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Technical report

Detection of pathogenic *Leptospira* spp. by RPA-NALFIA targeting *lipL32* gene

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Background: Lack of available sensitive point-of-care tests is one of the key challenges limiting the early point-of-care diagnosis of leptospirsis. Previously, a Recombinase Polymerase Amplification (RPA) and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 12a (CRISPR/Cas12a) *lipL32* detection platform with high sensitivity and specificity was developed. However, its turnaround time is between one and two hours, and two reactions are required.
Objective: To develop the RPA in combination with a nucleic acid lateral flow immunoassay (NALFIA) detection platform to reduce the turnaround time and make it a one-step reaction.
Methods: RPA combined with nucleic acid lateral flow immunoassay (NALFIA) detection platform was designed to detect the *lipL32* gene of pathogenic *Leptospira* spp.
Results: In pure culture, the limit of detection (LOD) for RPA-NALFIA was 10^5 cells/mL, whereas quantitative polymerase chain reaction (qPCR) and RPA-CRISPR/Cas12a FBDA/LFDA achieved 10^1 and 10^2 cell/mL, respectively, and none of the diagnostic tests indicated cross-reactivity with other infectious illnesses. In order to detect leptospirosis in clinical samples, the RPA-NALFIA LOD did not achieve the standard as expected. The further modification of the test to reach acceptable LOD is still needed.
Conclusion: A single-reaction RPA-NALFIA targeting the *lipL32* gene was capable of detecting pathogenic *Leptospira* spp. within an hour without the need for costly laboratory equipment, but improvements are necessary.

Keywords: Detection, lateral flow, *leptospira* spp., leptospirosis.

Leptospirosis is a zoonotic disease that affects global health, with over a million cases per year and 58,900 deaths.[1] The disease is caused by pathogenic spirochete *Leptospira* which can adapt to a broad spectrum of mammalian hosts and environment.[1]

Leptospirosis shared clinical signs and symptoms with other infectious diseases, including dengue, sepsis, and malaria. Therefore, it is challenging to diagnose. Current insensitive diagnostic techniques are one of the most significant obstacles to lowering the effect of leptospirosis. Currently, the World Health Organization (WHO) recommends three standard methods: the microscopic agglutination test (MAT), blood culture, and quantitative polymerase chain reaction (qPCR).[1] However, the majority of leptospirosis cases are admitted to rural hospitals that lack the laboratory equipment necessary to perform these tests. Consequently, new diagnostic tests for leptospirosis are still urgently required.[2]

We recently developed a recombinase polymerase amplification (RPA) in combination with a clustered regularly interspaced short palindromic repeat (CRISPR)/ CRISPR-associated protein 12a (CRISPR/Cas12a) fluorescence-based diagnostic

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assay (FBDA) targeting the *lipL32* gene, which pathogenic species-specific gene plays a crucial role in inflammatory responses by binding to Toll-like receptor 2.[3, 4] We demonstrated RPA-CRISPR/Cas12a potential utility in leptospirosis screening with 85.2% sensitivity and 100.0% specificity with the turn-around time of 1 hour and 25 minutes. In addition, a pilot study was conducted employing a lateral flow detection assay (LFDA) readout.[5] However, RPA-CRISPR/Cas12a still required two reactions, which raised the risk of contamination and the turnaround time for the second reaction. Therefore, the purpose of this work was to develop an RPA combination with nucleic acid lateral flow immunoassay (NALFIA) that is an hour-long, single-reaction test.

Materials and methods

Ethics statement

The study protocol has been approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No. 655/63). Furthermore, the study was performed under the international guidelines for human research protection of the Declaration of Helsinki, The Belmont Report, CIOMS Guideline, and International Conference on Harmonization in Good Clinical Practice.

Culture of Leptospira

For the direct culture of *Leptospira interrogans*, which performed as the study's positive control and limit of detection test, 1 mL of fresh whole blood was added to 4 mL of Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium and cultured for two weeks at 30 °C. The culture was inspected using dark field microscopy to confirm the presence of *Leptospira* spp. After confirmation, *Leptospira* cells were counted in a Petroff-Hauser chamber as previously described in.[6, 7] DNA were then extracted at 10^8 cells/mL using the High Pure PCR Template Preparation Kit (Roche, USA): then store at - 20° C.

Detection by qPCR assay

The qPCR targeted at the *lipL32* gene was performed as described previously. Using the forward primer (5'- AAG CAT TAC CGC TTG TGG TG -3'), reverse primer (5' - GAA CTC CCA TTT CAG CGA TT -3'), and Taqman probe (5' -FAM- AA AGC CAG GAC AAG CGC CG-BHQ1-3'), 242 base pair products were amplified and identified. In a final volume of 20 µL, the qPCR mixture contained 5 µL of extracted DNA, 10 µL of SsoAdvanced Universal Probe Supermix (Bio-Rad Laboratories, USA), 1 µL of each primer (10 µM), 0.4 µL of Taqman probe (10 µM), and 2.6 µL of nuclease-free water. In duplicate, qPCR reactions were conducted. As a negative control, a no template control (NTC) containing all of the aforesaid chemicals was utilized. *Leptospira interrogans* DNA extracted from EMJH culture was utilized as a positive control. The StepOnePlus Real-Time PCR System was utilized for amplification and fluorescence detection (Applied Biosystems, USA). The amplification procedure consisted of 10 minutes at 95 °C, followed by 45 cycles of 15 seconds at 95 °C and one minute at 60 °C. A result was considered negative if the threshold cycle (Ct) value was more than 40 cycles.

The RPA

The *lipL32* gene was amplified using the TwistAmp Basic Kit (TwistDx, United Kingdom) using the same primer set as the qPCR as previously studied in the prior work. [5] Briefly, rehydrated lyophilized RPA was mixed with 480 nM of each primer in rehydration buffer. Then, 14 mM of magnesium acetate (final concentration) and 5 µL of extracted DNA were added to the 25 µL total volume reaction mixture. The *lipL32* gene was amplified by incubation at 39 °C for 40 minutes, followed by inactivation at 75 °C for 5 minutes.

RPA-CRISPR/Cas12a FBDA and LFDA

The CRISPR/Cas12a FBDA and LFDA targeting the *lipL32* gene was performed as previous study.[5] The CRISPR/Cas12a reaction with 15µL total volume was composed of 30 nM of crRNA (5' UAAUUUCUACUAAGUAGAUUUCUGAGCGAGGA CACAAUC-3'), 330 nM of EnGen Lba Cas12a (Cpf1) (New England Biolabs, USA), 600 nM of fluorescent probe (5'-FAM-TTATTATT-BHQ1-3'), and 1X of NEBuffer 2.0. (New England Biolabs, USA). At 39° C, the CRISPR/Cas12a reaction was incubated for twenty minutes. Using a BluePAD Dual LED Blue/White Light Transilluminator (BIO-HELIX, Taiwan) with a 470 nm wavelength, the fluorescent signal was then detected. For LFDA, the DNA probe (5- FITC-AGGACCCGTATTCCCA-BIOTIN -3) was used at a concentration of 12 nM under the same conditions as FBDA. The reaction was incubated

for 30 minutes at 39 °C. The reaction was subsequently combined with 100 µL of running buffer and pipetted into a commercial lateral flow strip assay (Kestrelbioscience, Thailand). Uncleaved reporter molecules were caught at the first detection line (test line), whereas the random ssDNA cleavage activity of CRISPR/Cas12a only generated a signal at the second line (control line).

RPA-NALFIA

The RPA was performed using the same condition as described above with 0.48µM *lipL32* forward (5' -FITC-AAG CAT TAC CGC TTG TGG TG -32), reverse primer and reverse primers (5' -Biotin-GAA CTC CCA TTT CAG CGA TT -3'). Then the RPA product was mixed with 100 µL of running buffer and pipetted into the commercial lateral flow strip test (Kestrelbioscience, Thailand). The amplicons of the *lipL32* gene were captured at the first detection line (test line), whereas the negative results were not generating the band at the test line. Therefore, the visible band at the control line indicated that the test is valid.

Limit of detection (LOD) and cross-reactivity testing

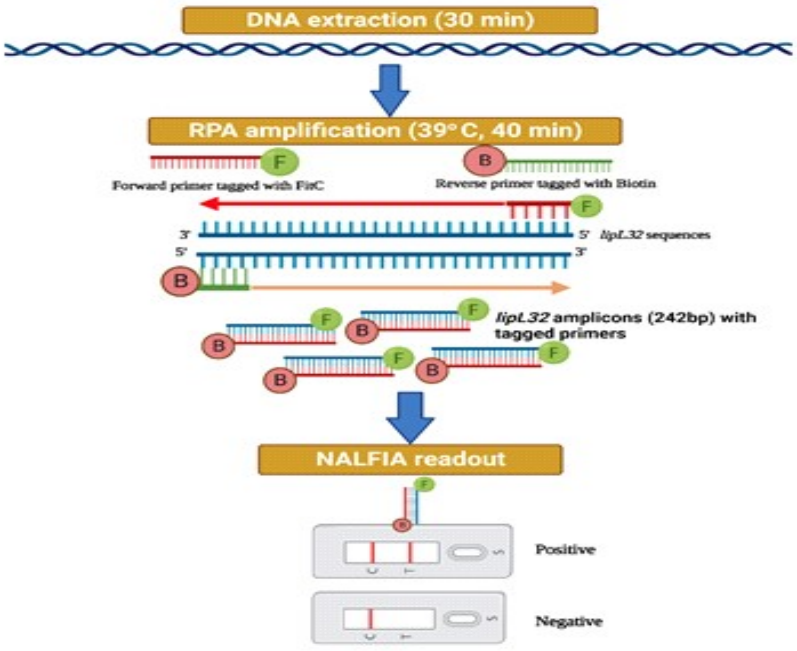
The LOD testing used genomic DNA extracted from *Leptospira interrogans* in EMJH cultures. The genomic DNA was diluted serially from 10^6 cells/mL to 1 cell/mL.[8] Determining the LOD involved detecting the signal in the tube containing the lowest cells. The specimens obtained from patients with an acute febrile illness, including acute viral hepatitis, scrub typhus, influenza, *Escherichia coli* septicemia, and dengue hemorrhagic fever, were tested to establish the analytical specificity of qPCR, RPA-CRISPR/Cas12a FBDA/LFDA and RPA-NALFIA.

Results

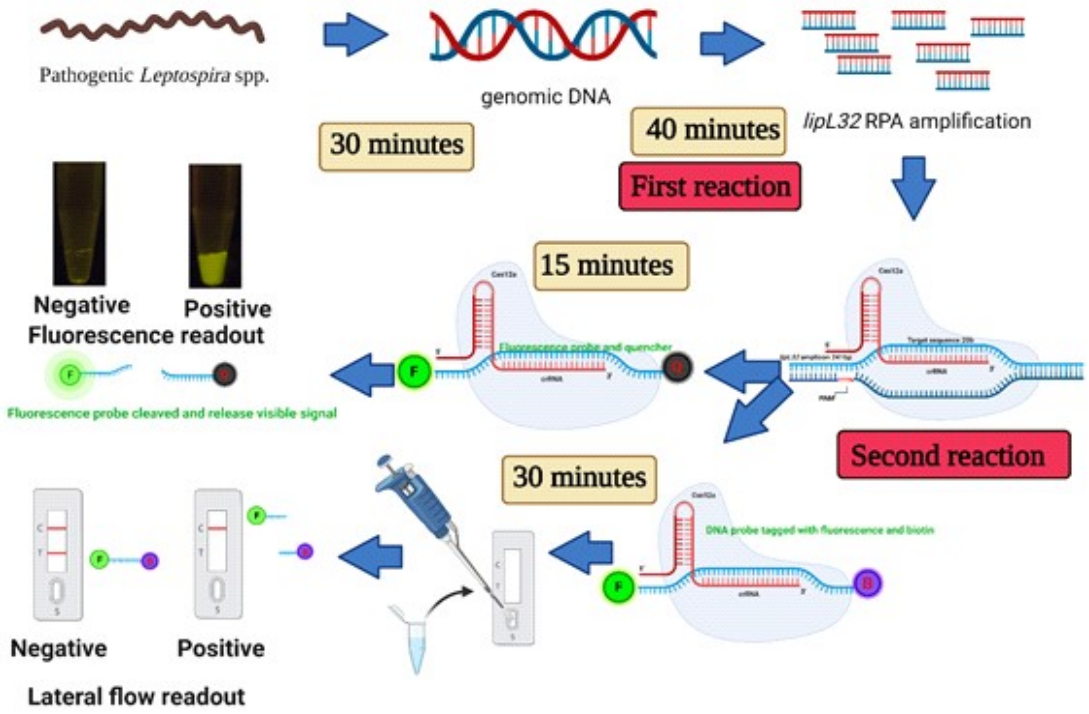
The LOD of qPCR, RPA-CRISPR/Cas12a FBDA/LFDA and RPA-NALFIA

The LOD of the qPCR, RPA-CRISPR/Cas12a FBDA/LFDA and RPA-NALFIA was tested by extracting DNA from a *Leptospira interrogans* culture that was serially diluted from 10^7 to 1 cell/mL. The schematic of the RPA-CRISPR/Cas12a FBDA/LFDA and RPA-NALFIA assay is shown in Figure 1A and B. The qPCR can detect *Leptospira* at 10^1 cell/mL, RPA/CRISPR-Cas12a FBDA/LFDA at 10^2 cell/mL whereas RPA-NALFIA at 10^5 cell/mL (Figure 1C).

A



B



C

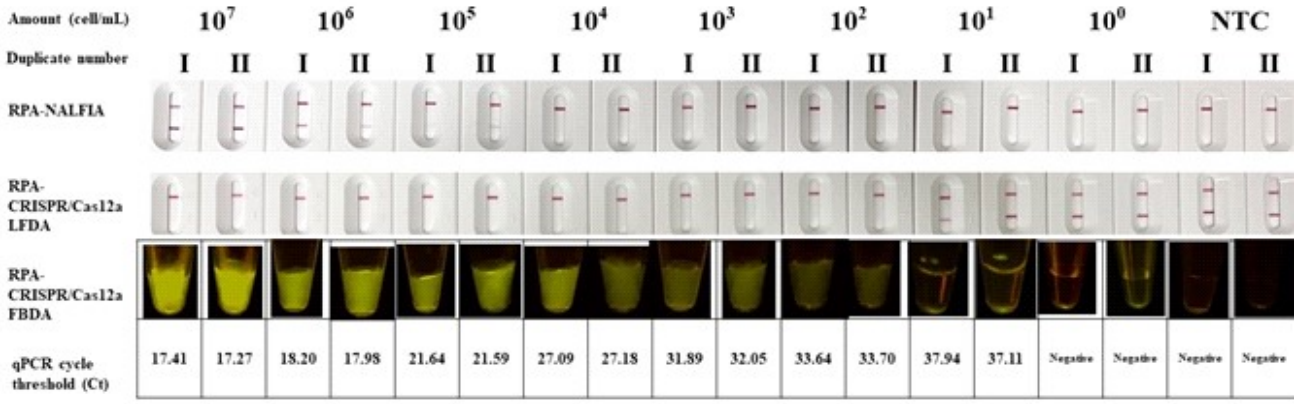


Figure 1. LOD testing using the qPCR, RPA-CRISPR/Cas12a FBDA/LFDA and RPA- NALFIA: (**A** and **B**) Schematics of the RPA-NALFIA and RPA-CRISPR/Cas12a FBDA/LFDA workflows, respectively (Created with BioRender.com); (**C**) LOD of each diagnostic methods. NTC is a non-template control.

Cross-reactivity testing

Five specimens from patients diagnosed with acute viral hepatitis, scrub typhus, influenza, *Escherichia coli* septicemia, and dengue hemorrhagic fever were analyzed using qPCR, RPA-CRISPR/Cas12a FBDA/LFDA, and RPA-NALFIA. The outcomes demonstrated no cross-reactivity (Figure 2).

Discussion

The RPA-NALFIA is a single-reaction and isothermal nucleic acid detection platform that can be used to diagnose many infectious diseases.[9 - 11] This study developed a pathogenic *Leptospira* spp. detection using the RPA-NALFIA assay targeting the highly conserved *lipL32* genes which exclusive to pathogenic *Leptospira* spp. [3, 4] We compared to RPA-CRISPR/Cas12a FBDA/LFDA and qPCR. The RPA-NALFIA unsuccessfully reach an acceptable LOD. According to a prior study, the presence of more than 1,000 *Leptospira* cell/mL was related with severe leptospirosis.[12] Consequently, LOD at 10^5 cell/mL may not achieve the benefit of

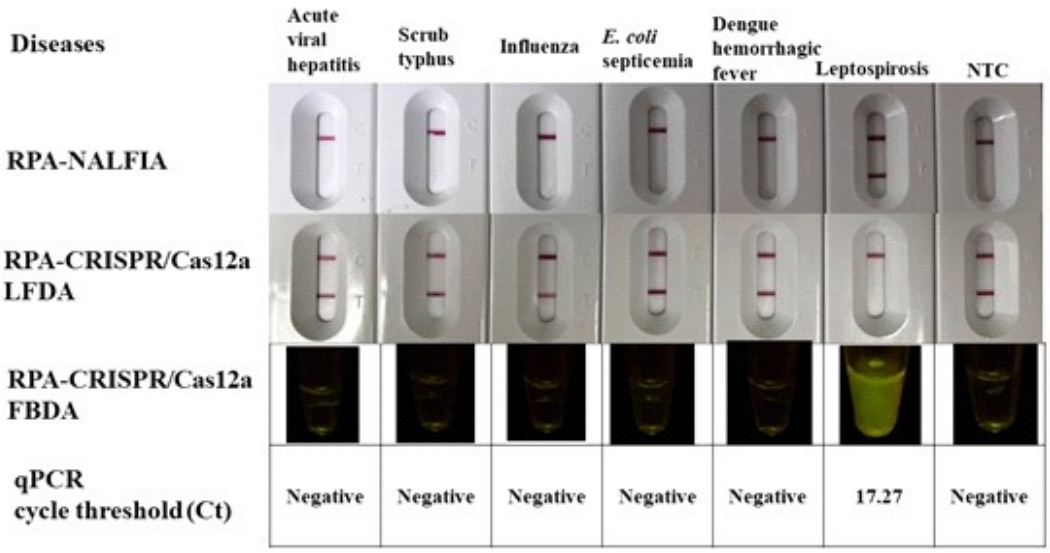


Figure 2. Cross-reactivity testing using the qPCR, RPA-CRISPR/Cas12a FBDA/LFDA and RPA-NALFIA. NTC is a non-template control.

RPA-NALFIA condition adjustments

In order to achieve the better LOD of the RPA-NALFIA, the RPA amplification time variation was performed ranging from 10 minutes to 60 minutes with the extracted DNA of a Leptospira culture at 10^7 cell/mL. Unfortunately, the false-positive results were observed with 50 minutes and 60 minutes amplification time (Figure 3).

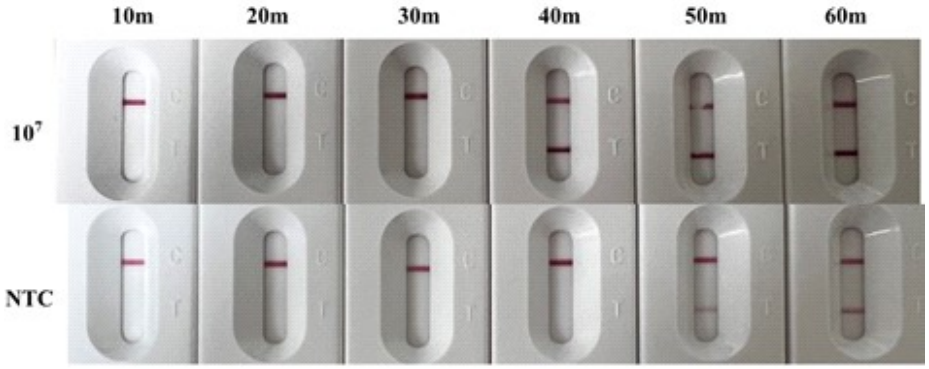


Figure 3. Adjustment of the RPA-NALFIA condition by varying the reaction time. NTC is a non-template control

early treatment to avert severity. In contrast to qPCR and RPA-CRISPR/Cas12a, FBDA/LFDA reached an adequate level of 10^1 and 10^2 cell/mL. Additionally, qPCR, RPA-CRISPR/Cas12a FBDA/FLDA, and RPA-NALFIA did not demonstrate cross-reactivity with other infectious illnesses. RPA condition adjustment to reach better LOD has caused the false positive due to unspecific amplification. Several studies have also encountered a similar issue.[13 - 15] The possible solution for future work is to design RPA primers with self-avoiding molecular recognition systems (SAMRS). [16] RPA-CRISPR/Cas12a FBDA/LFDA tolerance to false positives due to nonspecific amplification because CRISPR/Cas12a crRNA worked as a double check; hence, our earlier study demonstrated a specificity of 100.0%. [5]

A recent work demonstrated that the presence of PCR inhibitors in clinical samples can influence the sensitivity of qPCR for identifying *lipL32*.[17] The RPA's tolerance to PCR inhibitors makes it the optimal amplification platform for this study. [18]

The first strength of our study is that the RPA-NALFIA-based detection of *lipL32* requires less expensive laboratory equipment, such as a qPCR machine, and is therefore suitable for remote hospital settings. The only requirement for the isothermal reaction is a heat block. Second, compared to RPA-CRISPR/Cas12a FBDA/LFDA, RPA-NALFIA is a single reaction, which reduces the risk of contamination and turnaround time.

However, our research has a number of drawbacks. First, we must still make adjustments to our methods in order to reach our target LOD. Once the appropriate LOD has been determined, it is required to validate with clinical samples in order to determine the sensitivity and specificity.

Conclusion

Our study results may not have met the required LOD threshold. To achieve the optimal LOD, the RPA-NALFIA-based detection technique targeting the *lipL32* gene can be enhanced. This RPA-NALFIA platform could be useful in resource-limited settings.

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Conflicts of interest statement

The authors have each completed an ICMJE disclosure form. None of the authors declare any potential or actual relationship, activity, or interest related to the content of this article.

Data sharing statement

The present review is based on the references cited. Further details, opinions, and interpretation are available from the corresponding authors on reasonable request.

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