Fluorescent Treponemal Antibody-Absorption Double-Staining Procedure

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The fluorescent treponemal antibody-absorption double-staining step-by-step procedure and proposed reference reagents for the test are described. The test and reagents were evaluated in two separate laboratories on 265 fresh sera, and test results were compared with the reference fluorescent treponemal antibody-absorption test results performed in a third laboratory. The data indicate that the tests are comparable in the areas where the test is recommended for use. Problems with inadequate light filtration occurred, but these could be resolved. This test is recommended for use with microscopes equipped with incident illumination.

In 1979, Hunter et al. reported a double-staining (DS) procedure to be used with the fluorescent treponemal antibody-absorption (FTA-ABS) test for syphilis (1), enabling workers to perform the test using microscopes equipped with incident illumination. Although the initial report recommended the continued use of a fluorescein isothiocyanate label for the class-specific anti-human globulin, difficulties in obtaining the KP560 barrier filter to exclude rhodamine counterstaining when reading fluorescein fluorescence have made it necessary to reverse the dye labels. Two subsequent reports evaluated a FTA-ABS DS test in which a class-specific, rhodamine-labeled anti-human immunoglobulin G (IgG) globulin was used as a primary reagent and a fluorescein-labeled antitreponemal globulin was used as a counterstain reagent (6, 6a).

The present report describes some problems that developed with rhodamine filtration, the preparation of reference reagents, and the step-by-step directions for the test. In addition, a limited number of sera were examined with the reference reagents, and results were compared with those obtained with the FTA-ABS test.

MATERIALS AND METHODS

FTA-ABS test reagents. Treponema pallidum antigen, sorbent, control sera, and fluorescein-labeled anti-human globulin were reference reagents for the FTA-ABS test (5) and were obtained through the Biological Products Division, Centers for Disease Control. The reference FTA-ABS test conjugate is predominantly anti-IgG with anti-light chain activity (2).

FTA-ABS DS reagents. T. pallidum antigen and sorbent reagents were reference reagents for the FTA-ABS test above.

Serum for the reactive control was obtained from a patient with clinically documented secondary syphilis. Serum for the nonspecific control was obtained from a presumed normal individual. The sera were filter sterilized with Seitz EK filters, 0.01-μm pore size. The sera were added to vials in 1.0-ml volumes and lyophilized under vacuum. Samples of each serum were rehydrated in 1.0-ml volumes of sterile distilled water, and the rehydrated samples were compared to nonlyophilized samples on the day rehydrated and after storage at 2 to 8°C and at −20°C. For testing, the reactive serum was diluted 1:5 in phosphate-buffered saline (PBS) and 1:5 in sorbent for the 4+ staining. Serial PBS serum dilutions were made to establish a 1+ minimally reactive control dilution. The nonspecific control serum was diluted 1:5 in PBS and 1:5 in sorbent before testing (7).

Rhodamine-labeled anti-human IgG, which was prepared for the initial report (1), was goat antiserum to the Fc fragment of pooled normal human serum IgG. The IgG fraction of goat serum was separated by diethylaminoethyl-Sephadex chromatography with 0.1 M NaCl containing 0.05 M boric acid buffer. The pH was adjusted to 8.0 with NaOH. The globulin, containing 5 mg of IgG per ml, was labeled with tetramethyl rhodamine isothiocyanate to obtain a ratio of absorbance at 554 nm to that at 280 nm of 0.40 (5). The conjugate was absorbed with Treponema phagedenis biotype Reiter with 1 part packed cells to 5 parts conjugate. The conjugate and cells were incubated at 37°C for 2 h, followed by holding at 2 to 8°C overnight (3). The bacterial cells were removed by centrifugation at 17,300 × g for 30 min, and the supernatant was filtered with a 0.45-μm Millipore membrane filter.

Fluorescein-labeled antitreponemal globulin was
fractionated human serum obtained from a patient with clinical evidence of secondary syphilis. The IgG fraction, containing 28 mg of IgG per ml, was obtained as described above and labeled with fluorescein isothiocyanate. Absorbance readings obtained at 280 and 260 nm corresponded to a fluorescein/protein ratio of approximately 8.

The conjugates were diluted 1:2 in PBS containing 0.5% bovine serum albumin and either stored at 2 to 8°C or lyophilized. NaCl was added to each conjugate to a concentration of 0.1%. Tests and evaluation of these reagents were based on standard procedures described for the FTA-ABS test (7). For control of these evaluations, nonlyophilized reagents from a previous labeling (1) were appropriately diluted.

**Microscope equipment.** A Leitz Ortholux II microscope equipped with a dark-field condenser and a Xenon XBO-150 lamp was used for reading FTA-ABS test slides. A KP490 filter with a K510 barrier filter was used for reading fluorescein fluorescence. The readings were made with a 40x ocular and a 40x high-power objective.

Initially, FTA-ABS DS reagents were evaluated by using an Ortholux II microscope equipped for incident illumination with a HBO-50 lamp. The filter cube for reading rhodamine fluorescence was the Leitz N filter cube. With this cube, the filters for rhodamine excitation included the red-suppressing BG-38, the BG-36 band absorption glass filter to absorb the strong mercury line at 578 nm, the KP560 exciter filter, the K530 edge filter, the TK580 dichroic beam splitter, and the K590 barrier filter (1). A 6.3x ocular and a 100x/1.3 oil objective were used for FTA-ABS DS reading.

For this evaluation, two SM Lux microscopes equipped for incident illumination were kindly loaned to the Centers for Disease Control by E. Leitz and Co. These microscopes were each equipped with an HBO-50 lamp. Three filter systems were supplied for rhodamine excitation. The M filter cube included an interference band pass 546/14 narrow band filter, a BG-36 band absorption glass filter, a TK580 dichroic beam splitter, and a K580 long pass filter. The M2 and N2 filter cubes consisted of the same filters listed above for the M and N cubes, but filters were sandwiched together to avoid internal reflections and to increase intensity. A K2 cube was supplied for fluorescein excitation. The K2 cube included a K480 edge filter, two KP490 exciter filters, a TK510 dichroic beam splitter, and a K515 barrier filter. The SM Lux microscopes were equipped with 8x oculars and a 100x/1.3 oil objective.

**Sera.** A total of 265 sera were obtained from patients visiting the DeKalb County, Ga., Sexually Transmitted Disease Clinic. Sera were examined on the day of receipt or were kept at 2 to 8°C and examined within 24 h. Repeat tests were performed within 2 weeks with storage at 2 to 8°C or were performed on serum samples stored for approximately 1 month at -20°C.

**Evaluation.** Two individuals in two separate laboratories tested all sera in the FTA-ABS DS test. One individual in an additional laboratory served as control and tested all sera in the FTA-ABS test.

**RESULTS**

The Ortholux II microscope equipped with a N filter cube for reading rhodamine fluorescence was used in establishing acceptability of the proposed FTA-ABS DS reference reagents. We found that we did not obtain the same degree of reactivity and nonreactivity when reading FTA-ABS DS slides with the SM Lux microscopes equipped with the N2 or the M2 filter cubes. Slides treated with reactive serum were more brilliant and background staining was enhanced when viewed with the N2 or M2 filters than when read with the N filter cube. Additionally, the slide treated with the 1+ serum dilution resembled a 3+ staining intensity rather than the expected 1+ intensity. Slides treated with PBS only, sorbent only, or absorbed serum from non-syphilitic individuals had less staining than the 1+ control (indicating borderline or nonreactive results), but demonstrated low-grade staining sufficient to make slide reading difficult. Adding a second BG-36 filter in the lamp housing reduced background and treponemal staining to a level that made accurate reading possible, yet some low-grade fluorescence was still visible. FTA-ABS DS test results read with the M filter cube were comparable to test results read with the N filter cube. The M filter cube was selected for the evaluation reported in this manuscript.

The rehydrated and nonlyophilized samples of the reactive control demonstrated 4+ staining intensity when the serum was diluted 1:5 in PBS in 1:5 in sorbent. The 1+ minimally reactive control dilution of 1:1,900 was confirmed on the lyophilized and rehydrated serum samples. Rehydrated and nonlyophilized samples of the nonspecific control serum diluted 1:5 in PBS demonstrated 3+ staining intensity. When the nonspecific control serum was diluted 1:5 in sorbent, no fluorescence was observed. Samples of both reactive and nonspecific sera, frozen at -20°C, were comparable in reactivity to nonlyophilized and freshly rehydrated serum.

Titers of the rhodamine-labeled anti-human IgG after rehydration were determined by diluting serially in PBS containing 2% Tween 80 (5). Each dilution was tested on three smears incubated in the first step with the following: (i) reactive control serum diluted 1:5 in PBS; (ii) the 1:1,900 1+ serum dilution; and (iii) PBS only. The endpoint of the conjugate titration was 1:640, the highest dilution giving maximum (4+) fluorescence on the slides treated with the reactive (4+) control dilution. The selected working titer, 1:320, was one doubling dilution below the 4+ endpoint of the titration, and that dilution yielded a 1+ staining with the 1:1,900 min-
imally reactive dilution. No nonspecific staining was seen between the antigen and conjugate for three doubling dilutions below the 1:320 dilution. The conjugate titration is shown in Table 1. Counterstain reagent for these smears was the previously described nonlyophilized antitreponemal globulin (1).

The fluorescein-labeled antitreponemal globulin was serially diluted in PBS containing 2% Tween 80 and tested directly on T. pallidum antigen. The slides, incubated at 37°C for 20 min, demonstrated 4+ staining intensity through the 1:80 dilution with freshly rehydrated reagent and rehydrated reagent stored at 2 to 8°C and -20°C. For the counterstain working dilution, the selected titer was one doubling dilution below the 4+ endpoint.

The working dilutions of the two conjugate preparations and the selected 1+ dilution for the minimally reactive control were examined with 10 reference sera used in the evaluation of FTA-ABS test reagents (Table 2). Comparable results were obtained between the two test procedures and between the nonlyophilized and lyophilized FTA-ABS DS reagents.

The results of the larger evaluation are shown in Table 3. When testing sera from nonsyphilitic in the FTA-ABS DS test we found fewer borderline reactions than with the FTA-ABS test and no reactives. In comparing the two tests, results were essentially the same when we tested sera from syphilitic individuals, unless the sera were from patients with primary syphilis.

**DISCUSSION**

Microscopes equipped with incident illumination are a new development in microscopic technology. Currently, these microscopes are

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**Table 1. Rhodamine-labeled anti-human IgG conjugate titration**

<table>
<thead>
<tr>
<th>Conjugates</th>
<th>Controls</th>
<th>Reactive (++) control serum (1:10 in PBS)</th>
<th>Reactive (++) control serum (1:5 in PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control conjugate at working titer</td>
<td>-</td>
<td>4+</td>
<td>1+</td>
</tr>
</tbody>
</table>

New conjugate dilution:

1:40  
1:80  
1:160  
1:320  
1:640  
1:1,280  
1:2,560  

**Table 2. Comparison of the reference FTA-ABS results and the FTA-ABS DS results with proposed conjugate preparation stored under different conditions**

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Reference FTA-ABS test</th>
<th>Reference FTA-ABS DS test</th>
<th>Nonlyophilized, stored at 5°C</th>
<th>Proposed FTA-ABS DS conjugate lyophilized and rehydrated, then: Stored at 5°C</th>
<th>Stored at -20°C</th>
<th>Used immediately</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R</td>
<td>B</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>5</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>6</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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</tr>
<tr>
<td>7</td>
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</tr>
<tr>
<td>10</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

**Table 3. Comparison of the FTA-ABS DS procedure and the reference FTA-ABS test on 265 sera from syphilitic and nonsyphilitic individuals**

<table>
<thead>
<tr>
<th>Sera</th>
<th>FTA-ABS DS test</th>
<th>FTA-ABS test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>B</td>
</tr>
<tr>
<td>Syphilitic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Secondary</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Latent</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Late</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Treated, stage unknown</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Congenital</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Possible syphilis, stage unknown</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 4. Intensity of fluorescence**

<table>
<thead>
<tr>
<th>Reading</th>
<th>Intensity of fluorescence</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4+</td>
<td>Moderate to strong</td>
<td>Reactive (R)</td>
</tr>
<tr>
<td>1+</td>
<td>Equivalent to minimally reactive (1+) control</td>
<td>Reactive (R)</td>
</tr>
<tr>
<td>&lt;1+</td>
<td>Weak but definite, less than minimally reactive (1+) control</td>
<td>Borderline (B)</td>
</tr>
<tr>
<td>None to ±</td>
<td>Nonreactive (N)</td>
<td></td>
</tr>
</tbody>
</table>

Notes:

- B, Borderline.
- *R*, Reactive; *B*, borderline; *N*, nonreactive.
used in many direct immunofluorescence applications; however, with an indirect test it is difficult to take full advantage of the equipment when it is necessary to demonstrate the presence of antigen on nonreactive slides (7). When reading an FTA-ABS test slide, workers have difficulty switching from incident illumination to transmitted illumination because the numerical aperture of the recommended objective (1.3) is greater than that used with a dark-field condenser (1.15) (4). The FTA-ABS DS procedure eliminates this problem by providing a counterstain that reacts immunologically with all treponemes on the slide. Reading FTA-ABS slides totally above the stage with only incident illumination reduces reading time to half that normally used. In addition, the FTA-ABS DS test has an advantage over the FTA-ABS test read with transmitted light, since the use of a microscope with incident illumination eliminates any incorrect FTA results that could occur with incorrect positioning of the dark-field condenser.

The FTA-ABS DS procedure has been compared with the FTA-ABS test in this and several earlier studies (1, 6, 6a). In these evaluations, the FTA-ABS DS test has been slightly less sensitive than the FTA-ABS test when used to test sera from patients with syphilis and slightly more specific when testing sera from nonsyphilitics. These differences may be due to differences in conjugate specificity, which has been referred to earlier (6), or due to the competence level of reading immunofluorescence slides. Adjusting to reading rhodamine versus fluorescein fluorescence does not appear to be difficult, but this adjustment must be considered as a possibility for the test differences.

Reagents for the FTA-ABS DS test have been described and evaluated by the same procedures currently used for the reference FTA-ABS test reagents. These new reagents were found to be satisfactory and are recommended for reference standard reagents for the FTA-ABS DS test. Since this evaluation was completed, current FTA-ABS control sera have been found to be satisfactory for the FTA-ABS DS test. This may simplify transition from the FTA-ABS test to the FTA-ABS DS test.

Problems that developed with the filter cubes for rhodamine emission were unexpected. We prefer the older N or M filter cubes that will soon be replaced by the newer cubes, because background fluorescence was minimal and appropriate reactive and nonreactive results were obtained. We found that an additional BG-36 filter placed in the light path provided more adequate filtration than the N₂ and M₂ filters provided alone and that this combination was satisfactory for reading FTA-ABS DS test slides. A better solution could be to use a 100×/1.32-0.60 oil objective with an iris diaphragm to reduce the numerical aperture of the objective until appropriate readings of the FTA-ABS DS controls are obtained. Comparable readings with this objective and an N₂ filter cube have recently been reported (6a).

The recommended step-by-step methodology for the FTA-ABS DS test is as follows.

**FTA-ABS DS Procedure**

1. Identify prepared antigen slides with appropriate serum numbers.
2. Number the tubes to correspond to the sera and control sera being tested, and place them in racks.
3. Prepare reactive (4+), minimally reactive (1+), and nonspecific control serum dilutions in sorbent or PBS or both.
4. Pipette 200 μl of sorbent into a test tube for each test serum.
5. Add 50 μl of the heated test serum to the appropriate tube and mix eight times.
6. Cover the appropriate antigen smears with 30 μl of the reactive (4+) minimally reactive (1+), and nonspecific control serum dilutions.
7. Place slides in an incubator at 35 to 37°C for 30 min.
8. Rinse procedure:
   a. Place slides in slide carriers and rinse slides with running PBS for approximately 5 s.
   b. Place slides in staining dish and soak for 5 min in PBS. Agitate vigorously (50×).
   c. Rinse slides in running distilled water for approximately 5 s.
9. Gently blot slide with bibulous paper to remove all water drops.
10. Dilute rhodamine-labeled anti-human IgG globulin to its working titer in PBS containing 2% Tween 80.
11. Place approximately 30 μl of the diluted conjugate on each smear.
12. Dilute fluorescein-labeled anti-treponemal globulin to its working titer in PBS containing 2% Tween 80.
13. Place approximately 30 μl of the diluted conjugate on each smear.
14. Repeat steps 8 through 11.
15. Place slides in an incubator at 35 to 37°C.
for 20 min.
19. Repeat steps 10 and 11.
20. Mount slides immediately by placing a small drop of mounting medium on each smear and applying a cover glass.
21. Examine slides as soon as possible. If a delay in reading is necessary, place slides in a darkened room and read within 4 h.
22. Study smears with a microscope equipped with incident illumination. The following lamps and optics have been satisfactory: an HBO-200 or an HBO-50 lamp with a 100×/1.3 oil achromatic objective and a 6.3× ocular. The filters for rhodamine excitation include the red-suppressing BG-38, the BG-36 band absorption glass filter to absorb the strong mercury line at 578 nm, the KP560 exciter filter, the K530 edge filter, the TK580 dichroic beam splitter, and the K590 barrier filter. The filter system for fluorescein consists of a BG-38 red-suppressing filter, the K480 edge filter, two KP490 exciter filters, the TK510 dichroic beam splitter, and the K515 barrier filter.
23. Locate and focus treponemes with the fluorescein filter system.
24. After the treponemes have been located, dial in the rhodamine filters to read specific fluorescence.
25. Using the minimally reactive (1+) control slide as the reading standard, record the intensity of fluorescence according to the chart in Table 4. Retest all specimens with intensity of fluorescence of 1+ or borderline. When a specimen initially read as 1+
is retested and is subsequently read as 1+ or greater, the test is reported as "reactive." When a specimen initially read as 1+ is retested and is subsequently read as 1+ or −, it is reported as "borderline." Borderline readings that repeat as borderline are reported "nonreactive." All other results on retest are reported "nonreactive."

LITERATURE CITED