Problems Affecting Performance of the Fluorescent Treponemal Antibody-Absorption Test for Syphilis

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Immunofluorescent staining of *Treponema pallidum* was studied to clarify the effect of three factors on the results of the fluorescent treponemal antibody-absorption test: (i) heat inactivation of sera at 56°C for 30 min before testing, (ii) use of multicircle slides, and (iii) tungsten illumination to visualize and assess unstained treponemes on reactive as well as nonreactive smears. It was found that serum inactivation before testing was not necessary for detection of immunoglobulin G antibody, but an immunoglobulin M prozone was detected in unheated serum. On multicircle slides, it was demonstrated that a false-positive reaction could be obtained in 30 s at 37 and 25°C if a smear where a nonreactive serum had been placed was crossed by a strongly reactive serum from another circle. Tungsten illumination proved necessary for correct assessment of unstained treponemes on all fluorescent treponemal antibody-absorption test smears, reactive or nonreactive. The possible role of these factors in incorrect fluorescent treponemal antibody-absorption test results is discussed.

A standard procedure for performing the fluorescent treponemal antibody-absorption (FTA-ABS) test was described in 1968 (19). Since that time, variations in technique have been described, but certain changes that have been made in the earlier technology could adversely affect the FTA-ABS test results. For example, in the 1969 *Manual of Tests for Syphilis* (18) the FTA-ABS test procedure for serum states that the serum should be heated at 56°C for 30 min before testing is begun; however, O'Neill and associates (15) and Duncan et al. (6) have stated that neither serum nor spinal fluid requires heating. In the United States, the automated fluorescent treponemal antibody procedure (12) is not in current use, but the trend in many laboratories may be to use one of a variety of multispot slides to facilitate large-volume testing. With these slides, the problem of serum runover could result in false-positive or reversible FTA-ABS test readings. Additionally, the *Manual of Tests for Syphilis* (18) states that the presence of treponemes should be verified on nonreactive FTA-ABS test slides, but it does not specifically state that all slides should be checked under tungsten illumination. These three factors and their effects on the FTA-ABS tests are reported in this paper.

MATERIALS AND METHODS

Sera used in this study were characterized serum samples obtained from the Venereal Disease Serum

Bank, Bacteriology Division, Center for Disease Control, or were submitted for evaluation to the Bacterial Immunology Branch, Center for Disease Control.

The effect of heating serum at 56°C for 30 min before it is tested was evaluated by examining heated and unheated serum samples from syphilitic and non-syphilitic subjects in quantitative immunofluorescent (IF) tests. Sera were serially diluted in phosphate-buffered saline, pH 7.2, and were tested against *Treponema pallidum* antigen with class-specific fluorescein-labeled anti-human immunoglobulin G (IgG) and with fluorescein-labeled anti-human IgM (8, 17). Additionally, using the reference FTA-ABS test conjugate, the FTA-ABS test was performed on heated and unheated samples of 92 stored sera and 66 fresh non-frozen sera held for less than 1 week at 5°C. The FTA-ABS test conjugate has been described as a reagent that is predominantly anti-human IgG with anti-light-chain activity (9). The reference FTA-ABS test conjugate, *T. pallidum* antigen, control sera, and sorbent were obtained from the Biological Products Division, Center for Disease Control.

FTA-ABS tests performed on multicircle microscope slides were evaluated to determine the speed with which staining could be detected should serum runover occur at either 37 or 25°C and the number of incorrect readings that were made when no visible serum runover was recorded. Six-place multicircle slides were obtained from Cell-Lines, Inc. Wells were 1 cm in diameter and arranged in two rows of offset circles. Sera of known staining intensity were used to establish valid staining times. The antigen slides, serum dilutions, and phosphate-buffered saline were incubated at 25 or 37°C for 30 min before they were used. The reactive control at 4+ and 1+ dilutions and phosphate-buffered saline only as a negative control

were added to the antigen smears in a random and blind fashion. Slides were placed in a moist chamber and incubated for 0.5, 1, 2, 5, 10, or 20 min at the appropriate temperature. Slides were then washed and processed by the procedure described in the manual (18). Serum and conjugate were added very carefully to prevent runover between circles, except in one experiment in which the conjugate was intentionally laked over all circles. Class-specific anti-human IgG and anti-human IgM conjugates were substituted for the reference FTA-ABS test conjugate. When slides were examined with a microscope, treponemes were verified under tungsten illumination on all nonreactive slides.

RESULTS

In quantitative IF tests for IgG antibody in 31 sera, no difference in staining results was found between sera that had been heated at 56°C before testing and unheated sera. Two types of IgM staining were observed when sera were not heated (Table 1). A prozone or inhibition of staining in the lower serum dilutions was seen in 20 sera obtained from patients without a known history of rheumatoid disease. No prozone was observed in 11 sera, 9 of which were obtained from patients with rheumatoid arthritis (Table 2).

Heated and unheated samples of 92 stored sera and 66 fresh sera were compared in the FTA-ABS test with the reference conjugate preparation. No reportable differences in test results were found (Table 3).

When serum dilutions and antigen slides had been preheated at 25 or 37°C before the serum was added to the smear, IgG and IgM staining could be accomplished with approximately the

TABLE 1. Examples of IgM IF staining demonstrated with heated and unheated sera from two syphilitic patients

Reciprocal of serum dilution	IF staining in the following patients			
	Syphilitic nonrheumatoid		Syphilitic rheumatoid	
	Serum heated for 30 min at 56°C	Serum unheated	Serum heated for 30 min at 56°C	Serum unheated
5	4+	B ^a	4+	4+
25	4+	1+	4+	4+
50	4+	4+	3+	4+
100	4+	3+	2+	3+
200	3+	3+	2+	2+
400	3+	2+	2+	2+
800	2+	1+	1+	1+
1,600	1+	B	1+	1+
3,200	B	N ^b	B	B

^a B, Borderline.

^b N, Nonreactive.

TABLE 2. Inhibition of *T. pallidum* IF staining in 31 unheated sera

Serum category	No. of Sera	IgM inhibition		IgG inhibition	
		Yes	No	Yes	No
Syphilitic					
With RA ^a	6		6		6
Without RA	17	17			17
Nonsyphilitic					
With RA	3		3		3
Without RA or RA unknown	5	3	2		5

^a RA, Rheumatoid arthritis.

TABLE 3. FTA-ABS test results with serum samples heated at 56°C or with unheated sera

Serum Category	No. of sera	FTA-ABS test results with:			
		Sera heated at 56°C		Sera unheated	
		No. re-active	No. non-reactive	No. re-active	No. non-reactive
Fresh, without histories	66	38	28	38	28
Stored Sera					
Untreated primary	24	19	5	19	5
Untreated secondary	12	12		12	
Treated secondary	10	10		10	
Untreated latent	8	8		8	
Treated latent	14	13	1	13	1
Presumed normals	24		24		24

same speed (Table 4). The 4+ reactive control serum produced 2+ or 3+ fluorescent staining within 30 s when slides were incubated at either temperature. At 37°C the 1+ control dilution stained *T. pallidum* with borderline intensity within 5 min.

In a single experiment, 72 FTA-ABS tests were prepared with 12 of the six-circle slides. Neither serum nor conjugate crossed visibly from one circle to another. Among these 72 smears, only one discrepant reading was obtained. The smear was first read as reactive, but repeated examination indicated that the majority of treponemes were nonreactive. Table 5 gives the results of a repeat examination of the smear, with the original reading and serum pattern shown in Table 6 for each smear on the multicircle slide. Most of the treponemes found in 10 fields of the repeated smear were not stained, but reactive 4+ treponemes were pres-

TABLE 4. *IgG and IgM staining times for T. pallidum demonstrated by IF*

Incubation time (min)	Results under the following conditions ^a											
	IgG						IgM					
	37°C			25°C			37°C			25°C		
	4+ Dilu- tion	1+ Dilu- tion	PBS	4+ Dilu- tion	1+ Dilu- tion	PBS	4+ Dilu- tion	1+ Dilu- tion	PBS	4+ Dilu- tion	1+ Dilu- tion	PBS
0.5	2+	N	N	2+	N	N	3+	N	N	3+	N	N
1	3+	N	N	2+	N	N	3+	N	N	3+	N	N
2	3+	N	N	2+	N	N	3+	N	N	3+	N	N
5	4+	B	N	3+	N	N	4+	B	N	3+	N	N
10	4+	1+	N	3+	B	N	4+	1+	N	4+	N	N
20	4+	B	N	3+	B	N	4+	1+	N	4+	B	N

^a Abbreviations: N, nonreactive; B, borderline; PBS, phosphate-buffered saline.

TABLE 5. *Reexamination of 10 microscopic fields from an FTA saline smear on a multicircle slide*

Field	No. of unstained treponemes	No. of 4+ stained treponemes
1	23	6
2	24	5
3	46	8
4	29	2
5	30	0
6	25	2
7	17	0
8	22	8
9	20	5
10	35	2

ent in 8 of the 10 fields counted. The reactive 4+ control serum on the adjacent smear had not reached maximum intensity. Uniform staining of all treponemes on the smear was not observed.

When the conjugate was intentionally laked on four six-place slides, only one reading of 24 was discrepant.

DISCUSSION

The FTA-ABS test has been described as a test principally for syphilitic IgG (8-10). In IF saline tests for IgG antibody, no difference in staining was observed between sera heated at 56°C and unheated sera. Also, no reportable differences were found in FTA-ABS test results on heated and unheated sera. These data support the recommendation that heating the serum is not necessary for the multispot FTA-ABS test (15) for serum IgG and the FTA-ABS (IgG) test (7) for spinal fluids. Eliminating the heating step in the current FTA-ABS test may offer no particular advantage other than an advantage to reference laboratories where sera are frequently submitted with no information regarding previous heating. Nonetheless, we recommend that the requirement for heating at 56°C for 30 min be dropped.

TABLE 6. *Original reading and serum pattern for smear on multicircle slide^a*

Original reading			Serum pattern		
N	N	N	PBS	PBS	PBS
2-3+	4+ ^b	B	R	PBS ^b	1+

^a Abbreviations: N, nonreactive; R, reactive; B, borderline; PBS, phosphate-buffered saline.

^b Repeated smear.

Interestingly, an IgM prozone in unheated serum is reported. Earlier workers (2, 4) have found an IgM prozone, and Reimer and associates (17) reported a tendency for prozones when purified IgM from a patient with primary syphilis was combined with several minus IgM sera. We found no inhibition of IgM staining with low dilutions of sera that had been heated at 56°C or with sera from patients with a history of rheumatoid arthritis. The possibility that heating sera at 56°C aggregates most IgG (17), causing a rheumatoid-type reaction in unabsorbed serum, warrants further study. We suggest that failure to find IgM antibody in FTA-ABS tests on whole sera (14, 16) may be due to the variety of instructions for heating sera in FTA-ABS test procedures or to the quality of some sorbing reagents.

Early multispot slides made of Teflon apparently were effective in preventing serum runover (15). With the coating now used, discrepant results may occur more easily, but they were not observed in the study reported here unless runover occurred. When slides are removed from the incubator after the first step in the test, serum and slides are at optimal temperature, and runover in handling slides for washing could change a nonreactive smear to a reactive smear in 30 s. This possibility was demonstrated here with IgG and IgM staining times (Table 4). Kellogg (11) reported that direct IF staining

could be accomplished in 2 to 3 min. Similar data for indirect staining have not been emphasized, although some manufacturers of multispot slides have given specific directions for slide rinsing. These recommendations should be followed explicitly, but in light of the present data they probably need to be reviewed. In FTA-ABS tests performed on multispot slides, inaccurate results can be obtained in both the serum and conjugate steps if runover occurs; therefore, manufacturers should include precautionary information for their users.

The discrepant reading reported in Tables 5 and 6 could not have resulted from serum runover because both reactive and nonreactive treponemes were present on the smear. With multicircle slides, the number of smears in the saline rinse was increased, and this probably results in an increased number of free treponemes (stained as well as unstained) in the rinse water. Problems with free organisms during bulk rinse procedures are not limited to FTA-ABS test procedures, but this finding emphasizes the necessity for viewing all FTA-ABS test smears with tungsten illumination. In addition, the number of smears in a single volume of rinse solution should be kept low.

The FTA-ABS test has been subject to a number of clinical evaluations, including many in which specificity of the procedure has been demonstrated (3, 5, 13). Recently, false-positive FTA-ABS test results have been reported with increasing frequency (A. Luger, World Health Organization/Venereal Disease Treponematoses Research, unpublished working document, 77.354). We believe that some false-positive reports could be associated with the technical factors described here. Certainly reversible FTA-ABS tests (1) could be easily explained by serum runover. Another possible source of problems may be inadequate standardization of reagents. Although a well-established program for premarket evaluation of reagents exists in the United States, the same standards are not required elsewhere. The problems we have described are not technical factors requiring extensive expertise, but simple procedures that, if not understood, can lead to erroneous FTA-ABS test results.

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