Evaluation of the Bio-EnzaBead Test for Syphilis

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The sensitivity and specificity of the Bio-EnzaBead test for syphilis and the fluorescent treponemal antibody-absorption (FTA-ABS) test were determined by examining 262 serum samples, including 202 serum samples from patients with confirmed syphilis in various stages. Overall correlation with patient history was 95.8% with both tests. False-negative Bio-EnzaBead tests occurred in 9 of 86 (10.5%) cases of late-latent syphilis (>2 years) and in 1 of 38 (2.6%) cases for which the stage of disease could not be determined. False-negative FTA-ABS tests occurred in 5 of 86 (5.8%) cases of late-latent syphilis (>2 years) and in 2 of 38 (5.3%) cases for which the stage of disease could not be determined. One false-positive test occurred with Bio-EnzaBead, and the cause could not be determined. The reproducibility of the Bio-EnzaBead test was excellent when spectrophotometric readings were calibrated against either air or substrate blanks. The Bio-EnzaBead test for syphilis is a suitable alternative to the FTA-ABS test.

Several investigators have examined the enzyme immunoassay procedure as a diagnostic treponemal test for syphilis (1, 3, 6). The syphilis Bio-EnzaBead test developed by Litton Bionetics Laboratory Products Division, Charleston, S.C., now part of Organon Teknika Corporation, has been evaluated by Stevens and Schmitt (4) as a confirmatory treponemal test for syphilis and has been granted provisional test status by the Centers for Disease Control (Reagents Evaluation Program Supplement no. 28, 1984). The test is a modification of the enzyme-linked immunosorbent assay and uses ferrous metal beads as a solid-phase carrier for antigen prepared from *Treponema pallidum* (Nichols strain). The presence of treponemal antibody in a test serum sample is indicated by enzyme-substrate color intensity which may be read visually or spectrophotometrically and compared with a cutoff value established with control sera.

Previous evaluations of the syphilis Bio-EnzaBead test used visual estimations of color intensity measured on a scale of weakly positive (1+) through highly positive (4+) to determine the reactivity of specimens (4). A color intensity of 1+ or greater was considered positive, while specimens with a definite color intensity of less than 1+ were retested. Retests read at 1+ or greater were reported positive, while those read at less than 1+ were reported negative. The test was reported unsatisfactory if the control well color intensity was equal to or greater than that of the test well. When the syphilis Bio-EnzaBead test was compared with the fluorescent treponemal antibody-absorption (FTA-ABS) test, the sensitivity (96.7%), specificity (96.1%), and overall reliability (96.3%) for 1,338 serum samples supported a recommendation to consider the Bio-EnzaBead test as an alternative to the FTA-ABS test (4). However, this study included only 218 serum samples from documented cases of syphilis in various stages, and Bio-EnzaBead test reliability was measured against the FTA-ABS result rather than against clinical diagnosis.

The present study was undertaken to determine the effectiveness of the Bio-EnzaBead test for confirmation of syphilis by using sera found reactive by nontreponemal tests in a public health laboratory or sera referred by physicians to resolve diagnostic problems. We compared the Bio-EnzaBead and FTA-ABS tests to clinical diagnosis and examined the sensitivity, specificity, and reliability of both tests by using 262 serum samples, 202 of which were obtained from patients with documented cases of syphilis, as determined by the patient's physician and case records maintained by the Iowa State Department of Health. We examined the reproducibility of the Bio-EnzaBead test read visually and spectrophotometrically by using an air blank and a substrate blank. Interpretations of spectrophotometrically read tests were based upon optical density cutoff values at 690 nm established by the kit manufacturer. Spectrophotometric reading with a substrate blank was the method chosen after several trial runs, and this method, together with visual reading, provided the data for our evaluation.

MATERIALS AND METHODS

Sera. Whole clotted blood and serum specimens for diagnostic or prenatal syphilis serology were referred to the Hygienic Laboratory, University of Iowa, from physicians throughout Iowa. Aproximately 100 serum samples that were from patients with documented syphilis and that were stored at −70°C were included in the study to ensure that specimens from all stages of disease were represented. A total of 262 serum samples from patients for whom clinical histories could be obtained were included in the study, and 202 of these represented cases of syphilis. The remaining 60 serum samples were reactive when examined by nontreponemal tests but nonreactive in treponemal tests, and the clinical histories of these patients did not support a diagnosis of syphilis. Stages of disease were classified according to the criteria published in Quality Assurance Guidelines for STD Clinics—1982, Centers for Disease Control, Atlanta, Ga.

Reproducibility. The sera for the reproducibility tests were taken from six pools prepared by Bionetics Laboratory Products and designated A through F. Pools A (4+), B (3+), C (2+), and D (1+) were reactive in FTA-ABS and Bio-EnzaBead tests. Pools E and F were nonreactive in both treponemal tests for syphilis. A panel of 60 coded samples was prepared from these pools by Bionetics Laboratory

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Products and submitted to the Hygienic Laboratory for reproducibility comparisons between visual readings and spectrophotometric readings with both an air blank and a substrate blank. Sets of 30 samples each were examined on consecutive weeks.

Histories. A questionnaire was sent to each physician who submitted a specimen whenever the serum sample tested weakly reactive or greater. The questionnaire asked the physician to determine whether the patient had syphilis and, if infected, to indicate the stage of the disease. Physicians were asked whether reinfection had occurred, whether treatment was administered (including dates), and whether other diseases or circumstances were present which might affect the results of the treponemal tests. Questionnaires were compared with state health department records to confirm the stage of the disease. The histories were used to determine the sensitivity, specificity, and reliability of the treponemal tests.

Statistical analysis. Significance was determined from the McNemar test for correlated proportions (2). Reproducibilities are presented as percentages of true values determined from patient histories.

Serological testing. All sera were tested by the Venereal Disease Research Laboratory (VDRL) slide screening test with commercial reagents (Fisher Scientific Co., Pittsburgh, Pa.) and by standard techniques (5). All sera demonstrating any degree of reactivity were retested by the VDRL slide quantitative test (5). The FTA-ABS test was performed with sorbent and conjugate from Beckman Diagnostic Reagents Division and antigen from Clinical Sciences, Inc., by the standard procedure (5), and the Bio-EnzaBead test was performed in accordance with instructions supplied in the kit provided by the manufacturer, except that sera were heated for 30 min at 56°C prior to preparation of the test dilutions. Serum dilutions (1:101) were prepared by placing 1.0 ml of diluent supplied with the test kit into glass tubes (12 by 75 mm) and adding 0.01 ml of patient serum. The control sera of low, high, and negative reactivities supplied with the kit were reconstituted to working dilutions as described in the kit instructions. To prepare test plates, we transferred 0.2 ml of each serum dilution and each serum control to a 96-well microtiter plate. Antigen beads were placed in an empty microtiter plate in accordance with the record sheet supplied with the kit. Beads were transferred to test plates by using the magnetic transfer device and were incubated for 90 min at 37°C while wash solutions and conjugate were prepared. With the magnetic transfer device, beads were removed from test plates and washed 12 times by successive transfers into and out of 0.3 ml of wash solution in each well of a microtiter plate. After being washed, beads were transferred to plates containing 0.2 ml of conjugate and were incubated for 90 min at 37°C while substrate was prepared and dispensed into another microtiter plate. Beads were washed in fresh wash solution and transferred to plates containing 0.2 ml of substrate. Trays were incubated at room temperature (20 to 25°C) for 10 min, and the enzyme-substrate reaction was terminated by the addition of 25 μl of 1.25% sodium fluoride solution. Beads were removed, and the plates were read visually or spectrophotometrically for intensity of the green color which developed. For visual readings, color intensity comparable to that of weakly reactive control serum was considered 1+, and any color intensity less than 1+ was recorded as ±. Negative sera demonstrated no color when read against a white background. Sera with 1+ through 4+ color intensities were considered reactive. Sera read as ± were retested. Sera read again as ± and negative sera

were considered negative. Tests were repeated when the color intensity of the control well was equal to or greater than that of the test well. For spectrophotometric readings at 690 nm with both an air blank and a substrate blank, the cutoff was calculated by using a ratio times delta ($R \times D$) value, where R is equal to the optical density of the substrate following incubation with the antigen-coated bead divided by the optical density of the negative control bead, and D is equal to the optical density of the antigen-coated bead minus the optical density of the negative control bead. The test was considered in control for an air blank calibration at 690 nm when the negative control serum had an $R \times D$ of <0.035 and the weakly reactive control serum had an $R \times D$ of ≥0.044. Test specimens were considered nonreactive at an $R \times D$ of <0.035, equivocal at an $R \times D$ of ≥0.035 and <0.044, and reactive at an $R \times D$ of ≥0.044. Specimens testing equivocal were retested. On the basis of our experience with the reproducibility test sera, we used these same criteria for the evaluation of tests read spectrophotometrically and calibrated against a substrate blank.

RESULTS

Of the 262 specimens included in this study, 239 were reactive in the VDRL test, 199 were reactive in the FTA-ABS test, 193 were reactive in the Bio-EnzaBead test, and 204 were reactive in either or both treponemal tests. The FTA-ABS test was reactive with sera from four patients with late-latent syphilis when the Bio-EnzaBead test was nonreactive, while the FTA-ABS test was positive for one clinically normal patient whose serum was nonreactive in the Bio-EnzaBead test. The remaining discrepancy between tests occurred with a serum sample from one patient whose stage of disease had not been determined but who was diagnosed as having syphilis. The FTA-ABS test was borderline reactive, while the Bio-EnzaBead test was reactive.

When the FTA-ABS results were compared with patient history, 195 true-positive and 56 true-negative tests occurred, for a reliability of 95.8%. Seven false-negative FTA-ABS results read as borderline included five cases of late-latent syphilis and two cases of syphilis for which the stage could not be determined. The four false-positive FTA-ABS results involved sera from one pregnant subject, two patients with lupus erythematosus, and one patient with no known cause for a biological false-positive test. When Bio-EnzaBead results calibrated to a substrate blank were compared with patient history, the 192 true-positive and 59 true-negative tests resulted in a reliability of 95.8%. False-negative Bio-EnzaBead tests ($n = 10$) occurred in late-latent syphilis ($n = 9$) and syphilis for which the stage could not be determined ($n = 1$). One false-positive Bio-EnzaBead test occurred, and the cause could not be determined. When Bio-EnzaBead was read visually, the reliability was 85.8%. False-negative tests occurred in primary syphilis ($n = 3$), neurosyphilis ($n = 1$), congenital syphilis ($n = 2$), late-latent syphilis ($n = 24$), and syphilis for which the stage could not be determined ($n = 6$). A summary of laboratory results of the nontreponemal and treponemal tests compared with patient history is presented in Table 1.

The method for comparison of syphilis history and treponemal test results and our definitions of sensitivity, specificity, and reliability are shown in Table 2. Our analysis of these data by use of this method is presented in Table 3. There was no statistical difference between the sensitivity and overall reliability of the FTA-ABS and Bio-EnzaBead

TABLE 1. Results of VDRL, FTA-ABS, and Bio-EnzaBead tests compared with patient history

Disease	No. of serum samples tested	No. of serum samples with the indicated results[a] in the following test:								
		VDRL			FTA-ABS		Bio-EnzaBead			
							Substrate blank		Visual reading	
		R	WR	N	R	N	R	N	R	N
Primary syphilis	17	14	2	1	17	0	17	0	14	3
Secondary syphilis	23	17	3	3	23	0	23	0	23	0
Early-latent syphilis (<1 to 2 yr)	24	22	1	1	24	0	24	0	24	0
Late-latent syphilis (>2 yr)	86	66	15	5	81	5	77	9	62	24
Late-latent syphilis (cardiovascular)	1	1	0	0	1	0	1	0	1	0
Cardiovascular syphilis	2	1	1	0	2	0	2	0	2	0
Neurosyphilis	3	3	0	0	3	0	3	0	2	1
Congenital syphilis	8	5	3	0	8	0	8	0	6	2
Syphilis (stage undetermined)	38	28	8	2	36	2	37	1	32	6
Not syphilis	60	17	32	11	4	56	1	59	1	59

[a] R, Reactive; WR, weakly reactive; N, nonreactive.

tests ($P > 0.3$). The Bio-EnzaBead test was significantly more specific than the FTA-ABS test ($P < 0.01$). There was no significant difference between tests for any stage of syphilis ($P > 0.2$). When the Bio-EnzaBead test was read visually, the sensitivity and overall reliability of the test were significantly lower than those obtained with the spectrophotometer calibrated to a substrate blank ($P < 0.01$).

The reproducibility data are presented in Table 4. Visual test readings corresponded exactly with expected readings for 67% of samples, while 98% of samples were read within one gradation of the expected result. The one reading which deviated by two gradations occurred when a 1+ serum sample was read as negative. Additionally, two serum samples with 1+ reactivity were read as ± and would be reported nonreactive. The reliability of visual readings for the reproducibility test sera was 95%. No discrepancies occurred with spectrophotometric readings calibrated to an air blank for 100% reliability. When a substrate blank was used, the test reliability was 98%. The single discrepant serum sample had an $R \times D$ value 0.011 units above the cutoff.

DISCUSSION

The syphilis Bio-EnzaBead kit provides sufficient reagents to test 96 serum samples together with necessary controls. We used the accessory package, which includes flat-bottom microtiter plates and clear covers. Control and antigen beads are packaged 24 per vial, and beads are stable for 28 days after the package is opened. Our laboratory receives an average of 10 serum samples per day for treponemal antibody testing. This specimen volume corresponds to approx-

TABLE 2. Method for comparison of syphilis history and treponemal test results

Disease	Result[a] in treponemal test:		
	Reactive	Nonreactive	Total
Syphilis	A	B	T1
Not syphilis	C	D	T2
Total	T3	T4	N

[a] Abbreviations: T, total; A/T1, sensitivity; A/T3, true-positivity; D/T2, specificity; D/T4, true-negativity; C/T3, relative false-positivity; C/T2, absolute false-positivity; B/T4, relative false-negativity; B/T1, absolute false-negativity; (A + D)/N, reliability.

imately one vial of beads per day, with minimal waste of reagents occurring when sera are batch tested once or twice each week. The test is convenient to perform and requires approximately the same amount of setup time as the FTA-ABS test.

Required support equipment for the test includes the magnetic transfer device, the model LBI-30 multichannel recording spectrophotometer, and a bead dispenser. Optional equipment includes a Hamilton digital diluter to facilitate the preparation of the initial serum dilution of 1:101. The Spirit 80 (Mannesmann Tully Corp.) printer was set up on a stand over the spectrophotometer so that only 45 cm by 50 cm of bench space was required. The paper printout was conveniently arranged to correspond to the well pattern of the microtiter plate for easy comparison of visual versus spectrophotometric readings. Plate position and absorbance value were printed for each well read. The automated readout and manual $R \times D$ calculations require only 10% of the time necessary to read and record the results of the FTA-ABS test.

The advantages of the syphilis Bio-EnzaBead test over the FTA-ABS test include speed of performance, brief serologist training time (3 to 5 days), elimination of expensive microscopy equipment, and elimination of fatigue and eye strain associated with microscopy. A further advantage of the syphilis Bio-EnzaBead test is elimination of the subjective evaluation of fluorescence intensity to determine the endpoint between reactive and nonreactive sera. This last advantage is lost when Bio-EnzaBead is read visually.

The specimens included in this study represented patients with all stages of syphilis. Sera were inactivated at 56°C for 30 min prior to performance of the VDRL and FTA-ABS tests. The syphilis Bio-EnzaBead test instructions indicate that serum inactivation is not required. While we did not perform direct comparisons on heated versus unheated sera, we experienced no problem testing heated serum, on the basis of results obtained with the reproducibility test sera. It is possible that heating sera may reduce the number of elevated readings obtained with negative control beads, a problem observed by previous investigators (S. A. Larson, E. A. Hambie, and D. D. Cruce, personal communication). Use of heated sera may reduce the risk of exposure to human immunodeficiency virus, although the Bio-EnzaBead procedure offers minimum biohazards, since sera are diluted, bead transfers are made magnetically, and all test materials are disposable.

TABLE 3. Comparative summary of FTA-ABS and Bio-EnzaBead results

Test	No. of serum samples with indicated result/total no. of serum samples tested (%)								
	Sensitivity	Specificity	Reliability	True-positivity	True-negativity	Relative false-positivity	Relative false-negativity	Absolute false-positivity	Absolute false-negativity
FTA-ABS	195/202 (96.5)	56/60 (93.3)	251/262 (95.8)	195/199 (98.0)	56/63 (88.9)	4/199 (2.0)	7/63 (11.1)	4/60 (6.7)	7/202 (3.5)
Bio-EnzaBead									
Substrate blank	192/202 (95.0)	59/60 (98.3)	251/262 (95.8)	192/193 (99.5)	59/69 (85.5)	1/193 (0.5)	10/69 (14.5)	1/60 (1.7)	10/202 (5.0)
Visual reading	166/202 (82.2)	59/60 (98.3)	225/262 (85.9)	166/167 (99.4)	59/95 (62.1)	1/167 (0.6)	36/95 (37.9)	1/60 (1.7)	36/202 (17.8)

TABLE 4. Reproducibility of Bio-EnzaBead readings[a] of 60 coded serum samples

Serum pool[b] (no. of trials)	Reading expected	No. of serum samples with indicated Bio-EnzaBead reading:										
		Visual reading						Air blank		Substrate blank		
		4+	3+	2+	1+	±	N	R	N	R	E	N
A (6)	4+	5	1						6		6	
B (8)	3+	4	1	3					8		8	
C (8)	2+		1	5	2				8		8	
D (16)	1+			6	7	2	1	16		16		
E (12)	N						12		12		1	11
F (10)	N						10		10	1	1	8

[a] N, Negative; R, reactive; E, equivocal.
[b] The code was made available at the conclusion of the trials.

The model LBI-30 spectrophotometer performed well, as determined by a comparison of duplicate readings of the first three test panels. The only problem encountered occurred when the absorbance of a test serum well was equal to or less than the absorbance of the control bead well. This situation introduced a zero into the denominator of the formula, making calculation of $R \times D$ impossible and occasionally resulting in an apparent false-negative test result. When this occurs, the appearance of color in the test antigen well may be used to visually screen for these "numerical false-negative" tests so that they may be repeated. If numerical false-negative results are obtained on this repeat test, results should be reported on the basis of a visual reading.

Both treponemal tests demonstrated some lack of sensitivity with sera from patients with late-latent syphilis, but there was no statistical difference between the tests for diagnosis of any stage of syphilis. The syphilis Bio-EnzaBead test produced significantly fewer false-positive tests than did the FTA-ABS test. The overall reliability of the two treponemal tests was identical in this study when Bio-EnzaBead tests were read spectrophotometrically.

Our results indicate that visual readings are highly specific but lack the sensitivity and overall reliability required of a diagnostic treponemal test for syphilis. When substrate blanks were used for instrument calibration and tests were read spectrophotometrically, the sensitivity and overall reliability were equivalent to those of the FTA-ABS test, while the specificity was significantly greater.

The reproducibility of the syphilis Bio-EnzaBead test was determined by testing 60 coded serum samples prepared from six serum pools of known reactivity. Reproducibility was excellent overall, whether tests were read visually or spectrophotometrically with air or substrate blanks. Visual readings were poorest on sera from pool D, which gave 1+ reactivity. Of these 16 serum samples, 3 were read as equivocal or nonreactive because of an inability to visually detect color in the wells. No discrepancies occurred when the spectrophotometer was used with air blank calibration, and only one discrepancy occurred with substrate blank

calibration. This latter discrepancy occurred when one sample from nonreactive pool F was read as reactive. No cause could be determined, and an insufficient quantity of serum was available to repeat the test.

We were particularly interested in the reproducibility of the nonreactive, weakly reactive, and highly reactive control sera within each plate and throughout the study. Thoughtful distribution of control sera recommended by the kit manufacturer demonstrated no evidence of systematic error, thereby ensuring that each run was in control. We did observe an increase in absorbance values when negative control beads were tested against highly reactive sera, but the mean of optical densities for these negative control beads (0.017) was well below the mean of optical densities for weakly reactive sera tested against antigen control beads (0.063). The reproducibility of $R \times D$ values for control sera was acceptable.

Serum from one pregnant female and two patients with lupus erythematosus gave false-positive results with the FTA-ABS test but not the syphilis Bio-EnzaBead test. Because of the higher specificity and equal sensitivity, the Bio-EnzaBead test is recommended as an alternative to the FTA-ABS test as a treponemal antibody test for the diagnosis of syphilis.

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