

TYPING GROUP A HEMOLYTIC STREPTOCOCCI BY M PRECIPITIN  
REACTIONS IN CAPILLARY PIPETTES\*‡

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Two methods of typing group A hemolytic streptococci have been described: slide agglutination and anti-M precipitin tests. The first, developed by Griffith (1), is more extensively used. It has the distinct advantages of requiring small amounts of serum and culture, and of a quick reaction time when all conditions are favorable. It has certain disadvantages: The granular growth of some strains of streptococci is so marked that much subculturing is necessary before they are suitable for testing, and many days or weeks may elapse before the final result is available. Cross reactions are not uncommon, and make it impossible to differentiate certain types unless specially prepared sera are used which have been absorbed with regard to the antigenic relationships among types. This is due, in part, to the presence in group A streptococci of two antigens related to type specificity, so called M and T, which in a given microorganism do not always correspond (2, 3). Antibodies for one or both of these antigens have been used for the identification of types by the agglutination method. It is now evident that several types contain common or closely related T antigens, but that members of each type elaborate a distinct type-specific M substance (4). So far, we have only once encountered the antigenic combination consisting of two types with a common M substance and distinct T antigens. Probably a thorough understanding of the usual antigenic make-up of each type will eventually result in more satisfactory slide agglutination tests.

Anti-M precipitin typing is more precise in that a single antigen-antibody system is involved, and there is relatively little crossing among types when

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highly potent and well absorbed sera are employed. Furthermore, the type-specific protective antibody runs parallel with the anti-M precipitin content of a serum (5).

From an epidemiological viewpoint the anti-M precipitin technique has so far yielded satisfactory results in our hands. Because a bacterial extract is employed, the handicap resulting from spontaneous agglutination of streptococci is avoided and definite results are obtained promptly. Until recently the reactions were carried out in small test tubes, and required 0.05 cc. of immune serum and the concentrated extract from about 5 cc. of culture for each tube. This made application of the method impractical on a large scale, because the quantities of serum and culture required were too large.

In order to make the method more practical, a technique has been developed in which the reaction is carried out in capillary pipettes of such a size that about 250 tests can be made with 1 cc. of serum. The crude streptococcal extract previously used for grouping is also employed for typing by this method. As capillary pipettes have been used for precipitin tests since the early days of immunology (6, 7) and the principles underlying typing by the M precipitin reaction have already been described (8), this communication is made mainly with the object of defining the conditions whereby satisfactory classification of streptococci can be performed by the precipitin reaction.

#### *Apparatus*

*Capillary Pipettes.*—For anti-M precipitin typing the pipettes are made from capillary tubing about 1.0 mm. external diameter,<sup>1</sup> which is broken into 7.5 cm. lengths. The outer surface is carefully cleaned with soft paper tissue. The inner surface of the pipettes is not cleaned, because chemical treatment interferes with capillary action. The clean pipettes are placed in Wassermann tubes and sterilized in dry heat.

*Serum Containers.*—Convenient containers consist of two parts: (1) a small screw-cap glass vial 45 mm. long and 15 mm. in diameter, with neck having an inside diameter of 9 mm.; (2) an inner container made of glass tubing with an external diameter of 7 to 8 mm., and a bore of about 5 mm. From this tubing heated in a blow flame, a goblet-shaped cup is made about 20 mm. deep, standing on a solid glass stem and foot. The stem is cut so that the combined height of the cup and stem is 42 mm. (see Fig. 1).

<sup>1</sup> The capillary tubing is made by the Kimble Glass Company, Vineland, New Jersey, and can be obtained from laboratory supply houses. It is described by that Company as No. 46485 capillary pipette tubing, made of neutraglas (N-51A glass), not individually gauged, and varies in outside diameter from 0.7 to 1.0 mm. with 0.2 mm. wall. There are approximately 500 thirty-four inch lengths to the pound. For confirmatory tests and group classification, larger tubes with outside diameter of 1.2 to 1.5 mm. are used; 1 pound contains approximately 300 thirty-four inch lengths. In order to have reasonably quick service on capillary tubing at this time, it will be necessary for those ordering this tubing to obtain a priority of AA2X or better.

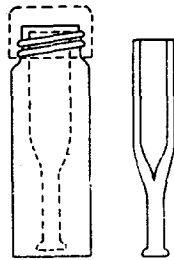


FIG. 1. Serum container in screw cap vial; detail to show shape.

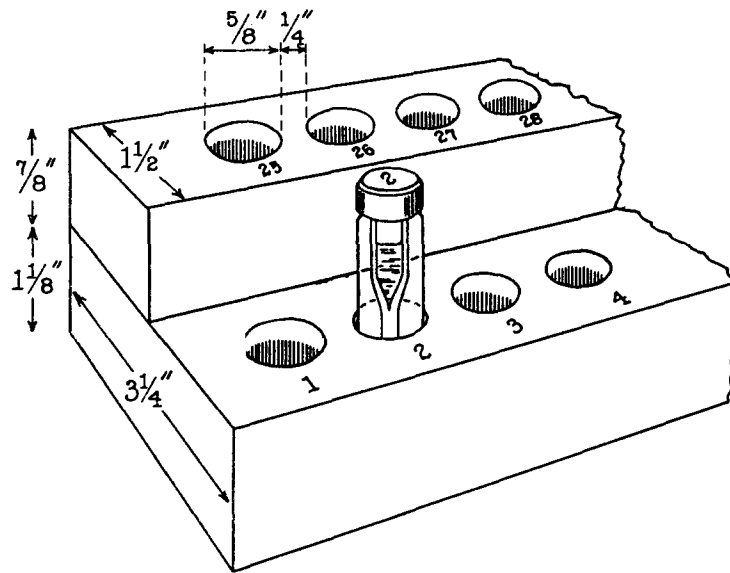


FIG. 2. Vial holder with one serum container in place.

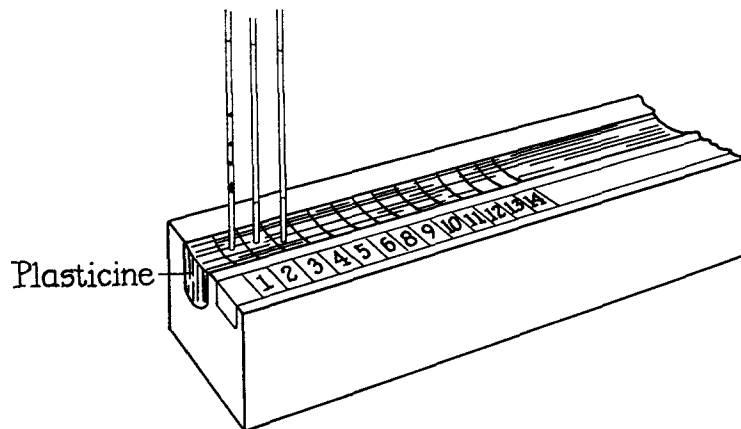


FIG. 3. Capillary pipette stand with three pipettes in place; precipitate in first tube (serum Type 1).

The inner container, which holds about 0.2 cc., is placed in the vial and the cap loosely attached; they are wrapped in paper and autoclaved.

*Vial Holder.*—Two rows of vials are held in a wooden block made from two boards of the dimensions shown in Fig. 2, and of a length to fit conveniently in a refrigerator. Holes,  $\frac{5}{8}$  of an inch in diameter,  $\frac{3}{4}$  of an inch deep, with their margins  $\frac{1}{4}$  of an inch apart, are bored as shown. A small piece of plasticine in the bottom of the hole holds the vials firmly so that they do not turn when the caps are unscrewed.

*Capillary Pipette Stand.*—This consists of a wooden block 10 inches long,  $1\frac{1}{4}$  inches wide, and  $\frac{7}{8}$  of an inch thick, in which is cut a groove  $\frac{1}{4}$  of an inch wide and deep. This is filled with plasticine. Labels are written on narrow strips of ruled paper fastened in front of the groove. Corresponding lines are drawn on the plasticine (see Fig. 3). One stand is used for each extract when the complete set of sera is employed.

### Materials

*Bacterial Extract.*—The crude extract, containing both group-specific C and type-specific M substances, is prepared from a heavy growth in broth. A modified Todd-Hewitt broth (9, 10) gives excellent results, but most streptococci grown in Difco tryptose phosphate broth have yielded satisfactory extracts. 40 cc. of broth in a 50 cc. centrifuge tube are heavily inoculated, and then incubated overnight, centrifuged, and the supernatant fluid discarded. The bacterial sediment is mixed with 0.4 cc. of  $N/5$  HCl. A loopful of the suspension gives an orange red color with a drop of 0.01 per cent solution of thymol blue and turns Congo red paper blue. The mixture is transferred to a pointed 15 cc. centrifuge tube and heated in a boiling water bath with occasional shaking for 10 minutes; then cooled and centrifuged. The clear supernatant fluid is decanted into a second centrifuge tube, a small drop of 0.01 per cent solution of phenol red is added, which colors the solution a distinct yellow. About 0.3 to 0.33 cc. of  $N/5$  NaOH in  $M/15$  phosphate buffer of pH 7.0 is added drop by drop.<sup>2</sup> Less than 0.4 cc. is required because about a quarter of the HCl has been taken up by the streptococci. The correct reaction, pH 7.0, is reached when the first pink color appears; but a range between pH 7.0 and 7.8 is satisfactory. The reaction may be more accurately checked by mixing a loopful of the extract with a drop of 0.01 per cent phenol red and comparing the color with that formed by this indicator and a loopful of buffered phosphate solution of known pH. If too alkaline, the extract is readjusted with  $N/20$  HCl. The cloudy solution is again centrifuged, and decanted. It is tested first with group A serum,<sup>3</sup> and if this gives a positive reaction it is further tested with

<sup>2</sup> The NaOH in  $M/15$  phosphate buffer solution of pH 7, used for neutralizing the HCl solution of streptococci, gives a salt concentration suitable for the formation of immune precipitates. It is conveniently prepared by dissolving 1 gm. of  $Na_2HPO_4$  in 100 cc. of  $N/5$  NaOH. This solution is balanced against the  $N/5$  HCl used for extracting the streptococci.

<sup>3</sup> *Group Classification.*—Capillary pipettes are substituted for test tubes in group classification provided especially strong antisera are available. The capillary, 1.5 mm. in diameter, is held horizontally while drawing in serum and extract, and is kept in this position until after it is inserted in the plasticine of the pipette holder. In

all of the type sera.<sup>4</sup> If it is negative with group A serum it may be tested for reactions with the other group sera.

*Preparation of Precipitating Sera.*—This serum is prepared by immunizing rabbits with heat-killed vaccines of streptococci known to be rich in M antigens (11). After the serum is thoroughly absorbed with a strain of heterologous type to remove non-type-specific precipitins, it must react strongly with homologous M extracts in order to be useful in this reaction (2). The absorbed sera, preserved with merthiolate 1:10,000 are conveniently stored in small dropping bottles from which about 0.2 cc. are transferred, by means of the droppers, to the small serum containers. Care is taken not to draw the precipitate which sometimes accumulates in the bottom of these containers into the capillary pipettes while performing the tests.

*Procedure of Capillary Pipette Typing.*—A container of capillary pipettes and a tube with a few drops of extract at its lip are conveniently mounted horizontally in plasticine. A capillary pipette is dipped first into the serum until a column about 2 cm. long has been drawn in by capillary action. It is next dipped into the streptococcal extract until a similar volume has been taken up. This procedure may be a source of error because traces of each immune serum used may be carried over into the common tube of extract, but no cross reactions traceable to this have been observed. If an air bubble gets in between serum and extract, the capillary pipette is discarded and a new test set up. The pipette is inverted until a column of air is present at each end; the film of serum and extract is removed with paper cleaning tissue; and the pipette inserted vertically in the appropriate plasticine square in the holder so that the serum is at the top of the column. The air spaces above and below the mixture allow a clear view of the whole column, including any precipitate which may fall to the bottom. Similar pipettes are set up with each serum to be tested (see Fig. 3).

Readings are made with a hand lens immediately, after 2 hours' incubation at 37°C., and again after overnight refrigeration. If the rack is held against a black background with a light, preferably from a fluorescent lamp, falling obliquely across the tubes, accurate readings are easier to obtain. The scale used is as follows:  $\pm$ , just visible; +, a few fine masses visible with a lens; ++, usually beaded throughout, easily seen without lens; +++ to +++++, column filled with masses of precipitate.

*Confirmatory Tests.*—Doubtful results are checked in comparison with known extracts by several different methods: (1) By a ring test, either in a capillary pipette about 1.5 mm. in diameter, or in a small test tube as previously described (11). (2) By diluting the serum 1:2, 1:4, 1:8, 1:16 with physiological salt solution and testing

this way layering of serum over extract is obtained, and the reaction is read as a ring test within a few minutes.

Prozone phenomena due to mixing may interfere if the reading is delayed beyond a half hour or if the finer capillaries are used for the test. In case of negative or doubtful reactions, the usual test tube method (11) is employed.

<sup>4</sup>In the presence of an epidemic due to hemolytic streptococci, both time and material may be saved by testing with the type serum covering the epidemic strain and with three to six other type sera as controls. Only if these give negative results need all of the sera be employed. Similar economies may be effected in testing repeated cultures from the same patient.

these dilutions in the 1.0 mm. capillary pipettes. (3) By setting up a series of dilutions in small test tubes in the usual manner (8).

*Cross Reactions.*—With properly absorbed serum significant cross reactions are rare; They are usually characteristic of a particular sample of serum rather than of complicating relationships among types. The precipitates are usually small compared with those formed by homologous serum and extracts. If strong cross reactions occur, the serum is removed from current use for further absorption or study.

#### RESULTS

Positive reactions indicate the presence in a strain of the M substance of a given type. Negative reactions may indicate that the strain belongs to some as yet unidentified type, since there is no doubt that numerous others exist in addition to those identified by Griffith. Recent studies here (4) and at Irvington House (12) have established at least 16 more types, with still other strains for which it has so far been impossible to prepare satisfactory antisera.

Negative reactions may also be due to loss of type-specific M substance after isolation of the strain. In some strains and types the M substance remains constant, but in others it may disappear rapidly during subculture on artificial media. The capacity to form M substance may usually be restored by animal passage, but this is often a laborious and expensive procedure. For these reasons, it is preferable to test strains as soon as possible after isolation from the patient or else to preserve the cultures either frozen or in the dried state (13). Many so called "carrier strains" also seem to produce only small amounts of M substance, possibly due to degradation of the streptococci by their host. For such strains, the M-precipitin reaction is less satisfactory than it is for known pathogenic strains.

This method of type classification does not furnish any information as to the T antigens of group A streptococci, but is solely dependent upon the M antigen-antibody reaction. The reliability of the method has been established in a large series of parallel tests in which the new method has been compared with the one previously in use (11). The errors are apparently the same in both. It has also been useful in studies analysing the antigenic components of group A hemolytic streptococci. The details of these studies will be subsequently reported.

#### SUMMARY

A method is described for performing type-specific anti-M precipitin tests on group A hemolytic streptococci in 1 mm. capillary pipettes. These tests require so much less precipitating serum than was formerly used that the method now seems to be practical.

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