

A FINAL REPORT ON THE CULTIVATION OF THE  
TUBERCLE BACILLUS FROM THE SPUTUM  
BY THE METHOD OF PETROFF.

By ROBERT A. KEILTY, M.D.

(From the McManes Laboratory of Pathology of the University of Pennsylvania,  
Philadelphia.)

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The purpose of this paper is to present a final report on the isolation of the tubercle bacillus from the sputum according to the method of Petroff.<sup>1</sup> From my early experience<sup>2</sup> it seemed to me that the method offered many opportunities, but my success at first was not great. In the present work an attempt has been made to control the possibilities which might influence a favorable or an unfavorable result. In a later communication Petroff<sup>3</sup> reports having isolated and cultivated the tubercle bacillus from 129 sputa out of 135 specimens. I have not been able to obtain this positive percentage, but I believe that after a little preliminary experience the results should be nearly as good.

Those accustomed to working with the tubercle bacillus realize that the results are variable, and that a method which will simplify or advance the present knowledge of tuberculosis will be of value. The method as devised by Petroff seems to open up many avenues of study in relation to the clinical course of tuberculosis and the tubercle bacillus.

According to my experience there are two important points to be realized. First, the demonstration of the tubercle bacillus unde-

<sup>1</sup> Petroff, S. A., A New and Rapid Method for the Isolation and Cultivation of Tubercle Bacilli Directly from the Sputum and Feces, *J. Exp. Med.*, 1915, **xxi**, 38.

<sup>2</sup> Keilty, R. A., A Study of the Cultivation of the Tubercle Bacillus Directly from the Sputum by the Method of Petroff, *J. Exp. Med.*, 1915, **xxii**, 612.

<sup>3</sup> Petroff, S. A., Some Cultural Studies on the Tubercle Bacillus, *Bull. Johns Hopkins Hosp.*, 1915, **xxvi**, 276.

monstrable in sputum by other means. By microscopic examination of the cultures this is possible even though they become contaminated later by a diffuse growth of organisms. Second, the isolation of the tubercle bacillus and its study in pure culture.

The method of Petroff briefly is as follows: About 5 cc. of fresh sputum in a sterile bottle are mixed with sterile 3 per cent sodium hydroxide solution and incubated at 37°C. for 30 minutes. This should be frequently shaken and the mucin thoroughly broken up. A longer period in the incubator may be necessary for this. Place a piece of sterile litmus paper in the bottle and neutralize with sterile normal hydrochloric acid, centrifugalize at high speed for 10 minutes, and plant the sediment on three tubes of the gentian violet egg veal medium. Incubate as nearly as possible between 38° and 39°C. Variations in temperature will retard the growth decidedly.

*Preparation of the Medium.*—Many different modifications of egg media have been tried in the laboratory, and I find that where the whole egg is used there is not much difference. Possibly where starch is added the luxuriance of the growth is increased. From this I have lately used the medium of Petroff exclusively with some modification in the sterilization.

*Meat.*—500 gm. of fresh lean veal are finely ground up and infused cold over night in 500 cc. of a 15 per cent solution of glycerin in water. Squeeze out either by press or by twisting in gauze, and filter.

*Eggs.*—Break one dozen eggs into a large vessel and beat thoroughly. Filter through gauze. Add one part by volume of meat juice to two parts of egg.

*Gentian Violet.*—Add sufficient 1 per cent alcoholic solution of gentian violet to make a dilution of 1:10,000 to one-half of the medium and tube. Tube the other half without the gentian violet.

*Sterilization.*—Petroff advised the sterilization of all vessels, presses, and eggs before breaking, as recommended for Dorcet's medium. It was found difficult to prevent breaks in technique, and later experience proved that absolute sterility is unnecessary.

Make up the medium in the morning after the infusion of the veal over night. Tube from 3 to 5 cc. and pack in the inspissator at about 1 p.m. Bring the temperature up to 95°C. as quickly as possible, avoiding bubbles, and keep it at this point for 1 hour. Temperatures over 100°C. will spoil the medium. Repeat the process in the inspissator at 95°C. on 2 successive days. In the early work there was considerable trouble with contamination of the medium, but with the technique carefully watched these troubles have been entirely eliminated. This should give about 125 tubes, one-half with and one-half without gentian violet. Considerable water of condensation will result. This is allowed to evaporate for a few days without dryness and the tubes are sealed as follows:

*Paraffin Mixture.*—Seven parts of paraffin at 55°C., one part of yellow bee's wax, and one part of petrolatum. Heat over the Bunsen as hot as possible with-

out combustion. Flame the top of the tube and cotton plug, remove the latter with sterile forceps and flame it. Immerse while burning into the hot paraffin mixture and plug, allowing the excess to drain back into the mixture. Heat the paraffin after every dozen tubes. We have never had an infection by using this method. When the tubes are in use heat before and after removing the plug. In this way tubes may be kept for as long as 8 months and frequently opened without much drying of the medium or contamination. I have had an opportunity of observing cultures obtained from many different laboratories, and find that the technique of sealing varies considerably and in some cases at least is impracticable for frequent use of the tube. For this reason and because drying was one of my earliest difficulties the method has been included in some detail.

*Results.*—Twenty-eight consecutive specimens were studied, most of which were kindly furnished by Dr. Baldwin Leuke, from the wards of the Philadelphia General Hospital. These were not selected in any way. They were collected during the night in sterile bottles and started the next day. The fact that they were not absolutely fresh has no doubt influenced the result somewhat. Fresh sputum as suggested by Petroff is important. In 2 of the 28 cases the tubes were broken and lost in the centrifuge and 1 was too old so that 25 cases were finally studied. Of the 25 cases, 23 were said to be advanced, 1 case, a student, undoubtedly negative, and 1 case suspected but probably negative. On examination by smear, 18 cases were positive and 7 negative for tubercle bacilli after 5 minutes' examination. Of the 18 positive cases, 12 showed cultural evidences of growth, and of the 7 negative cases, 1 showed growth. Of the 12 positive cases, 4 were obtained in pure culture and 8 showed microscopic evidence of growth; that is, colonies were too minute to be made out grossly or were overgrown by contamination, but upon microscopic examination showed groupings in numbers sufficient to exclude any doubt that they were carried over from the sputum originally. Where any doubt existed they were classed as negative. Of the 4 pure cultures, 2 averaged 10 to 14 organisms to a field upon the original sputum examination. 2 showed 1 to several fields. Of the 18 positive sputa, only 4 had many organisms to a field and 2 of these gave pure cultures, while 1 had microscopic evidence in culture and 1 had to be incubated 24 hours because of a thick ropy sputum. Of the 4 pure cultures, 1 case showed 2 contaminations out of 4 tubes inoculated. The other 3 cases grew with-

out contamination. Case 20, a negative case, was several days old when the specimen was received. 3 cases showed early microscopic evidence of growth, 1 on the 3rd day and 2 on the 4th day. This number probably would have been increased if the tubes had been examined more frequently. The first appearance of gross growth averaged 14 to 21 days. This result was dependent upon opportunities for observation and in some cases the time may be shorter. In transplants from young cultures I have frequently observed growth in 3 or 4 days and good heavy growth in 10 days on the plain egg medium.

The colonies first visible under a magnifying glass are pin-point. They dot up like minute mushrooms, tending to heap up; if scattered they will grow with umbilicated centers. They spread over one another with a typical dry hilly pattern if close together. When pure growth is first determined they should be immediately transferred to plain egg medium upon which they grow luxuriantly. They often fill the entire surface of the medium at the end of a month when they should again be transplanted. There seems to be a great variation in the rapidity of growth of different cultures. This depends upon the culture, the medium, and the incubation. The medium must be made and sterilized correctly. One batch, upon which no growth took place, was ruined by oversterilization. This is, however, an old experience with egg media. The moisture in the tube has a great deal to do with growth. The cultures will not thrive with visible water of condensation; on the other hand, they will not grow on a dry glazed surface. Finally there are unexplainable reasons for failures to grow. One series, No. 380, a subculture, was planted on ten tubes of plain egg on Jan. 23, 1916. The following note was made on Feb. 22: five tubes grew profusely, three scantily, and two were negative. All were subjected to the same conditions. For this work, therefore, it is advisable to use at least three tubes.

*Contamination.*—In the original planting three tubes of gentian violet medium were used. Where contaminations occurred they showed up, as a rule, at the end of 24 hours as small colonies, as diffuse, moist, smeary growths, or as localized colonies. In the majority of cases this contamination seemed to be held in check thereafter by the gentian violet; while in others the organisms continued

to grow, and in a few instances completely liquefied the medium. The contaminated cultures should be watched carefully and where growth is checked it may be possible to pick out a single colony of tubercle bacilli, transplant it, and thus obtain a pure culture. Where this is not possible, at least evidence of microscopical growth may be obtained.

*Brief History of the Pure Cultures.*

*No. 326.*—Dec. 20, 1915. Sputum incubated for 20 minutes; neutral in reaction; centrifugalized for 20 minutes at high speed.

Dec. 22. Two tubes are contaminated and discarded.

Dec. 23. A third tube shows microscopic growth with small white colonies contaminating, a large coccus. A small bit of uncontaminated material transferred to a plain egg tube No. 326 C.

Jan. 23, 1916. No. 326 C has a scattered growth in raised, dry colonies.

Feb. 5. A profuse dry growth of acid-fast bacilli. Transplanted.

Mar. 15. Culture growing profusely.

*No. 328.*—Dec. 20, 1915. Sputum incubated for 20 minutes; neutral in reaction; centrifugalized for 20 minutes.

Dec. 22. No growth.

Jan. 4, 1916. Shows the first evidence of growth, one small colony of pure acid-fast bacilli. Transferred to three tubes of plain egg medium, Nos. 328 A, B, and C.

Jan. 23. No. 328 C is growing well as multiple, whitish colonies tending to spread. Original tube did not continue to grow.

Mar. 15. Culture continues to grow after transplanting.

*No. 364.*—Jan. 6, 1916. Sputum incubated for 6 hours; slightly acid in reaction; centrifugalized 30 minutes at high speed.

Feb. 9. Two or three minute colonies the color of the medium; acid-fast bacilli in pure culture. Transplanted to plain egg medium, Nos. 364 A, B, and C.

Mar. 1. Has grown into dry, raised, granular colonies not tending to spread but to heap up.

*No. 365.*—Jan. 6, 1916. Sputum incubated 6 hours; slightly acid in reaction; centrifugalized for 30 minutes.

Feb. 9. Small raised colonies the color of the medium. Transplanted to plain egg medium, Nos. 365 A, B, and C.

Mar. 1. Original gentian violet tube and No. 365 C growing profusely. Nos. 365 A and B did not grow.

*Swabs.*—In twelve cases, Nos. 1 to 9 and 13 to 15 inclusive, sterile cotton swabs were thoroughly rubbed over the tonsils, fauces, tongue, and gums. The swabs were replaced in tubes and the latter

filled with 3 per cent sodium hydroxide. They were then incubated for 30 minutes. The sodium hydroxide was poured off except enough to cover the cotton swab. This was neutralized and the fluid centrifugalized. Both the sediment and the cotton removed from the applicator were planted on gentian violet egg tubes. The purpose in this portion of the work, the report of which is merely preliminary, is to obtain a method for the demonstration of the tubercle bacillus where sputum is not obtainable; for example, in children and in the insane.

In one instance, No. 371 from Case 14, the preliminary smear being negative, a pure culture was obtained. The history of this culture is as follows: The swab was obtained on Jan. 8, 1916; the smear from this was negative. On Jan. 17, one minute colony with pure acid-fast bacilli was present. It was transplanted to plain egg medium No. 371 X. On Mar. 15, No. 371 X has a good profuse growth of acid-fast bacilli in pure culture. The specimen of sputum from this case showed microscopic evidence of growth in 72 hours, but pure culture was not obtained owing to contamination.

This is a preliminary report as regards the swab, but it has possibilities with improved technique which will be reported on later. It may therefore be concluded that it is possible to isolate the tubercle bacillus in pure culture by Petroff's method from a swab of the throat and mouth.

As a control on the sputum work Dr. J. D. Paul of the resident staff of the Episcopal Hospital of Philadelphia examined some sputa in the laboratory of that Hospital. He selected ten cases from the dispensary service and I am indebted to him and to the Director of the Laboratory, Dr. C. Y. White, for the following report. The work was carried on entirely independently of me. From the ten cases four cases in pure culture were recovered. This gives a higher percentage of positive cases than my own work. In addition he recovered a pure culture by applying the technique to a specimen of pus from a tuberculous knee.

The method has not been studied in regard to feces, and the results with tissue have been negative as far as obtaining growth is concerned. In regard to tissue, small pieces are completely digested in the sodium hydroxide after incubation for 24 hours. From the sediment after centrifugalization the tubercle bacillus can be demonstrated

even when it is impossible or requires considerable time to demonstrate it in a section. From this result a 3 per cent sodium hydroxide solution is as good as antiformin for this purpose.

TABLE I.  
*Summary of Results.*

No. of specimen.	No. of culture.	Date.	Type of case.	Smear.	Organisms per field.	Tubes planted.	Contaminations in 24 hrs.	Contaminations in 1 wk.	First growth.	Type of result.	Result.
		1915							1916		
1	326	Dec. 20	Adv.*	+	14	4	2	0	Jan. 23	Pure.	⊕
2	327	" 20	"	-	0	3	0	0			-
3	328	" 20	"	+	10	3	0	0	" 4	"	⊕
4	336	" 22	"	+	Occ.	3	1	0	Dec. 26	Micro.	+
5	337	" 22	"	+	"	3	1	0	" 26	"	+
6	338	" 22	"	+	"	3	3	0			-
		1916									
7	355	Jan. 4									
8	356	" 4									
9	357	" 4	"	-	0	3	1	0			-
		1916									
10	363	" 6	"	+	Occ.	3	0	3	Feb. 8	"	+
11	364	" 6	"	+	1	3	0	0	" 8	Pure.	⊕
12	365	" 6	"	+	Occ.	3	0	0	" 8	"	⊕
13	367	" 8	"	+	"	3	0	0	" 9	Micro.	+
14	368	" 8	"	+	"	3	2	0	Jan. 11	"	+
15	369	" 8	"	-	0	3	1	3			-
16	375	" 16	?	-	0	3	3	0			-
17	376	" 17									
18	377	" 17	Adv.	+	15	3	0	0			-
19	378	" 17	"	+	15	3	3	0	Feb. 16	"	+
20	401	Feb. 23	?	-	0	5	5	0			-
21	412	" 27	Adv.	+	Occ.	3	1	3			-
22	413	" 27	"	-	0	3	3	0			-
23	414	" 27	"	-	0	3	0	0	Mar. 23	"	+
24	415	" 27	"	+	Occ.	3	0	0	" 23	"	+
25	426	Mar. 3	"	+	"	3	0	0			-
26	427	" 3	"	+	4	3	2	0	" 23	"	+
27	428	" 3	"	+	4	3	0	0			-
28	431	" 4	"	+	Occ.	3	0	0			-

\* Adv. indicates advanced; ?, suspected; Occ., occasional; Micro., microscopic evidence of growth; ⊕, pure culture.

## DISCUSSION AND SUMMARY.

In summing up the practicability of the Petroff method which is in reality a refinement and improvement upon well known methods, it has possibilities both from the standpoint of diagnosis and the study of the tubercle bacillus. It is not a difficult technique, but requires considerable attention to detail. The results should be better in larger series of cases carried on in laboratories where more time is available for close observation of the cultures during the early periods of development. It is the early transplanting of minute colonies even in the presence of contaminations which results in pure cultures.

As to the practical value of the method, my experience would lead me to conclude that where sputum is obtainable in suspected cases of tuberculosis in which the tubercle bacillus cannot be demonstrated, an opportunity for diagnosis would be missed if this technique were not applied. If the result is negative it means nothing; if positive an otherwise obscure case may be cleared up. The Petroff method offers an easy opportunity of isolating large numbers of pure cultures (Table I).

## CONCLUSIONS.

1. The method of Petroff offers an opportunity for the isolation of the tubercle bacillus in pure culture from sputum.
2. The method is available for the demonstration of the tubercle bacillus from sputum when otherwise not demonstrable.
3. The method is easy and rapid, but requires detail and constant observation.
4. The plain egg veal medium is the best medium thus far devised for the luxuriant growth of the tubercle bacillus.
5. The method supersedes antiformin, having all its good qualities and others in addition.
6. It is possible by applying the method to isolate the tubercle bacillus from a swab of the throat.
7. By using the digestive powers of sodium hydroxide it is possible to demonstrate more readily the presence of the tubercle bacillus in tissue, thus saving time on animal inoculation.
8. Finally, if positive results are obtained from an otherwise negative case the method opens up one more avenue of diagnosis.