Fluorescent-Antibody Techniques for the Identification of Group D Streptococci: Direct Staining Method

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Fluorescent-antibody (FA) techniques were employed in an attempt to develop a rapid test for the identification of group D streptococci. Fresh isolates were obtained from sewage and feces of sheep, cattle, horses, rabbits, chickens, geese, and rats. Identification to species were made by the conventional physiological, biochemical, and serological tests. Both whole and disrupted cells of representative strains of each species were used for the preparation of the group D streptococcus vaccine. Globulin fractions of individual and pooled antisera were labeled with fluorescein isothiocyanate, and the resulting conjugates were tested with homologous and heterologous antigens. The specificity of the conjugates and staining was assessed by adsorption and inhibition tests utilizing controls with homologous and heterologous antigens. Employing the direct staining method and individual and pooled conjugates, it was possible to obtain 84 and 85% positive FA reactions, respectively, with group D streptococcal strains. Trypsinization of the smears prior to staining eliminated all FA cross-reactions observed with non-group D streptococci and staphylococci. These findings suggest that the direct staining method will be of value in the rapid identification of group D streptococci.

The identification of group D streptococci has been inconclusively pursued for the past three decades. Current methodology is based on physiological, biochemical, and serological reactions. The techniques used for these characterizations are long, involved, quite vulnerable to error during laboratory manipulations, and lacking in standardization. Conventional serological methods for identifying group D streptococci by the precipitin test require at least several days. Likewise, the difficulties of producing a potent group D antiserum for serological tests as well as demonstrating the group antigen have been reported (11, 21-23). To compound the problem, commercially produced group D antisera quite often demonstrate extremely low levels of group-specific antibody (24).

The intracellular location of the group D antigen offers an explanation for these obstacles as well as for the refractory nature of most group D streptococcal strains to the serological demonstration of the group antigen (3, 8, 24). Shatlock (23) surmised that disruption of the cell affords liberation of the antigen and thus indirectly contributes to its antigenicity. This was confirmed by her observation that "group D antisera are more readily prepared in rabbits by using as a vaccine disintegrated rather than whole organisms."

The possible involvement of nutritional conditions affecting the synthesis of the group antigen has been suggested, since media which support excellent growth of the organisms do not always produce cells with substantial quantities of antigen (8). Medrek and Barnes (14) demonstrated that greater yields of the antigen were obtained when 0.5 to 1.0% glucose was incorporated into the medium.

The extensive literature on immunofluorescence illustrates the significance of fluorescent-antibody (FA) techniques in diagnostic microbiology, serology, and histology. Employing these FA techniques, it was suggested that rapid identification of group D strepto-
cocci could be made even though attempts for the past 13 years have been disappointing (2, 15, 18–20).

The purpose of this study was to develop an FA system for the rapid and economical identification of group D streptococci employing the direct staining method.

**MATERIALS AND METHODS**

**Organisms.** Two hundred and fifteen group D streptococcal strains were employed for performing the precipitin test. These included 31 stock cultures [9 obtained from ATCC and 22 from the Streptococcus Laboratory, Clinical Bacteriological Unit, Center for Disease Control (CDC) Atlanta, Ga.] and 184 freshly isolated strains from sewage and feces of sheep, cattle, horses, rabbits, chickens, geese, and rats. From the 215 group D streptococcal strains, 120 were tested for FA reaction. Also examined were 36 stock cultures of streptococci of groups A, B, C, F, G, K, L, N, Q, and staphylococci (obtained from CDC and the State Department of Health, Hartford, Conn.). Ten freshly isolated streptococcal strains of groups A, C, and G were provided by the Streptococcus Laboratory, Department of Microbiology, University of Massachusetts, Amherst. One Escherichia coli and 13 salmonella cultures of groups B, C, D, E, E, E, G, I, K, L, and O obtained from the U.S. Environmental Protection Agency, Office of Water Program, Edison, N.J., were also examined.

**Vaccine preparation.** Both whole Formalin-killed and disrupted cells of three freshly isolated strains (Streptococcus faecalis 28, S. liquefaciens 961, and S. faecium 1064) and four stock cultures (S. zymogenes D-76, S. zymogenes 12958, S. durans 6036, and S. bouis 9806) were utilized for immunization.

Formalin-killed whole cell antigen was prepared by the Lancefield method (11) modified by the substitution of Todd-Hewitt broth with glucose-Lemco broth. The strains used for the preparation of the cell-disrupted vaccines were grown in Todd Hewitt broth containing 1% glucose. After incubation for 24 hr at 37 C, the cultures were centrifuged at 1,000 rev/min for 1.5 hr. The cell sediment was washed twice in buffered saline and treated with 1.2% formaldehyde solution for 18 hr at 5 C. The formalized suspension of 36 ml to which 30 g of sterile glass beads was added was disrupted in a Bronwill cell-disintegrator (B. Braun Melsungen Apparatbau, type 286) for 8 min. The disrupted cell solution was filtered through no. 41 Whatman filter paper. A final dilution of 1:20 from both whole and disrupted cell antigen was used for injections.

**Antisera.** Strains of each of the six species of group D streptococci were injected intravenously into two to three rabbits (total of 15 rabbits). Amounts of 0.5 ml of vaccine were injected for 3 successive days, and 1.0-ml amounts were injected for 9 successive days (omitting weekends) with a 5-day rest period. The rabbits were bled, and antisera with titers of 1:640 or higher (estimated by slide agglutination and precipitin tests) were used for precipitin tests and for preparation of the conjugates. Where necessary, one or two additional 5-day courses of injections were provided.

Both individual and pooled antisera were tested; the rationale for pooling was to increase the number of reactions which could be identified by the test antisera. In Table 1 are listed the individual antisera used for preparation of pooled antisera.

**Precipitin test.** Hot hydrochloric acid extracts (10) were prepared from the organisms grown on Todd-Hewitt and glucose-Lemco broths. Precipitin ring tests were used for group identification. The tubes were examined immediately and after 10 min at room temperature. Commercial and individual and pooled laboratory-prepared group D antisera were tested.

**Preparation of globulin.** Globulin fractions of individual and pooled antisera were prepared by the method described by Cherry (1) with 70% saturated ammonium sulfate. A Sephadex G-25 fine gel column or dialysis against 0.85% NaCl solution was used to remove the ammonium sulfate from salt-fractionated serum globulins.

Total protein concentrations were determined by the biuret method with optical density readings at 540 nm (Beckman DB-G spectrophotometer).

**Conjugation of globulin fractions.** Globulin fractions were labeled with fluorescein isothiocyanate (FITC) by the method of McKinney as described by Cherry (1).

Unreacted fluorescent material was removed from the conjugate by dialysis against pH 7.6 phosphate-buffered saline (PBS) at 5 C for several days. All traces of precipitate were removed by centrifugation.

Merthiolate, as a preservative, was added to the conjugate to a final concentration of 1:10,000. The conjugate was refrigerated at 5 C for immediate use and frozen at −60 C in 1-ml samples for storage. The bound fluorescein-to-protein (F/P) ratio of the conjugate was determined by Hebert and Pittman at the CDC, as described by Hebert et al. (7).

Labeled individual globulins and pooled conjugate were tested. The pooled conjugate consisted of individual conjugates prepared from antisera of rabbits 5 and 6 (S. faecalis no. 28), 3 and 17 (S. liquefaciens no. 961), 8 (S. zymogenes no. 12958), 10 (S. zymogenes D-76), 1 and 2 (S. faecium no. 1064), 13 (S. durans no. 6036), and 7 and 15 (S. bouis no. 9806). Two control conjugates (labeled globulin from normal rabbit serum which failed to react in precipitin reactions with streptococcal antigens of group D) were also examined.

**Preparation of smears.** Streptococcal D cultures were grown at 37 C in glucose-Lemco broth for 6 hr with the exception of S. bouis which required 18 hr of incubation. Sediments from these cultures were resuspended in PBS, and their turbidity was adjusted to that of a McFarland no. 1 standard. The streptococci of groups A, B, C, F, G, K, L, N, and Q; staphylococci; E. coli; and salmonellae were similarly adjusted to a McFarland standard no. 4. Smears were then made, allowed to air-dry, and fixed either in heat or with 95% ethanol for 2 min.
Table 1. Individual antisera used for the preparation of laboratory-prepared pooled D antiserum

| Vaccine             | Species            | Strain no. | Rabbit no. | Agglutination titer       |
|---------------------|--------------------|------------|------------|---------------------------|
| D antiserum         | Streptococcus faecalis | 1, 2       | 1:5120     | 1:1280                    |
|                     | S. liquefaciens    | 6, 10      | 1:640      | 1:2560                    |
|                     | S. zymogenes       | 7, 10      | 1:1280     | 1:1280                    |
|                     | S. faecium         | 12, 14     | 1:2560     | 1:20480                   |
|                     | S. durans          | 12, 14     | 1:2560     | 1:20480                   |
|                     | S. bovis           | 7          | 1:1280     |                           |

Direct fluorescent-antibody staining. The direct staining technique consisted of covering the smears with immune conjugate and allowing them to stand 30 min at room temperature in a moist atmosphere. The smears were washed as described by Thomason and Wells (26), mounted under a cover slip with pH 7.6 buffered glycerol-saline, and examined by fluorescent microscopy. (At the present time the pH of the mounting medium is adjusted to 8.6 to 9).

Determination of staining titer of the conjugate for the direct method. Smears from selected group D streptococcal strains were stained with 1:10 to 1:2,560 dilutions of conjugate prepared in PBS. These were examined, and the highest staining intensity of the homologous strain with 4+ fluorescence intensity was determined as the staining titer of the conjugate. The appropriate dilution of the conjugate for routine use, the working dilution, was arbitrarily chosen to be twice the concentration of the conjugate in the staining titer (e.g., if the staining titer was found to be 1:80, a 1:40 working dilution was used). The staining titer and the working dilution were determined for each lot of conjugate prior to use. The control conjugate was used at the same dilution as the test reagent.

Establishing specificity of staining by the direct method. Controls with homologous antigen consisted of unstained smears and those stained with labeled normal rabbit globulin. Adsorption of conjugates with cells of each of the six species of group D streptococci was performed. Strains used for adsorption were grown for 18 hr at 37°C in glucose-Lemco broth, centrifuged, and washed three times in PBS. Conjugates in their working dilution were adsorbed with packed cells in a 2:1 ratio for 2 hr at 37°C. The adsorbed conjugate was separated from the cells by centrifugation and was used for staining. Inhibition tests were performed by employing the one-step procedure (5, 16). Labeled and unlabeled antisera were titered to determine optimal concentration for the reaction. Smears were stained in the usual manner with a mixture of equal parts of conjugate and each of the unlabeled antisera, prepared against the group D streptococcal species.

Controls with heterologous antigens (non-group D streptococci, staphylococci, E. coli, and salmonellae) consisted of smears stained with labeled normal rabbit globulin, smears stained with group D conjugate, and trypsinized smears stained with D conjugate to which normal rabbit serum was added as described by Moody et al. (17). Adsorption of group D conjugate was performed with streptococcal cells of groups A, C, and G in the manner described above, modified by the substitution of glucose-Lemco broth with Todd-Hewitt Broth. Inhibition tests with group D conjugate and normal rabbit serum were performed as reported for controls with homologous antigen.

Trypsinization. A drop of 0.1% trypsin solution (12, 13) was added to fixed smears and allowed to air-dry prior to staining with the conjugate to which normal rabbit serum had been added in an amount equal to 0.1 of the volume of the working dilution of the conjugate used.

The direct staining method, including trypsinization, required approximately 1 hr to perform.

Fluorescent microscopy and photography. The optical system used for examining the smears consisted of a Leitz-Ortholux microscope fitted with dark-field condenser; 10 × ocular and achromatic oil immersion objective (95 ×; NA 1.0); a lamp housing with 150-w high-pressure xenon bulb, and appropriate ultraviolet filters (red-suppression filter BG 38, blue filter BG 12, and suppression filter K 530). Photomicrographs of fluorescence were originally taken on 35-mm high-speed Ektachrome B color reversal film using exposures of approximately 3 to 6 min.

Fluorescence intensity measurement. Fluorescent-antibody reactions were read visually and recorded as described by Moody et al. (15). Fluorescence intensities of 2+ or greater were considered positive; intensities less than 2+ were considered negative reactions.

RESULTS AND DISCUSSION

Comparison of commercial and laboratory-prepared D antisera. A comparison of precipitin reactions with commercial group D antiserum and that prepared in our laboratory exposed the limitations of commercial anti-serum (Table 2). A higher proportion of negative precipitin reactions for S. faecium, S. durans, and S. bovis was observed with the commercial antiserum than with that prepared in the laboratory. Of 215 strains tested, only 49.8% were positive with commercial D antiserum as opposed to 82.2% with laboratory-prepared antiserum. Most probably the higher
percentage of positive reactions obtained with the laboratory-prepared antiserum resulted from the pooling effect of the high titer sera prepared with the freshly isolated strains in our collection.

**Conjugate characterization.** The bound F/P ratio of the conjugate no. 3 was determined as 30. However, since the high F/P ratio has been reported as a major cause of nonspecific staining, we performed studies to determine the optimum for this system F/P ratio. The results of our preliminary studies showed that an F/P ratio of 20 was the optimum. Serum fractionation with 35% final concentration of (NH₄)₂SO₄, resulted in a reasonably pure globulin preparation. Cellulose acetate strip electrophoresis (CASE) of this conjugate showed that it was 86.8% of gamma globulin. No unreacted fluorescent material was detected when the CASE membrane was examined under a Wood's light prior to staining with Ponceau S. The staining titers of the individual and pooled conjugates employed in this study ranged from 1:20 to 1:320.

**Staining group D streptococci by the direct method.** Since the direct staining method has been reported as the simplest and the most reliable of all the staining procedures (9) and has been employed in previous studies with group D streptococci, it was naturally the first method to be evaluated in this study.

FA reactions from 120 strains of group D streptococci, 12 strains of *S. equinus*, and 6 strains of *S. avium* (Q) obtained with individual and pooled conjugates were studied. It will be noted that most of the individual conjugates (1, 2, 6–8, 10, 12–14, 16) demonstrated type specificity (i.e., the individual conjugate stained only its homologous strain or strains of its homologous species). Conjugates 17 and 11 reacted, to some extent, with all group D species. Group-specific reactions were obtained with conjugate 3 and the pooled conjugate. The best results were achieved with antiserum 3, which had been prepared by disrupted-cell vaccine of a freshly isolated *S. liquefaciens* strain 961.

Summarized in Table 3 are the per cent positive FA reactions by group D streptococci with no. 3 and the pooled conjugates employing the direct staining method. On the basis of 20 strains of each of the six species, it was possible to obtain 84% positive FA reactions with conjugate no. 3, whereas 85% of the strains gave positive FA reactions with the pooled conjugate. Among the individual species, however, *S. faecium*, *S. durans*, and *S. bovis*, which have been reported as difficult to designate serologically, gave encouragingly high rates of positive FA reactions with both the no. 3 and the pooled conjugates. However, we suppose that the considerably higher percentage of positive reactions (90%) obtained with strains of *S. bovis* and the pooled conjugate was due to the presence of the type specific for *S. bovis* conjugate 7, which was included in the pooled conjugate.

Only 7 of the 12 *S. equinus* strains under study were FA-positive. From the six *S. avium* (Q) strains studied, three were FA-positive.

It has been observed by many investigators (18, 19) that the immunological response of different rabbits to antigens may be quite different. This was borne out in our studies whereby significant variations in the FA reactions appeared to be influenced by the test rabbits. It was found that when 60 group D streptococcal strains were tested with the antisera of two different rabbits immunized with the same vaccine and immunization schedule, 93% yielded positive FA reactions with one of the antisera, while 46% were positive with the other.

Table 3. Per cent positive FA reactions by group D streptococci with individual and pooled conjugates employing direct staining method

| Streptococcus species | Conjugate | No. 3 | Pooled |
|-----------------------|-----------|-------|-------|
| *S. faecalis*          |           | 90    | 90    |
| *S. liquefaciens*      |           | 100   | 100   |
| *S. zymogenes*         |           | 75    | 85    |
| *S. faecium*           |           | 85    | 65    |
| *S. durans*            |           | 100   | 80    |
| *S. bovis*             |           | 55    | 90    |

Avg 84 85
the same conjugate, tested on various group A strains, varied within 1 to 3 dilutions). A similar situation was observed during the studies with group D streptococci.

The fluorescence achieved by *S. faecalis* stained with conjugate 3 is shown in Fig. 1. If observed in natural color, it would show the typical morphology of *S. faecalis*, giving brilliant yellow-green fluorescence with the cells sharply outlined.

For establishing specificity of staining with the group D conjugates, negative controls with homologous antigen were utilized. Unstained group D streptococcal cells exhibited a fluorescence intensity of 1+ which was considered as the autofluorescence of the cells. Negative fluorescence observed after staining with labeled normal globulin from the test rabbits assured that the rabbits did not already possess group D streptococcal antibodies. A 1+ fluorescence resulted following adsorption and inhibition procedures which indicated the group D specificity of the conjugate and the validity of the direct staining. As shown in Table 4, the 2+ to 4+ fluorescence (positive), obtained when conjugate 3 was applied to the six species of group D streptococci, were reduced to 1+ (negative) when the conjugate was adsorbed with the cells of the individual species. Inhibition tests were performed with conjugate 3 (titer 1:640) which was diluted 1:10 and the following antisera: no. 3 (*S. liquefaciens* 961; titer 1:1,280); no. 8 (*S. zymogenes* 12958; titer 1:1,280); no. 10 (*S. zymogenes* D-76; titer 1:640); no. 11 (*S. zymogenes* D-76; titer 1:1,280); and no. 12 (*S. zymogenes* 12958; titer 1:640) which were diluted 1:5 and 1:10.

Several reports in the literature indicated that group D streptococci serologically cross-react with other bacteria as staphylococci and streptococci of groups A, C, E, F, G, L, M, N, and P (15, 18). In addition to the bacteria reported, common contaminants in water such as *E. coli* and salmonellae were included in the study. As will be noted from Table 5, FA cross-reactions occurred among staphylococci and streptococci of groups A, C, E, F, and G but not with streptococci of groups B, K, L, N, E. *coli*, and salmonellae of groups B, C, D, E, E, E, G, I, K, L, and O. The existence of the cross-reactivity was not surprising since labeled normal globulin used for the direct staining also reacted with these organisms, giving a wide range of fluorescence from 0 to 4+. Various techniques were utilized to eliminate cross-reactions between these bacteria and group D streptococci. As will be seen in Table 5, when inhibition technique was used, cross-reactions were reduced but not satisfactorily. Adsorption of the conjugate with the

**Fig. 1. Streptococcus faecalis stained with conjugate 3.**
cells of the cross-reacting bacteria initially reduced the fluorescence to 2+. However, it also restricted the fluorescence of the group D streptococci to 1+ and therefore proved to be an unsuitable technique.

Only trypsinization of the smears and addition of unlabeled normal rabbit serum to the conjugate used for staining eliminated interaction with potential cross-reacting strains (Table 5). Heterologous antigens exhibited 0 to 1+ fluorescence, whereas group D streptococcal strains maintained their 4+ fluorescence. Apparently the cross-reactions, observed with staphylococci and groups A, C, F, and G streptococci, appeared to be due, at least in part, to nonspecific proteins on the surface of the cells. Digestion of these proteins by trypsin eliminated the nonspecific fluorescence.

Since the group D antigen is a glycerol-teichoic acid, it remains intact to react with specific streptococcal D conjugate after trypsinization.

The unconjugated group D antisera were also tested for cross-reactions with the heterologous streptococcal A, B, C, F, G, K, L, and N group extracts. No cross-reaction was observed.

It was also observed that freshly isolated streptococcal D strains gave a higher percentage of positive precipitin and FA reactions than did stock cultures obtained from various culture collections. Thus, age and storage conditions must also be taken into account as contributing factors in the application of the FA techniques. Although more work is necessary and is progressing to determine the nature of the common cross-reacting antigen and antibody between group D and other streptococcal groups and staphylococci by agar-gel diffusion and immunoelectrophoresis, the present findings, nevertheless, suggest that the direct staining method described herein is specific, sensitive, rapid, simple, and economical and is applicable for rapid identification of group D streptococci.

### Table 4. Staining group D streptococci with adsorbed group D conjugate 3

| Streptococcus species | Strain no. | FA reaction with conjugate 3 before adsorption | FA reaction with conjugate 3 adsorbed with |
|-----------------------|-----------|-----------------------------------------------|------------------------------------------|
| S. faecalis           | 28        | 2+                                            | S. faecalis 28                           |
| S. liquefaciens       | 961       | 4+                                            | S. liquefaciens 961                       |
| S. zymogenes          | D-76      | 4+                                            | S. zymogenes D-76                         |
| S. faecium            | 29        | 3+                                            | S. faecium 29                             |
| S. durans             | 6056      | 3+                                            | S. durans 6056                            |
| S. bovis             | 90        | 3+                                            | S. bovis 90                               |

### Table 5. FA cross-reactions among various bacteria and group D streptococci with conjugate 3 employing the direct staining method

| Group tested  | No. of strains | Non-trypsinized cells + conjugate | Labeled normal globulin | Inhibition with normal serum + conjugate | Adsorption with A streptococci cells + conjugate | Trypsinized cells + conjugate |
|---------------|----------------|----------------------------------|------------------------|------------------------------------------|-------------------------------------------------|-------------------------------|
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