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Review Article

Molecular Diagnostic Tools for *Treponema pallidum*

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ABSTRACT

Syphilis, a common sexually transmitted disease, is caused by *Treponema pallidum subsp. pallidum*. Owing to the chameleonic behavior of syphilis, ocular involvement still presents a therapeutic problem. Direct detection of *Treponema pallidum* in the vitreous offers a potential diagnostic method because serodiagnosis has considerable limitations. The worldwide identification of *T. pallidum* substypes has occurred since the advent of molecular typing approaches. The purpose of this article is to provide more information on the development of a molecular approach for *Treponema pallidum* detection. A body of literature was gathered using automated database searches in Google Scholar, PubMed, and ScienceDirect. Although prior studies have focused on other genes, such as polA, 16S RNA, and the whole genome, there are still some that use the study of the arp and *T. pallidum* repeat (tpr) genes to subtype. Whole blood, vaginal ulcers, skin biopsies, and other samples can be used in molecular methods. Comparing quantitative reverse trascription-polymerase chain reaction (qRT-PCR) to traditional methods, such as reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody (IFA) assay, and virus isolation, qRT-PCR has the advantage of being faster and more sensitive. Quick molecular methods, particularly polymerase chain reaction (PCR) results, will enable early detection of primary, secondary, and latent syphilis, which will lead to prompt treatment and prevention of disease progression as well as a reduction in the amount of time that the patient's sexual partners are exposed to the illness.

Keywords: *Treponema pallidum*, syphilis, detection tool, molecular method, and gene.

Highlights: This review describes the development of molecular methods for the detection of *Treponema pallidum* with various target genes. The benefit of this study is that it can be used as a reference for developing molecular methods for future research on *Treponema pallidum* detection.

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INTRODUCTION

Syphilis is a condition caused by infection with *Treponema pallidum subsp. pallidum* (*T. pallidum*) which cannot be grown in a dish. Multistage sexual transmission is still a public health issue, with an estimated 12 million new cases annually.[1] Syphilis first appeared in the 15th century, and by the 20th century, its frequency had decreased. However, the predominance has risen once more during the past few decades in America, Europe, and Asia.[2] Syphilis and an elevated risk of Human Immunodeficiency Virus (HIV) transmission in homosexuals have been significantly correlated in Makassar, Indonesia. Syphilis should be regarded as a major risk factor due to the increased prevalence of syphilis and HIV double infection among homosexuals, which can promote the spread of both illnesses.[3]
Treponema pallidum is one of the small main bacterial infections of humans that cannot be cultured on synthetic medium, making the diagnosis of syphilis challenging.[4] *Treponema pallidum* may currently only be detected in clinical samples using technologies that are either insensitive, such as dark-field microscopy.[5] The cornerstones of laboratory diagnosis for *T. pallidum* are serologic testing. However, for some forms of disease, they are either too sensitive, specific, or both.

Polymerase chain reaction (PCR) can selectively amplify the copy number of a target gene by more than 106-fold. The use of PCR to identify numerous fastidious or slowly proliferating bacterial pathogens has already been demonstrated, and has the potential to significantly improve our ability to identify infectious illnesses caused by these microbes. Therefore, the goal of this study is to learn more about how the molecular method for *T. pallidum* detection has been developed.

MATERIALS AND METHODS

Using automated database searches in Google Scholar, PubMed, and ScienceDirect, a body of literature was gathered. The goal was to compile data on genetic diagnostics for *T. pallidum* that had been published in the last 22 years, from 2001 to the most recent year. This is because there has not been much research on *T. pallidum* molecular diagnostics. An extensive literature search was conducted based on this idea, and references to pertinent publications were examined.

RESULTS AND DISCUSSION

Treponema pallidum linier gene

In addition, 3 *T. pallidum* repeat (*tpr)* genes had many several single nucleotide polymorphisms (SNPs) and single nucleotide indels that caused frameshift gene alterations, and eight genes had tiny indels and SNPs.[6] In the prior study, A molecular subtyping method for *T. pallidum* was developed by Pillay et al. based on the analysis of the *arp* and *tpr* repeat genes.[7] To identify these genes, though, they must either be directly amplified from clinical specimens or propagated in rabbit testis. *tpr* gene's restriction fragment length polymorphism analysis enables strains to be categorized and given letter names based on a combination of fragment sizes.[8] Genome of *T. pallidum* showed in Figure 1.

Molecular techniques for *Treponema pallidum* detection

Certain *T. pallidum* genes have been identified using molecular techniques such as PCR amplification, with varying degrees of sensitivity. The PCR detection method appears to be more precise. It was possible to confirm that the amplification product was unique to the *tp47* gene using the *tp47* hybridization probe. Additionally, it was demonstrated that *tp47* is extremely specific to *T. pallidum* and distinct from other saprophyte spirochetes and microorganisms often detected in sexual diseases, and that it

shares no homology with other bacterial or eucaryotal proteins.[9] Sensitivity of PCR method ranged from 42 to 100%.

A final diagnosis should be made using a combination of serological findings and clinical symptoms along with epidemiological, geographic, and anamnestic information because of the significant epidemiological overlap between venereal syphilis and endemic treponemal diseases in Cuba, and the inability of serology to differentiate between syphilis and other endemic treponematoses (including yaws and bejel). At research laboratories in wealthy countries, PCR that can distinguish between syphilis and treponematosis in clinical samples is now accessible. Routine access to these technologies is now possible.[10]

The platform should be considered when interpreting Ct values, and quantification should only be attempted after direct comparison with established standards. Most likely, the samples from the analytical panel that yielded Rotor-Gene false-negative results had little DNA. Carry-over contamination may account for these inconsistencies, because the false-positive samples in both panels were positioned after a high-load positive sample. Less than one working day was required to complete the assay from the time the sample was received until the findings were reported. Hence, this assay is excellent for use in ordinary diagnostic laboratories.[11]

PCR amplification of target DNA from *T. pallidum* is a sensitive method that can detect a single copy of the treponemal chromosome. In actuality, nested PCR procedures showed a sensitivity that was very close to the theoretical maximum. Despite this excellent sensitivity, the minimal number of clinical samples analyzed by PCR is limited on PCR detection. This restriction is crucial for biological samples containing a few treponemes, such as blood and cerebrospinal fluid.[12]

Confirm the previous study's finding that it might be appropriate to use PCR as the standard diagnostic assay for syphilis with an early phase stadium. There are several reasons why *T. pallidum* PCR (Tp-PCR) is preferred. First, compared to our study's expanded definition, Tp-PCR was more accurate than dark-field microscopy (DFM). Second, it might be challenging to obtain high-quality DFM results, particularly when the test is not conducted consistently. Finally, Tp-PCR results may be more consistent and testing less expensive if conducted on a regular basis because it is less dependent on human judgment than DFM.[13]

The programmatic priority is the discovery of *T. pallidum*, although the clinical supervision of individuals with yaw suspension benefits from the detection of *Haemophilus ducreyi*. The loop-mediated isothermal amplification (LAMP) assay might offer quick, molecular detection of the presence of *T. pallidum* and *H. ducreyi* because the median time to amplification was less than 15 min for both *T. pallidum* and *H. ducreyi*. To ensure that cases of yaws are not overlooked, the performance of the *T. pallidum* component must be improved by assay optimization, especially in the case of co-infection. Because qPCR requires very expensive equipment, particularly thermal cyclers, it can be up to ten times as expensive as a tube scanner that can carry out the Loop-Mediated Isothermal Amplification (LAMP) assay; implementing qPCR at the point of care is operationally difficult. LAMP may be a substitute molecular assay to help the development of yaw eradication efforts, since real-time PCR is only available in a few national number and reference laboratories worldwide. The cost-effectiveness evaluation of the LAMP assay was not performed; however, such an evaluation should consider the cost of the assay itself, the cost per assay, and how well each assay performs in comparison to the others to determine the cost per case detected. According to our results, the *T. pallidum* and *H. ducreyi* Loop-

Mediated Isothermal Amplification (TPHD-LAMP) test, however, may be a more affordable option than qPCR, particularly at the point of care.[14]

In this study, *T. pallidum* DNA was found in tissues from genitourinary ulcers, enabling the laboratory detection of primary syphilis. Genetic studies of the 23S rRNA gene target allowed for laboratory detection of the high incidence of *T. pallidum* strains exhibiting macrolide resistance in Shanghai.[8] The genome *T. pallidum* showed in Figure 1.

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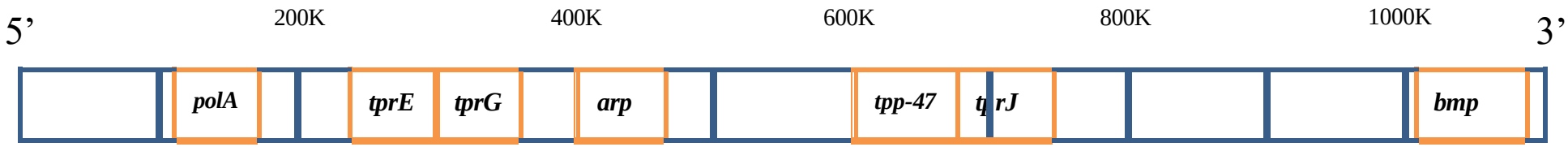

Figure 1. Genome *T. pallidum.*

Table 1. Molecular method for *T. pallidum* in The Previous Study.

Species	Specimen	Detection	Method	Primer	Amplicon (bp)	References
Treponema pallidum	Skin biopsy	*tp47* gene	Polymerase Chain Reaction	sense KO3A (5’-GAAGTTT GTCCCAGTTGCGGTT-3’) anti-sense KO4 (5’-CAGAGCCATCA GCCCTTTTA-3’)	260	Buffet M. et al.[9]
Treponema pallidum subsp. endemicum	Genital/anal ulceration or skin lesions	*polA*, *tp47*, and 16S rDNA loci	Real Time PCR	Not available	Not available	Noda AA, et al.[10]
Treponema pallidum	Genital ulcer swab	*polA* gene	Taqman real-time PCR assay	forward primer 5’-GGTAGAAGGGAGGGCTAGTA-3’, reverse primer 5’-CTAAGATCTCTATTTTCTATAGGTATG-3’and the Taqman probe 5’-ACACAGCACTCGTCTTCAACTCC-3’	116	Koek AG et al.[11]
Treponema pallidum subspecies pertenue	Swabs yaws-like ulcers	*PolA and 16S ribosomal RNA*	TPHD-LAMP assay	Not available	Not available	Becherer L, et al.[14]
Treponema pallidum	Genital ulcers	*arp and tpr genes*	PCR	Not available	Not available	Martin IE, et al.[8]
Treponema pallidum	blood	*Not available*	Taqman-locked nucleic acid (LNA) qPCR	TP forward primer 5’-AGGTCATTATCGTGGTGTTAC-3’ TP reverse primer 5’-CAATCCATCCGTTTCACAATC-3’ TP Probe ROX-CACACCATTCGCACACG-Eclipse	103	Zhou L, et al.[15]
Treponema pallidum	Genital ulcer and whole blood	*DNA polymerase I gene*	PCR-RFLP	*polA*-forward (5’-TGCGCGTGTGCGAATGGTGTGGTC-3’) reverse primer *polA*-reverse (5’-CACAGTGCTCAAAAACGCCTGCACG-3’)	377	Sutton MY, et al.[16]
Treponema pallidum	Blood and CSF	*arp, tpr subfamily II genes, tprC gene,tprD gene locus, tprE, tprG, and tprJ*	RFLP	Sense: 5’-CAGGTTTTGCCGTTAAGC-3’ antisense: 5’-AATCAAGGGAGAATACCGTC-3’	735 - 1575	Marra CM, et al.[17]

Treponema pallidum* ssp. *pallidum	TPA isolate Street Strain 14	*Complate genome*	Oligonucleotide array	Not available	Not available	Matějková P, et al.[18]
Treponema pallidum	Swab of suspected early syphilis lesion	*47kDA lipoproptein*	PCR	Reverse 5'-AGG GGA AGG TGC TGACCATAG-3' & Forward 5'-GGGAGTGAAATCCGCAGAGAG-3' and hydrolysis probe 5'-AGCCTAAGC TTGTCAGCGATCAAG C-3'	Not available	Shields M, et al.[19]
Treponema pallidum	Genital swabs, whole blood, sera, and cerebrospinal fluid	*Treponemal repeat genes, arp and tpr.*	PCR	Not available	Not available	Martin IE, et al.[20]
Treponema pallidum	Swab supernatant, whole blood, and blood serum	*polA and tmpC*	Nested PCR	Not available	Not available	Flasarová M, et al.[21]
Treponema pallidum subsp. pallidum	Genital, anal, oral or rectal lesion swabs and tissue sample.	*Whole genome*	Nested PCR and DNA sequencing	Not available	Not available	Grillová L, et al.[22]
Treponema pallidum* ssp. *pallidum	Treponemal cells	*Whole genome*	DNA sequencing	Not available	Not available	Pětrošová H, et al.[23]
Treponema pallidum* sp. *pallidum	Serum from whole blood	*polA and antibody*	Simple PCR and IgG/IgM ELISA	Forward primer 5'-AGACGGCTGCACATCTTCTCCA-3'; and reverse primer 5'-AGCAGACGTTACATCGAGCGGA-3'	209	Casal CAD, et al.[24]
Treponema pallidum subsp. pallidum	Isolate Propagation	*tprK*	DNA sequencing	Forward primer (5'-AGTAATGGTTTTCGGCATCG-3') and reverse primer (5'-CCATACATCCCTACCAAATCA-3')	175	LaFond RE, et al.[25]
Treponema pallidum	Blood and Tissues	*flaA*	Real-Time PCR	Forward primer 5'-GCGGTTGCACAGTGGGAG-3' and reverse primer 5'-CAGCATGGGCGACAGGAT-3'	61	Salazar JC, et al.[26]
Treponema pallidum subsp. pallidum	Bacterial pellets and swab samples	*tprK*	PCR amplification	Forward primer 5'-ATATTGAAGGCTATGCGGAGCTG-3' and reverse primer 5'-TACCCCACACTCGTAATACCC-3'	182 - 1380	LaFond RE. et al.[27]
Treponema pallidum	Genital ulcers	*polA*	PCR	Forward (F1) primer (5'- TGCGCGTGTGCGAATGGTGTGGTC-3') (nucleotides 1759 to 1783, corresponding to amino acids CACANGVV) (in *polA* 1390 to 1413) and reverse (R1) primer (5'-CACAGTGCTCAAAAACGCCTGCACG-3') (nucleotides 2111 to 2135, corresponding to amino acids RAGVFEHCA); than for forward (F2) primer (5'-CGTCTGGTCGATGTGCAAATGAGTG-3') (nucleotides 1539 to 1563, corresponding to amino acids TSG RCANEC) and reverse (R2) primer (5'-TGCACATGTACACTGAGTTGACTCGG-3')	377 and 395	Liu H, et al.[28]

Treponema pallidum	Genital ulcers	*arp and tpr*	PCR	Forward primer ARP1A 5′-CAAGTCAGGACGGACTGTCCCTTGC-3′) and reverse primer ARP2A (5′-GGTATCACCTGGGGATGCGCACG-3′), than forward primer B1 (5′-ACTGGCTCTGCCACACTTGA-3′) and reverse primer A2 (5′-CTACCAGGAGAGGGTGACGC-3′)	Not available	Pillay A, et al.[29]
Treponema pallidum	Swabs and blood	*Tpp47*	PCR	Tps (5'-TTCGATGCAGTTTCTCGCGCCAACC-3'); Tpe (5'-CTACTGGGCCACTACCTTCGCACG-3'); KO5 (5'-CCCGTTCGCAATCAAAGTCAGCCT-3'); and KO3B (5'-GACGCGAGCTACACCAATCTGATG-3')	1103	Grange PA, et al.[30]
Treponema pallidum	(ano)genital ulcer and skin scraping	*polA*	Real-Time PCR	Forward primer 5'-GGTAGAAGGGAGGGCTAGTA-3', and reverse primer 5'-CTAAGATCTCTATTTTCTATAGGTATGG-3'. Then for TaqMan probe 5'-FAM-ACACAGCACTCGTCTTCAACTCC-BHQ1-3'	Not available	Heymans R, et al.[31]
Treponema pallidum	Whole blood	*polA*	PCR	forward primer 5'-TGCGCGTGTGCGAATGGTGTGGTC-3' and reverse primer 5'-CACAGTGCTCAAAAACGCCTGCACG-3'	377	Marfin AA, et al.[32]

Molecular method for *T. pallidum* in the previous study are showed in Table 1. Quantitative Reverse Transcription PCR (qRT-PCR) has the benefit of being quicker and more sensitive when compared to conventional techniques like reverse transcription-polymerase chain reaction (RT-PCR), Enzyme-linked Immunosorbent Assay (ELISA), Indirect Fluorescent Antibody (IFA), and culture virus. Such assays were consequently created recently, and have swiftly emerged as one of the most crucial techniques for pathogen detection. It is extremely difficult to build a multiplex qRT-PCR assay because each target pathogen requires the use of different optimized primer pairs, probes, and ion concentrations. In fact, the planning and improvement of the reaction system require more attention. qRT-PCR allows the measurement of numerous fluorophores in a single tube and the execution of multiplex assays, allowing for the simultaneous testing of multiple target sequences in a simple reaction. Its superior analytical capacity is evident when a number of potential infections need to be detected quickly. It is a good alternative to conventional approaches because it can run tests concurrently rather than sequentially. Multiplex PCR has been frequently used to simultaneously detect several diseases.[15]

For RFLP analysis of the *tpr* gene, MseI was used to digest the unpurified PCR amplicons from the second stage of nested PCR amplification. Restriction fragments were detected by ethidium bromide staining after separation by electrophoresis on a agarose gel (2%) at 100 V for 60 min.[16]

The Centers for Disease Control and Prevention (CDC) technique has been utilized in several earlier studies to examine the prevalence of *Treponema pallidum* subtype prevalence in communities. For instance, during a syphilis outbreak in Phoenix, Arizona, Sutton, and colleagues identified CDC and *T. pallidum* subtypes using DNA taken from vaginal ulcer swabs and blood samples.[16]

The improved typing technique described here has biological, clinical, and epidemiological value. This marks a considerable improvement in the concept of the molecular epidemiology of syphilis and provides a chance to discover more about the pathophysiology of diseases of the central nervous system.[17]

The protein family (tpr*)* consists of 12 *T. pallidum* replicate proteins, which are only present in this bacterium and share sequence similarities with Treponema denticola's main sheath proteins. Sequence alterations were found to affect 8 of the 12 *tpr* genes (66%), a larger percentage than the rate for the entire genome (13.1%). The *tpr* genes contained locations exhibiting either interstrain, intrastrain, or both variability. The *tpr* genes contained a total of 53 SNPs and 38 intrastrain variable nucleotide locations, with at least one allele matching the Nichols genome sequence (V1–V7 regions of *tprK* were excluded from the analysis). Given the high degree of DNA similarity among *tpr* genes, it is anticipated that differences might be overestimated because of the limits of the hybridization approach for repetitive sequences.[18]

Improved specimen collection may increase the sensitivity of the PCR assays. Serum was extracted from carefully chosen secondary lesions using the edge of a large-bore needle or scalpel. This procedure may also increase the sensitivity of the PCR.[19]

Based on the examination of the repeat genes *arp* and *tpr*, a molecular substyping strategy was devised for *T. pallidum* was devised before. Therefore, direct amplification from clinical samples is necessary to characterize these genes. According to this approach, strains can be categorized based on the number of sixty-base pair tandem repeats that are present in

their *arp* genes; up to this point, repeat counts ranging from 3 to 22 have been recorded. The genes *of tpr*, notably *trpE, tprG*, and *tprJ* of *tpr* subfamily two, allow restriction fragment length polymorphism analysis, which permits the classification and letter designation of starins based on a combination of fragment sizes.[20]

Developing effective and affordable typing methods that may be applied in any laboratory with access to sequencing resources, wherever in the world. These techniques would offer data on the frequency of different strain types and variations over time, as well as trends among antibiotic-resistant strains and whether specific strain types are linked to particular patient groups.[22]

Strain Mexico A's high quality entire genome sequence was discovered utilizing the "next generation" sequencing method (Illumina). Despite the fact that this strain's genome had fewer significant rearrangements than those of other treponemal genomes, two genes incorporated sequences from the subspecies pallidum or pertenue. The apparent mosaic nature of these two genes is probably due to the concurrent infection of a single host, Pallidum and Pertenue, which recombinate with one another.[23]

PCR may be a promising option for syphilis diagnosis in the mother and for contributing to the early detection of the disease, especially primary, secondary, and latent syphilis), which, if undiagnosed and untreated, can have harmful effects on the fetus. Studies of this strategy have also demonstrated its value as a method for control of maternal and congenital syphilis epidemiology in Brazil, lowering public spending on treatable and avoidable diseases. This study found a discrepancy in the results of IgM FTA-Abs (fluorescent treponemal antibody absorption) versus IgG ELISA and PCR versus IgG ELISA due to the low sensitivity of polymerase chain reaction and FTA-Abs. It should be noted that the majority of patients had indeterminate syphilis according to clinical diagnosis and that 19 of the women in this group had received treatment for the disease before the study pregnancy, which could be explained by the serological finding of antibodies from an earlier infection or by the presence of a minor number of circulating treponemes.[24,33-36]

The distribution of germs in the different components of the blood under investigation also suggests that samples (serum) are a less trustworthy source than whole blood or plasma for detecting circulating spirochetes. The decreased amounts in serum could be due to fibrin, fibrin breakdown products, spirochetal adhesion to cellular components, or the clot itself.[26]

When ABI 310 equipment is utilized for analysis, the theoretical detection limit of polA PCR is 200 organisms per milliliter, or two organisms per PCR. This cap was established using data from a two hunderd-ml sample that underwent DNA isolation and yielded 100 ml of eluted DNA. The flow PCR procedure ran a PCR with 5 ml of the collected DNA. The sensitivity increased to approximately one organism per PCR, making it comparable to that of Rabbit Infectivity Testing (RIT). However, it is vital to remember that the sampling technique has a cap on the sensitivity of detection. The detection of organisms at concentrations less than one per 5 ml may be hampered by sampling errors.[28,37-40]

STRENGTH AND LIMITATION

This review article describes the development of molecular methods for the detection of *T. pallidum* targeting various genes. In addition, this article also describes the primers used in the molecular method so that it can be used as a reference for the detection of *T. pallidum* to develop a diagnostic system. This article is limited to laboratory research reports and does not include molecular methods used for patient diagnostics.

CONCLUSIONS

Early detection of primary, secondary, and latent syphilis, made possible by quick molecular methods, particularly PCR results, will result in prompt treatment and a reduction in the amount of time the patient's sexual partners are exposed to the disease, as well as the prevention of disease progression. Thus, it is sufficient to check for this disease using a whole blood specimen.

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CONFLICT OF INTEREST

The authors claim to have no conflicts of interest.

ETHICAL CLEARANCE

This study did not require an ethical review because the data were obtained from the existing literature and did not use human subjects.

FUNDING

There is no funding for this study.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest in writing this manuscript.

AUTHOR CONTRIBUTION

All authors contributed to the preparation of this review article. Author WS in this study was in charge of collecting data, analyzing data, and writing article reviews. Meanwhile, NW is the principal investigator who provides study ideas and validates the data.

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