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Comparative Evaluation of Nine Different Enzyme-Linked Immunosorbent Assays for Determination of Antibodies against *Treponema pallidum* in Patients with Primary Syphilis

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Nine different enzyme-linked immunosorbent assays (ELISAs) with a sonicate or recombinant proteins of *Treponema pallidum* as antigen have been evaluated comparatively by testing 52 highly selected sera from patients with primary syphilis, all negative in the microhemagglutination test for *T. pallidum* (MHA-TP). Eight tests exhibited greater sensitivity (48.5 to 76.9%) than the commonly used Venereal Disease Research Laboratory test (44.2%). Higher sensitivity could be related to (i) the volume and dilution of the serum, (ii) the design of the assay (capture and competitive tests showed higher sensitivity than sandwich-based assays), and (iii) the ability to detected specific immunoglobulin M antibodies. The specificity of the ICE Syphilis and the Enzygnost Syphilis tests was 99.5 and 99.8%, respectively, as determined by routine testing of 2,053 unselected sera in comparison with the MHA-TP test. ELISAs tested offered high sensitivity in patients with primary syphilis; however, recommendations to use these tests as screening assays do need further data on specificity and reactivity in late stages of the disease.

The serological detection of specific antibodies to *Treponema pallidum* is of particular importance in the diagnosis of syphilis, since the natural course of the infection is characterized by periods without clinical manifestations. Although syphilis rates are declining in the United States after an epidemic between 1986 and 1990 (1), the incidence of syphilis in Europe has increased since 1992, especially in the countries of the Russion Federation, where peaks of 263 cases per 100,000 have been reported (19).

In Europe, screening is based mainly on treponemal antigen tests such as the microhemagglutination assay for *T. pallidum* antibodies (MHA-TP), whereas in the United States the Rapid Plasma Cardiolipin antigen test (RPR) or the Venereal Disease Research Laboratory test (VDRL) is recommended as a screening test (23). Cardiolipin tests such as the RPR or VDRL, although technically simple and cheap, are labor-intensive, may occasionally give false-negative reactions due to the prozone phenomenon (9), and are insensitive with samples from patients with early or late-stage infection. *T. pallidum*-specific tests such as the MHA-TP also lack sensitivity in the very early stage of the disease; however, they offer the highest sensitivity for late stages of the disease (10, 12).

Unfortunately, neither lipoidal tests, e.g., VDRL, nor the MHA-TP can easily be automated; results are usually read subjectively and recorded manually. The potential of fully automated tests was first demonstrated in 1975 using an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of syphilis (21). Since then, ELISAs using as antigen the axial filament of *Treponema phagedenis* biotype Reiteri (20) and cardiolipin, cholesterol, and lecithin (18), as well as a sonicate of purified *T. pallidum* organisms (2–4, 8, 17), have been developed.

Serum immunoglobulin responses to individual *T. pallidum* polypeptides have been studied by Western blotting (14). During primary syphilis, the earliest responses are against TpN47 and some of the flagellar proteins, followed by TpN15 and TpN17. Antibodies against TpN15, TpN17, TpN44.5 (TmpA), and TpN47 appear to be diagnostic for acquired syphilis (10). With the availability of individual *T. pallidum* antigens produced with recombinant DNA techniques, new tests were developed. The use of recombinant *T. pallidum* antigens in place of poorly defined mixtures of antigens from the Nichols strains of *T. pallidum*, which may be contaminated with rabbit testicular components, has the potential for improving the specificity of serological assays. Tests based on antigens produced with recombinant DNA techniques from single genes like TmpA (7); the 4D antigen, a ring-forming decamer on the outer membrane (15); or a combination of different recombinant proteins have become available (23, 24). Most of the new tests have been evaluated in comparison with a standard test (3, 11, 13, 22, 24), e.g., the MHA-TP, the VDRL, or the Capital G test, an ELISA-based test commercially available for more than 10 years. However, no comparative evaluation of these tests has been published yet.

This report assesses the performance characteristics of nine ELISAs, commercially available in Europe. Sensitivity was evaluated with a panel of 52 highly selected sera (all MHA-TP negative) from patients with primary syphilis diagnosed by dermatologists. These sera were selected because (i) MHA-TP is used as a screening assay in many Western countries and (ii) sera from patients with early latent or secondary syphilis with titers of at least 1:640 by MHA-TP and 1:4 by VDRL were reactive in all ELISAs tested, allowing no differentiation of sensitivity (data not shown). In addition to the routinely used screening assay (MHA-TP; Fujirebio, Tokyo, Japan), the following tests were performed: the VDRL (Biomerieux, Lyon, France), the Fluorescent Treponemal Antibody-Absorption (FTA-ABS) test (Biomerieux), the Captia Syphilis M ELISA (Trinity, formerly Centocor US), the ICE Syphilis (Murex, Dartford, United Kingdom), the Trepanostika TP (Organon,

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TABLE 1. Characteristics of ELISAs for detection of *T. pallidum* antibodies[a]

Trade name (company)	Trepanostika (Organon)	ICE Syphilis (Murex)	Enzygnost Syphilis (Behring)	Pathozyme Syphilis Competition (Omega)	Bioelisa Syphilis (Biokit)	Trep-Chek (Phoenix)	TmpA-ELISA (Eurodiagnostica)	Captia Syphilis G (Trinity)	Captia Syphilis M (Mercia)	19S IgM FTA-ABS
Predilution (μl; serum + diluent)	No	No	No	No	Yes (10 + 200)	Yes (20 + 380)	Yes (20 + 400)	Yes (10 + 200)	Yes (20 + 1,000)	No
(Prediluted) serum (μl)	30	50	25	25	100	100	100	210	100	
Diluent (μl)	0	50	0	0	0	0	0	0	0	
Dilution	1:1	1:2	1:1	1:1	1:21	1:20	1:21	1:21	1:51	1:10
Antigen	*T. pallidum* lysate	Rec. TpN15 Rec. TpN17 Rec. TpN47	*T. pallidum* lysate	*T. pallidum* lysate	Rec. TpN15 Rec. TpN17	Cocktail of Rec. antigens	Rec. TmpA	*T. pallidum* lysate	*T. pallidum* lysate	Whole treponemes
Method	Competitive	Capture	Competitive	Capture	Sandwich	Sandwich	Sandwich	Sandwich	Capture	Immunofluorescence
No. of incubations	2	3	2	2	3	3	3	3	3	2
Duration of all incubations (min)	105	120	120	115	120	75	150	90	150	60
No. of washings	1	2	1	1	2	2	2	2	2	1
Cutoff	NC × 0.7	NC + 0.200	NC × 0.7	Low positive control	NC + 0.300	Cutoff control	NC + 0.100	NC × 2	Low positive control	
Detection of Ig	IgM + IgG	IgM + IgG	IgM + IgG	IgM + IgG	IgM + IgG	IgG	IgM + IgG	IgG	IgM	IgM

[a] Rec, recombinant; NC, negative control; ∗, overall dilution factor of IgM fraction of serum.

TABLE 2. Sensitivity of ELISAs for syphilis in comparison with the IgM specific immunofluorescence test (19S IgM FTA-ABS)[a]

Trade name (company)	Trepanostika (Organon)	ICE Syphilis (Murex)	Enzygnost Syphilis (Behring)	Pathozyme Syphilis Competition (Omega)	Bioelisa Syphilis (Biokit)	Trep-Chek (Phoenix)	TmpA-ELISA (Eurodiagnostica)	Captia Syphilis G (Trinity)	Captia Syphilis M (Mercia)	19S IgM FTA-ABS
No. nonreactive/total no.	12/52	13/52	16/52	16/52	17/41	15/41	16/31	24/31	7/52	5/52
Sensitivity (%)	76.9	75.0	69.2	69.2	67.3	63.4	48.5	22.6	86.5	90.4
Sensitivity (95% confidence interval)	63.2–87.5	61.1–86.0	54.9–81.3	54.9–81.3	52.9–79.7	46.9–77.9	30.2–66.9	9.6–41.1	74.2–94.4	79.0–96.8

[a] Comparative evaluation was performed with 52 sera from patients with primary syphilis, all nonreactive in the MHA-TP test.

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TABLE 3. Phi coefficients of agreement between each assay

Test (no.)	Trepanostika (1)	ICE Syphilis (2)	Enzygnost (3)	Pathozyme (4)	Bioelisa (5)	Trep-Chek (6)	TmpA (7)	Captia G (8)	Captia M (9)
1									
2	0.95								
3	0.82	0.87							
4	0.86	0.87	0.91						
5	0.90	0.78	0.93	0.82					
6	0.85	0.90	1.00	0.89	0.85				
7	0.72	0.72	0.72	0.72	0.69	0.77			
8	0.43	0.43	0.43	0.43	0.54	0.47	0.55		
9	0.59	0.59	0.47	0.47	0.45	0.46	0.31	0.26	
10	0.13	0.11	0.19	0.35	0.20	0.09	0.24	0.14	0.25

Veedik, Belgium), the Enzygnost Syphilis (Behring, Marburg, Germany), the Pathozyme Syphilis Competition (Omega Diagnostics, Alloe, United Kingdom), the TmpA enzyme immunoassay (Eurodiagnostica, Apeldoorn, The Netherlands), the Bioelisa Syphilis (Biokit, Barcelona, Spain), the Trep-Chek (Phoenix, Mississauga, Canada), the SelectSyph-G (Trinity), and the 19S FTA-ABS test, a fluorescent *T. pallidum* absorption test using an isolated immunoglobulin M (IgM) fraction of the serum. Separation of IgM antibodies was done by ion-exchange chromatography (IgM/IgG Trennsystem II; Bios Labordiagnostik, Munich, Germany). All sera were tested in duplicate. Each ELISA was performed according to the recommendations of the manufacturer using a Plato 3300 robotic microplate processor (Rosys, Hombrechtikon, Switzerland). Details of the characteristics of the different ELISAs are summarized in Table 1. Sufficient material was available to test all 52 sera in 10 assays; however, Trep-Chek could be evaluated with only 41 sera of the selected panel, and both SelectSyph-G and TmpA enzyme immunoassay could be evaluated with only 31 sera.

Specificity for two of the ELISAs, the ICE Syphilis and the Enzygnost Syphilis, was determined by routine testing of 2,053 unselected sera in comparison with the MHA-TP.

Sensitivities of nine different ELISAs are summarized in Table 2 together with the results of the 19S IgM FTA-ABS test, which has been used in our laboratory for more than 20 years. Coefficients of agreement between assays are shown in Table 3. VDRL and FTA-ABS were positive for only 23 of 52 (44%) and 39 of 52 (75%) patients tested, respectively (data not shown in Table 2), indicating that most sera were from patients in the very beginning of the disease. Not surprisingly, ELISAs using a greater volume of serum and/or a lower dilution yielded higher sensitivity. In three test kits, undiluted serum is used. The main influence on sensitivity can be attributed to the design of the assay: sandwich-based tests, where the solid phase is coated with the antigen and after incubation with serum the bound immunocomplex is detected by an anti-human IgG or IgM conjugate, all have a lower sensitivity. Competitive ELISAs also have a surface-bound antigen, but antibodies present in serum have to compete with added, labeled *T. pallidum* antibodies, resulting in low optical density (OD) values, if specific antibodies are present in tested serum. Capture ELISAs have anti-human IgG or IgM molecules bound to the microtiter well. After incubation with serum, a part of the human immunoglobulins is bound to the solid phase. In the second incubation, specificity is achieved with a complex of antigen and labeled anti-*T. pallidum* antibodies. ELISAs based on capture or competitive assays all had greater sensitivity than sandwich assays.

Two assays, the ICE Syphilis and the Trepanostika, do need intensive washings after incubations, e.g., dispensing at least 500 μl of wash buffer and simultaneously sucking off all but 280 μl, which results in a heavy turbulence of the liquid. Simple well filling and emptying of cavities, which otherwise worked well in all other tests, resulted in poor reproducibility of results.

Recombinant antigens used in ELISAs do not necessarily result in better performance than that of tests with a purified *T. pallidum* sonicate as antigen. Fujimura et al. (5) have shown that highly different ODs were obtained using ELISAs with different cloned antigens. A test based on a single cloned protein, the TmpA (7), offered a rather limited sensitivity (48.5%) in this evaluation. However, results of this test tended to become negative after treatment (23). Gerber et al. (6) have shown that ELISAs based on a combination of cloned antigens resulted in better sensitivity than assays with single cloned antigens.

High sensitivity could be obtained with ELISAs using a purified *T. pallidum* sonicate as antigen, as demonstrated by the Trepanostika (76.9%), the Enzygnost Syphilis (69.2%), or the Pathozyme Syphilis (69.2%) test. The ICE Syphilis ELISA (sensitivity, 75%) uses three recombinant *T. pallidum* antigens (TpN15, TpN17, and TpN47) applied as a coating to the wells of microtiter plate strips. The wells are also coated with anti-human IgG and anti-human IgM. The antitreponemal component of the captured antibodies is detected by labeled antigen (TpN15, TpN17, and TpN47). This test demonstrated the highest ODs compared to all other assays and also the highest antibody index, e.g., the best discrimination power, calculated by dividing the OD of the test serum by the cutoff. However, specificity (99.5%) was shown to be lower than that with Enzygnost Syphilis (99.8%). Evaluation of specificity was done by testing 2,053 unselected sera, submitted for routine screening, in three assays. Fifty-eight sera reacted with three tests. A further 10 were reactive only in the ICE Syphilis test, 4 were reactive only in the Enzygnost Syphilis test, and none were reactive with the MHA-TP only. The sera reactive with the three tests were tested by the FTA-ABS test and were all found positive. Sera reactive with only one of the two ELISAs were also tested with the FTA-ABS, but were all negative. Review of patient records enabled us to rule out the risk of infection, and none had a history of a previous infection.

Assays measuring IgG and IgM antibodies exhibited a higher sensitivity in this selected serum panel, as expected. Isolated reactivity to IgM antibodies (reactive only in the Captia Syphilis M or the 19S IgM FTA-ABS test) could be detected in five sera, two further were reactive in IgM tests and the FTA-ABS, and another three were reactive in IgM tests, FTA-ABS, and the VDRL. Sequential serum specimens of four of five isolated IgM reactive sera were available and found to be reactive to MHA-TP, FTA-ABS, and VDRL within the next 2 weeks. Thirty-eight sera were positive in IgM tests and

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in at least four different ELISAs, and for two patients all tests were negative (seronegative stage). The two tests (Trepchek and Captia Syphilis G), in which only specific IgG antibodies can be detected, had sensitivities of 63.4 and 22.6%. The highest sensitivity of all ELISAs compared was demonstrated by the single IgM-specific assay, the Capita M. Unfortunately, this test cannot be recommended as a screening assay, as specific IgM can be detected only rarely in late syphilis and the test specificity is no higher than 91% (16).

This evaluation with sera from patients in the very early stage of the infection clearly demonstrates the superiority of specific IgM tests. However, Young et al. (23) found specific IgG antibodies in all seven sera from patients with primary syphilis. The different results can be related to the selection of the sera: in that study, the seven sera were all reactive in the MHA-TP test and the ICE Syphilis test and six of seven were reactive in the VDRL test. In contrast, only sera with negative MHA-TP results were selected in this evaluation.

Sensitivity of all but one of the ELISAs was superior compared to the VDRL test and/or the MHA-TP test. In addition, ELISAs are ideally suited for the detection of large numbers of specimens, because they can be automated, the results are read objectively, and reports are generated electronically, reducing the risk of transcriptional errors. However, in using any one of the ELISAs tested as a screening assay, one has to consider the facts that (i) special precautions should be taken in handling (e.g., washing of plates), (ii) more data for specificity should be evaluated (only data for two tests are presented in this study), and (iii) insufficient data are available at present to verify proper reactivity in late syphilis. Compared to standard screening tests, more handling steps (preparation of serum dilution; dispensing the appropriate volume of diluted serum into the wells of the microtiter plate; dispensing of negative, positive, and cutoff controls; addition of conjugate, substrate, and stop solution; washings; incubation at elevated temperatures; and optical readings of the plates) are necessary for performing ELISAs. Screening large numbers of samples per day makes robotic processors unavoidable. Finally, all tested ELISAs are more expensive than the hemagglutination tests, the VDRL tests, or the RPR tests.

In syphilis, where latent infections now predominate, a screening test should be able to detect all stages of the disease. Our evaluation demonstrates that in early infection specific IgM tests are still the most sensitive ones.

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