JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1985, p. 387–389
0095-1137/85/030387-03$02.00/0

Vol. 21, No. 3

Four-Step Enzyme-Linked Immunosorbent Assay for Detection of *Treponema pallidum* Antibody

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Received 16 August 1984/Accepted 16 November 1984

Further studies of a four-step enzyme-linked immunosorbent assay procedure to detect *Treponema pallidum* antibody are described. High-titered antibody, produced in rabbits by intravenous injection of *T. pallidum*, was used to coat polyvinyl chloride microtiter plates. To these plates a known concentration of *T. pallidum* was added, followed in successive steps by serial dilutions of human sera and appropriately diluted peroxidase-labeled anti-human immunoglobulin G antibody. *o*-Phenylenediamine was the substrate. A total of 340 sera were obtained from the DeKalb County Sexually Transmitted Diseases Clinic, Atlanta, Ga., and examined within 3 days of receipt. Ninety-six percent test agreement between the enzyme-linked immunosorbent assay and the fluorescent treponemal antibody absorption-double staining test was obtained. A total of 372 additional sera stored at −20°C were examined. The overall sensitivity of the enzyme-linked immunosorbent assay with sera from patients with various stages of syphilis was 96%. With sera from uninfected individuals, the specificity of the enzyme-linked immunosorbent assay was 95%. No antigen instability was noted with the two antigen preparations used during this evaluation.

The enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G (IgG) antibodies to *Treponema pallidum* has been reported previously (3, 5–7). These reports describe a three-step method in which the antigen is bound directly to the microtiter plate and antibody and anti-human conjugate are added successively.

The *T. pallidum* ELISA method described in this report differs from the three-step methods in that it involves the use of two antibodies, one bound to the plate and one linked to the enzyme. This ELISA method is similar to that described by Yolken for *Escherichia coli* enterotoxin (9). We have compared the sensitivity and specificity of this four-step ELISA for the detection of *T. pallidum* antibody with those of the fluorescent treponemal antibody absorption-double staining (FTA-ABS-DS) test (2, 4).

MATERIALS AND METHODS

Coating antibody. Anti-*T. pallidum* antibody was obtained from a rabbit after six intravenous injections of the *T. pallidum* Nichols strain in 0.5% formalinized saline. Progressively increasing amounts of inoculum containing 5.4×10^8 treponemes per ml were used (0.2, 0.25, 0.5, 1, 1.5, and 2.0 ml) on days 1, 5, 10, 13, 16, and 20. Antibody obtained from bleeding on day 27 was used without further purification. The immunofluorescence titer was ≥5,120. The optimal dilution for coating plates was determined by block titration (8) to be 1:10,000.

Antigen. *T. pallidum* Nichols strain was propagated in rabbit testicular tissue, extracted in phosphate-buffered saline (PBS) containing 0.075 M sodium citrate, and sonicated on a Bronson sonifier three times for 15-s intervals at 50% output as described previously (3). The optimal dilution was determined by block titration to be 1:350, and although sonicated, there were approximately 4×10^7 treponemes per ml before dilution.

Conjugate. Goat anti-human IgG conjugated with horseradish peroxidase was obtained from Miles Laboratories, Elkhart, Ind.

Sera. Fresh sera in this study consisted of 340 sera obtained from the DeKalb County Sexually Transmitted Diseases Clinic, Atlanta, Ga., and examined within 3 days. Patient histories were unavailable for the patients without syphilis. However, these sera had nonreactive nontreponemal test results, and the individuals had no clinical symptoms of syphilis. A total of 372 sera stored for up to 4 years were obtained from the serum bank of the Sexually Transmitted Diseases Program, Centers for Disease Control, Atlanta, Ga. Clinical documentation indicated that 35 sera were obtained from individuals with primary syphilis, 68 were obtained from individuals with secondary syphilis, and 82 were obtained from individuals with latent syphilis; 187 sera were obtained from individuals presumed not to have syphilis.

ELISA procedures. A schematic diagram of this ELISA is shown in Fig. 1. This is a modification of that used by Voller et al. (8) and Yolken et al. (9). The steps for the four-step ELISA are as follows.

(i) Rabbit anti-*T. pallidum* antibody, diluted 1:10,000 in 0.05 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.6), was used to coat the wells of polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.); 50-μl volumes were plated in each well. The plates were incubated overnight at 4°C, washed once in PBS–0.05% Tween 20 (PBS-T), allowed to air dry, wrapped in foil, and frozen at −20°C until use. Plates stored for up to 2 months have been satisfactory.

(ii) *T. pallidum* antigen was appropriately diluted in PBS (pH 7.2) and plated in 50-μl volumes per well. The optimal dilution of the suspension was determined by block titration. The plates were incubated in a moist chamber at room temperature overnight.

(iii) After the plates were washed three times with PBS-T wash buffer, sera were added in 100-μl amounts. Based on previous studies in our laboratory (3, 6), twofold serial dilutions starting at 1:100 were prepared with Titertek mul-

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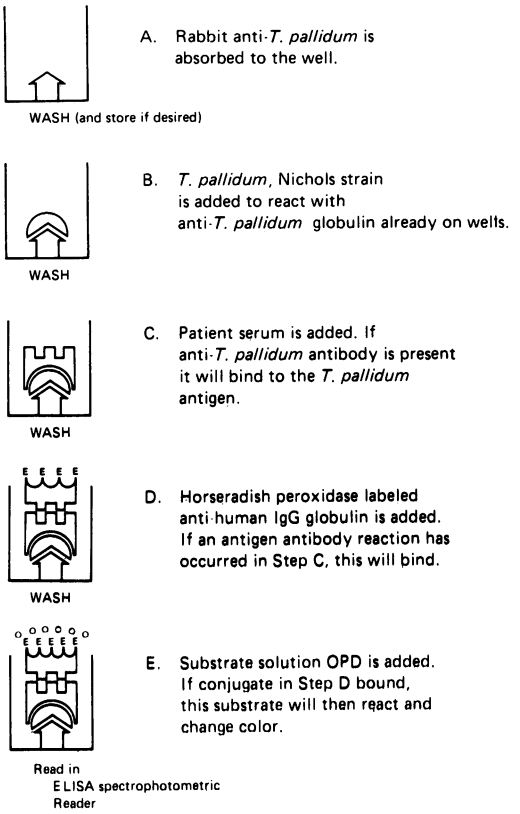

FIG. 1. Schematic diagram of the ELISA for *T. pallidum* antibody. (Modified from Voller et al. [8].)

tichannel pipettors (Flow Laboratories, Inc., McLean, Va.). The diluent was 1% normal rabbit serum–PBS-T. The plates were incubated overnight at 4°C.

(iv) The plates were again washed three times, and horseradish peroxidase-labeled anti-human IgG conjugate diluted in the 1% normal rabbit serum–PBS-T buffer was added to wells in 100-μl amounts. The plates were incubated in moist chambers at 37°C for 1 h.

(v) After the plates were washed three times with wash buffer, *o*-phenylenediamine substrate was added in 100-μl amounts and allowed to react in the dark for 30 min at room temperature. The reaction was stopped by the addition of 25 μl of 8 N H_2SO_4 per well. The absorbance of each reaction was read at 490 nm in a Dynatech MR580 MicroELISA reader with a buffer control as a blank. The cutoff between positive and negative values was set at 0.2 on the basis of control sera and previous ELISA results (3). Control sera for this study were portions of the same sera used previously (3).

FTA-ABS-DS. The FTA-ABS-DS test was performed as previously reported (2, 4). *T. pallidum* antigen for the FTA-ABS-DS test was fixed to microscope slides in acetone for 10 min. Human sera diluted in FTA-ABS sorbent were added to the smears and incubated at 37°C for 30 min. The slides were washed in PBS and distilled water, and then rhodamine-labeled anti-human IgG, appropriately diluted, was added to each smear. The slides were incubated at 37°C for 30 min, and the washing step was repeated. Fluorescein-labeled anti-treponemal globulin was added as the counterstain. The slides were incubated for 20 min at 37°C. After being washed and mounted, the slides were read with a Leitz Ortholux II microscope equipped with incident illumination.

TABLE 1. Comparison of reactivity of ELISA and FTA-ABS-DS tests

Serum group	No. of sera with given reaction[a]			
	FTA-ABS-DS R, ELISA R	FTA-ABS-DS R, ELISA N	FTA-ABS-DS N, ELISA R	FTA-ABS-DS N, ELISA N
Fresh[b]	9	3	10	318
Syphilis[c]	177	6	0	2
Nonsyphilis[c]	1	0	9	177

[a] R, Reactive; NR, nonreactive (in agreement in 684 of 712 cases [96%]).
[b] Histories unavailable; sera held for 1 to 3 days at 2 to 8°C.
[c] Clinically documented sera held at −20°C.

RESULTS

Table 1 shows a comparison of the reactivity of the various sera on the basis of the ELISA and FTA-ABS-DS test results. For 684 of the 712 sera tested (96%), the results were in agreement for both tests. A definite statement cannot be made concerning the 13 of 340 fresh sera that showed discrepant reactivity between ELISA and the FTA-ABS-DS test. Available data place them in the nonsyphilis category, and reactivity in all these 13 cases was low in either test system (R1+ in the FTA-ABS-DS test, and endpoint titer of 100 in ELISA). For categorized sera from syphilis patients, 179 of 185 sera gave results which agreed for the two treponemal tests. Of the six sera that were nonreactive in ELISA and reactive in the FTA-ABS-DS test, two were categorized as being from individuals with primary syphilis, one was categorized as being from an individual with secondary syphilis, and three were categorized as being from individuals with latent syphilis. For sera from nonsyphilitic individuals, 178 of 187 showed agreement between the test results for the two tests. The nine sera that were reactive in ELISA and nonreactive in the FTA-ABS-DS test were reactive only at the initial endpoint titer of 100. Agreement in results for the ELISA and the FTA-ABS-DS test for the 712 sera was as follows: nonsyphilis, 95%; syphilis, 97%; total stored sera, 96%; and total fresh sera, 96%.

Table 2 shows a comparison of ELISA and FTA-ABS-DS test reactivity for the 372 stored sera for which case histories were available and categorization could be made. The percentage of reactive results on sera from syphilitic individuals is slightly higher in the FTA-ABS-DS test in all three categories (100, 100, and 98% in the FTA-ABS-DS test compared with 94, 99, and 94% in ELISA for the primary, secondary, and latent categories, respectively). The percentage of reactive results on sera from nonsyphilitic individuals is slightly lower in the FTA-ABS-DS test (0.5% for the FTA-ABS-DS test compared with 5% in ELISA). The

TABLE 2. Comparison of test reactivity by category for 185 sera from syphilitic individuals and 187 sera from nonsyphilitic individuals

Syphilis category	No. of sera (%) with reactivity in:		Total no. of reactive sera
	ELISA	FTA-ABS-DS	
Primary	33 (94)	35 (100)	35
Secondary	67 (99)	68 (100)	68
Latent	77 (94)	80 (98)	82
Nonsyphilis	9 (5)	1 (0.5)	187

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results compare favorably between the tests. Sensitivity is based on the number of syphilis sera showing reactivity, whereas specificity is based on the number of nonsyphilis sera showing nonreactivity. The sensitivity was 96% for ELISA and 99% for the FTA-ABS-DS test. The specificity was 95% for ELISA and 99% for FTA-ABS-DS test.

DISCUSSION

The results of this study indicate that the four-step ELISA for *T. pallidum* IgG antibody could be an alternative to other treponemal tests for syphilis antibody. The sensitivity and specificity were excellent and were comparable to the sensitivity and specificity of the FTA-ABS-DS test, with a 96% agreement between the two tests. This would give laboratories the choice between using a test that is read microscopically or the ELISA, which is read with the automatic plate reader. ELISA and FTA-ABS-DS results would not be expected to agree completely with every serum tested because of inherent test differences such as antigen-binding sites (5), and with larger serum evaluations the percentages for agreement, sensitivity, and specificity could vary slightly.

The specificity based on categorized sera from nonsyphilis patients was 95%, only slightly lower than that of the FTA-ABS-DS test at 99%. Future evaluation of this new treponemal ELISA will be focused on obtaining an even higher specificity through studies of reagents and use of larger populations. The sensitivity for the categorized sera from syphilis patients was 96% for ELISA, which is an excellent correlation with the FTA-ABS-DS test sensitivity at 99%. Percentages for sera in the primary, secondary, and latent categories were 94, 99, and 94%, respectively, further emphasizing the earlier importance of using IgG in the diagnosis of *T. pallidum* infection.

There appeared to be several advantages to this four-step ELISA which contributed to the specificity and sensitivity. The first was the storage time and stability of the coated plates. Coating antibody is simple to prepare and is used at a high titer, resulting in minimal reagent cost. Both sensitivity and specificity should be enhanced when the plate is coated with the specific antibody to the specific antigen (*T. pallidum*) and then blocked with PBS-T to minimize background reactions (1) that could occur with the antigen step (9). Stability within the test is created by the availability of a constant reagent throughout many ELISAs. This constancy would help to create part of the controlled system that must exist for optimum reproducibility. Plates were stored for up to 2 months for this study and presently have been stored for 4 months without difficulty.

A second advantage seen in the four-step ELISA is the storage time and stability of the antigen. Two lots of *T. pallidum* antigen in PBS were used for 2 months during this evaluation and stored at 2 to 8°C. Antigens have remained stable for an additional 2-month period with no drop in antigen titer over time. No variability or problems with nonspecificity have been noted so far, possibly because of the position of the antigen as second in the sandwich. A relatively high dilution of antigen can be used in this procedure, resulting in conservation of a critical reagent. Although the antigen is not difficult or time consuming to prepare, test reproducibility is generally enhanced when the same preparation of reagent can be used over a long period.

Finally, the main advantage of the ELISA procedure is that the use of automatic plate readers eliminates the subjectivity of visual interpretation. Therefore, based on the sensitivity and specificity obtained and on the advantages of this particular procedure, the test results with the four-step ELISA support the potential consideration of the assay as a confirmatory test for the serodiagnosis of syphilis.

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