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Development of real-time polymerase spiral reaction assay for rapid and visual detection of *Treponema pallidum*

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ABSTRACT

Treponema pallidum, a bacterium, is the cause of the sexually transmitted disease syphilis. At present, the treatment of syphilis is relying on serum-based detection methods and darkfield observation of *T. pallidum*. However, these methods of identification are less effective in the initial stage of infection. In this study, we have developed a real-time polymerase spiral reaction (PSR) detecting the *polA* gene of *T. pallidum* DNA. The PSR reaction conditions were optimized at 62°C for 45 min. The *polA* gene displayed a distinct melt peak with a Tm value of 80 ± 0.5°C in the real-time melt curve analysis of the PSR reaction. The detection limit of the developed method was found to be 100×10^{-12} µg/µL. The specificity was evaluated by utilizing several bacterial species, and this method was 100% specific only to *T. pallidum.* The developed method is rapid, efficient, and can be used as an additional method for diagnosing *T. pallidum* infection.

ARTICLE HIGHLIGHTS

- Two visual interpretation methods real time-PSR and PSR-dye for the detection of Treponema pallidum, with a reaction time of less than 45 minutes.
- PSR methods do not require expensive equipment, and effectively prevent the aerosol contamination.
- A simple, inexpensive PSR method provides efficient role in managing the infection, especially in high prevalence.

1. INTRODUCTION

Treponema pallidum, a spirochete bacterium, causes the sexually transmitted disease syphilis [1]. Pathogenic *T. pallidum* is classified into 3 major subspecies, namely *T. pallidum* subspp. *pallidum* (syphilis), *T. pallidum* subspp. *pertenue* (yaws), and *T. pallidum* subspp. *endemicum* (bejal) based on their clinical manifestation in all stages of infection (primary, secondary, and tertiary) [2]. Currently, the diagnosis of syphilis is based on darkfield microscopy and serological methods. Dark field microscopy is primarily utilized for the direct identification of primary syphilis. Oral chancre and blood specimens cannot be used directly in this method because it is highly operator dependent. In such situations, *T. pallidum* cannot be directly detected [3]. Currently, the serological methods of identification are relying on the treponemal and non-treponemal assay. The treponemal assays are: Fluorescent treponemal antibody absorption test, *T. pallidum* hemagglutination assay (TPHA), and *T. pallidum* particle agglutination assay, were used to detect IgM and IgG antibodies can both be found in patient samples. Non-treponemal assays include the rapid plasma reagin test and the venereal disease research laboratory (VDRL) test, which detect antibodies to the cardiolipin and lecithin antigens of *T. pallidum* [4]. In cases of primary syphilis, the window period may lead to false-negative results. Subsequent follow-up visits are necessary for up to 3 months. However, it results in delayed diagnosis, severe health complications, and continued transmissions [5].

Various molecular methods such as classical polymerase chain reaction (PCR) [6], nested PCR [7], quantitative PCR [8], Multiplex PCR [9], and TaqMan real-time multiplex PCR [10] were used to detect *T. pallidum* DNA in blood, urine, semen as well as a specimen from genital ulcers. Unfortunately, these techniques are laborious and require specialized equipment, which makes them difficult for on-site detection.

Currently, different isothermal amplification assays have evolved, which can rapidly identify DNA/RNA without the requirement of sophisticated instruments. Among the isothermal amplification assays, loop-mediated isothermal amplification (LAMP) is the highly used method for many bacterial pathogen detections, including *T. pallidum* in clinical samples [2,5,11]. Despite the advantages, LAMP requires 4-6 primers, various optimization steps, and high risks of contamination causing false-positive amplification [12].

Polymerase spiral reaction (PSR) is a unique isothermal approach that efficiently amplifies DNA in the range of 60–65°C (under isothermal

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conditions) with high specificity and sensitivity [13]. The forward and reverse primers are designed to enclose a 20–22 bp unrelated exogenous sequence (botanic origin) at 5' end with the remaining sequences corresponding to the target region at 3' end [14]. The PSR method has several advantages over other isothermal methods, including that it only uses two primers; it is quick, very sensitive, accurate, and deliverable in resource-limited areas. Due to these advantages, PSR has been used to detect many bacterial, viral accurately, and fungal pathogens causing diseases such as brucellosis [15], vibriosis and gastrointestinal illness [16], foodborne diseases by *Salmonella* and *Staphylococcus aureus* [17], and tuberculosis [18]. PSR has been used for viral and fungal diseases caused by the West Nile virus (mosquito-borne virus) [19], as well as *Candida albicans* [20].

Real-time melt curve analysis is used for observing DNA melting behavior. This method is an in-tube approach (closed), requiring only DNA and an intercalating DNA-binding dye such as SYBR green. These dyes emit fluorescence when integrated into a double-stranded DNA but are inactive in a single-stranded form. The fluorescence signal is collected at a suitable temperature and a melt curve graph is generated. Real-time analysis allows the identification of a single sequence base change between samples [21,22]. The advantages of real-time melt curve analysis provide amplification of nucleic acids directly with real-time visualization, the absence of post-processing steps, and rapidity [15]. Melt curve-based real-time PSR is already reported for rapid detection of *C. albicans* [20], *Vibrio parahaemolyticus* [16], *Salmonella* [17], and *Mycobacterium tuberculosis* [18].

This study has developed the real-time PSR targeting the *polA* marker gene to detect *T. pallidum* DNA. The marker gene *polA* encodes for DNA polymerase I, a highly specific marker gene to identify the subspecies of *T. pallidum* [23]. The melting curve-based PSR used in this study uses the identification GC content of the gene (*polA*). The study used the real-time PSR for the 1st time to detect the *T. pallidum.* So far, this technique is not employed for the real-time detection of *T. pallidum.*

2. MATERIALS AND METHODS

2.1. DNA Isolation

The seropositive blood samples were extracted from *T. pallidum*-affected patients from Madras Medical College, Chennai, Tamil Nadu, India. Genomic DNA was isolated using QIAamp DNA Blood Mini Kit (Qiagen, Germany) as per the manufacturer's instruction. The DNA concentration was quantified using a Bio photometer (Eppendorf, Hamburg, Germany), and it was stored at −80°C. The isolated DNA was confirmed further by amplifying and sequencing 16S rDNA and *polA* regions.

2.2. Bacterial Strains

Bacterial species such as *Escherichia coli* (MTCC 1302), *Staphylococcus epidermidis* (MTCC 10623), *S. aureus* (MTCC 1430), *Leptospira interrogans* serovar *canicola* (ATCC 23606), and *Stenotrophomonas maltophilia* (MTCC 7528) were used for specificity analysis of the study. These bacterial strains were maintained as a 50% glycerol stock at −80°C and plated on appropriate medium and temperature. The pGEM®-T Easy Vector (Promega, USA) was used to clone the target gene (*polA*) of *T. pallidum* for PSR optimization reactions. All media and chemicals were purchased from HiMedia Pvt. Ltd, India.

2.3. Designing of PSR Primers

The PSR primers were generated to specifically target the sequence of *polA* gene (GenBank Accession number CP004010) of *T. pallidum* using the online primer explorer V5 tool (https://primerexplorer.jp/e/). PSR-specific primers consist of forward and reverse primers with two auxiliary accelerated primers designed and tested using *in silico* program (https://genome.ucsc.edu/cgi-bin/hgPcr). All the primers are listed in Table 1.

2.4. Development of PSR Assay and Real-Time Analysis

The PSR assay was carried out in a 25 µL mixture with target DNA (50–100 ng/µL), 40 µM of both forward and reverse primers, 2.5 µL of 10× isothermal reaction buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, and 0.1% Tween 20), 1.4 mM dNTPs, 8 mM $MgSO_4$, 0.8M betaine, 8U of Bst DNA polymerase (New England BioLabs, USA). The amplification was carried out at 62°C for 45 min. The assay without template DNA is considered a negative control. The amplified products were visualized on 2.5% agarose gel. Visual detection of PSR amplified products was analyzed using hydroxy naphthol blue (HNB) dye and SYBR Safe DNA stain.

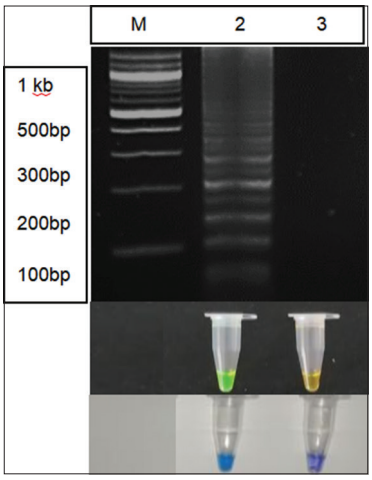

Figure 1: Polymerase spiral reaction optimization of *polA* amplicons visualized on 2.5% agarose gel and visual detection with SYBR safe gel stain and hydroxy naphthol blue dye. Lane M: 100 bp ladder, Lane 2: *polA* amplicon, Lane 3: Negative control.

Table 1: Primers used in this study.

Target gene	PSR primers (5'-3')	
polA gene	Forward primer	5' acgaattcgtacatagaagtatagATTGGTCCTAAGACGGCT 3'
	Reverse primer	5' gatatgaagatacatgcttaagcaGCGGAATACAACAGGAATC 3'
		Exogenous sequence

PSR: Polymerase spiral reaction

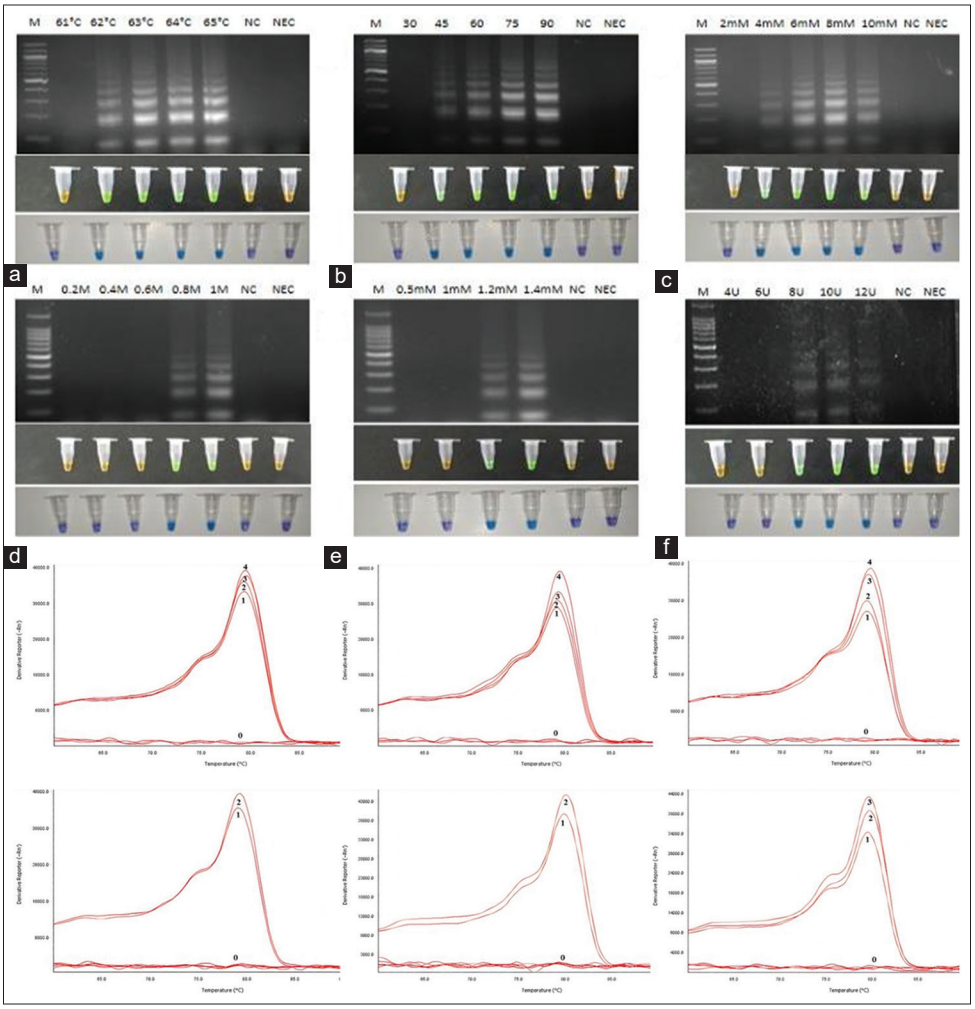

Figure 2: Optimization of polymerase spiral reaction (PSR) and real-time analysis for the amplification of *polA* amplicon. PSR amplified products of pGEM-T/*polA* at various reaction temperatures, time, MgSO4, Betaine, dNTP, and Bst polymerase visualized on 2% agarose gel, colorimetric detection, and real-time analysis. (a) Reaction temperature: Lane M: 100 bp ladder, Lane 1–5: *polA* (61°C, 62°C, 63°C, 64°C, and 65°C). (b) Reaction time: Lane M: 100 bp ladder, Lane 1–5: *polA* (30, 45, 60, 75, and 90 min). (c) MgSO4: Lane M: 100 bp ladder, Lane 1–5: *polA* (2, 4, 6, 8, and 10 mM). (d) Betaine: Lane M: 100 bp ladder, Lane 1–5: *polA* (0.2, 0.4, 0.6, 0.8 and 1M). (e) dNTP: Lane M: 100 bp ladder, Lane 1–4: *polA* (0.5, 1, 1.2, and 1.4 mM). (f) *Bst* polymerase: Lane M: 100 bp ladder, Lane 1–5: *polA* (4, 6, 8, 10, and 12U); Lane 6: Negative control, Lane 7: Nonextraction control for all reactions.

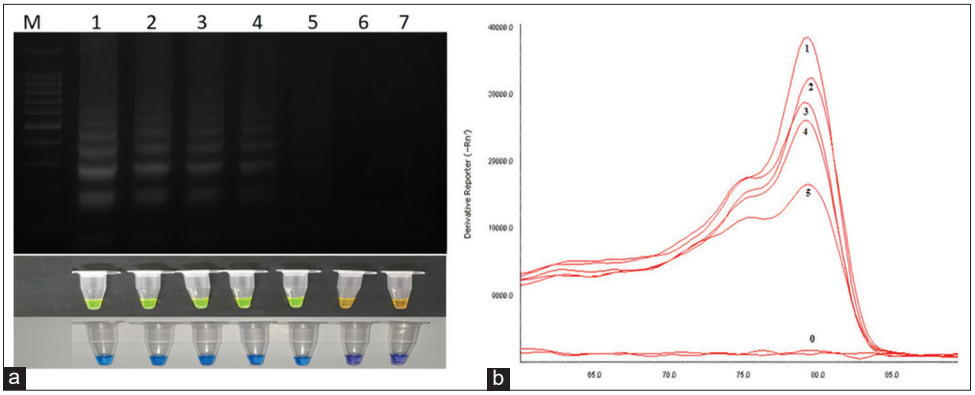

Figure 3: Detection limit analysis of *polA* gene using agarose gel and real-time analysis of *Treponema pallidum* genomic DNA. (a) Detection limit analysis of *polA* amplicon visualized on 2.5% agarose gel. Lane M: 100 bp ladder, Lane 1–5: *polA* amplicon (100, 100 × 10^{-3}, 100 × 10^{-6}, 100 × 10^{-9}, 100 × 10^{-12}) µg/µL, Lane 6: Negative control, and Lane 7: No extraction control. (b) Real-time analysis of *polA* amplicon. Real-time peak 1–5: *polA* amplicons (100, 100 × 10^{-3}, 100 × 10^{-6}, 100 × 10^{-9}, 100 × 10^{-12}) µg/µL, and real-time peak 0: Negative control and no extraction control.

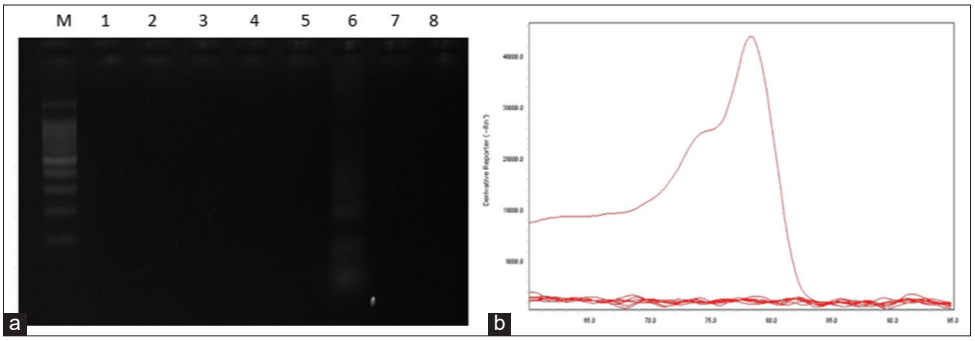

Figure 4: Specificity analysis of *polA* gene with other organisms using agarose gel and real-time analysis of *Treponema pallidum* genomic DNA. (a) Specificity analysis of *polA* amplicon visualized on 2.5% agarose gel. Lane M: 100 bp ladder, Lane 1: *Escherichia coli*, Lane 2: *Staphylococcus epidermidis*, Lane 3: *Staphylococcus aureus*, Lane 4: *Stenotrophomonas maltophilia*, Lane 5: *L. canicola*, Lane 6: *T. pallidum* (DNA isolated from blood), Lane 7: negative control, and Lane 7: no extraction control. (b) Real-time analysis of *polA* amplicon. Real-time peak 1: *polA* amplicon, and real-time peak 0: Non amplified products, negative control and no extraction control.

Subsequently, the amplification of PSR assay was also detected by real-time melt curve analysis in QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Applied Biosystems™, USA). The real-time PSR assay was carried out as described above. The real-time PSR reaction was performed at 62°C for 45 min and followed by the melt curve analysis at 95°C for 15 s, 60°C for 1 min, and 95°C for 1 s. The results were analyzed in QuantStudio™ Real-time PCR software v1.4. The fluorescence intensity produced in each reaction was measured and reflected by melt curve peaks.

2.5. Optimization of Real-Time PSR

The optimization of PSR assay was performed at a template DNA concentration of 100 ng/µL, 2–10 mM $MgSO_4$ (New England BioLabs, USA), 0.5–1.4 mM dNTP (New England BioLabs, USA), 0.2–1 M Betaine (Sigma-Aldrich, St Louis, USA), 4–12 U *Bst* polymerase (New England BioLabs, USA), 61–65°C reaction temperature, and 30–90 min reaction time. The PSR amplified products were visualized on 2.5% agarose gel. The optimization products were also detected visually by HNB dye and SYBR Safe DNA stain. Meanwhile, the amplified products were subjected to real-time melt curve analysis as described above. All the reactions were carried out in triplicates. Non-template DNA control was included in all the reactions.

2.6. Detection Limit and Specificity Assay of PSR

The detection limit of the developed PSR assay was checked using artificially contaminated healthy serum samples. Blood serum was prepared from the healthy seronegative volunteer (TPHA negative). The serum was artificially contaminated or spiked with different *T. pallidum* genomic DNA concentrations isolated from infected blood samples (TPHA and VDRL positive). The concentration from (100, 100×10^{-3}, 100×10^{-6}, 100×10^{-9}, 100×10^{-12}) µg/µL were prepared, and 1 µL from each dilution was used as a DNA for PSR and real-time melt curve assays. The specificity of this assay was tested using different bacterial strains such as *E. coli, S. epidermidis, S. aureus, L. canicola,* and *S. maltophilia* for its cross-reactivity with *T. pallidum* DNA.

3. RESULTS

3.1. Development of PSR Assay

The development of the PSR assay revealed a Ladder-like pattern in the gel-like LAMP amplification in the positive reaction. In contrast, the negative reaction did not show any amplification. The amplified products were run on 2.5% agarose gel [Figure 1].

Visual discernment of PSR amplicons was detected by the addition of HNB dye and SYBR Safe gel stain. HNB is a metal ion indicator. The concentration of Mg2+ ions decreases significantly during amplification and induces HNB dye to change color from violet to sky blue in all the positive products. No color change was noticed in the negative products. Similarly, SYBR safe gel stain is a fluorescent dye that binds to the minor groove of double-stranded DNA. The fluorescence intensity increases and the DNA concentration is detected by green fluorescence in positive amplicons, whereas the negative amplicons remain the original orange color [Figure 1].

Real-time melt curve-based PSR was also established for the *polA* gene detection in a single reaction. The melting temperature (Tm) of DNA is mainly based on the GC content of the amplified product. Different amplicons showed distinct Tm values that can be used to differentiate the specific target amplicon. The *polA* gene was found to have a GC content of 43.6%. A distinct melt peak was obtained with the Tm value of 80 ± 0.5°C.

3.2. Optimization of PSR and Real-Time PCR

The developed PSR-based assay was optimized for temperature, time, $MgSO_4$, dNTP, betaine, and *Bst* polymerase. The assay was optimized for temperatures varying from 61°C to 65°C at 1°C increment and the amplification was started at 62°C, but not at 61°C. The optimum time required for the assay ranged from 30 min to 90 min with a time interval of 15 min and the amplification result was observed within 45 min onward but showed no amplification 30 min. Various concentrations of $MgSO_4$ ranging from 2 mM to 10 mM were tested, of which the amplification started at 4 mM but did not show any amplification in 2 mM. Different concentrations of betaine were checked from 0.2 M to 1 M, and the amplification was observed only at 0.8 M and 1 M. The optimal dNTP concentrations were tested from 0.5mM to 1.4 mM; the optimum concentrations were observed at 1.2 mM and 1.4 mM. Finally, the concentrations of *Bst* polymerase from 4 U to 12 U were tested, and amplification was observed starting from 8 U, but not in 4 U and 6 U concentrations. The amplified products were observed on a 2.5% agarose gel and confirmed by real-time melt curve analysis [Figure 2]. The PSR and melt curve results indicate that the methods had similar amplification in all the optimized conditions.

3.3. Detection Limit and Specificity Analysis of PSR

The artificially contaminated or spiked serum samples were used at different concentrations ranging from (100, 100×10^{-3}, 100×10^{-6}, 100×10^{-9}, 100×10^{-12}) µg/µL. The detection limit of PSR assay was found to be 100×10^{-12} µg/µL, while the LAMP assay was able to detect up to 100×10^{-9} µg/µL. Thus, the PSR assay detection limit is 10 times more sensitive than the LAMP assay [Figure 3]. The specificity of PSR was tested with various bacterial strains such as *E. coli, S. epidermidis, S. aureus, L. canicola,* and *S. maltophilia* for its cross-reactivity with *T. pallidum* DNA. PSR specificity was reported by amplification only with *T. pallidum* DNA [Figure 4]. The above results demonstrate the high specificity and detection limit of the developed method.

4. DISCUSSION

Syphilis is a global public health condition caused by *T. pallidum* subspp. *Pallidum*, especially during co-infection with human immunodeficiency virus. As indicated by the World Health Organization, 17.7 million people all around 15–49-years-old had syphilis in the year 2018 [24]. Serological and molecular techniques are currently used to diagnose this disease. However, the sensitivity of these methods is less because of the low level of pathogenic bacterial load in the patient's blood.

The standard marker genes used for the positive detection of *T. pallidum* are *polA* (DNA polymerase I), *T. pallidum* repeat family, Basic membrane protein, *T. pallidum* 47 kDa protein, acidic repeat protein, and *23s* rRNA [25]. The marker gene *polA* of *T. pallidum* has high cysteine content and low homology with other bacterial species [26]. Hence, in this study, *polA* marker genes were selected as a specific target gene for real-time PSR assay for sensitive and rapid detection of *T. pallidum*.

PSR is a potentially useful substitute for available molecular methods, which require sophisticated equipment and time. The advantage of the PSR assay developed in this study requires only two primers and a single enzyme (*Bst* polymerase) [16]. This method minimizes the optimization of the reaction and eliminates the non-specific amplification, which is most common when multiple primers are used, like LAMP assay. The optimal PSR conditions were obtained at 62°C for 45 min, with the reagent concentrations of 4 mM $MgSO_4$, 0.8M betaine, 1.2 mM dNTP, and 8 U *Bst* polymerase. Similar to our observation, a recently developed PSR assay for the detection of *Salmonella* in pork has shown the amplification at 64°C for 60 min with the addition of 8 mM $MgSO_4$, 0.8M betaine, 1.4 mM dNTP, and 8 U *Bst* polymerase and also, the detection of *S. aureus* in meat established at 66°C for 60 min with the reagent concentration of 4.0 mM $MgSO_4$, 0.8 M betaine, 1.4 mM dNTP, and 8.0 U *Bst* polymerase [27].

The developed PSR assay is also more sensitive than the earlier published PSR assay for the detection of *Salmonella* [28], *S. aureus* [29], *Clostridium perfringens* [27], and *Brucella* [30] showed the detection limit of 100 fg, 77 fg, 80 fg, and 11.8 fg respectively. Recently, many PSR assays have been developed to identify pathogens in various fields and have proven to achieve 10 times higher sensitivity than LAMP and PCR. Thus, the developed PSR assay serves as a promising diagnostic tool for the detection of *T. pallidum.*

5. CONCLUSION

This is the first study to detect *T. pallidum* using PSR assay. This could play an essential and efficient role in managing the infection, especially in high prevalence because of its high sensitivity, specificity, and less time-consuming. Since it provides a precise and sensitive, user-friendly, rigorous, rapid, equipment-free technology that can deliver fast results, it can be a pathway for clinical screening, on-site diagnosis, and fundamental quarantine procedures.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. FUNDING

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

The Institutional Ethics Committee of Madras Medical College (EC Reg. No. ECR/270/Inst./TN/2013) has approved the study for collecting human samples.

11. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

12. PUBLISHER'S NOTE

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