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PCR detection for syphilis diagnosis: Status and prospects

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Syphilis, a re-emerging public health problem worldwide caused by *Treponema pallidum subsp pallidum (T. pallidum)*, usually induces systemic and chronic inflammation in hosts who do not receive timely therapy after exposing to high-risk factors such as leprous sexual contact. Before the treatment, rapid and accurate detection of syphilis is essential. However, the existing detection methods, which focus on the treponemal or non-treponemal antibody test, both have inherent limitations. For instance, both of them cannot distinguish the stage and severity of syphilis. Non-treponemal test such as RPR, which is generally deemed to be used for assessing treatment response, is influenced by biological false positives. Therefore, it is imperative to seek out a new and effective diagnostic test. With recent advancements in molecular biology and whole-genome sequencing, the molecular diagnosis has increased in popularity, especially the use of polymerase chain reaction (PCR). Here, we firstly present a mini-review on the research of PCR detection methods used for syphilis diagnosis over the past decade, and we then compare these methodologies to assess their potential and the challenges faced. This information can provide a fresh perspective to help researchers address the current challenges.

KEYWORDS
PCR, syphilis diagnosis, *Treponema pallidum*

1 | INTRODUCTION

1.1 | Conventional detection of syphilis

The syphilis spirochete *Treponema pallidum* is well known to be a "great imitator"[1] for mimicking dermatosis lesions and as a "stealth pathogen"[2] for circumventing the immune system easily and disseminating into the blood quickly after initial infection without any specific symptoms, especially during co-infection with human immunodeficiency virus (HIV).

It has been reported[3] that the acquisition and transmission of HIV infection would be facilitated by primary syphilis since they have the same susceptible population, men who have sex with men (MSM), and the same route of infection, which often leads to a vicious cycle between aggravation of the patients' condition and dissemination of the pathogen. The natural course of syphilis composed of primary, secondary, and latent stages, usually last for decades. People infected with *T. pallidum* will gradually deteriorate if they do not receive diagnosis and treatment in time, and eventually may

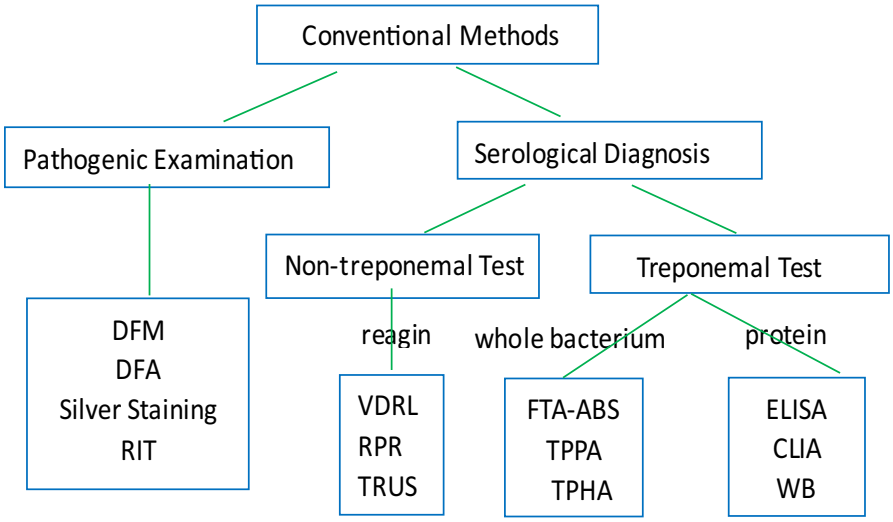

FIGURE 1 Summary of diverse conventional methods in syphilis. CLIA, chemiluminescent immunoassay; DFA test, direct fluorescence antibody test; DFM test, dark-field microscopy test; ELISA, enzyme-linked immunosorbent assay; FTA-ABS, fluorescent treponemal antibody absorption test; RIT, rabbit infectivity test; RPR, rapid plasma reagin; TPHA, *Treponema* pallidum hemagglutination; TPPA, *Treponema* pallidum particle agglutination; TRUST, tolulized red unheated serum test; VDRL test, venereal disease research laboratory test; WB, Western blotting

have severe complications, such as cardiovascular and late neurological syphilis[4] which cause severe psychological and physical impairment. In addition, a WHO survey[5] revealed that cases of syphilis, along with other sexually transmitted diseases, such as *Chlamydia*, *Neisseria gonorrhoeae*, and *trichomonas vaginalis*, are increasing globally, with one million individuals newly infected with one or more of these illnesses every day. Hence, effective and rapid detection of *T. pallidum* is pivotal in preventing propagation.

Conventional diagnostic methods[6] consist of pathogenic detection and serological test. The former directly detects *T. pallidum* and includes dark-field microscopy (DFM), silver staining, direct fluorescence immunoassay (DFA), and the rabbit infectivity test. They have been recognized as the gold standard in syphilis diagnosis. However, *T. pallidum* still encounter difficulties in extracorporal cultivation even though a new article[7] expounded *T. pallidum* can be co-incubated in rabbit epithelial cell for 180 days; the sensitivity of rabbit infectivity test in current post-antibiotic era is no longer highly sensitive,[8] and high false-positive or false-negative result of microscopy test usually occurs when laboratory workers lack experiences to distinguish *T. pallidum* from commensal *treponemas*. All these factors make pathogenic detection arduous in extensive clinical application. The latter methods include non-treponemal and treponemal tests, which are used for preliminary screening and diagnosis confirmation, respectively. Although non-treponemal tests are economical and practical, cross-reaction occurs frequently in case of pregnancy, malignancy, and autoimmune diseases such as rheumatoid arthritis since reagin antibody is not very specific in early syphilis. Treponemal tests are costly, time-consuming, and technically difficult to perform because this bacterium is strictly endobiotic.[6,9] Furthermore, both pathogenic detection and serological tests have drawbacks. On the one hand, they cannot distinguish the stage and severity of syphilis; on the other, non-treponemal test such as RPR, which is generally deemed to be used for assessing treatment response, cannot afford accurate information about the curative effect to doctors. Figure 1 is a summary of the traditional tests used for syphilis diagnosis.

In the past decades, researchers have proposed constructive solutions, such as reverse algorithm and neotype recombinant antigen,[10] to overcome the flaws as well as improve the accuracy and sensitivity of these diagnostic methods. Indeed, these tactics have achieved the purpose to some degree but still have limitation.[11] PCR, a crucial technique in molecular diagnosis, has been suggested to be a useful supplement in diagnosing early-stage syphilis, particularly in those with evident erythema.[12] Some researchers[13] postulated that PCR would increase the rate of detection of syphilis in patients whose symptoms were generally masked by acquired immune deficiency syndrome (AIDS). We now provide a summary of the research status of some PCR method.

1.2 | Status of PCR frequently used in diagnosing syphilis

Since its invention by American scientist Mullis et al[14] in 1985, PCR technology has played a very important role in many fields, especially in diagnostic procedures. With the introduction of whole-genome sequencing[15] of *T. pallidum* and the continuing development of molecular biology, an increasing number of PCR methodologies have been promoted to adapt to the vast clinical and laboratory requirements and to overcome the limitations of routine PCR. Although the type of PCR is various, here we just select several tests including routine PCR, nested PCR, real-time PCR, and multiplex PCR to talk mainly for they are the ones with the fastest development, widest application, and deepest exploration over the past decade. The status of each test can be found in Table 1.

Matt Shields' study[16] showed that the sensitivity of routine PCR ranged from 84.6% to 89.1%, and the specificity ranged from 93.1% to 100% for primary syphilis, while in the secondary stage of disease, the sensitivity declined to 50%. This result implied that routine PCR should be used only for early-stage syphilis, which was supported by Gayet-Ageron et al[17] However, an other research[18] showed that routine PCR could also be used effectively for secondary syphilis, obtaining a sensitivity and specificity that reached 81.1% and 100%,

TABLE 1 The research status of frequently used PCR methods for syphilis diagnosis

Type of PCR	Syphilis stage	Patient size	Specimen type	Primer target	Sensitivity (%)	Specificity (%)	Reference no.	Published (y)	Remarks
routine PCR	Early (55)	288	Swab	Tpp47	89.1	99.1	[16]	2010	Useful for early syphilis without serological response
	Secondary (22)				50	100			
routine PCR	Early (170)	273	Ulcer	——	87.0	93.1	[17]	2015	Just compared with DFM
routine PCR	——	124	Cerebrospinal fluid	Tpp47	75.8	86.8	[41]	2016	47-PCR, high sensitivity, polA-PCR, high specificity
				polA	69.7	92.3			
routine PCR	Primary (13)	149	Skin lesion	——	84.6	100	[18]	2018	Also useful at secondary stage
	Secondary (33)				81.8	100			
Nested PCR	Primary (87)	329	Swab	Tpp47	82 (swab)	95 (swab)	[21]	2012	Swab specimens from lesion are more useful
	Secondary (103)		Blood		≈21 (blood)	≈94 (blood)			
	Latent (40)								
Nested PCR	Primary (195)	315	Blood	Tpp47	90.3	100	[22]	2013	Nested PCR to improve the specificity
Nested PCR	Neurosyphilis	40	Cerebrospinal fluid	Tpp47	42.5	97	[19]	2016	Low sensitivity
Nested PCR	Primary (84)	262	Whole blood	Tpp47	53.6	——	[20]	2018	No discrepancy between the two target genes (P = 1.0)
	Secondary (97)			polA	62.9				
	Latent (81)				7.4				
Type of PCR	Syphilis stage	Patient size	Specimen type	Primer target	Sensitivity (%)	Specificity(%)	Reference No.	Published (yr)	Remarks
Real-time PCR	Primary (716)	849	Ulcer or skin	polA	87.0 (compared with DFM)	93.1	[42]	2010	Efficient only in primary syphilis
	Secondary (133)				72.8 (compared with STI)	95.5			
					75 (compared with serology)	97.0			
Real-time PCR	Optic neuritis (1)	5	Aqueous humor	Pol 1	3 (only positive in retinitis)	——	[23]	2011	Samples limited
	Chorioretinitis (1)								
	Retinitis (3)								

(*Continues*)

TABLE 1 (Continued)

Type of PCR	Syphilis stage	Patient size	Specimen type	Primer target	Sensitivity (%)	Specificity (%)	Reference no.	Published (y)	Remarks
Real-time PCR	Early infection	99	Ulcer	Tpp47	100	97.14	[43]	2011	Higher sensitivity of blood in secondary phase
			Blood		34.1	100			
Real-time PCR	HIV(+) with early syphilis	122	Cerebrospinal fluid	polA, Tpp47 bmp	58	67	[44]	2013	Limited utility in neurosyphilis
Real-time PCR	Secondary	48	Urine	polA	16	——	[24]	2015	Urine considered to be useless here
Multiplex real-time PCR	Primary	15	Swab	Tpp47 TaqMan-LNA probe	100	100	[25]	2010	More sensitive than serology and DFM
Multiplex real-time PCR	Co-infection with HCV, HIV, and HBV	328	Blood	——	82.6	——	[26]	2015	Large-scale screening for co-infection
				TaqMan-LNAprobe					
Type of PCR	Syphilis stage	Patient size	Specimen type	Primer target	Sensitivity (%)	Specificity (%)	Reference No.	Published (yr)	Remarks
Multiplex real-time PCR	——	17	Paraffin-embedded biopsies	polA TaqMan probe	100	100	[27]	2017	qPCR is more sensitive than routine PCR
Multiplex PCR	Seven sexually transmitted diseases	76	Semen	Different for each organism	100	100	[28]	2014	Compared to single PCR
Multiplex PCR	Nine sexually transmitted diseases	295	Urine (146)	Different for each organism	98	97	[29]	2016	Just compared to FilmArray
			Swab (149)						
LAMP	Secondary	642	Peripheral blood	Bmp	82.1	100	[30]	2017	Compared to PCR

TABLE 2 Factors that might influence the PCR results and how we could approach them

Factor	Component	Comparative analysis result	Personal proposals
Syphilis stage	Primary Secondary Latent period Tertiary	PCR is more sensitive in the primary stage than the secondary stage, especially when with ulcers.	Using PCR diagnosis sooner rather than later
Specimen type	Ulcer, tissue sample, whole blood, serum, plasma, cerebrospinal fluid, urine, semen, vitreous humor	The best specimens are ulcer and tissue. Serum is better than whole blood. Other non-invasive samples' value needs to be improved.	Ulcer secretion acquisition is hard because of antibiotic abuse. Therefore, improving the DNA concentration of blood products and urine may be a solution.
Target gene	polA Tpp47 bmp tpr arp	polA and Tpp47 are mostly used, and they show no distinction in sensitivity or specificity.[45]	Search for new target genes that can improve the sensitivity as well as specificity.
DNA extraction	Physical methods Chemical methods	No comparison exists, but use of a chemical extraction kit is common.	Larger tissue with lower elution buffer to extract more DNA and reduce contamination and degradation during the whole process.
PCR type	Routine PCR Nested PCR Real-time PCR Multiplex real-time PCR Multiplex PCR LAMP	According to related references, routine PCR, multiplex PCR, and LAMP might be suitable for screening because of their high sensitivity, while the rest might be more appropriate for confirmation.	Choose one or more type of PCR based on the intention of the examination and the state of illness, but the patient should be tested with other methods such as CLIA or TPPA.
Co-infected with other pathogens	HIV Chlamydia Haemophilus ducreyi Trichomonas vaginalis	The results are not affected by other causative agents in early stages even though syphilis symptoms might be masked.	Use multiplex and single PCR as screening tools; the suspected cases should be examined by other confirmation methods.
Set criteria	Reaction system Temperature Circle	Changing the reaction condition may turn a negative result to a positive one even in the same specimen.	Use an optimal temperature and reaction system according to the specimen and PCR type.
Others	Technician Equipment Statistical analysis	No related data.	Train technicians and maintain equipment diligently; a skilled operator and a precise machine can enhance detection rate.

STD, sexually transmitted disease.

respectively. Additionally, routine PCR has been reported to detect atypical cases in tonsillar, vertebral, and ocular syphilis. It is widely accepted that routine PCR could be an effective supplement for early diagnosis, especially suitable for the clinical sample cases, such as chancre secretions.

Nested PCR is both more specific and sensitive, relative to routine or single PCR with the use of a probe, which can improve the accuracy of amplification products. The specificity of nested PCR reaches 95%, while the sensitivity is lower at 70%.[19-22] Indeed, Wang Guini et al[20] recently showed that sensitivity of nested PCR can be superior, especially in the early or infectious stage of syphilis, and they also reported that the *T. pallidum* DNA load correlates with the RPR titers. These discoveries might indicate that nested PCR is a useful tool for early diagnosis and prognosis in syphilis; however, the applicability of PCR still needs further study since the sample sizes in their study were limited.

Real-time PCR is well known for its ability to calculate the quantity of pathogens according to a standard curve, which is used to estimate the severity of the infection. Admittedly it is difficult to truly calculate bacterium DNA load to some extent since the accuracy depends more on the type of sample and DNA extraction protocol. But real-time PCR is still studied widely because it is a fast and easy format to perform in the laboratory. In this review, five studies about real-time PCR for syphilis diagnosis are mentioned, in which two of the studies have small sample sizes and use non-invasive specimens, such as the aqueous humor[23] or urine[24] The sensitivity of these non-invasive specimens is lower than that of ulcer secretions or skin, which means that they are not appropriate for screening.

Multiplex real-time PCR is more convenient than single real-time PCR because it can simultaneously detect several pathogens and assess their quantities without interfering with each other, and it is thus often applied in patients who are suspected of having co-infection, especially in areas where multiple indistinguishable diseases are epidemic and higher diagnostic standards are needed

such as blood donation. This approach greatly reduces the costs and time of diagnostic testing since it can simultaneously monitor and separate several amplifications in a single well according to their different fluorophore. Laura et al[25] conducted a test mainly for herpes simplex virus 1/2 (HSV1/2) and unexpectedly identified 15 cases of syphilis. These patients all underwent serological testing and DFM as soon as the results were confirmed by PCR, but the sensitivity of these methods is lower than PCR, at only 83.3% and 50%, respectively. However, the negative serum results turned positive a few days later. This indicates that multiplex real-time PCR is more sensitive than serological testing in primary syphilis and might be a valid screening method; this finding is consistent with that of researches.[26,27]

In areas where multiple infections are epidemic, we may simply want to know whether the population is infected or not. Multiplex PCR is a clear choice to meet this need, as it is a rapid and accurate method that can be used to simultaneously screen for multiple sexually transmitted agents. Two scientific research teams[28,29] thoroughly investigated ways to improve the detection rate of several diseases concurrently. The results show that multiplex PCR has a favorable consistency with single PCR or FilmArray; thus, multiplex PCR is a complementary point-of-care choice in the future but still needs further research and practice.

The main difference between loop-mediated isothermal amplification (LAMP) and the aforementioned PCRs may be homothermal (60-65°C) amplification, which can simplify the performance process and enhance the amplificaCtion efficiency. The LAMP can be widely generalized in a low-income area where advanced medical facilities are lacking because of its simplicity, rapidity, and low cost. As a matter of fact, it has been reported that similar methods can detect many pathogens such as *Plasmodium* and *Mycobacterium ulcerans* which are epidemic in developing regions with high sensitivity and specificity, even when DNA concentration is negligible. However, it has not been widely used in clinical and laboratory settings for the diagnosis of syphilis, since it is a new type of technology that appeared after the new millennium. There is only one article, written by Xiao Yongjian et al[30] that investigates LAMP and syphilis, and the findings indicate that LAMP has better sensitivity and specificity in peripheral blood in secondary syphilis.

2 | DISCUSSION AND PROSPECTS

Sensitivity and specificity are inevitably referred to when discussing the efficacy of diagnostic methods, as we did for PCR. What influences the sensitivity and specificity of PCR and how can we improve these values? Some researchers have given us useful guidance. For instance, some scholars[31] emphasized the importance of primer selection, while some researchers[32] focused on the type of specimen. SM Bruisten[9] systematically stated how to combine all parameters optimally and showed a very useful guideline about PCR procedure. Marios Arvanitis et al[33] compiled a table that comprehensively summarized which elements affected the PCR diagnosis of fungal disease. The various diagnosis values of each type of PCR are presented in Table 1 clearly, and the reasons for these discrepancies may be related to syphilis stage, specimen type, DNA purity, and other factors. Table 2 includes a personal explanation and assessment of factors that might interfere with the efficiency of PCR according to the related references mentioned above. This is the first time that this type of comparison of syphilis diagnostic techniques has been presented.

Despite the diagnostic applications of PCR described, PCR can still be applied to perform other functions. In fact, it has also been used in the vaccine and bacterial resistance fields. For example, Lithgow Karen et al[34,35] utilized real-time PCR to detect the quantity of *T. pallidum* in New Zealand rabbits, and this method provided the precise immune effect of recombinational proteins Tp0751 and FlaB3. Although *T. pallidum* has been sensitive to penicillin since it first appeared, we should continue to be vigilant at drug-resistant strains because of antibiotic abuse and gene mutations. It has been reported that point mutations of A2058G and A2059G in 23SrRNA[36] might be a cause of azithromycin resistance, and these mutations are usually detected by real-time PCR.

Similar to the idiom stating where there is light there is a shadow, we acknowledge that there are still many obstacles to overcome. The sensitivity of PCR decreases among individuals in the secondary and latent stages, which may be the primary limitation that has caused some researchers to think that PCR is unnecessary since it adds little value over serology detection.[37] We disagree with this line of thinking because the samples have mainly been from the second stage when the serum antibody is higher than the *T. pallidum* DNA loading. Besides, the optimal samples for PCR detection are ulcer secretion whose acquirement has a little bit difficulty in the current post-antibiotic era and specimen obtained easily, such as whole blood, serum, urine, can hardly reach the clinical requirements.

As Winston Churchill's description "difficulties mastered are opportunities," what we should do at this moment is to seek solutions rather than denying the application value of PCR. Fortunately, progress never stops. Technology research and development of PCR emerges in endlessly, which aims at promoting efficiency by labeling different targets[38] or simplifying operation by using novel probes.[39] Moreover, a specimen urine has been considered as useless,[24] but now has been demonstrated to contain an uncovered candidate biomarkers of *T. pallidum*,[40] as stated, "garbage is the wealth of been misplaced." What's more, PCR has been verified it is helpful in the early phase by many researchers above-mentioned and the LAMP has also demonstrated capability in detecting *T. pallidum* in peripheral blood. Therefore, we prefer to think that PCR detection is a very potential test for syphilis diagnosis still needs improvement. We speculate that the relatively low sensitivity in the secondary and latent stages may be because of the following: (a) An excess of *T. pallidum* quickly diffuses to body organs to avoid the immune response rather than staying in the blood; (b) a large number of antibodies may indirectly kill most pathogens, reducing the pathogen quantity; (c) the optimal target gene for *T. pallidum* has still not been identified; and (d) the DNA extracted from blood or serum may be compromised because the components of blood are

complex, leading to inhibition of the *T. pallidum* PCR by iron ions. Therefore, rather than discarding PCR as a diagnostic tool for syphilis, we suggest further development and promotion of its use, which cannot be accomplished without the endeavors and teamwork of all researchers, and we outline several suggestions in Table 2.

3 | CONCLUSION

Based on the literature, we believe that routine PCR and multiplex PCR can be used as supplemental methods for the screening of syphilis, especially in the early stage when the serological reaction is negative, while nested PCR and real-time PCR are more appropriate for confirmation. However, all types of PCR diagnostic methods for syphilis require further development and improvement to enhance their sensitivity and specificity. Ulcer secretions are the best samples to obtain the DNA of this pathogen; however, their widespread use is restricted by asymptomatic cases caused by antibiotic abuse. The challenge we are now facing is how to improve the purity of DNA acquired from peripheral blood or other tissues, especially from most convenient samples such as peripheral blood and urine. This review aimed to summarize the research status of different PCR methods used for syphilis diagnosis and the current challenges associated with them. These pieces of information presented can provide researchers with a fresh perspective to overcome the current limitations. We believe that with scientific and technological progress and persistent cooperation, we can make PCR testing for syphilis more effective and practical.

CONFLICT OF INTEREST

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS

Chenglong Zhou and Xiaohong Zhang wrote the article; Tie Zhao and Junxia Duana designed and arranged the tables; Feijun Zhao modified and organized the article.

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