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Toluidine Red Unheated Serum Test, a Nontreponemal Test for Syphilis

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We have shown that a modification of the color-coded antigen developed by Kasatiya and Lambert (Appl. Microbiol. **28**:317–318, 1974) can be used as a substitute for other nontreponemal antigens used in screening tests for syphilis. The antigen is based on the Venereal Disease Research Laboratory antigen, with EDTA, choline chloride, and toluidine red toner added. Performance of the toluidine red unheated serum test (TRUST) is identical to that of the rapid plasma reagin 18-mm circle card test (U.S. Department of Health, Education, and Welfare, Public Health Service Publication no. 411). In a series of preliminary evaluations, the TRUST antigen was found to be stable over a period of 6 months at 4°C. In a comparison of TRUST with the rapid plasma reagin card test, the qualitative agreement was 100%, whereas agreement between these two tests and the Venereal Disease Research Laboratory slide test was 99.7%. The quantitative agreement ±1 twofold dilution between TRUST and the rapid plasma reagin card test was 100%; between TRUST and the Venereal Disease Research Laboratory slide test it was 75.0%; and between the rapid plasma reagin card and Venereal Disease Research Laboratory slide tests it was 60.0%.

In the mid-1970s, Kasatiya and Lambert (1) and Lambert and Ste-Marie (2) reported on the use of a color-coded antigen in the automated reagin test for syphilis. For several years, this automated reagin test toluidine red toner antigen has been used successfully in the Quebec Public Health Laboratory, Canada. Recently, we chose to modify this antigen for use in an 18-mm circle card macroscopic test to satisfy the need for an inexpensive flocculation test that is easy to perform and read and that gives results comparable to the Venereal Disease Research Laboratory (VDRL) slide (5) and rapid plasma reagin (RPR) card (4) tests for the screening and serodiagnosis of syphilis.

Our version of the Canadian test, the toluidine red unheated serum test (TRUST), is performed with modified VDRL antigen stabilized with EDTA and choline chloride plus toluidine red toner which has been ground to a particle size of ca. 1.4 μm. The toluidine red toner is added for the same reason charcoal is added to the standard RPR card test, to enable the laboratorian to visualize the antigen-antibody reaction. Procedures for TRUST are the same as for the standard RPR card test; with both tests Brewer diagnostic cards are used. The complete antigen preparation, its stability, and an initial evaluation of TRUST are described herein.

MATERIALS AND METHODS

Toluidine red toner. The toluidine red pigment used in this evaluation was kindly supplied by N. G. Lambert and P. S. Harbec of the Quebec Public Health Laboratory, Quebec, Canada, and was obtained from Dominion Color Co., Ltd., Quebec, Canada. Two additional lots of pigment were used for comparison. Lot 4-08018 was supplied by C. J. Rickey of Hercules Corp., and lot 9-31235 was supplied by William J. Hart of Ciba-Geigy Corp., Oakbrook, Ill. A 0.25% suspension of toluidine red toner in distilled water was prepared by wetting and grinding the pigment with distilled water in the capsule of a Torit dental amalgamator by the procedure of Kasatiya and Lambert (1) (P. S. Harbec, personal communication). That is, 0.05 g of toluidine red was placed into the capsule of a dental amalgamator. The mortar was placed into the capsule, and distilled water from a test tube containing 16 ml of distilled water (tube 1) was added to the bevel line. The capsule was shaken for five periods of 30 s

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each, after which the pasty dye was drawn off and placed into a tube containing 4 ml of distilled water (tube 2). The capsule was again filled with water from tube 1 and shaken for three periods of 30 s. The pasty suspension was again drawn off and added to tube 2. The capsule was refilled with distilled water from tube 1 a third time and shaken for two periods of 30 s. With the mortar still in place, all residue pigment was transferred to tube 2. Distilled water from tube 1 was used to rinse the capsule, thus recovering as much dye as possible. The remainder of the distilled water from tube 1 was then added to the dye suspension to make a final volume of ca. 20 ml. The dye suspension was shaken vigorously and stored at 4°C.

TRUST antigen base. The TRUST antigen base was prepared from centrifuged VDRL suspension resuspended in a solution similar to that used to prepare RPR card antigen (4). Initially, 100 ml of VDRL suspension was prepared and checked according to the Centers for Disease Control reference procedure (5). That is, 8 ml of VDRL buffered saline was pipetted to the bottom of a 250-ml, round, glass-stoppered bottle, and then 10 ml of VDRL antigen was added directly onto the buffered saline while the bottle was continuously rotated. Finally, 82 ml of the buffered saline was added, and the suspension was shaken from bottom to top ca. 30 times in 10 s. The VDRL suspension was then centrifuged in an angle centrifuge at room temperature at 2,000 × *g* for 15 min. The resulting supernatant was decanted from the sediment, and any remaining drops of supernatant were wiped away with cotton gauze. The sediment was suspended with a volume of solution (described below) equal to the amount of antigen centrifuged. This was done by expelling the solution directly onto the sediment with a pro-pipette and swirling. All suspensions were combined and shaken gently to resuspend. The resuspending solution was prepared on the day of use and contained 5.0 ml of 0.25 M EDTA, 25.0 ml of 40% choline chloride, 50.0 ml of 0.02 M phosphate–0.2% thimerosal solution, and 20.0 ml of distilled water (Table 1).

A 15-ml amount of well-shaken dye suspension was drawn off and added to 100 ml of antigen suspension, i.e., 0.15 ml of dye suspension to 1 ml of antigen suspension, resulting in a 0.03% final concentration of toluidine red. The TRUST antigen was swirled to mix and then stored at 4°C for 1 week to stabilize. The antigen was then divided into small screw-capped vials and stored at 4°C.

Reference reagents manufactured at the Centers for Disease Control were used throughout this study, with the exception of initial practice lots of antigen, which were made from various sources of commercial VDRL antigen.

TRUST procedure. Antigen from the storage vial was gently mixed and transferred to a dispensing vial (Hynson, Westcott and Dunning, RPR antigen dispensing bottle 8705-09). After the antigen had warmed to room temperature (23 to 29°C), a 20-gauge needle (3/4 in. [19.05 mm] in length) with the bevel removed and which had been calibrated to deliver 0.017 ml or 60 drops/ml[3] (Hynson, Westcott, and Dunning dispensing needle, 8735-09) was attached. Before we proceeded with the actual testing of specimens, the antigen was checked with control sera which were known to give nonreactive, minimally reactive, and reactive results with either the standard RPR card test or the TRUST. In-house controls were prepared as previously described (5). The TRUST was performed with unheated serum at a room temperature of 25 to 27°C. A serum sample of 0.05 ml was placed in the 18-mm circle of a Brewer diagnostic card (Hynson, Westcott, and Dunning, 8718-49) with an automatic pipette. The sample was spread with a wooden stirrer (toothpick), and 1 drop of TRUST antigen was delivered to the test circle

TABLE 1. Resuspending solution preparation

Resuspending solution and reagents	Ingredient (amt)	Special instructions
Stock reagents		
0.02 M phosphate–0.2% thimerosal solution (pH 6.9 ± 0.1)	Secondary sodium phosphate, anhydrous (1.42 g); primary potassium phosphate (1.36 g); thimerosal (1.00 g); distilled water q.s. to 500.0 ml	Store in dark at room temp. May be used for 3 mo
40% Choline chloride solution	Choline chloride (40.0 g), distilled water q.s. to 100.0 ml	Filter with qualitative-grade coarse porosity filter paper. Store in dark at room temp. May be used for 1 yr. Discard if solution turns yellow
EDTA solution (pH 7.0)	EDTA, disodium (9.3 g), distilled water q.s. to 100.0 ml	Adjust pH to 7.0 with NaOH. Store at room temp. May be used for 1 yr
Resuspending solution for 115 ml of TRUST antigen	EDTA solution (5.0 ml), choline chloride solution (25.0 ml), phosphate-thimerosal solution (50.0 ml), distilled water (20.0 ml)	Prepare on same day antigen suspension is made

without stirring. The card was then rotated at 100 rpm for 8 min under a humidity cover. The card was removed after rotating and rocked gently or tilted or both, and the degree of flocculation was observed by using either fluorescent or tungsten lamp illumination. Readings were reported as follows: slight but definite clumping to large flocculation was reported as reactive; smooth, no particulate matter was reported as nonreactive. Reactivity was reported in the same manner as the standard RPR card test; i.e., a minimally reactive serum was reported as reactive instead of weakly reactive as in the VDRL. The quantitative test was performed with reactive sera by making serial dilutions in 0.05 ml of 0.9% NaCl. When the titers of test sera were greater than 1:32, 2% normal human serum in 0.9% NaCl was used for dilution. All dilutions were made on the card with an automatic pipette. For reporting purposes, the endpoint titer was the last dilution which gave a reactive reading.

Preliminary evaluation of TRUST. The TRUST antigen was tested for use on a paraffin-ringed glass slide with the same sample amounts for both serum and antigen as that used for the card test. One of us, P.S.H., designed a glass slide for use with the TRUST antigen. This slide was 7.6 by 11.5 mm and 2 mm thick with 12 wells of 18-mm diameters on the surface which was painted with dull black enamel. The wells were etched out, and the under surface was painted with white lustered enamel. The slides were rotated for 4 min at 180 rpm and 8 min at 100 rpm and then examined.

The stability of the TRUST antigen stored at 4, 26, and 37°C was determined by testing a panel of 18 sera. Of these 18, 11 sera gave various degrees of reactivity as determined by the RPR card and VDRL slide tests, whereas 7 of the sera were nonreactive by these tests. These sera were dispensed into 1-ml samples and frozen. On each antigen testing date, a new vial of serum was tested. Antigen was tested after 1 week and 1, 2, 3, and 6 months of storage in 1-dram vials. The sensitivity, specificity, and reproducibility of the TRUST were compared to those of both the standard RPR card test and the VDRL slide test. A total of 317 sera obtained from the DeKalb County Sexually Transmitted Diseases Clinic, Ga., were evaluated in the three nontreponemal tests without prior knowledge of the patient history or results in the other nontreponemal tests. While this study was in progress, 15 sera selected on the basis of their reactivity in the VDRL test were randomly placed in each day's testing to measure repeatability of the titers in all three test procedures. Each serum was tested three times in each procedure.

RESULTS

Experimental lots of TRUST antigen made with commercial VDRL antigen gave satisfactory results, as did the Centers for Disease Control reference VDRL antigen which was used to make the TRUST antigen for the actual testing of sera from the DeKalb County Sexually Transmitted Diseases Clinic. Satisfactory results were judged as those in which the TRUST yielded the same reactivity as the RPR 18-mm circle card test with Centers for Disease Control reference control serum. In addition, the reactive results were required to be easily distinguished from nonreactive results. The antigen initially had a grainy appearance which smoothed out after 1 week of stabilization at 4°C. The use of the paraffin-ringed glass slides as a matrix for the TRUST was abandoned. Agglutination was immediately visible in the reactive wells when the slide was observed macroscopically; however, nonreactive wells could not be distinguished from minimally reactive wells owing to their extremely grainy appearance, even after the antigen had been stabilized. The slide developed by P.S.H. is still under evaluation at the Quebec Public Health Laboratory and is preferred over the card in that laboratory. Results reported here are based solely on the use of the 18-mm circle card.

Color varied slightly among the lots of pigment tested, but test reactivity did not appear to be affected. It was found, however, that adding

TABLE 2. Stability study at 4°C for lot 4-08018

Serum	Stability at week 0 of:		Stability of TRUST at following times					
	VDRL	RPR	0 wk	1 wk	1 mo	2 mo	3 mo	6 mo
2	R-32[a]	R-32	R-16	R-32	R-64	R-32	R-128	R-32
4	R-1	R-4	R-2	R-2	R-4	R-4	R-2	R-4
6	R-256	R-256	R-128	R-256	R-256	R-128	—[b]	R-128
7	W-0	R-2	R-1	R-1	R-1	R-2	R-1	R-1
9	R-2	R-4	R-4	R-2	R-8	R-2	R-4	R-4
10	W-0	R-2	R-1	R-1	R-1	R-1	R-1	R-1
12	R-4	R-16	R-16	R-16	R-16	R-16	R-8	R-8
13	W-0	R-2	R-1	R-1	R-1	R-1	R-1	N
15	R-2	R-4	R-4	R-4	R-4	R-4	R-4	R-2
17	R-8	R-16	R-32	R-16	R-16	R-16	R-8	R-32
18	W-0	R-1	R-1	R-1	R-1	R-1	N	N

[a] Reciprocal of dilution end-point titer. R, Reactive; W-0, weakly reactive; N, nonreactive.
[b] —, Unable to test specimen.

more than 15 ml of the pigment suspension to the antigen base resulted in graininess which could confuse the reading of test results between minimally reactive and nonreactive sera. Recovery of the pigment from the amalgamator capsule was found to be more effective by using a measured sample of distilled water to rinse the Pasteur pipette residue and capsule residue into tube 2.

The initial reactivity of the TRUST antigen in the stability study was comparable with that of the VDRL slide and RPR card tests (Table 2). Reactivity increased by one twofold dilution after 1 week of storage in two cases. At 6 months of storage at 4°C, all of the sera retained the original degree of reactivity within one twofold dilution except for specimen 18, which dropped from a reciprocal of dilution endpoint titer of R-1 to nonreactive after 3 months, and specimen 13, which dropped from R-1 to nonreactive after 6 months. That antigen stored at 20 and 37°C generally gained reactivity over that of the first week of storage, i.e., it increased in titer by approximately one twofold dilution with those sera originally reactive. Even though nonspecificity did not appear to increase, the antigen became coarser, and thus, it became more difficult to distinguish between minimally reactive and nonreactive. Those sera originally nonreactive were still not reactive, except for serum 3, which was minimally reactive at weeks 2 through 12.

In the initial study of 317 patient sera, 270 were nonreactive in the TRUST, RPR card, VDRL slide, and fluorescent treponemal antibody-absorption tests and therefore were considered normal. A total of 21 sera were reactive in at least one of the nontreponemal tests, whereas 26 were reactive only in the fluorescent treponemal antibody-absorption test and were considered to be from individuals with treated syphilis. All reactive sera obtained in the nontreponemal tests were classified on the basis of clinical history or findings or both as follows: primary syphilis, 3 sera; secondary syphilis, 5 sera; early latent syphilis, 6 sera; late latent syphilis, 1 serum; congenital syphilis, 1 serum; biological false-positive (BFP), 4 sera; and syphilis stage unknown, 1 serum. The qualitative agreement between the TRUST and RPR card procedures was 100%, and the agreement between these two tests and the VDRL slide test was 99.7%. All three nontreponemal tests were 100% sensitive, detecting all 17 sera classified as syphilis. The specificity of the TRUST and the RPR card test was 98.9% (3 of 274) and that of the VDRL slide test was 98.5% (4 of 274). The quantitative agreement (±1 dilution) among the TRUST, RPR card test, and VDRL slide test was as follows: 100% between the TRUST and the RPR card test; 75% between the TRUST and the VDRL slide test; and 60% between the RPR card and the VDRL slide tests (Table 3). Endpoint titers in the TRUST generally fell between those of the VDRL slide test and RPR card test. Lack of quantitative agreement as defined was due to twofold higher titers or greater obtained in the TRUST and RPR card test compared with the VDRL slide test. When the serological response of three successfully treated patients from the DeKalb County Sexually Transmitted Diseases Clinic was followed for several months in all three tests, the TRUST and the RPR card test were of equal reactivity, but the VDRL slide test was generally lower in reactivity than the other two tests. The patients were not followed long enough for the serology to become nonreactive. When the repeatability of the three tests was measured, the titer of each serum was always repeatable within one twofold dilution. These sera had been randomly placed in each day's run and could not be distinguished from the other sera. Nonreactive test results with the TRUST antigen were remarkably smooth, and there was very little problem in distinguishing minimally reactive from nonreactive test results.

Interlaboratory comparison of the manufacture of TRUST antigen and test results with split serum specimens were easily reproducible and will be reported later.

TABLE 3. Comparison of quantitative results among the TRUST, VDRL slide, and RPR card tests

Tests compared	Titer comparison at the following dilution:[a]			
	−1	Equal	+1	≥+2
TRUST titer vs RPR card titer	11 (55)[b]	8 (40)	1 (5)	
TRUST titer vs VDRL titer		10 (50)	5 (25)	5 (25)
RPR card titer vs VDRL titer		4 (20)	8 (40)	8 (40)

[a] No results were obtained at a dilution of ≥−2.
[b] Number of sera tested. Number in parentheses is the percent.

DISCUSSION

By modifying the color-coded antigen of Kasatiya and Lambert (1) for use on Brewer diagnostic cards, the need for an expensive autoanalyzer and the large amount of antigen it uses is eliminated. Because the performance of the TRUST is identical to that of the RPR 18-mm circle card test, it is essentially a familiar technique for most laboratorians to perform. Satisfactory test results have been obtained with the

use of blood plasma (3); therefore, the time and expense of drawing a serum tube for the screening of syphilis can be further reduced.

The antigen is easily prepared and made from relatively inexpensive VDRL antigen, and it appears to be stable for long periods. These factors contribute to the lower cost of performing the TRUST in comparison with some other screening tests for syphilis. The TRUST may also be performed quantitatively, thereby providing a means to aid in the serodiagnosis of syphilis and in the follow-up of treatment schedules.

The TRUST may be used as a substitute for the RPR 18-mm circle card test, thus fulfilling the needs of cost-conscious laboratories in the United States and laboratories in many developing countries, many of which are critically short of equipment, well-trained technicians, proper storage conditions, and reagents. The TRUST antigen appears to be both as sensitive and as specific as the antigens for the VDRL slide and RPR card tests; the antigen is also stable because the reactivity does not appear to increase or decrease appreciably under adverse conditions of antigen storage. The TRUST antigen is a candidate for an inexpensive, satisfactory substitute for other more expensive nontreponemal tests for syphilis.

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