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Establishment and Evaluation of a One-Step Microplate Chemiluminescence Immunoassay to Detect IgG Antibody Against *Treponema Pallidum*

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Background: The serological detection of specific antibodies against *Treponema pallidum* is of particular importance in the diagnosis of syphilis. The chemiluminescence immunoassay (CLIA) has been widely used for clinical diagnosis because they remit no radical waste products, cause no enzyme precipitation, and exhibit an excellent sensitivity. *Methods:* A one-step CLIA was established to detect *T. pallidum* IgG antibody based on microplate coated with a mixture of recombinant *T. pallidum* antigens TpN15, TpN17, and TpN47. The Chinese national reference substances standard panel for *T. pallidum* diagnosis was applied to test the accuracy, stability, interference, and cross-reactivity of the established CLIA. The validation of efficacy for clinical application was performed by comparing the established method with the marketed *T. pallidum* particle agglutination (TPPA) kit and the Abbott ARCHITEC Auto System. *Results:* The established method met the requirement of the Chinese national reference substances standard for *T. pallidum* diagnosis. When compared with TPPA ($n = 1{,}052$), the specificity, sensitivity, and overall concordance were 99.7%, 99.0%, and 98.8% respectively, showing a great agreement with a kappa value of 0.81. When compared with the Abbott ARCHITEC Auto System ($n = 352$), the results showed that the specificity, sensitivity, and overall concordance were 98.6.0%, 96.6% and 98.6% respectively, and a high-degree agreement was observed (kappa value = 0.95). *Conclusion:* The established rapid, specific, sensitive, and stable microplate CLIA method to detect IgG antibody against *T pallidum* will provide an efficient alternative to the treponemal tests and wide application in clinical laboratory. J. Clin. Lab. Anal. 29:493–497, 2015.

Key words: *Treponema pallidum*; chemiluminescence; antibody; IgG; diagnosis

INTRODUCTION

Syphilis caused by the spirochete *Treponema pallidum* is a reemerging disease that is sexually transmitted and can progress in stages. The incidence of syphilis has increased worldwide (1). The diagnosis of syphilis during the early primary stage can be accomplished by dark-field microscopy of primary-chancre samples for the presence of spirochetes, but this is possible only when lesion is present, and this is not the case in the majority of patients. The serological detection of specific antibodies against *T. pallidum* is of particular importance in the diagnosis of syphilis, since the natural course of the infection is characterized by periods without clinical manifestations. Antibody detection by serological assay is regarded as the "gold standard" for diagnosing syphilis and for monitoring treatment efficacy (2).

The most common screening tests are the Rapid Plasma Cardiolipin antigen test (RPR) and Venereal Disease Research Laboratory (VDRL) tests, both of which test for the presence of antilipoidal antibodies (2). The RPR or the VDRL, although technically simple and cheap, are labor-intensive, may occasionally result in false-negative reactions due to the prozone phenomenon (3) and are insensitive with samples from patients with early- or late-stage infection. Confirmatory tests include FTA-Abs

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(fluorescent treponemal antibody absorption test), MHA-TP (microhemagglutination assay for *T. pallidum*), and TPHA (*T. pallidum* hemagglutination assay), which use crude *T. pallidum* antigens (4).

Here we established a one-step enhanced chemiluminescence immunoassay (CLIA) method using luminol/peroxidase system for detection of IgG Ab against *T. pallidum* based on microplate coated with recombinant *T. pallidum* antigens, a combination of TpN15, TpN17, and TpN47. The evaluation of efficacy for clinical application was also performed by comparing the established microplate CLIA method with the marketed *T. pallidum* particle agglutination (TPPA) kit (Fujirebio, Tokyo, Japan), as well as the Abbott ARCHITEC I1000 Auto System (Abbott Diagnostics, Wiesbaden, Germany) for detection of IgG Ab against *T. pallidum*.

MATERIALS AND METHODS

Reagents and Serum Samples

The methods for comparison were TPPA kit (lot number VN10813) and CLIA kit run on Abbott ARCHITEC I1000 Auto System (lot number 09131LP03). The chemiluminescence analyzer, HC-CLIA-III, for microplate relative light unit (RLU) reading were produced by Hongcheng Biotechnology Co. Ltd., China. The Chinese national reference substances standard panel for *T. pallidum* diagnosis was purchased from Chinese Food and Drug Inspection Institute (Beijing, China; lot number 240013-200901). Purified recombinant *T. pallidum* antigens, TpN15, TpN17, and TpN47 for microplate coating, as well as for horse radish peroxidase (HRP) labeling were purchased from the Fapon Biotech Inc., Shenzhen, China. A sum of 352 serum samples (279 male, 73 female) were collected from Syphilis patients and non-Syphilis patients in the Fuzhou Infectious Disease Hospital, Fujian province, while 350 serum samples (173 male, 177 female) from Fuzhou General Hospital, Fujian province, and 350 serum samples (35 male, 315 female) from Fujian Provincial Maternity and Child Care Center. Sera were stored at −30°C until use.

Development of a One-step Microplate CLIA Method to Detect IgG Antibody Against *T. Pallidum*

HRP-labeled recombinant *T. pallidum* antigens and sodium iodide enhanced chemiluminescence using luminol/peroxidase system were employed in the methods. Briefly, microplates were coated with 100 μl (5 mg/l) of purified recombinant *T. pallidum* antigens, a mixture of TpN15, TpN17, and TpN47 in 0.05 mol/l pH 9.6 carbonate buffer and kept at 4°C for overnight. Then wells were washed with a 0.01 mol/l pH7.4 PBS for three times, and blocked at room temperature for 2 h with PBS containing 0.5% BSA. The brief procedure for one-step double-antigen sandwich CLIA method was described as below: first, 50 μl of serum samples or controls were added to the microplates wells that were covered with the purified recombinant *T. pallidum* antigen mixture, then 50 μl of HRP-conjugated antigen mixture (1:3,000 dilution) was added to each microplate well followed by the mixture kept at 37°C for 60 min. The microplates were then washed with PBS for five-rinse cycles. Finally, 100 μl of enhanced luminal reagent substrate solutions was added and kept in dark for 5 min, and finally the result for the RLU was read in a chemiluminescence analyzer, HC-CLIA-III.

The cut-off RLU for assigning negative or positive results for IgG Ab against *T. pallidum* were defined as the mean plus two times the standard deviation of the RLU of the uninfected sera ($n = 120$). The negative sera were defined as those that yielded RLU less than or equal to the cutoff, while the positive sera were defined as those that gave RLU greater than this value.

The Accuracy and Stability for the Established Microplate CLIA Method

The Chinese national reference substances panel for *T. pallidum* diagnosis, including 20 vials for negative test, 10 vials for positive test, 1 vial for precision test, and 4 vials for sensitivity test were used for testing the accuracy and stability of the established CLIA method for IgG Ab against *T. pallidum*. Three lots of reagent preparation for the microplate CLIA method (lot no. 111110, no. 111120, and no.111130) were analyzed for accuracy test. For the stability test, the microplate CLIA reagent (lot no.111110) was kept at 42°C for 3 days to accelerate degradation, and the procedure for both tests was described above.

The Interference and Cross-Reactivity of the Tests

For the interference test, a serial with different final concentration of cholesterol, triglyceride, hemoglobin, and bilirubin were added separately to the known *T. pallidum* IgG Ab positive or negative serum samples, followed by testing the antibody to *T. pallidum* in the samples as described above. Serum samples with positive or negative antibody without adding of these interference substances were parallelly detected as controls.

For the cross-reactivity test, a collection of serum samples with strong-positive antibody to other pathogens were added separately to the known *T. pallidum* IgG Ab positive and negative serum samples, followed by testing the antibody against *T. pallidum* in the samples as described above. The other pathogens included cytomegalovirus (MV); rubella virus; *Toxoplasma gondii*; herpes simplex virus (HSV) type I and II; hepatitis A,

TABLE 1. Accuracy and Stability Test Results of the Established Microplate CLIA Method by Using the Chinese National Reference Substances for *T. pallidum* Diagnosis

		Accuracy			Stability
	Nat. Ref. Stand.	Lot 111110	Lot 111120	Lot 111130	Lot 111110
Neg. Concord.	20/20	20/20	20/20	20/20	20/20
Pos. Concord.	10/10	10/10	10/10	10/10	10/10
Sen. Concord.	No. 1 +	+	+	+	+
	No. 2 +	+	+	+	+
	No. 3 +/–	–	–	–	–
	No. 4 –	–	–	–	–
Pre. Concord.	Intra. CV ≤ 15.0%	4.68%	5.56%	5.77%	8.91%
	Intra. CV ≤ 20.0%		12.8% among three lots of reagents		

Nat. Ref. Stand., national reference standard; Neg., negative; Pos., positive; Sen., sensitive; Pre., precision; Concord., Concordance; Intra. CV, intraassay coefficient of variation; Inter. CV, interassay coefficient of variation.

B, C, and E virus (HAV, HBV, HCV, and HEV); human immunodeficiency virus (HIV); and Epstein–Barr virus (EBV). Serum samples with positive or negative antibody against *T. pallidum* without adding of serum with positive antibody to the other pathogens were parallelly detected as controls.

Microplate CLIA Test, TPPA Test, and Abbott ARCHITEC I1000 Auto System Test

To evaluate the efficacy for clinical application, a total of 1,052 serum samples collected from three hospitals were parallelly detected for the presence of IgG Ab to *T. pallidum* by the established microplate CLIA method as described above and the Fujirebio TPPA method. A sum of 352 serum samples collected from the Fuzhou Infectious Disease Hospital was additionally detected by the Abbott ARCHITEC I1000 Auto System. All methods were performed according to the instructions of the manufacturers. The microplate CLIA, TPPA, and Abbott Auto System tests were performed under blinded conditions. A sample with different results by the various methods used in this study was retested by a technologist who was unaware of the previous results. The significance of the differences in the results was analyzed by the X^2-test and kappa value.

RESULTS

The Accuracy and Stability of the Test

The accuracy and stability of the test results showed that the established microplate CLIA kit met the requirement of the Chinese national reference substances standard for *T. pallidum* diagnosis (Table 1). The intraassay coefficients of variation for the three lots of reagents were 4.68%, 5.56%, and 5.77% for accuracy test, while 8.91% for stability test. The interassay coefficient of variation among three lots of reagent was 12.8%.

TABLE 2. Comparison of *T. pallidum* IgG Ab in Clinical Serum Samples Detected by the Microplate CLIA and TPPA

		TPPA		
		+	–	Total
Microplate CLIA	+	290	10	300
	–	3	749	752
	Total	293	759	1052

The Interference and Cross-Reactivity of the Tests

A final concentration of cholesterol 2.0 mg/ml, triglyceride 2.0 mg/ml, hemoglobin 10 mg/ml, and bilirubin 0.15 mg/ml, all are supposed to be a high level in clinical samples, showed no interference with the *T. pallidum* IgG Ab detection by the established microplate CLIA method, as the RLU remitted by the samples with adding of interference substances show no increase compared with samples without adding of the interference substances (data not shown). Moreover, all the serum samples with strong-positive antibody to any pathogens tested showed no interference with the established microplate CLIA for detection of IgG Ab to *T. pallidum* (data not shown).

The Comparison Test

The microplate CLIA, TPPA, and Abbott Auto System tests were performed for evaluation of clinical efficacy. When compared with TPPA ($n = 1{,}052$), the specificity, sensitivity, and overall concordance for microplate CLIA method were 99.7%, 99.0%, and 98.8%, respectively (Table 2). The two methods showed a great agreement with a kappa value of 0.81. When compared with the

TABLE 3. Comparison of *T. pallidum* IgG Ab in Clinical Serum Samples Detected by the Microplate CLIA and Abbott ARCHITEC I1000 Auto System

		Abbott		
		+	−	Total
Microplate CLIA	+	56	3	59
	−	2	291	293
	Total	58	294	352

TABLE 4. Samples With Different Results of *T. pallidum* IgG Ab Detected by Three Methods

Sample Serial No.	TPPA	Microplate CLIA (S/co)	Abbott (S/co)
23	+ (week)	− (0.14)	− (0.53)
25	–	+ (1.08)	− (0.57)
140	–	+ (4.96)	− (0.09)
142	–	+ (1.27)	− (0.09)
208	–	− (0.13)	+ (3.32)
351	–	− (0.20)	+ (1.17)

Abbott ARCHITEC I1000 Auto System ($n = 352$), the results showed that the specificity, sensitivity, and overall concordance for the microplate CLIA method were 98.6.0%, 96.6%, and 98.6% respectively (Table 3), and a high-degree agreement with the compared method was observed (kappa value = 0.95). A total of six samples, a rather low number, with different results by two of the three methods were summarized in Table 4. All these samples with discrepant results were negative or weak positive (S/co<5.0) for the *T. pallidum* IgG Ab detected by the three methods.

DISCUSSION

Recombinant *T. pallidum* antigens can be produced economically with large quantities in *E. coli* culture. Recombinant antigens have shown promise for syphilis serodiagnosis. A variety of *T. pallidum* proteins have been tested including TpN44.5 (TmpA, Tp0768), TpN15 (Tp0171), TpN17 (Tp0435), and TpN47 (Tp0574) (5–8). These antigens are sometimes used in combination in commercial tests. These tests have often been shown to identify individuals with active syphilis as well as those who have been treated successfully. From screening hundreds of serum samples, it has been determined that the sensitivity and specificity of tests employing some of these antigens can be as high as 99.7% (5–9).

In this report we have established a one-step microplate CLIA method for detection of IgG antibody to *T. pallidum* based on microplate coated with recombinant *T. pallidum* antigens, a combination of TpN15, TpN17, and TpN47, and HRP-labeled *T. pallidum* antigens, as well as a luminol/peroxidase system. The Chinese national reference substances panel for *T. pallidum* diagnosis was used to evaluate the method, as well as by comparing with the TPPA method and the Abbott ARCHITEC I1000 Auto System. Tests for accuracy, stability, interference, and cross-reactivity were also carried out, with the accuracy and the stability test results showing that the established microplate CLIA method met the requirement of the Chinese national reference substances standard for *T. pallidum* diagnosis (Table 1). The intraassay and interassay coefficients of variations for three lots of reagent were between 4.68% and 12.8%, meeting the requirements of the national reference standard for *T. pallidum* diagnosis. The microplate CLIA method showed no reaction with samples containing a serial of high concentration of interference substances including cholesterol, triglyceride, hemoglobin, and bilirubin. The reagents prepared for microplate CLIA method showed stability after being kept at 42°C for acceleration of degradation. Besides, antibodies to other ten pathogens, such as MV, rubella virus, *Toxoplasma gondii*, and so on, do not cross-react with *T. pallidum* antigens used in the microplate CLIA kit.

To evaluate the efficacy of the established microplate CLIA method for clinical application, we compare it with other two methods, TPPA and Abbott System, for detection of the IgG antibody against *T. pallidum* in clinical samples. When compared with the TPPA ($n = 1,052$), the specificity, sensitivity, and overall concordance for microplate CLIA were 99.7%, 99.0%, and 98.8%, respectively. The two methods showed a great agreement with a *kappa* value of 0.81. When compared with the Abbott ARCHITEC I1000 Auto System ($n = 352$), the results showed that the specificity, sensitivity, and overall concordance for the microplate CLIA were 98.6.0%, 96.6%, and 98.6% respectively, and a high-degree agreement with the compared method was observed (kappa value = 0.95). A total of six samples, a rather low number, with discrepant results by two of the three methods were negative or weak positive (S/co<5.0) for the *T. pallidum* IgG Ab detected by the three methods. Taken together, we demonstrated here that the recombinant *T. pallidum* antigens, a combination of TpN15, TpN17, and TpN47, have excellent sensitivity and specificity for syphilis serodiagnosis.

The CLIA method has been widely used for clinical diagnosis because they remit no radical waste products, cause no enzyme precipitation, and exhibit an excellent sensitivity (10–12). The CLIA method is known for sensitive, specific, and accuracy. Although the TPPA is usually cheaper, the microplate CLIA method can be easily automated and provides objective results while the TPPA test results are read subjectively and recorded manually

(11). Here we have succeeded in establishing a rapid, specific, sensitive, and stable CLIA method for detection of IgG Ab against *T. pallidum*, providing an efficient alternative to the treponemal tests and wide application in clinical laboratory.

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