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Laboratory Diagnosis of Syphilis With Automated Immunoassays

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The serological detection of specific antibodies to *Treponema pallidum* is of particular importance in the diagnosis of syphilis. The purpose of this study was to evaluate diagnostic performances of automated immunoassays in comparison with *T. pallidum* hemagglutination test (TPHA) and Western Blot (WB). The retrospective study was performed with different panels of sera: 244 clinical and serological characterized syphilitic sera and 203 potentially interfering samples. All the sera were tested by Enzygnost Syphilis, ARCHITECT Syphilis TP, TPHA, and homemade WB. The diagnostic performances of the two assays were very similar: both Enzygnost Syphilis and ARCHITECT Syphilis TP performed with a sensitivity of 99.2%, whereas the specificity was 98.5 and 98.4%, respectively. Considering the suitability for automation, both immunoassays may represent a good choice as a screening test. However, the use of a confirmatory test, such as TPHA or WB, remains a must in order to avoid false-positive results. J. Clin. Lab. Anal. 23 : 1—6, 2009.

Key words: syphilis serology; *Treponema pallidum*; automated immunoassays; TPHA; Western Blot

INTRODUCTION

Syphilis, which is caused by the spirochete *Treponema pallidum* subsp. *pallidum*, is a chronic bacterial infection that remains a public health concern worldwide. The WHO estimated that 12 million new cases of venereal syphilis occurred in 1999, more than 90% of them in developing countries, with a rapidly increasing number of cases in Eastern Europe (1,2). Congenital syphilis is of particular concern in developing nations as it may lead to spontaneous abortion, stillbirth, death of the neonate, or disease in the infant; a recent report from Tanzania estimates that up to 50% of stillbirths are caused by syphilis (3). Of particular importance to worldwide health is the recognition that syphilis infection greatly increases the transmission and acquisition of human immunodeficiency virus (HIV) (4,5). These factors, along with the highly destructive nature of late disease, make syphilis an important public health concern.

Moreover, recent outbreaks have been reported in several cities in Europe and North America among men who have sex with men (MSM) (6–8). Outbreaks among MSM are associated with a rise in unsafe sexual behavior, perhaps a consequence of improved antiretroviral treatment for HIV in recent surveys; 37–52% of MSM reported multiple risk behaviors (6–9).

The serological detection of specific antibodies to *T. pallidum* is of particular importance in the diagnosis of syphilis, as the natural course of the infection is characterized by periods without clinical manifestations (10,11). Serological tests are divided into nontreponemal and treponemal tests and neither alone is sufficient for diagnosis, as nontreponemal tests can be used to monitor therapy, but owing to their low specificity the positive results obtained by these methods need to be confirmed by treponemal tests. On the contrary, as the positivity at treponemal tests lasts throughout the life, treponemal tests cannot be used in the follow-up of patients. Consequently, the quest for simple, reliable,

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and money-saving diagnostic methods continues. Nontreponemal tests include the Venereal Disease Research Laboratory and the Rapid Plasma Reagin (RPR) card tests. Treponemal tests include the serum fluorescent treponemal antibody absorption test, the *T. pallidum* hemagglutination test (TPHA) (Radim, Pomezia, Italy), the enzyme-linked immunoassay (ELISA), and the Western Blot (WB) assay; both ELISA and WB tests can be based on either whole-cell lysate (12–15) or recombinant (16–20) treponemal antigens. More recently, chemiluminescent immunoassays set up with recombinant antigens have been evaluated (21,22). The purpose of this study was to evaluate the diagnostic performances of Enzygnost Syphilis (Dade Behring, Marburg, Germany) in comparison with ARCHITECT Syphilis TP (Abbott Japan Co., Tokyo, Japan). Both tests are fully automated, but differ in the antigen composition and principle of the method. As confirmatory tests, TPHA and WB were used.

A retrospective study was performed with three panels of sera: the first included 244 clinical and serological characterized syphilitic sera, whereas the second included 74 samples obtained from patients suffering from potentially interfering diseases. Finally, we evaluated Enzygnost Syphilis performances using a third panel of 129 sera selected from among the 9,210 samples submitted between 1st February 2007 and 30th April 2007 to the Microbiology Laboratory of the St. Orsola Hospital in Bologna for routine screening for syphilis: all 129 sera had been scored reactive by ARCHITECT Syphilis TP, but negative by TPHA and WB.

MATERIALS AND METHODS

Study Group

For this retrospective study, sera were obtained from three different groups of subjects as follows: the first panel of specimens was obtained from 244 patients suffering from different stages of syphilis. The staging of the disease was done following the clinical and laboratory criteria proposed by Norris and Larsen (23). The second panel of 74 sera was obtained from patients suffering from some of the most common biological conditions possibly resulting in false-positive reactivity in syphilis serology. In this group the following specimens were included: sera obtained from culture-confirmed Lyme disease ($n = 10$), sera from subjects with a clinical diagnosis of infectious mononucleosis detected as positive by the Paul–Bunnel–Davidsohn agglutination ($n = 10$), sera from pregnant women ($n = 14$), sera from patients with cytomegalovirus acute infection (IgM positive and low avidity) ($n = 20$), and sera from patients with acute toxoplasmosis (IgM positive and low avidity) ($n = 20$). Finally, the third panel of sera consisted of 129 sera selected from among the 9,210 samples submitted between 1st February 2007 and 30th April 2007 to the Microbiology Laboratory of the St. Orsola Hospital in Bologna for routine screening for syphilis. All 129 sera included in this study had been previously scored reactive by ARCHITECT Syphilis TP, but negative by TPHA and WB. In particular, 43 of these samples were from blood donors.

All the samples used in this study had been frozen after being tested by ARCHITECT Syphilis TP, TPHA, WB, and RPR. Before testing by ELISA, all the sera were allowed to thaw at room temperature and prior to use they were mixed thoroughly by vortexing.

Enzygnost Syphilis

Enzygnost Syphilis is a competitive one-step enzyme immunoassay for the in vitro determination of antibodies to *T. pallidum*. *T. pallidum*-specific antibodies (IgG and/or IgM) contained in the sample and the peroxidase conjugate-labelled antibodies compete for binding to the treponemal antigens coated onto the wells of the plate. Unbound serum antibodies and conjugate antibodies are washed out and the enzyme activity of the bound conjugate is then determined. The intensity of the resultant color is inversely proportional to the concentration of *T. pallidum* antibodies in the sample. The assay was processed by automated instrumentation (Genesis RSP 200/BEP III) and the results were interpreted following the manufacturer's instructions.

ARCHITECT Syphilis TP

ARCHITECT Syphilis TP is a two-step immunoassay for the qualitative detection of IgG and/or IgM to *T. pallidum* in human serum or plasma using chemiluminescent microparticle immunoassay (CMIA) technology. In the first step, sample microparticles coated with recombinant *T. pallidum* antigens (TpN15, TpN17, and TpN47) and diluent are combined. Anti-*T. pallidum* antibodies present in the sample bind to the coated microparticles. After washing, the acridinium-labelled anti-human IgG and IgM conjugate is added in the second step. Following another wash cycle, pretrigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of anti-*T. pallidum* antibodies in the sample and the RLUs detected by the ARCHITECT immunoassay optical system. The test was performed and interpreted following the manufacturer's instructions.

Source of *T. pallidum* for WB

T. pallidum subsp. *pallidum* (Nichols strain) was originally obtained from the Statens Serum Institute, Copenhagen, Denmark, and maintained by passage in the testicles of adult male New Zealand white rabbits every 10–14 days. The animals were given antibiotic-free food and water ad libitum. Treponemes were extracted from the infected testicles and prepared for use as antigens as described elsewhere (24), after the animal had been euthanatized with thiopental (Pentothal). Briefly, each treponemal suspension was prepared from infected rabbit testicles 10–14 days after inoculation. In order to be used as a working stock of antigen, the treponemes were diluted in phosphate-buffered saline (PBS) to 1×10^9 organisms per ml, as determined by dark-field microscopy examination.

SDS-PAGE and WB

Separation of *T. pallidum* polypeptides was performed with a Laemmli buffer system by using a 12% w/v acrylamide gel (24,25). The WB procedure was performed according to Towbin et al. (26). After electrophoretic transfer, the blots were incubated overnight at room temperature with sera diluted 1:100 in PBS containing 0.05% v/v Tween 20. Antigen–antibody complexes were detected by using a peroxidase-labelled rabbit antiserum to human IgG (DAKO, Copenhagen, Denmark) diluted 1:500 in PBS-Tween and 4-chloro-1-naphthol (Bio-Rad, Hercules, CA) as already described (24). A WB test was considered positive when at least three bands out of TpN47, TmpA, TpN17, and TpN15 were clearly recognized. A test was considered negative when no bands or fewer than the three above-mentioned *T. pallidum* antigens were recognized (19,27). The reader was blinded of the identity and of the clinical status of the serum samples when the WB strips were examined. The apparent molecular weight of each band was determined by plotting the positions of SeeBlue Plus2 Prestained Molecular Weight Standard (Invitrogen, Carlsbad, CA). Positive and negative control sera were run with each group of specimens. The positive control was prepared by pooling 20 sera from patients suffering from primary or secondary syphilis: each serum used gave a positive result both in WB and in TPHA, and the pool obtained was retested by both methods, giving a value of 1:1,280 in TPHA and a clear recognition of the four specific *T. pallidum* bands in WB. As a negative control, we used a pool of 50 samples from healthy blood donors: each serum was tested by TPHA showing a negative result; the pool obtained was retested, being negative by TPHA (titer <40), and no bands were detected by WB.

TPHA and RPR

TPHA and RPR (Radim) testing was performed following the manufacturer's instructions. TPHA titers ≥ 80 were considered as positive

RESULTS

Results obtained by all the diagnostic methods in the three different groups are summarized in Table 1. When used to define the immune response against *T. pallidum* in sera obtained from syphilis patients, both Enzygnost Syphilis and ARCHITECT Syphilis TP showed very good diagnostic performances compared with WB and TPHA findings. In fact, both Enzygnost Syphilis and ARCHITECT Syphilis TP performed with a sensitivity of 99.2% (Table 2).

In particular, two sera obtained from patients with previously treated late latent syphilis were negative by

TABLE 1. Reactivities Obtained by ELISA, CMIA, WB, TPHA, and RPR With Syphilis Specimens, Specimens Obtained From Patients Suffering From Potentially Cross-Reacting Conditions, and Specimens With False-Positive CMIA Syphilis Results

	% Reactivity (no. of samples reactive or equivocal/total no. tested)				
Specimens category (no. of samples)	Enzygnost Syphilis	ARCHITECT Syphilis TP	WB	TPHA	RPR
Syphilis ($n = 244$)	99.2% (242/244)	99.2% (242/244)	100.0% (244/244)	97.1% (237/244)	57.4% (140/244)
Lyme disease ($n = 10$)	0% (0/10)	0% (0/10)	0% (0/10)	10.0% (1/10)	0% (0/10)
Mononucleosis ($n = 10$)	10.0% (1/10)	0% (0/10)	0% (0/10)	0% (0/10)	10.0% (1/10)
Pregnant women ($n = 14$)	0% (0/14)	0% (0/14)	0% (0/14)	0% (0/14)	14.3% (2/14)
Toxoplasmosis ($n = 20$)	0% (0/20)	5.0% (1/20)	0% (0/20)	0% (0/20)	5.0% (1/20)
Cytomegalovirus infection ($n = 20$)	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)	5.0% (1/20)
False-positive CMIA results ($n = 129$)	1.6% (2/129)	100.0% (129/129)	0% (0/129)	0% (0/129)	0% (0/129)

ELISA, enzyme-linked immunoassay; CMIA, chemiluminescent microparticle immunoassay; WB, Western Blot; TPHA, *T. pallidum* hemagglutination test; RPR, Rapid Plasma Reagin.

TABLE 2. Sensitivities and Specificities of Enzygnost Syphilis and ARCHITECT Syphilis TP

	Enzygnost Syphilis[a]	ARCHITECT Syphilis TP[b]
Sensitivity (%)	99.2	99.2
Specificity (%)	98.5	98.4

TPHA, *T. pallidum* hemagglutination test; WB, Western Blot; RPR, Rapid Plasma Reagin.
[a]Specificity was calculated by considering the sum of negative sera found in the two panels of potentially interfering samples (73+127) on the total amount of samples belonging to the second (74) and third panels (129) of sera (200/203 negative samples).
[b]From 1st February 2007 to 30th April 2007 the total number of sera submitted to the Microbiology Laboratory of the St. Orsola Hospital in Bologna for routine screening for syphilis was 9,210. ARCHITECT Syphilis TP scored 1,065 samples as positive; 936 sera were confirmed positive by TPHA and/or WB, whereas 129 samples were negative by TPHA, WB, and RPR. Therefore, ARCHITECT Syphilis TP found 8,145 negative sera out of the total amount of 8,274 samples scored negative by confirmatory tests. Specificity was calculated by considering the sum of negative sera found in the panel of interfering sera and in the routine group during the study period (73+8,145) on the total amount of samples belonging to the second panel of sera (74) and the routine negative (8,274) samples (8,218/8,348 negative samples).

Enzygnost Syphilis: these specimens were weakly reactive by WB, TPHA (titers = 80), and CMIA, whereas they were negative by RPR. ARCHITECT Syphilis TP scored two different sera as negative: one sample had been drawn from a patient with previously treated late latent syphilis and it was weakly reactive by WB, ELISA, and TPHA (titer = 80), and negative by RPR. The second specimen had been obtained from an HIV patient with a very recent acquired primary infection: this serum was scored borderline by ELISA, weakly reactive by WB and TPHA (titer = 80) and RPR. A second sample obtained from the same patient 10 days later was scored positive by all the assays (TPHA titer = 640).

Regarding specificity, Enzygnost Syphilis initially scored 5 samples as reactive (2 positive and 3 borderline) obtained from the 74 patients suffering from a potentially cross-reacting condition. These 5 samples were retested and only 1 (obtained from a patient with infectious mononucleosis) was confirmed to be reactive.

WB showed no false-positive reactions in this group of 74 sera, whereas both ARCHITECT Syphilis TP and TPHA scored one sample each as positive: when retested these samples were confirmed as positive. In particular, ARCHITECT Syphilis TP scored one sample obtained from a patient suffering from acute toxoplasmosis as positive, whereas one sample obtained from a Lyme disease patient had a TPHA titer = 80.

Finally, we tested by Enzygnost Syphilis 129 specimens selected from among the 9,210 samples submitted between 1st February 2007 and 30th April 2007 to the Microbiology Laboratory of the St. Orsola Hospital in Bologna for routine screening for syphilis: all these sera were scored reactive by ARCHITECT Syphilis TP but negative by TPHA and WB. It is interesting to underline that 43/129 sera were obtained from healthy blood donors. All these 129 were tested twice by ARCHITECT Syphilis TP and all of them were confirmed as positive.

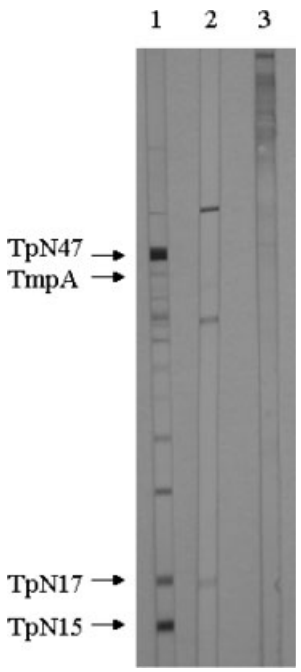

Fig. 1. WB analysis of human sera. The positions of the four diagnostic *T. pallidum* proteins are indicated on the left. Lane 1: syphilis patient's serum (TPHA 1/640). Lane 2: serum obtained from a patient with false-positive CMIA and Enzygnost Syphilis results (TPHA negative); this serum recognizes only one diagnostic antigen (TpN17). Lane 3: blood donor's serum scored positive by ARCHITECT Syphilis TP (TPHA and Enzygnost Syphilis negative); no diagnostic antigens were recognized. WB, Western Blot; TPHA, *T. pallidum* hemagglutination test; CMIA, chemiluminescent microparticle immunoassay.

When analyzed by WB, all 129 samples gave only very faint reactions compared with the positive control (Fig. 1). The most frequently recognized antigen was TpN47 (25 sera), as expected, followed by TmpA (14 sera). TpN17 and TpN15 were hardly recognized by two of these sera, confirming that TpN17 and TpN15 are the most specific antigens among the diagnostic ones. Only

nine sera recognized two bands (in particular, six sera recognized both TpN47 and TmpA, one sera recognized both TpN47 and TpN17, and two sera recognized both TmpA and TpN15). No sera recognized more than two bands; therefore, no samples were scored as borderline. Enzygnost Syphilis initially scored 6/129 sera as positive or borderline. These six samples were retested and only two were confirmed as being weakly positive. No specimens from blood donors were reactive when tested by ELISA.

Considering the sum of negative sera in the two panels of potentially interfering samples, Enzygnost Syphilis performed with a specificity of 98.5% (200/203 negative samples) as reported in Table 2.

As reported in Table 2, the specificity of ARCHITECT Syphilis TP (98.4%) was calculated by considering the sum of negative sera found in the second panel of interfering sera and in the routine screening on the total amount of negative sera studied during the 3-month study period.

DISCUSSION

Syphilis is still a public health problem worldwide (1) and recently outbreaks have been reported in several European countries (6–8).

Serological testing is a crucial element of any control program for syphilis (28) and, used both in screening for asymptomatic infections and as an adjunct to clinical diagnosis, remains the principal tool for the diagnosis of syphilis, as it has been since the 1930s (29). Last year Tridapalli et al. reported findings on the importance of serological testing for the control of congenital syphilis in Italy. None of the infants with syphilis infection had any evident clinical signs at birth; therefore, congenital disease would have been missed if serological tests had not been performed for both mother and infant pair at the time of delivery (30).

There is a huge choice of test reagents, manufactured and/or supplied by different companies. Decisions on which test a laboratory should use are based on many factors including cost, ease of use, suitability for automation, as well as performance characteristics.

Several different immunoassays set up with whole-cell lysate (12–15) or recombinant *T. pallidum* antigens (16–22) are described in the literature.

In this study, both Enzygnost Syphilis and ARCHITECT Syphilis TP performed with a sensitivity of 99.2%, higher than TPHA (97.1%), but lower than WB (100.0%).

Specificity, too, is a crucial factor in the serodiagnosis of syphilis. Increasing the sensitivity may lead to overdiagnosis because of potential similarity with other nontreponemal proteins. As syphilis is a sexually transmitted disease it is important to avoid false-positive results that can lead to unpleasant situations for the patients and their relatives.

At present, in the Microbiology Laboratory of the St. Orsola Hospital in Bologna all sera giving reactive results by CMIA are further analyzed by TPHA. If TPHA test is negative, WB test is also performed, in order to better characterize serum antibody reaction. During a 3-month study period, we selected 129 sera from among a total of 9,210 samples submitted to our laboratory for routine screening for syphilis: all 129 sera were reactive by ARCHITECT Syphilis TP but negative by TPHA and WB. It is noteworthy to underline that 43 out of these 129 samples were obtained from healthy blood donors.

ARCHITECT Syphilis TP is set up with recombinant antigens (TpN15, TpN17, and TpN47); the lack of specificity shown in the group of 129 samples was not owing to the presence of possibly cross-reacting antibodies against treponemal antigens, as analyzed by WB. Only a few faint reactions against TpN47 (25 cases), TpN17, and TpN15 (2 cases for each protein) were observed when analyzing these sera in comparison with the positive control. The source of the false positivity of this method does not appear to be cross-reacting antibodies against treponemal antigens, but at present is still unknown. On the other hand, Enzygnost Syphilis is set up with a detergent extract antigen of *T. pallidum*, and the false-positive reactions shown by this assay were owing to the presence of cross-reacting antibodies in the samples studied, as expected. In particular, 2 sera (no blood donor samples) from the group of 129 and 1 sample drawn from a mononucleosis patient were reactive by Enzygnost Syphilis. When analyzed by WB, 1 sample showed a weak reaction against TpN47 and treponemal flagellar proteins and another one reacted against TpN17 and a flagellar protein (the 2 sera from the group of 129), whereas the serum obtained from the mononucleosis patient reacted against TpN47, TmpA, and treponemal flagellar proteins.

Considering the suitability for automation, the good sensitivity and specificity of both Enzygnost Syphilis and ARCHITECT Syphilis TP make them good choices as screening tests in blood donor banks or microbiology laboratories.

It is nevertheless important to recognize the limits of the screening tests for the diagnosis of syphilis and the use of confirmatory tests, such as TPHA or WB, remains a must in order to avoid false-positive results.

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