Recombinase Polymerase Amplification Combined with a Lateral Flow Strip for the Detection of *Plasmodium knowlesi*

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Abstract. The aim of this study was to develop a recombinase polymerase amplification (RPA) combined with a lateral flow (LF) strip method for specific diagnosis of *Plasmodium knowlesi*. With incubation at 37°C, the 18S rRNA gene of *P. knowlesi* was successfully amplified within 12 minutes. By adding a specifically designed probe to the reaction solution, the amplified RPA product can be visualized on a LF strip. The RPA assay exhibited high sensitivity with limits of detection down to 10 parasites/μL of *P. knowlesi*. Nonetheless, it was demonstrated that all *P. knowlesi* (N = 41) and other *Plasmodium* sp. (N = 25) were positive while negative samples (N = 8) were negative. Therefore, a combination of RPA and LF strip detection is a highly promising approach with the potential to be suitable for use in resource-limited settings.

According to World Malaria Report 2016, malaria causes the most deaths (estimated 235,000 to 639,000 per year) worldwide among parasitic infections.¹ People staying in low-resource setting rural areas are at high risk of getting the disease.² *Plasmodium knowlesi* is newly recognized as an important human pathogen in addition to *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium falciparum*. *Plasmodium knowlesi* is the main cause of malaria in Malaysia, and cases have been reported throughout Southeast Asia, including cases in Thailand, Indonesia, Singapore, Philippines, Vietnam, Cambodia, Indonesia, and Myanmar.³ The World Health Organization (WHO) Malaria Policy Advisory Committee recognized the importance of this parasite and constituted an Evidence Review Group on *P. knowlesi* to discuss strategy control, mitigation, and appropriate preventive methods.¹

Humans are a good reservoir for *P. knowlesi*; however, it has been proved that it remains a zoonosis that naturally infects long-tailed and pig-tailed macaques.³ A wide variety of reservoirs threaten the effort of eliminating malaria infection. In addition, the lack of adequate diagnostic approaches contributes to this factor too.⁴ Nonetheless, *P. knowlesi* could be misdiagnosed as *P. malariae* or *P. falciparum* due to their morphological similarities. Following a case-control study, *P. knowlesi* malaria patient with parasitemia of more than 35,000 parasites/μL should be managed using WHO guidelines for the treatment of severe malaria to prevent deadly complications.⁵ In this regard, a rapid and accurate diagnosis method is a major tool to be used to eradicate and control malaria infections.

Traditionally, microscopic examination is the gold standard for malaria diagnosis. However, adequate expertise is required for interpretation of smears, particularly in samples with low parasitemia. Polymerase chain reaction (PCR) is an alternative method to diagnose malaria specifically by amplification of the DNA due to a designed gene-specific primer. However, the PCR-based method cannot be widely used for diagnosis because of the laborious operation, long reaction time, expensive instrument, and PCR reagents required.

Recombinase polymerase amplification (RPA) is a recently developed nucleic acid amplification-based isothermal method.^{6,7} With the aid of a simple instrument, such as a heating block, the RPA amplification reaction can be performed at a constant low temperature. This is a rapid method because the result can be obtained within 15 minutes. In this study, we developed a lateral flow RPA (LF-RPA) molecular technique to diagnose *P. knowlesi* malaria efficiently and rapidly. The amplified RPA amplicon is loaded onto a lateral flow (LF) strip and a significant band on the strip can be visualized after 5 minutes incubation in assay buffer. Combination of LF strip and RPA technology promptly gives us a result within 20 minutes. This is the first work to demonstrate that RPA detection for *P. knowlesi* results can be read out with LF strips.

To conduct the LF-RPA assay, blood samples were obtained from the University of Malaya Medical Center and Sarawak State Health Department. DNA was extracted from whole blood using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany). The samples were previously examined under microscope and diagnosed with the BinaxNOW Malaria Kit (Alere, Ontario, CA) before DNA extraction. This study was approved by the Medical Research and Ethics Committee of the Ministry of Health Malaysia (NMRR-15-672-23975) and the Medical Ethics Committee of the University of Malaya Medical Center (MEC Ref. No. 817.1). Negative samples involved in this assay consisted of non-*Plasmodium* sp. samples.

The RPA assay was performed using the primers and probes listed in Table 1. The *P. knowlesi* small subunit ribosomal RNA (18S rRNA) and *Plasmodium* genus-specific gene was used as the target gene and the primers and probes were designed based on the manufacturer's recommendations (TwistDx, Cambridge, United Kingdom). Endpoint detection of the amplification product was performed by LF technology that required a specifically designed LF probe. The probes consisted of an oligonucleotide backbone with a 5'-carboxyfluorescein (5'-FAM), a tetrahydrofuran (THF) residue that replaces a nucleotide, and a polymerase extension blocking group, C3-spacer at the 3' end. For the detection of *Plasmodium* species genus, the LF probe was used with a reverse amplification primer, which was biotin-labeled at the 5' end. Another oligonucleotide primer that was involved in the reaction (equidirectional with the probe) is a conventional primer. To detect the *P. knowlesi* genus-specific gene, the LF probe designed is similar to *Plasmodium* genus gene, which contains a FAM

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TABLE 1
Primer and probe sequence in this study

Name	Sequence (5'-3')
Pk F	CCGTTCTCATGATTTCATGGTCCAGGGTT
Pk R	(B)CCTGAACACCTCATGTCGTGGTAGAAATAG
Pk LF Probe	(F)CCGTTCTCATGATTTCATGGTCCAGGGTT (H)AGTTTTTTCGGTCCC[3]
Plas genus F	CATGGCTATGACGGGTAACGGGGAATTAGA
Plas genus R	(D)AATTGGGTAATTTACGCGCTGCTGCCTTC
Plas genus Probe	(F)CATGGCTATGACGGGTAACGGGGAATTAG A(H)TTCGATTCCGGAGAG[3]

Features: F = FAM-dT; H = Tetrahydrofuran; 3 = C3-spacer; B = biotin; D = digoxigenin. The primer sequence for PK R was labelled with biotin-tag (B) at 5' end. The PK LF probe and Plas genus probe used in this study were particularly created with an insertion of an abasic nucleotide analogue (a tetrahydrofuran residue, H) flanked by a dT-FAM (F) at 5' end and carbon blocker (C3 spacer, [3]) at 3' end. Plas genus R primer was labelled with digoxigenin-tag (D) at 5' end.

group, THF, and C3-spacer. However, this probe was used together with reverse amplification primer which was digoxigenin-labeled at the 5' end. Another oligonucleotide primer that is involved in the reaction is a forward amplification primer. As advised by the manufacturer, both primer and probe were diluted to 10 μ M as the working solution throughout the entire study. The RPA assay was conducted using the commercial TwistAmp nfo kit (TwistDx). The 50- μ L RPA reaction mixture was made of 11.2 μ L of water, 1 $\times$ rehydration buffer, 400 nM each of the forward and reverse primers, and 120 nM of the FAM-tagged probe was added to the RPA dried pellet. After mixing all these components, 4 μ L of DNA was added to the reaction mixture followed by 2.5 μ L of 280 mM magnesium acetate, which was pipetted into the tube caps instead of the reaction mixture directly. Tube cap was closed during the spinning process and the reaction was initiated. The reaction tube was then incubated in a heating block with constant temperature at 37°C for approximately 15 minutes. After incubation, 1 μ L of the RPA amplification product was diluted in 99 μ L of dilution buffer (provided with the kit). One Hybridetec-2 LF strip (Milenia Biotec, Giessen, Germany) was placed vertically into the diluted mixture and incubated at room temperature with the final result read at 5 minutes. For a positive sample, LF-RPA amplicon was observed as two test lines (TL1 and TL2) on the LF strips (Figure 1). TL1 indicates a genus-specific gene and TL2 shows *P. knowlesi*-specific gene detection. A control line, which is immobilized with antirabbit antibodies serves as the RPA assay control. The test was considered invalid if the control line was absent.

To investigate the effect of temperature on RPA amplification, the RPA product was incubated at 30°C and 37°C, and it was found that *P. knowlesi* was successfully amplified after 15 minutes incubation at both temperatures, suggesting that the LF-RPA is not sensitive to aberration in the temperature profile during the reaction.

For the evaluation of the signal enhancement, amplified RPA products were tested in different dilution buffers labeled as buffers A (50 mM Trizma-HCl and 150 mM NaCl), B (50 mM Trizma-HCl, 150 mM NaCl, 0.05% Tween-20), and C (25 mM Trizma-HCl, 150 mM NaCl, pH 7.2). We found that buffer B was the most suitable dilution buffer as the band of amplified RPA product was more visible with less background compared with other dilution buffers (Figure 2). The formation of antigen-antibody complex on strips can be affected by pH and ionic strength of the assay buffer. It was noted that Tris

buffer is important in DNA solubilization. Here, buffers with different concentrations of Tris were tested. Buffer with addition of Tween-20 showed a promising result as it may allow a better flow of the antigen-antibody-nanoparticle matrix through the strips. No visible adsorption was observed in both TL1 and TL2 for the blank sample. These observations were made by Liu et al.,⁸ that the presence of surfactants had a great influence in decreasing the background signal.

The reaction time was determined by stopping the reaction at 5, 10, 12, and 15 minutes by immediate dilution and analysis on the LF dipsticks. A signal on the LF dipstick can be detected after 12 minutes of amplification reaction at 37°C and < 5 minutes of incubation in assay buffer, although the band is weaker in comparison to longer amplification times. To standardize the result, 12 minutes of RPA reaction time and 5 minutes incubation in assay buffer were used in the subsequent analysis. Compared with other molecular diagnostic approaches, LF-RPA highlighted the significance of diagnostic advantages associated with sample amplification and result read-out time. Moreover, the dried pellet form used in this study helped to eliminate time for reaction mixture preparation.

To determine the detection limit of the developed LF-RPA assay in this study, DNeasy Blood and Tissue kit (Qiagen) was used to extract and purify the DNA from *P. knowlesi* strain A1H1 culture. A 10-fold serial dilution of the stock (10^5 parasites (p)/ μ L to 1 p/ μ L) was performed with sterile distilled water. One microliter of each of the diluted DNA was used as the template. In the work presented here, the detection limit of *P. knowlesi* RPA was as low as 10 copies (Figure 3). The DNA template from each of the serial dilutions was tested in duplicate and repeated twice to ensure the accuracy of the result.

The specificity of LF-RPA was tested using DNA templates from other non-*P. knowlesi* parasites such as *Toxoplasma gondii*, *Sarcocystis* sp., *P. falciparum*, *P. vivax*, and *P. ovale*. The results show that LF-RPA was 100% specific for all *P. knowlesi* samples where both test lines and control line

FIGURE 1. Detection of recombinase polymerase amplification (RPA) amplified product on lateral flow strip. TL1 indicates a genus-specific gene and TL2 shows *Plasmodium knowlesi*-specific gene detection. A sample was interpreted as negative if only the CL was visible. Representative image of specificity of RPA on genomic DNA extracted from patient samples. CL = control line; Strip 1 = negative control (distilled water); Strip 2 = *Plasmodium vivax*; Strip 3 = *Plasmodium falciparum*; Strip 4 = *Toxoplasma gondii*; Strip 5 = *Sarcocystis* sp. Strip 6 = *P. knowlesi*; TL1 = test line 1; TL2 = test line 2. This figure appears in color at www.ajtmh.org.

FIGURE 2. Effects of different concentration of Tris in running buffer A (50 mM Trizma-HCl and 150 mM NaCl), buffer B (50 mM Trizma-HCl, 150 mM NaCl, 0.05% Tween-20), and buffer C (25 mM Trizma-HCl, 150 mM NaCl, pH 7.2) on recombinase polymerase amplification product. Strip 1, 3, and 5: Negative control (distilled water); 2, 4, and 6: *Plasmodium knowlesi* sample. This figure appears in color at www.ajtmh.org.

were present within 5 minutes of incubation at room temperature (Figure 1). Meanwhile, only TL1 and control line were present when tested with non-*P. knowlesi* strains (11 *P. falciparum*, 12 *P. vivax*, and 2 *P. ovale*) (Figure 1). In further experiments, DNA extracted from eight healthy donors was used as template in the LF-RPA reaction. The LF-RPA did not detect any of the negative DNA samples.

With the aid of nucleic acid LF immunoassays, double stranded amplification products could be detected within a short time by labeling with three antigenic tags during the enzymatic reactions.⁹ These antigenic tags are FAM, digoxigenin, and biotin labeled, which had been incorporated into specifically designed primer and probe. The end product of RPA could be detected in a convenient LF strip. The

FIGURE 3. Determination of the detection limit of *Plasmodium knowlesi* recombinase polymerase amplification (RPA). A 10-fold serial dilution of the stock (10^5 *P. knowlesi* parasites (p)/ μ L to 1 p/ μ L) was performed with sterile distilled water. The DNA template from each of the serial dilutions was tested in duplicate and repeated twice. Strip 1, 2, 3, 4, 5, and 6 indicate RPA assay using 10^5 to 1 p/ μ L as template. This figure appears in color at www.ajtmh.org.

instrument-free detection is particularly suited to point-of-care use.

The LF-RPA has limit of detection (10 p/ μ L) surpasses the analytical sensitivity of microscopy examination (200 p/ μ L)¹⁰ and rapid diagnostic tests such as BinaxNow Malaria kit (> 100 p/ μ L).¹¹ On top of that, the LF-RPA is either more sensitive or equivalent in the detection of *P. knowlesi* than other molecular diagnostic methods such as loop-mediated isothermal amplification (LAMP). By using *P. knowlesi* β -tubulin gene as the target gene, Iseki et al.¹² managed to detect *P. knowlesi* down to 200 copies of DNA only. However, using 18S rRNA as the target gene, the sensitivity of LAMP was improved and managed to detect *P. knowlesi* DNA down to 10 copies by Lau et al.¹³ Real-time PCR was another approach used to detect *P. knowlesi* because of its low detection limit ability. Divis et al.¹⁴ reported that the analytical sensitivity for amplification of *P. knowlesi*-specific assay was 10 copies/ μ L using a TaqMan real-time PCR assay.

To date, LF-RPA in the detection of *P. knowlesi* has not been published. However, Kersting et al.⁷ developed a rapid LF-RPA for the detection of *P. falciparum* and the specificity was 100%. Their result indicated that the detection limit was as low as 100 fg of genomic DNA *P. falciparum*, which corresponds to approximately four parasites per reaction. Here, we developed a LF strip detection on *Plasmodium* sp. and *P. knowlesi* in a combination with RPA technology. This is the first LF-RPA technology in malaria detection.

However, there is some limitation of LF-RPA technology in this study. Some false positive results may be generated because of the inhibitory effect arising from blood, anticoagulants from the tube, and residuals from lysis buffer used during the DNA extraction. These inhibitor factors have an influence on the enzymatic amplification by direct binding to the enzymes or hindering the binding of cofactors to the enzyme.^{15–17} To eliminate false positive occurrences, uniform sampling and handling methods are suggested. Blood samples can be collected in the same type of tubes. DNA extraction from all samples was prepared using the same protocol. Besides, blood samples may be kept in a form of direct-blood spot on the filter paper. In future, as reported by Li et al.¹⁸ in their isothermal thermophilic helicase-dependent amplification of five human *Plasmodium* sp., a direct-blood method without further sample preparation is expected to be used in LF-RPA technology.

In conclusion, the LF-RPA assay developed here provides a robust *P. knowlesi* DNA amplification system that is more advanced than currently available DNA diagnostic methods. LF-RPA is a simple-to-conduct method requiring minimal infrastructure and the results are easily interpreted by untrained personnel, and it has the potential for point-of-care diagnosis of *P. knowlesi* in endemic field settings with further improvement.

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