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METHOD OF CULTURING SPIROCHAETA PALLIDA

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The culture of virulent forms of *Spirochaeta pallida* (syphilis) bacteria has heretofore been considered impossible. While there is no difficulty in culturing the bacteria they undergo some sort of mutation which renders them incapable of reproducing the disease.

While this factor is undoubtedly a fortunate one from the standpoint of natural control of the disease, it has been proven to be extremely disadvantageous in the development of suitable methods of artificial control.

It is now proven that the bacteria may be developed in virulent form with considerable ease providing only that a suitable culture medium be supplied. The most suitable form of culture medium may be prepared from reproductive tissue, which preferably is the testicular tissue of the rabbit.

It is rather surprising that no successful use of such tissue has been made heretofore because *Spirochaeta pallida* strains have for a long time been carried forward by inoculation of the testicles of live rabbits.

In carrying out the invention rabbits were selected that were known to be negative to the Wassermann reaction and are anesthetized and the testicles removed under surgical asepsis. These are then cut into small pieces and put into a sterile ball mill in the presence of a sufficient normal salt solution to accommodate grinding. The mill is run continuously for about fifty hours, during which time a creamy liquid is produced. This is removed from the mill at the conclusion of the grinding and filtered through a Seitz filter.

The filtrate is then tested for specific gravity and pH. Then enough normal salt solution is added to bring the specific gravity and pH of the liquid to substantially that of the normal blood serum of the rabbit.

At the conclusion of this operation the medium is tested for sterility in any usual manner. If contaminated it is refiltered until it is sterile. It is then stored in suitable sterile containers in a refrigerator.

The inoculation of the medium with a pure strain of *Spirochaeta pallida* involves considerable control and preparation.

The rabbits to be employed are given two tests, one week apart, and are only employed if both tests give negative reactions to the Wassermann, Kline and Kahn tests.

Such a rabbit is inoculated with a salt solution of the Nichols' strain of *Spirochaeta pallida* by making a needle puncture through the skin well forward to each testicle and then introducing the needle into the anterior pole of the testicle. Both testicles are inoculated at the time without withdrawing the needle from the skin until the operation is complete.

After ten, fifteen, and twenty days the rabbits give positive syphilis reactions, and they develop typical chancres at the site of the inoculation.

Before such rabbit is to be used as a source of *Spirochaeta pallida* for inoculation of the culture medium, its blood must show a positive reaction to syphilis, it must present a chancre at the site of inoculation, and these testicles, when excised, must yield typical *Spirochaeta pallida* by darkfield examination.

The term "typical *Spirochaeta pallida*" means bacteria possessing a highly refractile body that is spiral in physical conformation and which possesses a slender body that averages from 8 to 14 micra in lengths and the turns of which are short and sharp and which are easily recognizable to the trained eye.

If the rabbit responds suitably to these reactions the testicles are aseptically removed under ether anesthesia and the rabbit retained for future observation.

These testicles are now cut in thin layers and at right angles to the long axis of the testicles. A suitable amount of this chancrous material is incorporated with the culture medium and shaken with it in a sterile container.

In order to determine the increase of bacteria in the medium, the bacteria are counted from time to time. This may be done accurately by removing a known small quantity of the liquid medium by the use of a No. 23 caliber spinal needle attached to a tuberculin syringe. This measured quantity is then placed on a slide for darkfield counting using a hundred fields as a basis.

The medium container is then sealed by melted paraffin over the surface of the medium or by sealing the mouth of the container securely by using sheet paraffin. This is not only to prevent contamination and evaporation, but it also excludes the oxygen. It is then incubated at 37.5° C.

Further counts are made at weekly intervals. After a maximum count is reached, which generally occurs at the end of the third or fourth week, the first cycle is terminated.

In order to prove the virulence of this growth, two rabbits having negative reactions as heretofore described are inoculated in both testicles with medium from the culture growth. On the

twelfth day they are examined for serological reactions and if the operations have been properly carried out they will be found to give positive reactions to all tests. They should again be tested about the seventeenth and twenty-sixth days.

These rabbits will develop typical chancres at the site of inoculation.

In a particular test these rabbits were anaesthetized in the twenty-sixth day with ether and both testicles removed. Darkfield tests were made and both showed positive and typical darkfield findings. The spirochete were present in great numbers and were typical as required for morphological findings. They were very motile and highly refractile. They were from 8 to 14 micra in length and the spirals were short and sharp.

In actual practice this cycle has been repeated until three complete successive cycles have been carried out without any outside bacterial introductions. The successive cycles showed the same results and were carried out in the same manner, although tests were made on inoculated rabbits at somewhat different periods of time than those given.

Likewise, a single medium has been retained and used effectively on successive rabbits up to as long as fourteen weeks. At the end of this time the medium still contained living active spirochete. The reproduction cycle of the bacteria is far less than fourteen weeks.

The medium may be used effectively in producing a more powerful antigen as described in my copending applications, Serial No. 144,541, filed May 24, 1937, and Serial No. 9,914, filed March 7, 1935.

The spirochete may be centrifuged from the medium and washed with doubly distilled water to free them from the medium. They are then dried in vacuo at 45° C. and are then killed by elevating the temperature just above the thermal death point.

They are then ground for fifty hours in a ball mill with normal salt solution, enough to accommodate grinding. The resulting creamy liquid is filtered through a Berkefeldt filter or its equivalent.

Sufficient normal salt solution is then added so that each cc. of the antigen contains 1 mgm. of the dried bacteria. After usual sterility tests, a preservative is added and the material placed in the usual containers adapted to hypodermic administration.

I claim:

1. The method of developing virulent *Spirochaeta pallida* which comprises inoculating a medium for culture of virulent *Spirochaeta pallida* comprising testicular material which has been prepared by subdividing the testicle, grinding in the presence of normal salt solution, filtering to remove suspended matter, and adjusting the filtrate with normal salt solution to substantially the normal pH and specific gravity of the blood serum and then incubating the inoculated culture to develop the virulent *Spirochaeta pallida*.

2. The method as set forth in claim 1, in which the testicular material is of rabbit origin.

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