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TEST FOR SYPHILIS AND THE PROCESS OF
PREPARING THE REAGENT USED IN PER-
FORMING THE TESTGeorge Franklin Laughlen, Toronto, Ontario,
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1 Claim. (Cl. 167—78)

The invention relates to a test for syphilis and the process of preparing the reagent used in performing the test, as described in the present specification and pointed out in the claim for novelty attached thereto.

The objects of the invention are to prepare an agglutination reagent for the purpose of making a simple, rapid and efficient test on blood serum or other body fluid for the presence of syphilis; to furnish hospitals, clinics and other places with a reagent for blood testing and therefore of special value in cases of blood transfusions when speed in the testing of blood donors and quick reading is of the utmost importance; and generally to provide a reliable test for syphilis which can be performed in a few minutes' time, is inexpensive, can be used with the minimum of equipment, and which may be accomplished by any medical practitioner or technician without special training.

It is well known that there are many tests for syphilis which employ antigen made from beef heart and cholesterol and that they are frequently diluted with sodium chloride solutions before being employed in testing. It is also known that in one or more methods balsam of tolu is added and in one or two other tests dyes have been added. The method of carrying out the tests in microscope slide is also employed in other methods.

In this test greater visibility has been secured by the addition of a water insoluble stain which colours or adheres to the suspended particles of the antigen but does not colour the liquid in which they are suspended, thus affording a good contrast when agglutination occurs. Greater sensitivity and speed have resulted from coarsening the particles in the antigen suspension. Cholesterol, which does this to some extent, is supplemented by the addition of stain particles and balsam.

The method employs drops on exposed slides, which results in concentration and speedier reactions, and the readings which are readily made in the gross can conveniently be confirmed by the microscope. It employs a reagent which is difficult to prepare, but which is fairly stable and can be secured ready for use without further alteration or addition, thus avoiding any technical procedures that can be considered difficult.

It offers advantages over other methods in the following ways:

1. It is more rapid and easier to perform.
2. The readings are more distinct because they

depend upon an agglutination of coloured particles in an unstained medium.

3. As it resembles the methods employed in typing blood, hospital interns and others are familiar with the technique.

4. As the reagent is stable for at least several weeks it is ready for use in emergencies.

5. It affords a convenient means of grading the degrees of positivity.

6. Accuracy has not been sacrificed to secure speed or simplicity.

7. The test requires only small amounts of materials.

All the equipment necessary is the agglutination reagent, a capillary pipette, the patient's serum or spinal fluid, a known positive and a known negative serum for control; a platinum loop, and glass slides. The blood serum should be free from red blood cells, as these interfere with the readings. Two or three tests may be done on each slide. The blood serum and reagent may be used at room temperature, but reactions occur more quickly if they are cold. Citrated blood serum may be used instead of plain serum, but too much dilution should be avoided. For each test one drop of reagent is placed on one section of a glass slide, and to this a large loopful of the patient's serum equal in amount to the drop of reagent, or two loopfuls of spinal fluid are added. These are mixed, and then jarred or tilted repeatedly and observed at short intervals by holding the slide near a strong light and looking through it towards a dark object, but not directly at the light.

Readings may be made by looking toward the cross-bar of a window, if a lamp is not available. The mixture appears at first as a white turbid fluid in which small reflecting granules are seen on tilting the slide. With non-syphilitic specimens the appearance does not alter appreciably until drying is about complete. Slightly coarser granules may then be observed, but there is no true clumping or agglutination. Excess drying should be avoided, and if it occurs inside of ten minutes the test should be repeated using larger amounts of the materials. With the positive control and specimens from cases of syphilis coarse particles soon develop if the slide has been sufficiently jarred or tilted back and forth. These particles increase in size and become distinctly red in colour, and, as drying progresses, they tend to accumulate at the margins of the drop. This reaction is readily recognized with the naked eye. Gross readings may be supplemented by microscopic examination, but microscopic read-

ings are not necessary, nor are they more accurate. Negative readings should never be made until at least ten minutes have elapsed. Positive readings can often be made within one minute. The time required for the reaction to become visible is an index of the degree of positivity of the specimen. Reactions occurring in two minutes or less are strongly positive; those which are definite but occur after a longer interval of time are positive.

If positive blood serum dries, it still retains its ability to cause the reaction. While whole blood and haemolyzed blood may be used, they are not so satisfactory as serum or citrated plasma. Many chemicals, such as acetic acid, interfere with the reaction. Before infected samples are tested, the serum should be cleared as much as possible.

The ease with which tests can be compared with known negative or known positive ones enables nearly all readings to be either positive or negative, rather than doubtful. No difficulty arises in reading strongly positive or negative tests if the above instructions have been followed. Some difficulty may arise if the specimens have been badly preserved, or if the blood is taken during the course of treatment, or if bile is present in excess.

A history of the recent treatment should accompany all specimens from cases under treatment, and such a history is also required by laboratories doing Wassermann or precipitation tests.

It is recommended that the agglutination reagent be secured ready for use from a central laboratory where it can be made in quantity by technicians experienced in its preparation. It has been found that great care must be exercised to prepare a uniform product, as variability may occur when the prescribed technique has been followed. Some practice is necessary to properly modify and dilute the antigen and adjust it to the proper sensitivity. Small quantities of the reagent are as difficult to prepare as larger amounts and the preparation of small quantities would add greatly to the trouble and expense. Most institutions, whether large or small, would find it more economical and satisfactory to obtain the finished product at regular monthly or more frequent intervals from a central distributing station.

The reagent is prepared as follows:

Four steps are necessary—

1. Preparing the antigen.
2. Modifying the antigen.
3. Diluting the modified antigen.
4. Adjusting the sensitivity to a certain standard.

Preparation of the antigen.—This is made in the same way as the precipitation reagent or antigen that is used in the Kahn test, and it is prepared as follows:

(1) *Extraction with ether.*—Twenty-five grams of finely powdered dried beef heart muscle are placed in a 250 c. c. Erlenmeyer flask. One hundred c. c. of anaesthetic ether are added and the flask is shaken at frequent intervals for ten minutes. At the end of this period the ether is filtered off. Gentle pressure is applied to the beef heart in the funnel by means of a spatula to assure as complete removal of the ether as possible. The filtration is completed when further pressure does not cause drops of ether to pass through the funnel. The moist beef heart is now transferred to the original flask and 75 c. c. of fresh ether added. The flask is again shaken

at frequent intervals during a ten minute period, and the ether filtered off as in the previous case. The heart muscle is returned to a flask a third time and again covered with 75 c. c. ether. The flask is shaken from time to time during a ten-minute period and filtration carried out as previously.

The moist powder is now transferred to the Erlenmeyer flask for the fourth and last ether extraction. Seventy-five c. c. ether are added to the flask, the mixture shaken for ten minutes, and final filtration carried out. The first three ether filtrations may be carried out with the same filter paper. For the last filtration, however, a fresh layer of filter paper is employed. After the ether is removed as completely as in the earlier filtrations, the moist heart muscle is spread upon a white sheet of paper or a clean glass plate and dried in the incubator at 37° C. for about ten minutes or for a somewhat longer period at room temperature. When the material is dry and free from ether odor, it is ready for extraction with alcohol.

(2) *Extraction with alcohol.*—The ether extraction being completed, the powdered muscle is reweighed and placed in a 250 c. c. flask. An amount of 95 per cent. alcohol is then added in the proportion of 5 c. c. of alcohol per gram of powder. The mixture is thoroughly shaken for ten minutes and extracted for three days at room temperature (21° C.) without shaking. At the end of this period it is shaken for five minutes and the alcoholic extract is filtered off. This extract is kept at room temperature in the dark in tightly corked flasks. This alcoholic extract is the antigen which is cholesterolized by the addition of 6 mg. of pure cholesterol per c. c. of antigen. It is necessary that pure ethyl alcohol be used in its preparation.

Modification.—This must be carried out just before the saline dilution is to be made. One-half c. c. or a multiple quantity of the cholesterolized antigen, is placed in a small test tube, and to this is added some fat stain of the class consisting of scarlet red also called Sudan IV and listed as Number 258 of the Color Index and Sudan III which is listed as Number 248 of the Color Index. This class of stains has the property of being practically insoluble in water and freely soluble in fatty or oily substances. The quantity is small, probably about 1 to 2 mg. but it is not necessary to have an exact amount, as a small excess is permissible. The tube containing the antigen and stain is placed in a water bath at approximately 46° C. for 3 to 4 minutes, and is shaken when warm to secure saturation of the stain. The tube should be plugged with a cork covered by tinfoil to lessen evaporation during the warming. At the end of this time 2 drops of Tincture Benzoini Co., Friar's balsam, are added and the tube is again shaken. It is now ready for dilution, which should be carried out at once.

Dilution of the modified antigen.—Before modifying the antigen as described above, the proper quantity (5 c. c. for each half c. c. of antigen) of 1.5 per cent (the strength may vary for different lots of antigen) saline should be measured into a small tube and placed in a water bath at approximately 46° C. This is done first because the larger amount of fluid requires a longer time to arrive at the temperature of the bath. By the time the modification of the antigen is complete the saline and cholesterolized antigen are ready for mixing. The saline is poured quickly into the antigen and back and forth four or five

times. The mixture is again placed in the water bath for two minutes, when it is taken out and allowed to cool.

An experienced technician at this stage will be able to predict if the diluted antigen, or agglutination reagent, as it is now called, is likely to be satisfactory. If satisfactory it should present a sufficient opacity and denseness. Thin, deep red emulsions are usually too sensitive. If any particles other than a few heavy portions of undissolved stain are observed throughout the solution it may as well be discarded. The colour should be reddish-white, rather than a deep red. If unsatisfactory emulsions are obtained the trouble should be sought in the temperature of the bath or in the method of mixing. More stable or more dense emulsions can be obtained by increasing slightly the amount of cholesterol or Friar's balsam. Loss by evaporation must be avoided during these processes. The mixture should be set aside for fifteen or twenty minutes, when the coarse particles can be removed by decanting the supernatant fluid.

If the mixture at this stage is satisfactory, sedimentation occurs slowly and is not complete in twenty-four hours. This is an inactive stable emulsion. It has been found necessary to prepare such an emulsion of sufficient density to partially inhibit the agglutination when positive syphilitic serum is added, and later to adjust its sensitivity to a certain standard by the addition of an activating agent (electrolyte). For this purpose sodium chloride or ammonium sulphate may be used, but care must be taken not to add an excess of these, as the process cannot be reversed and most of the success of the test depends not only on the proper preparation of the reagent but also on increasing its sensitivity as much as possible without causing it to become non-specific. Weak reagents would indicate with strongly positive sera but not with weakly acting ones and too sensitive reagents would cause difficulty in distinguishing negative tests.

Anyone attempting to adjust the sensitivity of the freshly prepared emulsion should have some knowledge of its physical character. It is an emulsion (water, alcohol and balsam) in which are suspended particles derived from the beef heart, particles of stain, and cholesterol. There are also salts in true or molecular solution. The particles of meat, stain, cholesterol and balsam cohere. These coherent particles tend to agglutinate in the presence of electrolyte, but the colloidal nature of the solution prevents such action. In other words, in molecular solutions these particles would agglutinate. In the reagent, which is both molecular and colloidal, sufficient denseness of the colloid suspension is obtained to prevent agglutination unless other reagents are added. When non-syphilitic serum is added to this reagent there is a lessened tendency to agglutination, but when syphilitic serum is added

the union of an antibodylike substance with the coherent particles gives rise to agglutination which is prompt and definite.

Adjusting the sensitivity.—Several positive sera should be available for this purpose. They should be labeled to indicate the time in which a positive reading can first be made with each of them when using a standard agglutination reagent. A strongly positive serum which reacts in one minute is necessary as well as some which require a longer time. At least one negative serum is also necessary.

Each specimen of reagent must be sensitized to react in one minute with the strongly positive, and must remain free from agglutination for at least ten minutes with negative sera.

Tests are carried out as outlined under technique, using a known strongly positive serum and the freshly prepared reagent. When it has been determined by this means that the reagent is below the proper degree of sensitivity, 9 per cent sodium chloride is added, about 1 drop for each c. c. of reagent.

This tube is shaken and set aside for several hours, as the full effect of adding the electrolyte is not obtained immediately. At the end of this time partial clearing of the solution should have occurred. It is again tested in the same way, and if it does not give positive readings in one minute with strongly positive serum, more saline must be added. It is allowed to stand and is tested again. These processes are repeated until the reagent has developed the necessary degree of sensitivity. It is then tested with negative serum, and must remain free from agglutinations for ten minutes or longer. Further checking against weakly positive, strongly positive, and negative sera should also be carried out on two or three successive days, to be sure that no change has taken place on standing.

Saturated ammonium sulphate solution C. P. may be used instead of 9 per cent saline to activate the reagent, and 1 or 2 drops are usually sufficient for a 10 c. c. quantity. There is greater danger of producing over-sensitivity when using this activating agent.

The reagent should be kept at room temperature in tubes or bottles closed by corks covered by tinfoil. Rubber corks should not be used. It should also be kept in the dark to prevent fading, which however does not greatly impair its efficacy.

What I claim is:

The process of preparing a test reagent for syphilis comprising adding a compound tincture of benzoin and a fat stain of the class consisting of Scarlet Red and Sudan III to cholesterolized antigen and diluting the mixture with an amount of an aqueous solution of an electrolyte sufficient to adjust the reagent to a desired standard of sensitivity.

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