TECHNICAL NOTE

A MODIFICATION OF THE OAKLEY TUBE METHOD OF THE AGAR DOUBLE-DIFFUSION PRECIPITATION TEST FOR MYCOBACTERIAL ANTIBODIES

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It being probable that application, in leprosy investigations, of the double-agar diffusion precipitation test would be found profitable, there is offered—on request—the present note on technique. It describes a method, in which Oakley tubes are used for the reactions, which is reasonably simple and for most purposes more practicable than would be the Ouchterlony plate (petri-dish) method, although to the practiced worker the latter may perhaps sometimes be preferable for special purposes, as when reactions to multiple antigens are to be compared.

We have observed that many human sera which contain mycobacterial antibodies—evidently in low concentration—fail to give positive reactions in the plate test do so very nicely in the tube test. The reason is not certain, but it is my opinion that the greater area available in plates for diffusion of the reacting substances makes that technique less sensitive than the other, for in the tubes the diffusion areas are relatively restricted.

The technique of the test is, in summary, as follows: In a reaction tube of specified dimensions, previously coated inside with a thin layer of dried agar, the test is made with three superimposed columns. In the lower part of the tube is a relatively long column of agar containing the antigen; second is a short "neutral" column of agar without any reactive substance, in which the reaction occurs; and above that the tube is filled with the serum being tested. The precipitation reaction occurs in the neutral agar column, as a result of the diffusion of (*a*) antigen from the agar-antigen column below, and of (*b*) antibody from the serum column above.

Instead of simply presenting a re-write of the technique described in recently published articles ([1,2]), I have chosen to give a slightly modified technique based on a mimeographed protocol currently in use in this laboratory. Features which might affect the applicability of the method in places where facilities are limited, including certain of the specifications originally given in footnotes, are given special consideration.

MATERIALS

1. Reaction tubes, 10 cm. long with 3 mm. internal diameter.
2. Capillary pipettes, with delivery tips 10 cm. long.
3. Agar, any specially washed, purified product. The agar solutions are made with distilled water or 0.9 per cent saline as directed. Those used for the test columns (Items 2 and 3 of Procedures) should contain 1 M (7.5%) glycine.
4. Reagents: Antigen; antiserum; glycine (glycocol, m.w. 75.05); 1N NaOH (for adjusting the pH of the agar solutions).
5. Equipment: (*a*) Water bath, maintained at about 50°C to keep the agar solutions melted while the tubes are being prepared. (*b*) Hot air oven heated to about 50°C, for drying the agar lining of the reagent tubes (Item 1 of Procedure). (*c*) Incubator at 37°C for developing the reactions.

Comments.—The tubes specified (Items 1 and 2) are made of soft glass (2). Pyrex would be more expensive to buy and more difficult to manipulate, although it would be more durable in repeated usage. The tubes and the Wintrobe capillary pipettes used in our work are obtained from the Aloe Scientific Co., St. Louis, Missouri. However, entirely usable tubes can easily be made on a do-it-yourself basis from ordinary soft glass tubing of appropriate sizes. For the reagent tubes, stock of 3 mm. inside diameter is cut so that the length will be 10 cm. after one end is closed by flaming. To make the capillary pipettes, for which stock tubing 5 mm. inside diameter is satisfactory, the central section of a piece is heated red in a wing flame and drawn out to produce a coarse capillary section small enough to fit readily into the reagent tube and long enough to reach the bottom of it. With proper cleaning, these pipettes and tubes may be reused many times.

Regarding the agar, we use a purified product supplied by the Difco Laboratories, Detroit, Michigan, under the name "Special agar (Noble)." Inquiries have been made to ascertain the availability of specially purified agars in other countries. Dr. J. Ross Innes, of London, reported that a good grade of agar produced in New Zealand is readily available in England, but that the only specially purified agar obtainable there is the Difco product. Drs. R. Chausinand, of Paris, and L. M. Bechelli, of São Paulo, both reported in effect that purified agar is not made in their countries but when needed is obtained from the United States.

It is a matter of fact that any agar which gives a clear, solid product can be employed. In our earlier work we used, with good results, shredded bulk agar dialyzed against distilled water until a clear product was obtained. The commercial product specified is used now because it is convenient.

Finally, regarding equipment, the water bath need not be a special apparatus; it could be merely some simple container on a tripod, a small flame being applied beneath it as required. If a regular hot-air oven is lacking, a paraffin oven may be used even if the temperature should be as high as 56° or 58°C. All that is necessary is to dry to a film the agar which coats the inside surface.

PROCEDURE

1. *Internal coating of reaction tubes.*—As a preliminary measure the reaction tubes must be coated internally with a thin film of dried agar, to ensure that the two agar columns of the test set-up will adhere to the wall of the tube.

Invert a batch of clean tubes in a beaker containing a simple 0.1 per cent solution of agar in distilled water (without glycine), enough to cover the tubes, and autoclave the loaded beaker in the ordinary

way. This drives the air out from the tubes and causes the agar solution to be drawn up. Subsequently, when the tubes have partially cooled but while the agar is still fluid, the filled tubes are emptied of as much of the solution as possible—done by simply shaking it out into the sink. At once, before the agar can gel, the tubes are placed, inverted in a container with the bottom covered with an absorbent substance such as filter paper, in a hot air oven at 50°C (or in a paraffin incubator at its usual temperature) to dry for 12 hours or overnight. The tubes so prepared keep indefinitely if kept free from contaminating dust, so they may be prepared in large numbers.

2. *Antigen-agar column.*—The first step in preparing for the test itself is the preparation of the antigen-agar mixture and its introduction into the lower part of the reaction tube.

This mixture is made in an ordinary sterile tube or flask, maintained at 50°C in the water bath. The agar is a 2 per cent sterile solution, made with 0.9 per cent saline and adjusted to pH 8.0, with glycine added in the concentration indicated above. The antigen, also heated to 50°C, is then added in the quantity needed for the desired dilution.[1] The mixture is maintained at 50°C until the reagent tubes are loaded.

With a sterile capillary pipette, fill the reagent tubes with the agar-antigen mixture (the first agar column) to a depth of approximately 4.5 cm., taking care not to include any air bubbles. Tubes with this column can be stored in the refrigerator for several weeks, if desired, and therefore they can be prepared in advance of performing the actual tests. (To do this would ensure uniformity of this element of the test when, over a material period of time, considerable numbers of sera are to be tested with a given antigen.)

As it tends to increase the speed as well as improve the visual quality of the test, we now add 0.15 cc. of a 0.1 per cent Tween 80 aqueous solution per each 3.0 cc. of antigen-agar mixture in the mixing flask, prior to adding the antigen-agar column to the tubes.

3. *Agar diffusion or reaction column.*—The last step in preparation for the actual performance of the test is to introduce above the antigen-agar column a short column of agar without reagent (i.e., the neutral reaction zone). For this is used a sterile 1 per cent agar solution, made as before with 0.9 per cent saline, with glycine added, and adjusted to pH 8.0, and as usual maintained in the 50°C water bath. With a sterile capillary pipette enough of the solution is introduced to the reagent tube to make a column 0.5 cm. long, particular care being taken to avoid air bubbles. At least 10-20 minutes should be allowed for this second agar layer to gel before the next step is taken.

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[1]The suitable proportions of antigen and agar solution (2%) will be determined by experience. It is recommended that with concentrated, unheated culture-filtrate antigens of mycobacteria, 4 ratios to agar, 3:1, 1:1, 1:3 and 1:20, be prepared.

Previously, longer columns were used, up to as much as 3 cm. The individual precipitate bands were correspondingly separated. It has been found, however, that although in the short column the precipitate bands are closer together and their separation may not be so easily seen, the test is more sensitive.

About the use of glycine in the antigen-agar and reaction-agar columns, the purpose is to inhibit nonspecific precipitates that sometimes occur with human serum. If necessary, it could be omitted as not absolutely essential although helpful. In fact, we did not use it in the first year of our work; but we do now because it has proved useful on occasion. The only precaution in its use is that it should not be autoclaved more than the usual pressure and time (15 lb., 15 min.).

4. *Test serum column.*—The test preparation is completed by filling the tube to the top with clear, sterile patient's serum—undiluted, nonhemolyzed and nonlipemic—which is run in above the second agar layer with a sterile capillary pipette. (Unused serum is preserved in the refrigerator for replacement in case of very weak reactions, as described below.) The tubes may be placed in any clean container such as a beaker or a "Mason" fruit jar; and, after being covered for protection from dust, they are incubated at 37°C.

Sterility.—Regarding sterility in the procedure, it is obviously not an entirely sterile technique. The test would be difficult to set up quite aseptically, but an effort is made to introduce as little contamination as possible. The tubes are autoclaved (the reagent tube with the coating agar), as are the other agar solutions; the antigens are sterile from filtering, and the sera are obtained sterile and kept so.

OBSERVING AND READING REACTIONS

The tests, maintained in the incubator, should be observed at 24-hour intervals for 7 days, with record of the number of clearly-defined precipitate bands in each tube.

In most tests for antibody to *M. tuberculosis*, the reactions are found to be positive within the first 48 to 72 hours of incubation. Depending entirely upon the antibody titer of each serum, visible precipitate bands will appear in any one, several or all of the 4 tubes used for the test. The absence of precipitate bands in any one or more tubes of the test series is due to either an excess antigen or excess antibody inhibition, whichever the case may be.

Readings are made for each of the 4 tubes in the test according to the key given below.

Four-plus (4+) reactions: In the strongest (4+) reactions, three or more sharply-defined, dense bands of precipitate develop after 24 to 48 hours of incubation. If the tubes are kept in the refrigerator, these bands will persist for several weeks.

Three-plus (3+) reactions: Here again three or more bands of precipitate will develop, but they are usually slower to appear and never become as dense and well-defined as the bands seen in the 4+ reactions. These reactions may appear as of 1+ or 2+ grade at 24 hours, and progress to the 3+ grade the next day (i.e., after 48 hours).

Two-plus (2+) reactions: In these reactions at least two bands of precipitate appear at some time between 24 and 48 hours. They appear as distinctly separate bands, but they lack the sharp delineation seen in 3+ and 4+ reactions. These reactions may tend to fade with time.

One-plus (1+) reactions: One definite, but poorly-delineated precipitate band appears at any time between 24 and 72 hours. It never differentiates into more than one band. In many cases it may appear and subsequently fade out.

Negative (−) reactions: Total absence of precipitate.

EXPLANATION

If one assumes: (1) That the greater bulk of visible precipitate is due to antibody; (2) that the greater the concentration of a reagent, the more rapid is its rate of diffusion into the agar reaction column; and (3) that antigen or antibody excess will result in desolution of visible precipitate:

Then the following can be assumed to be indicative of the serum antibody titer: (1) Consideration of the antigen concentration in each positive tube. (2) The speed of the reaction. (3) The degree of delineation and the density of the resultant precipitate bands. (4) Persistence or disappearance of precipitate bands with increase of time. (5) Increase in the number of bands as the incubation time is prolonged.

REFERENCES

1. Parlett, R. C. and Youmans, G. P. An evaluation of the specificity and sensitivity of a gel double-diffusion test for tuberculosis. American Rev. Resp. Dis. **80** (1959) 153-166.
2. Parlett, R. C. and Rehr, C. A. Further studies on gel diffusion tests in tuberculosis. I. Methods for standardization of the antigen and detection of small amounts of antibody. American Rev. Resp. Dis. **80** (1959) 886-894.