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Rapid and visual detection of leptospira interrogans using polymerase spiral reaction assay

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ABSTRACT

Leptospirosis is a waterborne zoonotic disease caused by pathogenic species of the genus *Leptospira* that affects humans and animals. Current diagnostic methods, such as culture and serology-based techniques, have limitations, including low sensitivity and time-consuming nature. This study presents the first implementation of the polymerase spiral reaction (PSR) assay for the rapid and visual detection of *Leptospira interrogans*. The assay utilizes Lipl32, a highly conserved gene that encodes an outer membrane lipoprotein, as a marker gene to detect pathogenic *Leptospira* spp. For visual detection, the assay was evaluated using a colorimetric dye and nucleic acid stain. The assay was optimized for various reaction parameters. The optimal conditions were determined to be 60°C for 60 min with 6U of Bst Polymerase, 5 mM of Mg^{2+}, 2.5 mM of Betaine, and 10 mM of dNTPs. An artificial contamination study in tap water established the assay's detection limit at 16 GEq/mL. Notably, the PSR assay showed high specificity and sensitivity compared to loop-mediated isothermal amplification (LAMP), requiring only a single primer pair in contrast to the six primers required for LAMP.

ARTICLE HIGHLIGHTS

1. Rapid and Sensitive Detection: Developed a Polymerase Spiral Reaction (PSR) assay for quick and accurate detection of Leptospira, addressing the limitations of current methods.
2. Comparable Results: PSR assay exhibited results similar to the LAMP assay, confirming its reliability through distinctive patterns on agarose gel and visual indicators.
3. Optimized Parameters: Successfully optimized assay conditions, including temperature, polymerase concentration, MgSO4, Betaine, dNTP concentration, and incubation time for robust and specific amplification.
4. High Sensitivity: Demonstrated the assay's high sensitivity with a Limit of Detection (LOD) of 16 GEq/mL, surpassing the limitations of existing detection methods.
5. Practical Advantages: Elimination of the need for a thermal cycler, in-tube detection using end-point indicators (HNB and Hi-SYBr Safe Gel Stain), and simplified procedure enhance the assay's practicality.
6. Specificity Confirmation: Specificity testing validated the assay's reliability, ensuring positive amplification only in the presence of L. interrogans genomic DNA.
7. Potential for Resource-Limited Settings: The PSR assay's simplicity, speed, and sensitivity make it a promising tool for Leptospira detection in resource-limited settings, addressing the urgent need for improved diagnostic methods.
8. First PSR-Based Assay: This study pioneers the use of PSR for Leptospira detection, presenting a novel, rapid, and apt technique with potential applications in public health and disease prevention.

1. INTRODUCTION

Leptospirosis is a widespread zoonotic disease that affects both humans and animals, with cattle, rodents, and domesticated animals serving as primary sources of infection. The disease is transmitted through the release of urine from infected animals. Leptospirosis has biphasic symptoms that are similar to other febrile diseases such as dengue fever and chikungunya, making a clinical diagnosis difficult [1-3]. Therefore, there is a need to increase the clinical sensitivity of diagnostic methods for leptospirosis [4].

Current laboratory diagnosis of leptospirosis relies on culture and serology-based methods, which are time-consuming, technologically demanding, and cannot detect the disease during its early acute phase [5,6]. The gold standard test for leptospirosis is the microscopic agglutination test (MAT), but it has limitations, including low sensitivity in the early stages of the disease. Molecular techniques such as polymerase chain reaction (PCR), loop-mediated isothermal Loop-mediated isothermal amplification' is the correct expansion of LAMP offer higher sensitivity and faster turnaround time [7].

PCR-based methods provide higher sensitivity for detecting

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Leptospira DNA in patient samples than MAT [8]. They are beneficial in low-resource settings. Isothermal amplification techniques such as loop-mediated amplification (LAMP) and Nucleic acid sequence-based amplification are superior to conventional PCR methods as they do not require thermal cyclers and allow for a straightforward interpretation of results. PSR, a novel isothermal amplification method that uses a single enzyme and a pair of primers in an isothermal setting, has effectively detected various bacteria and viruses [9-11]. However, its potential in detecting *Leptospira* remains unexplored.

To address these limitations, this study developed a new assay based on the PSR that targets Lipl32, a gene encoding an outer membrane protein specific to pathogenic *Leptospira*. The PSR assay demonstrated rapid and sensitive detection of *Leptospira*, even at low concentrations of the target template, with results obtained within 60 min, even at low target concentrations. In addition, the PSR assay eliminates the need for gel electrophoresis. It prevents aerosol contamination in the surroundings by combining with end-point indicators such as hydroxynaphthol blue (HNB) and Hi-SYBr safe gel stain. This PSR assay has shown high specificity and sensitivity and has the potential to become a useful diagnostic tool for the detection of leptospirosis, particularly in resource-limited settings.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

The pathogenic strain of *Leptospira interrogans* serovar Canicola was obtained from Dr. K. Natarajasrinivasan at the Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. *Leptospira* Medium Base (HiMedia, M1009), supplemented with *Leptospira* Enrichment Medium (HiMedia, FD066) and 5-fluorouracil (HiMedia, RM1502), was used to culture *Leptospira* at 28°C for 4 weeks. DNA was extracted from the culture using the method described below. In addition, DNA was extracted from *Leptospira meyeri* serovar Semarangaa (a non-pathogenic strain) and nine non-Leptospiral species, which were used as negative controls in this study [Table 1].

Table 1: Bacterial strains used in the study.

S. No.	Bacterial species	Strain/source
1.	*Leptospira interrogans* serovar Canicola	Bharathidasan University
2.	*Leptospira meyeri* serovar Semarangaa	Bharathidasan University
3.	*Vibrio cholerae* (0139)	MTCC 3906
4.	*Shigella flexneri*	MTCC 1457
5.	Methicillin-resistant *Staphylococcus aureus* subspp. *aureus* Rosenbach	ATCC 33592
6.	*Staphylococcus aureus*	ATCC 25923
7.	*Staphylococcus spidermidis*	ATCC 12228
8.	*Enterobacter faecalis*	ATCC 19433
9.	*Escherichia coli*	ATCC 11229
10.	*Stenotrophomonas maltophilia*	ATCC 17666
11.	*Pseudomonas aeruginosa*	ATCC 35032

2.2. Genomic DNA Isolation and Primer Designing

This study used the QIAmp DNA Mini Kit to extract genomic DNA from bacterial cultures following the manufacturer's instructions. The quality and concentration of the extracted DNA were determined using ultraviolet (UV)-Visible spectrophotometry (Eppendorf BioSpectrometer®, Germany) and were stored at −20°C until further use. The gene that encodes for the pathogenic *Leptospira*-specific outer membrane protein Lipl32 was chosen as the target for primer design. Based on the criteria described in earlier studies [11], the PSR assay primers were designed using Primer3 and were synthesized by Eurofins Scientific Pvt Ltd (Chennai, India). The primer sequences are listed in Table 2. To compare the specificity and sensitivity of the developed PSR assay, a LAMP assay targeting Lipl32 was employed to detect *Leptospira*.

2.3. Development of PSR Assay

In this experiment, a PSR assay was set up in a final volume of 25 µL. The components of the reaction mixture included 2.5 µL of 10× isothermal amplification buffer, 1 µL of dNTP mix (10 mM), 1.5 µL of $MgSO_4$ (10 mM), 4 µL of Betaine (5 M), 1 µL of each of the Ft and Bt primers (4.0×10^{-8} mM), 1 µL of Bst Polymerase (8U/µL), 12 µL of nuclease-free water, and 1 µL of target DNA. The LAMP assay was also set up as a positive control, using FIP and BIP primers at a concentration of 4.0×10^{-8} mM and F3, B3, LF, and LB primers at 5×10^{-9} mM each. The PSR and LAMP assays were conducted for 60 min at 65°C in a dry bath, and the products were visualized through electrophoresis on a 2.0% agarose gel to confirm the amplification.

2.4. In-tube Detection of PSR Products

HNB, a metal indicator dye, and Hi-SYBr safe gel stain were employed in the colorimetric PSR assay to visualize the amplified products. The assay was performed using a 25 µL reaction mix comprising 2.5 µL of 10× Isothermal amplification buffer, 1 µL dNTP mix (10 mM), 1.5 µL $MgSO_4$ (10 mM), 4 µL of Betaine (5M), 1 µL each of the Ft and Bt primers (4.0×10^{-8} mM), 1 µL of Bst Polymerase (8U/µL), 1 µL of DNA template, 1 µL Hydroxynaphthol Blue (120 µM) (Sigma Aldrich V001758), and 12 µL of nuclease-free water. Positive reactions were indicated by a blue color change, while negative reactions retained the original purple color of the HNB dye. Hi-SYBr safe gel stain (HiMedia ML053) was added to the reaction mix to allow visual detection of the PSR-amplified products. Positive reactions displayed a bright green color when viewed under UV light, while negative reactions retained the dye's orange color. Furthermore, the PSR-amplified products were visualized by electrophoresis on a 2% agarose gel.

2.5. Optimization of PSR Assay

The optimal conditions for the PSR assay were determined by testing different reaction conditions, including temperature and amplification time. A temperature gradient of 60–65°C for 60 min was used for optimization. The concentration of Bst polymerase was set at 6, 8, 10, and 12 U/reaction; the concentration of Mg^{2+} was set at 2.5, 5, 10, and 12 mM, respectively. The concentration of Betaine ranged from 1 M to 5 M. The concentration of dNTP was optimized at 2.5, 5, 10, and 12 mM, respectively. The incubation time was also optimized at the established optimal temperature from 30 to 120 min. The amplified products were analyzed on 2% agarose gels stained with ethidium

Table 2: List of primers used in this study.

Assay	Primer	Sequence (5'-3')	Reference
PSR	Ft	ACGAATTCGTACATAGAAGTATAGACTTGGATCCGTGTAGAAAG	This study
	Bt	GATATGAAGATACATGCTTAAGCACCTGGTTTGTAGGTAGTGAA	
LAMP	F3	TGTTTGGATTCCTGCCGTAA	[12]
	B3	GCAGCTTTGGCGATTTGG	
	FIP	CTCCATCGCCTGGTTCACCGTCGCTGAAATGGGAGTTCG	
	BIP	AGCGGCTACCCCAGAAGAAAAAAGGCATAATCGCCGACATTC	
	LF	TCGCCTGTTGGGGAAATCA	

Ft: Forward primer, Bt: Reverse primer, F3: Forward outer primer, B3: Reverse outer primer, FIP: Forward inner primer, BIP: Reverse inner primer, LF: Loop primer, PSR: Polymerase spiral reaction, LAMP: Loop-mediated isothermal amplification.

bromide. The PSR assay was tested for cross-reactivity using non-pathogenic species of *Leptospira* and non-*Leptospira* strains from different genera [Table 1].

2.6. Limit of Detection (LOD) of the PSR Assay

An artificial contamination study was carried out to determine the assay's LOD. Bacterial cells for the study were harvested by pelleting 5 mL of *L. interrogans* serovar Canicola culture by centrifugation and resuspended in 1 mL of sterile water. Tap water was spiked with 10-fold serial dilutions (10^{-1} to 10^{-6}) of the cell suspension and used as a template for the assay. The tap water used in the study was checked for leptospiral contamination by a LAMP assay targeting the Lipl32 gene. The bacterial load in each dilution was determined by quantitative PCR (qPCR) (Initial denaturation at 95°C – 5 min, followed by 35 cycles of denaturation at 95°C – 30 s, annealing at 61°C for 30 s, extension at 72°C for 15 s, followed by a final extension at 72 for 2 min). PSR assay was performed using the optimized parameters, and the results were visualized by agarose gel electrophoresis and HNB and Hi-SYBr safe gel stain.

3. RESULTS

3.1. Development of the PSR Assay

The developed PSR assay for detecting *Leptospira* was evaluated and compared to the LAMP assay. Agarose gel electrophoresis revealed a ladder-like pattern for the amplified products of both assays, while negative samples did not show any such pattern. Furthermore, adding a metal indicator dye, HNB, to the reaction tubes facilitated visualization of the amplified products, with blue color indicating positive amplification and violet color indicating negative amplification [12]. In addition, Hi-SYBr safe gel stain was used to visualize the products, with positive samples producing a green fluorescence and negative reactions remaining orange in color [13]. These findings prove that the PSR assay, like the LAMP assay, can be confirmed in-tube [Figure 1].

3.2. Optimization of the PSR Assay

The PSR assay was systematically optimized for temperature, Bst polymerase, $MgSO_4$, Betaine, dNTP concentration, and incubation time. Temperature optimization revealed that amplification occurred at all temperatures between 60°C and 65°C; however, 60°C was deemed the most appropriate for further experiments. Bst polymerase concentration of 6U per tube was sufficient for optimal amplification, while 5 mM $MgSO_4$ and 2.5 mM Betaine were the optimal concentrations for these components. The ideal dNTP concentration was determined to be 10 mM, and maximal amplification was

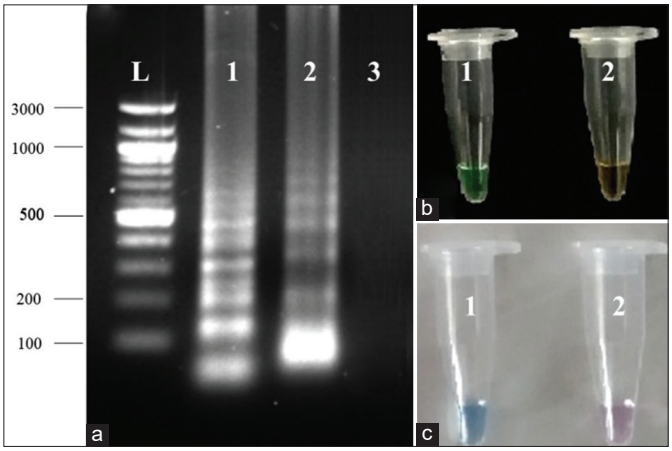

Figure 1: Polymerase spiral reaction (PSR) and LAMP reactions for *Leptospira interrogans* serovar Canicola. (a) Results of agarose gel electrophoresis: L: 100bp DNA Ladder; (1) amplification products of Lipl32 gene by PSR; (2) amplification products of the Lipl32 gene by LAMP; (3) Non-template control, (b) PSR-amplified products using Hi-SYBr safe gel (Green: Postive; Orange: Negative), (c) PSR-amplified products using hydroxynaphthol blue (Blue: Positive; Violet: Negative).

achieved after 60 min of incubation [Figure 2]. Analysis of the PSR amplicons on a 2% agarose gel indicated successful amplification. Specificity testing revealed that amplification occurred only with genomic DNA from pathogenic *L. interrogans* and not with DNA from non-pathogenic *Leptospira* or non-Leptospiral strains. These results confirm the high degree of specificity of the PSR assay, as illustrated in Figure 3.

Furthermore, positive amplification was confirmed using a metal indicator dye HNB and Hi-SYBr safe gel stain. Blue-colored and green-fluorescent samples indicated successful amplification, while negative samples remained violet-colored and orange, respectively. In-tube confirmation of positive amplification was thus demonstrated for the PSR assay, comparable to the LAMP assay, as shown in Figure 1.

3.3. LOD of the PSR Assay

The LOD of the PSR assay was determined after the extraction of DNA from the artificially contaminated samples. qPCR results revealed that the dilutions ranged from 1.6×10^4 to 1.6 GEq/mL. The detection limit of the developed assay was 1.6×10^1, i.e., 16 GEq/mL [Figure 4]. None of the negative controls showed any amplification, which further confirms the specificity of the developed PSR assay.

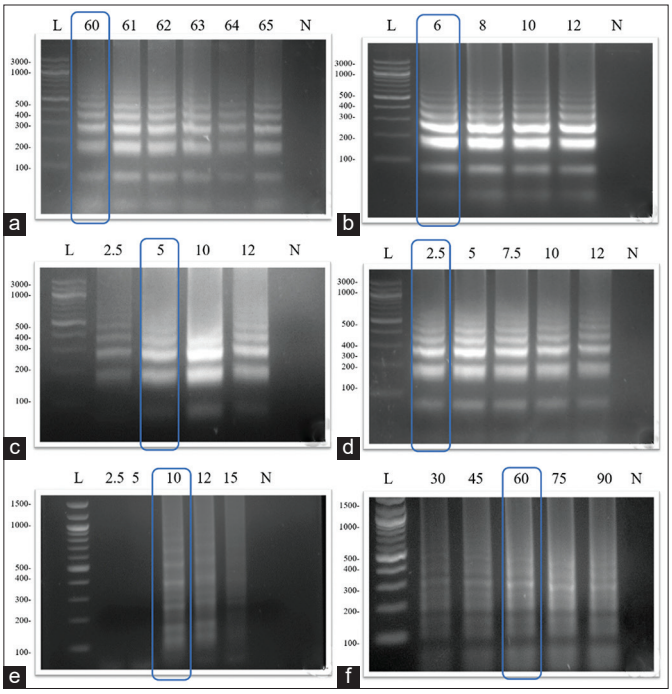

Figure 2: Optimization of reaction conditions of polymerase spiral reaction (PSR) assay. (a) PSR assay incubation temperature 60–65°C, (b) Bst DNA polymerase concentration 6 to 12U, (c) $Mg2^{+}$ concentration 1 to 5 mM, (d) Betaine concentration 2.5 to 12 mM, (e) dNTP concentration 2.5–12 mM, (f) PSR assay incubation time 30–120 min. The optimum conditions have been marked in the figures, (l) 100 bp DNA ladder; (N) Non-template control.

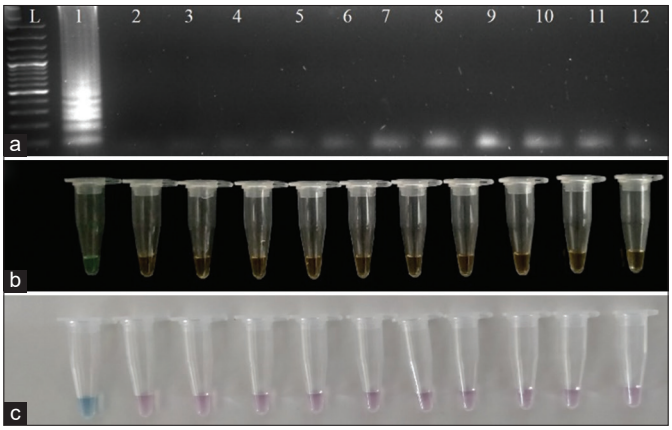

Figure 3: Specificity of polymerase spiral reaction assay using genomic DNA visualized by (a) Gel electrophoresis, (b) Hi-SYBr safe gel stain, (c) Hydroxynaphthol blue dye. Lane 1: *Leptospira interrogans* serovar Canicola, Lane 2: *Leptospira meyeri* serovar Semarangaa, Lane 3: *Vibrio cholera*, Lane 4: *Shigella flexneri*, Lane 5: Methicillin-resistant *Staphylococcus aureus*, Lane 6: *Staphylococcus aureus*, Lane 7: *Staphylococcus epidermidis*, Lane 8: *Enterococcus faecalis*, Lane 9: *Escherichia coli*, Lane 10: *Stenotrophomonas maltophilia*, Lane 11: *Pseudomonas aeruginosa*, Lane 12: Negative control.

4. DISCUSSION

Leptospirosis is a looming threat in both developed and developing nations. The existing detection methods suffer from many drawbacks, including low sensitivity, a large number of false positives, a need for trained personnel, and the time-consuming nature of the tests. A large number of outbreaks occur during the monsoon, especially in the farming community, which is exposed to stagnant water for long periods. Considering the severity of the disease and the diagnostic challenges that it presents, there is an urgent need for a highly sensitive detection method to diagnose the disease early [4,7]. Detecting the causative agent in its early stage is essential from a public health perspective as it can prevent misdiagnosis and identify and prevent the outbreak at the source. This study reports a rapid and sensitive PSR assay to detect *Leptospira* in response to the need for an accurate and efficient diagnostic tool.

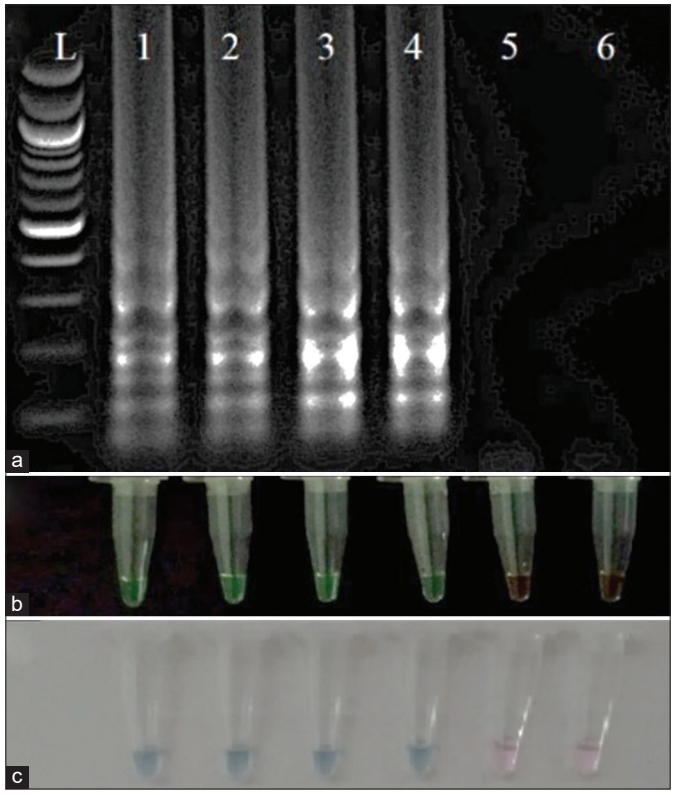

Figure 4: Limit of detection of polymerase spiral reaction assay visualized by (a) Gel electrophoresis (b) Hi-SYBr safe gel stain (c) HNB Dye. Lane 1: 1.6×10^{4}, Lane 2: 1.6×10^{3}, Lane 3: 1.6×10^{2}, Lane 4: 1.6×10^{1}, Lane 5: 1.6 GEq/mL of *Leptospira interrogans* serovar Canicola in tap water; Lane 6: Non-template control.

Our artificial contamination study revealed that the PSR assay could detect up to 16 GEq/mL of *Leptospira*, showcasing its sensitivity within a quick 60-min timeframe. Although LAMP-based assays have been explored for *Leptospira* detection, PSR offers the novel advantage of using only one pair of primers, simplifying the assay while reducing the risk of non-specific amplification [14,15]. Moreover, PSR eliminates the need for a thermal cycler and gel electrophoresis, making it a practical and cost-effective detection technique, particularly in resource-limited settings.

By incorporating end-point indicators such as HNB and Hi-SYBr safe gel stain, in-tube detection of PSR amplicons can be achieved without compromising the surrounding environment through aerosol contamination. Additionally, Bst polymerase, which is less prone to inhibition than Taq polymerase, is used in the PSR assay, further enhancing its accuracy [16,17].

Our study is the first to report a *Leptospira* detection assay based on PSR. The PSR assay is a rapid and simple method that is particularly suitable for resource-limited settings. While our study has shown promising results, further field testing is required to assess the assay's applicability. Given the numerous advantages of the PSR assay over conventional methods, it has the potential to become the preferred method for detecting *Leptospira*.

5. CONCLUSION

Our study successfully developed a Polymerase Spiral Reaction (PSR) assay for the rapid and sensitive detection of Leptospira, addressing the limitations of existing detection methods. The PSR assay exhibited results comparable to the LAMP assay, with a distinctive ladder-like pattern on agarose gel and visual confirmation using metal indicator dye HNB and Hi-SYBr Safe Gel Stain.

Optimization of the PSR assay parameters, including temperature, Bst Polymerase concentration, MgSO4, Betaine, dNTP concentration, and incubation time, revealed robust amplification at 60°C with 6U of Bst Polymerase, 5mM MgSO4, 2.5mM Betaine, and 10mM dNTP. Specificity testing confirmed the assay's reliability, with positive amplification only in the presence of L. interrogans genomic DNA.

The Limit of Detection (LOD) analysis demonstrated the PSR assay's sensitivity, detecting Leptospira concentrations as low as 16 GEq/mL. This surpasses the limitations of current methods, especially in resource-limited settings. The assay's ability to provide quick and accurate results within 60 minutes further positions it as a valuable tool for early and efficient Leptospira detection.

In comparison to traditional PCR, the PSR assay eliminates the need for a thermal cycler, making it more accessible. The use of end-point indicators such as HNB and Hi-SYBr Safe Gel Stain enhances the assay's practicality by enabling in-tube detection and eliminating the need for gel electrophoresis. The assay's high sensitivity, specificity, and simplicity make it a promising choice for Leptospira detection, paving the way for potential applications in real-world settings. Further field testing will be crucial to validate its practical utility.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to 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

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All relevant data has been disclosed in the article. Any additional data required can be made available on request.

12. PUBLISHER'S NOTE

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