


Latex Agglutination Test for Detecting Antibodies to Treponema pallidum

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We have developed an automated latex agglutination test for Treponema pallidum (TPLA) for measurement of the concentration of treponemal antibodies in syphilitic sera. The assay needs neither a complicated pretreatment of the sera nor special techniques. Intra- and interassay precision studies showed high reproducibility. Potentially interfering substances, such as bilirubin, hemoglobin, triglycerides, and rheumatoid factor, did not affect the results. The results obtained with the TPLA test showed a strong correlation with those from conventional methods.

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This test for antibodies to T. pallidum may be useful for the mass screening of syphilitic infections.

Indexing Terms: syphilis · infection · screening

Syphilis is transmitted by direct, usually sexual, contact. Diagnosis of syphilis is currently based on several criteria: history, clinical appearance, serological tests, and identification of Treponema pallidum in lesions or tissue (1). Among these criteria, serological tests play an important role. The tests currently available are classified according to the antigens used.

Among the tests in which T. pallidum is used as the antigen—such as the T. pallidum immobilization test (2), the T. pallidum hemagglutination (TPHA) test (3, 4), and the fluorescent treponemal antibody-absorption

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(FTA-ABS) test (5)—those most commonly used for clinical serodiagnosis are the FTA-ABS and the TPHA tests. These tests are also recommended by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (Atlanta, GA) (6).

The most commonly used nontreponemal test is the rapid plasma reagin (RPR) test, in which cardiolipin is used as the antigen. The RPR test, which is a variant of the Venereal Disease Research Laboratory (VDRL) test (7), is easy to perform as the VDRL test but cannot be used on an automated analyzer (8). The disadvantage of the RPR test, compared with the treponemal tests, is its lesser specificity, i.e., a greater occurrence of false-positive reactions (9–11).

The FTA-ABS test is regarded as a confirmatory test for doubtful cases. However, the performance of the FTA-ABS test requires highly trained examiners and equipment. It is also time-consuming and expensive (1).

The TPHA test, developed by Rathlev (3) and Tominaga et al. (4), has high sensitivity and specificity, but is not sensitive enough for the diagnosis of primary syphilis (12–14). The animal erythrocytes that are used in the TPHA test may show nonspecific agglutination with high titers of heterophile antibodies. In addition, the quality may vary even in batches from the same manufacturer. This is a concern because internal quality control and proficiency testing are necessary for standardization (1, 15).

To overcome these problems, we have developed the T. pallidum latex agglutination (TPLA) test, using latex particles immobilized with purified T. pallidum antigens.

Materials and Methods

Sera

We tested 102 syphilitic patients’ sera by at least two kinds of serodiagnostic tests and took into account the clinical appearance of the patients. The sera were grouped as follows: (a) untreated primary syphilis (35 samples); (b) treated and untreated secondary syphilis (42 samples); (c) treated and untreated late latent syphilis (23 samples), and (d) congenital syphilis (2 samples). Sodium azide (1 g/L) was added to each of the samples, which were then stored at 4 °C. Nonsyphilitic sera that were nonreactive in the RPR test and the TPHA test (n = 76) were assayed and were also stored at 4 °C. In addition, 20 false-positive serum samples were tested: They were nonreactive in the TPHA test but were reactive in other tests, such as the RPR test. All serum samples were obtained from the Osaka Prefectural Ban- dai Clinic. A pool of rheumatoid factor-positive sera (from the Center for Diagnostic Products, Milford, MA) was determined with the use of WHO Standard Serum.

TPLA Test Kit

Latex preparation. We isolated and purified the treponemal antigens by the methods of Matsumoto et al. (submitted for publication). Antigen-immobilized latex particles were prepared by the following procedure. We mixed 1 mL of the 100 g/L suspension of polystyrene latex particles (Sekisui Chemical Co., Ltd., Osaka, Japan; diameter: 0.400 μm) with 4 mL of purified antigen solution at a final protein concentration of 15 mg/L. The mixture was then incubated at 4 °C on an automated stirrer at 700 rpm for 60 min. The antigen-immobilized latex particles were then blocked with bovine serum albumin (BSA, Fraction V, reagent grade; Miles Inc., Kankakee, IL) by adding 20 mL of phosphate-buffered saline (23 mmol/L KH2PO4 and 13 mmol/L Na2HPO4 · 12 H2O, adjusted to pH 6.5, and NaCl, 126 mmol/L) containing 10 g/L BSA. The mixture was stirred at 4 °C for 90 min, then washed three times by centrifugation with 25 mL of sodium phosphate buffer (NaPB: 100 mmol/L Na2HPO4 · 12 H2O and 100 mmol/L NaH2PO4 · 2 H2O, adjusted to pH 7.4) containing 10 g/L BSA and 1 g/L sodium azide (reagent grade; Nacalai Tesque Inc., Kyoto, Japan), and resuspended in 10 mL of the same buffer. Afterwards, the suspension was slightly dispersed by ultrasonication (Astrazon Model W-385; Heat Systems Ultrasonics Inc., New York, NY), and was resuspended to a concentration of 2.5 g/L in NaPB containing 10 g/L BSA. It was stored at 4 °C.

Dilution buffer. NaPB (100 mmol/L, pH 7.4) containing 2.5 g/L BSA, 1 g/L sodium azide, and 2.5 g/L polyethylene glycol (PEG 500 000; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a dilution buffer for the serum samples.

Standard syphilitic serum. Syphilitic serum standards containing various concentrations of antibodies to T. pallidum were made by diluting pooled human syphilitic serum with pooled normal human serum.

After dilution, standard syphilitic sera were freeze-dried and stored at 4 °C. The concentrations of antibodies to T. pallidum were determined by calibrating them by comparison with the diluted samples of International Standard for Human Syphilitic Serum established by the WHO Expert Committee on Biological Standardization in 1957 (16). In this test, we used five standard syphilitic sera, ranging in concentrations from 55 to 931 titer units (TU): 1280 TU is equivalent to 2.10 International Units of International Standard for Human Syphilitic Serum, and is equivalent to a TPHA titer of 1280 when it is determined by the commercial TPHA test kit.

Other Reagents

The FTA-ABS test kit (FTA-ABS set "Eiken;" Eiken Chemical Co., Ltd., Tokyo, Japan), the TPHA test kit (Serodia-TP; Fuji Rebio Co. Ltd., Tokyo, Japan), and the RPR test kit (RPR Test "Kaketsuken;" The Chemo-Serotherapeutic Research Institute, Kumamoto, Japan) were used. Bilirubin, hemoglobin (Sigma Chemical Co., St. Louis, MO) and triglycerides (International Re-
agents Corp., Tokyo, Japan) were used. All materials were of reagent grade.

Apparatus
All determinations were monitored with a fully automated Hitachi 7050 analyzer (Hitachi, Ltd., Tokyo, Japan) at a wavelength of 570 nm. The reaction was carried out at 37 °C in a 0.5-cm-pathlength cuvette.

Procedure
The assay protocol for all the experiments was performed by a Hitachi 7050 loaded with a programmable calculator. A sample (20 μL) of serum and 350 μL of dilution buffer were dispensed into the cuvette and kept at 37 °C. After incubation for 300 s and addition of 50 μL of latex reagents, the absorbance change between 80 s and 320 s was measured at 570 nm. All data were calculated via computer programs loaded onto the programmable calculator. The absorbance change for each standard syphilitic serum was corrected for corresponding blank values. A nonlinear standard curve was fitted by the method of curve-fitting approximation. The concentration of antibodies to T. pallidum in each of the unknown samples was calculated from the absorbance change found on the calibration curve.

Results
Calibration Curve
Five concentrations of freeze-dried standard syphilitic sera (55, 116, 235, 500, and 931 TU) and isotonic saline as a blank (0 TU) were assayed in duplicate (Figure 1). The absorbance values ranged from 0.03 to 0.50 as the antibody titer of standard syphilitic sera varied. The detection limit, defined as 3 SD for the reagent blank signal, was 13.5 TU (data not shown).

We determined the intraassay CV for the TPLA test by analyzing four serum samples having different concentrations of T. pallidum antibodies, in 10 replicates in the same run. The interassay CV was estimated by determining T. pallidum antibodies in serum samples at four concentrations on 10 different occasions (Table 1).

Analytical Recovery
To determine the analytical recoveries of the TPLA test, we assayed three serum samples (samples A, B, and C) after adding different concentrations of T. pallidum antibodies, ranging from 0.0 to 130.1 TU. Recovery ranged from 94.7% to 100.7% (mean 98.3%) (Table 2).

Effects of Interfering Substances
We studied the effects of bilirubin, hemoglobin, triglycerides, and rheumatoid factor on this test. After adding three components to sample A and rheumatoid factor to sample B, we determined the recovery of T. pallidum antibodies. As shown in Table 3, none of these substances interfered with the results.

Linearity
The linearity of the TPLA test was assessed by diluting three serum samples with isotonic saline in the following volume ratio (serum:isotonic saline): 1:1, 1:3, 1:7, 1:15, 1:31. We measured the antibodies to T. pallidum in the undiluted samples, from which we calculated the expected values of the diluted samples. Correlation between the observed and expected values were as follows: Sample A: r = 1.000; sample B: r = 0.999; sample C: r = 0.999 (Figure 2).

Table 1. Intra- and Interassay Precision of TPLA Test

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.5</td>
<td>0.8</td>
<td>2.5</td>
<td>31.1</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>72.9</td>
<td>1.6</td>
<td>2.1</td>
<td>66.9</td>
<td>1.8</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>483.8</td>
<td>22.0</td>
<td>4.7</td>
<td>467.0</td>
<td>5.6</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>762.2</td>
<td>17.0</td>
<td>2.2</td>
<td>755.2</td>
<td>14.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Ten preparations of four serum samples with different concentrations of antibodies were measured.

Four serum samples containing different concentrations of antibodies were assayed in five separate runs.

Table 2. Analytical Recovery of the TPLA Test

<table>
<thead>
<tr>
<th>Antibodies added, TU</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>61.7</td>
<td>227.9</td>
<td>416.3</td>
</tr>
<tr>
<td>25.9</td>
<td>88.3 (100.0)</td>
<td>253.4 (99.8)</td>
<td>435.1 (98.4)</td>
</tr>
<tr>
<td>58.5</td>
<td>115.6 (96.2)</td>
<td>280.1 (97.8)</td>
<td>484.8 (97.9)</td>
</tr>
<tr>
<td>130.1</td>
<td>188.3 (98.2)</td>
<td>360.8 (100.7)</td>
<td>517.7 (94.7)</td>
</tr>
<tr>
<td>Mean</td>
<td>98.4</td>
<td>99.4</td>
<td>97.0</td>
</tr>
</tbody>
</table>

* (Amount measured/amount expected) × 100.
Table 3. Effects of Potentially Interfering Substances

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Antibodies measured, TU</th>
<th>Recovery* %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Bilirubin</td>
<td>210 mg/L</td>
<td>61.0</td>
<td>95.9</td>
</tr>
<tr>
<td>+ Hemoglobin</td>
<td>15 000 mg/L</td>
<td>61.6</td>
<td>96.8</td>
</tr>
<tr>
<td>+ Triglycerides</td>
<td>850 mg/L</td>
<td>63.2</td>
<td>99.4</td>
</tr>
<tr>
<td>Sample B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Rheumatoid factor-</td>
<td>2512 kIU/L</td>
<td>99.1</td>
<td>100.8</td>
</tr>
<tr>
<td>positive serum</td>
<td>1330 kIU/L</td>
<td>98.3</td>
<td>100.0</td>
</tr>
<tr>
<td>480 kIU/L</td>
<td>98.6</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>102 kIU/L</td>
<td>98.6</td>
<td>100.3</td>
<td></td>
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</tbody>
</table>

* (Amount measured/amount expected) x 100.

Distribution of TPLA Values

Analysis of the distribution of TPLA values by disease stage (Figure 3) revealed characteristic profiles for each stage. The mean ± SD treponemal antibody concentration in 76 normal sera was 0.5 ± 1.6 TU with a range of 0.0–12.7 TU; that of false-positive sera was 0.7 ± 2.5 TU with a range of 0.0–11.2 TU. The antibody concentrations from 102 patients' sera ranged from 26.2 to 1162.3 TU. Therefore, we set the positive–negative borderline at 20 TU. We show the sensitivity and specificity of the TPLA test and of other tests according to the stage of syphilis in Table 4. The TPLA test showed strong specificity and sensitivity compared with the FTA-ABS test and the TPHA test in all stages.

Comparison with Conventional Tests

Using 102 syphilitic patients' sera, 76 normal sera, and 20 false-positive sera as determined by the TPLA test, we compared the results of the present method with those of three conventional methods. We used the TPHA test kit, the FTA-ABS test kit, and the RPR test kit. Results of the methods comparison experiments are shown in Figure 4.

Discussion

We have reported a new type of serodiagnostic test for syphilis. During the study, the following advantages became apparent. First, the TPLA test can be used on a fully automated analyzer, which saves personnel time and secures precision. We can also use this test on other automated analyzers that can calculate the results by the method of curve-fitting approximation (data not shown). Therefore, this test seems suitable for mass screening.

Second, because the agglutination reaction was based on the binding of antibodies in the syphilitic serum to the antigens immobilized on the latex particles, high specificity was expected. Because the degree of agglutination was detected as the absorbance change, the concentration of T. pallidum antibody was obtained as successive values.

We used the standard syphilitic sera adjusted to the International Standard for Human Syphilitic Serum established by the WHO Expert Committee on Biological Standardization so that we could compare the results
of this test with those of conventional methods. The higher sensitivity resulted in superior precision and the opportunity to maintain high analytical performance with use of photometric equipment (Tables 1, 2, and 4).

Potentially interfering substances showed no influence on this assay; the TPLA test thereby avoided the usual nonspecific agglutination (Table 3).

The TPLA test showed T. pallidum antibodies of at least 26.2 TU in all the syphilitic sera and 0.0–12.7 TU in the nonsyphilitic sera tested. The positive–negative borderline was therefore defined at 20 TU, although this will require further confirmation on a larger scale.

Among syphilitic serum samples, the sensitivity of the TPLA test was 100% each for primary, secondary, late latent, and congenital syphilis. These results are comparable with those obtained with the TPHA test and the FTA-ABS test.

Serodiagnostic tests for syphilis are commonly used in the evaluation of patients with genital lesions. We also studied an additional seven serum samples from untreated subjects with darkfield microscopy-positive chancres (data not shown). The sensitivity of the TPLA test was 71% (5 of 7). The percentages of the RPR test (5 of 7, 71%) and the FTA-ABS test (4 of 7, 57%) were not significantly different from that of the TPLA test. The two RPR and FTA-ABS test-negative sera from individuals with primary syphilis were the same as the TPLA test-negative samples. The TPLA test proved to be sensitive for detecting antibodies in the sera of patients with primary syphilis. Because we did not determine the IgM concentration, we have yet to confirm the sensitivity of the test to IgM antibodies by using an HPLC separating system (M. Matsumoto et al., in preparation).

The 100% specificity of the TPLA test compared favorably with the conventional tests (Table 4). This specificity, as well as the high sensitivity, was due to the use of the nonantigenic latex particles and purified treponemal antigens.

It was difficult to compare the results of the TPLA test with those of other conventional tests directly because only the value of the TPLA test was quantitative. The results of the TPHA test, the FTA-ABS test, and the RPR test were expressed as reciprocal titers, intensities of fluorescence, and dilution rates, respectively. Because the TPLA test has similar characteristics to those tests, however, the results of the comparison showed a high correlation.

In conclusion, the use of latex particles on a fully automated analyzer, and the short reaction time (10 min compared with about 2 h for the TPHA test) make

| Table 4. Comparison of the TPLA Test with Other Serological Tests |
|-------------------------|-----------------|-----------------|----------------|-----------------|
| Diagnosis               | No. of serum samples | TPLA  | TPHA  | FTA-ABS | RPR  |
| Primary                 | 35               | 35 (100)*   | 35 (100) | 35 (100) | 33 (94.3) |
| Secondary               | 23               | 23 (100)   | 23 (100) | 23 (100) | 22 (95.7) |
| Late latent             | 42               | 42 (100)   | 42 (100) | 42 (100) | 36 (85.7) |
| Congenital              | 2                | 2 (100)    | 2 (100)  | 2 (100)  | 2 (100)   |
| Normal sera             | 76               | 0           | ND      | 0          |
| False-positive sera     | 20               | 0           | 0       | 0          | 17 (85.0) |

ND, Not determined.

* No. positive detected (% positive).
the TPLA test useful for mass screening. We are now studying the possibility of using the TPLA test to monitor syphilitic patients treated with antibiotics and patients with untreated early primary syphilis.

References

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Adsortion Losses from Urine-Based Cannabinoid Calibrators during Routine Use
Jennifer A. Blanc,¹ Victor A. Manneh,¹ Roberta Ernst,¹ Donald E. Berger,¹ Steve A. de Kecker,² Chip Chase,³ Joan M. Centofanti,¹ and Anthony J. DeLizza¹,4

The major metabolite of cannabis found in urine, 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid (Δ⁹-THC), is the compound most often used to calibrate cannabinoid immunoassays. The hydrophobic Δ⁹-THC molecule is known to adsorb to solid surfaces. This loss of analyte from calibrator solutions can lead to inaccuracy in the analytical system. Because the calibrators remain stable when not used, analyte loss is most probably caused by handling techniques. In an effort to develop an effective means of overcoming adsorption losses, we quantified cannabinoid loss from calibrators during the testing process. In studying handling of these solutions, we found noticeable, significant losses attributable to both the kind of pipette used for transfer and the contact surface-to-volume ratio of calibrator solution in the analyzer cup. Losses were quantified by immunoassay and by radioactive tracer. We suggest handling techniques that can minimize adsorption of Δ⁹-THC to surfaces. Using the appropriate pipette and maintaining a minimum surface-to-volume ratio in the analyzer cup effectively reduces analyte loss.

Indexing Terms: abused drugs · sample handling · variation of source of

Cannabinoids are hydrophobic molecules subject to adsorption to solid surfaces from aqueous solutions such as urine (1). The major metabolite of cannabis found in urine, 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid (Δ⁹-THC, 1, see Figure 1), is the principal analyte measured when testing for cannabis use. Most immunoassay methods are calibrated and monitored for quality control with solutions containing this metabolite. When cannabinoid metabolites are lost from the calibrator or control solution through adsorption, the accuracy of the test measurements is affected. Although commercial preparations of Δ⁹-THC are chemically stable (unpublished data), Δ⁹-THC concentrations in calibrators and controls have been observed to decline during normal use (2). Unused calibrators and controls do not exhibit this decline, suggesting that the loss of drug occurs during use and may be attributable to handling techniques.

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Nonstandard abbreviations. Δ⁹-THC, 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid; Δ⁹-THC, 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid; dpm, disintegrations per minute; TLC, thin-layer chromatography; GC/MS, gas chromatography with mass spectrometry; NIDA, National Institute on Drug Abuse.