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Treponemal Antibody-Absorbent Enzyme Immunoassay for Syphilis

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An enzyme immunoassay for the diagnosis of syphilis (ELISA-SY) was developed with solid-phase extracts of *Treponema pallidum*, specimen diluent containing Reiter treponeme absorbent, and three 30-min incubations. The ELISA-SY results were determined in comparison with a standardized positive control and reported as a percentage of strong positive control. In tests with 1,005 serum samples from a venereal disease clinic and other sources, 98.2% agreement was found with fluorescent treponemal antibody-absorption (FTA-ABS) results, and 98.3% agreement was found with *T. pallidum* passive hemagglutination (PHA) findings. Only 1 of 29 sera originally considered to be biologically false-positive was positive by ELISA-SY; the latter specimen was also positive by PHA and FTA-ABS tests performed in our laboratories. Serum samples from clinically diagnosed syphilitics (16 primary-stage isolates, 7 secondary-stage isolates, and 3 latent-stage isolates) were all positive by ELISA-SY, FTA-ABS, and PHA. Serum samples from 51 newborns suspected of having syphilis on the basis of positive cardiolipin flocculation tests showed 98% agreement of ELISA-SY results with FTA-ABS and PHA findings. Sera from all 61 patients with a variety of autoimmune and other diseases known to be associated with biologically false-positive reactions for syphilis were negative by this ELISA-SY. The specificity of the ELISA procedure for *T. pallidum* antibody was also confirmed immunologically by blocking experiments.

The serological detection of specific antibodies to *Treponema pallidum* is of particular importance in the diagnosis of syphilis, since the natural course of this infection is characterized by periods without significant clinical manifestations and the organism cannot be readily cultured in vitro. Although the nontreponemal (cardiolipin) antibody tests are extremely valuable for screening purposes, their relative lack of specificity for syphilis, i.e., treponemal antibody specificity, is a distinct disadvantage. Because of this, more specific serological tests with *T. pallidum* as antigen have been introduced and are widely used for confirmation of the screening results. Principal among these are the fluorescent treponemal antibody-absorbent (FTA-ABS) and passive hemagglutination (PHA) methods (9). In both of these, an extract of a nonpathogenic treponeme (*Treponema phagedenis* Reiter) is added to each specimen as an absorbent to render the procedures even more specific for *T. pallidum* antigens (15).

In recent years, enzyme-linked immunoassays (ELISAs) have proven quite valuable in the diagnosis of a wide variety of infectious and immunological diseases (3, 12, 16). Several laboratories have applied such procedures to syphilis with considerable success by using *T. pallidum* extracts as antigen (4, 7, 14, 17, 18). Our group has developed a series of 16 diagnostic ELISA procedures, all of which use similar reagents and format (5, 6, 10). The present report describes the results obtained with a rapid simple assay of this type for the detection of antibodies to *T. pallidum* that incorporates the *T. phagedenis* Reiter absorbent in the specimen diluent.

MATERIALS AND METHODS

***T. pallidum* antigens.** Washed suspensions of *T. pallidum* Nichols from 7- to 10-day infected rabbit testes were obtained from Lee laboratories, Grayson, Ga., and stored frozen. When needed, the suspension was thawed and extracted twice with 0.2% sodium deoxycholate in 0.1 M citrate-buffered 0.1 M NaCl (pH 7.4) for 1 to 2 h at 4°C. The extracts were clarified by centrifugation, and the protein content was estimated by A_{280} and A_{260} (8). The antigen solutions were stored at −70°C until used.

ELISA reagents. *T. pallidum* antigen was appropriately diluted in 0.1 M sodium bicarbonate (pH 9.6) and coated overnight at 4°C onto small plastic disks containing isothiocyanate groups (2). The disks were transferred to 0.1% gelatin and rotated at 4°C for 8 h to block unused binding sites. They were then sequentially washed with phosphate-buffered saline (pH 7.4), 0.01 M sodium bicarbonate buffer (pH 9.6), and finally 0.1% gelatin solution in 0.01 M sodium carbonate before being lyophilized. The dried antigen-coated disks were stored with desiccant at 4°C until use. They were stable for at least 16 months under these conditions.

The specimen diluent contained 83% (vol/vol) Reiter treponemal absorbent, 10% normal goat serum, 5% normal rabbit serum, 2% rabbit testicular extract, and 0.05% Tween 20. The Reiter treponemal absorbent was prepared from 7- to 10-day-old cultures of *T. phagedenis* in spirolate broth (BBL Microbiology Service, Cockeysville, Md.) containing 10% heat-inactivated normal rabbit serum by the method of Stout et al. (15). Rabbit testicular extract was prepared by high-speed homogenization of minced normal rabbit testes (Pelfreeze, Rogers, Ark.) in 0.05 M phosphate-buffered saline, (pH 7.4), followed by an overnight extraction at 4°C. The extract was clarified by centrifugation and filtration. The specimen diluent ingredients were mixed and sterilized through filters (pore size, 0.22 μm).

Goat antibodies to human immunoglobulin G were immunospecifically purified so as to include both heavy- and light-chain specificities. The latter were retained to accommodate other classes of syphilitic antibodies which are known to occur (1, 4, 11, 13). The anti-immunoglobulin G was conjugated to calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) with glutaraldehyde (3). The concentrations of this conjugate and the *T. pallidum* extract on the disks were adjusted so

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TABLE 1. Correlation of qualitative treponemal antibody test results with 1,005 serum samples form various sources

Assay procedure and result	No. giving result by ELISA-SY		% Agreement
	+	−	
FTA-ABS			
+	179	8	98.2
−	10	808	
PHA			
+	183	11	98.3
−	6	805	

that the standardized strong positive syphilitic control serum gave absorbance readings of approximately 1.0 in the assay.

The strong positive control for each test set was adjusted to have the same potency in the assay as a primary reference syphilitic serum pool. The latter was stored at −70°C in numerous aliquots. The weak positive control serum was calibrated to yield a positive value which was slightly above the cutoff point.

ELISA-SY procedure. In the ELISA procedure for the diagnosis of syphilis (ELISA-SY), 10 μl of serum sample was mixed with 500 μl of specimen diluent and incubated with an antigen-coated disk at 37°C for 30 min with gentle agitation. The disk was rapidly washed five times with 0.001 M Tris-buffered saline (pH 7.2 to 8.0) and then incubated as above with 500 μl of antibody-enzyme conjugate. After the disk was washed again, it was transferred to a clean vial and incubated as described above with 1.0 ml of the substrate *p*-nitrophenyl phosphate. The reaction was stopped with 0.1 ml of 3 M NaOH, and the A_{405} was read. Each assay included the strong positive control in triplicate, the weak positive control, and the negative control. Test results were determined by dividing the absorbance readings of the test and control serum samples by the average absorbance of the strong positive control and multiplying by 100. The result is the percentage of the strong positive control.

Serum samples. The bulk of the serum specimens were obtained from local sources and included specimens from commercial blood donors, volunteer blood donors, and patients attending a local venereal disease clinic. In addition, cord blood from newborns suspected of having congenital syphilis on the basis of screening test results was obtained from Jackson Memorial Hospital through the kindness of R. Hellman. We are deeply indebted to S. Larsen of the Centers for Disease Control (CDC), Atlanta, Ga., for making available to us several panels of carefully documented sera, including biologically false-positives, sera from patients clinically diagnosed as having primary syphilis, and a collection of quality control sera. Unless otherwise specified, biologically false-positive sera are reagin test reactive but treponemal antibody test nonreactive, as revealed by the original investigators who sent us the samples.

Other assays. Immunofluorescence assays (FTA-ABS) were performed with reagents from Difco Laboratories, Detroit, Mich. The PHA test for syphilis was obtained from the same source. Both assays were carried out by procedures specified by the CDC. The rapid plasma reagin test (RPR card) was from Hynson, Westcott and Dunning, Baltimore, Md. All test sets used were approved by the CDC.

RESULTS

Normal values. The normal cutoff values of ELISA-SY were determined by assaying 299 fresh serum samples which were negative by other treponemal antibody tests. The results showed a mean value of 20 and a standard deviation of 8. Further studies with 465 blood donor serum samples revealed similar results. Based on these findings, an ELISA-SY value of 45, representing the mean with 3 standard deviations, was established as the upper limit for negative results. Values of ≥50 were considered positive. Those which were ≥45 and <50 and were equivocal were retested. Equivocal results were considered negative when they were not found to be positive in the repeated assay.

Comparison with other treponemal antibody assays. A total of 1,005 serum samples were assayed for treponemal antibodies at Cordis Laboratories by ELISA-SY, FTA-ABS, and PHA. Excellent agreement was found among these three assays (Table 1 and 2). ELISA-SY showed 98.2% agreement with FTA-ABS and 98.3% agreement with PHA. There were 19 discrepant results, but the ELISA-SY findings disagreed with both of the other assays in only eight cases (0.8%).

The distribution of quantitative ELISA-SY values in FTA-ABS-positive and -negative sera are shown in Fig. 1. The 1,266 specimens depicted include the above-mentioned 1,005 in addition to 261 serum samples for which FTA-ABS findings were observed elsewhere. It may be seen that the positive ELISA-SY values ranged widely, from the cutoff of 50 to as high as 410. Of the FTA-ABS-positive specimens which were ELISA-SY negative, two were reported to be biologically false-positive by the original investigators who sent us the samples, and one was negative by PHA. Of the FTA-ABS-negative specimens which were ELISA-SY positive, three were also positive by PHA.

Sera (172 samples) from a venereal disease clinic were assayed by ELISA-SY within 1 to 3 days after the sera were harvested. The sera were stored at 2 to 8°C and were not frozen prior to assay. With these fresh sera, there was complete agreement among the ELISA-SY, FTA-ABS, and PHA results, with 26 samples being positive and 146 being negative.

Serum samples from 14 patients with primary syphilis were tested blindly by ELISA-SY, FTA-ABS, and PHA. All were positive by all methods. A panel of 40 specimens assembled by the CDC was assayed blindly and singly by ELISA. It was not known that this panel consisted of only 20 serum samples which had been randomly split or that the panel included serum specimens of patients in different stages of syphilis. The results (Table 3) revealed complete agreement with the FTA-ABS findings and showed very good quantitative reproducibility of the duplicate specimens.

Sera (51 samples) from newborns suspected of having congenital syphilis because of positive RPR screening test results were assayed by ELISA-SY, FTA-ABS, and PHA. The results (Table 4) show agreement among ELISA-SY and the other tests in 50 of 51 specimens.

A panel of 29 serum specimens found to be biologically

TABLE 2. Discrepancies in qualitative treponemal antibody test results with 1,005 serum samples

No. of specimens	Result as determined by:		
	ELISA-SY	PHA	FTA-ABS
5	−	+	−
4	+	+	−
6	+	−	−
2	−	+	+
2	−	−	+

false-positive by the CDC was assayed in our laboratories in parallel by ELISA-SY, FTA-ABS, and PHA. Of 29 specimens, 28 were negative by ELISA-SY and 1 was positive by all three procedures (Table 5). One additional specimen was positive by PHA, and two more were positive by FTA-ABS.

Specificity. The specificity of the ELISA-SY procedure was further studied in two ways. First, serum samples from 61 patients with a variety of diseases often associated with biologically false-positive reactions were studied (systemic lupus erythematosus, rheumatoid arthritis, infectious mononucleosis, thyroiditis, and myeloma). All showed strongly positive reactivities of the autoantibodies, etc., associated with their diseases. In addition, 30 serum samples from individuals with extremely high levels of antibodies to three unrelated infectious agents (*Toxoplasma gondii*, herpes simplex virus, and cytomegalovirus) were tested by ELISA-SY. All of the 91 specimens were negative by ELISA-SY (Table 6). However, 13 of the 85 specimens tested were positive by a syphilis screening procedure with cardiolipin as antigen (RPR). These 13 therefore represent additional biologically false-positive reactors which were negative by ELISA-SY.

The immunological specificity of the ELISA for syphilis was also confirmed by blocking experiments. Sera (four samples) which were positive by ELISA-SY (values of 240, 105, 76, and 55) were preincubated for 30 min with a variety of antigens at 1 mg/ml prior to the ELISA-SY. The antigens used were *T. pallidum* sonic extracts, *T.*

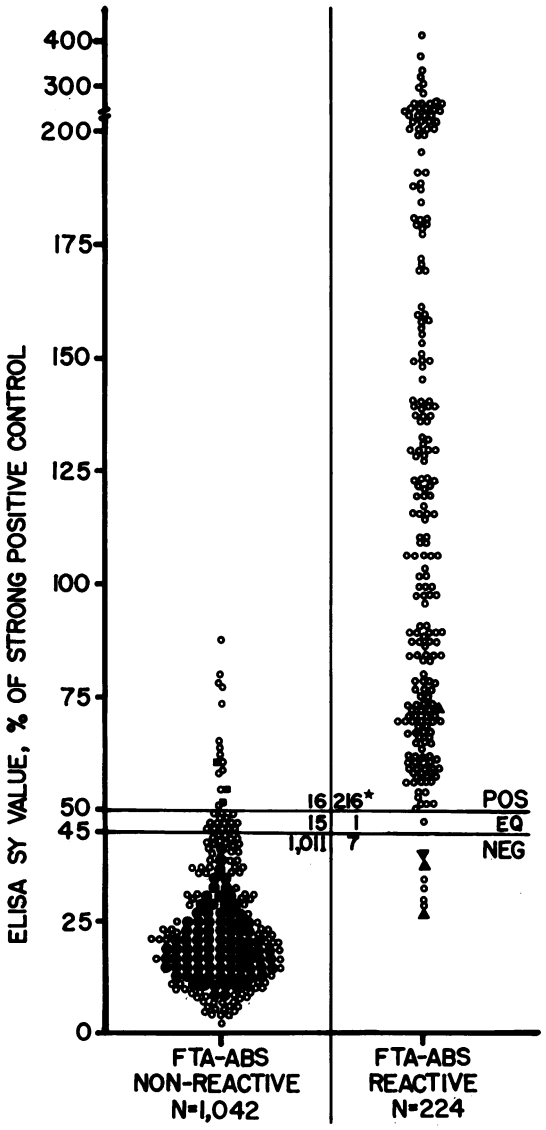

FIG. 1. Distribution of ELISA-SY values of 1,266 serum samples from several sources and disease categories in relation to FTA-ABS test results performed at Cordis Laboratories. Symbols: ●, 10 sera each showed this value by FTA-ABS and ELISA-SY; ○, one serum each showed this value by FTA-ABS and ELISA-SY; ■, positive by PHA for one serum each; ▼, negative by PHA for one serum each; ▲, biologically false-positive for one serum each; ★ and N, number of sera. POS, Positive; EQ, equivocal; NEG, negative.

TABLE 3. ELISA-SY results with 40 carefully documented specimens from the CDC[a]

Clinical group and duplicate sample ELISA-SY values	Qualitative result by ELISA-SY[b]	Result by FTA-ABS[c]
Normal		
26;37	N	N
17;14	N	N
30;24	N	N
17;19	N	N
22;22	N	N
31;31	N	N
10;11	N	N
24;23	N	N
Primary syphilis		
66;69	+	$1–2^+$
126;148	+	2^+
Secondary syphilis		
102;111	+	$1–2^+$
288;301	+	4^+
108;88	+	$2–3^+$
213;208	+	4^+
257;245	+	3^+
194;203	+	3^+
67;73	+	2^+
Latent syphilis		
309;307	+	4^+
85;144	+	$2–3^+$
65;67	+	$1–2^+$

[a] The 40 specimens consisted of 20 serum samples divided into randomly coded duplicates. The ELISA-SYs were done singly.
[b] N, Negative; +, positive.
[c] FTA-ABS results were as determined by the CDC. N, Negative. The CDC results ranged from 1 to 4^+, where 1 is the minimal reactive control and 4^+ is maximal fluorescence.

The immunological specificity of the ELISA for syphilis was also confirmed by blocking experiments. Sera (four samples) which were positive by ELISA-SY (values of 240, 105, 76, and 55) were preincubated for 30 min with a variety of antigens at 1 mg/ml prior to the ELISA-SY. The antigens used were *T. pallidum* sonic extracts, *T.*

TABLE 4. Correlation of qualitative treponemal test results with 51 serum samples from newborns suspected of having syphilis on the basis of RPR reactivity

Assay procedure and results	No. giving result by ELISA-SY		% Agreement
	+	−	
FTA-ABS			
+	38	1	98.0
−	0	12	
PHA			
+	38	1	98.0
−	0	12	

TABLE 5. Correlation of qualitative treponemal antibody test results with 29 serum samples considered to be biologically false-positive by the CDC

Assay procedure and result[a]	No. giving result by ELISA-SY[b]	
	+	−
FTA-ABS		
+	1	2
−	0	26
PHA		
+	1	1
−	0	27

[a] The assay was performed at Cordis Laboratories.
[b] Discrepancies were found among ELISA-SY, PHA, and FTA-ABS for two specimens. One specimen was negative by ELISA-SY but positive by PHA and FTA-ABS; the other was negative by ELISA-SY and PHA but positive by FTA-ABS.

phagedenis Reiter sonic extracts, extracts of bovine heart, cytomegalovirus, and *Toxoplasma gondii*, calf thymus deoxyribonucleoprotein, and calf thymus DNA. The last four antigen preparations had been previously shown to significantly block the ELISAs for antibodies to these antigens by the same format and procedure (5, 6). Only the *T. pallidum* extracts significantly blocked the ELISA-SY reactivity (Table 7).

Reproducibility. Positive sera (four samples; ELISA-SY values of 70, 81, 117, and 178) and negative sera (three samples; ELISA-SY values of 22, 18, and 18) were assayed by ELISA-SY in replicates of six on five different days in one laboratory. In 210 total repeat tests, the four positive serum samples always showed positive results, with coefficients of variation of 9, 11, 5, and 8%. The three negative serum samples always showed negative results, with coefficients of variation of 13, 15, and 14%. The good duplicate reproducibility of 20 serum samples assayed blindly at the CDC is also shown above (Table 3).

These same seven specimens were coded and assayed by four different operators in four different laboratories in replicates of six for each specimen. In 168 total repeat tests in the four laboratories, all four positive serum samples were positive on every occasion, with coefficients of variation of 8, 6, 9, and 16%. All three negative serum samples were always negative, with coefficients of variation of 14, 7, and 10%.

DISCUSSION

Serological tests for syphilis play a significant role in the diagnosis of this infection since the organisms cannot yet be readily cultivated in vitro and can be visualized by direct microscopy for only a relatively short time during the course of the prolonged infection. The present ELISA procedure with the Reiter absorbent in the specimen diluent revealed results which were in very good agreement with the two other most widely used treponemal tests for detecting *T. pallidum*-specific antibodies. Unlike these other procedures (FTA-ABS and PHA), the ELISA-SY does not require any pretreatment of the serum (such as heat inactivation) or parallel testing with uncoated erythrocytes. In addition, the ELISA-SY results are based on objective numerical absorbance readings, whereas the other tests require subjective interpretation. The ELISA-SY results agreed with FTA-ABS findings in 98.2% of the cases and with PHA in 98.3% of the 1,005 specimens assayed by all procedures. Of the 19 discrepancies in the 1,005 specimens, only eight cases were seen in which the ELISA-SY findings disagreed with both of these other methods. It is possible that these discrepancies were caused by differences in the physical form or presentation of the *T. pallidum* antigen used in the tests or that the subjectivity of readout in the non-ELISA methods was contributory.

The specificity of the ELISA-SY procedure for *T. pallidum* and syphilis was supported in a number of ways in addition to the very good agreement among all three treponemal test results. Serum samples from 61 patients with a variety of autoimmune and other diseases which are often associated with biologically false-positive cardiolipin screening tests for syphilis were all negative by ELISA-SY. Most of these patients had very high levels of the autoantibodies, etc., associated with their illnesses. In fact, 13 of the 56 serum samples tested (23%) showed positive RPR results,

TABLE 6. ELISA-SY and RPR test results with 91 serum samples from patients with autoimmune and other diseases

Disease (no. of patients)	Antibodies detected[a]	Antibody values		Result of test for syphilis by:			
		Range in patients	Normal	ELISA-SY		RPR	
				+	−	+	−
Systemic lupus erythematosus (18)	Anti-DNA	506–1,308 (IU/ml)	<160 (IU/ml)	0	9	3	6
	Anti-nucleoprotein	322–8,086 (IU/ml)	<25 (IU/ml)	0	9	3	6
Rheumatoid arthritis (8)	RF	134–1,682 (IU/ml)	<10 (IU/ml)	0	8	2	5[c]
Thyroiditis (9)	Anti-thyroglobulin	22–2,127 (%)[b]	<10 (%)	0	9	0	5[c]
Infectious mononucleosis (15)	Heterophil antibody	37–148 (%)	<15 (%)	0	15	5	10
Toxoplasmosis (10)	Anti-toxoplasma	221–612 (IU/ml)	<30 (IU/ml)	0	10	0	9[c]
Herpes simplex virus infection (10)	Anti-herpes simplex virus	108–144 (%)	<12 (%)	0	10	0	10
Cytomegalovirus infection (10)	Anti-cytomegalovirus	191–244 (%)	<18 (%)	0	10	0	10
Myeloma (11)[d]				0	11	0	11

[a] The following CORDIA assays measured the antibodies indicated: CORDIA N, anti-DNA; CORDIA NP, anti-nucleoprotein; CORDIA RF, immunoglobulin M rheumatoid factor (RF); CORDIA TG, anti-human thyroglobulin; CORDIA IM, Paul-Bunnel heterophil antibody; CORDIA T, antibodies to *Toxoplasma gondii*; CORDIA HS, antibodies to herpes simplex virus; CORDIA CMV, antibodies to cytomegalovirus.
[b] %, Percentage of positive control.
[c] Insufficient quantities of sera were available for testing all the specimens by RPR.
[d] Abnormally elevated levels of immunoglobulin G (seven samples), A (two samples), and M (two samples) were found.

TABLE 7. Blocking experiment to demonstrate the immunological specificity of ELISA-SY

Blocking antigen[a]	Residual ELISA-SY activity (% of control) for sample[b]:				
	A	B	C	D	Mean ± SD
T. pallidum	65	43	46	46	50 ± 10
T. phagedenis	91	96	92	104	96 ± 6
Bovine heart	88	99	91	109	97 ± 9
Cytomegalovirus	94	98	100	105	99 ± 5
Toxoplasma gondii	95	94	94	100	96 ± 3
Calf thymus NP	85	85	87	120	94 ± 17
Calf thymus DNA	87	97	88	102	94 ± 7

[a] Blocking antigens were prepared as described elsewhere (5, 6) or by the same procedure as for *T. pallidum*. NP, Deoxyribonucleoprotein.

[b] Four serum samples (A, B, C, and D) were preincubated with blocking antigens (1 mg/ml) at 37°C for 30 min before the ELISA-SY. The ELISA-SY values were converted to the percentage of that treated similarly with phosphate-buffered saline (control). Specimens A, B, C, and D showed ELISA-SY control values of 240, 105, 76, and 55, respectively.

indicating that they would be considered biologically false-positive results. In addition, 30 serum samples with extremely high titers to three other microbial agents (*Toxoplasma gondii*, herpes simplex virus, and cytomegalovirus) were all clearly negative by ELISA-SY. A panel of 29 specimens which were biologically false-positive revealed only 1 which was reactive by ELISA-SY. The same specimen was also reactive by FTA-ABS and PHA in our laboratory. Finally, immunological absorption studies with a number of other antigens, including the Reiter spirochete and extracts of bovine heart, revealed that only *T. pallidum* extracts were capable of blocking the ELISA-SY reactivity in positive specimens.

Although ELISA tests with *T. pallidum* extracts were described some time ago (4, 7, 14, 17, 18), these assays are not yet widely used diagnostically. It has been suggested by Hunter et al. (7) that poor antigen stability may be one factor in this delay. The present ELISA-SY does not suffer from this problem, and the disks, as well as the other reagents, are stable for at least 1 year 4 months when stored appropriately at 2 to 8°C. Other quite similar diagnostic ELISAs which have been developed in our laboratory (5, 6, 10) have revealed documented stability for up to 4 or 5 years.

Many of the sera tested in this study of necessity had been stored for some time prior to assay, mostly frozen. It has been suggested that fresh sera (fewer than 4 days old) stored at 2 to 8°C may give more reliable results in other syphilis serological assays (9). It is of interest that there were no discrepancies in the three treponemal antibody tests with all 172 serum samples that were assayed within 3 days after collection. Further studies are required to confirm this.

Although a small number of serum samples from individuals with clinically diagnosed syphilis were found to yield satisfactory results with the present ELISA-SY, additional investigations with larger numbers of patients at various stages of the disease are planned.

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