Identification of Group A Streptococci by Direct Fluorometry

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A simple direct fluorometric method for rapid identification of group A streptococci is described. The method permits the detection of the organism in mixed cultures without the aid of a microscope and is amenable to automated processing of specimens. Experience with the indirect fluorometric method revealed that non-trypsinized cells from a 10-fold dilution of overnight broth cultures could be stained with uniform brilliance with fluorescent antibody (1:15 dilution) and that fluorescent antibody dissociated from such cells at 55°C for 20 min gave serologically specific fluorometric values. With this information, it was possible to develop a simpler fluorometric test which gave results comparable to those obtained by conventional cultural-precipitin grouping techniques. In the direct test described, cultures from throat swabs were incubated overnight, and cells from a 10-fold dilution were stained with specific fluorescent antibody (1:50 dilution) and then rinsed. The stained specimens were transferred to a continuous-filter paper strip (Whatman 3 MM) and read serially in a Turner 110 fluorometer with Corning 5840 and Wratten 2A filters in place. The reagents used required careful standardization and testing to assure that fluorometric readings above a specified value would be indicative of the presence of group A streptococci.

In many laboratories, fluorescent antibody (FA) is used as a screening device in the identification of group A streptococci. Several state health departments are now examining over 200,000 throat swabs a year for group A streptococci by FA. Because of this increasing work load in public health laboratories, the use of automated FA tests in the future seems to be indicated. A specific fluorescent reagent used in a quantitative electronic readout technique could provide accurate, reproducible identification of group A streptococci (by FA) without having to use the human eye for interpretation. An indirect fluorometric method, described by Hochberg et al. (2), presented a possible means for identifying streptococci quantitatively without using a microscope. This method measures the FA dissociated from group A streptococci after staining. The present study was undertaken to define some of the conditions under which the Hochberg indirect fluorometric technique can be performed. The effects of the following conditions on the results of the technique were examined: cell concentration, volume and titer of FA reagent, nonspecific staining (NSS), trypsinization of specimen, and the dissociation of fluorescent reagent from the stained specimen. After defining these conditions, a technique which permits direct fluorometric readings of stained organisms on a paper matrix was developed. Results were obtained which demonstrate the feasibility of automating the latter technique.

MATERIALS AND METHODS

Fluorometric equipment. A Turner model 110 fluorometer, with a light source consisting of an ultraviolet lamp with major emission at 360 nm, was used. For duplicating Hochberg's method, Wratten 47-B plus 2-A primary filters were used in combination with a Wratten 2A-12 secondary filter. In other experiments, a combination of the Corning 5840 primary and Wratten 2A secondary filters was substituted. A 1% neutral density filter was used for both systems. The range setting (light intensity) of the fluorometer was 3X for indirect fluorometry and 30X for direct fluorometry. The lower limit for positive identification was 10 fluorescent units. Any reading below 10, which is within the machine's limits of error, was considered negative.

Cultures. Stock strains from the National Streptococcal Disease Center were used for checking specificity and cross-reactions of FA conjugates. NSDC stock cultures of seven group A, five group C, and four group G streptococci and two Staphylococcus aureus strains and swabs from patients with sore
thor was used to standardize both the indirect and direct fluorometric techniques.

Quantitation of organisms. Cultures or throat swabs were incubated in 5 ml of Todd-Hewitt broth for 15 hr at 37 C before testing. Serial 10-fold dilutions were prepared in 0.01 m phosphate-buffered saline (PBS), pH 7.2. Blood-agar pour plates were inoculated in triplicate with 0.1 ml of the 10^−1, 10^−2, and 10^−4 dilutions. The above procedure was repeated 10 times.

Streptococcal particles in the above dilutions were counted with a model B Coulter counter set with an upper threshold of 90, a lower threshold of 10, amplification of 2, aperture current of ½, and 30-µm aperture. For these determinations, the dilutions were prepared in 5 ml of PBS containing 1% Tween 80. Quantities of 0.5 ml per dilution were tested in triplicate. This procedure was repeated on each of 9 subsequent days. The calculated numbers of organisms per milliliter that would be expected in 15-hr broth cultures, as determined by the two methods, are given in Table 1.

Preparation of FA. Conjugate specific for group A streptococcus was prepared by labeling globulin of group A streptococcus antiserum with fluorescein isothiocyanate (Isomer I; dye content, 95%; prepared by Sylvania Chemical Co.) according to methods described by Moody et al. (4), except that labeling was carried out at 25 C for 4 hr. The conjugate was absorbed with group C streptococci, and residual cross-reactions were eliminated by mixing appropriate dilutions of conjugate and normal rabbit serum. A negative control conjugate was prepared by absorbing a portion of the specific group A conjugate with group A streptococci. Fluorescein-protein (F/P) ratios of the conjugates were determined. Protein was measured by a biuret method (1). The fluorescein content of the conjugate was determined by measuring the optical density at 490 nm on a Beckman DU spectrophotometer and by using a standard curve for fluorescein diacetate in alkaline solution (3). The F/P values for the group A and negative control conjugates were 10.8 and 11.1 µg of fluorescein per mg of protein, respectively. Both conjugates contained 0.75 g per cent protein.

To determine the concentration of conjugate for use in the conventional microscopic FA test, smears of representative strains of groups A, C, and G streptococci and of S. aureus were stained with varying dilutions of each conjugate. All group A strains stained with a 4+ fluorescence at a 1:50 dilution of group A conjugate. No greater than 1+ reactions were demonstrated with all other strains. The negative control conjugate gave no greater than 1+ fluorescence reactions with strains of groups A, C, and G streptococci and S. aureus.

Indirect fluorometric procedure. In preliminary experiments, the procedure described by Hochberg (2) was followed (Fig. 1). It was difficult to demonstrate sufficient differences between fluorometric values obtained with group A and non-group A streptococci. Therefore, attempts were made to determine the source of difficulties and to establish conditions for obtaining clear-cut positive and negative fluorometric reactions.

Examination of smears from stained bacterial sediment on the fluorescence microscope showed that maximal fluorescence of streptococci had not resulted. Earlier work in our laboratories had shown that maximal staining was dependent upon adequate FA being available for the amount of antigen to be stained.

Maximal fluorescence of group A streptococci could be demonstrated by staining bacterial sediment from a 10^−1 dilution of the 15-hr broth culture. Fluorometric readings from conjugate eluted from such suspensions were increased, but only to levels slightly above the base line of 10. To obtain readings of eluted conjugate higher in the positive range, the conjugate used for staining cells in a dilution had to be no greater than 1:15.

RESULTS

Effect of temperature and time on dissociation of conjugate from group A streptococci. Group A streptococci (representing several strains) from 10^−1 dilutions of 15-hr broth cultures were stained for 20 min at 37 C with 0.25 ml of a 1:15 dilution of group A conjugate. The cells were then centrifuged and rinsed twice with 0.01 m PBS, pH 7.2. Smears of the stained cells were prepared for examination on the fluorescence

<table>
<thead>
<tr>
<th>Strains</th>
<th>No. of organisms* (0.5 ml)</th>
<th>Theoretical no. of organisms per ml in undiluted broth culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coulter counter (0.5 ml)</td>
<td>Four plate (0.1 ml)</td>
</tr>
<tr>
<td>ANT-16</td>
<td>161 × 10^4</td>
<td>36 × 10^4</td>
</tr>
<tr>
<td>AT1-4</td>
<td>390 × 10^4</td>
<td>78 × 10^4</td>
</tr>
<tr>
<td>AT2-3</td>
<td>222 × 10^4</td>
<td>51 × 10^4</td>
</tr>
<tr>
<td>AT2-2</td>
<td>480 × 10^4</td>
<td>77 × 10^4</td>
</tr>
<tr>
<td>AT2-2-1</td>
<td>382 × 10^4</td>
<td>53 × 10^4</td>
</tr>
<tr>
<td>C-23</td>
<td>642 × 10^4</td>
<td>97 × 10^4</td>
</tr>
<tr>
<td>G-2</td>
<td>380 × 10^4</td>
<td>62 × 10^4</td>
</tr>
</tbody>
</table>

* Means of triplicate counts of a 10^−4 dilution from 10 runs. The coefficient of variation for the mean counts ranged from 3.1 to 13.9%, being under 10% in all but 3 of 14 means shown.
Broth and cells

Centrifuge at 2,000 rev/min for 15 min, decant

Cells

Add 5 ml of 0.1% trypsin, incubate 30 min at 37°C

Trypsinized cells

Centrifuge at 2,000 rev/min for 10 min, decant, add 5 ml of PBS, centrifuge, decant

Washed trypsinized cells

Add 4 drops 1:50 group A conjugate, incubate 20 min at 37°C

Labeled trypsinized cells

Add 5 ml of PBS, centrifuge at 2,000 rev/min for 15 min, decant, repeat

Washed trypsinized labeled cells

Dissociation. Suspend in 5 ml of demineralized distilled water, incubate 10 min at 30°C; centrifuge at 2,000 rev/min for 10 min

Eluted FA

Read total fluorescence* on fluorometer

Cells

Add negative control FA, incubate 20 min at 37°C

Nonspecifically stained cells

Add 5 ml of PBS, centrifuge at 2,000 rev/min for 10 min, decant, repeat

Washed nonspecifically stained cells

Dissociate as above, centrifuge at 2,000 rev/min for 10 min

Eluted FA

Read nonspecific fluorescence*

Cells

* specific fluorescence units = total fluorescence minus nonspecific fluorescence

FIG. 1. Hochberg indirect fluorometric technique.

microscope. The packed cells were suspended in distilled demineralized water for 10 min at 30°C to effect dissociation of conjugate. The eluted conjugate was collected after centrifugation. One portion of the packed cells was rinsed once with PBS. Smears of rinsed and unrisned cells were made and examined microscopically to determine whether fluorescence remained. Examination of stained organisms prior to dissociation showed that maximal (4+) staining had been attained. Organisms in smears, rinsed and unrisned, made after dissociation with distilled demineralized water retained fluorescence at 3 to 4+ levels, indicating that complete dissociation had not taken place. Fluorometric readings of the conjugate eluted from such cells were no higher than the base-line level. The next experiments were performed to determine whether dissociation of conjugate could be enhanced if carried out at higher temperatures or in increased time. The dissociation step was done at 30, 37, and 55°C. Samples of the eluted conjugate were collected after 10, 20, and 30 min and read fluorometrically. Dissociation was enhanced as time and temperature were increased (Fig. 2). Dissociation was markedly greater if carried out at 55°C than at 30 and 37°C, even for 20 min. Examination of smears after elution for as long as 2 hr at 55°C revealed that organisms remained brightly fluorescent; therefore, only partial elution of conjugate presumably had been accomplished.

Effect of trypsinization of cells on fluorometric values. Conventional FA tests do not require trypsinization of specimens, provided the conjugate has been treated adequately to remove cross-reactions with related organisms. Eliminating this step in the fluorometric procedure would save considerable time. Therefore, duplicate tubes of packed cells of 10⁻¹ dilutions of 15-hr broth cultures of strains of groups A, C, and G streptococci and S. aureus were prepared. The packed cells in one tube of each pair were suspended in
0.1% trypsin in PBS (pH 8) and held 30 min at 37 C. The trypsinized cells, as well as the corresponding nontrypsinized cells, were centrifuged and stained with FA for 20 min at 37 C. The preparations then were treated according to the steps shown in Fig. 1. The use of trypsinized cells did not appear to affect the specificity or sensitivity of the fluorometric reaction (Table 2). The fluorometric units attributed to specific fluorescence in every case were well above the base line of 10 for group A streptococci and well below the base line when other strains of streptococci and staphylococci were tested. Microscopic examination of smears after staining with group A conjugate and rinsing corresponded with these observations.

**Modified indirect fluorometric technique.** Results obtained in the above experiments suggested the feasibility of modifying the Hochberg technique by (i) omitting the trypsinization of specimens and (ii) dissociating conjugate from stained cells at 55 C for 20 min. This procedure (Fig. 3) was evaluated with 280 unknowns in which pure cultures of 66 strains of groups A, C, and G streptococci and *S. aureus* were introduced. Group A was used in 216 of the unknowns and the other strains in 64. Specimens were coded, tested, and results recorded without knowledge of their contents. Fluorometric readings were based upon values given in Fig. 4 which are representative of those obtained with some of the strains tested in the unknowns by the same procedure. A value of 10 or above for specific fluorescence was considered positive for presence of group A. Readings below 10 were recorded as negative. Of 216 unknowns containing group A,

![Graph showing Effect of increased time and temperature on dissociation of fluorescent reagent for group A streptococci as determined by fluorometric readings](image)

**TABLE 2. Effect of trypsinsed streptococci on fluorometric readings**

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Fluorescence units after dissociation of</th>
<th>Units of specific fluorescence</th>
<th>FA microscopy results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A conjugate</td>
<td>Normal control conjugate</td>
<td></td>
</tr>
<tr>
<td>Trypsin treated</td>
<td></td>
<td></td>
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<tr>
<td>A-NT-16</td>
<td>31</td>
<td>12</td>
<td>19</td>
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<tr>
<td>A-T1-4</td>
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<td>A-T12-1</td>
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<td>15</td>
</tr>
<tr>
<td>A-T5-3</td>
<td>28</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>C-23</td>
<td>11</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>C-25</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>G-2</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>G-17</td>
<td>13</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>42-bp</td>
<td>17</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>64-bp</td>
<td>16</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Nontrypsinized</td>
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<td>A-NT-16</td>
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<td>A-T12-1</td>
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</tr>
<tr>
<td>64-bp</td>
<td>17</td>
<td>9</td>
<td>8</td>
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</tbody>
</table>

* Units of specific fluorescence were obtained by subtracting fluorochrome units following dissociation of normal control conjugate from the units following dissociation of group A conjugate.

204 gave fluorometric readings of 10 or above. Readings on the 12 remaining specimens containing group A were below 10. Of the 64 specimens that contained no group A, 46 gave readings below 10; readings from the 18 remaining specimens were 10 or above. Therefore, reporting of the presence or absence of group A streptococci was correct in 250 of the 280 unknowns tested.

The accuracy of the modified indirect procedure was further determined with 85 cultures freshly isolated from throat swabs. These cultures included 52 strains of group A streptococci, 17 strains of groups B, C, and G streptococci, and 15 miscellaneous α or nonhemolytic streptococci. Cultures were coded and tested as described above. Forty-seven cultures were positive by both fluorometric and culture-precipitin tests, and 32 were negative by both tests—an agreement of 93% of the 85 cultures. In one case, the fluorometric reading was 10 when only α-streptococci were isolated by culture. In five cases, group A streptococci were not detected fluorometrically.
Broth culture
- Make $10^{-1}$ dilution in PBS (pH 7.2), centrifuge at 3,000 rev/min for 10 min

Washed cells
- Stain 20 min at 37 C with 0.25 ml of group A FA

Labeled cells
- Wash 2X with PBS (pH 7.2), centrifuge at 3,000 rev/min for 10 min

Washed labeled cells
- Dissociation. Suspend in 5 ml of demineralized distilled water, incubate 20 min at 55 C, centrifuge at 3,000 rev/min for 10 min
- Eluted FA
  - Read total fluorescence* on fluorometer

Cells
- Stain 20 min at 37 C with 0.25 ml of negative control FA

Nonspecifically stained cells
- Add 5 ml of PBS, centrifuge at 3,000 rev/min for 10 min, decant, repeat

Washed nonspecifically stained cells
- Dissociate as above, centrifuge at 3,000 rev/min for 10 min
- Eluted FA
  - Read nonspecific fluorescence*

Cells
* specific fluorescence units = total fluorescence minus nonspecific fluorescence

Fig. 3. Modified indirect fluorometric technique.

Fig. 4. Fluorometric values determined for representative strains of groups A, C, and G streptococci and S. aureus by the modified Hochberg indirect fluorometric technique.

The fluorometric readings were 9, 9, 8, 6, and 3, respectively. In the latter specimen, only one colony of group A streptococci was cultured. Based on an 0.05-level chi-square test, these differences between the two methods were not statistically significant.

**Direct fluorometric technique.** The feasibility of using a direct fluorometric procedure that would be more adaptable to automation than the indirect procedure was investigated. In its simplest form, the procedure consisted of staining the packed cells from a specimen in the test tube, rinsing, applying the stained organisms to a holding matrix, and taking a direct fluorometric reading of the stained material. The best reproducibility of results was obtained when Whatman 3 MM filter paper was used as a matrix for reading the specimens. Other materials tested included Whatman filter paper no. 1, 2, 4, 11, 41, and 42, cellulose acetate electrophoresis strips, filters (Millipore Corp., Bedford, Mass.), and Schleicher and Schuell filter paper no. 402 and T3402.

In carrying out the adopted procedure (Fig. 5), a 10-fold dilution of broth cultures which had incubated 15 hr at 37 C was prepared in 0.01 M filtered PBS (pH 7.2) and centrifuged for 10 min at 3,000 rev/min.

The pellet of cells was suspended in 0.25 ml of group A streptococcus conjugate (1:50 dilution) held for 20 min at 37 C, and then centrifuged for 10 min at 3,000 rev/min. The supernatant fluid was discarded; the cells were suspended in PBS and then centrifuged again. An 0.02-ml amount of the packed cells was transferred to a designated spot on a strip of filter paper for reading in the fluorometer. The dimensions of the filter paper
were 40 by 304 mm. To place specimens in appropriate locations for fluorometric readings, a template constructed so that 15 specimens could be spotted equidistant linearly on the filter paper was used. The specimens were air-dried and mounted horizontally on a paper chromatogram door of the fluorometer for reading. Each filter-paper strip contained a PBS control which served as a "blank" for adjusting the fluorometer to a zero reading before the actual specimen reading. Controls consisting of specimens stained with negative control conjugate were considered unnecessary, since fluorescence values no greater than one were demonstrated with representative strains of groups A, C, and G streptococci and S. aureus.

Stock strains of streptococci and staphylococci were tested. Fluorometric values are shown in Fig. 6. Values for specific fluorescence above 10 corresponded with presence of group A streptococci, whereas values no greater than nine were demonstrated with other organisms. Shaded areas show the amount of fluorescence that would be expected from staining with negative control conjugate.

Pure cultures isolated from 48 throat cultures were tested in the direct fluorometric procedure without knowledge of their identity as determined by cultural methods. The collection included 39 strains of group A streptococci, 7 strains of groups C and G, and 2 strains of α-streptococci.

Thirty-five specimens were positive by both methods and six were negative by both. Four specimens were positive by culture but negative fluorometrically. Three non-group A strains (two group C, one group G) were positive fluorometrically. Based on an 0.05-level chi-square test, these differences were not statistically significant.

**DISCUSSION**

A fluorometric method was developed which permits identification of fluorescein-labeled group A streptococci by taking direct readings in a fluorometer and without the use of a microscope. The simplicity of the method appears to make it highly amenable to automation. Our experience with the indirect fluorometric method of Hochberg et al. (2) provided a basis for development of a direct test. The indirect method consists of trypsinizing centrifuged bacteria from overnight broth cultures, staining with group A streptococcus FA, rinsing, then eluting the FA that had reacted with the group A streptococci, and taking a reading of total fluorescence on a fluorometer. Cells are then treated with a normal conjugate and rinsed, and the conjugate is eluted and read as nonspecific fluorescence. If the difference between the two fluorometric values lies in a certain range, the presence of group A streptococci is indicated.

Our results indicated that trypsinization of the
specimen could be circumvented if optimal proportions of cells to FA were maintained. For example, more uniform and intense staining among individual streptococci could be demonstrated by staining cells from a 10-fold dilution of a 15-hr broth culture with a 1:15 dilution of conjugate. The manner in which the conjugate was prepared, treated to eliminate cross-reactions, and standardized undoubtedly was an important factor in assuring specificity.

Another important observation was that more efficient elution of conjugate from stained nontrypsinized cells occurred at temperatures as high as 55 C, as judged by fluorometry. Fluorescence microscopic examination of such cells after elution and washing, however, revealed that complete dissociation was never accomplished, since the organisms continued to exhibit 3 to 4+ fluorescence. Nevertheless, corresponding fluorometric values from the eluted conjugate were sufficiently high and the nonspecific fluorometric values sufficiently low that reliable specific fluorescence values could be obtained. These results further substantiate the validity of the Hochberg method as modified in the above manner.

By applying the observations obtained with the indirect technique, a more direct fluorometric procedure was developed. The feasibility of identifying FA-stained group A streptococci applied to filter paper strips by direct fluorometry was demonstrated. A more dilute conjugate (1:50) could be used than with the indirect test which would be expected to permit greater specificity of the reaction as well as greater economy. Screening of various grades and kinds of matrices for implanting the stained bacteria, which would permit maximal specific and minimal nonspecific fluorescence, and consistent readings from area to area where replicates of samples were implanted were necessary before reproducibility could be demonstrated. Whatman 3 MM filter paper possessed these qualities.

From results obtained with pure cultures, it may be assumed that a high degree of specificity can be expected from the direct fluorometric procedure. Based upon the assumption that reactions can be obtained by both the direct fluorometric technique and the conventional culture-precipitin tests for group A streptococci, our preliminary comparison indicated that differences between the two tests were not statistically significant.

These results and those given in a separate report (Webb and Moody, submitted for publication) provide evidence for the feasibility of detecting group A streptococci from throat swabs by an automated fluorometric procedure.

LITERATURE CITED