THE CULTIVATION OF THE TUBERCLE BACILLUS

Thesis submitted for the

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by

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PREFACE

This work was carried out during my appointment as Resident Physician in Knightswood Hospital and Sanatorium, Glasgow, and Resident Medical Officer in the Burgh Isolation Hospital and Sanatorium, Paisley.

The work was commenced in October, 1934, and was completed in March 1936.

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INTRODUCTION

The importance of early diagnosis and immediate treatment of pulmonary tuberculosis is nowadays being insisted upon by all Public Health Authorities, and it is everywhere acknowledged that the prospects of recovery are enhanced in proportion as treatment is instituted early. In Britain, the highest death rate from pulmonary tuberculosis is found in young adults aged 15 - 25 years. Many of them are not notified as tubercular until the disease is far advanced and beyond all hope of cure. With early diagnosis, the death rate could be reduced considerably.

One of the first considerations of a case of suspected pulmonary tuberculosis is examination of the sputum. The direct detection of tubercle bacilli in the sputum by microscopic examination is conclusive but what of a negative finding? A large Public Health Authority in this country has found that, by cultural methods, a positive result is obtained in sputum from cases of pulmonary tuberculosis in ten per cent of cases which were negative to the ordinary methods of examination. Here lies the importance of successful cultivation of tubercle bacilli from the sputum. For this reason alone it is imperative to have a reliable culture medium.

During the past four years while acting as Medical Officer in various Sanatoria I have carried out work on the cultivation of the tubercle bacilli from sputum.

In the following pages I propose to discuss and compare the principal media in use in Britain and abroad and to describe a modification introduced by myself which has certain advantages over existing media.

2. HISTORICAL

On the 24th March 1882 Robert Koch (1) announced the discovery of the tubercle bacillus in his Lecture to the Physiological Society of Berlin.

Much work had been done in the earlier part of the nineteenth century by such men as Laennec (2), Bayle (3) and Villemin (4) in proving that tuberculosis was a morbid and infective condition but it remained to Koch to demonstrate the actual organism. By the application of alkaline methylene blue followed by bismarck brown he found in sections of tuberculous material a characteristic bacillus, hitherto unknown. After many failures he succeeded, by the method of Tyndall, in growing the bacillus on ox or sheep serum which had been sterilised by heat at 58°C. and finally stiffened at 65°C. The pure cultures were inoculated into a large number of animals and in practically all cases reproduced tuberculosis. By these discoveries Koch proved for the first time in history the postulates which, although now named Koch's postulates, had been suggested by his teacher Henle. A specific bacterium was the cause of tuberculosis, this organism could be cultivated from the infected tissues and when inoculated into an animal caused the specific disease and could again be recovered.

Ehrlich (5) immediately improved upon the staining method for the tubercle bacillus. In May 1882, Ehrlich published a paper in which he showed for the first time that the bacillus was acid-fast. Using a solution of gentian violet, to which aniline oil was added, he found that even after the additions of strong solutions of nitric acid the tubercle bacillus retained the dye. When the rest of the preparation was treated with a

counterstain, it took the counterstain, while the tubercle bacilli retained the original colour. Koch accepted this method as superior to his own and it is now known under the name of Ziehl-Neelsen. According to Bulloch (6) Ziehl used carbolic acid instead of aniline oil; Neelsen advised the use of fuchsin instead of gentian violet and of sulphuric acid in place of nitric acid as used by Ehrlich. Neither Ziehl nor Neelsen published the method which bears their names and it is only referred to in a footnote of a paper by Johne (7) in 1885.

Koch in addition to using congealed blood serum also cultivated the tubercle bacillus on gelatin prepared with agar to which beef-tea and peptone had been added. The growths were small and sparse and could not be compared with those on congealed serum. He also used a liquid culture medium. In his famous publication in the Berliner Klinische Wochenschrift (April 1882) he stated that Klebs, Schueller and Toussaint had tried to grow microorganisms from tuberculous material in a liquid culture medium but always got a cloudiness of their medium after a few days with numerous bacteria. In Klebs experiments small motile rods developed and in Schueller's and Toussaint's they were micrococci. Koch himself found it difficult to grow the tubercle bacillus in a fluid medium but when he succeeded, while he got a weak growth his medium remained clear. Growth only showed itself at the end of from three to four weeks.

The discovery of the tubercle bacillus by Koch immediately led to a world wide research into the bacteriology of the organism. In 1887 Nocard and Roux (8) discovered the value of glycerin in the cultivation of the tubercle bacillus and grew

it on a medium containing glycerin agar. They noted differences in growth and morphology of the organism in cases of human and animal tuberculosis. This along with the work of Rivolta (9) in 1889 and Maffucci (10) in 1890 led up to the announcement in 1896 by Theobald Smith (11) that there existed three different types of the tubercle bacillus, namely human, bovine and avian. He used congealed dog's serum as the basis of his medium. Theobald Smith was the first person to find that the human type grew more quickly and more luxuriantly than the bovine type on artificial media.

Finally Koch, at the British Congress on Tuberculosis in 1901 stated that "human tuberculosis differs from bovine and cannot be transmitted to cattle". He carried the matter further, stating that bovine tuberculosis was so rarely transmitted to man that the dangers of milk containing bovine tubercle were negligible, an error which took years to correct. The result of Koch's contention was the establishment of the Royal Commission (12) which carried on research for nine years. This investigation proved that the bacillus of bovine tuberculosis was especially dangerous to children, but that the mortality of infection by the bovine bacillus, for all ages was only six per cent.

Up until this time all culture media used in the isolation of the tubercle bacillus had been made of serum from various animals with or without glycerin. In an attempt to find a medium which would grow the bacillus quicker and more luxuriant, Dorset (13) in 1902 evolved a medium containing hen's eggs. His results showed that his medium was superior to those previously used, A. S. and F. Griffith (14) in 1907 experimented with a variety of media and they also found that an egg medium was

the most successful and could always be relied upon for the production of primary cultures. They also found that of the numerous congealed sera media, that of the cat was best.

The chief source of worry to the bacteriologist in the cultivation of the tubercle bacillus is the contamination by other bacteria. With a view to eliminate this Petroff (15) in 1915 produced a medium containing egg, veal-juice and gentian violet. He stated in his original article that the work of Churchman, Simons and Krumweide on the effect of various dyes upon the growth of micro-organisms, suggested the use of gentian violet in the isolation of the tubercle bacillus. Gentian violet was selected as the most favourable stain on account of its inhibitory action on many organisms.

In 1922 Calmette (16) produced a glycerol potato medium. This medium was further improved by Corper (17) in 1927 who, after a comparison of Dorset's egg medium, Petroff's gentian violet medium and Calmette's glycerol potato medium, evolved an improved glycerol potato medium with crystal violet to inhibit contamination.

Most of the media about this time all had the same aim in view, namely, to give a profuse and early growth and to inhibit contamination. Examples of these are the media of Petragnani (18), Sweeny-Evanoff (19) and Löwenstein (20).

Since then there has been a desire for a more simplified medium with greater stress on the pre-treatment of the tuberculous material for inoculation. A good example of this is the nutrient agar egg medium of Herrold (21). Herrold emphasized the fact that simplified cultural methods were needed for a more general use of this valuable method of control of tuberculosis as well as to stimulate more work on the incidence and clinical classification of human tuberculosis in all its forms. He proved that

the cultural method was much more certain than smears and not only as delicate as, but also much more prompt than the inoculation of guinea-pigs.

At the present time, the media chiefly used for the cultivation of the tubercle bacillus are those of Corper, Herrold, Löwenstein, Petragnani, Petroff and Sweany-Evanoff.

3. THE TUBERCLE BACILLUS

Morphology: (22)

Tubercle bacilli are slender non-motile organisms which vary in length from 0.5 μ to 8.0 μ, the usual limits being 2.5 μ - 3.5 μ, and in breadth from 0.3 μ to 0.5 μ. In the tissues they are seldom perfectly straight, but are generally slightly curved and not infrequently bent at an angle. The bacilli do not usually stain uniformly, but show clear spaces which give the stained portions the appearance of cocci. Sometimes deeply stained oval bodies are seen at the poles or in the length of the bacilli. Both the clear spaces and darkly stained bodies have been regarded as spores but it is now generally believed that the former are due to segmentation of the chromophil substance and that the latter are chromatin granules such as are seen in other bacteria. The tubercle bacillus proliferates by elongation and transverse fission and not by spore formation. The fact that it is speedily killed by sunlight also indicates that the tubercle bacillus has no spores. Microscopically, an enveloping or capsular waxy substance can often be made out around each bacillus, especially those which have been artificially cultivated on serum for several generations.

In tuberculous tissues and exudates the number of bacilli is very variable. They are generally numerous in early, acute or progressive lesions and often absent, microscopically, in chronic lesions. The bacilli occur singly, or two are attached end to end and often form an angle; in large groups or in masses so compact that individual organisms are not seen; one may also

see a short chain of bacilli.

Staining Methods:

It has already been noted that the tubercle bacillus is surrounded by a fatty or waxy capsule containing a lipoid substance. It is to this sheath that the peculiar staining reactions of the bacillus are attributed. The bacillus shows little affinity for ordinary stains, not combining with them unless exposed for a long time or heated. Koch originally stained the bacilli in sections with alkaline methylene blue, counterstaining with Bismarck brown. The first practically useful method was that of Ehrlich who found that if the bacilli were stained with aniline and fuchsin or gentian violet, they subsequently resisted decolorisation with nitric acid. Hence, the designation of the bacilli as acid-fast. The stain in use at present rests on this principle of intense staining followed by decolorisation vigorous enough to remove the stain from everything on the film but tubercle bacilli. The tubercle bacillus can also be stained by Gram's method. Stained in this way the bacilli are often very granular, sometimes resembling a short chain of cocci. For general use Gram's method cannot be recommended.

Methods:

1) Ziehl-Neelsen's method for film preparations.

2) Ziehl-Neelsen's method for section preparations.

Film preparations are smears taken with a platinum loop from the fluid containing the bacteria. The smear is spread over the surface of a glass slide, dried at room temperature and fixed by heating over a bunsen flame. Section preparations are portions of infected tissue treated preferably with paraffin and cut with a microtome to produce very thin sections of the tissue.

The Ziehl-Neelsen method is deservedly the most popular. The stain consists of basic fuchsin with phenol as the mordant. The dye is basic and its combination with an acid produces a compound which is yellowish brown in colour. Any strong acid can be used as a decolorising agent, but sulphuric acid in a twenty per cent solution is usually employed. In order to show what has been decolorised and to form a contrast with the red stained bacilli, the preparation is counterstained with methylene blue.

Ziehl-Neelsen's (strong) Carbol Fuchsin has the following composition:-

Basic Fuchsin	1 part
Absolute Alcohol	10 parts
Solution of Carbolic Acid (1 : 20)	100 parts

Dissolve the dye in the alcohol and then add the solution to the carbolic acid.

1) Film preparations:- Smears are made, dried and fixed as described above.

1) Flood the slides with filtered carbol fuchsin and heat until steam rises. Allow the preparation to stain for five minutes, heat being applied at intervals to keep the stain hot. The stain must not be allowed to evaporate or dry on the slide.

2) Wash with water.

3) Immerse the slide in 20 per cent sulphuric acid. The red colour of the preparation is changed to yellowish brown. After about a minute in the acid remove the slide, wash with water and place it in the acid again. This process should be repeated several times. The object of the washing is to remove the compound of acid with stain and allow fresh acid to gain access to the preparation. The decolorisation is finished when, after washing, the smear is a faint pink.

4) Wash the slide well in water.

5) Treat with 95 per cent. alcohol for two minutes.

6) Wash with water.

7) Counterstain with Löffler's methylene blue for ten to thirty seconds.

8) Wash, blot, dry and mount.

Acid-fast bacilli stain bright red, while the tissue cells and other organisms are stained blue.

Other organisms are "acid-fast" in addition to the tubercle bacillus. The most important in diagnostic work is the smegma bacillus, which is frequently found in urine. Treatment with spirit in addition to acid will decolorise this type of organism, whereas the tubercle bacillus is both acid-and alcohol-fast. The decolorisation with spirit is therefore important when examining urine for the presence of tubercle bacilli.

It should be noted that in films stained by Ziehl-Neelsen's method, red-stained organisms in the midst of hyaline material must not be regarded as tubercle bacilli, as such material may be resistant to decolorisation.

Sections:-

1) Sections are treated with xylol to remove paraffin, then with spirit, 50 per cent alcohol, and finally washed in water.

2) Stain with Ziehl-Neelsen's stain as described for films, but heat gently, otherwise the section may become detached from the slide.

3) Wash with water.

4) Decolorise with 20 per cent sulphuric acid as for films. The process takes longer owing to the thickness of the section and care must be exercised in washing to retain the section on the slide.

5) Wash well with water.

6) Counterstain with methylene blue for a half to one minute.

7) Wash with water.

8) Wipe the slide dry all round the section, blot with filter paper and treat with a few drops of absolute alcohol. Pour on more absolute alcohol, wipe the slide again and immerse it in xylol.

9) Mount in Canada balsam.

Much's granules:- (23)

It often happens that ordinary microscopic examination of a smear preparation of chronic tuberculosis lesions stained by Ziehl-Neelsen method fails to reveal acid-fast organisms, although that material is infective, as shown by its ability to produce tuberculosis in guinea pigs. By employing a modification of Gram's method, Much (1908) described (1) rod-shaped granular organisms, (2) isolated granules, which he believed represented a virulent non-acid-fast form of tubercle bacilli. Further investigation has failed to confirm Much's findings. Among others, Bittrolf and Momose (24) were unable to demonstrate by Much's method any other form than that stained by the Ziehl-Neelsen method. A. S. Griffith's experience has been that acid-fast bacilli can generally be found if the search is sufficiently prolonged, in tuberculous tissues which according to Much ought not to contain them. Also, direct cultures from such material always yield colonies proportionate in number to the scanty acid-fast elements seen microscopically.

Cultivation:-

The tubercle bacillus is aerobic and grows at temperatures ranging from 29° to 42°C., the optium being 37° to 38° for mamalian bacilli and 40°C for avian: it requires for initial cultures from tuberculous tissue solid media containing

albuminous substances or glycerin. The media should have a neutral reaction (optimum pH7.0 to 7.6) and a dry surface; a soft moist surface is unfavourable, but a little moisture of condensation at the foot of the tubes is advantageous. Growth is slow and colonies are usually not visible to the naked eye until the eighthor tenth day and sometimes later. The medium must, therefore, be prevented from drying. The presence of glycerin favours the growth of human, avian and some bovine strains, but is not essential.

Powers of resistance:

The tubercle bacillus grows best at the temperature of the human body, 37° to 38°C, but growth is not abolished at 29° to 42°C. From a practical standpoint it is important to mention that they are not killed when exposed to moist heat of 50°C. for less than twelve hours, but heating to 55°C for four to six hours does destroy them. They are also killed when exposed to moist heat of 60°C for a half-hour, and in fifteen minutes at 70°C.; in five minutes at 80° to 90°C., and in one minute at 95°C. With sputum, conditions are different: the mucus protects the bacilli and it requires more time to destroy them with heat. Five minutes boiling is sufficient to kill the bacilli under all circumstances.

Dry heat is less potent in destroying tubercle bacilli; circulating steam requires a half-hour for this purpose, while bacilli in dried sputum can withstand a temperature of 100°C for an hour. On the other hand, cold does not destroy their virulence, and freezing with subsequent thawing does not harm them very much. Calmette found that even liquid air does not destroy the viability or virulence of tubercle bacilli.

As has already been mentioned, the lipoid substance contained in the tubercle bacilli protect them from the effects

of desiccation and from the bactericidal action of the normal body cells, although for growth and proliferation they require moisture. When dried and pulverised by being converted into dust, as is often the case with tuberculous sputum eliminated by patients, most of the bacilli die, but some have been found to resist desiccation at ordinary temperatures for months.

It has been proved that light, especially sunlight, decomposes the fatty substance in the bacilli and thus destroys them completely. When cultures are exposed to direct sunlight for a couple of hours, the vitality as well as the virulence of the tubercle bacilli is destroyed; in sputum, the bacilli are protected by the mucus, and it requires a longer time for their destruction. Some writers maintain that their virulence is destroyed with only partial loss of vitality.

Chemistry and Bio-chemistry:

There have been many chemical analyses of the composition of tubercle bacilli as well as the chemical changes in the medium in which the bacilli are grown. Special efforts have been made to ascertain the identity of the lipoids composing the capsule of the bacilli and to explain their acid-fast property. The more recent aspects of the problem are surveyed in Long's Harvey Lecture (25). He has shown that if tubercle bacilli are extracted as completely as possible with alcohol and petroleum ether and are then treated with $\frac{N}{1}$ HCL, a subsequent extraction with the organic solvents removes a further quantity of lipin substances and destroys the acid-fastness of the bacilli which had resisted the primary extraction. Long contends that it is the manner of distribution of the lipin substances in the bodies of the bacilli, rather than its inherent acid-fast character which confers the typical staining properties on the tubercle

bacillus.

The respiratory metabolism of the tubercle bacillus and especially the influence of changes in the culture medium on the respiration of the bacillus have been studied by Loebel, Shorr and Richardson (26); of particular interest is their finding that glycerol, an important constituent of media for growing tubercle bacilli, increases notably their consumption of oxygen.

The correlation between individual chemical constituents of the tubercle bacillus and its antigenic properties and the injury done to the tissues of the host has shown several important facts. Chiefly that the protein fraction and the lipoid fraction produce degenerative changes in the host. Laidlow and Dudley (27) have obtained a glycogen fraction and also a complex carbohydrate in the nature of a gum. The latter gives specific precipitation with an immune serum.

"Theobald Smith reaction curve":

In 1903 a biochemical test for distinguishing human from bovine tubercle bacilli was introduced by Theobald Smith. He stated that when tubercle bacilli are grown on the surface of glycerin broth with an initial acidity, with phenol phthalein as indicator, the subsequent changes in the reaction of the broth differ with the type of bacillus. He found that with cultures of bovine tubercle bacilli the acidity of the medium steadily diminished until the neutral point was reached or even passed. With cultures of human tubercle bacilli there was first a diminution of acidity, not, however, so great as with bovine bacilli, and then a reversal of the reaction, although the final compared with the initial reaction always showed a diminished acidity. The test is laborious, and requires a long time for its performance.

Serology:

It has not been found possible to differentiate between human and bovine bacilli by agglutination reactions. According to the results of Tulloch (28), G. S. Wilson (29) and A. S. Griffith, bovine and human strains are serologically identical, since all bovine and human strains were able to absorb the agglutinin both from human and bovine sera. Typical avian strains produce a specific agglutinin with which human and bovine strains do not combine. So, it is possible to distinguish the common avian bacillus from mammalian bacilli by the absorption of agglutinin test.

Types:

The Royal Commission on Tuberculosis set up in this country recognised three types of tubercle bacilli. These are the human, the bovine and the avian.

Human: The human variety grows quickly and luxuriantly on culture media; the addition of glycerin enhances the growth. On glycerin bouillon growth is seen during the first week and within three weeks there is a wrinkled or verrucose growth on the surface of the media. Morphologically, the human bacilli, when grown on serum cultures, appears as long, straight or curved rods.

The virulence of human bacilli is low in various animals. Guinea-pigs are very susceptible and may be infected in various ways, even by rubbing the bacilli into the shaved skin of the abdomen. Rabbits are less susceptible. Cattle are infected when large doses are injected intravenously. But with subcutaneous infection there is produced only an infiltration at the point inoculated, which soon suppurates and heals. The regional lymph glands swell up and at times become calcified. Feeding calves with human bacilli never produces any progressive disease. Pigs

dogs, cats and sheep are not affected by human bacilli, while monkeys are very susceptible. Some species of birds are also susceptible.

Bovine: Bovine bacilli are difficult to cultivate and the addition of glycerin inhibits their growth. On glycerin bouillon growth is slow. A thin pellicle spreads over the surface of the culture media within four to eight weeks and rarely forms verrucose thickening. Morphologically, they are shorter, thicker and more evenly stained than the human type, but the various intermediate forms makes it impossible to differentiate human from bovine bacilli on morphological characters. The bovine bacilli are more virulent for rabbits, calves and swine than the human. Guinea pigs are killed or become acutely and progressively sick when infected with small doses of bovine bacilli. The bovine type causes fatal generalised miliary tuberculosis in the rabbit, whereas the human type in the same amount never causes generalised miliary tuberculosis. Cattle are very susceptible to the bovine bacilli and after intravenous inoculation perish from generalised tuberculosis within three or four weeks. Intraperitoneal, intraocular and intramammary inoculation also caused generalised and fatal tuberculosis. Feeding cattle with even small doses of pure culture of bovine bacilli causes tuberculous disease of the intestines, followed by tuberculous lymphangitis and lymphadenitis of the mesentery; the disease spreads to other lymph glands, serous membranes and lungs. Inhalation produces caseous pneumonia. Pigs, sheep, goats, cats and monkeys are very susceptible; dogs, rats and mice are not affected by the bovine bacillus. Some species of birds are susceptible but the domestic fowl shows complete resistance.

Avian: On glycerin agar and on serum their growth is more luxuriant, appears more moist than in mammalian bacilli and they produce an orange pigment. They grow at a temperature of 41°C. which stops the growth of mammalian tubercle bacilli. Rabbits rats and mice are the only mammals susceptible to inoculation with avian tubercle bacilli. Fowls are very susceptible when fed with portions of organs containing avian bacilli, but they may consume large quantities of phthisical sputum without becoming tuberculous. The parrot, however, is susceptible to both human and bovine bacilli, as well as to avian. Tuberculosis can cause severe epidemics among domestic fowls.

There have been reported several cases of avian tuberculosis in humans and Löwenstein suggests that it may be acquired by ingestion of eggs from tuberculous hens which have been found to contain tubercle bacilli. The cases observed in man run a course not unlike a septicaemia with high continuous fever. The lungs may appear free from changes but the spleen is enlarged. The bone marrow and the kidneys are the organs chiefly affected. Erythraemia and leukaemia may occur in patients with avian tuberculosis.

Tubercle bacilli of cold-blooded animals: Certain diseases observed in worms, lizards, frogs, turtles, snakes and fish are similar to human tuberculosis and in many cases acid-fast bacilli have been isolated. Various workers conclude that these acid-fast bacilli are not connected with tubercle bacilli, but they are saprophytes which may be found in healthy animals and in the soil. Others, however, consider them as true pathogenic bacilli of cold-blooded animals, or such as have become attenuated in their virulence by long residence in and adaptation to growth at a lower temperature.

Effects produced in the human body: (30)

"When a few tubercle bacilli gain a foothold in a tissue, a focus of reaction occurs, resulting in the formation of what is known as a "tubercle follicle." At a very early stage the centre of the focus consists of a collection of swollen cells, often called "epithelioid" or "endothelioid", whilst around them there is a zone of round cells, chiefly lymphocytes. The endothelioid cells are usually oval, spindle-shaped, or irregular in form, have fairly abundant protoplasm and a faintly staining nucleus. Very soon there is evidence of the action of the bacilli in producing damage to the cells. The central cells become swollen and lose their outline, their nuclei cease to stain, and ultimately they become fused into a homogeneous, or slightly granular, structureless material. This is an example of Weigert's coagulative necrosis and sometimes a certain amount of fibrinous exudate is added to the necrotic material; the ultimate result is a necrotic centre surrounded by endothelioid cells and these again by small round cells. Another common constituent of the tubercle follicle is the giant-cell, which occurs amongst the endothelioid cells. It is a large cell with somewhat irregular outline, and contains numerous oval or rounded cells resembling those of the endothelioid cells. The nuclei are often arranged at the periphery, whilst the centre part of the cell may show signs of degeneration, being granular or hyaline in appearance. The typical follicle is sometimes described as containing a central giant-cell surrounded by a zone of round cells.

Tubercle nodules are non-vascular in their substance, though considerable vascularity may be present at their margin, especially those of chronic nature; and formerly it was believed by some that the central necrosis was due to a want of blood supply. We know now, however, that it is the result of the

direct action of the tubercle toxins on the cells and that the toxic action produces also the non-vascularity, as it leads to necrosis of the capillary walls and thrombosis.

While the initial reaction conforms generally to the description given above, the ultimate results vary greatly and thus tuberculous lesions come to present very different characters. The following examples show this.

(a) Acute Miliary Tuberculosis: Sometimes there occurs an eruption of small tubercle nodules throughout the organs - an acute miliary tuberculosis. These nodules may be nearly all of the same character and therefore of the same age, and thus are due to an extensive dissemination of bacilli about the same time. One would be inclined to regard such an occurrence as unlikely in view of the non-vascular nature of the tubercle nodules. But a tuberculous focus may implicate, or actually form in, the wall of a vessel, usually a vein, and then ulcerate into it, leading to a discharge of bacilli into the circulation. For example, one may sometimes find a caseous bronchial gland which has thus eaten into a pulmonary vessel. In some cases tubercle spreads from the abdominal lymphatic glands to the thoracic duct; tubercles form in its wall, and then undergo softening and ulceration. From such lesions bacilli may be carried up the duct and thus reach the veins. The former is, however, the commoner method by which miliary tuberculosis is set up, though the site of the vascular lesion is often difficult to find.

(b) Caseous Lesions: Frequently the necrotic action of the bacilli is most in evidence and thus large areas of caseation result, these being surrounded by a connective tissue in which giant-cell systems are usually to be found. Large caseous masses are specially common in lymphatic glands, and often occur in the

kidneys, epididymus, suprarenals, etc. The caseous material may have a firm consistence, as in the tuberculous masses in the brain, though frequently it is pulpy or semi-fluid. In the case of chronic tubercle of bones, notably the vertebrae, the caseous material has a tendency to become softened; it attracts chemotactically polymorphonuclear leucocytes, and becomes a caseous pus. This increases in amount and burrows along muscles, as occurs in lumbar and psoas abscesses; owing to the absence of the signs of acute inflammation these are known as "cold abscesses".

(c) Tuberculous granulation tissue: Again, tubercle may lead to the formation of abundant granulation tissue. This is a noteworthy feature of tuberculosis of the joints, where the synovial membrane becomes covered with a layer of pinkish and somewhat gelatinous granulation tissue. Here again the tubercles become obscured by the newly formed tissue, but giant-cell systems are to be found in it on microscopic examination.

(d) Fibrotic Lesions: The growth of tubercles may be slow and localised, and much fibrous material may form around them, fibroid tubercles thus resulting. In the lungs, in connection with these, there is often a considerable spread of fibrous tissue into the parts beyond, apparently due to the diffusion of toxic products along the lymphatics. The lesion thus comes to be one of chronic interstitial pneumonia, and its real nature may not be apparent on naked eye examination; it is a prominent feature in chronic fibroid phthisis.

(e) More acute Inflammatory Changes: Tubercles, when abundant, may give rise to fibrinous exudation. This is well illustrated in the case of tuberculous meningitis, where there is a growth of tubercles along the lines of the arterial branches, which is followed by exudation into the subarachnoid meshwork around. In

such cases, polymorphonuclear leucocytes, many of them degenerated, may be found in the cerebro-spinal fluid obtained by lumbar puncture. Tuberculosis of serous cavities also is often attended by fibrinous and serous exudates, and in the case of the pericardium the exudate may be haemorrhagic in type.

The diversity of tuberculous lesions may be partly explained by varying degrees of resistance or relative immunity of the tissues, and it may be said in a general way that the formation of tubercles and connective tissue proliferation indicate a relative immunity, whilst diffuse caseation and exudative processes occur in the converse condition. A comparison of the pulmonary tuberculous lesions in the young child and in the adult shows that the latter has acquired a certain degree of immunity. The relative immunity of the adult is generally ascribed to infection in the early years of life and some writers have even attempted to distinguish stages of tuberculosis corresponding with those of syphilis. One may say that tuberculosis in the earliest years of life is usually a diffuse and rapidly fatal disease, that there follows a period up to puberty when caseating glandular tuberculosis becomes common and that this is accompanied by metastatic tubercle in bones, joints and internal organs; whilst in the adult the outstanding lesion (apart from the sequelae of the previous) is pulmonary phthisis, which is often of a chronic nature and often undergoes healing. Miliary tuberculosis, resulting usually from intimal tubercle of veins, is in a sense an accidental phenomenon which may occur at any period, though it is commonest in the early years of life."

It has been shown that when tubercle bacilli are killed by heat and injected into a susceptible animal, tubercular nodules are found to develop. This proves that the dead bacilli must have contained toxins. These toxins cannot be secreted by

the bacilli since they have been killed, but are produced by the actual constituents of the protoplasm of the bacteria. In other words they are endotoxins. The injection of dead bacteria or their products also leads to the development of specific antibodies which are associated with the state of immunity. It was with this aim that Koch in 1890 first used the products of disintegrated tubercle bacilli under the name of "tuberculin" or "Heilmittel". He showed that in tuberculous animals there was a supersensitiveness to tuberculin. This showed itself in a "local" reaction at the site of injection, by a "general" reaction, and also by a "focal" reaction around the tuberculous lesions. Thus a tuberculous focus in the body could be recognised. Koch also used his tuberculin therapeutically thinking that the focal reaction led to encapsulation of the tuberculous lesion and cure. But it was soon proved by Virchow (31) that the "Heilmittel" of Koch often led to disastrous consequences for the patient. Since then much smaller doses of tuberculin have been used therapeutically and favourable results have been claimed by Calmette, Heimbeck (32) and others.

4. THE CULTURE MEDIA DESCRIBED SERIATUM

After a review of the various types of media recommended for the isolation of the tubercle bacillus it was decided to use the following six media:

(a) Petragnani's medium
(b) Löwenstein's "
(c) Herrold's "
(d) Corper & Uyei's "
(e) Petroff's "
(f) Sweany-Evanoff's "

Petragnani's medium is a rich medium and gives excellent growths of tubercle bacilli; contamination is rare on account of the malachite green which is used as an inhibitory agent. It was found that when the amount of malachite green was reduced contaminating organisms frequently appeared. It is costly to make and is only useful for the cultivation of the tubercle bacillus.

Löwenstein's medium is similar in composition to Petragnani's medium but being less rich it gives a poorer growth. Again, it is only useful for the cultivation of the tubercle bacillus due to the inhibitory malachite green.

Herrold's egg yolk agar medium also gives very good growths of tubercle bacilli and is cheap and easy to make. Herrold uses his medium for the cultivation of stock strains of Brucella organisms, Neisserian strains and B. Lepri as well as for tubercle bacilli. Care is required in preparation to prevent contamination owing to the lack of any inhibiting dye.

The medium of Corper-Uyei is a good potato medium for the cultivation of the tubercle bacillus but requires careful

preparation. The potatoes should be free from surface defects and the medium autoclaved at the correct pressure and time, otherwise the medium becomes broken and collapses. Without the inhibitory crystal violet, contamination is frequent.

The media of Petroff and Sweany-Evanoff are similar in composition and, when free from contamination, give good growths of tubercle bacilli. Sterile ground veal is too costly for practical use and with fresh veal contamination is frequent.

a) Petragnani's Medium:

The chief components are milk, peptone, potato meal, raw potato, whole egg, egg yolk, glycerol and malachite green. The potato meal used is the same as the potato flour in Löwenstein's medium; both are derived from powdered potato which has been dried at a low temperature. These substances are obtained from British Drug Houses Ltd.

Preparation: Mix together in a beaker 300 c.c. of fresh milk, 2 g. of peptone, 12 g. of potato meal, 2 finely grated potatoes, each about the size of a hen's egg. Place the beaker in a water bath at 100°C., stirring the mixture constantly for ten minutes. Allow the beaker to remain in the water bath for one hour, remove and cool the mixture to 60°C. Now add 8 whole eggs, 2 egg yolks, 24 c.c. of glycerol and 20 c.c. of a 2 per cent aqueous solution of malachite green. Stir well to ensure thorough mixing, filter through sterile muslin and tube. Slant and coagulate the medium at 90°C. for one hour; sterilise by inspissation at 80°C. for 20 minutes on two successive days.

b) Löwenstein's Medium:

This consists of mineral salts, whole egg, potato flour, asparagin and malachite green. Löwenstein formed the opinion that peptone was not conducive to the growth of the tubercle bacillus and replaced the peptone by asparagin as a source of

nitrogen. Asparagin is the amide of amino-succinic acid and has the following chemical formula $CHNH_2 - COOH$ / $CH_2 - CONH_2$

Malachite green is inhibitory to contaminating organisms. Löwenstein also used congo red as an alternative antiseptic.

Preparation: The following solution is prepared:-

Monopotassium phosphate	0.6 g.
Magnesium sulphate	0.06 g.
Magnesium citrate	0.15 g.
Asparagin	0.9 g.
Distilled Water	147 cc.

To 120 cc of this solution add 6 g. of potato flour and put in the Koch steriliser for two hours (shaking occasionally) at 100°C. Cool to 50°C., add 4 whole eggs and 0.06 g. of malachite green dissolved in 10 ccs of distilled water. Filter through sterile muslin, bottle and slant. The medium is coagulated at 90°C. for one hour, and sterilised by inspissation at 80°C. for 20 minutes on two successive days.

c) Herrold's Medium:

This medium contains nutrient agar with egg yolk. Herrold (1931 a & b; Woolsey, 1931) found that the tubercle bacillus grew profusely on egg yolk agar, slightly less on whole egg agar and not at all on egg white agar. This seemed to show that egg white, although not bactericidal, may be bacteriostatic. From a practical point of view, in Herrold's medium, the addition of egg white causes flaking with a resulting rough surface of the medium. C. I. Woolsey found the results of cultures of tubercle bacilli on Herrold's medium and of inoculation into guinea pigs agreed in 93 percent of 130 cases; in 5 of the nine cases in which the results disagreed the cultures on Herrold's medium were the more reliable.

A moist atmosphere and a temperature 34°C. to 36°C. are essential for the optimal growth of tubercle bacilli on Herrold's medium.

Preparation: The following broth is prepared:-

Lab. Lemco	3 g.
Peptone	10 g.
Sodium Chloride	5 g.
Dextrose	0.33 g.
Distilled water	2 litres

To this broth add 2% agar and prepare as for ordinary agar with pH 7.5. To 150 c.c. of this nutrient agar, which is cooled to 55 - 60°C., add one egg yolk. Shake well to ensure thorough mixing, tube and allow to set in a sloping position. Incubate overnight to ensure that the medium is sterile.

d) Medium of Corper and Uyei

Corper and Uyei carried out quantitative seeding tests with tubercle bacilli on the following media - Calmette's potato medium, Dorset's egg medium, Petroff's gentian violet medium, glycerol agar and Long's non-protein medium. They found that Calmette's potato medium was superior to the rest in favouring the growth of bacilli when present in small numbers.

After further experiments they produced their crystal violet potato medium. This medium proved superior to Petroff's gentian violet egg medium and possessed an advantage over plain potato medium in being less favourable for growth of contaminating organisms, without affecting the growth of the tubercle bacilli. They used crystal violet to replace the pre-war gentian violet of uncertain composition. They also found that crystal violet was a suitable material for incorporation in egg media, as recommended by Petroff to inhibit the development of contaminators during the primary isolation of tubercle bacilli from contaminated

sources. The concentration of crystal violet required is about the same in egg media as that recommended for gentian violet. Preparation: Fresh, clean, large potatoes, preferably without surface defects, are cut into cylinders of 3 ins. or more in length with a cork borer of 5/8th inch. The cylinders are then cut longitudinally in halves. These are then soaked in 1 per cent sodium carbonate solution, containing crystal violet in a concentration of 0.003 per cent (1 to 75,000) for 1 or 2 hours. The dye solution must be added to the sodium carbonate solution immediately before use, since prolonged standing causes a decolorisation of the crystal violet. At the end of this time the potato cylinders are gently wiped with a towel to free them from excess liquid. They are then introduced into a sterile culture tube containing 1.5 c.c. of 5 per cent glycerol bouillon. The tubes are plugged with cotton wool and sterilised in the autoclave for 30 minutes at 15 lbs. pressure. It was found that the medium must be autoclaved at correct pressure and time; if the pressure is raised or heating prolonged, the nutrient effect of the potato is lost and no growth results.

e) Petroff's Medium:

This medium contains veal, glycerol and whole egg, with gentian violet to inhibit contamination. Petroff compared a series of media containing gentian violet, methyl violet, methylene blue, crystal violet and fuchsin in dilutions varying from 1 to 1,000 to 1 to 100,000. He found that tubercle bacilli grew well on egg-meat juice media containing all of the above stains even in a dilution as low as 1 to 5,000 with the exception of methylene blue, which gave negative results below a dilution of 1 to 25,000. In media containing this stain the tubercle bacilli grew rather slowly, and the individual organisms were impregnated with the

stain. From these results gentian violet was selected as the most favourable stain.

Preparation:

Two parts of egg (white and yolk).

One part of meat-juice.

Gentian violet sufficient to the proportion of 1 to 10,000.

Meat juice:- 500 gm. of beef or veal are infused in 500 c.c. of a 15 per cent solution of glycerol in water. Twenty-four hours later the meat is squeezed in a sterile meat press and collected in a sterile beaker.

Eggs:- Sterilise the shells of the eggs by immersion for ten minutes in 70 per cent alcohol or by pouring hot water upon them. Break the eggs into a sterile beaker and after mixing the eggs well, filter through sterile gauze. Add one part by volume of meat juice.

Gentian Violet:- Add sufficient 1 per cent alcoholic gentian violet to make a dilution of 1 to 10,000.

Tube about three cubic centimetres in each sterile test tube and inspissate for three successive days: on the first day at 85°C., until all the medium is solidified, changing the places of the tubes if necessary; on the second and third days for not more than one hour at 75°C.

f) Medium of Sweany and Evanoff:

This medium is composed of veal, milk, glycerol and egg. Preparation: All apparatus and gauze are sterilised on the previous day. Clean fresh eggs are sterilised in alcohol at least 30 minutes before using. It has been found that fresh milk and cream, in quantities of 5 c.c. or less, can be thoroughly sterilised in live steam by two sterilisations for 45 minutes on two successive days, without coagulating the protein.

(1) Soak one pound of veal overnight in an equal amount of sterilised whole milk with 15 per cent glycerol added.

(2) Strain through sterile gauze and add twice the amount of well-beaten eggs prepared as described above.

(3) Filter through sterile gauze and tube, having the operation shielded from dust as much as possible.

(4) Inspissate for one hour at 85°C. immediately and one hour at 75°C. on two succeeding days.

(5) Incubate three days in incubator and three days at room temperature.

AUTHOR'S MEDIUM AND TECHNIQUE

The following egg yolk agar medium is the result of an attempt to find a reliable simple medium for the cultivation of the tubercle bacillus from infected tissues especially sputum.

Preparation: The following broth is prepared:

Fresh Horse Flesh (free from fat and minced)	1 lb.
Tap Water	800 c.c.

Put into a two litre flask and mix well using a glass rod. Heat the mixture in the steam steriliser until a temperature of 80°C. is reached. 6.4 g. anhydrous sodium carbonate dissolved in 800 c.c. cold tap water is added. Allow to cool until the temperature falls to 45°C.

Add:		
	Sodium Citrate	3 g.
	Glucose	2 g.
	Chloroform	18 c.c.
	Liquor Trypsin Co.	18 c.c.

The mixture is then incubated at 37°C. for six hours, the liquid being frequently stirred. After digestion is completed, add 13 c.c. of pure strong hydrochloric acid, steam for twenty minutes and then filter. When the broth is cool adjust the reaction with normal solution of caustic soda to pH 7.6. The broth is then bottled and sterilised by steam for one hour.

To this broth add 2% agar and prepare as for ordinary agar. To 300 c.c. of this nutrient agar which is cooled to 55 - 60°C., add two egg yolks. Shake well to ensure thorough mixing. The medium is then poured into sterile screw capped bottles as recommended by McCartney (33) and allowed to settle in a sloping position. Incubate overnight to ensure that the medium is sterile.

To avoid contamination from the egg yolk the following

method recommended by C. I. Woolsey (34) is carried out:- the whole eggs are immersed in methylated spirits for half-an-hour and then the outside of the eggshell is sterilised by means of the bunsen burner flame. The upper half of the shell is removed using a sterilised glass rod and the egg white discarded. The yolk in the remaining half shell is whipped up with a sterilised platinum loop and any remaining egg white removed. The egg yolk is then poured direct into the nutrient agar broth. This also eliminates any need to strain the medium through muslin.

This medium is similar to Herrold's egg yolk agar medium but has been found to give an earlier and more luxuriant growth of tubercle bacilli than Herrold's medium. Also, it does not produce so much water of condensation in the culture bottle and so the medium is firm and does not collapse when the culture bottle is in the upright position.

The medium described does not contain any inhibitory substances such as malachite green, congo red, crystal violet or gentian violet and so it can be used for the cultivation of other bacteria, e.g. pneumococcus, staphylococcus, streptococcus and Neisseran strains. The initial broth is used for blood cultures and the nutrient agar for subcultures and the making of blood agar plates. It is a good medium for use in an Isolation Hospital where the laboratory staff is limited.

TECHNIQUE

The specimens are collected in sterile stoppered sputum flasks (fig.1) and treated within two hours. Using a sterile 2 c.c. syringe with cannula attached (Fig.II) 1 c.c. of sputum is delivered into a sterile centrifuge tube (Fig III) containing an equal volume of a 6 per cent. solution of sulphuric acid (17 c.c. of sulphuric acid (specific gravity 1.84) in 500 c.c. of distilled water). The contents are mixed with a sterile glass

Figure I.

Figure II.

Figure III.

rod until no large particles remain. By using the syringe with cannula, an accurate amount of sputum can be measured directly into the bottom of the centrifuge tube, so preventing any sputum adhering to the sides of the tube. In previous attempts this precaution was not taken, the sputum being allowed to flow along the side of the centrifuge tube; as all the sputum thus did not come into contact with the sulphuric acid in the tube, contaminating bacteria were not destroyed.

After incubation of the mixture for 30 minutes at 37°C., 10 c.c. of normal saline are added, the tube is shaken and then centrifuged at 3,000 r.p.m. for 10 minutes. The supernatant fluid is poured off and the sediment resuspended in a second 10 c.c. charge of normal saline. The tube is thoroughly shaken, allowed to stand for 5 minutes, again shaken and centrifuged at 3,000 r.p.m. for 10 minutes. The supernatant fluid is poured off and the sediment used for the inoculation of the culture bottles. Before inoculating the media, the sediment is stirred with a few drops of normal saline to render it more fluid. Using a platinum loop 4 m.m. in diameter, the sediment is carefully streaked on the surface of the medium. After inoculation, the bottles are incubated at 37°C. and observed at weekly intervals for signs of visible growth.

Smears also are prepared from the treated sediments and stained by the Ziehl-Neelsen method. If tubercle bacilli are not found on the first examination, the slides are again examined on two successive occasions.

Two bottles of each medium are inoculated with sediment from each specimen.

6. COMPARISON OF RESULTS

First Series of 100 cases. (Table I)

In this series the following six media were compared:

Petragnani

Löwenstein

Herrold

Corper & Uyei

Petroff

Sweany-Evanoff.

By direct microscopic examination of stained smears 92 positive results were obtained out of the 100 cases examined. A negative was only accepted on the results of 3 examinations. The cultural findings with the various media were as follows:

Petragnani's Medium:

After 5 weeks' incubation the total number of positives obtained was 100 as against 92 by the microscopic method. In 27 cases this medium showed visible growth at the end of 7 days. Colonies were visible in 10 - 12 days and almost always by the end of the third week. On this medium growth usually occurred earliest and in luxuriance exceeded that obtained with the other media. Contamination was rare.

Löwenstein's Medium:

This medium also gave 100 per cent positive results at the end of 5 weeks. Twelve specimens showed visible growth at the end of one week. Colonies frequently were visible in 14- 16 days but were much smaller and more discrete than those obtained by Petragnani's method,

Herrold's Medium.

Good results were also obtained on this medium, 96 cultures being positive after 5 weeks' incubation. In 13 specimens this

medium showed growth within 7 days. In 10 - 14 days colonies were often apparent and equalled those on Petragnani's medium in luxuriance.

Medium of Corper and Uyei:

This medium was slightly less reliable than microscopic examination, yielding, after 5 weeks, 89 positives. Colonies were not usually visible until the third week, but, once formed, the growth was rapid and luxuriant. Contamination was rare.

Media of Petroff and of Sweany and Evanoff:

On these media colonies were slow to grow but eventually became fairly luxuriant. Five weeks' incubation produced 77 and 75 positive results on the respective media.

In comparing the 8 sputa which gave negative results by the microscopic method, the cultures showed the following:-

Petragnani	8 positive
Löwenstein	8 "
Herrold	7 "
Corper-Uyei	6 "
Petroff	2 "
Sweany-Evanoff	3 "

In the 5 specimens where very few bacilli were found microscopically (2 - 3 bacilli in the whole smear) the cultural findings were:-

Petragnani	5 positive
Löwenstein	5 "
Herrold	5 "
Corper-Uyei	4 "
Petroff	3 "
Sweany-Evanoff	4 "

The rate at which growth appeared on the various media is shown in the following table:-

Medium	Period of incubation				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
Petragnani	27	84	95	100	100
Löwenstein	12	54	91	95	100
Herrold	13	80	89	94	96
Corper-Uyei	3	46	64	75	89
Petroff	-	15	46	54	77
Sweany-Evanoff	-	17	39	56	75

In every case the growth showed the tubercle bacilli to be apparently of the human type. On all the media, with the exception of that of Herrold, the growth was dry, wrinkled or verrucose and of a yellowish tint. On Herrold's medium the growth showed smooth white colonies with a marked tendency to coalesce.

Second Series of 50 Cases (Table II)

In this series Herrold's medium was compared with the Author's medium. From the previous series it was found that a simple egg medium, which could be used for the cultivation of other organisms, would be the most useful for routine use.

By direct microscopic examination of stained smears 47 positive results were obtained out of the 50 cases examined. A negative was only accepted on the result of 3 examinations. The cultural findings with the two media were as follows:-

Author's Medium:

After 5 weeks incubation the total number of positives obtained was 50 as against 47 by the microscopic method. In 17 cases this medium showed visible growth at the end of 7 days. Colonies were usually visible in 10 - 12 days and almost always

by the end of the third week. On this medium growth usually occurred earlier and in luxuriance exceeded that obtained by Herrold's medium. There was not the same amount of water of condensation as with Herrold's medium so that the medium was firmer and did not collapse readily.

Herrold's Medium:

This medium was equal to the microscopic method, yielding, after 5 weeks, 47 positives. In 8 specimens this medium showed growth at the end of one week. In 10 - 14 days colonies were often apparent.

In comparing the 3 sputa which gave negative results by the microscopic method, the cultures showed the following:

Author	3 positive
Herrold	0 "

In the 8 specimens where very few bacilli were found microscopically (2 - 3 bacilli in the whole smear) the cultural findings were:

Author	8 positive
Herrold	8 "

The rate at which growth occurred on the two media is shown in the table below:

Medium	Period of incubation				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
Author	17	42	48	50	50
Herrold	8	33	36	40	47

Third Series of 100 cases (Table III)

In this series the following four media were compared:

Author

Petragnani

Löwenstein

Corper-Uyei

The media of Petroff and Sweany-Evanoff were omitted in this series owing to frequency of contamination and slowness of growth. The author's egg yolk agar medium was used in place of Herrold's egg yolk agar medium.

By direct microscopic examination of stained smears 90 positives results were obtained out of the 100 cases examined. The cultural findings on the various media were as follows:-

Author's Medium:

After 5 weeks incubation the total number of positives obtained was 100 as against 90 by the microscopic method. In 29 cases this medium showed visible growth at the end of 7 days. Colonies were visible in 10 - 12 days and almost always by the end of the third week. On this medium growth usually occurred earliest and in luxuriance exceeded that obtained with the other three media. (See photo). Growth began as small yellowish-white smooth colonies which quickly joined together to form a sago-like mass of growth (Photo).

Petragnani's Medium:

This medium also gave 100 per cent positive results at the end of 5 weeks. 23 specimens gave positive results at the end of 7 days. Colonies were visible in 10 - 12 days and almost always by the end of the third week. There were some strains of the tubercle bacillus which gave poor growths with Petragnani's and Löwenstein's media while giving good growths with the Author's medium. This may have been due to the strong inhibitory action

Coloured Photographs showing growth on the four principal media after 4 weeks incubation from a sputum containing human tubercle bacilli.

AUTHOR

PETRAGNANI

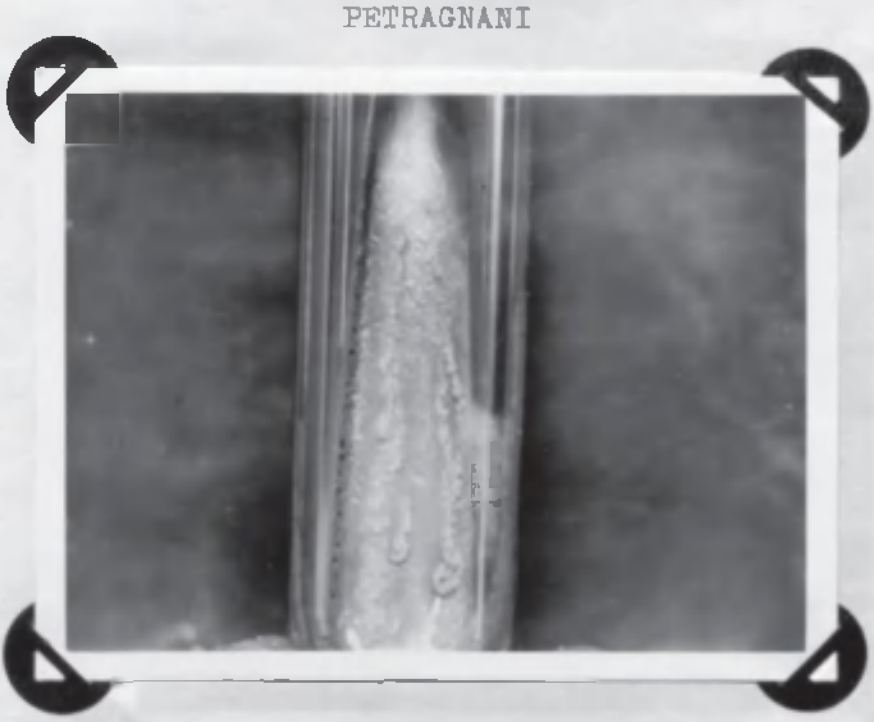
LOWENSTEIN

CORPER-UYEI

Photographs showing growth on the four principal media after 4 weeks incubation.

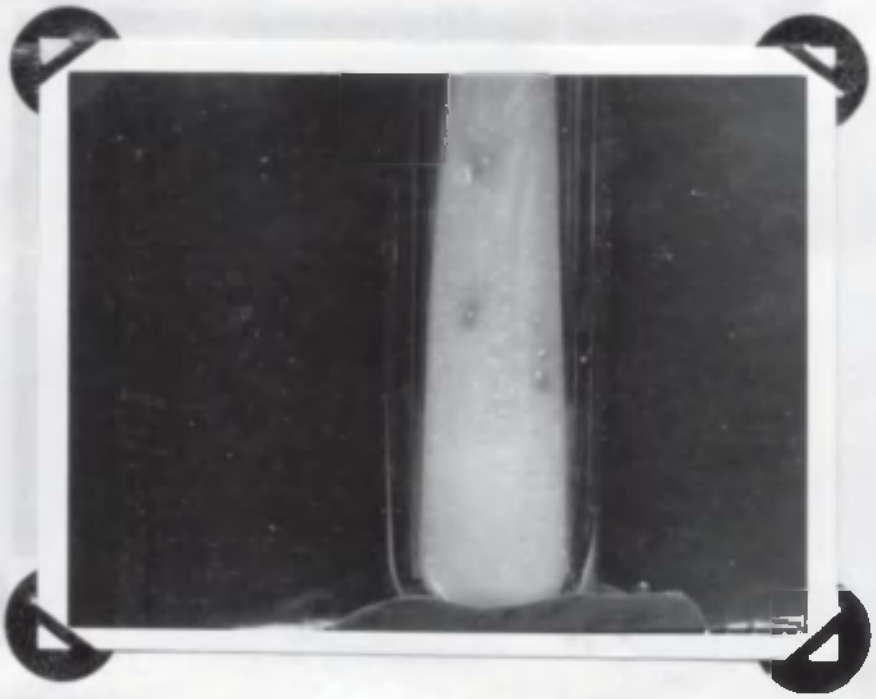
AUTHOR

PETRAGNANI

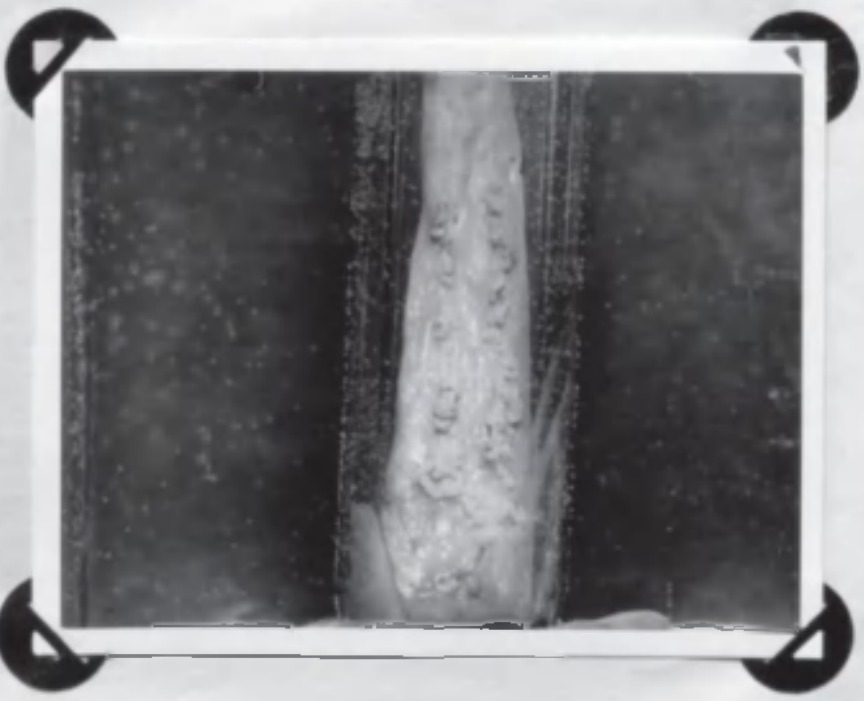


Photographs showing growth on the four principal media after 4 weeks incubation (contd.).

LOWENSTEIN

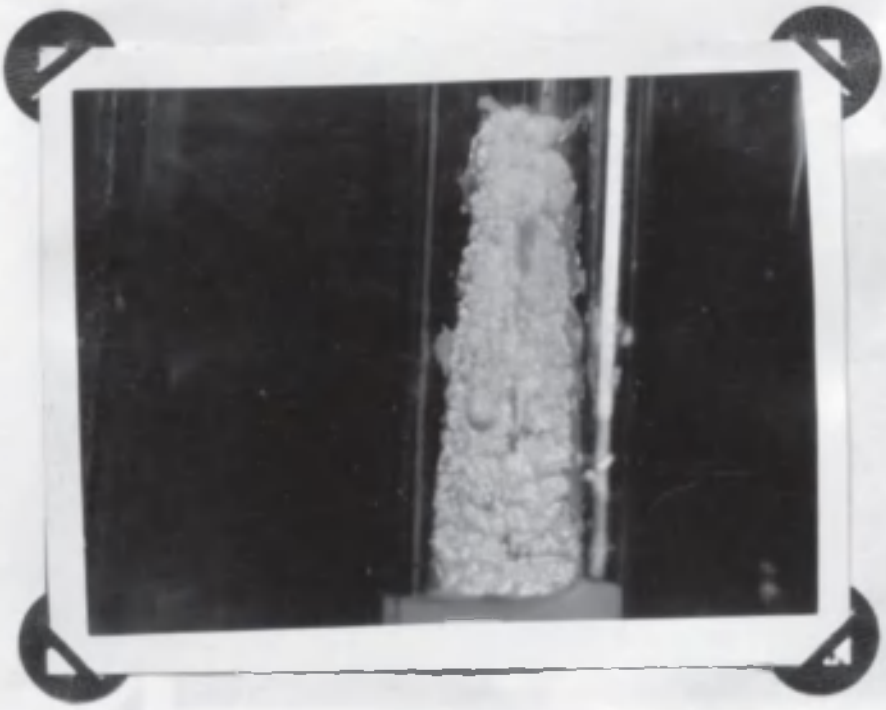


CORPER-UYEI

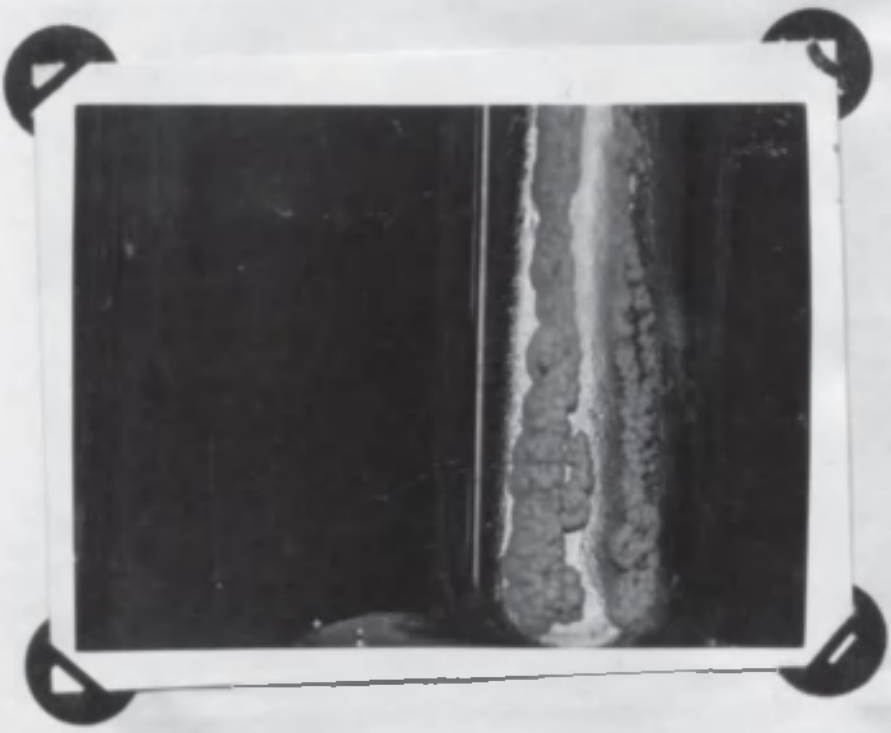


Photographs showing growth on the four principal media after 5 weeks incubation.

AUTHOR



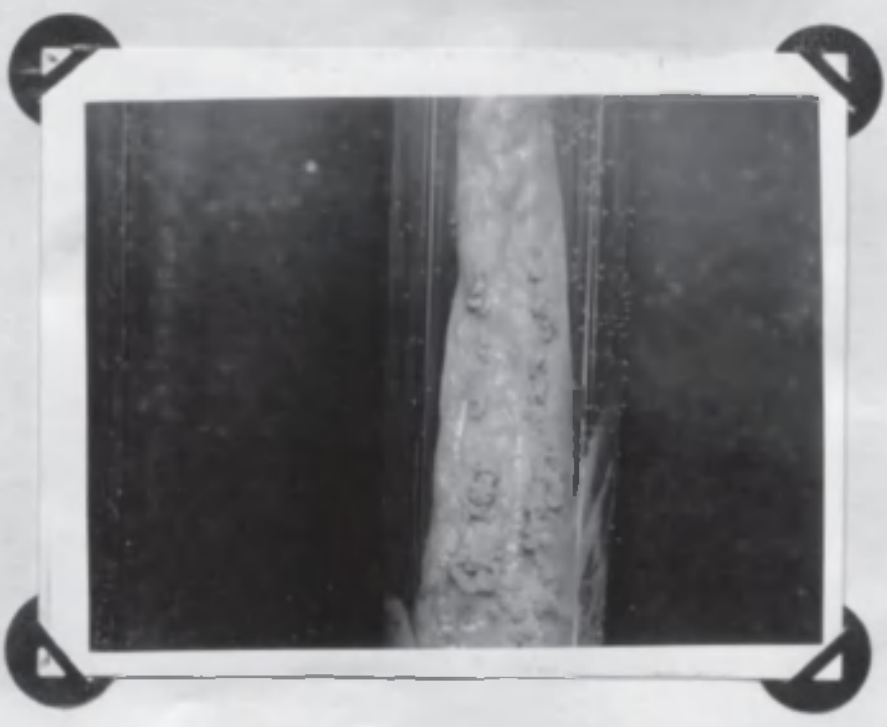
PETRAGNANI



Photographs showing growth on the four principal media after 5 weeks incubation (contd.).

LOWENSTEIN

CORPER-UYEI



Coloured Photographs showing growth on the four principal media after 5 weeks incubation from a subculture of bovine tubercle bacilli.

AUTHOR

PETRAGNANI

LOWENSTEIN

CORPER-UYEI

of the malachite green used in Petragnani's and Löwenstein's media.

Löwenstein's Medium:

Good results were also obtained on this medium, 97 cultures being positive after 5 weeks incubation. 9 specimens showed visible growth at the end of one week. Colonies frequently were visible in 14 - 16 days but were small and discrete compared with the two previous media.

Corper-Uyei's Medium:

This medium was again slightly less reliable than microscopic examination, yielding after 5 weeks 89 positives. Two specimens showed visible growth at the end of 7 days. As before, colonies were not usually visible until the third week, but once formed, the growth was rapid and luxuriant.

In comparing the 10 sputa which gave negative results by the microscopic method, the cultures showed the following:

Author	10 positive
Petragnani	10 "
Löwenstein	7 "
Corper-Uyei	6 "

In the 14 specimens where very few bacilli were found microscopically, the cultural findings were:

Author	14 positive
Petragnani	14 "
Löwenstein	14 "
Corper-Uyei	9 "

The rate at which growth appeared on the above four media is shown in the following table:

Medium	Period of Incubation				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
Author	29	84	93	100	100
Petragnani	23	80	90	96	100
Löwenstein	9	67	85	93	97
Corper-Uyei	2	32	64	77	89

SUMMARY AND CONCLUSIONS

In Series I six media have been compared as to their value in the cultivation of tubercle bacilli from sputum, following preliminary treatment with 6 per cent sulphuric acid according to the Author's modification of the Corper-Uyei method.

The medium of Petragnani gave the best results, being superior to the microscopic method as regards the total number of positive results and in producing the earliest possible growth. Because of the excellence of the results obtained, this medium is particularly recommended for the primary cultivation of tubercle bacilli following treatment by sulphuric acid.

The medium of Löwenstein is also highly recommended as it yielded results almost as good as those of Petragnani. These two media were superior to the other four with regard to the number of positive results obtained.

The media of Herrold and Corper-Uyei also gave very good results but greater care was necessary in their preparation. These four media, therefore, all gave reliable results and any one of them may be used with confidence in the routine isolation of tubercle bacilli from sputum.

The media of Petroff and Sweany-Evanoff, owing to frequency of contamination and tardiness of growth, were inferior to the other four.

In Series II the Author's medium has shown itself to be superior to Herrold's medium in giving an earlier growth and a greater number of positive results.

In Series III four media have been compared as to their value in the cultivation of tubercle bacilli. The results showed

that the Author's medium was superior to the media of Petragnani, Löwenstein and Corper-Uyei. Thus the Author's medium has been proved superior to the six media most frequently used at the present time, namely, Petragnani, Löwenstein, Herrold, Corper-Uyei, Petroff and Sweany-Evanoff.

In this study the use of culture media has proved itself superior to direct microscopic examination. Of the 250 specimens examined only 229 positives were obtained by the microscopic method as against 250 positives by the culture method. This shows 8.4 per cent of microscopically negative sputa were positive.

Comparison with guinea-pig inoculation was not carried out in this investigation, but the positive cultural findings in 21 specimens with no tubercle bacilli demonstrable microscopically and in 27 others where they were very scantily present, suggest that the culture method will give as good results as guinea-pig inoculation, besides being cheaper, quicker and less fallacious.

Conclusions:

The principal media for the cultivation of the tubercle bacillus in use in Britain and abroad have been compared with the Author's medium. The Author's medium has been shown to be superior to these media.

A simple egg medium containing no inhibitory dye is the best medium for the routine isolation of the tubercle bacillus.

8.4 per cent positive cultural results have been obtained where microscopic results were negative. Cultural methods are cheaper and quicker than animal inoculation.

All Public Health Authorities should adopt cultural methods in all cases of suspected pulmonary tuberculosis with a view to instituting early treatment and reducing the high death rate in young adults.

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APPENDIX

Tables corresponding to Series

TABLE 1.

Period of Incubation - 5 weeks

Specimen Number	Microscopic Finding	Petragnani	Löwenstein	Herrold	Corper-Uyei	Petroff	Sweany-Evanoff
1	+	++++	+++	+	++++	(c) ++	+++
2	+	++++	+++	+	+++	+++	(c)++
3	+	++++	+++	+++	++++	(c) ++	-
4	++	++++	++++	+++	++++	++++	++++
5	+	++++	+++	+	++++	(c)++++	(c)++++
6	++	++++	++++	++++	++++	++++	-
7	+	++++	++++	++++	+	(c)	++
8	++	++++	++++	++	++++	(c)	++++
9	+	++++	+++	++	-	++	(c)
10	-	+++	+++	+	+++	++	++
11	++	++++	++++	++++	++++	++++	(c)++++
12	+	++++	+++	+++	++++	++++	(c)++++
13	+(few)	++++	+++	++++	+++	+++	++++
14	+(few)	++++	+++	++	++++	++	++++
15	+	++++	+++	++	++++	+++	+++
16	+	++++	+++	++	-	-	-
17	++	++++	++++	++++	++++	++++	++++
18	+	++++	+++	+++	+++	-	-
19	-	++++	++++	++++	+	-	-
20	+(v.few)	++++	+++	+++	++++	+++	++
21	+	++++	+++	++++	++++	++++	(c)++
22	-	+++	++	++	++	-	-
23	+(few)	++++	+++	+++	++++	-	-
24	+	++++	++++	++++	++	-	++++
25	-	++++	+	-	-	-	-
26	+	++++	+++	++++	++++	++++	+++
27	-	+++	++	+	++	++	+
28	+	++++	++++	++++	++++	++++	++

TABLE 1 (continued).

Specimen Number	Microscopic Finding	Petragnani	Löwenstein	Herrold	Corper-Uyei	Petroff	Sweany-Evanoff
29	-	+++	++	+	-	-	-
30	+(few)	++++	++++	++++	++++	++++	+++
31	+(few)	++++	++++	++++	++	++	++++
32	+(few)	++++	++++	++++	++++	++++	+++
33	+	++++	++++	+	++++	++++	++++
34	+	++++	++++	++++	++++	+	+++
35	+	++++	++++	++++	++++	++	+
36	+	++++	+++	+++	++	+++	+++
37	+	+	+++	++++	++++	-	-
38	+	++++	++++	++++	++++	-	-
39	+	++++	+++	++++	++++	++++	+++
40	+	++++	++++	+++	++++	-	++++
41	-	+++	++	++	++	-	++
42	-	+++	+++	++++	++	-	-
43	+(v.few)	+++	+++	+	+++	-	+
44	+	+++	+++	++	+++	+	+++
45	+	++++	+++	++++	++++	-	++++
46	+	++++	+++	++++	++++	++	++
47	++	++++	++++	++++	++++	+	++
48	+(few)	++++	++++	++++	++++	++	++
49	+(few)	++++	+++	+++	-	-	-
50	+(few)	+++	++++	+++	+++	++	++++
51	+	++++	++++	++++	++++	++	++
52	+(few)	++++	+++	++++	++++	+++	++++
53	+(few)	++++	++++	++	++++	++	++
54	+	++++	+++	-	-	-	-
55	+	++++	++	++++	++++	++	+
56	+(few)	++++	++	++++	+	+	+
57	+	++++	+++	++++	++++	+++	++
58	+(few)	++	+++	+++	++	+	-
59	+	++++	++	++++	++++	++	++

TABLE 1 (continued).

Specimen Number	Microscopic Finding	Petragnani	Löwenstein	Herrold	Corper-Uyei	Petroff	Sweany-Evanoff
60	+	++++	+++	+++	+	+	-
61	++	++++	++	+++	+	++	-
62	++	++++	++	+++	++++	++	++
63	+(few)	++++	++	++++	+++	++	-
64	+	++++	+++	++++	++++	+++	-
65	+	++++	+++	+++	++++	++	++
66	+(v.few)	++++	+++	++	-	-	-
67	+	++++	+++	+++	++++	++	+
68	+(v.few)	++++	+++	++++	++++	+++	++
69	+(v.few)	++++	++	+++	+++	++	+++
70	+	+++	+++	++	-	+	++
71	+	++++	+++	++	-	++++	+++
72	+	+++	+++	+++	++++	++++	+++
73	+	+++	+++	+	+	++	++
74	+	++++	+++	++++	++++	++++	++
75	+	++++	+++	++++	++++	++++	+++
76	++	++++	++++	+++	-	++++	+
77	+	++++	++++	+	++++	++++	-
78	++	++++	++++	++++	++++	++++	++
79	+	++++	+++	++++	++++	+++	+
80	+	++++	+++	+++	++++	++++	+++
81	+	++	+	-	-	-	+
82	+	++++	-	++	++	-	++
83	+	++++	++	++	++++	++++	+++
84	+	++++	++	+++	++++	++++	+++
85	+	+++	++	+	++++	++	++
86	+	++++	++	++++	+++	++++	++
87	+	++++	+++	++++	++++	+++	+++
88	++	++++	+++	+++	+++	++++	+
89	+	++++	+++	+	++++	++++	-
90	++	++++	++++	++++	++++	++++	++

TABLE 1 (continued).

Specimen Number	Microscopic Finding	Petragnani	Löwenstein	Herrold	Corper-Uyei	Petroff	Sweany-Evanoff
91	+	++++	++++	++++	++++	++++	+
92	+	++++	+++	+++	++++	++++	+++
93	+	++	-	-	++	+	+
94	+	++++	+++	+	++++	++	+++
95	+	++++	+++	++++	+++	+++	++
96	+	++++	+++	+++	++++	++	-
97	++	++++	++++	+++	++++	++++	++++
98	+	++++	+++	+	++++	++++	++++
99	++	++++	++++	++++	++++	++++	-
100	+	++++	++++	++++	+	-	++
Positive	92	100	100	96	89	77	75
Negative	8	-	-	4	11	23	25

TABLE II

Period of incubation - 5 weeks.

Specimen Number	Microscopic Finding	Author	Herrold
1	+	++++	++++
2	+	++++	+++
3	+	++++	++++
4	+	++++	+++
5	+	++++	++++
6	++	++++	+++
7	++	++++	++++
8	+	++++	+++
9	+(v.few)	++	+
10	+	++++	+++
11	+	+++	+
12	++	++++	+++
13	++	+++	+++
14	+	++	+
15	+	+++	++
16	+	++++	++
17	+	++++	+++
18	+	++++	+++
19	+	++++	+++
20	+	++	++
21	+	++++	+++
22	+(v.few)	++	+
23	+	++++	+++
24	-	++	-
25	+(v.few)	++	+
26	+(v.few)	++	+
27	+	+++	++
28	++	++++	+++
29	+	++++	+++

TABLE II (continued).

Specimen Number	Microscopic Finding	Author	Herrold
30	+	++	+
31	+	++++	+++
32	+(v.few)	++	+
33	+	++++	++++
34	-	++	-
35	+	++	++
36	+	++	+
37	+	+	++
38	++	++++	+++
39	+	++++	+++
40	+(v.few)	++	+
41	+	++++	+++
42	+	+++	+
43	+	++++	+++
44	+	++	++
45	+	++++	+++
46	+(v.few)	++	+
47	+(v.few)	++	+
48	+	++++	++++
49	-	++	-
50	+	++	++
Positive	47	50	47
Negative	3	-	3

TABLE III

Period of incubation - 5 weeks.

Specimen Number	Microscopic Finding	Author	Petragnani	Löwenstein	Corper-Uyei
1	++	++++	++++	++++	++++
2	++	++++	++++	+++	++++
3	++	++++	+++	+++	++++
4	+	++++	++++	+++	+
5	+	++++	++++	+++	++++
6	+	++++	++++	++++	++++
7	-	++++	+++	+++	++++
8	++	++++	++++	+++	++++
9	++	++++	++++	+++	++++
10	++	++++	++++	+++	++++
11	+	++++	++++	+++	++++
12	++	++++	++++	++	+
13	++	++++	++++	++++	++++
14	+	++++	++++	++++	++++
15	++	++++	++++	++++	-
16	++	++++	++++	++++	+++
17	++	++++	++++	+++	++++
18	-	++++	++++	++++	+
19	+	++++	++++	+	++++
20	+	++++	++++	++++	++++
21	+	++++	++++	++++	++++
22	++	++++	++++	++++	+++
23	++	++++	++++	++++	++++
24	++	++++	++++	++++	++
25	++	++++	++++	+++	++++
26	++	++++	++++	++++	++++
27	++	++++	++++	+++	++++
28	++	++++	++++	++++	++++

TABLE III (continued).

Specimen Number	Microscopic Finding	Author	Petragnani	Lowenstein	Corper-Uyei
29	+	++++	++++	++++	++++
30	+	++++	++++	++++	++++
31	++	++++	++++	++++	++++
32	++	++++	++++	++++	++++
33	++	++++	++++	++++	++++
34	++	++++	++++	++++	++++
35	++	++++	++++	++++	++++
36	++	+++	+++	++++	++++
37	++	++++	+++	++++	++++
38	++	++++	++++	++++	++++
39	++	++++	+++	+++	++++
40	++	++++	++++	++++	++++
41	++	++++	++++	++++	++++
42	++	++++	++++	++++	++++
43	+	++++	++++	++++	++++
44	+	++++	++++	++++	++++
45	++	++++	++++	+++	+++
46	++	++++	++++	++++	++++
47	++	++++	++++	++++	++++
48	++	+++	+++	++++	++++
49	+(v.few)	+++	+++	+	-
50	+(v.few)	+++	+++	+	-
51	-	+++	++	+++	-
52	+	++++	++++	+++	+
53	+	++++	+++	+++	+
54	+	++++	++++	++	++
55	+	+++	+++	+	+
56	+	++++	++++	+++	+
57	+	++++	++++	+++	+
58	-	++++	+++	+++	+

TABLE III (continued).

Specimen Number	Microscopic Finding	Author	Petragnani	Löwenstein	Corper-Uyei
59	-	+++	+++	++	+
60	+	++++	+++	++	++
61	+	+++	+++	+	++
62	+(v.few)	+++	+++	++	-
63	+(few)	+++	+++	+++	+
64	+	++++	+++	++	+
65	+(few)	+++	+++	++	+
66	+(few)	+++	+++	++	+
67	+(v.few)	+++	+++	+	-
68	+	+++	+++	++	+
69	+	++++	++++	++	-
70	+	+++	+++	++	+
71	+	++++	++++	++	+
72	-	++	++	-	-
73	+(few)	+++	++	++	++
74	+(few)	+++	++	++	++
75	+	+++	++	++	++
76	+(few)	+++	+	+	++
77	+(v.few)	++	++	++	++
78	+(v.few)	++	++	++	++
79	+	+++	++	++	+
80	-	+++	+	-	++
81	+(v.few)	++	++	++	+
82	+(v.few)	++	++	++	++
83	+(v.few)	++	++	++	++
84	+(v.few)	+++	+	+	++
85	+	++++	+	+	+++
86	+(few)	+++	+	+	++
87	+(few)	+++	+	+	++

TABLE III (continued).

Specimen Number	Microscopic Finding	Author	Petragnani	Löwenstein	Corper-Uyei
88	-	++	+	-	-
89	+	+++	++	++	+
90	+	++	+	+	++
91	-	+++	+	+	+
92	+(v.few)	++	+	+	+++
93	+(v.few)	+++	+++	++	-
94	+(v.few)	+++	+	+	++
95	+(v.few)	+++	+	+	++
96	-	+++	+	+	-
97	++	++++	++++	++++	++++
98	+	++++	++++	++++	++++
99	+	++++	++++	+++	+
100	+	++++	++++	+++	++++
Positive	90	100	100	97	89
Negative	10	-	-	3	11

EXPLANATION OF SIGNS

MICROSCOPIC RESULTS:

+	=	acid-fast bacilli present.
++	=	numerous acid-fast bacilli present.
+ (v. few)	=	2 - 3 acid -fast bacilli per smear.
+ (few)	=	10 - 20 " " " " "
-	=	acid-fast bacilli absent.

CULTURE-BOTTLE RESULTS:

c	=	overgrowth by contamination
(c)+	=	positive before overgrowth.
+	=	one or two small colonies.
++	=	several fair sized colonies.
+++	=	numerous colonies.
++++	=	surface of medium covered with growth.