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Interlaboratory Comparison of the Toluidine Red Unheated Serum Test Antigen Preparation

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The toluidine red unheated serum test (TRUST) antigen, a macroscopic flocculation test antigen developed by Pettit et al. (J. Clin. Microbiol. 18:1141–1145, 1983) by modifying the color-coded antigen of Kasatiya and Lambert (Appl. Microbiol. 28:317–318, 1974), was compared with the Venereal Disease Research Laboratory (VDRL) slide and rapid plasma reagin (RPR) 18-mm circle card tests for sensitivity, specificity, and reproducibility. Two lots of TRUST antigen were prepared by two laboratories in the Centers for Disease Control. Both laboratories performed the TRUST and VDRL slide test on serum samples from 1,102 patients attending the DeKalb County, Georgia, Sexually Transmitted Disease Clinic. In addition, one laboratory performed the RPR card test. Reactive sera were quantitated in the three nontreponemal tests and confirmed with the fluorescent treponemal antibody absorption test. The sensitivity in untreated syphilis for all nontreponemal tests involved was 98.4%. The specificity for these tests was 98.6%. The qualitative reproducibility among the four lots of TRUST antigen, VDRL slide, and RPR card tests was 98.2%. Only 20 sera showed discrepant results. Intralaboratory reproducibility of the two TRUST antigens was 100% for one laboratory and 99.6% for the other. Interlaboratory reproducibility for the four lots of TRUST and the VDRL slide test was 99%. Quantitative agreement ± 1 dilution between the TRUST and RPR card test was 92.3%, and quantitative agreement ± 1 dilution for the TRUST and RPR card test versus the VDRL slide test averaged 50%. The TRUST appeared to be comparable to the RPR card test in all parameters compared.

In 1974, Kasatiya and Lambert (2) reported on the use of a color-coded antigen in the automated reagin test for syphilis. Recently, this antigen was modified by Pettit et al. (3) for use in an 18-mm circle card macroscopic test to satisfy the need for a flocculation test that is inexpensive and easy to perform and produces results comparable to those with the Venereal Disease Research Laboratory (VDRL) slide and rapid plasma reagin (RPR) card tests for screening and serodiagnosis of syphilis.

The modified version of the color-coded antigen test, the toluidine red unheated serum test (TRUST), uses an antigen prepared from VDRL antigen suspension. A 0.25% toluidine red pigment suspension is added as a visualizing agent. This red pigment allows the antigen-antibody reaction to be observed macroscopically in the same manner as charcoal in the standard RPR card test. The test is performed on Brewer diagnostic cards (Hynson, Westcott, and Dunning catalog no. 8718-49), using the same method as in the RPR card test (3).

This report presents the comparison of TRUST antigen preparation by two laboratories in the Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga.: laboratory 1, Evaluation Branch, Biological Products Program, and laboratory 2, the Treponemal Research Branch, Sexually Transmitted Disease Laboratory Program.

MATERIALS AND METHODS

Specimens. All serum samples used in this study were obtained from 1,102 blood samples collected from patients attending the DeKalb County, Georgia, Sexually Transmitted Disease Clinic. The blood samples were centrifuged, and the resulting serum samples were divided between two laboratories. Before testing, serum samples were stored at 2 to 8°C in tubes (13 by 100 mm) tightly fitted with paraffin corks. Testing was done within 18 to 24 h after the serum was collected.

TRUST antigen suspension. Two 115-ml lots of TRUST antigen suspension were prepared by each laboratory, using two lots of toluidine red pigment: lot no. 4-08018 supplied by C. J. Rickey of Hercules Inc., Glens Falls, N.Y., and lot no. 9-31235 supplied by William J. Hart of Ciba-Geigy Corp., Oakbrook, Ill. The TRUST suspensions were prepared by resuspending the sediment of two 100-ml centrifuged VDRL antigen suspensions in resuspending solution equal to the volume of VDRL suspension centrifuged. The VDRL antigen suspension was prepared with reference VDRL test reagents supplied by the Biological Products Program, Center for Infectious Diseases, Centers for Disease Control. The resuspending solution (200 ml) was prepared on the day of use by combining the following: EDTA (0.25 M solution), 10 ml; choline chloride (40% solution), 50 ml; phosphate (0.02 M) merthiolate (0.2% solution), 100 ml; and distilled water, 40 ml.

The two lots of 0.25% toluidine red pigment suspension were prepared by the method of N. G. Lambert et al. as modified by Pettit et al. (3). Then 15 ml of each of the two toluidine red pigment suspension lots was added to each 100 ml of resuspended sediment to form the two lots of TRUST antigen suspension. The suspensions were put in vials in 4-ml aliquots and stored at 2 to 8°C. Pettit et al. have indicated that TRUST antigen is stable for up to 6 months at 4°C. (3). VDRL, RPR 18-mm circle card, and fluorescent treponemal antibody absorption (FTA-ABS) test reagents and reference reactive control serum were supplied by the Biological Products Program, Center for Infectious Diseases, Centers for Disease Control.

Test performance. Each laboratory tested a serum sample from each patient in the TRUST, using two lots of TRUST

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TABLE 1. Distribution of reactivity among 1,102 serum samples qualitatively tested by the TRUST, VDRL slide, and RPR card tests by two laboratories

Laboratory	Test	No. of samples that were:		
		Nonreactive	Weakly reactive	Reactive
1	TRUST 1	1,011		91
	TRUST 2	1,011		91
	VDRL	1,009	15	78
2	TRUST 3	1,019		83
	TRUST 4	1,015		87
	VDRL	1,017	14	71
	RPR	1,013		89

TABLE 3. Results of TRUST, VDRL slide, and RPR card tests on 1,024 nonsyphilitic serum samples

Laboratory	Test	No. of samples that were:		% Specificity
		Nonreactive	Reactive[a]	
1	TRUST	1,009	15	98.5
	VDRL	1,009	15	98.5
2	TRUST	1,011	13	98.7
	VDRL	1,010	14	98.6
	RPR	1,007	17	98.4

[a] Includes VDRL weakly reactive results.

antigen, and in the VDRL slide test. In addition, one laboratory tested all serum samples in the RPR card test. Serum samples that were reactive in any of the nontreponemal tests were quantitated and confirmed with the FTA-ABS test. The patients were categorized as to stage of syphilis, untreated and treated, based on FTA-ABS results and clinical histories. On the days of testing, all reagents were allowed to equilibrate to room temperature (25 to 27°C). The TRUST was performed on fresh, unheated sera. A 0.05-ml serum sample was placed in an 18-mm circle of a Brewer diagnostic card (Hynson, Westcott, and Dunning catalog no. 8718-49) with a semiautomatic pipetor capable of delivering 50 to 100 μl. The sample was spread over the entire circle with a wooden stirrer. One drop (1/60 ml) of TRUST antigen was delivered to the test circle with a 4-ml plastic vial fitted with a 20-gauge needle without bevel, calibrated to deliver 1/60 ml per drop. The card was placed under a humidity cover on a clinical rotator and rotated for 8 min at 100 rpm. After rotation, the test was read without magnification. Any degree of flocculation was read as reactive. No flocculation or slight "roughness" was read as nonreactive. The quantitative tests were performed on reactive serum samples by making serial twofold dilutions in 0.05 ml of 0.9% saline on the card, using a semiautomatic pipette system. Specimens with endpoint titers greater than 32 were diluted 1:16 in 2% normal serum in 0.9% saline and tested quantitatively on the card until an endpoint was reached. The endpoint titer was read as the highest dilution producing a reactive result.

The VDRL, RPR card, and FTA-ABS tests were performed according to standard procedures outlined in the 1969 *Manual of Tests for Syphilis* (4). The TRUST and VDRL slide tests were performed by one laboratorian in laboratory 1. The VDRL slide test, the RPR card test, and the TRUST, using two lots of TRUST antigen, were performed by a different laboratorian in laboratory 2.

RESULTS

The qualitative reactivity of each test performed in laboratories 1 and 2 is shown in Table 1. The greatest number of reactive (not weakly reactive) results, 91, was obtained with the TRUST in laboratory 1. A total of 93 sera were reactive to some degree (78 reactive and 15 weakly reactive) in the VDRL slide test performed in laboratory 1. All sera showing any degree of reactivity were further tested in the FTA-ABS test. Of the 93 sera showing some degree of reactivity, 78 were reactive in the FTA-ABS test. Test agreement was determined as follows: percent agreement = [(no. of test results in agreement)/(no. of sera tested)] × 100. The test agreements on 1,102 sera tested were as follows: 100% for TRUST 1 and 2 (1,102 of 1,102); 99.6% for TRUST 3 and 4 (1,098 of 1,102); 99.2% between the TRUST in different laboratories (1,093 of 1,102); 99.3% between the VDRL slide tests in different laboratories using suspensions prepared from the same lot of antigen (1,094 of 1,102); 99.1% between the TRUST and the VDRL slide test (1,092 of 1,102); 98.9% between the VDRL slide and RPR card tests (1,090 of 1,102); and 98.2% among the TRUST, VDRL, and RPR card tests, with 20 sera showing discrepant results (1,082 of 1,102). The TRUST showed that it was as reactive as the VDRL slide and RPR card tests.

The 78 serum samples reactive in the FTA-ABS test were classified according to the stages of syphilis and were grouped into untreated and treated categories (Table 2). VDRL weakly reactive results were considered reactive. Sensitivity was defined as the ability of each test to indicate the presence of syphilis in a syphilitic serum specimen. The following formula was used to measure the ability of each test to produce a reactive result in the presence of syphilis: sensitivity = [(true positive)/(true positive + false negative)]

TABLE 2. Sensitivity of the TRUST, VDRL, and RPR card tests on 78 serum samples from patients with untreated and treated syphilis in the primary, secondary, and latent stages

Laboratory	Test	No. of reactive[a] results (% sensitivity) among serum samples from patients at the following syphilitic stage:							
		Primary		Secondary		Latent		All stages	
		Untreated (7)[b]	Treated (6)	Untreated (20)	Treated (8)	Untreated (10)	Treated (27)	Untreated (37)	Treated (41)
1	TRUST	7 (100)	6 (100)	20 (100)	7 (88.8)	10 (100)	24 (90)	37 (100)	37 (91)
	VDRL	7 (100)	6 (100)	20 (100)	7 (88.8)	10 (100)	26 (96.3)	37 (100)	39 (95)
2	TRUST	6 (87.5)	5 (85.7)	20 (100)	7 (88.8)	10 (100)	22 (84.4)	36 (97.4)	34 (85.4)
	VDRL	7 (100)	5 (85.7)	20 (100)	7 (88.8)	9 (90)	22 (84.4)	36 (97.4)	34 (85.4)
	RPR	6 (87.5)	5 (85.7)	20 (100)	7 (88.8)	10 (100)	24 (90)	36 (97.4)	36 (89)

[a] Includes VDRL weakly reactive results.
[b] Numbers in parentheses with "untreated" and "treated" indicate the total number of serum samples.

TABLE 4. Comparison of quantitative titers ± one dilution in the TRUST, VDRL slide, and RPR card tests on 91 serum samples

Comparison	No. of discrepant results[a]	Agreement of quantitative titers			
		Equal	± one dilution	± two dilutions or greater	% Agreement[b]
Intralaboratory agreement					
TRUST antigen lot 1 vs lot 2		91			100
TRUST antigen lot 3 vs lot 4	1	66	22	2	96.7
Interlaboratory agreement					
TRUST antigens lots 1 and 2 vs lot 3	9	49	31	2	87.9
TRUST antigens lots 1 and 2 vs lot 4	8	45	35	3	87.9
VDRL vs VDRL same lot	8	34	36	12	76.9
Agreement among tests					
TRUST and VDRL	6	12	33	40	49.4
TRUST and RPR card	4	59	25	3	92.3
RPR card and VDRL	7	12	30	42	46.2

[a] Sera reactive in one test and nonreactive in the other.
[b] Titers that were equal or differed by ± one dilution.

× 100 (1). In laboratory 1, the sensitivity of the TRUST and the VDRL slide test was 100% on serum samples from untreated patients and 91 and 95%, respectively, on serum samples from treated patients. In laboratory 2, the sensitivity of the TRUST and the VDRL slide test was the same on serum samples from untreated and treated patients at 97 and 85%, respectively. The average sensitivity of the TRUST and the VDRL slide test was 98.7% for both on untreated patients and 88.2 and 90%, respectively, on serum samples from treated patients. The TRUST was as sensitive as the VDRL slide and the RPR card tests on serum samples from untreated and treated patients in the secondary stage of syphilis. It was as sensitive as the VDRL slide test and more sensitive than the RPR card test on serum samples from untreated patients, regardless of stages of syphilis. It was less sensitive than the VDRL slide test and slightly more sensitive than the RPR card test on serum samples from treated syphilitic patients, regardless of stages.

The specificity of each test was based on results obtained on 1,024 nonsyphilitic serum samples (Table 3). Specificity was defined as the ability of each test to indicate the absence of syphilis in a nonsyphilitic specimen. The following formula was used to measure the ability of each test to produce a negative result in the absence of syphilis: specificity = [(true negative)/(true negative + false positive] × 100 (1). The VDRL weakly reactive results were considered reactive. The average number of serum samples producing reactive results in the TRUST and the VDRL test was 14 and 14.5, respectively. There were 17 serum samples producing reactive results in the RPR card test. The specificities of the TRUST, VDRL slide, and RPR card tests were very close at 98.6, 98.65, and 98.4%, respectively. The RPR card test was slightly less specific than the VDRL slide test and the TRUST.

All serum samples showing any degree of reaction were quantitated, and the titers were compared. The titer was determined by the highest serum dilution producing a reactive result. The quantitative agreement ± one dilution within a laboratory, between laboratories, and among tests was measured (Table 4). Quantitative agreement in this study is defined as titers that are equal or differ by ± one dilution. The agreement of titers between TRUST antigens within laboratory 1 and laboratory 2 was 100 and 96.7%, respectively. The agreement of the VDRL slide test in laboratory 1 with the VDRL slide test in laboratory 2 was 76.9%. The agreement among tests was as follows: 49.4% between TRUST and the VDRL slide test; 46.2% between the RPR card and VDRL slide tests; and 92.3% between the TRUST and the RPR card test. The low percentage of agreement between the TRUST and the VDRL slide test and between the RPR card and VDRL slide tests is due primarily to the method used to determine titers. The TRUST and the RPR card test are read as reactive and nonreactive, whereas the results in the VDRL slide test are read as reactive, weakly reactive, and nonreactive. Dilutions producing weakly reactive results in the VDRL slide test were not considered in determining endpoint titers; however, they were considered as reactive on undiluted serum samples. Since the weakly reactive results did not play a role in determining titers except on undiluted sera, the VDRL agreement with the TRUST and the RPR card test was very low. The agreement would have been very close if the titers had been determined by the highest dilution producing any degree of reactivity, which would have included weakly reactive results.

DISCUSSION

Our study, involving two different lots of toluidine red pigment, indicates that the TRUST antigen can be successfully prepared in one's own laboratory with performances comparable to those of other standard nontreponemal antigens.

The TRUST antigen was easily prepared, using relatively inexpensive VDRL antigen. The antigen base was prepared from sediment of centrifuged VDRL antigen suspension resuspended in a resuspending solution similar to the resuspending solution used to prepare RPR card antigen (4). Since the preparation of TRUST antigen and the test technique are essentially the same for the RPR card, the TRUST could easily be substituted for the RPR card test.

Our results on 1,102 serum samples confirmed the results obtained on the initial evaluation of 317 serum samples by Pettit et al.: the TRUST antigen is as sensitive and specific as the antigens for the VDRL slide and RPR card tests (3).

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