

## Research Article

# Syphilis Diagnose Evaluation for Nested Real-Time PCR Method by Comparison with Serological Treponemal Antibody Testing

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## Abstract

**Objective:** To explore the diagnostic concordance between a novel nested real-time PCR (NR-PCR) in identifying *T. pallidum* DNA in various biosamples from syphilis patients and serological treponema antibody testing. **Method:** A total of 401 various biospecimens collected from 264 participants were tested for the presence of *T. pallidum* DNA by NR-PCR. Diagnostic concordance of NR-PCR was evaluated by comparison with serological testing. **Results:** The TP-DNA positive rate by NR-PCR for various syphilis stages ranged from 38.5% to 87.8%, and a significant difference was observed ( $\chi^2 = 29.80$ ,  $P = 0.000$ ), and the TP DNA positive rate for various sample types ranged from 50% to 92.0%, significant difference was observed too ( $\chi^2 = 37.2$ ,  $p < 0.001$ ). We observed 72.1% agreement (289/401), and kappa of 0.25 for NR-PCR by comparison with serological testing, syphilis diagnostics rate exists a significant difference between the two tests ( $\chi^2 = 44.62$ ,  $p < 0.01$ ), with a sensitivity of 70.9% (95%CI 66.0~75.3), specificity of 83.8% (95% CI 68.9~92.3), PPV of 98%, NPV of 22.6%, +LR of 4.4, and -LR of 0.35 for NR-PCR assay and with a sensitivity of 97.7% (95% CI 95.13~98.05), specificity 22.6% (95% CI 16.4~30.0), PPV 70.9% and NPV 83.8% for serology, and CSF was found to be TP DNA positive regardless of syphilis stages. **Conclusion:** It was concluded from the study that pol A NR-PCR assay in syphilis diagnosis revealed a fair agreement compared with serological testing, and NR-PCR can be used as an auxiliary procedure for neurosyphilis diagnosis.

**Keywords:** Syphilis; *T. pallidum*; Amplification; Molecular technique; PCR

## Introduction

Syphilis continues to be a public health problem in the world, especially in developing countries, it is noteworthy that syphilis started to increase in the 2000s in several Western European men and a large proportion of cases reported among men who have sex with men (MSM), particularly HIV-positive MSM [1], and congenital syphilis can still occur in high-income countries with a high rate of antenatal screening [2], and syphilis have been associated with increased HIV acquisition and transmission [3]. Early diagnosis of *T. Pallidum* infection is helpful to reduce pathogen transmission to sex partners. Conventional syphilis diagnostic tests can be divided into direct or indirect [4]. The direct methods include *T. pallidum* detection in fluids or tissues under dark field microscopy (DFM), as well as molecular biology techniques such as Polymerase Chain Reaction (PCR) [5]. *T. pallidum* PCR is more efficient in syphilis diagnosis based on ulcerative lesions such as indurated chancres and condyloma lata in early syphilis, which contains numerous *T. pallidum*. Indirect methods (serologic) consist of non-treponemal and treponemal tests [4], and both are important in diagnosing various stages of syphilis. Nevertheless, PCR is a preferable choice for the identification of *T. pallidum* infection when serology results are negative. Gayet-Ageron et al. conducted a meta-analysis, which indicated nested PCR (nPCR) is the preferred diagnostic approach over reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qPCR) for swabs of early syphilis [6]. Gama et al. investigated the higher sensitivity of qPCR in identifying *T. pallidum* ssp. *Pallidum* in paraffin-embedded skin biopsies by immunohistochemistry compared with conventional PCR and concluded that qPCR is a rapid and highly accurate method for detecting syphilis in tissue specimens [4]. Wang et al. described that the polA gene nPCR method can detect *T. pallidum* DNA in fluid tissue specimens and concluded that the detection levels in patients with primary and secondary syphilis were significantly higher than those with latent syphilis [7]. We developed NR-PCR assay as a universal detection for syphilis, which generated a 64.2% detection rate in whole blood and 92.0% detection rate in Cerebrospinal Fluid (CSF) [8], but concordance assessment of NR-PCR DNA using various biospecimens from syphilis patients compared with serological treponemal antibody testing in identifying *T. pallidum* infection is limited. The present study evaluates the sensitivity and specificity of novel NR-PCR in detecting *T. pallidum* DNA in various specimens of syphilis patients and determines the concordance of NR-PCR DNA-positive performance compared with serological treponemal antibody testing.

## Methods

### Participants and specimens

**Inclusion criteria for participants:** Patients with suspected syphilis after exclusion of HIV infection were enrolled after providing their informed consent as we described previously [8]. All procedures used in this study were approved by the medical ethics committee of the Guangzhou Institute of Dermatology (N20121A031001\_1).

### Syphilis diagnosis

Syphilis was diagnosed and treated according to syphilis management guidelines [9]. Patients confirmed as having syphilis were given a treatment course with benzylpenicillin 2,400,000 U, intramuscular injection, once a week three times, and the syphilis stage was determined by clinicians using the CDC case definitions [10].

### Specimen collection and preservation

All biospecimens were collected before treatment according to described procedures as we reported previously [8]. Briefly, tissue exudates were collected with two cotton swabs (Tianli, Co., Ltd. Jiangsu, China) from suspected early syphilis, and one swab was immediately placed in 1 ml of sterile PBS, and another one for DFM detection was transferred immediately onto a glass slide for *T. pallidum* examination within half an hour. Approximately 3 ml of whole blood was collected from each syphilis patient and placed in dry tubes. Then, 1 ml of the blood was divided into five 0.2 ml aliquots for DNA extraction, the remaining 2 ml of blood was centrifuged at 2500g at room temperature, and the supernatant (serum) was collected for treponema antibody testing. For latent syphilis, we collected 50-100  $\mu$ l earlobe peripheral blood with Mitsubishi needle and micropipettes, and the blood was added 0.9% NaCl up to 200  $\mu$ l. For suspected neurosyphilis patients who were without contraindications, 2 ml of Cerebrospinal Fluid (CSF) was collected. All samples were stored at -80°C until NR-PCR was performed. Negative controls from non-syphilis patients were also used in this study.

### Dark-field microscopy (DFM)

DFM at 1000 x magnification was performed to directly detect *T. pallidum* in swab samples. The detection of one typical motile organism by at least two independent experienced observers constituted a positive result.

### Serum treponemal antibody testing

We performed treponemal antibody and non-treponemal antibody testing with *T. pallidum* particle agglutination (TPPA) (Fuji Co Ltd., Guangzhou) and non-treponemal antibody with Rapid Plasma Regain (RPR) (Wantai, Guangzhou, China).

## DNA extraction

DNA in various biospecimens was extracted using a TIAN Amp Micro DNA Kit (TianGen Biotech Co., Ltd., Beijing, China), following the manufacturer's instructions as described previously [8]. DNA samples were stored at -80°C until amplification. Reference DNA was donated by Dr. Yin Yueping (National Venereology Reference Laboratory, Nanjing, China) and quantified by NanoDrop ND-1000 ultraviolet spectrophotometer (Thermo Scientific Co., Guangzhou, China).

## PCR primer design

**Primers and TaqMan Minor Groove Binder (MGB) probes:** All PCR assays were performed at the School of Public Health, Sun Yat-sen University, Guangzhou, China. For the NR-PCR assay, we designed two pairs of primers specific to the target *T. pallidum* gene, polA (partial coding sequence (CDS); GenBank Accession no. **TPU57757.1**). The sequence of primer pairs for PCR, MGB probe and NR-PCR was based on the literature [11, 12] and modified, a list of primers is shown in S1 (Table 1), All primer sequences were synthesized by Applied Biosystems (Guangzhou, China).

Groups	NR-PCR results		
	positive	negative	Positive rate (%)
Total samples (N=401)	264	137	65.8
Seronegative samples (N=30)	0	30	0%
Seropositive sample (N=364)	258	106	70.9
Serodiscrepant samples (N=7)	6	1	85.7
Total patients (N=264)			
Seropositive patients (n=227)	178	49	78.4
Serodiscrepant patient (N=7)	6	1	85.7
Seronegative patients (N=30)	0	0	0
NR-PCR Positive rate of various sample types			
Swabs	27	11	71.1
Serum	98	49	66.7
The whole blood	70	70	50.0
peripheral earlobe blood	23	1	92.0
Cerebrospinal fluid (CSF)	46	5	90.2

**Table 1:** NR-PCR results for various samples and patient in different serological test results161; Note : Chi-square test. Seropositive (e.i., both non-treponemal and treponemal test were positive), and serodiscrepant (e.i., either non-treponemal or treponemal serological test positive).

## NR-PCR assay

Samples were tested for the presence of treponemal DNA using Taq Man Master Mix (ABI Co., Ltd., Guangzhou, China) by classic PCR, outer primer pair F1/R1, and homemade NR-PCR protocol as described previously [8]. And for all specimens, we considered Ct values < 40 as positive for *T. pallidum* DNA. Ct values were calculated as previously described [13]. For details of PCR mixtures, and protocols see S1.

## Statistical analysis

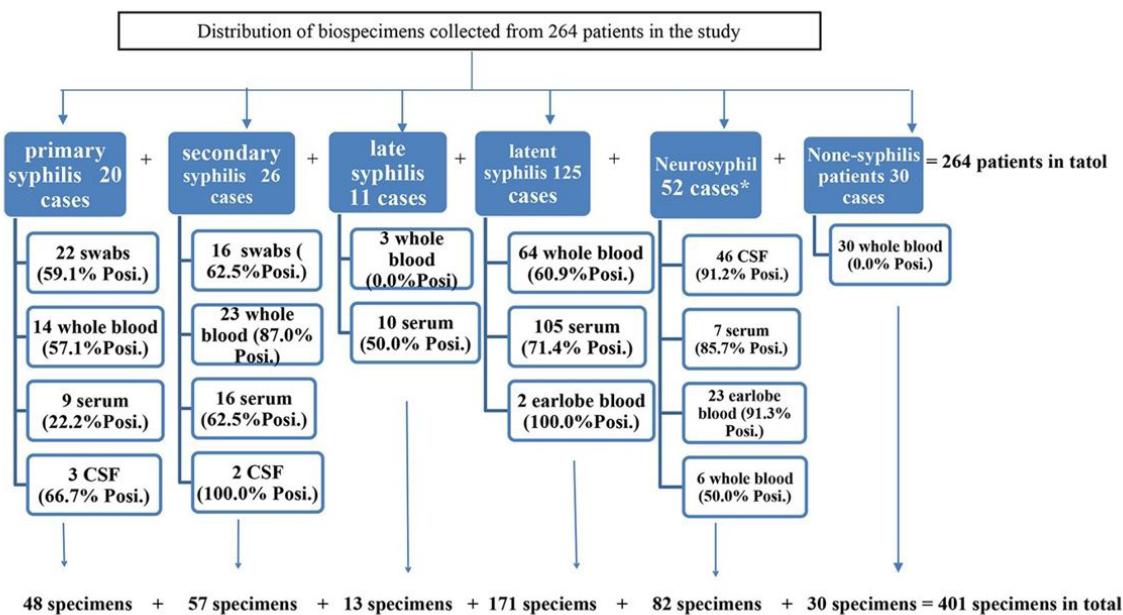
Data were analysed by SPSS 17.0 (SPSS, Chicago, IL, USA), and correlations between results of NR-PCR and serology were tested using the two-sided Fisher's exact test. Agreement between NR-PCR and serology was assessed by calculation of the kappa coefficient. Sensitivity, specificity and predictive values were determined individually for NR-PCR and serology. We considered differences with  $P < 0.05$  statistically significant.

## Results

### Features of participants and biospecimens

A total of 264 participants were recruited for the study, ages from 22 to 58 (median: 35), and All participants underwent both serum RPR and TPPA test, an HIV antibody test as well. Of 264

patients, 234 patients were considered as syphilis including 227 patients with seropositive and 7 patients with zero discrepant (i.e., RPR or TPPA was positive only, but DFM was positive) and 30 with syphilis seronegative were considered as non-syphilis. Of the 234 syphilis patients, 20 were primary syphilis presented with a single hard chancre, 26 were secondary syphilis, 11 were late syphilis, 125 were latent syphilis, and 52 were neurosyphilis. Of the 234 syphilis cases, 28 cases showed serum RPR titers within 0-4, 189 were within 8-128, and 17 were at 128 or higher. A total of 401 biospecimens were collected from 264 participants. Of the 401 biospecimens, 371 biospecimens were collected from syphilis patients and 30 from non-syphilis. Details of the distribution of various biospecimens, participants, and TP DNA positive rate (% in parentheses) for every specimen type are shown in (Figure 1).



**\*Note:** A total of 6 CSF samples were not included in the statistics due to poor custody.

**Figure 1:** Distribution of 401 biospecimens collected from 264 participants.

Serology and NR-PCR results on the set of collected samples and syphilis patients of the 401 biological samples, 364 samples were syphilis seropositive, 30 samples were seronegative, and 7 sample was zero discrepant. A total of 264 biological samples were TP DNA positive, and the overview TP DNA positive rate was 65.8% (264/401). The TP DNA positive rate for samples of seropositive, zero discrepant and seronegative is 70.9%, 85.7% and 0.0% respectively, and a significant difference in TP DNA positive rate was observed among the three seroprevalence groups ( $\chi^2 = 37.2$ ,  $p < 0.001$ ). In addition to late syphilis, all any other stages of syphilis have NR PCR positive findings in CSF. It suggested that NR-PCR can be used as an auxiliary procedure of neurosyphilis diagnosis and peripheral ear lobe blood PCR DNA positive rate is higher than that of both serum and the whole blood and is a convenient way to sample for syphilis diagnose. An overview of serological and NR-PCR results is shown in (Table 1).

**Concordance assessment of *T. pallidum* DNA in different biospecimens from the same syphilis patient assayed by NR-PCR**

Of the 234 syphilis patients, 184 patients were TP DNA positive; the positive rate was 78.63% (184/234). Of the 184 patients, the 64 patients were with parallel samples (including 28 patients with parallel swabs and the whole blood or serum, 23 patients with parallel cerebrospinal fluid and earlobe blood, and 13 with serum and the whole blood). And NR-PCR positive results of 64 patients with parallel samples are shown in (Table 2). NR-PCR positive rate exist no significant difference for blood samples when compared with swabs ( $p = 0.0548$ ), and for blood sample when compared to serum ( $p = 0.10$ ), however, NR-PCR positive rate exist significantly difference for earlobe blood when compared with CSF ( $P = 0.0023$ ).

Sample Types	Blood sample		Ear lobe blood samples		Total no. of patients
	<sup>a</sup> +(%)	-	<sup>b</sup> +(%)	-	
<b>Swabs</b>					
+	9 (56.3)	7	NA	NA	16
	2(20.0)	10	NA	NA	12
<b>CSF</b>					
+	NA	NA	19(95.0)	1	20
	NA	0	0	3	3
<b>Serum</b>					
+	6(75.0)	2	NA	NA	8
	1(25.0)	4	NA	NA	5
<b>Total no. of patients</b>	18(28.1)	23	19(29.7)	4	64

**Table 2:** NR-PCR results of 64 patients with parallel whole blood and swab sample or CSF and ear lobe blood (N=64) 174; Note: Fisher exactly test; NA= None available. %=<sup>a</sup> or <sup>b/c</sup>.

**Analysis for NR-PCR positive rate of samples from different RPR titer and different syphilis stages**

The NR-PCR positive rate of samples for primary syphilis is 52.1% (25/48), 80.7% (46/57) for secondary syphilis, and 38.5% (5/13) for late syphilis, and 87.8% (72/82) for neurosyphilis, and 67.8% (116/171) for latent syphilis, and significant difference of positive rate was observed among samples of various syphilis stages ( $\chi^2 = 29.80$ ,  $P = 0.000$ ), and so as that between any two syphilis stages (all  $p < 0.01$ ), nevertheless, no significant difference was observed for different sample types (i.e., swabs, serum, the whole blood and earlobe blood, and CSF) of each syphilis stage (all  $p > 0.05$ ) with exception of latent syphilis in which serum PCR positive rate (71.4%) is higher than that of the whole blood (62.12%,  $\chi^2 = 5.85$ ,  $P = 0.02$ ). The relationship between serum RPR titers and NR-PCR positive rate of the patient samples was also analyzed. Significant differences in positive rate were observed among the various RPR titration groups ( $\chi^2 = 54.5$ ,  $p < 0.01$ ), and so as that among swabs, blood and CSF for syphilis patients with RPR 1:4 and below ( $\chi^2 = 6.81$ ,  $p = 0.035$ ), in which positive rate of CSF was higher than that of other samples ( $\chi^2 = 4.73$ ,  $p = 0.03$ ). and so, like that for samples of RPR from 1:8 to 1:64 ( $\chi^2 = 19.63$ ,  $p = 0.001$ ) in which CSF NR-PCR positive rate is higher than that of blood samples ( $\chi^2 = 9.86$ ,  $p = 0.0017$ ), but no significant difference of positive rate was observed among various samples types of patients with RPR equal to and more than 1:128 ( $\chi^2 = 1.20$ ,  $p = 0.75$ ). The NR-PCR positive rate analysis among different specimens of various syphilis stages and various RPR titration groups is shown in (Table 3).

Syphilis stage	a No. of samples	b N. of PCR Posi (%†)	N. of NR-PCR Posi. (%‡)				
			c Swabs	d Serum	e Whole blood	f Ear lobe	g CSF
Primary syphilis	48	25(52.1)	13(59.1)	2(22.2)	8(57.1)	-	2(66.7)
Secondary syphilis	57	46(80.7)	14(87.5)	10(62.5)	20(87.0)	-	2(100.0)
Late syphilis	13	5(38.5)	-	5(50.0)	0(0.0)	-	-
Neurosyphilis	82	72(87.8)	-	6(85.7)	3(50.0)	21(91.3)	42(91.3)
Latent syphilis	171	116(67.8)	-	75(71.4)	39(60.9)	2(100.0)	
Non-syphilis	30	0(0.0)	-	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
Subtotal	401	264(65.8%)	-	-	-	-	-
RPR titration groups							
RPR negative	37	6(16.2)	3(100.0)	0	2(6.25)	-	1(100.0)
≤ 1:4	36	22(61.1)	1(20.0)	2(40.0)	2(40.0)	6(75.0)	11(84.6)
1:8~1:64	300	209(69.7)	23(76.7)	84(65.6)	56(60.2)	16(100.0)	30(90.9)
≥ 1:128	28	27(96.4)	-	12(92.3)	10(100.0)	1(100.0)	4(100.0)
Subtotal	401	264(65.8)					

**Table 3:** The positive rate of NR-PCR in different biological samples of various syphilis stages and RPR titration (N=401); Note : Chi-Square test or Fisher's exactly test; %†=b/a\*100% ; %‡=c/total samples of the type which is shown in Fig.1, b=c + d + e + f + g “-” means no data.

#### Assessment of diagnostic concordance of NR-PCR by comparison with serological antibody testing

Of the 401 biospecimens, the concordance in syphilis diagnosis between positive NR-PCR assay and treponemal serological testing was analyzed. it suggested a 72.1% (289/401) agreement, kappa of 0.25, with a sensitivity of 70.9% (258/364, 95%CI 66.0-75.3), specificity of 83.8% (31/37, 95%CI 68.9-92.3), PPV of 98% (258/264), NPV of 22.6% (31/137), +LR of 4.4, and -LR of 0.35 for NR-PCR assay. for the subsample set without zero discrepant results, the agreement was 71.8%, with a specificity of 100%, and a kappa of 0.24. And with a sensitivity of 97.7% (95% CI 95.13-98.05), specificity 22.6% (95% CI 16.4-30.0), PPV 70.9% and NPV 83.8% for serology. Syphilis diagnostics rate exists a significant difference between the two tests ( $\chi^2 = 54.5$ ,  $p < 0.01$ ) (Table 4).

NR-PCR	Serological test			Kappa	OR (95%CI)	PPV (%)	NPV (%)	Sensi. (95%CI)	Speci. (95%CI%)
	+	-	subtotal						
+	258	6	264						
-	106	31	137	0.25	12.5(5.1-31.0)	70.9	86.5	70.9(66.0-75.3)	83.8(68.9-92.3)
Total	364	37*	401						

**Table 4:** Analysis of the concordance between serological and NR-PCR tests for syphilis diagnosis (N=401); Note : Chi-square test.  $\chi^2=54.5$ ,  $p < 0.01$ , 95%CI: Wilson score confidence interval; \*including 7 patients with serodiscrepant.

#### Discussion

In the present study, a total of 264 patients, 227 were seropositive syphilis patients with a 78.4% NR-PCR positive rate, and 37 patients were seronegative with and 16.2% positive rate. Of the 227 patients, the overview NR-PCR positive rate for all the 364 specimens was 70.9% (64 patients had more than one sample). The NR-PCR positive rate of various syphilis stages ranged from

38.5% to 87.8%, and the late syphilis specimen had the lowest (38.5%), and neurosyphilis had the highest positive rate (87.8%). The NR-PCR positive rate of various sample types ranged from 50% to 92.0%, and the whole blood had the lowest (50.0%) and peripheral earlobe blood had the highest (92.0%) in addition, CSF was observed to be TP DNA positive in every syphilis stage, this implied that treatment for central nervous system syphilis infection should be considered simultaneously during the management of early syphilis.

Sutton's group was the first to describe the presence of *T. pallidum* DNA in genital ulcer swabs or whole blood specimens from syphilis patients [12]. Several studies have reported the application of PCR methods in syphilis of all stages, which showed that the DNA-positive rate varies with biosamples [7,15-17]. NR-PCR assay is sensitive to a minimum of 2 *T. pallidum* strain Nichols cells/ml with primers and probes specific to the polA gene [13], and generated positive results ranging from 64.2% to 92% in various biospecimens of syphilis patients [8] and 71.24% in samples of pre-treatment for all stages of syphilis [18], these results are comparable to that reported by Wang's et al, but TP DNA positive rate of 67.8% in latent syphilis in the present study was much higher than 7.4% reported by Wang et al. [7] with nested PCR procedure. Peng et al. reported sensitivity and specificity of the PCR assay for early syphilis are 83.3% and 92.9% respectively [16], Gayet-Ageron's group assessed the application of RT-PCR in diagnosing syphilis from diverse biospecimens, the global sensitivity of *T. pallidum* by PCR was 65% for primary syphilis, 53% for secondary syphilis, thus, it can be concluded that syphilis PCR provides better sensitivity in lesion swabs from primary syphilis and displays only moderate sensitivity in blood from primary and secondary syphilis [15]. in the present study, NR-PCR had comparable sensitivity (59% for swabs) for primary syphilis and higher sensitivity (80.7%) for secondary syphilis.

Concerning the two tests in the study, sensitivity, specificity, and positive and negative predictive values were 70.9 (95% CI 66.0 - 75.3), 83.8% (95% CI 68.9-92.3), 97.7%, 22.6% respectively for NR-PCR. The sensitivity and positive predictive value of NR-PCR are comparable with the results reported by Vrbova et al. [19], but with lower specificity and negative predictive value of NR-PCR, and higher sensitivity and negative predictive value of serology.

As we know, several studies previously reported syphilis diagnosing agreement for nested PCR and serological testing for early syphilis. Grange's group found no agreement between *T. pallidum* in blood using nested PCR and syphilis serological diagnosis, with a sensitivity of only 29% for peripheral blood mononuclear cells and 14.7% for a serum for PCR [17], Leslie's group reported polA gene-based TaqMan PCR assay, compared with serology, showed 95% agreement, with 80.39% of sensitivity,

and 98.40% of specificity [11]. This is the first study to evaluate the agreement for both NR-PCR and serological tests for all syphilis stages. The overview TP DNA positive rate was 65.8%, with 72.1% agreement, kappa of 0.25. Though the agreement rate is higher than that reported by Vrbova et al. [19], lower than that reported by Leslie [11], in which PCR showed 95% agreement by comparison with serological testing. This difference may due to differences in both sample composition and stages of syphilis, in this study, 55% of patients were with latent syphilis and 42% of specimens were collected from latent patients, the disequilibrium of the sample is partly attributed to syphilis screening increasing year by year in Guangzhou, China [20], and nearly 90% of the reported syphilis cases were latent syphilis in recent years, rather than the risk population in the STI clinic described in Leslie's study [11]. In a word, the fair kappa value of the study indicated that NP-PCR assay and serological test are both clinically needed as reported by else [20]. And should be independently used for the diagnosis of *T. pallidum* infection in the clinic, and NR-PCR has deserved priority recommendation especially in diagnosing neurosyphilis and detecting TP DNA in peripheral earlobe blood and CSF specimens.

There were some limitations in the study. More than two-thirds of the sample were from latent syphilis, but None of the CSF specimens, such imbalance as relatively few samples of swabs and earlobes blood may lead to result bias, and thus interpretation should be with caution, However, NR-PCR is still a useful diagnostic tool in diagnosing latent syphilis, neurosyphilis, and *T. pallidum* early infection, especially when serological results are negative.

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## Contributors

Zhang Y drafted the manuscript. Ye XD and Hu BJ conceptualized the research design and revised the draft. Dai XN, Shen XC, Xie ZM and Li QQ analyzed the data and Cao WL handled samples. Ren ZF completed all of the experiments, and all of the authors were involved in revising the manuscript and approving the final version.

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## Institutional Review Board Statement

All procedures used in this study were approved by the medical ethics committee of the Guangzhou Institute of Dermatology (N20121A031001\_1).

## Informed Consent Statement

“Written informed consent has been obtained from the patient(s) to publish this paper” if applicable.

## Conflicts of Interest

“The authors declare no conflict of interest.” “The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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