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Performance of novel infection phase-dependent antigens in syphilis serodiagnosis and treatment efficacy determination

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ABSTRACT

Objective: The objective of this study was to screen new antigens for syphilis serodiagnosis.
Methods: First, we determined whether the *Treponema pallidum* proteins Tp0971, Tp0768 and Tp0462 were infection phase-dependent antigens by observing serum reactivity differences in New Zealand rabbits infected with activated or inactivated *T. pallidum*. A non-infection phase-dependent antigen, the Tp92 membrane protein, was used as the negative control. Next, Tp0971-, Tp0768- and Tp0462-based ELISA was performed on 2138 human serum samples and compared with the *T. pallidum* passive particle agglutination assay (TPPA) and LiZhu™ Tp-ELISA. In addition, another 60 paired serum samples from patients at follow-up were analysed to evaluate the relationships between titre changes and differences in the A450 nm values of the Tp0971, Tp0768, Tp0462 and Tp92 antibodies measured by ELISA.
Results: Compared with Tp92 (negative control), Tp0971, Tp0768 and Tp0462 were determined to be infection phase-dependent antigens. Compared with those of the TPPA, the sensitivities of Tp0971-, Tp0768- and Tp0462-based ELISA were 96.4%, 96.9% and 93.0%, respectively, and the specificities were 97.7%, 95.4% and 98.9%, respectively, resulting in consistencies of 97.1%, 96.2% and 95.9%, respectively. Compared with those of the LiZhu™ Tp-ELISA, the consistencies of Tp0971-, Tp0768- and Tp0462-based ELISA were 95.1%, 94.2% and 94.0%, respectively, with kappa values of 0.902, 0.884 and 0.880, respectively. Tp0971, Tp0768 and Tp0462 demonstrated high sensitivities and specificities, as well as high conformity to the TPPA and LiZhu™ Tp-ELISA. Moreover, a significantly positive Spearman rank correlation coefficient (0.82,$^*P < 0.05$) was found between the difference in the A450 nm values of the Tp0971 antibody and the RPR titre change.
Conclusion: The infection phase-dependent antigens Tp0971, Tp0768 and Tp0462 are promising for syphilis diagnosis, and Tp0971 may be utilized to monitor curative effects during syphilis treatment.

1. Introduction

Syphilis, a chronic systemic disease caused by the invasive spirochete *Treponema pallidum* subsp. pallidum (*T. pallidum*), is transmitted mostly through sexual contact, the placenta or blood transfusions. Syphilis remains a serious public health concern despite being a preventable infection [1]. Timely, accurate and reliable diagnostic methods, especially for early syphilis, are important for clinical management.

T. pallidum cannot be continuously cultivated in vitro. Whole-genome analysis of *T. pallidum* [2] has provided new opportunities for exploring its proteome. The confirmation of syphilis relies primarily on serologic diagnosis [3]. The *T. pallidum* passive particle agglutination assay (TPPA) has been established to validate syphilis diagnosis

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because of its superior sensitivity and specificity [4]. However, this method is time consuming and costly, and it also fails to evaluate treatment efficacy and requires automatic operation [5]. Rapid plasma reagin (RPR) is the foundation of non-treponemal tests, in which the titre change reflects disease activity and is thus used to monitor syphilis treatment. Nevertheless, RPR can be positive in other diseases, such as systemic lupus erythematosus (SLE), malaria [6] and mononucleosis, leading to high false-positive rates. Recently, enzyme immunoassays (EIAs) and chemiluminescence assays (CIAs) based on *T. pallidum* outer membrane proteins and flagellins have been developed and applied in syphilis serological diagnosis because of their unique advantages [7]. Representative diagnostic antigens include TPN15 (Tp1071), TPN17 (Tp0435), TPN47 (Tp0574), TPN44.5 (TmpA, Tp0768) [8], Tp0663 [9], Tp0965 [10] and Tp0821 [11], as well as the flagellins FlaB1, FlaB2, and FlaB3 [12]. Although these antigens show good performance in syphilis serological diagnosis, treponeme-specific tests, including enzyme-linked immunosorbent assay (ELISA), are incapable of evaluating syphilis treatment [3]. Hence, it is of great importance to seek new specific and sensitive recombinant antigens for syphilis serodiagnosis. This approach will facilitate the development of novel commercial syphilis tests to rapidly diagnose active (vs. treated) infection in easy-to-obtain samples (including blood) and in all disease stages [22].

In our previous animal experiments, we found that the antigens produced in response to live or inactivated *T. pallidum* inoculation are different. Thus, novel antigens (e.g., Tp0134 and Tp0470) may be synthesized and then secreted into the cytoplasm during live *T. pallidum* infection. These antigens may regulate signal transduction and mediate the interactions between *T. pallidum* and the host cell, resulting in some clinical symptoms, such as hard chancre, syphiloderm and chronic inflammation [14]. These newly synthesized antigens are only generated by live *T. pallidum* during the infection phase and are not membrane proteins; thus, these antigens are called infection phase-dependent antigens. The characteristics of these proteins (Tp0971, Tp0768 and Tp0462) are in complete accordance with those of infection phase-dependent antigens, as confirmed via our animal experiments. Because these proteins show peculiarities, we hypothesized that they may serve as promising diagnostic antigens for syphilis; as expected, these proteins showed increased immune activity in serum samples from both New Zealand rabbits and patients [15–18]. In the current study, we produced three infection phase-dependent antigens (Tp0971, Tp0768 and Tp0462) and assessed their diagnostic value through antibody detection in 2138 clinical serum samples, with the aim of appraising their diagnostic value for improving syphilis treatment efficacy.

2. Materials and methods

2.1. T. pallidum Nichols strain propagation and genomic DNA isolation

The *T. pallidum* Nichols strain used in this study was a generous gift from Tianci Yang (Zhongshan Hospital, Medical College of Xiamen University, Xiamen, China) and was maintained in the Pathogenic Biology Institute, Medical College, University of South China. The genomic DNA of the *T. pallidum* Nichols strain was prepared using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. Cloning, expression, and purification of recombinant proteins

The genome sequences of Tp0768, Tp0971 and Tp0462 were obtained through GenBank (http://www.ncbi.nlm.nih.gov/). The primers and cleavage sites were as follows: Tp0768, forward 5'CGCGGATCCATGAATGCTCATACGCTTGTGT3′ (*BamHI*) and reverse 5'CCGGAATTCTCATCGAGAGGCTCCTTCTT 3' (*EcoRI*); Tp0971, forward 5'CGCGGATCCATGAAGAGGGTGAGTTTGCTC3' (*BamHI*) and reverse 5'CCGCTCGAGCTACCACTGAGGCCCCTTC3' (*XhoI*); and Tp0462, forward 5'CGCGGATCCGTGCGCCGCATAGTCTGT3' (*BamHI*) and reverse 5'CCGCTCGAGTCAGTGTTGCCCGTTTTTGA3' (*XhoI*). The regions encoding recombinant proteins were amplified by PCR from *T. pallidum* genomic DNA and were cloned into the expression vector pET30a. Expression of the Tp92 protein was performed as previously described [13]. The sequences of all constructs were confirmed by DNA sequencing.

For protein expression, *Escherichia coli* BL21 (DE3) strains harbouring the expression vectors were grown overnight at 37 °C in Luria-Bertani medium supplemented with 50 μg/mL kanamycin. The cultures were transferred to new medium at a ratio of 1:100 to achieve an A600 of 0.6. The cultures were then induced with isopropylthio-β-D-galactoside (IPTG; 1.0 mmol/L, 0.5 mmol/L and 0.5 mmol/L for Tp0768, Tp0462 and Tp0971, respectively), followed by an additional 4 h of culture at 20 °C for Tp0768 and Tp0462 and at 37 °C for Tp0971. Uninduced and negative groups were established simultaneously.

Protein purification was performed as previously described [9,12]. The concentrations of the purified recombinant proteins were estimated by using a BCA protein assay kit (Pierce, Rockford, IL, USA), and the proteins were then used for ELISA experiments.

2.3. Infection phase-dependent antigen identification

A total of 18 male New Zealand rabbits (Department of Laboratory Animals, University of South China) were raised at 18–20 °C without antibiotics. The rabbits were randomly divided into 3 groups of 6 each, as follows: a live Tp-inoculated group, an inactivated Tp-inoculated group and an untreated group. From the first immunization day to week eight, 1 mL of ear vein blood was collected from the rabbits in each experimental group ($n = 6$) once per week, and serum was obtained for ELISA (J&L Biological, Shanghai, China). Ninety-six-well ELISA plates (Costar, Corning, NY, USA) were coated with the purified recombinant proteins at final concentrations of 5 μg/mL, 3 μg/mL and 10 μg/mL for Tp0971, Tp0768 and Tp0462, respectively; a concentration of 3 μg/mL was used for the outer membrane protein Tp92 (negative control). The plates were then incubated with 5% skim milk overnight at 4 °C and washed 3 times with PBS-Tween (PBST, PBS with 0.05% Tween-20). Rabbit serum was diluted 1:200, added to each well in a volume of 100 μL and incubated at 37 °C for 2 h. After washing with PBST 5 times, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000 dilution; Proteintech Group Inc., Wuhan, China) was added, followed by incubation at 37 °C for 30 min. After another wash, 3,3′,5,5′-tetramethylbenzidine (TMB) dye was added, the samples were incubated for 20 min, and the reaction was stopped by adding 2 mol/L H_2SO_4. The absorbance of each reaction was measured at 450 nm using a Multiskan MK3 microplate reader (Thermo, Waltham, MA, USA). Each sample was assayed in triplicate.

2.4. Clinical serum samples

A total of 2138 clinical serum samples were obtained from patients hospitalized in the Regional Affiliated Hospitals of the University of South China between September 2014 and September 2016. Patients were diagnosed with syphilis according to clinical presentation, laboratory tests and history of sexual contact, as follows: primary syphilis presented with typical chancre and a positive syphilis serology test; secondary syphilis presented with a generalized cutaneous rash and/or mucosal lesions and a positive syphilis serology test; latent syphilis presented with no clinical symptoms, no treatment history for syphilis during the previous 2 years and a positive syphilis serology test; and congenital syphilis in infants presented with positive syphilis serological results (nontreponemal and treponemal) and/or direct *T. pallidum* detection by dark-field microscopy, fluorescent antibodies, or polymerase chain reaction. There were 1075 samples obtained from patients with primary ($n = 231$), secondary ($n = 263$), tertiary ($n = 81$), congenital ($n = 30$) and latent ($n = 470$) syphilis. Furthermore, 1063 syphilis-negative samples were obtained from

Table 1
Characteristics of the patients whose serum samples were used in this study (n = 2138).

Parameter	Number of patients (% of total or median)
Sex	
Male	854 (39.9%)
Female	1284 (60.1%)
Age, years	
Male	0–92 (median 48.5)
Female	0–82 (median 42.5)
Patients with syphilis (n = 1075)	
Primary syphilis	231 (10.8%)
Secondary syphilis	263 (12.3%)
Tertiary syphilis	81 (3.8%)
Congenital syphilis	30 (1.4%)
Latent syphilis	470 (22.0%)
Patients with non-syphilitic conditions (n = 1063)	
Pregnant women	314 (14.7%)
Physical examination	316 (14.8%)
Leptospirosis	20 (0.9%)
EBV infection	154 (7.2%)
Rheumatic disease	182 (8.5%)
Typhoid fever	77 (3.6%)

Abbreviations: EBV, Epstein-Barr virus.

pregnant women (n = 316) with negative syphilis serological results (TRUST and TPPA); normal controls (n = 314) with negative syphilis serological results (TRUST and TPPA); patients with leptospirosis (n = 20), whose laboratory-confirmed diagnosis relied on the microscopic agglutination test (MAT), with a 4-fold increase in MAT titres or a reciprocal MAT titre of N1:800; patients with Epstein-Barr virus (EBV) infection (n = 154) based on positive serological results (VCA-IgG/IgM); patients with rheumatic disease (n = 182) based on positive serological results (ANA/AKA/anti-CCP); and patients with typhoid fever (n = 77) based on positive serological results (the Widal test) (Table 1). All human sera were rescreened by the TPPA (Fujirebio, Tokyo, Japan) in a blinded manner, according to the manufacturer's protocol. All patients signed a written informed consent form before serum collection.

2.5. ELISA, TPPA, LiZhu™ Tp-ELISA and RPR testing

Ninety-six-well ELISA plates (Costar, Corning, NY, USA) were coated with the purified recombinant Tp0971, Tp0768 and Tp0462 proteins at final concentrations of 5 μg/mL, 3 μg/mL and 10 μg/mL, respectively, incubated with 5% skim milk overnight at 4 °C, and then washed 3 times with PBST. The clinical sera were diluted 1:100, added to each well in a volume of 100 μL and incubated at 37 °C for 1 h. After washing with PBST 5 times, HRP-conjugated goat anti-human IgG (1:10,000 dilution; Proteintech Group Inc., Wuhan, China) was added, followed by incubation at 37 °C for 30 min. After another wash, TMB dye was added, the samples were incubated for 20 min, and the reaction was stopped by adding 2 mol/L H_2SO_4. The absorbance of each reaction was measured at 450 nm using a Multiskan MK3 microplate reader (Thermo, USA). Testing with the TPPA (Fujirem, Japan), LiZhu™ Tp-ELISA (IgG/IgM, Livzon Reagent Factory, Zhuhai, China) and RPR (KeHua Bio-Engineering Inc., Shanghai, China) was then performed on all samples. The RPR test is a semi-quantitative assay. Each sample was assayed in triplicate.

2.6. ELISA and RPR tests on paired follow-up samples

ELISA and RPR tests were performed as described above. Sixty paired follow-up samples were obtained from patients hospitalized in the Regional Affiliated Hospitals of the University of South China between September 2015 and September 2016. The follow-up samples were all from patients with syphilis, according to clinical presentation, laboratory tests and history of sexual contact. Depending on the RPR titre changes, the samples were divided into the following 3 groups: no change (n = 20), two-fold titre change (n = 20) and four-fold titre change (n = 20). Each sample was assayed in triplicate.

2.7. Statistical analysis

SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. ELISA results were expressed as the mean ± the standard error of the mean (SEM). The cut-off values were defined as the mean plus two standard deviations of the absorbance values of the uninfected controls (n = 40). Absorbance values less than the equivocal ranges were defined as negative results, while values greater than the equivocal ranges were defined as positive results. Absorbance values within the equivocal ranges were not included in the final sensitivity and specificity calculations. Cross-tabs analysis was performed to evaluate the agreement of Tp0971-, Tp0768- and Tp0462-based ELISA results with those of the TPPA and LiZhu™ Tp-ELISA. Spearman rank correlation analysis was used to determine the correlations of the RPR titre change with the differences in the A450 nm values of paired samples in Tp0971-, Tp0768-, Tp0462- and Tp92-based ELISA. A P value < 0.05 was used as the alpha value to determine statistical significance for all analyses.

3. Results

3.1. Results of infection phase-dependent antigen identification

To determine whether the *T. pallidum* antigens were truly infection phase-dependent antigens, rabbit serum was collected weekly from the 6 animals in each experimental group (activated Tp-inoculated group, inactivated Tp-inoculated group and untreated group) from the first inoculation day to week eight. The samples were then reacted with 96-well plates coated with purified Tp0971, Tp0768, Tp0462 or Tp92 (control group) protein (Fig. 1a–d). The A450 nm absorbance values of the Tp0971, Tp0768 and Tp0462 antibodies increased from week 3 to 5 and then maintained their peak levels until week 8 only in the live Tp-inoculated group, while the values were unchanged in the inactivated Tp-inoculated and untreated groups. The absorbance differences observed between the live Tp-treated and inactivated Tp-treated groups and between the live Tp-treated and untreated groups from week 3 to 8 were significant (*, P < 0.05), but no discrepancy was observed between the inactivated Tp-treated and untreated groups at any time-point. Moreover, the A450 nm absorbance values of antibodies against the outer membrane protein Tp92 increased from week 3 to 5 and remained at their peak level until week 8, regardless of whether Tp was live or inactivated. The difference between the two groups was non-significant (*P > 0.05). These results demonstrated that the Tp0971, Tp0768 and Tp0462 proteins had the characteristics of infection phase-dependent antigens, while the control Tp92 protein did not.

3.2. Comparisons of Tp0971-, Tp0768- and Tp0462-based ELISA with the TPPA and LiZhu™ Tp-ELISA

We detected and compared the positive rates of Tp0971-, Tp0768- and Tp0462-based ELISA with those of the TPPA, LiZhu™ Tp-ELISA and RPR in 2138 serum samples. The results presented in Table 2 show that the positive rates of Tp0971-, Tp0768- and Tp0462-based ELISA in various syphilis infections fluctuated from 92.5–100%, 93.9–100%, and 89.6–100%, respectively. Table 3 shows the concordance rates of Tp0971-, Tp0768- and Tp0462-based ELISA with those of LiZhu™ Tp-ELISA and the TPPA. The data indicated that Tp0971-, Tp0768- and Tp0462-based ELISA had excellent concordance rates with LiZhu™ Tp-ELISA and the TPPA.

Finally, we verified the sensitivity, specificity, positive predictive

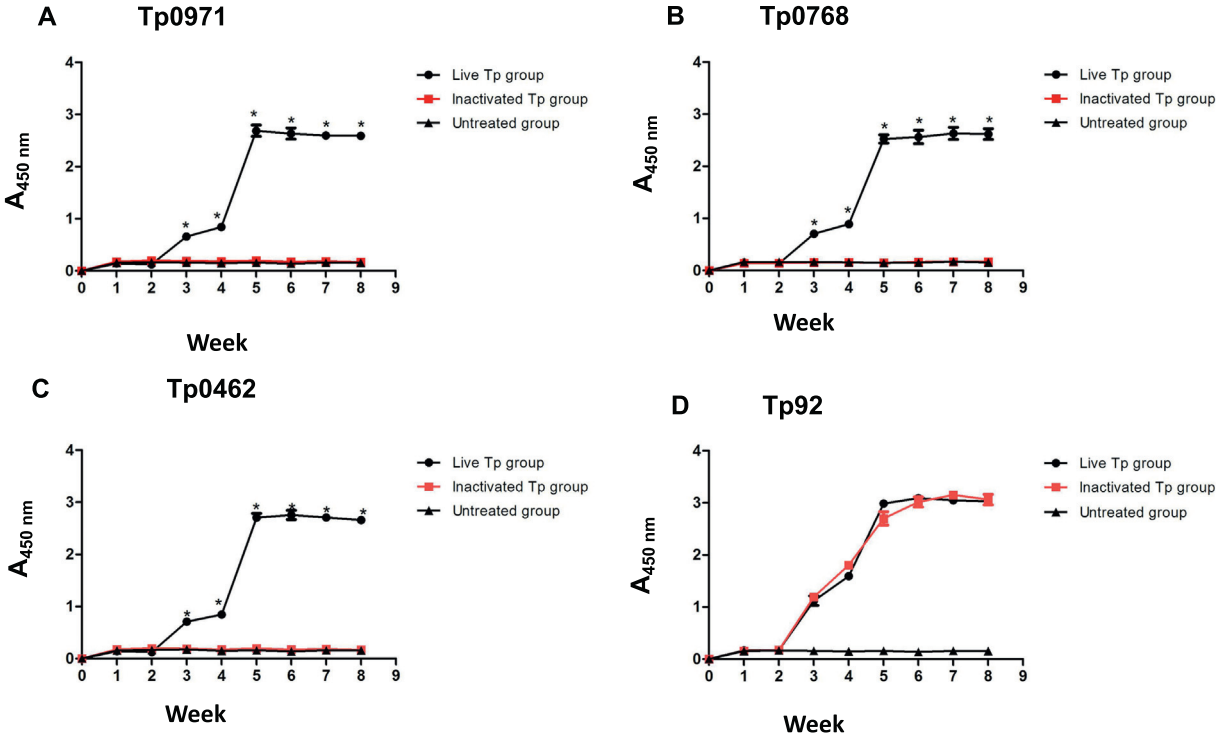

Fig. 1. A450 nm absorbance of the Tp0971, Tp0768, Tp0462 and Tp92 (control group) antibodies in rabbit sera from various inoculated groups. Male New Zealand rabbits (n = 18) were randomly divided into the following 3 groups: a live Tp-inoculated group, an inactivated Tp-inoculated group and an untreated group. After primary inoculation, rabbit serum was collected weekly from week 0 to week 8. The serum was reactive with the infection phase-dependent antigens Tp0971, Tp0768, and Tp0462 and with Tp92 (negative control) in 96-well plates. Single-track analysis of variance was used for statistical analysis. Pairwise comparisons were also performed for the live Tp-inoculated group, the inactivated Tp-inoculated group and the untreated group. *, $P < 0.05$.

value (PPV) and negative predictive value (NPV) of Tp0971-, Tp0768- and Tp0462-based ELISA compared with those of the TPPA. Table 4 shows that Tp0971-, Tp0768- and Tp0462-based ELISA had favourable sensitivity and specificity compared with those of the TPPA in diagnosing syphilis at different stages. As shown in Tables 2–4, Tp0971, Tp0768 and Tp0462 are promising antigens that can be used in syphilis diagnosis.

3.3. Venn diagram analysis of Tp0971-, Tp0768- and Tp0462-based ELISA

Venn diagram analysis, a method that is generally applied to estimate the joint effect of two or more factors, was adopted here to roughly predict the efficiency of combined Tp0971-, Tp0768- and Tp0462-based ELISA by positive serum sample intersection. As shown in Fig. 2, among 1075 syphilis-positive serum samples, 952 were triple-positive for Tp0971, Tp0768 and Tp0462, and 58, 28 and 20 were double-positive for Tp0768 and Tp0971, Tp0462 and Tp0768 and Tp0462 and Tp0971, respectively. For single antigens, positive reactions for Tp0971, Tp0768 and Tp0462 were quite low, with absolute numbers of 6, 4 and 0, respectively. Additionally, only 7 samples showed a negative response to all antigens. Therefore, we can deduce that multiple combined antigens may enhance diagnosis more effectively than single antigens.

Table 2
Positive rates for various methods of syphilis detection in 2138 serum samples.

Sample type (n)	Positive samples, n (%)					
	TPPA	LiZhu™ Tp-ELISA	RPR	Tp0971	Tp0768	Tp0462
Patients with syphilis (n = 1075)						
Primary syphilis (231)	231 (100)	229 (99.1)	230 (99.6)	224 (97.0)	217 (93.9)	207 (89.6)
Secondary syphilis (263)	263 (100)	26 3 (100)	263 (100)	260 (98.8)	263 (100)	258 (98.1)
Tertiary syphilis (81)	81 (100)	80 (98.8)	81 (100)	75 (92.5)	81 (100)	81 (100)
Latent syphilis (470)	470 (100)	466 (99.1)	470 (100)	447 (95.1)	451 (96.0)	424 (90.2)
Congenital syphilis (30)	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)
Patients with non-syphilitic conditions (n = 1063)						
Pregnant women (316)	0 (0)	12 (3.8)	14 (4.4)	7 (2.2)	6 (1.9)	0 (0)
Physical examination (314)	0 (0)	15 (4.8)	8 (2.5)	0 (0)	12 (3.8)	0 (0)
Leptospirosis (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
EBV infection (154)	0 (0)	11 (7.1)	3 (1.9)	12 (7.8)	12 (7.8)	6 (3.9)
Rheumatic disease (182)	0 (0)	9 (4.9)	2 (1.1)	5 (2.7)	19 (10.4)	6 (3.3)
Typhoid fever (77)	0 (0)	2 (2.6)	0 (0)	0 (0)	0 (0)	0 (0)

Patients diagnosed with syphilis (n = 1075, TPPA+) and non-syphilitic controls (n = 1063, TPPA-).

Table 3
Comparison of Tp0971-, Tp0768- and Tp0462-based ELISA results with the TPPA and LiZhu™ Tp-ELISA in 2138 serum samples.

ELISA based on	TPPA				LiZhu™ Tp-ELISA			
	+	−	CR	Kappa value	+	−	CR	Kappa value
Tp0971								
+	1036	24	97.1%	0.941	1036	24	95.1%	0.902
−	39	1039			81	997		
Tp0768								
+	1042	49	96.2%	0.923	1042	49	94.2%	0.884
−	33	1014			75	972		
Tp0462								
+	1000	12	95.9%	0.919	1000	12	94.0%	0.880
−	75	1051			117	1009		

Note: + represents a positive result, and – represents a negative result. TPPA, *T. pallidum* passive particle agglutination assay; CR, concordance rate. The LiZhu™ Tp-ELISA detects IgG and IgM.

Table 4
Comparison of the diagnostic performance of Tp0971-, Tp0768- and Tp0462-based ELISA results with the TPPA in patients diagnosed with syphilis (n = 1075, TPPA +) and non-syphilitic controls (n = 1063, TPPA-).

	TPPA		Sensitivity	Specificity	PPV	NPV
	+	−				
Tp0971						
+	1036	24	96.4%	97.7%	97.7%	96.3%
−	39	1039				
Tp0768						
+	1042	49	96.9%	95.4%	95.5%	96.8%
−	33	1014				
Tp0462						
+	1000	12	93.0%	98.9%	98.8%	93.3%
−	75	1051				

Note: + represents a positive result, and – represents a negative result. TPPA, *T. pallidum* passive particle agglutination assay; PPV, positive predictive value; NPV, negative predictive value.

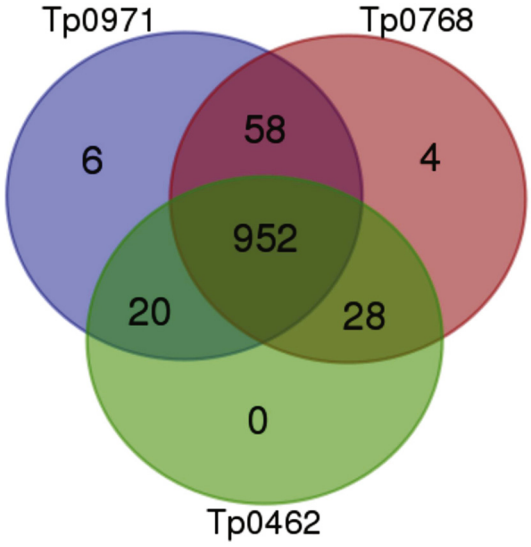

Fig. 2. Venn diagram of the interactions among Tp0971-, Tp0768- and Tp0462-based ELISAs. Serum from syphilis patients (n = 1075) was tested by Tp0971-, Tp0768- and Tp0462-based ELISA. The number represents the "positive" results measured by each ELISA.

3.4. Correlation between the A450 nm absorbance values of the Tp0971, Tp0768 and Tp0462 antibodies and RPR titre changes

Interestingly, the absorbance values of the clinical samples varied (data shown in the data article) in the Tp0971-based ELISA tests. We assumed that this variation might be related to antibody levels. Consequently, an experiment to verify our inference was essential. We performed a correlation analysis between the differences in A450 nm values for Tp0971-, Tp0768-, Tp0462- and Tp92-based ELISA and the RPR titre changes. As shown in Fig. 3, the differences in A450 nm values for Tp0971-based ELISA (r = 0.82) and Tp0768-based ELISA (r = 0.52) suggested a significant correlation with the RPR titre changes (P value = 0.02, < 0.05), while Tp0462-based ELISA (r = 0.12) presented only weak positivity. In addition, the control Tp92-based ELISA (r = −0.15) showed a negative correlation with the RPR titre change. Therefore, we can draw a preliminary conclusion that the antibody levels of Tp0971 and Tp0768, which are represented by the A450 nm absorbance values obtained in recombinant protein-based ELISA, are positively correlated with the serum RPR titre change, especially for Tp0971. All serum samples were collected from patients (n = 60) in pairs before and after syphilis treatment. Thus, TP0971 might be a potential target for evaluating syphilis prognosis.

4. Discussion

The current approach to the serodiagnosis of syphilis does not satisfy clinical needs. For example, it cannot distinguish between past and present infections. In addition, clinical needs shorten the test window period. Therefore, we are screening new antigens for the diagnosis of *T. pallidum*. We have already screened approximately 200 antigens; however, only 5 antigens were identified as infection phase-dependent antigens, and 3 of those antigens performed better than the other antigens in syphilis serodiagnosis. However, we may have missed some other infection phase-dependent antigens, so we intend to identify additional antigens that support improved syphilis diagnosis.

To the best of our knowledge, this is the first study of infection phase-dependent antigens from *T. pallidum*. In a previous study [19], live and inactivated *T. pallidum* Nichols was inoculated subcutaneously in the backs of New Zealand rabbits to observe antigen-antibody reactions. There was no significant difference in antibody levels between the live and inactivated *T. pallidum* groups during the process of screening for infection phase-dependent antigens of *T. pallidum*. In addition, antibodies specific to the Tp0134 and Tp0470 antigens were noted to be present at high titres in the live *T. pallidum* group. These antibodies were not bacterial membrane proteins of *T. pallidum* and were not observed in the inactivated *T. pallidum* group. Subsequent large-scale screening demonstrated that Tp0971, Tp0768 and Tp0462 had characteristics similar to those of infection phase-dependent antigens, contradicting the previous hypotheses that Tp0768 was a membrane protein [16,20] and that Tp0971 was an inner-membrane protein of *T. pallidum* [17]. The RPR test differs from ELISA because of its unique role in syphilis screening [21]; the RPR is primarily established to evaluate the efficacy of syphilis treatment and to detect reinfection.

In this study, we found high sensitivities and specificities of Tp0971, Tp0768 and Tp0462 according to the results of Tp0971-, Tp0768- and Tp0462-based ELISA, respectively, suggesting that these proteins represent promising antigens for syphilis diagnosis. However, misdiagnosis (i.e., false positive or false negative) was noted for all three recombinant antigens, as revealed by Venn diagram analysis. This result suggests that a combination of multiple recombinant antigens or specific polypeptide antigens may provide a better diagnosis than a single antigen. Furthermore, 7 syphilis cases could not be detected using Tp0971, Tp0768 and Tp0462, which may have been due to individual differences and limitations of the ELISA method, indicating that Tp0971-, Tp0768- and Tp0462-based ELISA cannot be used as the gold standard for syphilis diagnosis. In addition, we did not assess the

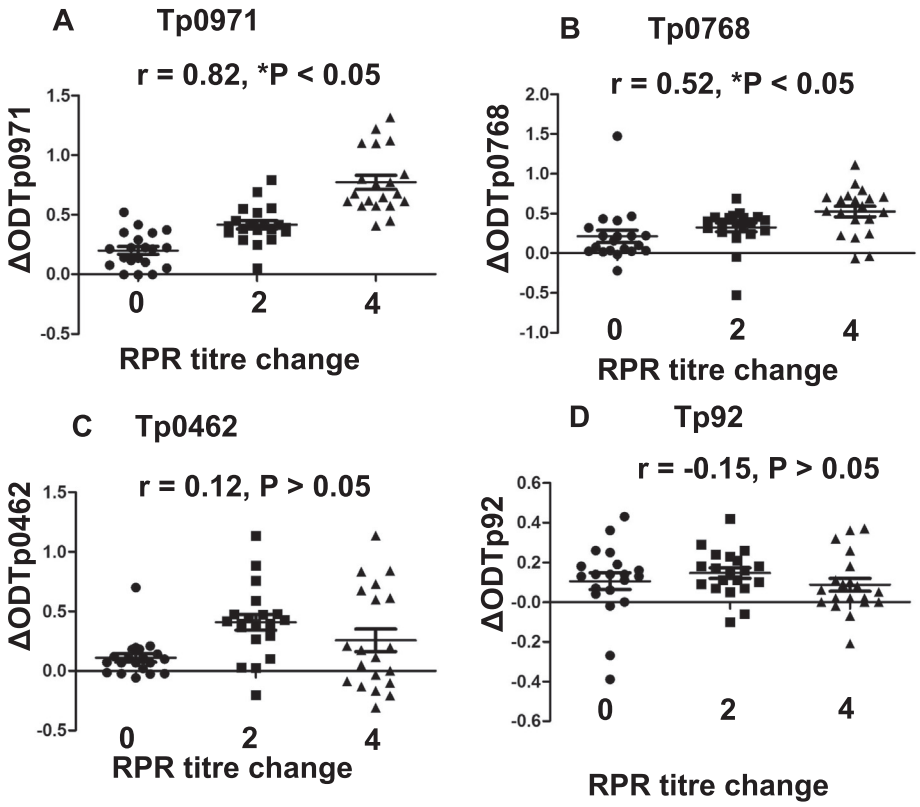

Fig. 3. Scatter distribution of the correlation between the A450 nm differences in the Tp0971 (a), Tp0768 (b), Tp0462 (c) and Tp92(d) antibodies measured by ELISA and RPR titre changes for paired follow-up samples. ΔODTp0971, ΔODTp0768, ΔODTp0462 and ΔODTp92 represent the differences in A450 nm values for paired follow-up samples in the Tp0971-, Tp0768-, Tp0462-, and Tp92- based ELISAs. In addition, 0, 2, and 4 represent no change, a two-fold titre change and a four-fold titre change in RPR titres, respectively.

true-positive (negative) and false-positive (negative) sample rates because we set the TPPA test as the relative "gold standard test". Tp0971-, Tp0768- and Tp0462-based ELISA showed high agreement with TPPA testing and LiZhu™ Tp-ELISA in syphilis diagnosis. Furthermore, the Spearman correlation coefficients of the difference in A450 nm values for Tp0971 showed a highly significant positive correlation (0.82,*P< 0.05) with the RPR titre change before and after syphilis treatment. This finding suggested that Tp0971 may be utilized to evaluate the curative effect of syphilis treatment. One limitation of this study is that it was performed in the distinct geographical area of Hunan Province, China. It remains unclear whether the diagnostic accuracy of serum samples from syphilis patients is affected by age, gender or regional distribution differences. In a subsequent study, we will expand the source of serum samples while evaluating various detection methods by double-blind detection.

In summary, ELISAs based on the infection phase-dependent antigens Tp0971, Tp0768 and Tp0462 demonstrated high sensitivity and specificity for syphilis diagnosis at various stages. Compared with LiZhu™ Tp-ELISA and the TPPA, these assays showed high concordance rates. Moreover, Tp0971 may be utilized to evaluate the curative effect of syphilis treatment.

However, ELISAs based on the infection phase-dependent antigens Tp0971, Tp0768 and Tp0462 failed to show a significant diagnostic advantage over traditional methods of syphilis diagnosis. A single antigen may not perform as well as a combination of antigens. Because of the recognized rate of false positivity in treponemal tests, this change is increasingly leading to inaccurate case identification, especially during screening of people in settings with a low syphilis prevalence [22]. Future studies should aim to identify the immunodominant epitopes of the Tp0971, Tp0768 and Tp0462 proteins, synthesize polypeptides and establish corresponding ELISAs through bioinformatics analysis to improve the sensitivity and specificity of syphilis diagnosis and to evaluate the curative effect of syphilis treatment with synthesized polypeptides.

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Declarations of interest

None.

References

[1] B. Sousa-Pinto, A. Freitas, C. Lisboa, Syphilis hospitalisations in Portugal over the last decade, Eur. J. Clin. Microbiol. Infect. Dis. 35 (2016) 169–174.

[2] C.M. Fraser, S.J. Norris, G.M. Weinstock, O. White, G.G. Sutton, R. Dodson, M. Gwinn, E.K. Hickey, R. Clayton, K.A. Ketchum, E. Sodergren, J.M. Hardham, M.P. McLeod, S. Salzberg, J. Peterson, H. Khalak, D. Richardson, J.K. Howell, M. Chidambaram, T. Utterback, L. McDonald, P. Artiach, C. Bowman, M.D. Cotton, C. Fujii, S. Garland, B. Hatch, K. Horst, K. Roberts, M. Sandusky, J. Weidman, H.O. Smith, J.C. Venter, Complete genome sequence of *Treponema pallidum*, the

syphilis spirochete, Science 281 (1998) 375–388.
[3] A.C. Sena, B.L. White, P.F. Sparling, Novel *Treponema pallidum* serologic tests: a paradigm shift in syphilis screening for the 21st century, Clin. Infect. Dis. 51 (2010) 700–708.
[4] F. Xia, Y.H. Xu, X.L. Wang, Analysis on the clinical value of methods used for the detection of *Treponema pallidum* antibody, Zhonghua Liu Xing Bing Xue Za Zhi 37 (2016) 863–867.
[5] M. Kingston, P. French, S. Higgins, O. McQuillan, A. Sukthankar, C. Stott, B. McBrien, C. Tipple, A. Turner, A.K. Sullivan, K. Radcliffe, D. Cousins, M. FitzGerald, M. Fisher, D. Grover, S. Higgins, M. Kingston, M. Rayment, A. Sullivan, UK national guidelines on the management of syphilis 2015, Int. J. STD AIDS 27 (2016) 421–446.
[6] R.C. Maves, K. Dean, N. Gadea, E.S. Halsey, P.C. Graf, A.G. Lescano, False-positive rapid plasma reagin testing in patients with acute Plasmodium vivax malaria: a case control study, Travel Med. Infect. Dis. 12 (2014) 268–273.
[7] K.W. Hoover, J.D. Radolf, Serodiagnosis of syphilis in the recombinant era: reversal of fortune, J. Infect. Dis. 204 (2011) 1295–1296.
[8] R. Sun, D.H. Lai, R.X. Ren, S. Lian, H.P. Zhang, *Treponema pallidum*-specific antibody expression for the diagnosis of different stages of syphilis, Chin. Med. J. 126 (2013) 206–210.
[9] M. Xu, Y. Xie, C. Jiang, Y. Xiao, X. Kuang, F. Zhao, T. Zeng, S. Liu, M. Liang, L. Li, C. Wang, Y. Wu, A novel ELISA using a recombinant outer membrane protein, rTp0663, as the antigen for serological diagnosis of syphilis, Int. J. Infect. Dis. 43 (2016) 51–57.
[10] A.V. Runina, A.S. Starovoitova, D.G. Deryabin, A.A. Kubanov, Evaluation of the recombinant protein tp0965 of *Treponema pallidum* as perspective antigen for the improved serological diagnosis of syphilis, Vestn. Ross. Akad. Med. Nauk 2 (2016) 109–113.
[11] Y. Xie, M. Xu, C. Wang, J. Xiao, Y. Xiao, C. Jiang, X. You, F. Zhao, T. Zeng, S. Liu, X. Kuang, Y. Wu, Diagnostic value of recombinant Tp0821 protein in serodiagnosis for syphilis, Lett. Appl. Microbiol. 62 (2016) 336–343.
[12] C. Jiang, J. Xiao, Y. Xie, Y. Xiao, C. Wang, X. Kuang, M. Xu, R. Li, T. Zeng, S. Liu, J. Yu, F. Zhao, Y. Wu, Evaluation of FlaB1, FlaB2, FlaB3, and Tp0463 of *Treponema pallidum* for serodiagnosis of syphilis, Diagn. Microbiol. Infect. Dis. 84 (2016) 105–111.
[13] F. Zhao, Y. Wu, X. Zhang, J. Yu, W. Gu, S. Liu, T. Zeng, Y. Zhang, S. Wang, Enhanced immune response and protective efficacy of a *Treponema pallidum* Tp92 DNA vaccine vectored by chitosan nanoparticles and adjuvanted with IL-2, Hum. Vaccin. 7 (2011) 1083–1089.
[14] L. Arriaga-Pizano, E. Ferat-Osorio, G. Rodriguez-Abrego, I. Mancilla-Herrera, E. Dominguez-Cerezo, N. Valero-Pacheco, M. Perez-Toledo, F. Lozano-Patino, F. Laredo-Sanchez, J. Malagon-Rangel, H. Nellen-Hummel, C. Gonzalez-Bonilla, G. Arteaga-Troncoso, A. Cerbulo-Vazquez, R. Pastelin-Palacios, P. Klenerman, A. Isibasi, C. Lopez-Macias, Differential immune profiles in two pandemic influenza a(H1N1)pdm09 virus waves at pandemic epicenter, Arch. Med. Res. 46 (2015) 651–658.
[15] S.A. Lukehart, S.A. Baker-Zander, R.M. Lloyd, S. Sell, Characterization of lymphocyte responsiveness in early experimental syphilis. II. Nature of cellular infiltration and *Treponema pallidum* distribution in testicular lesions, J. Immunol. 124 (1980) 461–467.
[16] M. McKevitt, M.B. Brinkman, M. McLoughlin, C. Perez, J.K. Howell, G.M. Weinstock, S.J. Norris, T. Palzkill, Genome scale identification of *Treponema pallidum* antigens, Infect. Immun. 73 (2005) 4445–4450.
[17] R.K. Deka, C.A. Brautigam, F.L. Tomson, S.B. Lumpkins, D.R. Tomchick, M. Machius, M.V. Norgard, Crystal structure of the Tp34 (TP0971) lipoprotein of *Treponema pallidum*: implications of its metal-bound state and affinity for human lactoferrin, J. Biol. Chem. 282 (2007) 5944–5958.
[18] C. Brautigam, R. Deka, Z. Ouyang, M. Machius, G. Knutsen, D. Tomchick, M. Norgard, Biophysical and bioinformatic analyses implicate the *Treponema pallidum* Tp34 lipoprotein (Tp0971) in transition metal homeostasis, J. Bacteriol. 194 (2012) 6771–6781.
[19] C. Jiang, F. Zhao, J. Xiao, T. Zeng, J. Yu, X. Ma, H. Wu, Y. Wu, Evaluation of the recombinant protein TpF1 of *Treponema pallidum* for serodiagnosis of syphilis, Clin. Vaccine Immunol. 20 (2013) 1563–1568.
[20] D. Šmajs, M. McKevitt, L. Wang, J.K. Howell, S.J. Norris, T. Palzkill, G.M. Weinstock, BAC library of *T. pallidum* DNA in *E. coli*, Genome Res. 12 (2002) 515–522.
[21] C. Liu, Q. Ou, H. Chen, J. Chen, S. Lin, L. Jiang, B. Yang, The diagnostic value and performance evaluation of five serological tests for the detection of *Treponema pallidum*, J. Clin. Lab. Anal. 28 (2014) 204–209.
[22] E.N. Kersh, Lukehart S.A. Lukehart, Biomedical Research Priorities for Modern Syphilis Clinical Management, Diagnosis, and Vaccines: Overview and Commentary for Unit 1, Sex. Transm. Dis. 45 (2018) S7–S9.