical College and the Connaught Military Hospital Laboratory, and also to test the results of the two periods of centrifuging. We arranged therefore to test as follows:

200 specimens by the Petroff method trying both rates of centrifuging.

200 specimens by the Jungmann method trying both rates of centrifuging.

In these latter 200 cases the Connaught Laboratory used the 3 ml. of 10 vol. $\text{H}_2\text{O}_2$ and the Royal Army Medical College the method used by the working party, \textit{i.e.}, one-tenth of this.
Specimens selected were divided into:

(a) those from cases originally sputum positive now sputum negative, and
(b) from cases still sputum positive.

Ten cases were investigated a week, each sputum specimen being divided into two, one-half being examined by the Royal Army Medical College and the other half by the Connaught Laboratory.

The Petroff method used by both laboratories was as follows: The specimen of sputum was mixed with an equal volume of 4 per cent. sodium hydroxide and allowed to stand for thirty minutes until digestion was completed. Phenol red indicator was then added. Incubation was only performed if the sputum was particularly viscous.

Equal quantities of the mixture were centrifuged at fifteen and thirty minutes respectively at 3,000 r.p.m. The deposit was neutralized by 8 per cent. hydrochloric acid and the supernatant discarded into lysol.

Both the fifteen- and thirty-minute deposits were then inoculated on to two Lowenstein-Jensen slopes each and incubated for six weeks at 37° C.

Both unconcentrated and concentrated sputa were checked by Ziehl-Neelsen staining.

The Jungmann method used was to add to a measured quantity of sputum 0.6 of its volume of each of Solutions A and B. The container was then shaken and divided into two further universal containers for the different centrifuging periods: after the first centrifuging the supernatants were discarded, and the volume adjusted with sterile saline solution up to the neck of the container and a second centrifuging done—both at 3,000 r.p.m.

Plating on Lowenstein-Jensen slopes and incubation were carried out as before.

Solution A=Ferrous sulphate, 20 grammes.
Concentrated sulphuric acid, 20 ml.
Aqua dist., 180 ml.

Solution B=Hydrogen peroxide (made up fresh for use).
10 volumes (Connaught).
20 volumes of 5/100 (R.A.M. College).

RESULTS

Royal Army Medical College

Petroff Method.—Of the 200 cases tested, of which 114 (57 per cent.) were eventually found sputum positive by direct staining, culture by this method showed positive:

In 128 by the fifteen-minute technique, and
In 125 by the thirty-minute technique (67 and 65 per cent. respectively),
i.e., a very slight swing in favour of the fifteen-minute centrifuging.

The Jungmann Method.—Of the 200 cases tested, again 114 (57 per cent.) were positive eventually by sputum staining. Of these cases, however, only
65 were positive on culture after thirty minutes' centrifuging and 80 after fifteen minutes' centrifuging (35 and 42 per cent.).

This suggested that the low category for the Jungmann method by the working party was upheld and that, at any rate with this method, the fifteen minutes' centrifuging was an advantage.

The Connaught Laboratory

Petroff Method.—This laboratory only succeeded in finding 100/200 sputa ultimately positive by staining (50 per cent.).

Of these 200, 115 (57 per cent.) were found positive on culture after fifteen minutes' centrifuging and 117 (58 per cent.) after thirty minutes' centrifuging. The difference of 1 per cent. is not worth considering.

Jungmann Method (using neat H₂O₂).—Again only 100/200 sputa were finally found positive by staining. Of these 200, only 69 (34.5 per cent.) were found positive on culture after fifteen minutes' centrifuging and only 7 (3.5 per cent.) after thirty minutes' centrifuging (excluding doubtful positives and interference by debris in the inoculum by this method).

Again the difference of centrifuging time has greatly improved the picture. The over-all picture with the strong H₂O₂ is, however, very unsatisfactory.

It would appear, therefore, that the shorter time of centrifuging is of advantage only in certain methods of homogenization, apparently in the more inferior methods.

Summary

An attempt has been made to define the relative values of Jungmann's and other methods of homogenization in the culture of M. tuberculosis from sputum, in consideration of comments on the report of the working party of the Public Health Laboratory Service, on the Laboratory Diagnosis of Tuberculosis (Monthly Bulletin of Ministry of Health and Public Health Laboratory Service, August 1952, Vol. 11) which appeared in the Journal of Clinical Pathology, 1953, Vol. 6, 124-7.

An attempt has also been made to evaluate the claim that fifteen minutes' centrifuging is superior to thirty minutes', made in the same working party report.

This work was undertaken by the Pathology Department of the Royal Army Medical College and the laboratory of the Connaught Military Hospital.

Two hundred cases were investigated by each laboratory by the Petroff homogenization method and 200 by the Jungmann method, in which latter method the Connaught Laboratory used ten times stronger H₂O₂ as suggested by Anderson, Hughes and Knox. All sputa were spun both for the fifteen- and thirty-minute periods.

Of these 400 sputa, approximately 200 were positive by staining, but all patients had originally been positive.

It was found that by the Petroff method the difference in successful culture
between the two centrifuging periods was negligible, but that using both methods of Jungmann the fifteen minutes' centrifuging had a distinct advantage—particularly so where the stronger H₂O₂ was used.

As regards the value of the methods, we cannot but agree that Jungmann's method is by no means the best—and that with the higher strength H₂O₂ it is even less efficacious.

CONCLUSIONS

A trial of Jungmann's homogenization method for culture of *M. tuberculosis* was carried out alongside Petroff's method by two laboratories. All specimens received two types of centrifuging, one for fifteen minutes and one for thirty minutes.

It was found that there is no difference worthy of note between the results from the two periods of centrifuging, provided a reliable homogenization method was used.

Jungmann's method did not prove reliable in our hands, but was definitely improved by limiting the centrifuging to fifteen minutes, *i.e.*, the effect of the prolonged centrifuging may well be on the homogenization mixture rather than on the bacteria.
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