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ORIGINAL ARTICLE

Clinical Evaluation of a New Chemiluminescence Assay for the Detection of *Treponema Pallidum* Antibodies

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SUMMARY

Background: **A new chemiluminescence assay, the Anti-TP-II assay, is going to be commercially available in clinical laboratories in China and other countries. This study examined the performance of the new assay for the detection of TP infection and compared it with that of the Anti-TP assay by using large amounts of clinical samples.**
Methods: **The precision, accuracy, anti-interference ability, and the clinical sensitivity and specificity of the Anti-TP-II assay were evaluated. In addition, compared with those of the Anti-TP assay, the false positive and false negative rates of the Anti-TP-II assay were evaluated for 2,436 clinical routine samples and 711 preselected Anti-TP assay reactive samples. Discrepancy of the samples was investigated with the recomLinec Treponema IgM/IgG kit or the Elecsys syphilis assay.**
Results: **The precision, accuracy, and anti-interference ability of the Anti-TP-II assay met the national standard of China, and there was an overall agreement of 96.75% (Kappa = 0.91) between the two assays. The sensitivity and specificity of the Anti-TP-II assay were 100% (95% CI: 94.13% to 100%) and 99.92% (95% CI: 99.70% to 99.99%), respectively. Compared with the Anti-TP assay, the Anti-TP-II assay significantly reduced the number of borderline samples and the false positive rate.**
Conclusions: **Considering its excellent performance, the Anti-TP-II assay is a good screening test for high-throughput laboratories and can replace the previous generation of reagents, the Anti-TP assay, with a superior specificity.**
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INTRODUCTION

Syphilis, caused by *Treponema pallidum* (TP), is a multistage sexually transmitted infection of significant importance for global health [1]. Estimates from the WHO indicate that approximately 22.3 million individuals worldwide had TP infection, with 7.1 million new cases in 2020. In recent years, owing to interventions by health authorities, the reported increase in the incidence of syphilis in China has been slowing, but it remains high and continues to grow steadily, threatening the public health in China [2-4]. The disease has a broad

clinical spectrum and is a challenge for clinicians due to the long incubation period and the sometimes complex interpretation of serological test results, and if left untreated, it can result in serious complications [5]. As such, there has been a great deal of research into clinical strategies for treating this disease, including diagnostic biomarkers and possible strategies for treatment and prevention [6,7].

However, currently, there is no gold standard test for identifying TP, because it can hardly be cultured in vitro or identified with simple laboratory stains [8]. At present, serological tests, which can be broadly categorized into nontreponemal tests (NTTs) and treponemal tests (TTs), are the most commonly used methods for the diagnosis of syphilis in clinical laboratories [9]. Driven by the increasing demand for syphilis screening, an increasing number of laboratories, especially high-volume clinical laboratories, have implemented automated TTs, such as enzyme-linked immunosorbent assay (ELISA), chemiluminescence assay (CIA), and electrochemiluminescence immunoassays (ECIA), as the first screening test. Compared with ELISA, both CIA and ECIA can quickly detect serum or plasma TP-specific antibodies with a greater sensitivity and specificity [10-12]. However, false negatives and false positives can occur due to defects in the antigen selection and false positive biological reactions [9,11,13]. Therefore, there is still room for improvement in these detection methods.

In March 2023, our emergency laboratory began to use the Anti-TP assay (Mindray, China), a CIA method that is a two-step indirect format assay using the recombinant treponemal TpN15, TpN17, and TpN47 antigens to determine the level of antibody to TP, for the initial screening of syphilis. Like other users [14], we found that a large portion of the samples with cutoff index (COI) values between 1 and 5 were false positives during the use. As the Anti-TP-II assay (Mindray, China), a two-step sandwich CIA assay using the same recombinant treponemal antigens to qualitatively determine the level of antibody to TP, is going to be commercially available in clinical laboratories in China and other countries, we intend to evaluate the performance of the Anti-TP-II assay for its reliable detection of TP infection and compare it with the Anti-TP assay by using a large number of clinical samples to clarify whether it can reduce the false positive rate.

MATERIALS AND METHODS

Methodological evaluation

The precision of the Anti-TP-II assay was determined by using five replicates of each of the same materials, and the assay was performed during 5-day evaluation periods according to CLSI EP15-A3 (user verification of precision and estimation of bias). Quality control (QC) samples at 2 levels (negative and positive) and patient samples at 3 levels (low, medium, and high) were used. The values of repeatability and intermediate precision were calculated. Accuracy was evaluated by the National Reference Panel for *Treponema pallidum* antibody (chemiluminescent testing, 370036-202002) (National Institutes for Food and Drug Control, China), which includes 20 TP antibody negative references, 10 TP antibody positive references, and 4 sensitivity references. These references are made from the plasma of TP-infected individuals, plasma of TP-related viruses (such as human immunodeficiency virus, hepatitis B virus, etc.) infected individuals, and plasma of normal individuals. This method is suitable for evaluating the performance of TP antibody CIA reagents, including the negative coincidence rate, positive coincidence rate, and detection limit. The evaluation of the anti-interference ability was conducted by using 16 common interfering substances and 14 infectious disease-positive cross reactants.

Comparison of the two syphilis assays by using clinical samples

First, 2,436 routine serum samples with unknown TP infection status were selected randomly in the West China Hospital from April 2023 to May 2023 to compare the consistency of the two assays. Then, 711 preselected Anti-TP reactive samples were collected from April 2023 to December 2023. All 3,147 samples were measured in parallel by using the Anti-TP assay and Anti-TP-II assay on a CL8000i immunoassay analyzer (Mindray, China). Samples showing consistent results in both assays were defined as either positive or negative. Discrepant samples between assays were stored at -20°C and subsequently confirmed with the recomLinec Treponema IgM/IgG kit (Mikrogen GmbH, Germany) or the Elecsys syphilis assay (Roche, Germany). Samples with a positive or negative RIBA result were considered positive or negative, respectively. Samples with an indeterminate outcome for RIBA were further confirmed by the Elecsys syphilis assay result on an E601 analyzer (Roche, Germany).

Both the CL8000i and the E601 analyzers automatically calculate the cutoff values based on the calibrations, and the results are given in the form of a COI. Samples were considered positive if the COI was ≥ 1 and negative if the COI was < 1. These assays were all carried out according to the package instructions, and all samples with a COI ≥ 0.9 were retested in duplicate. Samples with a COI in the range of ≥ 0.9 to < 3.0 were considered borderline.

Statistical analysis

The statistical analysis of the diagnostic indices and representations was performed by using MedCalc 22.002 (MedCalc Software Ltd., Belgium) and GraphPad Prism 10.1.1 (GraphPad Software Inc., USA). The kappa coefficient was calculated as a measure of agreement between the Anti-TP assay and the Anti-TP-II assay. Descriptive analysis was used to calculate the proportions and frequencies for all categorical variables.

Table 1. The Precision for Anti-TP-II assay.

Sample type	Level	n	Mean (COI)	Verified estimate		Acceptable standards *	
				Repeatability CV (%)	Intermediate precision CV (%)	Repeatability CV (%)	Intermediate precision CV (%)
QC samples	Negative	25	0.15	2.94	2.69	< 10.00	< 15.00
	Positive	25	7.87	2.44	1.77		
Patient samples	Low	25	0.39	1.14	2.32		
	Medium	25	0.84	1.35	2.16		
	High	25	2.22	1.08	1.54		

* - The acceptable standards are sourced from the guidelines for performance characteristics of immunological qualitative tests issued by the National Health Commission of China (WS/T 494-2017).

RESULTS

Precision, accuracy, and anti-interference ability of the Anti-TP-II assay

As shown in Table 1, for the 2 levels of QC samples and 3 levels of patient samples, the percent coefficient of variation (% CV) of repeatability and intermediate precision for the Anti-TP-II assay ranged from 1.08% to 2.94%. The results of the Anti-TP-II assay were in 100% agreement with those of the National Reference Panel for *Treponema Pallidum* antibody. The detection rate of negative references was 20/20, and the detection rate of positive references was 10/10. Among the four sensitivity references, the samples at level 1 and level 2 tested negative, while the samples at level 3 and level 4 tested positive. Both the precision and accuracy met the requirements of the health industry standards issued by the National Health Commission of China (WS/T 494-2017: Guideline for performance characteristics of immunological qualitative test). The anti-interference ability of the Anti-TP-II assay is shown in Table 2. In the presence of different interfering substances or cross reactants, all the detection results of the Anti-TP-II assay were acceptable.

Comparison of the Anti-TP assay and the Anti-TP-II assay

In 2,436 routine samples, a total of 61 positive results were found, hence, the prevalence of Anti-TP-positivity was 2.50% in our hospital. The sensitivity, specificity, and positive and negative predictive values of the Anti-TP and Anti-TP-II assays for routine samples are shown in Table 3.

The overall agreement between the Anti-TP-II and Anti-TP assays was 96.75% (3,045/3,147) (the kappa coefficient was 0.91, and the 95% CI was 0.89 to 0.93). The distributions of the COI values of the 3,147 samples for the Anti-TP and Anti-TP-II assays are shown in Figure 1. The numbers of borderline samples for the Anti-TP and Anti-TP-II assays were 162 and 51, respectively. According to our testing algorithms, 684 samples were identified as positive, and 2,463 were identified as negative. By using COI = 1.00 as the diagnostic threshold according to the manufacturer's claim, there were 96 false positive results for the Anti-TP assay and only 2 false positive results for the Anti-TP-II assay (Figure 2A). However, 4 false negatives were detected for the Anti-TP-II assay, while no false negatives were detected for the Anti-TP assay (Figure 2B).

DISCUSSION

At present, ELISA, CIA, and ECIA are routinely used to screen for TP infection. Compared with ELISA, CIA and ECIA, including sample preprocessing systems and result analysis systems from the same manufacturers, are fully automated and self-contained platforms that minimize operator involvement, have better reproducibility, and partly avoid the false positives/negatives caused by operator factors [15]. In this study, we are the first to conduct a comprehensive methodological and large-sample clinical evaluation of the Anti-TP-II assay to provide useful reference information for laboratory users.

In the present study, the Anti-TP-II assay showed a favorable reproducibility and intermediate precision (both % CVs < 3.00), and the sensitivity, specificity, PPV, and NPV were 100% (95% CI: 94.13% to 100%), 99.92% (95% CI: 99.70% to 99.99%), 96.82% (95% CI: 88.40% to 99.19%), and 100% (95% CI: 99.85% to 100%), respectively. The methodological performance parameters were consistent with those of other commonly used CIA or ECIA clinical procedures reported in previous studies [16-20] and met the requirements of the health industry standards in China. Therefore, the Anti-TP-II assay should be able to meet the clinical needs of most routine screening laboratories. The overall agreement between the Anti-TP-II and Anti-TP assays was 96.75% (the kappa coefficient was 0.91, and the 95% CI was 0.89 to 0.93), indicating that the agreement between the two assays was near perfect. There-

Table 2. The anti-interference ability of Anti-TP-II assay.

	Relative deviation of weakly positive samples (%)	Negative agreement (n, %)	Acceptable standards
Interfering substance			**Relative deviation < ± 20% or Negative agreement = 100%**
Bilirubin (25 mg/dL)	**-1.38**	**-**	
Triglyceride (3,000 mg/dL)	**-2.22**	**-**	
Hemoglobin (500 mg/dL)	**3.52**	**-**	
Total protein (12 g/dL)	**7.71**	**-**	
Alkaline phosphatase (1,200 U/L)	**1.90**	**-**	
Biotin (1,200 ng/mL)	**-1.45**	**-**	
Rheumatoid factors (1,200 IU/mL)	**-2.51**	**-**	
Immunoglobulin G (5.0 g/dL)	**-2.76**	**-**	
Immunoglobulin M (0.5 g/dL)	**-5.63**	**-**	
Doxycycline (200 mg/L)	**-1.62**	**-**	
Ceftriaxone sodium (750 mg/mL)	**-1.48**	**-**	
Erythromycin (500 mg/L)	**0.35**	**-**	
Probenecid (500 mg/L)	**2.48**	**-**	
Azithromycin (2,000 mg/L)	**-3.61**	**-**	
Antinuclear antibody (positive)	**-4.35**	**-**	
Human anti-mouse antibody (positive)	**7.89**	**-**	
Cross reactant			
Hepatitis A virus antibody positive	**-**	**7,100**	
Hepatitis B virus antigen positive and anti-HBc positive	**-**	**12,100**	
Hepatitis C virus antibody positive	**-**	**5,100**	
Human immunodeficiency virus antigen/antibody positive	**-**	**3,100**	
Human T-lymphotropic virus antibody positive	**-**	**4,100**	
Cytomegalovirus antibody positive	**-**	**5,100**	
Epstein-Barrvirus antibody positive	**-**	**9,100**	
Herpes simplex virus antibody positive	**-**	**6,100**	
Rubella virus antibody positive	**-**	**10,100**	
Varicella-zoster virus antibody positive	**-**	**5,100**	
Toxoplasma gondi antibody positive	**-**	**9,100**	
Leptospirosis antibody positive	**-**	**2,100**	
***Escherichia coli* antibody positive**	**-**	**4,100**	
Novel coronavirus antibody positive	**-**	**5,100**	

Table 3. The sensitivity, specificity, and positive and negative predictive values of Anti-TP and Anti-TP-II assay in random routine samples.

		Confirmed results		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
		positive	negative				
Anti-TP assay	**positive**	**61**	**8**	**100% (94.13% to 100%)**	**99.66% (99.34% to 99.85%)**	**88.39% (79.22% to 93.83%)**	**100% (99.84% to 100%)**
	negative	**0**	**2,367**				
Anti-TP-II assay	**positive**	**61**	**2**	**100% (94.13% to 100%)**	**99.92% (99.70% to 99.99%)**	**96.82% (88.40% to 99.19%)**	**100% (99.85% to 100%)**
	negative	**0**	**2,373**				

CI - confidence interval, PPV - positive predictive value, NPV - negative predictive value.

Table 4. Test results of different assays of the 4 "false negative" samples confirmed by our testing algorithms.

Sample	Results					
	Anti-TP assay (COI)	Anti-TP-II assay (COI)	RIBA IgM	RIBA IgG	ECIA (COI)	TPPA *
1	positive (1.91)	negative (0.47)	negative	indeterminate	positive (2.72)	reserve
2	positive (1.95)	negative (0.85)	indeterminate	negative	positive (1.97)	reserve
3	positive (1.41)	negative (0.09)	negative	indeterminate	positive (1.08)	negative
4	positive (3.72)	negative (0.10)	negative	positive	negative (0.13)	negative

*** - To further determine the results, we specifically added *Treponema pallidum* granule agglutination assay (TPPA) (Fujirebio, Japan) to these 4 samples.**

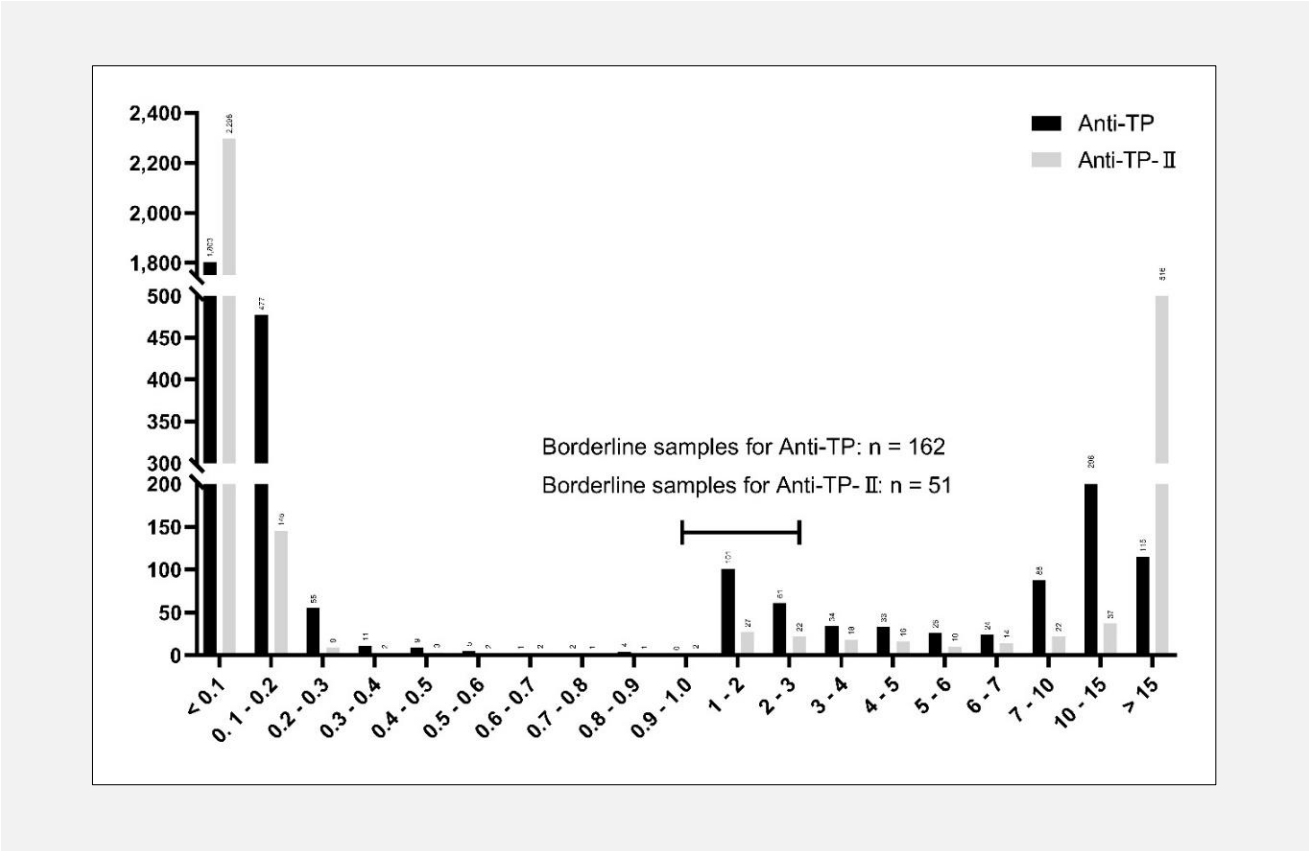

Figure 1. Distribution of the COI values of samples determined with Anti-TP and Anti-TP-II assay.

fore, it is feasible to replace the Anti-TP assay with the Anti-TP-II assay in clinical laboratories. However, more effort is still needed to explore its performance in areas with different syphilis incidence rates, different disease stages, and different patient populations.

In the clinical setting, we often face the problem that some samples with borderline results (0.90 < COI < 3.00) are difficult to define, which may result in a waste of time and money for duplicate detection. According to the distribution of the COI sequences among the 3,147 clinical samples, 162 and 51 borderline samples were subjected to the Anti-TP and Anti-TP-II assays, respectively, showing that the Anti-TP-II assay can reduce the number of borderline samples by approximately two-thirds. Moreover, compared with the Anti-TP assay, the Anti-TP-II assay greatly reduced the false positive rate. In 2,463 negative samples confirmed by our testing algorithms, there were 96 false positive results from the Anti-TP assay and only 2 false positive results from the Anti-TP-II assay. These data suggest that the Anti-TP-II assay has a better diagnostic performance in terms of specificity than the Anti-TP assay. According to the in-

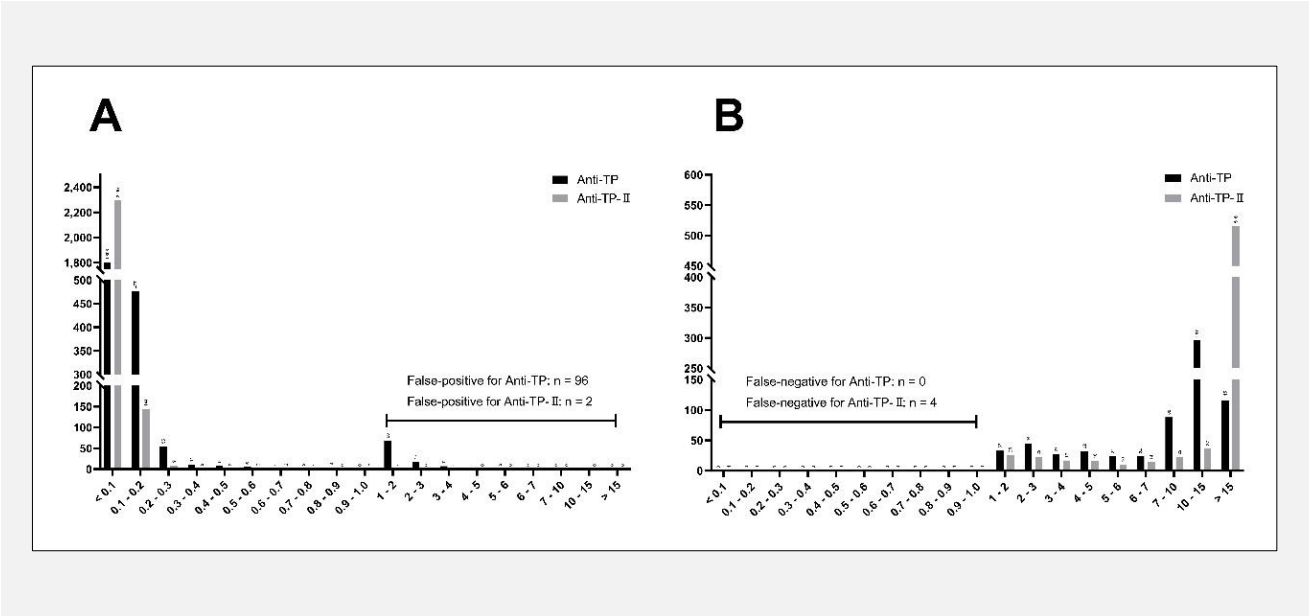

Figure 2. Distribution of the COI values of negative (A) and positive (B) samples determined with Anti-TP and Anti-TP-II assay.

structions provided by the manufacturer, the Anti-TP-II assay is a two-step sandwich assay, while the Anti-TP assay is a two-step indirect format assay. We believe that this new feature will greatly improve the specificity of the Anti-TP-II assay. The sandwich assay, which is usually the best assay configuration for a specific protein, has long been proven to be able to greatly enhance the specificity of immunometric assays [21]. Nevertheless, pregnant women and patients with autoimmune disorders, viral infections, and malignant neoplasms or tumors still reportedly have high rates of false positive serological results, according to sandwich assays [16, 17,22-24]. In this study, 2 false positive results were detected in samples from patients with malignant ovarian tumors and patients with systemic lupus erythematosus (SLE) by using Anti-TP-II assay. Although no potential cross-reactivity was observed in patients with viral infections, more studies are needed to evaluate the ability of the assay to use more samples from these special populations. Overall, compared to the Anti-TP assay, the Anti-TP-II assay was more efficient at providing evidence for clinical decision making, reducing costs, and the need for confirmatory testing.

Among the 684 positive samples confirmed by our testing algorithms, four false negatives were detected by the Anti-TP-II assay, while no false negatives were detected by the Anti-TP assay. The detailed test results of the 4 false negative samples are listed in Table 4. Based on these test results, regardless of which diagnostic algorithm was used, it is difficult to determine whether these 4 samples are true negative or true positive. Therefore, the appearance of these 4 “false negative” samples did not mean that the sensitivity of the Anti-TP-II assay was not as good as that of the Anti-TP assay. In fact, syphilis serodiagnosis is challenging because distinct clinical manifestations of the infection may influence the serological performance, and discordant results between tests also make clinical decisions difficult [25]. For such specimens, we often need to follow up for a longer period of time to determine whether the patient is infected.

In our study, 61 positive cases were detected in 2,436 random routine samples, revealing a prevalence rate of 2.50%. The prevalence of syphilis in China varies greatly among regions and population subgroups, reaching 14.9% in human immunodeficiency virus (HIV)-positive men, who have sex with men [26,27]. Starting in 2010, the Chinese government initiated a 10-year syphilis control plan, called the national syphilis control plan (NSCP), to address the emerging threat of syphilis. Our findings were approximately equivalent to the 2.38% reported in our previous research in 2016 [17], and our results revealed that the NSCP program contributed to progress in syphilis control in the Chengdu area of the Sichuan Province. However, the slight increase in the Anti-TP-positive rate also indicates that the NSCP should be sustained and strengthened to further control the syphilis epidemic in China.

In conclusion, the precision, accuracy, anti-interference ability, and the clinical sensitivity and specificity of the Anti-TP-II assay were excellent and met the required national standards of China, indicating that it is a good screening test for high-throughput laboratories and that it can replace the previous generation of reagents, the Anti-TP assay, with a superior performance.

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Ethics Statement:
The Ethics Committee of the West China Hospital of Sichuan University approved this study.

Declaration of Interest:
None.

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