The use of fluorescent-antibody and cultural-precipitin grouping procedures for identifying Group A streptococci from throat swabs was evaluated with paired throat swabs. The sensitivity of the fluorescent-antibody technic was equivalent to or greater than that of cultural-precipitin technics. The importance of a number of conditions and factors for successful use of fluorescent-antibody tests on a routine basis is emphasized.

FLUORESCENT-ANTIBODY IDENTIFICATION OF GROUP A STREPTOCOCCI FROM THROAT SWABS

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CONVENTIONAL cultural and serologic methods for detecting Group A streptococci and identifying the streptococcal group require at least 48 hours. An earlier laboratory diagnosis may contribute to the proper management of Group A streptococcal infections and, thus, help prevent rheumatic fever and other sequelae. A fluorescent-antibody method which may make this possible was described in 1958.[1] In the present investigation, the feasibility of using this method to detect streptococci from throat swabs was evaluated. The results of comparing conventional culture-precipitin grouping tests with fluorescent-antibody tests on throat swab specimens suggest that the fluorescent-antibody method is as specific and perhaps more sensitive than conventional methods.

Materials and Methods

General methods utilized for the preparation of reagents and for performing fluorescent-antibody tests to identify streptococci were given previously.[1] However, the methods used in the present investigation include slight modifications and are given in detail (see Addendum) inasmuch as they now have been evaluated in several other laboratories and are being considered as "recommended procedures."

Patients Examined

The study was done in Chicago in 1958 and 1959 during seasons of high streptococcal incidence. Patients examined in 1958 were at the following locations:

1. Northwestern University Medical School Rheumatic Fever Clinic: Adults and children reporting for routine follow-up examination or with intercurrent respiratory illnesses.
2. Children's Memorial Hospital Upper Respiratory Disease Clinic: Children either with sore throats or those examined periodically following Group A streptococcal infections.
3. Michael Reese Hospital Upper Respiratory Diseases Clinic: Children with sore throats.
4. Great Lakes Naval Training Center, Great Lakes, Ill.: Young male naval recruits reporting to sick call with sore throats.

In 1959 only patients at the Children's Memorial Hospital were examined.

Table 1—Effect of Broth Enrichment on Identification of Group A Streptococci by Fluorescent-Antibody and Culture-Precipitin Grouping Tests

Kind of Swab	Methods Compared	Number Examined	Per cent Postive	
			Direct	Broth
Cotton	FA: Direct vs. two-hour broth	63	3	46
	Conventional: Direct pour streak vs. two-hour broth pour streak	63	18	33
Dacron	FA: Direct vs. two-hour broth	163	11	37
	Conventional: Direct pour streak vs. two-hour broth pour streak	164	24	41

Collection of Throat Swabs and Identification of Group A Streptococci by Culture-Precipitin and Fluorescent-Antibody Grouping Technics

Physicians were instructed to roll duplicate cotton or dacron swabs simultaneously over identical pharyngeal areas. One swab was selected randomly for culturing and identifying Group A streptococci in the routine bacteriology laboratory by culture-precipitin grouping technics. Blood agar base (Difco) plates containing 5 per cent sheep blood were streaked directly and incubated 24 to 48 hours at 37° C (Direct Streak Plate). In addition, throat swabs from 92 of the 210 patients examined in 1958 and all of the patients in 1959 were cultured as follows: Poured-streaked plates were inoculated from broth rinsings of the swab immediately after collection (Direct Pour-Streak Plate) and from broth rinsings in which the swab had incubated two hours at 37° C (Two-Hour Pour Streak Plate). In all cases extracts were prepared from pure cultures of beta hemolytic streptococci and precipitin tests performed with antiserum for Groups A, B, C, D, F, and G streptococci.

Fluorescent-antibody tests were performed with the remaining swab by using (1) Direct Smears and (2) Two-Hour Broth Smears (see Addendum). Positive tests for Group A streptococci were recorded only if brilliantly fluorescent cocci in several chains consisting of at least three to five cocci were observed in the smear stained with Group A streptococcus conjugate and not in the control smear. Smears made immediately after collection of the throat swab often contained fluorescent aggregates of mucus and bacteria but were not called positive unless actual fluorescent cocci could be detected as described above. In smears made from broth cultures, positive specimens contained numerous fluorescent streptococcal chains which were differentiated readily from the massive numbers of bacterial contaminants. In general, not more than two or three minutes were required to examine

and record results for an average smear. All broth rinsings were saved in the refrigerator for retesting of specimens on which discrepant results from cultural and fluorescent-antibody tests were obtained.

Effect of Broth Enrichment upon Identification of Group A Streptococci

Decidedly more Group A streptococcus-positive specimens were detected from two-hour broth cultures than directly from the swab, regardless of the kind of swab used or test performed (Table 1). Direct smears for fluorescent-antibody tests were considered unsatisfactory, in general, because so few of the specimens were positive. It was also noted that positive fluorescent-antibody reactions on direct smears were not confirmed consistently in broth smears. Broth smears regularly contained fluorescent chains which were easily located, and the test was interpreted readily. Although more positive specimens were detected in direct tests by culture than by fluorescent-antibody tests, plating of broth was significantly advantageous for culturing. The data would seem to indicate that either cotton or dacron swabs offered advantages in certain cases, but, for reasons to be discussed later, these apparent advantages may be explained by certain uncontrolled factors.

Fluorescent-Antibody Versus Cultural-Precipitin Tests on Paired Throat Swabs

Of 496 paired swabs tested immediately after collection, a total of 129 positive reactions for Group A streptococci were obtained by cultural-precipitin and fluorescent-antibody methods. Fourteen of these pairs were positive by both methods (Table 2). Thirty-three were positive by fluorescent-antibody tests only, and 82 by cultural-precipitin tests only. More positive fluorescent-antibody than conventional tests were demonstrated among specimens from two sources (Northwestern and Great Lakes).

Of 313 paired swabs which were incubated in broth two to three hours and tested, 83 were positive by both fluorescent-antibody and cultural-precipitin

Table 2—Identification of Group A Streptococci from Swabs Immediately after Collection

Source of Patient and Cultural Method*	Results Categories of Specimens†			
	FA + Conv +	FA − Conv −	FA + Conv −	FA − Conv +
Michael Reese (1958) CS-DS	0	20	2	3
Northwestern (1958) CS-DS	0	33	5	0
Great Lakes (1958) CS-DS	0	55	13	9
Children's Memorial (1958) CS-DS	3	48	2	13
Children's Memorial (1959) CS-DS	0	45	2	16
Children's Memorial (1959) CS-PS	0	49	2	12
Children's Memorial (1959) DS-PS	11	117	7	29
Totals	14	367	33	82

* CS-DS: Cotton swab—direct streak plate conventional method.
CS-PS: Cotton swab—pour streak plate conventional method.
DS-PS: Dacron swab—pour streak plate conventional method.
† Number of paired throat swabs from which results of fluorescent-antibody (FA) and conventional (Conv) tests fell into the categories indicated.

Table 3—Identification of Group A Streptococci after Incubation of Swabs in Broth

Source of Patient and Cultural Method*		Results Categories of Specimens†			
		FA + Conv +	FA − Conv −	FA + Conv −	FA − Conv +
Michael Reese	(1958) CS-PS	1	18	5	1
Northwestern	(1958) CS-PS	0	12	1	0
Great Lakes	(1958) CS-PS	1	6	4	1
Children's Memorial	(1958) CS-PS	14	13	5	4
Children's Memorial	(1959) CS-PS	18	31	11	3
Children's Memorial	(1959) DS-PS	49	86	11	18
	Totals	83	166	37	27

* CS-PS: Cotton swab—pour streak plate conventional method.
DS-PS: Dacron swab—pour streak plate conventional method.
† Number of paired throat swabs from which results of fluorescent-antibody (FA) and conventional (Conv) tests fell into the categories indicated.

methods, and 166 were negative by both (Table 3). That is, in 249 (80 per cent) of 313 specimens examined, results from both methods were in agreement. Thirty-seven pairs of swabs were positive by fluorescent-antibody tests and negative by cultural-precipitin tests. Conversely, in 27 other pairs cultural-precipitin tests were positive and fluorescent-antibody tests negative. Of the 147 positive tests demonstrated by both methods, 120 (81 per cent) were positive by fluorescent-antibody and 110 (74 per cent) by cultural-precipitin tests.

Immediate Cultural-Precipitin Versus Delayed Fluorescent-Antibody Tests

Data obtained in this study make it possible to compare results of what may be considered commonly used cultural-precipitin grouping technics (immediate culturing of throat swab followed by grouping of cultures of beta hemolytic streptococci) with grouping from young (two-hour) broth cultures by fluorescent-antibody tests. Of 482 paired swabs examined, 172 (88 per cent) of the broth cultures gave positive fluorescent-antibody reactions, and 75 (38 per cent) were positive in conventional tests initiated immediately after collecting the throat swab. Fifty-two pairs of swabs were positive by both methods, and 287 negative by both. In all groups of specimens there were more positive fluorescent-antibody than cultural-precipitin tests.

Specificity of Fluorescent-Antibody and Cultural-Precipitin Tests

Divergent results obtained with certain paired swabs tested by fluorescent-antibody and cultural-precipitin tests, described in the foregoing, indicated a need for further examination of the specimens involved. Remaining saline suspensions from all swab washings were tightly stoppered and stored at 0° C to 5° C after sampling for the initial testing of specimens collected in 1959. After results of all initial tests were obtained and recorded, 31 specimens which gave positive fluorescent-antibody but negative cultural-precipitin tests were re-examined intensively by cultural-precipitin methods. Group A streptococci were isolated from 14 of the 31 specimens (47 per cent). Cultural failure in the remaining 17 specimens probably resulted during the storage period in saline for ap-

proximately four weeks. Conversely, 62 specimens which gave positive cultural-precipitin but negative fluorescent-antibody reactions were re-examined by staining additional smears with fluorescent-antibody. In all 62 cases Group A streptococci were demonstrated in a single additional smear. Control smears were negative.

In the course of the investigation, 156 cultures of beta hemolytic streptococci were isolated in pure culture and found to be Group A by precipitin tests. All were brilliantly stained with Group A streptococcus fluorescent-antibody absorbed with Group C cells and not by normal rabbit globulin conjugate absorbed with Group A cells. In addition, three Group C and four Group G strains were isolated and failed to stain with Group A fluorescent-antibody. Most specimens contained alpha streptococci; 17 strains were tested with fluorescent-antibody and none reacted. Similarly, two strains of gamma streptococci were tested and failed to stain.

Discussion

The results obtained by cultural-precipitin grouping tests for identifying Group A streptococci from throat swabs were compared with those obtained by fluorescent-antibody tests. Although cultural-precipitin tests cannot be considered 100 per cent accurate, they were accepted as standard procedures for the purpose of this study. It will be assumed that the technical skills employed were at least average. One of the most important observations in the study was that the holding of swabs in broth for two hours at 37° C resulted in considerably more positive cultural-precipitin and fluorescent-antibody tests for Group A streptococci than did testing immediately after collection of the swabs. Two reasons for these results may be that (1) multiplication of organisms occurred, or (2) mechanical or chemical inhibitors of growth or staining were removed by dilution in broth. The advantage gained by enrichment of throat swabs in broth was greater for fluorescent-antibody than for cultural tests.

Dacron swabs were used in one phase of the study based upon the assumption that a better recovery of organisms would be possible with a fiber less absorptive than cotton.[2] The results did not bear this out consistently. The fact that dacron swabs were much smaller than the cotton swabs used may have been a factor which affected the number of positive tests by culture or fluorescent-antibody methods. The qualities of swab fiber undoubtedly affect the ease with which organisms detach from the swab.

Since two-hour broth cultures gave considerably more positive cultural-precipitin and fluorescent-antibody tests, a better comparison of the true sensitivity of the two technics was possible. In all groups of patients more positive fluorescent-antibody tests than cultural-precipitin tests resulted. Eighty-one per cent of all positives were detected by fluorescent-antibody, while 74 per cent were positive from cultural isolations. The results of both tests were in agreement in 80 per cent of the specimens. It seems likely that the group represented by the 20 per cent disagreement reflects inherent deficiencies of either test. Regardless of the comparisons made in this study, there usually were some paired swabs in which one swab was positive and the other negative. There were some indications that disagreement of results from duplicate swabs occurred more often in specimens from which only a few beta hemolytic streptococci were cultured on plates. Yarashus and Siegel[3] have demonstrated that a closer correlation between cultural and fluorescent-antibody tests occurs when many colonies of Group A streptococci are found on the plate than when few are isolated. In another sense, the greater sensitivity of fluorescent-antibody tests over conven-

tional tests is based upon the fact that clear-cut specific fluorescence reactions are possible on smears containing massive bacterial contamination. Pure cultures of beta hemolytic streptococci are required for reliable results in grouping by precipitin tests.

The fluorescent-antibody tests appeared to be specific for Group A streptococci. It was possible to demonstrate positive reactions on all subcultures of Group A streptococci as well as on all broth sediments containing these organisms. By staining one additional smear of stored broth sediment in those cases originally called fluorescent-antibody negative and culturally positive, it was possible to demonstrate fluorescent chains of streptococci. Confirmatory culturing of those saline-washed broth sediments that gave positive fluorescence reactions originally was delayed; Group A streptococci could not be isolated from all, although several did reveal positive cultures. It is most likely that viability was reduced during the storage period in saline. None of the Group A strains stained with normal rabbit globulin conjugate absorbed with Group A cells.

Cross-reacting strains of Groups C and G streptococci were not encountered, although such strains have been found in other clinical studies.[4,5] Usually such cross-reactions are of lower intensity than specific Group A reactions but may pose a problem unless adequate control strains of Groups C and G are included with each series of tests. Because of the close serological relationships existing among strains of A, C, and G, complete removal of cross-reactions by absorption is not always possible. Therefore, a dilution of absorbed conjugate which no longer reacts with these unusual C and G strains, but one which stains representative Group A strains well, is recommended. Redys, et al.,[4] suggested that a combination of an inhibition test with Group C antiserum and Group A conjugate absorbed with Group C provided a satisfactory means of ruling out Groups C and G cross-reactions.

Wolfe and Cameron[6] observed occasional strains of Staphylococcus aureus in stained smears made from Trypticase Soy Agar slant throat cultures mailed to the state laboratory. Our conjugates were tested with approximately 90 strains of S. aureus. All became well stained with fairly high dilutions of both Group A and normal rabbit conjugates. Additional experiments indicate that conjugates prepared from globulin of a large number of nonimmunized rabbits consistently stain S. aureus strains.[13] The problem of removing cross-reacting antibody without affecting the Group A reaction has not been solved. Absorptive qualities of S. aureus strains are considerably variable. Thus, in reading the test, brilliantly fluorescing cocci should be observed in the smear stained with Group A conjugate but not in the control. If S. aureus is present, fluorescent cocci not in chains should be observed in both smears.

Warfield, et al.,[7] appear to have demonstrated Group A streptococci more successfully by direct staining of throat swab smears than by staining smears from incubated broth. Their conjugate was absorbed with Group C streptococci. It is not clear whether a control conjugate was used with each specimen to rule out the presence of S. aureus. Specimens which contained unchained fluorescent Group A streptococci would be particularly difficult to evaluate without the additional control conjugate which has been absorbed with Group A cells. In our experience, absorption of the Group A conjugate with Group C streptococci does not affect the reaction of the conjugate with S. aureus (over 80 strains). The absorbed conjugate prepared by Warfield, et al.,[7] did fail to stain two strains of S. aureus. Redys, et al.,[4] employed Streptosel broth for enrichment of throat swabs since it seemed to eliminate problems with S. aureus.

However, extreme caution in the preparation of the broth was indicated in order that growth of Group A streptococci would not be inhibited. Since hemolytic S. aureus is demonstrated in throat swab cultures and because of certain disadvantages of using inhibitory media, we feel at the present time the fluorescent-antibody test should be so controlled that S. aureus can be differentiated definitely from Group A streptococci in every specimen tested. A point not to be overlooked is the possibility that some fluorescent-antibody reactions could arise from nonbeta hemolytic Group A streptococci which would not be detected ordinarily in the search for beta hemolytic colonies. Such organisms have been encountered in nature, but their frequency is not known.

The procedures described here (see Addendum) were recommended as reliable for routine use in state public health laboratories approximately four years ago with the following qualifications:

1. That bacteriologists who plan to use the technic obtain thorough training in the performance of both culture-precipitin and fluorescent-antibody grouping technics.
2. That well-standardized reagents and reliable equipment be used according to tested procedures.
3. That the use of culture-precipitin grouping methods be continued at least until competency in the use of the fluorescent-antibody tests becomes well established, and that after this period representative isolants be grouped by precipitin tests as a control on new or unusual situations.

Bacteriologists from 63 state, territorial, and major metropolitan public health laboratories have received such training, equipment, and reagents.

Results received from 51 of the laboratories which tested 27,966 paired throat swabs by culture-precipitin and fluorescent-antibody methods were in agreement in approximately 95 per cent of the specimens. Of 6,223 pairs that were positive by either method, 5,574 (89 per cent) were positive by fluorescent-antibody and 5,408 (87 per cent) by culture-precipitin tests. All comparisons were made following incubation of the swabs in broth for approximately two hours. Despite the variable conditions existing in different laboratories, the correlation and sensitivity is high. In some cases it becomes necessary to modify the over-all procedure in order to accomplish the laboratory workload more efficiently.

For example, substantial testing indicates that Group A streptococci can be identified as to group by testing smears of colonies, or more satisfactorily, smears of young broth subcultures of colonies, with fluorescent-antibody.[12] Thus, the presence of Group A streptococci can be confirmed within 24 hours after the swab is collected. Modifications such as this should be evaluated by the individual laboratories involved.

Summary

The use of fluorescent-antibody and cultural-precipitin grouping procedures for identifying Group A streptococci from throat swabs was evaluated with paired throat swabs. Significantly more positive specimens were obtained by both methods when swabs were incubated in broth for two hours, rather than being tested immediately after collection. The sensitivity of the fluorescent-antibody technic was equivalent to or greater than that of cultural-precipitin technics. Furthermore, reliable reactions were possible with highly contaminated specimens and with those which failed to yield positive cultures. Under the conditions described, the fluorescent-antibody test was specific for Group A streptococci. The importance of using trained personnel, standardized reagents, properly controlled tests, and reliable equipment was suggested as necessary for successful use of fluorescent-antibody tests on a routine basis.

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REFERENCES

1. Moody, M. D.; Ellis, E. C.; and Updyke, E. L. Staining Bacterial Smears with Fluorescent Antibody. IV. Grouping Streptococci with Fluorescent Antibody. J. Bact. 75:553–560, 1958.
2. Hollinger, N. F., and Lindberg, L. G. Delayed Recovery of Streptococci from Throat Swabs. A.J.P.H. 48:1162–1169, 1958.
3. Yarashus, D., and Siegel, A. Unpublished results, 1961.
4. Redys, J. J.; Ross, M. R.; and Borman, E. K. Inhibition of Common-Antigen Fluorescence in Grouping Streptococci by the Fluorescent Antibody Method. J. Bact. 80:823–829, 1960.
5. Updyke, E. L. Unpublished material, 1961.
6. Wolfe, M. D., and Cameron, G. M. Fluorescent Antibody Technique versus Cultural Methods as a Routine Procedure for Finding Beta-Hemolytic Streptococci in Throat Cultures. Pub. Health Lab. 17:76–82, 1959.
7. Warfield, M. A.; Page, R. H.; Zuelzer, W. W.; and Stulberg, C. S. Immunofluorescence in Diagnostic Bacteriology. II. Identification of Group A Streptococci in Throat Smears. A.M.A. Am. J. Dis. Child. 101:160–163, 1961.
8. Lancefield, R. C. Micro-Precipitin-Technique for Classifying Streptococci. Proc. Soc. Exper. Biol. & Med. 38:473–478, 1938.
9. Gornall, A. G.; Bardawil, C. J.; and David, M. M. Determination of Serum Proteins by Means of the Biuret Method. J. Biol. Chem. 177:751–766, 1949.
10. Riggs, J. L.; Seiwald, R. J.; Burckhalter, J.; Downs, C. M.; and Metcalf, T. G. Isothiocyanate Compounds as Fluorescent Labelling Agents for Immune Serum. Am. J. Path. 34:1081–1097, 1958.
11. Marshall, J. D.; Eveland, W. C.; and Smith, C. W. Superiority of Fluorescein Isothiocyanate (Riggs) for Fluorescent Antibody Technique with a Modification of Its Application. Proc. Soc. Exper. Biol. & Med. 98:898–900, 1958.
12. Moody, M. D. Unpublished material, 1961.
13. Pittman, B., and Moody, M. D. "Staining Staphylococcus aureus with Fluorescein-Labeled Globulin from Nonimmunized and Streptococcus-Immunized Animals." Bacteriological Proc. Detroit, Mich.: American Society for Microbiology, 1960, p. 140.

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ADDENDUM

I. Preparation of Serums

A. Group A streptococcus antiserum

1. Prepare antiserum in rabbits by injecting a formalin-killed whole cell antigen of Group A streptococcus, preferably from a strain in which "M" protein can no longer be demonstrated.
2. Prepare a pool of antiserums which react strongly only with Group A streptococci in precipitin tests.[8]

B. Normal rabbit serums

Using nonimmunized rabbits, prepare a pool of serums which fail to give precipitin reactions with streptococcal Groups A, B, C, D, F, and G.

II. Preparation of Globulin

A. Mix equal parts of serum and saturated ammonium sulfate and allow to stand overnight at 0° C to 5° C.

B. Centrifuge and decant supernatant fluid.

C. Resuspend precipitate in cold half-saturated ammonium sulfate, centrifuge, discard supernatant fluid.

D. Repeat step C

E. Dissolve the final precipitate in distilled water and adjust to the volume of the original serum.

F. Remove ammonium sulfate by dialysis against 0.85 per cent sodium chloride at 0° C to 5° C.

G. Determine protein content by a biuret test[9] using a standard protein solution of bovine albumin (Armour Laboratories) for establishing a standard curve. If the protein content is at least 1.0-g per cent, proceed with labeling, or if less than 1-g per cent, concentrate at room temperature by suspending the globulin in dialysis tubing in front of a fan.

III. Labeling Globulin with Fluorescein Isothiocyanate

A. Method (modification of Riggs, et al., 1958[10])

1. Place globulin in Erlenmeyer flask and, while stirring, add an equal volume of 0.85 per cent NaCl.
2. Add carbonate-bicarbonate buffer (0.05 M concentration, pH 9.0) using a volume equal to 15 per cent of the volume of globulin-saline mixture.
3. Add acetone (Reagent grade) dropwise using a volume equal to 12 per cent of the volume of globulin-saline mixture.
4. Check the pH rapidly. If under pH 9.0, add a few drops of pH 11.0 carbonate buffer.
5. Suspend fluorescein isothiocyanate (0.05 mg per mg protein) in 1.5-ml acetone and add slurry to buffered globulin.
6. Stopper flask with rubber stopper and place on slowly rotating shaker overnight at 0°C to 5° C.
7. Sorb conjugate with Dowex 2— x 4 (Dow Chemical Company) by mixing equal volumes of conjugate and Dowex and incubating two hours at 37° C or overnight at 0° C to 5° C.
8. Collect sorbed conjugate by pipetting through glass wool.
9. Dialyze at 0° C to 5° C against 0.85 per cent sodium chloride buffered at pH 7.2 with 0.01 M phosphate buffer for approximately 48 hours or until unreacted fluorescein ceases to appear in the dialysate.

B. Method 2 (modification of Marshall, et al., 1958[11])

Method 2 is similar to Method 1 except that dry fluorescein isothiocyanate is added directly to the globulin-saline-buffer mixture. Extreme care is required to bring all particles of dye into solution at the beginning of the reaction period.

IV. Testing of Conjugates Before Absorption

Determine staining titers by testing several strains of Groups A, C, and G streptococci and Staphylococcus aureus using varying dilutions of conjugate as follows:

1. Make smears from cells grown two to five hours in Todd-Hewitt Broth and washed in 0.85 per cent NaCl. (Smears may be prepared and frozen until ready for testing conjugates.)
2. Fix smears gently with heat or with ethanol (95 per cent or absolute for one minute).
3. Stain 15 to 30 minutes at room temperature in a moist atmosphere.
4. Rinse ten minutes in buffered saline (pH 7.2).
5. Blot, mount with glycerol-saline and a coverslip, and examine on the fluorescence microscope.

V. Absorption of Conjugates with Streptococci

A. Grow large quantities of Group C and Group A streptococci in an enriched Todd-Hewitt Broth prepared as follows: Prepare two liters of enrichment solution by dissolving in distilled water 120-g dextrose, 80-g sodium bicarbonate, 32-g sodium phosphate dibasic, and 80-g sodium chloride; sterilize by filtration. Use 750 ml of sterile enrichment solution for each 15 liters of Todd-Hewitt Broth (Difco).

B. Add formalin (0.25 per cent final concentration) and refrigerate 48 hours.

C. Collect cells by centrifugation and wash packed cells three times with phosphate buffered saline (pH 7.2).

D. Mix two parts labeled globulin with one part of packed cells (Group A conjugate with Group C cells and normal rabbit conjugate with Group A cells). Allow to stand at 37° C for two hours or at 0° C to 5° C overnight with occasional stirring.

E. Centrifuge and collect absorbed conjugates. More complete removal of cells used for absorption may be obtained by filtration.

F. If necessary, repeat the absorption if a greater level of specificity is desired. Repeated absorptions may be expected to reduce homologous staining titers, however.

G. Check pH. Adjust to approximately 7.2-7.4 with phosphate buffer.

VI. Testing Absorbed Conjugates

A. Determine staining titers as indicated in step IV.

B. Dilutions of Group A conjugate selected for routine use should stain representative strains of Group A streptococci at a 3-4+ level of brilliancy. At this dilution occasional strains of Group C and G streptococci may stain at moderate levels of brilliancy, and with lower dilutions, more brilliantly. Hence, such possible cross-reactions must be known when the reagents are used. The normal rabbit and Group A conjugates should be used at the same dilutions.

VII. Preservation and Storage of Conjugates

Conjugates may be preserved with either Merthiolate or phenylmercuric borate (1: 10,000), dispensed in small amounts, and stored at 0° C to 5° C, frozen, or lyophilized

and held at room temperature for at least two years without serious deterioration.

VIII. Equipment for Fluorescence Microscopy

For examining the smears, use a good quality monocular microscope fitted with a cardioid darkfield condenser and oil immersion lens. For illumination use an Osram High Pressure HBO-200 Mercury Vapor Lamp. Proper wave lengths of light are obtained with a Schott BG-12 pass filter, 3-mm thickness, and a 2-mm Schott OG-1 barrier filter. Satisfactory photographs may be obtained with Super Anscochrome or Tri XXX film.

IX. Examination of Throat Swabs for Group A Streptococci by Fluorescent-Antibody Tests

A. Place throat swab into 1-ml Todd-Hewitt Broth and incubate two to five hours at 37° C.

B. Drain swab against side of tube, replace in sterile test tube, and store at 0° C to 5° C in case cultural tests are desired.

C. Centrifuge broth three to four minutes to pack sediment. Carefully decant supernatant fluid, resuspend sediment in 1-ml buffered 0.85 per cent NaCl, centrifuge, and discard supernatant fluid.

D. Using a capillary pipette and most of the sediment, prepare duplicate smears on a slide inscribed with two circular areas. Stopper tube with wax-coated cork and refrigerate in case additional smears are needed. Smears may also be prepared from a small saline suspension of beta hemolytic colonies from blood agar plates. If time permits, a young broth subculture of such colonies is desirable, in which case the broth would be handled as in step C.

E. Allow smears to air-dry and fix one minute in 95 per cent or absolute ethanol. Rinse off in buffered saline, pH 7.2, and blot gently.

F. Stain one smear each with appropriate dilutions of absorbed Group A streptococcus and normal rabbit conjugates for 15 to 30 minutes under a petri dish lid containing moist filter paper. If the fluorescence-inhibition test is used, each of the reagents is mixed with an equal volume of an appropriate dilution of Group C streptococcus precipitin grouping antiserum and the mixtures are used to stain smears.

Equally effective inhibition of cross-reactions, however, of the Group A and normal rabbit conjugates for Group C and G streptococci and S. aureus can be accomplished by using mixtures of dilutions of either conjugate and a dilution of serum from most normal rabbits (unpublished material).

G. Rinse ten minutes in buffered saline, then momentarily in distilled water. Blot gently.

H. Add a drop of glycerol-saline and a coverslip.

I. Examine on the fluorescence microscope with oil immersion. Not more than three minutes should be required to read a smear. Note the presence of fluorescent cocci, whether or not they are in chains, and the intensity of fluorescence.