Syphilis is a chronic infection caused by *Treponema pallidum* subsp. *pallidum*, and diagnosis with sensitive and specific methods is a challenging process that is important for its prevention and treatment. In the present study, we established a recombinant protein TpF1-based indirect immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) and a Western blot assay for human and rabbit sera. The 20-kDa recombinant protein TpF1 was detected by Western blotting performed with sera from rabbits immunized with recombinant TpF1 and infected with the *T. pallidum* Nichols strain and *T. pallidum* clinical isolates but was not detected by Western blotting 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 ELISAs 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 assays, 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 *T. pallidum* particle agglutination (TPPA) assay. These data indicate that the recombinant protein TpF1 is a highly immunogenic protein in human and rabbit infections and a promising marker for the screening of syphilis.

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 laboratories, 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 of these antigens can be used for the detection of the early stage of syphilis. It is imperative to evaluate more specific and sensitive recombinant antigens for the serodiagnosis of syphilis.

In a previous study, the researchers used isoelectric focusing (IEF) and nonequilibrium pH gel electrophoresis (NEPHGE) forms of two-dimensional gel electrophoresis (2DGE) to analyze the whole lysates of purified *T. pallidum subsp. pallidum* (Nichols strain) 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 responses 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.

**Syphilis** is a chronic multistage disease caused by the spirochete *Treponema pallidum subsp. pallidum* and usually transmitted by sexual contact or congenitally (1). Syphilis has been a public health problem in the last 2 decades, with an estimated 12 million new cases occurring per year worldwide (2). Furthermore, syphilis has been considered one of factors that facilitates 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 the 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 relies mainly on clinical investigation and serological tests. Traditional serological tests include two kinds of assays, nontreponemal tests, such as the rapid plasma reagin (RPR) and Venereal Disease Research Laboratory (VDRL) tests, and treponemal tests, such as the fluorescent treponemal antibody absorption (FTA-ABS) test, the microhemagglutination assay for *T. pallidum* (MHA-TP), and the *T. pallidum* particle agglutination (TPPA) assay. Serum samples are first tested using a nontreponemal test and positive samples are analyzed with a treponema-specific test (7, 8). 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 nontreponemal tests may result in false-positive detection in many situations, such as in patients with advanced age, pregnancy, and other bacterial infection (10).
of the recombinant protein TpF1 by expressing it in and purifying it from *Escherichia coli* as a His-tagged 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 those of *T. pallidum*, sera collected from individuals with these infections were also tested for 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 Weiming Gu (Skin Diseases and Sexual Transmitted Diseases Hospital, Shanghai, China). The *T. pallidum* clinical isolates nhgz-01 and nhgz-02 were obtained from 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 the University of South China and conducted in accordance with the regulations of the institution. *E. coli* strain JM109 (Invitrogen) was used for DNA cloning, and *E. coli* strain BL21(DE3) (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 the University of South China. Human serum samples were obtained from patients diagnosed with primary (*n* = 15), secondary (*n* = 35), or 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 the 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, typical chancre and positive syphilis serology test; secondary syphilis, generalized cutaneous rash and/or mucosal lesions and positive syphilis serology test; latent syphilis, 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* detected directly by DFM. Lyme disease sera (*n* = 30) were obtained from the 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 the serological results. Serological results were considered positive only when the enzyme immunosassay result was positive and Western blotting results showed more than five reactive bands. Sera from patients diagnosed with leptospirosis (*n* = 5) were obtained from the Hunan Provincial Centre for Disease Control and Prevention (Changsha, China). The laboratory-confirmed diagnosis of leptospirosis relied on the microscopic agglutination test (MAT) at a 4-fold rise in MAT titer or a reciprocal MAT titer of >1:800. All human sera were screened in a blinded manner by an RPR test (KeHua Bio-Engineering, Inc., Shanghai, China) and a TPPA assay (Fujirebio, Inc., Japan) according to the manufacturers’ protocols.

**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 BamH1 (5’-end) and XhoI (3’-end) restriction sites to give expression of N-terminal His-tagged proteins. The primers used were 5’-CCGGGATCCATGAACATGTGTACAGAT-3’ (for TP1) and 5’-CCGCTGCAGCTAGGCATTCAAGGGTTACAGAT-3’ (for TP2), which contained restriction sites (underlined) for BamH1 and XhoI, respectively. The sequence of the TpF1 construct within the pET28a vector was confirmed by DNA sequencing. *E. coli* BL21 (DE3) strains harboring the expression vectors were grown in Luria-Bertani medium supplemented with kanamycin 30 μg/ml. Protein expression was induced at 30°C for 4h with 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) after the A₆₀₀ reached 0.8. Bacteria were harvested and lysed in a buffer containing 50mM Tris-HCl (pH 7.6), 300mM NaCl, 10 mM imidazole, 20% glycerol, and 1% Triton X-100. His-tagged proteins were purified by affinity chromatography using Ni-nitritolactric acid (NTA) beads (Qiagen, Inc., Germany). Proteins were then washed with 25 mM imidazole and eluted with 250 mM imidazole in 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, and 10% glycerol. Eluted samples were further dialyzed against a buffer containing 50 mM Tris-HCl (pH 7.6) and 150 mM NaCl to remove the imidazole. Protein concentrations were estimated by using a bicinchoninic acid (BCA) protein assay kit (Pierce).

**Immunization with the recombinant protein.** Four adult male New Zealand White rabbits were immunized four times at 2-week intervals with 200 μg 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 2 weeks following each immunization.

**Western blot analysis.** The recombinant protein TpF1 (15 μg) was diluted 1:1 in 2× sample loading buffer containing 5% β-mercaptoethanol and boiled for 5 min at 100°C, subsequently separated on SDS-PAGE 12% (wt/vol) gels, and transferred to nitrocellulose membranes (0.45 mm) (Millipore) by using semidy Transfer-Blot SD (Bio-Rad). Membranes were blocked at room temperature for 2 h with phosphate-buffered saline (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-tagged monoclonal antibody (1:1,000), diluted in PBSTM. After being washed five times with PBS containing 0.05% Tween 20 (PBSTM), membranes were incubated for 1 h at 37°C with secondary antibodies (the dilution of secondary antibodies was as follows: horseradish peroxidase [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 chemiluminescence substrate (Pierce).

**ELISAs.** Purified recombinant TpF1 was diluted in carbonate buffer (0.1 M [pH 9.5]) at a concentration of 5 μg/μl, and 96-well plates (Costar) were coated with 100 μl per well overnight at 4°C. Plates were blocked for 2 h at room temperature with 200 μl PBSTM. Each serum sample was diluted (1:100 for human sera or 1:200 rabbit sera) in PBSTM, and 100 μl was added to each well. After incubation at 37°C for 2 h, the plates were washed five times with PBST, and then 100 μl 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 1 h, the plates were washed five times with PBST, and 100 μl of tetramethylbenzidine (TMB) liquid substrate was added to each well. After a 30-min incubation at room temperature, the reaction was stopped by the addition of 50 μl of 1 M 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 the 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 the cutoff value were defined as positive. The R value was calculated for correlation. Differences were considered to be significant at two-tailed *P* values of <0.05.

**RESULTS**

Production and identification of recombinant protein TpF1. The TpF1 gene was successfully amplified from the *T. pallidum*
Nichols strain genome (Fig. 1A) and then cloned into the expression vector pET28a. TpF1 was expressed as a 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 a Western blot assay with anti-His monoclonal antibodies, human syphilis sera, and uninfected control sera.

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 blotting (Fig. 2A). We observed stronger than expected reactivity with sera collected 30 days after infection, which was in contrast with previous findings (18). Furthermore, strong specific reactions against recombinant TpF1 were also observed with sera from 60 and 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 a Western blot assay. As expected, the recombinant TpF1 reacted strongly with anti-TpF1 rabbit sera but not with healthy 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, antisera 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 evoked a robust and specific immune response in an experimentally induced 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 blotting and TpF1-based ELISA. The serum samples contained primary (n = 15), secondary (n = 35), latent (n = 22), and congenital syphilis (n = 10). In the Western blot assay, the sensitivity of TpF1 was determined to be 100% for all stages of syphilis (Fig. 3, Table 1). In the ELISA, 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 humans (n = 30) and patients with other spirochetal diseases, including Lyme disease (n = 30) and leptospirosis (n = 5), were tested by Western blotting and TpF1-based ELISA. As shown in Fig. 3, none of these serum samples was found to yield positive reactivity to TpF1, resulting in a specificity of 100% for the Western blot assay and the ELISA (Table 1). Thus, the higher sensitivity and specificity of the recombinant protein TpF1 demonstrated that it can 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.** Lipoidal antigen-based tests, such as the RPR test, are often used for the
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/H11005 4), secondary (n/H11005 1), latent (n/H11005 2), and congenital (n/H11005 1) syphilis. In general, the absorbance values from TpF1-based ELISA did not correlate well with the RPR titer (Fig. 4A), reflecting the nontreponemal nature of the RPR test. In contrast, the reactivity observed with the TpF1-based ELISA correlated well with the TPPA titer (Fig. 4B), which was based on crude T. pallidum antigens and represents a treponema-specific test.

### DISCUSSION

The identification and characterization of antigens of *T. pallidum* are central to the improvement of current laboratory diagnostic methods for syphilis. TpF1 is an oligomeric protein, belonging to the bacteriominiferritin 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 160-kDa oligomers and 19-kDa monomers (22). Furthermore, it can be dissociated completely by boiling in SDS in the absence of reducing agents (23). In the present study, we 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 terminus (Fig. 1B). By Western blotting, we observed that the monomeric form of TpF1 also has reactivity with *T. pallidum* infection. In a prior study reported by McGill et al., the monomeric form of TpF1 had no

### 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 infection

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Results of RPR test</th>
<th>Results of Western blot assay</th>
<th>Results of TpF1-based ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive</td>
<td>No. negative</td>
<td>Sensitivity (%)</td>
</tr>
<tr>
<td>Human serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>11</td>
<td>4</td>
<td>73.3</td>
</tr>
<tr>
<td>SS</td>
<td>34</td>
<td>1</td>
<td>97.1</td>
</tr>
<tr>
<td>LS</td>
<td>20</td>
<td>2</td>
<td>90.9</td>
</tr>
<tr>
<td>CS</td>
<td>9</td>
<td>1</td>
<td>90.0</td>
</tr>
<tr>
<td>All syphilis samples</td>
<td>74</td>
<td>8</td>
<td>90.2</td>
</tr>
<tr>
<td>Le</td>
<td>0</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>LD</td>
<td>3</td>
<td>27</td>
<td>90.0</td>
</tr>
<tr>
<td>U</td>
<td>1</td>
<td>29</td>
<td>96.7</td>
</tr>
<tr>
<td>All negative samples</td>
<td>4</td>
<td>64</td>
<td>98.5</td>
</tr>
</tbody>
</table>

*PS, primary syphilis; SS, secondary syphilis; LS, latent syphilis; CS, congenital syphilis; Le, leptospirosis; LD, Lyme disease; U, uninfected control.*

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**FIG 4** Relationships of the reactivities of sera from syphilis patients to recombinant protein TpF1. (A) 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 indicate nonreactive reactivity in the RPR test. (B) The x axis shows the results for the TPPA assay 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.
reactivity with sera from infected rabbits or with syphilitic sera (18). A possible explanation for this discrepancy may be the differences in individual preparations of T. pallidum antigens. The antigen used in that study was composed of the lysates of T. pallidum. It is possible that the structure of native TpF1 is at very low levels, thus escaping detection by Western blotting. Another possibility is that some macromolecules derived from rabbit tissues 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 sera from eight patients with primary (n = 4), secondary (n = 1), latent (n = 2), and congenital (n = 1) syphilis whose sera 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 (the TPPA assay). Overall, the TPPA results correlated well with the TpF1-based ELISA results (Fig. 4B). However, there is a significant advantage in the preparation of recombinant proteins over the preparation of crude T. pallidum proteins. 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 amounts 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 that differentiate recently acquired infections from those acquired in the more distant past (27).

In this study, we demonstrated that the TpF1 has excellent sensitivity and specificity for serodiagnosis, exhibiting sensitivities of 98% and 100% for the TpF1-based ELISA and the Western blot assay, respectively (Table 1). In addition, sera from 30 individuals who tested negative for syphilis infection and 35 patients with other spirochetal infections that have no reactivity against TpF1 showed specificities of 100% for both the TpF1-based ELISA and the Western blot assay. The Western blot technique based on T. pallidum whole-cell lysate has been widely used over the past years, and it has been shown to be a reliable confirmatory test (11, 29, 30). In this study, the Western blot assay showed a 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, the Western blot assay is not suitable for high-throughput screening of syphilis, but it offers an additional, accurate treponemal test that can supplement current syphilis testing (33).

In conclusion, in this study we have identified an additional recombinant diagnostic candidate, TpF1, which exhibited high sensitivity (98.8%) for the 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 that can be developed into a diagnostic antigen for the screening of syphilis. Further studies need to be performed, however, before TpF1 can be definitively demonstrated to be helpful in the serodiagnosis of syphilis.

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