Evaluation of Recombinant Protein TpF1 of Treponema pallidum for Serodiagnosis of Syphilis

Running title: TpF1 for Serodiagnosis of Syphilis

Chuanhao Jiang†, Feijun Zhao†, Jinhong Xiao, Tiebing Zeng, Jian Yu, Xiaohua Ma, Haiying Wu and Yimou Wu*

1Pathogenic Biology Institute, Medical College, University of South China, Hengyang 421001, China;
2Clinical Laboratory, Hunan Provincial People’s Hospital, Changsha 410005, China;
3Department of Experimental Zoology, Medical College, University of South China, Hengyang 421001, China

†These authors contribute equally to this work.

*Corresponding author

Yimou Wu
Pathogenic Biology Institute, Medical College, University of South China, Hengyang 421001, Hunan, China.

TEL: +86-734-8281555; FAX: +86-734-8282907,
E-mail:yimouwu@sina.com

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Abstract

Syphilis is a chronic infection caused by *Treponema pallidum* subsp. *pallidum*, and the diagnosis of syphilis with sensitive and specific methods is challenging and important for the prevention and treatment of syphilis. In present study, we established a recombinant protein TpF1-based indirect immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) and Western blot assay for human and rabbit sera. The 20-kDa recombinant protein TpF1 was detected by Western blotting probed with sera from rabbits immunized with recombinant TpF1 and infected with *T. pallidum* Nichols strain and *T. pallidum* clinical isolates, but was not detected probed with sera from uninfected rabbits. The sensitivity of the recombinant protein was determined by screening sera from individuals with primary, secondary, latent and congenital syphilis (n=82). The specificity of the recombinant protein was determined by screening sera from uninfected controls (n=30), and individuals with potentially cross-reactive infections including Lyme disease (n=30) and Leptospirosis (n=5). The sensitivities of TpF1-based ELISA were 93.3%, 100%, 100%, and 100% for primary, secondary, latent, and congenital syphilis, respectively, and the specificities were all 100% for sera from uninfected controls and individuals with potentially cross-reactive infections. In Western blot assay, the sensitivities and specificities of TpF1 for human sera were all 100%. The reactivities of TpF1 with syphilitic sera were proportional to the titers of the treponemal test TPPA (*T. pallidum* particle agglutination). These data indicate that the recombinant protein TpF1 is a highly immunogenic protein in human and rabbit infections and a promising marker for screening of syphilis.
Introduction

Syphilis is a chronic multistage disease caused by the spirochaete *Treponema pallidum* subsp. *pallidum*, and usually transmitted by sexual contact or congenitally (1). Syphilis has been a public health problem in the past two decades, with an estimated 12 million new cases occurring per year worldwide (2). Furthermore, syphilis has been considered as one of factors to facilitate HIV infection and transmission, and congenital syphilis causes more than half a million stillbirths or neonatal deaths annually (3). In China, Syphilis has become one of the top five most reported infectious diseases and a most frequently reported sexually transmitted disease (STD), the incidence of which increased from 7.12 cases per 100,000 people in 2004 to 22 cases per 100,000 people in 2008 (4, 5).

While the direct visualization of *T. pallidum* can be performed by dark field microscopy (DFM), direct detection of *T. pallidum* is difficult, due to the fact that *T. pallidum* cannot be cultured *in vitro*, and DFM is highly operator dependent and is possible only for the early stage of syphilis when lesions are present (6). Hence, the diagnosis of syphilis is mainly relied on clinical investigation and serological tests. Traditional serological tests include two kinds of assays: a nontreponemal test, such as rapid plasma reagin (RPR) or Venereal Disease Research Laboratory (VDRL) test, and a treponema test, such as the fluorescent treponemal antibody absorption (FTA-ABS) test, the microhemagglutination assay for *T. pallidum* (MHA-TP), or the *T. pallidum* particle agglutination (TP-PA) test. Serum samples are first tested using a non-treponemal test and positive samples are analyzed with a treponema specific test.
The RPR and VDRL tests show median sensitivities of 86% and 78%, respectively, for primary syphilis and 73% and 71%, respectively, for late syphilis (9). Furthermore, these non-treponemal tests may result in false-positive detection in many situations, such as advanced age, pregnancy, and other bacterial infection (10).

Enzyme-linked immunosorbent assays (ELISAs) that use nonspecific lipoprotein, purified whole extracts from *T. pallidum*, or recombinant proteins as antigens for screening of syphilis have been widely used in clinical laboratory, since they are easy and quick to perform and also have the potential to be automated (11, 12). Several *T. pallidum* proteins have been tested including TpN15 (Tp0171), TpN17 (Tp0435), TpN44.5 (TmpA, Tp0768), TpN47 (Tp0574), Tp0453, Tp92 (Tp0326), and Tp0965 (13-17). Although these recombinant antigens are sometimes used in combination in commercial tests and exhibit high sensitivity, not all these antigens can be used for the detection of the early-stage syphilis. It is imperative to evaluate more specific and sensitive recombinant antigens for the serodiagnosis of syphilis.

In previous study, the researchers used isoelectric focusing (IEF) and nonequilibrating pH gel electrophoresis (NEPHGE) forms of two-dimensional gel electrophoresis (2DGE) to analyze the whole lysates of purified *T. pallidum* subsp. *pallidum* Nichols, and identified a set of antigens specifically reactive with infected human serum (18). The bacterioferritin protein TpF1 (Tp1038) is one of these antigens, which exhibited high antibody response with primary and other different stages of syphilis, suggesting that TpF1 might be useful in early diagnostic studies. The aim of this study was to further investigate the diagnostic potential of the
recombinant protein TpF1 by expressing in and purifying from *E. coli* as a His-tag fusion protein. Subsequently, the sensitivity of recombinant protein TpF1 was screened by using sera collected from individuals with syphilis. Since other spirochetal diseases such as Lyme disease and leptospirosis were expected to have antigens most similar to *T. pallidum*, sera collected from individuals with these infections were also tested for the specificity. These results showed that the recombinant protein TpF1-based ELISA was highly sensitive and specific for detecting *T. pallidum* infections. Thus, TpF1 is a promising candidate for automated commercial ELISAs for screening of syphilis.

**Materials and Methods**

**Bacterial strains, plasmids and DNA**

The *T. pallidum* Nichols strain was supplied by Dr. Weiming Gu (Skin Diseases and Sexual Transmitted Diseases Hospital, Shanghai, China). The clinical isolates of *T. pallidum* nhgz-01 and nhgz-02 were obtained from Dr. Diqing Luo (The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China). The *T. pallidum* Nichols strain and clinical isolates were propagated by intratesticular inoculation of adult New Zealand White rabbits as previously described (19). All animal experiments were approved by the Animal Welfare Committee of University of South China and conducted in accordance with the regulations of the institution. *E. coli* JM109 strain (Invitrogen, USA) was used for DNA cloning and *E. coli* BL21 (DE3) strain (Novagen, Merck Millipore, Germany) was used for protein expression. The expression vector pET28a was purchased from Novagen (Merck Millipore, Germany).
The genomic DNA of *T. pallidum* Nichols strain was prepared as previously described (20) using the QIAamp DNA minikit (Qiagen Inc., Germany).

**Human sera**

This study was approved by the Human Ethics Committee of University of South China. Human serum samples were obtained from patients diagnosed with primary (n=15), secondary (n=35), latent (n=22) syphilis and uninfected controls (n=30) at The Second Affiliated Hospital, University of South China. Congenital syphilis sera (n=10) were obtained from Maternal and Child Health Care Hospital, Changsha, China. The stages of syphilis were established by the clinical presentations and laboratory serology tests as follows: primary syphilis, presented typical chancre and positive syphilis serology test; secondary, generalized cutaneous rash and/or mucosal lesions and positive syphilis serology test; latent syphilis, presented no clinical presentations and no history of treatment for syphilis during the previous 2 years and positive syphilis serology test; congenital syphilis, infants with positive syphilis serological results and/or *T. pallidum* was detected directly by DFM. Lyme disease sera (n=30) were obtained from Chinese Centre for Disease Control and Prevention (Beijing, China). The criteria for the diagnosis of Lyme disease were based on clinical manifestations consistent with Lyme disease and serological results. Serological results were considered positive only when the enzyme immunoassay result was positive and more than five reactive bands by Western blot analysis. Sera from patients diagnosed with leptospirosis (n=5) were obtained from Hunan Provincial Centre for Disease Control and Prevention (Changsha, China). The
laboratory-confirmed diagnosis of leptospirosis relies on the microscopic agglutination test (MAT) with a fourfold rise in MAT titer or a reciprocal MAT titer of >1:800. All human sera were screened by RPR (KeHua Bio-Engineering Inc., Shanghai, China) and TP-PA (Fujirebio Inc., Japan) according to the manufactures’ protocols in a blinded manner.

**Cloning, Expression and Purification of TpF1**

The full-length TpF1 (TP1038) was amplified by PCR from a preparation of *T. pallidum* genomic DNA and cloned into the expression vector pET28a via BamHI (5’-end) and XhoI (3’-end) restriction site to give expression of N-terminal His-tag proteins. Primers used were TP1: 5’-CGCGGATCCATGAACATGTACAGAT-3’ and TP2: 5’-CCGCTCGAGCTAGGCTTTCAGGGTAGC-3’, containing restriction site for BamHI and XhoI, respectively. The sequence of the TpF1 construct within the pET28a vector was confirmed by DNA sequencing.

*E. coli* BL21 (DE3) strains harbouring the expression vectors were grown in Luria–Bertani medium supplemented with kanamycin 30μg/mL. Protein expression was induced at 30°C for 4h with 0.5mM isopropyl-B-D-thiogalactopyranoside (IPTG) after A_{600} reached 0.8. Bacteria were harvested and lysed in a buffer containing 50mM Tris-HCl (pH 7.6), 300mM NaCl, 10mM imidazole, 20% glycerol and 1% Triton X-100. His-tagged proteins were purified by affinity chromatography using Ni-NTA beads (Qiagen Inc., Germany). Proteins were then washed with 25mM imidazole and eluted with 250mM imidazole in 50mM Tris-HCl (pH 7.6), 300mM NaCl and 10% glycerol. Eluted samples were further dialysed against a buffer containing 50mM...
Tris-HCl (pH 7.6) and 150mM NaCl to remove the imidazole. Protein concentrations were estimated by using BCA protein assay kit (Pierce).

**Immunization with the recombinant protein**

Four adult male New Zealand White rabbits were immunized four times at 2-weeks intervals with 200μg of purified TpF1 recombinant protein emulsified in the Freund’s adjuvant (complete adjuvant at the first time and incomplete adjuvant at other times) (Sigma). Sera were collected prior to the initial immunization as negative controls, and antisera were collected at two weeks following each immunized.

**Western blot analysis**

The recombinant protein TpF1 (15μg) was diluted 1:1 in 2×sample loading buffer containing 5% β-mercaptoethanol and boiled 5 min at 100°C, subsequently separated on SDS-PAGE 12% w/v gels and transferred to nitrocellulose membranes (0.45mm) (Millipore) by using semidry Trans-Blot SD (BioRad). Membranes were blocked at room temperature for 2h with PBS containing 5% nonfat milk and 0.05% Tween-20 (PBSTM), and then, membranes were incubated overnight at 4°C with human sera (1:600) or rabbit sera (1:400) or anti-His tag monoclonal antibody(1:1,000) respectively, diluted in PBSTM. After washing five times with PBS containing 0.05% Tween-20 (PBST), membranes were incubated with secondary antibodies for 1h at 37°C (the dilution of secondary antibodies was as follows: HRP-conjugated goat anti-human IgG (Abcam) 1:10,000; HRP-conjugated goat anti-rabbit IgG (Abcam) 1:12,000, HRP-conjugated goat anti-mouse IgG (Millipore) 1:10,000). Finally, chemiluminescent detection was performed using the SuperSignal West Pico
ELISAs

Purified recombinant TpF1 was diluted in carbonate buffer (0.1M, pH 9.5) at concentration of 5μg/μL, and ninety-six well plates (Costar) were coated overnight at 4°C with 100μL per well. Plates were blocked for 2h at room temperature with 200μL PBSTM. Each serum sample was diluted (human sera 1:100 or rabbit sera 1:200) in PBSTM and added 100μL to each well. After incubation at 37°C for 2h, the plates were washed five times with PBST, and then 100μL of a 1:10,000 dilution of HRP-conjugated goat anti-human IgG or a 1:15,000 dilution of HRP-conjugated goat anti-rabbit IgG was added to each well. After incubation at 37°C for 1h, the plates were washed five times with PBST and 100μL of TMB liquid substrate was added to each well. After 30min incubation at room temperature, the reaction was stopped by the addition of 50μL of 1M sulfuric acid. The absorbance of each reaction was measured at 450 nm using an automatic ELISA reader (Bio-Rad). Each sample was assayed in triplicate.

Statistical analysis

The cutoff value was defined as the mean plus two standard deviations of the absorbance values of the uninfected controls (n=30). The cutoff value of TpF1-based ELISA was 0.4688. The absorbance values less than or equal to the cutoff value were defined as negative, while those greater than cutoff value were defined as positive. R value was calculated for correlation. Two-tailed p values< 0.05 were considered to be significant.
**Results**

**Production and identification of recombinant protein TpF1**

TpF1 gene was successfully amplified from the *T. pallidum* Nichols strain genome (Fig. 1A), and then cloned into expression vector pET28a. TpF1 was expressed as His-tagged protein in *E. coli* BL21 (DE3) and purified from cell-free supernatants by affinity chromatography using Ni-NTA beads. The soluble full-length protein TpF1 was produced as a single protein that was determined by SDS-PAGE analysis to have an estimated purity of >95% (Fig. 1B). The identification of recombinant protein TpF1 was performed by western blot assay with anti-His monoclonal antibody, human syphilis sera and uninfected control. These results showed that recombinant protein TpF1 reacted positively with anti-His monoclonal antibody and human syphilis, not with sera from uninfected control (Fig. 1C).

**Immunogenicity of recombinant protein TpF1**

To further evaluate the potential immunogenicity of the recombinant protein TpF1, immune sera obtained from rabbits that were experimentally infected with *T. pallidum* Nichols strain and *T. pallidum* clinical isolates (nhgz-01 and nhgz-02) were tested by western blot (Fig. 2A). We observed stronger reactivity with sera collected 30 days after infection than expected, which was in contrast with previous findings (18). Furthermore, strong specific reactions against recombinant TpF1 were also observed with sera from 60, 120 days after infection, whereas no reactions were observed with sera from uninfected rabbits.

In addition, the ability of the specific anti-TpF1 rabbit sera, generated by
immunizing rabbits with TpF1, was also tested by western blot assay. As expected, the recombinant TpF1 reacted strongly with anti-TpF1 rabbit sera, not with health control rabbit sera. Furthermore, specific anti-TpF1 antibodies appeared at 14 days after the first immunization and remained elevated throughout the immunization (Fig. 2B). After the fourth immunization, anti-sera were collected and determined with a high titer (1:25,600, data not shown) by TpF1-based ELISA. These results demonstrated that the *T. pallidum* protein TpF1 could evoke a robust and specific immune response in experimentally syphilis infection.

**Evaluation of recombinant protein TpF1 for human serum**

To determine the sensitivity of the recombinant protein for the diagnosis of syphilis, TpF1 was tested against a well-characterized panel of serum samples from patients with syphilis infection by western blot and TpF1-based ELISA. The serum samples contained primary (n=15), secondary (n=35), latent (n=22) and congenital syphilis (n=10). In western blot assay, the sensitivity of TpF1 was determined to be 100% for all stages syphilis (Fig. 3A, Table 1). In ELISA assay, the overall sensitivity was determined to be 98.9%, with sensitivities of 93.3%, 100%, 100%, and 100% for the detection of primary, secondary, latent, and congenital syphilis, respectively (Fig. 3B, Table 1).

To further investigate the specificity of the recombinant protein, sera obtained from uninfected human (n=30) and patients with other spirochetal diseases, include Lyme disease (n=30) and leptospirosis (n=5), were tested by western blot and TpF1-based ELISA. As shown in Fig. 3, none of these serum samples was found to yield positive
reactivity to TpF1, resulting a specificity of 100% for both western blot and ELISA (Table 1). Thus, the higher sensitivity and specificity of the recombinant protein TpF1 demonstrated that it could be developed into a diagnostic antigen for evaluating syphilitic patients.

Comparison of reactivity of recombinant protein TpF1-based ELISA with that of present syphilis diagnostic tests

The lipoidal antigen-based test such as RPR is often used for screening of syphilis. The TpF1-based ELISA was more sensitive than the RPR test in detecting syphilis (Fig. 4A). Eight serum samples that had a negative RPR result demonstrated reactivity against recombinant TpF1 (Fig. 4A); these sera were from patients with primary (n=4), secondary (n=1), latent (n=2), and congenital (n=1) syphilis. In general, the absorbance values from TpF1-based ELISA did not correlate well with the RPR titer (Fig. 4A), reflecting the non-treponemal nature of the RPR test. In contrast, the reactivity observed with TpF1-based ELISA correlated well with the TPPA titer (Fig. 4B), which was based on crude *T. pallidum* antigens and represents a treponemal specific test.

Discussion

The identification and characterization of antigens of *T. pallidum* is central to the improvement of current laboratory diagnostics for syphilis. TpF1 is an oligomeric protein, belonging to the bacterio-miniferritin family, in which 12 identical subunits form a nearly spherical shell by disulfide bonds, creating a very stable oligomer (21). But in the presence of β-mercaptoethanol, the oligomeric TpF1 can be dissociated into
160kDa oligomers and 19 kDa monomers (22). Furthermore, it can be dissociated completely by boiling in SDS in the absence of reducing agents (23). In present study, we have successfully cloned the TpF1 gene, expressed recombinant protein TpF1 in E. coli, and purified the recombinant protein by His-tag based affinity chromatography under native conditions. The size of the expressed recombinant protein was slightly larger than the predicted size, possibly due to the His-tag at the N-terminal (Fig. 1B). We observed the monomeric form of TpF1 reacted with anti-His tag monoclonal antibody and human patient sera by Western blotting (Fig. 1C). Furthermore, the recombinant protein TpF1 was able to react with sera from rabbits infected with T. pallidum Nichols strain and clinical isolates (Fig. 2A). These results suggested that the monomeric form of recombinant protein TpF1 also has reactivity with T. pallidum infection. In prior study reported by McGill et al, the monomeric form of TpF1 has no reactivity with sera from infected rabbits and syphilitic sera (18). A possible explanation for this discrepancy may represent differences in individual preparations of T. pallidum antigen. The antigen used in that study was the lysates of T. pallidum. It is possible that the structure of native TpF1 is stable and dose not easily to dissociate into the monomeric form during the sample preparation. Therefore, the monomeric form of TpF1 is at very low levels escaping detection by western blotting. Another possibility is that some macromolecules derived from rabbit tissues could also affect the immunoreactivity of TpF1.

Because T. pallidum cannot be cultured in vitro, diagnosis of syphilis has principally relied on serodiagnosis. Using recombinant T. pallidum protein to test for
Syphilis has advantages over lipoidal antigen or crude *T. pallidum* antigen. Lipoidal antigen-based screening misses up to 30% of sera from individuals with primary and latent syphilis (24). In the group of sera that we tested, there were eight patients with primary (n=4), secondary (n=1), latent (n=2), and congenital (n=1) syphilis who had no reactivity in the RPR test, yet had good reactivity with recombinant protein TpF1 (Fig. 4A). These sera were also positive with the crude *T. pallidum* antigen based test TPPA. Overall, the TPPA results correlated well with the TpF1-based ELISA results (Fig. 4B). However, there is a significant advantage in preparation of recombinant proteins over preparation of crude *T. pallidum* protein. Recombinant *T. pallidum* proteins can be produced economically and in large quantities in *in vitro* *E. coli* culture, but crude *T. pallidum* antigens must be extracted from treponemes grown within the rabbit animal model. These crude extracts contain large amount of proteins and other macromolecules, and most of them can influence the results of the test. Therefore, false-positive concordance often occurs when these kits are used (25). The use of purified recombinant proteins is an alternative for the detection of serum antibodies and will allow better standardization of the immunoassays. Furthermore, the use of a combination of recombinant antigens may enhance the sensitivity of an antibody-based assay. Several previous studies have found that recombinant antigens improve the serological diagnosis of *T. pallidum* infection (25-28). Moreover, recombinant antigens have the potential to be used in the creation of new tests capable of differentiating recently acquired infections from those acquired in the more distant past (27).
In this study, we demonstrate that the TpF1 has excellent sensitivity and specificity for serodiagnosis, exhibiting sensitivities of 98% and 100% for TpF1-based ELISA and Western blot assay, respectively (Table 1). In addition, sera from 30 individuals who tested negative for syphilis infection and 35 patients with the other spirochetal infections have no reactivity against TpF1, showed specificities of 100% for both TpF1-based ELISA and Western blot assay. The Western blot technique based on whole T. pallidum-cell lysate has been widely used over past years, and it has been shown to be a reliable confirmatory test (11, 29, 30). In this study, Western blot assay showed higher sensitivity and specificity for screening syphilis. However, due to the context of an increased volume of sera referred for laboratory screening, cheaper, quicker, and less labor-intensive diagnostic assays represent an attractive option for clinical diagnostic laboratory services (31, 32). For this reason, Western blot assay is not suitable for high-throughput screening of syphilis, but it offers an additional, accurate treponemal test that can supplement the current syphilis testing (33).

In conclusion, we have identified an additional recombinant diagnostic candidate TpF1 in this study, which exhibited high sensitivity (98.8%) for detection of all stages of infection and was extremely specific (100%) when tested against potentially cross-reactive sera. Taken together, the current results highlight the recombinant protein TpF1 as a promising antigenic marker of syphilis and can be developed into a diagnostic antigen for screening of syphilis. However, before the TpF1 can be definitively demonstrated to be helpful in serodiagnosis of syphilis, further studies need to be performed.
Acknowledgement

We thank Weiming Gu, Skin Diseases and Sexual Transmitted Diseases Hospital, Shanghai, China, for the gift of *T. pallidum* Nichols strain; Diqing Luo, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, for the gift of *T. pallidum* clinical isolates nhgz-01 and nhgz-02. We also thank Jun Zhou, The Second Affiliated Hospital, University of South China; Li Peng, Maternal and Child Health Care Hospital, Changsha, China; Kangning Wan, Chinese Centre for Disease Control and Prevention, Beijing, China; and Liang Cai, Hunan Provincial Centre for Disease Control and Prevention, Changsha, China, for providing serum samples for this study.

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References


Figure legends

Fig. 1. Expression, purification, and identification of recombinant protein TpF1. (A) Amplification of TpF1 gene from the *Treponema pallidum* Nichols strain genome. (B) SDS-PAGE analysis of the expression of TpF1 in E. coli BL21(DE3) harboring pET28a-TpF1 plasmid was induced with (+) or without(-) IPTG and the purified recombinant protein TpF1. (C) Western blot analysis of recombinant protein TpF1 with Anti-His monoclonal antibody (Anti-His), syphilitic sera (S), and uninfected human sera (C).

Fig. 2. Western blot analysis of the immunoreactivity of recombinant protein TpF1 with sera from rabbits (A) infected with *Treponema pallidum* Nichols strain, clinical isolates nhgz-01 and nhgz-02, (B) immunized with recombinant protein TpF1.

Fig. 3. Reactivities of sera from individuals at different stages of syphilis and individuals with other spirochetal diseases and control sera from uninfected individuals to the recombinant protein TpF1. (A) Western blot analysis of recombinant protein TpF1 with a pool of different human sera. (B) The reactivities of different human sera to recombinant protein TpF1 determined by TpF1-based ELISA, the y axis shows the absorbance values at 450 nm for reactivities of the sera with the recombinant protein TpF1. The sera are grouped as follows: PS, Primary Syphilis (n=15); SS, Secondary Syphilis (n=35); LS, Latent Syphilis (n=22); CS, Congenital Syphilis (n=10); Le, Leptospirosis (n=5); LD, Lyme disease (n=30); U, Uninfected
control (n=30). The overall mean absorbance of each group is represented by a horizontal line.

Fig. 4. Relationships of the reactivities of sera from syphilis patients to recombinant protein TpF1 and (A) to the RPR test, the x axis displays the value for the RPR test, shown as the reciprocal of the highest dilution that gave reactivity in the test, the eight values at 0 along the x axis were nonreactive reactivity in the RPR test, and (B) to the TPPA test, the x axis shown as the reciprocal of the highest dilution that gave reactivity in the test on a log2 scale. The y axis shows the absorbance values at 450 nm for reactivities of the sera with the recombinant protein TpF1.
A  Infected with Treponema pallidum

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B  Immunized with recombinant TpF1

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Table 1: Sensitivities and Specificities of RPR test, Western blot assay, and TpF1-based ELISA in the detection of different stages of syphilis and cross-reactive infections.

<table>
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<th>Human serum samples*</th>
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*PS, Primary Syphilis; SS, Secondary Syphilis; LS, Latent Syphilis; CS, Congenital Syphilis; Le, Leptospirosis; LD, Lyme Disease; U, Uninfected control.