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Evaluation of the Microenzyme-Linked Immunosorbent Assay with *Treponema pallidum* Antigen

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Whole-cell sonicates of *Treponema pallidum*, Nichols strain, were evaluated in an enzyme-linked immunosorbent assay (ELISA) for syphilis, and results were read in a Dynatek Microelisa Reader. The antigen was evaluated with sera from patients with syphilis, persons presumed normal, and biological false-positives. Two hundred and ninety-seven sera were tested by the ELISA with *T. pallidum* antigens, the Venereal Disease Research Laboratory (VDRL) slide test, the fluorescent treponemal antibody absorption (FTA-Abs) test, and the microhemagglutination assay for *T. pallidum* antibodies (MHA-TP). The results of all of the tests were compared. The ELISA, with 89.3% sensitivity, was less sensitive than the VDRL (93.3%) and FTA-Abs (100.0%) tests but more sensitive than the MHA-TP (76.0%). The ELISA was considerably more sensitive in primary syphilis than the MHA-TP. Specificity was as follows: ELISA, 98.5%; FTA-Abs test, 97.8%; MHA-TP, 98.2%; and VDRL test, 92.7%. The ELISA has good potential as a confirmatory test in the serodiagnosis of syphilis.

The enzyme-linked immunosorbent assay (ELISA) was developed independently in 1971 by Engvall and Perlmann (2) and van Weeman and Schuurs (10). In 1975, Ruitenberg et al. (8) and Voller et al. (12) published microtechniques that improved test methodology. Also in 1975, Veldkamp and Visser published a method for detecting treponemal antibody by using the ELISA (11). Since the ELISA procedure offers a number of advantages over current treponemal tests for syphilis, a study following Veldkamp and Visser's procedure and adapted to the microtechnique described by Walls et al. for toxoplasmosis (13) was investigated. Two advantages of the ELISA are the stability of the reagents and the availability of automation to interpret the results objectively.

Treponema pallidum, Nichols strain, was evaluated in the ELISA procedure, and the results were compared with those obtained with the Venereal Disease Research Laboratory (VDRL) test (6), the fluorescent treponemal antibody absorption (FTA-Abs) test (6), and the microhemagglutination assay for *Treponema pallidum* antibody (MHA-TP) (7).

MATERIALS AND METHODS

Sera. Thirty-two sera from documented "normal" people, seven from persons having diseases other than syphilis, and three from persons with a false-positive reagin test (VDRL) were obtained from the Centers for Disease Control (CDC) syphilis serum bank. All of the sera had been stored for several years at −20°C. An additional 25 sera from persons with diseases other than syphilis were obtained from the CDC serum bank. Seventy-two sera from known syphilitic individuals were obtained from the CDC syphilis serum bank.

One hundred and fifty-nine fresh sera from presumed normal individuals were obtained from specimens submitted to the Venereal Disease Serology Laboratory from a Public Health Service outpatient clinic as part of routine physicals and from healthy blood donors from two blood banks. Fresh sera from patients with syphilis and from patients with biological false-positive reactions were obtained from the Houston City Health Department, Houston, Tex., and the Fulton County Health Department, Atlanta, Ga.

All sera were tested in the VDRL test, the FTA-Abs test, the MHA-TP, and the ELISA as received and then stored at −20°C. The VDRL and FTA-Abs tests were done according to the 1969 *Manual of Tests for Syphilis* (6). The MHA-TP was performed according to the provisional technique of 1977 (7).

Antigens. For each antigen, 11 rabbit testes infected with *T. pallidum*, Nichols strain, were minced aseptically, and the tissue was transferred to a flask containing 15 ml of phosphate-buffered saline (PBS) with 0.075 M sodium citrate per testis (11). The extracting fluid was centrifuged at 2,000 rpm for 10 min to remove gross tissue debris. The supernatant containing the treponemes was held at 5°C for 2 to 3 days. The suspension was concentrated at 17,300 × *g* for 30 min at 5°C, and the sediment was washed three times in PBS. After the final centrifugation, the cells were suspended in 10 ml of pH 9.6 carbonate buffer, and the number of treponemes per milliliter was established as described for the *T. pallidum* immunobilization test (1). Satisfactory antigens contained 5.3×10^6 to 11.7×10^7 treponemes per ml. To disrupt the treponemes, the suspensions were sonicated on a Biosonik IV sonicator (Bronwill Scientific Inc., Rochester, N.Y.) at the

low setting, force of 4, for three 15-s intervals. Darkfield examination revealed no intact treponemes.

Protein determinations by the Lowry method (5) were made on all antigens. The working dilution contained approximately 3 μg of protein per ml of antigen; however, the optimal dilution was determined by block titration.

Substrate. A stock solution of *ortho*-phenylenediamine (Eastman Kodak Co., Rochester, N.Y.) was made by adding 100 mg to 10 ml of absolute methanol. The working solution was made by adding 1 ml of stock solution and 0.1 ml of 3% hydrogen peroxide to 99 ml of distilled water (13).

Conjugate. Goat anti-human immunoglobulin G (gamma chain) conjugated to horseradish peroxidase (Miles Laboratories, Elkhart, Ind.) was used. The optimal dilution was determined by block titration and found to be 1:1,000. The conjugate was diluted in PBS with 0.05% Tween 20 (PBS-T20) with 1% bovine serum albumin.

ELISA test procedures. One hundred microliters of antigen was placed in each well of Cooke Microelisa plates (Dynatech Laboratories, Inc., Alexandria, Va.). The plates were then sealed and incubated in a 37°C water bath for 3 h. At the end of the incubation, the plates were removed to a 5°C refrigerator and stored until needed. When used, the plates were washed three times with PBS-T20, and PBS-T20 was placed in columns 2 through 6 and 8 through 12. Two hundred microliters of a 1:100 dilution of serum was placed in columns 1 and 7, one-half row for each serum, including a reactive and nonreactive control. With a pipette of 100-μl capacity, a twofold dilution of each serum was made through the next five wells so that the final dilution was 1:3,200. The plates were then covered and incubated in the 37°C water bath for 2 h. The plates were washed as before, and 100 μl of the optimal conjugate dilution was added to each plate. The plate was covered and incubated in the 37°C water bath for 20 min. The plates were removed and washed, and 100 μl of working *ortho*-phenylenediamine substrate was added to each well. The plates were then incubated in the dark at room temperature (25°C) for 30 min. Twenty-five microliters of 8 N sulfuric acid was added to each well to stop the reaction. The plates were read on a Dynatech Microelisa Reader with a filter giving a wavelength of 488 nm. An optical density of 0.2 or above was considered reactive. The machine was blanked on the 1:3,200 dilution of the nonreactive control.

RESULTS

Table 1 shows a comparison of reactivity between the FTA-Abs test and the ELISA. Three nonsyphilis sera were reactive in the FTA-Abs test but not in the ELISA. One of these sera was from a person presumed normal, one was from a drug addict, and one was from a patient with smallpox. Two sera were nonreactive in the FTA-Abs test and reactive in the ELISA. These were from a patient with brucellosis and a presumed normal individual. There were eight sera in the syphilis category that were reactive in the FTA-Abs test but not in the ELISA. Most of these were in the primary category. No sera were reactive in the ELISA and nonreactive in the FTA-Abs test in the syphilis category. There was 95.6% agreement between the two tests.

There was 93.6% agreement between the MHA-TP and the ELISA. Three sera were reactive in the MHA-TP but not in the ELISA, and 13 were reactive in the ELISA but not in the MHA-TP (Table 2). All sera reactive in the MHA-TP were quantitated to obtain titers. These results were then compared with the

TABLE 1. Comparison of reactivity of ELISA and FTA-Abs test

Serum group	No. with given reaction[a]				Total
	FTA-R, ELISA-R	FTA-R, ELISA-NR	FTA-NR, ELISA-R	FTA-NR, ELISA-NR	
Syphilis	67	8	0	0	75
Nonsyphilis	0	3	2	217	222
Total	67	11	2	217	297

[a] R, Reactive; NR, nonreactive. In agreement 284/297 (95.6%) of the time.

TABLE 2. Comparison of reactivity of the ELISA and the MHA-TP

Serum group	No. with given reaction[a]				Total
	MHA-TP-R, ELISA-R	MHA-TP-R, ELISA-NR	MHA-TP-NR, ELISA-R	MHA-TP-NR, ELISA-NR	
Syphilis	54	3	13	5	75
Nonsyphilis	0	1	2	219	222
Total	54	4	15	224	297[b]

[a] R, Reactive; NR, nonreactive. In agreement 277/297 (93.3%) of the time.

[b] One serum could not be counted as either reactive or nonreactive in the MHA-TP, since it was reactive with nonsensitized cells.

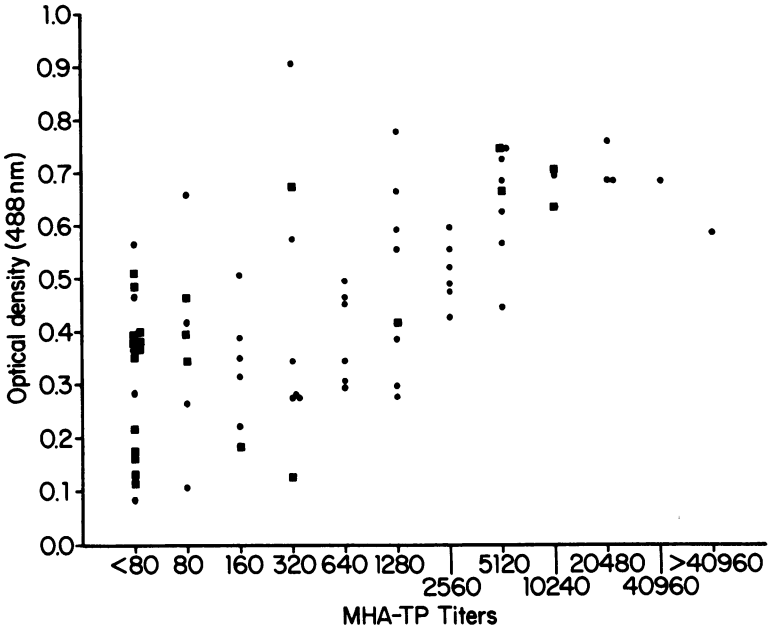

FIG. 1. Agreement of MHA-TP titers and optical densities of syphilitic sera. Symbols: (■) primary syphilis; (●) all other syphilis. *T. pallidum*, Nichols strain, as ELISA antigen.

optical density readings of the ELISA (Fig. 1). The MHA-TP and ELISA correlation coefficient was $r = 0.647$.

Table 3 shows the reactivity for all tests by category of syphilis and nonsyphilis. By stage of syphilis, tests had similar reactivity except when sera were from patients with primary syphilis. The ELISA was less reactive than the FTA-Abs test but more reactive than the MHA-TP.

The optical densities of the syphilitic sera in the ELISA were compared with the titers. The titers were determined by the last dilution showing an optical density equal to or greater than 0.2. The two values were then plotted (Fig. 2). The correlation coefficient was $r = 0.941$.

Three antigen preparations have been satisfactory for use in the ELISA procedure. Antigens have been satisfactorily held for up to 2

TABLE 3. Comparison of test reactivity by category of disease when testing 75 sera from syphilitic individuals and 222 sera from nonsyphilitic individuals

Category	No. with reactivity to:				
	FTA-ABS	ELISA	MHA-TP	VDRL	Total
Primary syphilis					
Untreated	22	17	9	17	22
Treated	2	1	2	1	2
Secondary syphilis					
Untreated	8	8	6	8	8
Treated	12	12	12	12	12
Latent syphilis					
Untreated	13	12	12	10	13
Treated	10	10	9	9	10
Treatment unknown	1	1	1	1	1
Questionable latent	3	3	3	0	3
Neurosyphilis	3	2	2	0	3
Cardiovascular syphilis	1	1	1	0	1
Nonsyphilis					
Presumed normal	1	1	1	0	178
Biological false-positive	1	0	0	7	15
Diseases other than syphilis	1	1	0	1	29

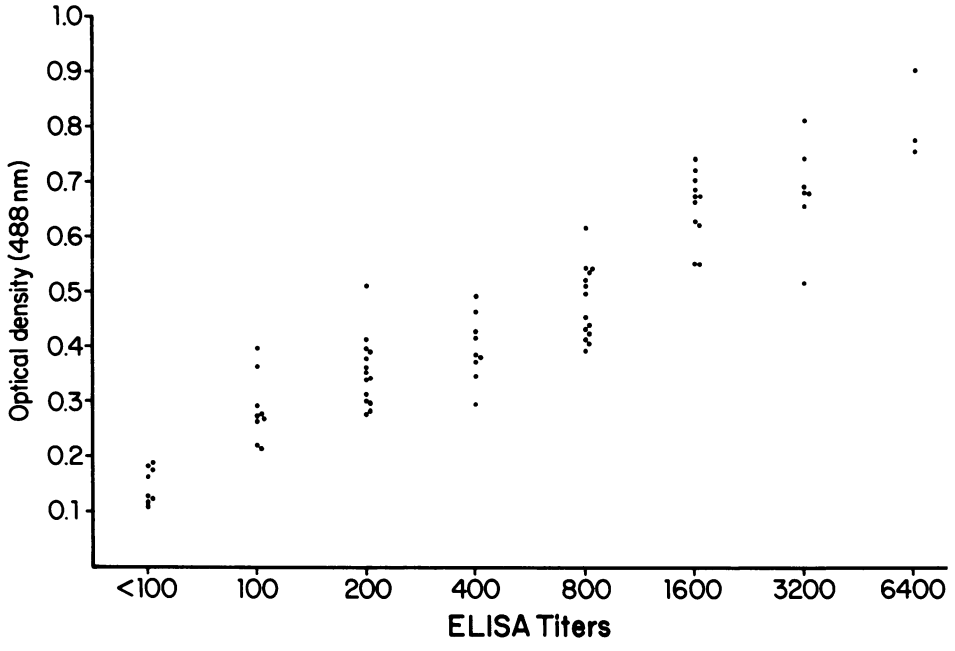

FIG. 2. Agreement of optical densities at 488 nm to corresponding reciprocal titers for each syphilitic sera run in the ELISA (*T. pallidum*, Nichols strain, as antigen).

months at 2 to 8°C in glass tubes and then diluted and plated at the time of use. Plated antigen tends to dry in the wells when held for several weeks at 2 to 8°C, even though the plates are sealed and stored in closed containers. No reactivity has been observed with the positive and negative control sera and the normal rabbit testicular tissue antigen.

DISCUSSION

The results of this study indicate that freshly extracted *T. pallidum* antigen may be disrupted by ultrasonic vibration and used successfully in an ELISA test for detection of syphilitic antibody. Veldkamp and Visser reported an ELISA procedure in which a *T. pallidum* sonic extract was used (11). They found 99% sensitivity and 98% specificity for the ELISA, whereas our data resulted in 89% sensitivity and 99% specificity. Veldkamp and Visser's study was based on 245 syphilitic and 262 nonsyphilitic sera; ours was based on 75 syphilitic and 222 nonsyphilitic sera. The differences in the sample sizes selected do not make an adequate comparison feasible. In addition, their procedure was a macro technique, whereas ours was a micro procedure.

Specificity of the ELISA compared favorably with that of both the FTA-Abs test and MHA-TP, at 98.5%. Sensitivity (89.3%) was not as good as with the FTA-Abs test, which was 100.0%, but was much better than with the MHA-TP, which had a sensitivity of only 76.0%. In the ELISA eight syphilitic sera were missed, with six of these in the primary category. The other two were a latent and a tertiary case of syphilis. In comparing ELISA optical densities with MHA-TP titers, the results indicate differences in the sensitivity of the two tests primarily when testing sera from patients with primary syphilis.

The final comparison was made between the optical densities of the ELISA and the corresponding titer. In this evaluation, correlation was good. At a titer of 1:3,200, some of the optical densities were slightly low in comparison with the titer. Several authors described a prozone effect (3, 4) with high levels of serum antibody. Grippenberg et al. (3) felt that this was apparently due to competition for antigenic sites by immunoglobulin M antibody, since no prozone effect was seen when the immunoglobulin G fractions were studied. The observed linear relationship between the optical density and the titer indicates a good possibility for using a standard curve to read titers.

The test proved to be reproducible within the parameters we have evaluated. When the false-positive sera and the nonreactive syphilitic sera were repeated, the original results were repeated. The only area where some reproducibility proved to be a problem was when optical densities were close to 0.2. This may be related to the automated read-out, since equipment may be first-stage models. Also, reproducibility in the

minimally reactive area has been a problem in all of the serological tests for syphilis (9). For qualitative testing in microliter plates, it is recommended that sera be run in duplicate and, when reactive results are not reproduced, that the test be repeated. An occasional well may be strongly reactive due to some error, and repeated testing allows a double check of that reading. It was found that aberrant wells were rarely too low, but usually too high.

The purpose of this study was to evaluate the ELISA as either a diagnostic or a confirmatory test for syphilis. The test results suggest that with sonicated, freshly extracted *T. pallidum* the test has good potential as a confirmatory test for syphilis. With automation, the usefulness of this test is enhanced, and it possibly could be used for a screening test where large-scale testing would be required. If automation were not available, the test would require too much time and expense as a screening test. The test could be done manually, including interpretation of the results, eliminating the need for special equipment.

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