Evaluation of recomWell Treponema, a novel recombinant antigen-based enzyme-linked immunosorbent assay for the diagnosis of syphilis

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Objective To evaluate the diagnostic performance of an enzyme immunosorbent assay (recomWell Treponema) for the diagnosis of syphilis. The novel recombinant antigens Tpn47, TpN17 and TpN15 were utilized.

Methods A total of 782 human serum specimens, belonging to four different categories (blood donors, $n = 200$; routine laboratory screening for syphilis, $n = 400$; syphilis patients, $n = 122$; potential cross-reactors, $n = 60$), were evaluated to compare the sensitivity and specificity of the recomWell Treponema kit with a standard whole *Treponema pallidum* cell lysate antigen-based ELISA (Syphilis Screening) and with micro-haemagglutination (MHA-TP).

Results The overall specificity and sensitivity of the recomWell Treponema IgG was 98.9% and 98.3%, respectively. The specificity and sensitivity of Syphilis Screening ELISA was 98.7% and 98.3%, respectively. The agreement between recomWell Treponema and Syphilis Screening was 100%, 97.8%, 95.9% and 95% among the blood donor specimens, screening samples, syphilis specimens and the potential cross-reactors, respectively. Values of concordance varying from 96.7% to 98.3% were found in the different groups of sera between recomWell Treponema and MHA-TP. In addition, recomWell Treponema demonstrated a good diagnostic performance when used to detect the IgM to *T. pallidum*. No false-positive sera were identified and, in 17/19 samples from primary infection, an IgM immune response was found.

Conclusions recomWell Treponema was shown to be a highly specific and sensitive method in all stages of syphilis screening and it can be considered as alternative to other ELISA tests based on native antigen preparations.

Keywords *Treponema pallidum*, serology, recombinant antigens, ELISA, syphilis

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INTRODUCTION

The serologic diagnosis of syphilis has for many years been carried out mainly with a two-step approach [1]. First, serum samples were screened with a flocculation assay using non-treponemal antigens to detect antibodies to cardiolipin (Venereal Disease Research Laboratory [VDRL] and rapid plasma reagin [RPR] test) and then, sera reactive in the screening tests were evaluated again to identify specific antibodies to *Treponema pallidum* antigens [2] (treponemal haemagglutination assay [MHA-TP] or fluorescent treponemal antibody absorption test [FTA-ABS]). Since there were limitations of the non-treponemal serologic tests [3] (i.e. their lack of sensitivity in early dark-field positive primary cases and in late syphilis, and the relatively high incidence of false-positive reactions), the quest for a specific serologic test for syphilis began many years ago and still continues. The use of the immunoenzymatic techniques in the serology of syphilis started in the mid 1970s [4] and, at present, many different enzyme-linked immunosorbent assays (ELISAs) are available and their usefulness as syphilis screening and diagnosis methods has been proved extensively. The sensitivity and specificity is comparable to treponemal tests like the MHA-TP and FTA-ABS, but as in these traditional tests, the sensitivity of ELISA is suboptimal in primary and congenital disease [5–10]. This report assesses the diagnostic performance (specificity and sensitivity) of a novel recombinant antigen-based ELISA (recomWell Treponema, Mikrogen, Martinsried, Germany) in comparison with a native antigen-based

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ELISA method (Syphilis Screening ELISA, Radim, Rome, Italy) and with MHA-TP (Fujirebio, Tokyo, Japan). The selection of syphilis sera to be evaluated was made by clinical and laboratory criteria proposed by Norris and Larsen [11].

MATERIALS AND METHODS

Study groups

This study involved a total of 782 human serum specimens. Two hundred sera were obtained from the blood bank of the St Orsola Hospital in Bologna, Italy; 400 sera were unselected samples submitted to the Microbiology Laboratory of the St Orsola Hospital for routine laboratory syphilis screening; 122 sera were obtained from patients attending the STD outpatient clinic of the University of Bologna and suffering from syphilis at various stages. In detail, eight sera were obtained from patients with a clinical diagnosis of suspected primary syphilis with only genital ulcer resembling the typical chancre but negative at the direct fluorescent assay (DFA) of the lesion fluid (suspected syphilis patients); 19 sera were obtained from patients with a clinical- and laboratory-confirmed diagnosis of primary syphilis; 24 sera were from patients with a clinical diagnosis of secondary syphilis; 67 sera were from patients with a diagnosis of latent syphilis; and four sera were from patients suffering from late syphilis. The clinical and laboratory criteria proposed by Norris and Larsen [11] have been followed to obtain the *T. pallidum* infection status of each individual patient.

Furthermore, a panel of 60 sera was obtained from patients suffering from some of the most common biological conditions known as possibly giving a false-positive reactivity in syphilis serology, such as serum specimens obtained from culture-confirmed Lyme disease patients [12–14] ($n = 20$) and from leptospirosis patients [15] ($n = 10$, kindly provided by M. A. Santos, INSA, Porto, Portugal), sera in which the presence of antinuclear antibodies has been detected [16,17] ($n = 11$, a gift of F. Cassani, Bologna, Italy), serum samples drawn from subjects with clinical diagnosis of infectious mononucleosis (showing a positive Paul–Bunnel–Davidsohn reaction; $n = 10$) and, finally, sera from healthy, pregnant women ($n = 9$).

RecomWell Treponema IgG and recomWell Treponema IgM testing

The recomWell Treponema test (Mikrogen, Martinsried, Germany) is a quantitative in vitro method for the detection of IgG or IgM antibodies against *T. pallidum* in human serum or plasma samples. This test, prepared with recombinant form of the *T. pallidum* antigens TpN47, TpN17 and TpN15, is based on the principle of an indirect 'sandwich' enzyme immunoassay. Briefly, serum samples, positive and negative controls, and cut-off samples were diluted 1 : 101 in diluting buffer and 100 μL of the diluted sera were pipetted in each well. Following incubation at 37 °C for 1 h, the wells were washed four times with a solution containing 0.05% (v/v) Tween-20 in phosphate-buffered saline (PBS; pH 7.0) and then 100 μL of anti-human IgG (or IgM) horseradish peroxidase-conjugated antibody solution (1 : 101 diluted in dilution buffer) were added to each well. The plate was incubated at 37 °C for 30 min and an additional washing cycle was performed. At the end, 100 μL of tetramethylbenzidine substrate solution were pipetted in each well; after 30 min incubation at room temperature the enzymatic reaction was stopped by adding 100 μL of stop solution to each well. The results were read at 450 nm after subtraction of the optical density (OD) values obtained at the reference wavelength of 650 nm. The gray zone (i.e. the range of values that cannot be considered as truly negative or positive) was calculated as follows: the lower limit corresponded to the OD value shown by the cut-off reference serum available in the kit and the upper limit corresponded to the OD cut-off value +20%. Samples with OD values above the gray range were considered positive; samples with OD values below the gray range were considered negative. Samples showing an OD value falling in the gray range were boundary cases and, following the manufacturer's instructions, they were tested again.

Syphilis Screening ELISA test

This is an enzyme immunoassay intended for the detection of total human immunoglobulins to a *T. pallidum* whole-cell native preparation. In detail, *T. pallidum* spp. *pallidum* (Nichols strain) was grown in testes of male New Zealand white rabbits for 10–14 days. At the end of this period the animals were euthanasized, the testes were spliced and resuspended in PBS (pH 7.2). The spirochetes were extracted from this tissue with gentle rotation under anaerobic atmosphere. The bacterial suspension was then washed once and the microrganisms were sonicated to disrupt the bacterial bodies. The obtained mixture of treponemal antigens was used to coat ELISA plates, following a standard procedure. This kit is based on a conventional competitive ELISA method that is capable of detecting the total Ig immune response to *T. pallidum* in human serum specimens [9,18–20].

Testing was performed following the manufacturer's instructions and all the specimens showing an equivocal result were tested twice.

MHA-TP

All the 782 samples were subjected to confirmatory testing by a quantitative MHA-TP method (Fujirebio, Tokyo, Japan). The MHA-TP was performed in accordance to the manufacturer's instructions, which had an established cut-off value corresponding to a 1 : 80 dilution.

RESULTS

IgG findings

Blood donor serum specimens

Preliminary experiments were made by testing 200 sera obtained from the St Orsola Hospital blood bank with recomWell Treponema IgG to assess the specifity of this new ELISA test; all the sera were also tested by Syphilis Screening ELISA and MHA-TP. No sample showed either a positive or equivocal result when tested by any technique, so the specificity of all the methods was 100% when applied to this group of sera. Consequently, the values of agreement between all these methods were 100% (Tables 1 and 2).

Sera submitted for routine laboratory screening for syphilis

Thirteen samples out of this group of 400 sera were positive when tested by recomWell Treponema IgG. (Table 3 reports details for discrepant results found among this group of specimens). The agreement between recomWell Treponema IgG and Syphilis Screening ELISA was 97.8% (Table 1). The sensitivity, specificity and agreement values obtained by comparing recomWell Treponema IgG to MHA-TP were 100%, 98.3% and 98.3%, respectively (see Table 2 for details).

Syphilis specimens

A total of 112 out of 122 samples were reactive when tested by recomWell Treponema IgG: of the 10 negative sera, eight were from patients with clinically suspected primary syphilis and two from patients showing a positive DFA slide with clinically confirmed primary syphilis. No negative result was obtained by the different tests used when sera from secondary, latent or late syphilis was studied. Detailed results for sera showing discrepant results are given in Table 3.

The agreement between recomWell Treponema IgG and Syphilis Screening ELISA was 95.9% (Table 1). The performances of recomWell Treponema IgG compared to MHA-TP gave sensitivity and specificity values of 98.2% and 88.9%, respectively, whereas the agreement was 97.5% (Table 2).

Table 1 Comparison of recomWell Treponema IgG and Syphilis Screening ELISA reactivities with syphilis specimens, healthy blood donor specimens, cross-reacting specimens and screening samples

Specimen category	No.tested	+ and +	+ and –	– and +	– and –	% Agreement
Syphilis	122	110	2	3	7	95.9%
Suspected syphilis	*8*	*0*	*0*	*2*	*6*	*75%*
Primary syphilis	*19*	*15*	*2*	*1*	*1*	*84.2%*
Secondary syphilis	*24*	*24*	*0*	*0*	*0*	*100%*
Early latent syphilis	*67*	*67*	*0*	*0*	*0*	*100%*
Late syphilis	*4*	*4*	*0*	*0*	*0*	*100%*
Blood donors	200	0	0	0	200	100%
Cross-reacting	60	1	1	2	56	95%
Screening samples	400	8	5	4	383	97.8%

The results obtained for sera drawn from different stages of syphilis are in italics.

Table 2 Comparison of recomWell Treponema IgG and MHA-TP reactivities with syphilis specimens, healthy blood donor specimens, cross-reacting specimens and screening samples

Specimen category	No.tested	+ and +	+ and –	– and +	– and –	% Agreement
Syphilis	122	111	1	2	8	97.5%
Suspected syphilis	*8*	*0*	*0*	*1*	*7*	*87.5%*
Primary syphilis	*19*	*16*	*1*	*1*	*1*	*89.4%*
Secondary syphilis	*24*	*24*	*0*	*0*	*0*	*100%*
Early latent syphilis	*67*	*67*	*0*	*0*	*0*	*100%*
Late syphilis	*4*	*4*	*0*	*0*	*0*	*100%*
Blood donors	200	0	0	0	200	100%
Cross-reacting	60	0	2	0	58	96.7%
Screening samples	400	6	7	0	387	98.3%

The results obtained for sera drawn from different stages of syphilis are in italics.

Table 3 Detailed results for sera from previous tables with discrepant results

Sample no.	Specimen category	recomWell IgG	Syphilis Screening ELISA	MHA-TP (titer)
202	Suspected syphilis	Negative	Positive	Negative
208	Suspected syphilis	Negative	Positive	80
215	Primary syphilis	Negative	Positive	80
220	Primary syphilis	Positive	Negative	Negative
224	Primary syphilis	Positive	Negative	160
390	Screening sample	Positive	Negative	Negative
410	Screening sample	Positive	Negative	Negative
527	Screening sample	Positive	Negative	Negative
654	Screening sample	Positive	Negative	Negative
703	Screening sample	Positive	Negative	Negative
544	Screening sample	Negative	Positive	Negative
599	Screening sample	Negative	Positive	Negative
631	Screening sample	Negative	Positive	Negative
706	Screening sample	Negative	Equivocal	Negative
387	Screening sample	Positive	Positive	Negative
479	Screening sample	Positive	Positive	Negative
735	Cross-reacting	Positive	Positive	Negative
770	Cross-reacting	Positive	Negative	Negative
761	Cross-reacting	Negative	Positive	Negative
780	Cross-reacting	Negative	Positive	Negative

Cross-reacting samples

Two out of the 60 samples belonging to the cross-reacting panel were reactive when tested by recomWell Treponema IgG: one was obtained from a Lyme disease patient and the second from a patient suffering from infectious mononucleosis. Table 3 shows detailed results. No positive reaction was obtained when these sera were tested by MHA-TP. The specificity of recomWell Treponema IgG in this group of sera was 96.7%. The same value was detected for the agreement between this immunoenzymatic method and MHA-TP (Table 2). Syphilis Screening ELISA performed with a specificity of 95%, whereas MHA-TP was shown to be 100% specific. The comparison between the two immunoenzymatic tests showed an agreement of 95%.

IgM findings

Blood donor serum specimens and cross-reacting samples

No sample belonging to these two groups of sera showed equivocal or positive result when tested by recomWell Treponema IgM.

Sera submitted for routine laboratory screening for syphilis

Six out the 13 sera positive by recomWell Treponema IgG were also positive by recomWell Treponema IgM. Moreover, two out of the 12 sera positive by Syphilis Screening ELISA and negative by the other tests were also positive or equivocal by recomWell Treponema IgM. When tested by MHA-TP, these sera were scored negative.

Syphilis specimens

The 17 sera from primary syphilis that were identified as positive by recomWell Treponema IgG, also gave positive results when analyzed by recomWell Treponema IgM. All these samples were also positive by Syphilis Screening ELISA. No specimen obtained from patients suffering from later syphilis stages gave positive result when tested by recomWell Treponema IgM.

DISCUSSION

The diagnosis of syphilis is based upon clinical symptoms and laboratory results [1,17]. As the lesion material is only available for direct examination during the early disease, the main laboratory diagnostic tool for stages later than primary syphilis is serology [21]. Recently, several different preparations of recombinant *T. pallidum* antigens have been described [22–25] and their diagnostic performance by ELISA for syphilis has been assessed [26,27]. In this study, the performance of recomWell Treponema, a novel ELISA kit containing a recombinant form of three *T. pallidum* antigens was evaluated. recomWell Treponema is based on the identification of the Ig response to the following recombinant polypeptides: TpN47, TpN17 and TpN15. This test is designed for the identification of a specific IgG (recomWell Treponema IgG) or IgM (recomWell Treponema IgM) immune response in syphilis. In this study the

overall diagnostic performance of this method was compared to the routinely used immunoenzymatic assay in our laboratory, Syphilis Screening ELISA, and to a non-ELISA confirmatory method, such as MHA-TP. A first evaluation, made on 200 sera obtained from the blood bank of the St Orsola Hospital, showed that recomWell Treponema IgG and recomWell Treponema IgM are specific methods, since none of these samples was identified as positive by either IgG or IgM evaluation. When applied to a panel of 60 potential cross-reacting sera, recomWell Treponema IgG performed with a specificity of 96.6%. This loss of specificity was due to a sample from a Lyme disease patient and to another sample from a patient suffering from infectious mononucleosis, positive by this immunoassay. recomWell Treponema IgM was shown to be 100% specific also in this group of specimens, since no sample gave a positive result. The specificity and sensitivity of Syphilis Screening ELISA were 98.7% and 98.3%, respectively. A good advantage of recomWell Treponema, compared to Syphilis Screening ELISA, is the possibility to discriminate between two different classes of immunoglobulins (IgG and IgM). The identification of the IgM immune response is generally considered not useful in the diagnostic procedure of sexually acquired syphilis, whereas the detection of this antibody subclass has been proved an important adjunctive tool in the laboratory investigation of congenital *T. pallidum* infection [11,28,29]. recomWell Treponema IgM could probably also be useful in this case, as suggested by the specificity and sensitivity shown in the present study. When applied to sera drawn from primary syphilis patients, recomWell Treponema IgM was shown to be able to identify a specific IgM immune response in 17/19 sera (89.5%). This loss of sensitivity of the ELISA method when applied to sera from primary syphilis is as expected, since all the diagnostic tests have suboptimal sensitivity when applied to primary syphilis patients.

The decrease of sensitivity is well explained by the criteria followed to classify a result on a serum as indicating early infection [11]: only in the case of a positive DFA slide could a patient be diagnosed as suffering from primary syphilis. Lacking this laboratory confirmation, only the diagnosis of suspected primary syphilis must be posed, resulting in a well known bias in the criteria for the selection of this group of specimens. The detection of an immune response is the most widely used method in the laboratory diagnosis of syphilis both for the identification of patients suspected to be suffering from secondary, latent and late syphilis, and for epidemiological purposes [1,3,11]. Quite recently, several native [7–10] or recombinant [22–24,26,27], antigen ELISA methods for the detection of antibody response to *T. pallidum* have been evaluated, and their overall sensitivity and specificity have been comparable to those of MHA-TP. In this study we showed that recomWell Treponema is a highly specific and sensitive method, capable of detecting IgG and IgM antibodies in human serum in all stages of syphilis, and it can be considered a good alternative to the other ELISA tests based on native *T. pallidum* antigen preparations. These advantages are principally due to the fact that the availability of recombinant antigens is not connected to the growth of treponemes in animals. Consequently, the diagnostic use of these polypetides can overcome the well known problem of keeping constant over time the antigenic properties of animal-derived *T. pallidum* proteins. In addition, large quantities of recombinant antigens can be produced in quite a simple way, contributing to lower costs for this new test.

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