Evaluation of an IgM/IgG Sensitive Enzyme Immunoassay and the Utility of Index Values for the Screening of Syphilis Infection in a High-Risk Population

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Background: Increasing interest in the use of enzyme immunoassays (EIA) for syphilis screening has generated a considerable need for data on the performance of such tests.

Methods: We compared the performance of 1 EIA, the TREP-SURE EIA to that of the Venereal Disease Research Laboratory (VDRL) and *Treponema pallidum* particle agglutination assay (TPPA) in the detection of infection with *Treponema pallidum*. In total, 674 specimens were tested by VDRL and EIA (356 VDRL-nonreactive and 318 VDRL-reactive). All specimens that were found to be reactive by either the VDRL or EIA were subsequently analyzed by TPPA.

Results: We found that the TREP-SURE EIA was marginally less sensitive than the VDRL test for screening, but was significantly more specific. All EIA-TPPA discordant specimens were analyzed by multiple tests, including Immunoglobulin M- and G-specific Western blots and an IgM-specific EIA. Signal-to-cutoff ratios (index values) generated by the TREP-SURE EIA were also investigated. It was found that these values may be instructive regarding the interpretation of test results, as they were found to correlate strongly with the probability of positivity on a TPPA assay. Specimens that reacted positively on the EIA with very high index values were found overwhelmingly to be reactive by TPPA, perhaps obviating the need for the testing of most EIA positive specimens with a secondary treponemal test.

Conclusions: An IgM/IgG sensitive EIA would be an effective alternative to VDRL for syphilis screening. Using the EIA index values may provide additional, helpful information to the diagnostic process.

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K.O.B. is an employee of Trinity Biotech, the EIA evaluated in this study. J.S.Q. is an employee of Phoenix Biotech, whose assays were used as part of the evaluation of the EIA, above.

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Infection by *Treponema pallidum*, the organism that causes syphilis can be a lifelong condition when left untreated. Infection includes an initial symptomatic stage followed by prolonged latency and in some cases serious, symptomatic disease.[1] Diagnosing infection with *T. pallidum* has been a challenging task for physicians and the clinical and/or public health laboratory for many reasons. The identification of infected individuals through symptom recognition and clinical screening is limited by the often asymptomatic nature and short duration of the primary symptomatic stage and poor specificity of the secondary stage. Following primary infection are long periods of clinical latency during which time organisms cannot be directly detected. For that reason, infection is most often diagnosed by serological means. Serologic diagnosis of syphilis is complicated for several reasons. *T. pallidum* contains many antigens which are common to several Treponemal species which do not cause syphilis. Hence, people may produce antibodies to treponemes other than *T. pallidum* which may cross react with *T. pallidum*-specific assays. Complicating the diagnostic scenario further, is that while a cure for syphilis is available, most patients treated for infections will remain reactive to treponemal-specific serological tests. For this reason, the diagnosis of current infection with *T. pallidum* requires assays which will only demonstrate reactivity if an individual is actively infected. This is currently accomplished with nontreponemal tests, the Venereal Disease Research Laboratory test (VDRL), and rapid plasma reagin test (RPR). Although nontreponemal tests are useful for the detection of current infection with *T. pallidum*, they lack the specificity of treponemal tests.[2,3] Moreover, the VDRL and RPR nontreponemal tests are very labor intensive and are not conducive to large scale testing due to multiple pipetting steps in the preparation of dilutions and a subjective, microscopic reading of the test results. Neither the VDRL nor the RPR can be run on automated platforms, further complicating their application for large scale testing.

Since immunoassays (most commonly "enzyme immunoassays," EIA) can be easily performed by automated methods, they are the preferred screening methods for laboratories that process high specimen volumes. Several EIAs have been evaluated.[4–9] Recently, there has been a widespread increase in the consideration of using such assays as screening tests.[10] Resistance to their use as screening tests derives from the fact that IA for *T. pallidum* antibodies are treponemal tests, and therefore cannot distinguish between previous or cured infection, and current, treated infection. Increases in the demand for syphilis testing have placed a strain on both clinical and public health laboratories. As a result, many laboratories that perform

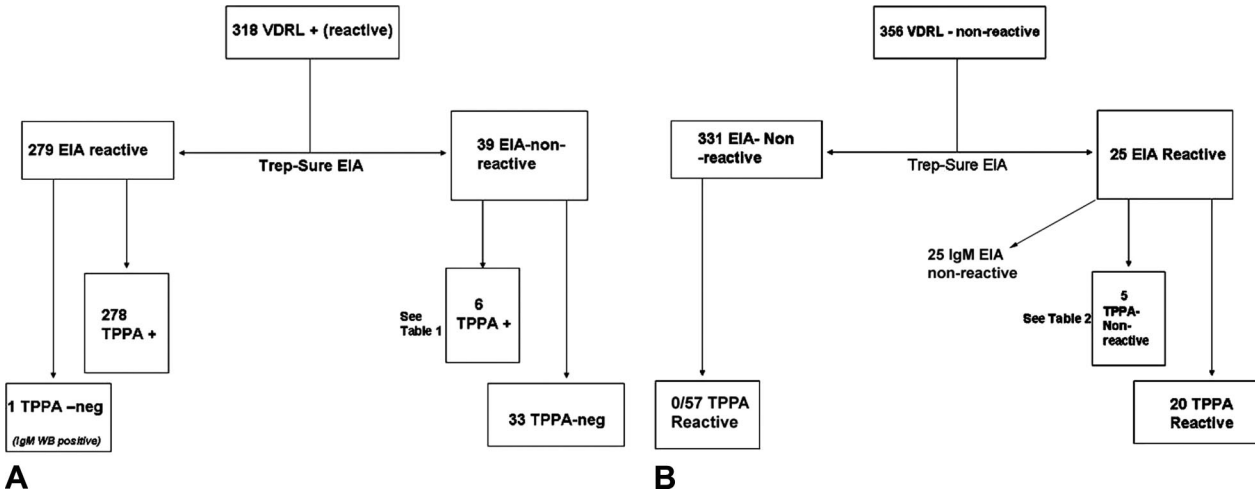

Figure 1. A, Flow chart showing the testing process and results for the analysis of VDRL-reactive specimens. B, Flow chart showing the testing process and results for the analysis of VDRL-nonreactive specimens.

syphilis testing at high volume have begun to consider the use of immunoassays for syphilis screening. For that reason, we have compared Food and Drug Administration (FDA)-cleared EIA for antibodies to *T. pallidum,* the TREP-SURE EIA (TS-EIA) (Trinity Biotech, Jamestown, NY), to the VDRL test for their ability to screen for syphilis infection. The TS-EIA is a sandwich EIA capable of detecting both Immunoglobulin G (IgG) and M (IgM). Our data indicate that the TS-EIA for syphilis was slightly less sensitive but more specific than the VDRL test. The TS-EIA was far easier for laboratory staff to perform, making it amenable to the processing of many specimens at once.

METHODS

Serum Specimens

Study specimens were deidentified remnant sera from clinical whole blood specimens collected from patients presenting to San Francisco municipal Sexually Transmitted Disease clinic. The VDRL testing population at this clinic is 69.3% men who have sex with men and 16.6% of the tested population is human immunodeficiency virus (HIV)-positive and 9.4% have had a documented case of early syphilis. Specimens were transported at ambient temperature to the laboratory where serum was prepared and stored refrigerated for no more than 5 days before testing by VDRL or EIA.

Serological Testing

VDRL (Becton-Dickinson, Sparks, MD) and *T. pallidum* particle agglutination assay (TPPA) (Fujirebio, Malvern, PA) testing were performed according to manufacturer's directions. Specimens were analyzed by the TrepSure (TS) EIA (Trinity Biotech, Jamestown, NY) according to manufacturer's specifications. Index values for the EIA were calculated according to the package insert, which are as follows: optical density (OD) values of a blank well are subtracted from the OD of a specimen well. The resulting OD value is divided by the mean OD of 3 cut-off calibrators included in the assay. The generated ratio is the Index Value for that particular specimen. Index scores less than 0.8 are considered negative, while those between 0.8 and 1.2 are considered "equivocal." Index scores greater than 1.2 are considered positive.

Western blotting was performed on a subset of specimens by the Trinity Biotech laboratory using the MarDx *T. pallidum* IgG Marblot Strip Test System, and the MarDx *T. pallidum* IgM Marblot Strip Test System (Trinity Biotech, Carlsbad, CA) according to manufacturer's recommendations. EIA for IgM was performed using the Captia Syphilis-IgM Capture assay (Trinity Biotech, Jamestown, NY) according to manufacturer's recommendations. TrepID EIA (Phoenix Bio-Tech, Mississauga, Ontario, Canada) was performed at Phoenix Bio-Tech laboratories according to manufacturer's instructions. The TrepID EIA tests for antibodies to 4 treponemal antigens simultaneously and differentially (each antigen in 4 separate wells). Reactivity for any 2 *T. pallidum* antibodies on the TrepID test indicates a "positive" specimen, while testing reactive for one or zero treponemal antibodies indicates a "negative" specimen.

The TrepSure EIA and the Captia Syphilis-IgM Capture Assay are approved by the US FDA for use in testing patient specimens. However, the MarDx *T. pallidum* Marblot Strip tests (both IgG and IgM) and the TrepID EIA are not FDA approved.

RESULTS

A total of 674 serum specimens collected from patients at the San Francisco municipal Sexually Transmitted Disease clinic were tested prospectively by VDRL. In all, 318 specimens found to be reactive by VDRL were tested by both TPPA and TS-EIA. As shown in Figure 1A, 279 were found to be reactive by TS-EIA and 39 were found to be nonreactive by TS-EIA. Of the 279 found to be reactive by TS-EIA, 278 were reactive by TPPA. The one specimen found to be nonreactive by TPPA while reactive by VDRL and positive by EIA was positive by Western Blot for IgM antibodies against *T. pallidum* antigens.

Of the 39 VDRL-reactive specimens found to be nonreactive by TS-EIA, 33 were nonreactive by TPPA, whereas 6 were reactive by TPPA. Because the 33 specimens that were found to be nonreactive by TS-EIA were found also to be nonreactive by TPPA, we hypothesize that those specimens

TABLE 1. Investigation of 6 VDRL Reactive Specimens Found to be TS-EIA Negative, TPPA Reactive

VDRL	TPPA	TS-EIA*	TS-EIA Index	TS-EIA Repeat†	IgM EIA‡	IgG WB§	IgM WB¶
WR	R	N	0.629	0.839	N	P	P
WR	R	N	0.222	0.451	P	P	P
WR	R	N	0.509	0.799	P	P	P
WR	R	N	0.384	0.395	P	EQ	P
R	R	N	0.431	0.51	P	P	P
WR	R	N	0.282	0.388	P	EQ	P

*Final interpretation of TrepSure EIA.
†TrepSure EIA index, repeat test.
‡CaptiaTM Syphilis-IgM Capture.
§MarDx T. PALLIDUM IgG Marblot Strip Test System.
¶MarDx T. PALLIDUM IgM Marblot Strip Test System.
R indicates reactive; WR, weakly reactive; N, negative; P, positive; EIA, enzyme immunoassay; WB, Western blots; VDRL, Venereal Disease Research Laboratory; TPPA, *Treponema pallidum* Particle Agglutination Assay; IgG, Immunoglobulin G; IgM, Immunoglobulin M; EQ, equivocal.

were falsely reactive by VDRL, which is a common and well-documented limitation of VDRL.[2,11]

The 6 specimens that were found to be both VDRL and TPPA reactive, while nonreactive by TS-EIA were analyzed by both IgG Western blot and an IgM-only Western blot. Results of all tests for these 6 discordant specimens can be found in Table 1. All 6 were positive for *T. pallidum* antibodies by both the IgG and IgM Western blots. Interestingly, the signal-to-cutoff ratios (index values) of these 6 specimens tested by the TS-EIA were notably higher than the mean signal-to-cutoff ratio seen for true negative specimens (a mean index value of 0.487 ± 0.188 compared to a mean of 0.045 ± 0.07 for true negative serum specimens [$P < 0.0001$]). Due to the presence of IgM and these elevated negative index values, these specimens were most likely from patients who were recently infected with *T. pallidum*, and were falsely negative by the TS-EIA.

In all, 356 serum specimens that were prospectively tested and found to be nonreactive by VDRL were subsequently analyzed by TS-EIA. As shown in Figure 1B, 331 of the specimens were found to be negative by TS-EIA, while 25 were positive. A sampling of 57 of the 331 VDRL and TS-EIA negative specimens were tested by TPPA and all were found to be nonreactive.

All 25 specimens that were VDRL-nonreactive and TS-EIA positive were analyzed by TPPA. Of those, 20 (80%) specimens were found to be TPPA reactive, whereas 5 (20%) were TPPA nonreactive. To determine whether the 20 reactive specimens were from recently infected individuals, we analyzed all 20 specimens by an IgM-specific EIA assay (Trinity Biotech, Jamestown, NY). None of the 20 specimens were reactive on an IgM EIA. The 5 TPPA-non reactive, TS-EIA positive specimens were also investigated further by a number of treponemal antibody tests (Table 2). All 5 were negative for *T. pallidum*-specific IgM by both Western blot and an IgM-specific EIA. Sufficient specimen volume was available for 4 of the 5 VDRL nonreactive, TPPA nonreactive, TS-EIA positive specimens for further testing. These 4 specimens were subjected to analysis by the TrepID EIA. On the TrepID test, 2 of the specimens were reactive for antibodies only to Tp47, while one was solely reactive for antibodies to Tp17. One specimen was found to be reactive to 2 treponemal antigens (Tp47 and Tp15). Omitting the specimen for which insufficient volume was available for confirmation testing, and assuming that the 3 specimens above are in fact negative for actual *T. pallidum* antibodies, the specificity of the TS-EIA would be calculated as 99.1%. Since the VDRL testing population at the clinic used in this study has a 9.4% rate of documented previous early syphilis cases, it is reasonable to assume that the 25 EIA reactive, VDRL-non reactive specimens (out of 356

TABLE 2. Investigation of Specimens Found to be TS-EIA Positive, TPPA Nonreactive

		TS-EIA						TrepID Results*				
VDRL	TPPA	EIA	EIA Index	EIA Repeat	IgM EIA	IgG WB (No. Bands)	IgM WB (No. Bands)	Tp15	Tp44	Tp17	Tp47	Trep ID Interpret
N	N	P	1.246	1.741	Neg	EQ (1)	Neg (0)	N	N	N	R	1 Ag Positive
N	N	P	4.316	5.108	Neg	EQ (1)	Neg (0)	R	N	N	R	Positive
N	N	P	3.191	3.445	Neg	EQ (1)	Neg (0)	N	N	N	R	1 Ag Positive
N	N	P	5.014	5.909	Neg	EQ (1)	Neg (0)	N	N	R	N	1 Ag Positive
N	N	I	1.024	0.204	Neg	Neg (0)	Neg (0)	nd	nd	nd	nd	nd
WR	N	P	3.455	6.438	Neg	EQ (1)	Neg (0)	nd	nd	nd	nd	nd

*Trep ID, Phoenix Bio-Tech.
N indicates nonreactive; I, indeterminate; P, positive; R, reactive; EQ, equivocal; Neg, negative; nd, not done; VDRL, Venereal Disease Research Laboratory; TPPA, *Treponema pallidum* Particle Agglutination Assay; IgG, Immunoglobulin G; IgM, Immunoglobulin M; EIA, enzyme immunoassay; WB, Western blots; Ag, Antigen.

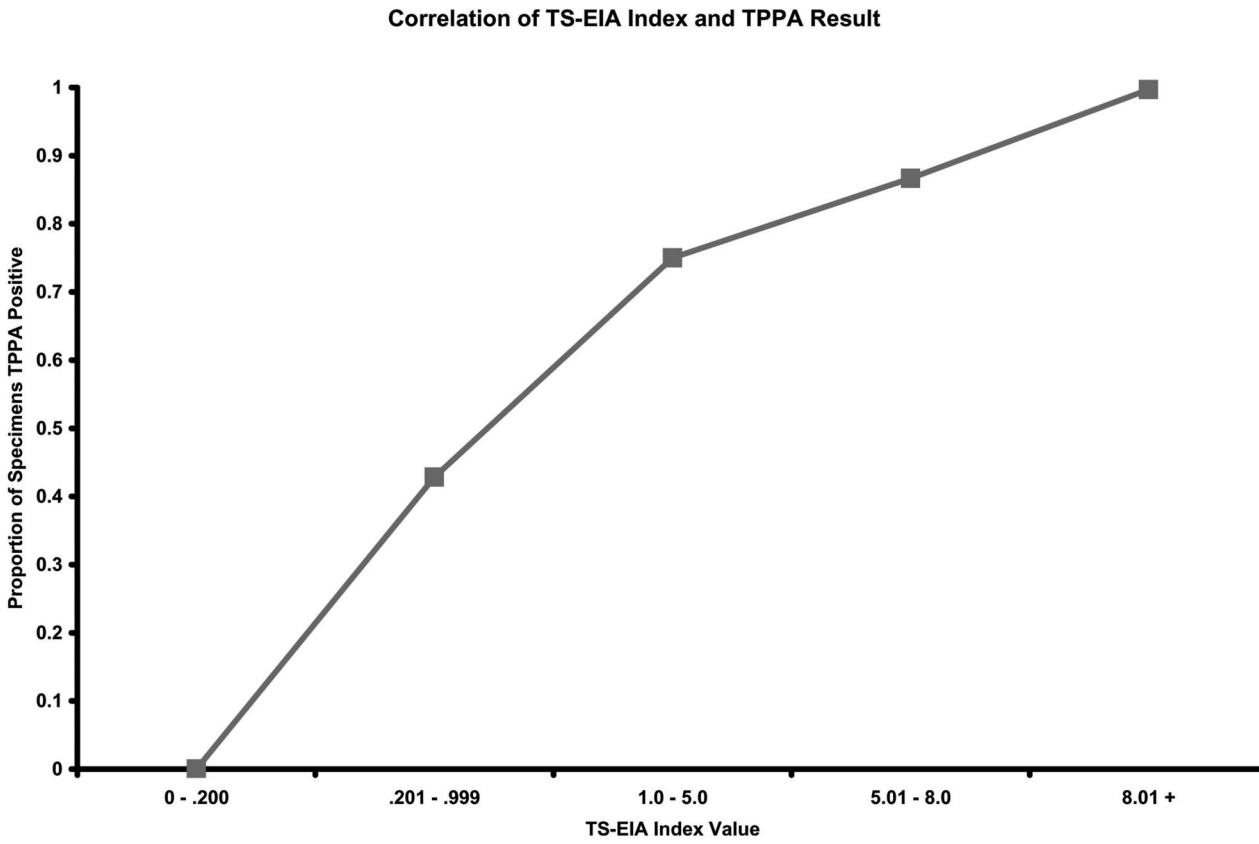

Figure 2. Graphical representation of the correlation between EIA index value and probability of TPPA positivity.

tested) were specimens from previously infected individuals. This is supported by the lack of detectable IgM in any of those specimens.

Figure 2 shows the relationship of index value (signal-to-cutoff ratio) and TPPA reactivity of 389 specimens tested by EIA, VDRL, and TPPA. Among 269 specimens with a TS-EIA index score of 8.0 or greater, 268 (99.6%) were reactive by TPPA while 13 of 15 (87%) specimens with EIA indices between 5.0 and 8.0 were TPPA reactive. Of 20 specimens, 15 (75%) specimens in the 1.0 to 5.0 range were reactive by TPPA. Six (43%) of 14 specimens with an index value in the range 0.200 to 0.999 were TPPA reactive. Specimens with an index of less than 0.200 were nonreactive by TPPA 100% (71/71 tested specimens) of the time.

We compared the time and work required to perform the TREP-SURE EIA to the time and work required to perform the VDRL. To assay 80 specimens by VDRL was found to take approximately 150 minutes (to resolve both reactive and non-reactive specimens). To assay the same 80 specimens by TREP-SURE EIA required approximately 120 minutes, but with incubation times (microbiologist free time) of both 60 and 30 minutes. For the TREP-SURE EIA, specimens are pipetted only once into the assay plate wells, while for the VDRL, specimens found to be reactive must be diluted (3–5 dilutions per specimen, each requiring multiple pipetting steps) and subsequently reanalyzed.

CONCLUSIONS

We have analyzed the performance of the TREP-SURE EIA to function as a screening test for infection with *T. pallidum*, relative to VDRL. We found that while the TREP-SURE EIA was somewhat less sensitive than the VDRL for screening our population, the TREP-SURE EIA was far more specific than the VDRL. We note that the high prevalence of HIV infection in the population that provided specimens for this work may have affected the results. HIV infection has been shown to cause false-positive VDRL results.[11]

The TREP-SURE EIA provided quantitative data in the form of a signal-to-cutoff ratio (an index value), which we have found strongly correlated with true infection and may be highly instructive in the interpretation of indeterminate results. Both very high and very low index scores on the TREP-SURE EIA appear to obviate the need for additional analysis by TPPA. Additionally, specimens found nonreactive by the TREP-SURE EIA, but that possessed high nonreactive index scores (>0.200) were found frequently to be specimens from truly infected individuals. This may allow for certain individuals found negative by EIA but with elevated index values to be flagged for repeat testing, minimizing the number of cases of syphilis missed. In a high prevalence setting such as the one utilized in this study, the secondary testing of high negative specimens would seem to be important, given the fact that 43% of such specimens (as defined above) would be found to be truly positive. This may not be true in communities of lower prevalence but further study might be warranted.

We note that screening for syphilis by EIA alone would result in many previously infected, but currently uninfected individuals to be flagged for treatment. However, when used in an algorithm that includes a nontreponemal test such as RPR or

VDRL as a confirmation test, it is quite possible that only currently infected individuals would be identified.

The time and manual labor required to perform the TREP-SURE EIA was substantially less than that for VDRL testing. Moreover, the VDRL is a test that is assessed by the microbiologist through the use of a microscope, while the TREP-SURE EIA results are determined by an automated plate reader. Hence, the overall efficiency for performing the TREP-SURE EIA is much greater than that required for the execution of the VDRL. While the VDRL appeared to be somewhat more sensitive than the TREP-SURE EIA, the superior specificity and the ease of execution of the TREP-SURE EIA make it a highly desirable candidate assay for laboratories accustomed to analyzing a high volume of patient specimens.

This study possessed several limitations. The specimens used in this study were all gathered at 1 clinical site, known for serving patients at high risk for sexually transmitted infections. Additionally, only 1 EIA was studied herein, making it unclear whether studies of other available EIA would have similar results. These data however do provide some guidance in the use of EIA as a screening test for syphilis and provide new information on the utility of the EIA index value in the interpretation of test results. Clearly, a great deal of education will be needed for both laboratorians and physicians who seek to go forward in the use of treponemal immunoassays for syphilis screening.

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