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Rapid Diagnosis of Plasmodium knowlesi Infection Utilizing the Loop-Mediated Isothermal Amplification Technique Coupled with a Lateral Flow Assay

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Abstract:

Objective: To develop a highly sensitive and specific molecular diagnostic assay for detecting *P. knowlesi* by combining LAMP with lateral flow assay (LFA).

Material and Methods: Six LAMP primers targeting the *P. knowlesi* 18S rRNA gene were designed, incorporating FITC and biotin labels into the FIP and LoopF primers. Specificity and sensitivity were evaluated using DNA from various *Plasmodium* species. Clinical samples were tested, and the results were visualized on LFA.

Results: The *Pk*LAMP-LFA assay demonstrated a sensitivity of 95.4% (95% CI: 84.5–99.4%) and a specificity of 100% (95% CI: 15.8–100%) compared to nested PCR. The limit of detection was 10 copies/μL. The assay produced results within 60 minutes and required only minimal equipment.

Conclusion: The *Pk*LAMP-LFA assay is a rapid, accurate, and field-deployable diagnostic tool for *P. knowlesi*, offering a practical solution for improved malaria detection and control in endemic regions.

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Introduction

Plasmodium knowlesi is becoming more prevalent in Southeast Asia[1]. It has a natural reservoir host involving the long- and pig-tailed macaque and the banded-leaf monkey (*Macaca fascicularis*, *M. nemestrina*, and *Presbytis melalophos*, respectively)[2]. The distribution of *P. knowlesi* infections has also been reported in Malaysia[3], extending to other regions of Southeast Asia[1]. In Thailand, the first case of *P. knowlesi* infection was documented in Southern Thailand in 2004[4], and since then, the number of patients has steadily increased[4,5]. Over the last decade, there has been a substantial decrease in total malaria incidence. While *P. falciparum* cases have decreased, *P. vivax* cases are rising. In early 2023, a report from the Department of Disease Control of Thailand revealed that *P. knowlesi* is now one of the top two causes of malaria infections[5]. The biology of *P. knowlesi* involves rapid multiplication every 24 hours of the erythrocytic cycle, as well as the ability to invade every stage of erythrocytes. This outcome can potentially result in a wide spectrum of symptoms and multiorgan failure, particularly in cases of hyperparasitemia[6]. Distinguishing the morphological features of *P. knowlesi* from *P. falciparum* during the asexual erythrocytic stages has posed challenges, especially during the ring stage to early trophozoites, while the other stages resemble those of *P. malariae*. The rapid multiplication and minor differences between *P. knowlesi* and *P. malariae* may lead to misdiagnosis and delayed treatment. Therefore, accurate diagnostic tests and timely treatment are needed[7–9]. Currently, rapid diagnostic tests (RDTs) are used to detect malaria parasites in field studies. However, RDTs are not specific to *P. knowlesi*, resulting in low sensitivity[10–11]. The World Health Organization (WHO) has recommended using molecular methods, including nested PCR, which is considered the gold standard for the diagnosis of *P. knowlesi*[8]. However, due to limitations, including cost, complexity, and extended assay time, nested PCR is not appropriate for routine field diagnostics[12]. Therefore, alternative methods, such as loop-mediated isothermal amplification (LAMP), can be employed to detect pathogens, such as bacteria, fungi, and parasites, in a cost-effective and timely manner, particularly in remote areas[13]. The LAMP assay operates at a constant temperature of 60 to 65 °C and requires a set of 4–6 distinct primers to adhere at 6–8 different regions on the target gene[14–16]. This nucleic acid amplification relies on the repeating polymerization of the target sequence based on the strand displacement activity of *Bst* DNA polymerase[14]. This enzyme demonstrates resistance to inhibitors commonly found in biological samples, including hemoglobin, and therefore, it does not require DNA purification. LAMP products are easily detectable via turbidity[15], various color indicators[17], agarose gel electrophoresis, and real-time turbidity measurement. Nonetheless, the detection methods have some limitations, such as potential interference from the hemoglobin color in the LAMP reactions, the non-specificity of the color indicators, or the requirement of more costly automated equipment[18]. LAMP combined with a lateral flow assay (LFA) is based on the principles of DNA hybridization technology and antigen-antibody reactions. Commonly, biotin and fluorescein isothiocyanate (FITC) are used as markers for primers. Dual labeling of the amplified DNA product is followed through interaction with anti-FITC antibodies in a lateral flow assay. Within 5 minutes, the resulting DNA complex becomes visible as a purple band on a strip test. Therefore, the combination of the LAMP technique and LFA has the potential for on-site diagnosis in a resource-limited setting[15,19]. Many studies have reported the potential of the LAMP-LFA platform as a diagnostic tool

for many pathogens, such as bacteria, fungi, protozoans, and viruses. A LAMP-LFA assay has been developed for detecting *Salmonella* in cases of food-safety monitoring[20]. It was applied for the rapid detection of SARS-CoV-2, offering a portable diagnostic solution during the COVID-19 pandemic[21]. It can be used for detecting *Toxoplasma gondii* in meat samples, indicating its utility in foodborne pathogen surveillance[22]; this method can also be used for the detection of *P. falciparum* and *P. vivax* in resource-limited and malaria-endemic regions[23].

However, the incidence of *P. knowlesi* malaria is less common in Thailand than in *P. vivax* and *P. falciparum*; the control of *P. knowlesi* malaria presents additional challenges due to its zoonotic nature. Managing *P. knowlesi* malaria involves multiple dimensions, including vector control, accurate diagnosis and treatment, as well as public health interventions.

This study developed a rapid diagnostic approach utilizing LAMP, illustrated by LFA techniques, to detect *P. knowlesi* in the field. The application demonstrated potential in improving the field diagnosis of *P. knowlesi*, representing a critical step in preventing severe disease progression and mortality.

Material and Methods

Clinical samples collection

Fifty-one malaria patients were used in this study. They were suspected of having either *P. knowlesi* or *P. knowlesi and/or P. malariae* infection by malaria clinic staff. Among these, 31 patients were identified at malaria clinics in Ranong and Chumphon under the Office of Disease Prevention and Control 11, Thailand, and 20 patients were received from Surat Thani Hospital, between 2019 and 2022. Microscopic examinations, including both thick and thin blood smears, were performed in all samples. Samples collected from the malaria clinic were stored as dried blood spot (approximately 50-80 µL) on filter paper, individually sealed in plastic bags with silica beads. For hospital cases, approximately 1 mL of leftover whole blood was collected for analysis.

Ethical approval for this study was obtained from the Ethics Committee of Prince of Songkla University, Thailand (HRECMT64-003).

Sample size calculation

Sample size was calculated to ensure sufficient precision in estimating the diagnostic accuracy of the *Pk*LAMP-LFA assay. Based on the expected sensitivity of 90%, specificity of 95%, a 95% confidence level, and a precision of ±10%, the minimum number of positive samples required to reliably estimate sensitivity was 35, and the minimum number of negative samples required to estimate specificity was 19. These values were calculated using the standard formula for proportions: , where Z=1.96 for 95% confidence, p is the expected proportion (sensitivity or specificity), and d is the desired precision. This approach is commonly used in diagnostic test evaluations[24].

Target gene and primer design

LAMP primers were designed to target *P. knowlesi* by amplifying the *18S rRNA* gene, known for its dispersed distribution across the chromosomes of the *Plasmodium* parasite, with typically 4-8 copies per genome, making it a well-established molecular target for malaria identification[25]. Nucleotide sequences for the *18S rRNA* gene from 5 human and 4 simian malaria strains were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). The primers for *P. knowlesi* detection were designed using Primer Explorer V5 software (http://primerexplorer.jp/e/), with 12 target sequences including *P. knowlesi* (L07560.1), *P. vivax* (X13926.1), *P. falciparum* (AF145334.1), *P. ovale* (L48987.1), *P. malariae* (M54897.1), *P. cynomolgi* (AB287290, MF582549), *P. inui* (FJ619079, AB287276), *P. coatneyi* (FJ619094, AB265790), and *P. simiovale* (AB287285) (Figure 1, Table 1). The

specificity of the LAMP primers was verified using Primer–BLAST to confirm that there was no cross–reactivity with other *Plasmodium* species (http://www.ncbi.nlm.nih.gov/tools/primer–blast/). F3 and B3 were external primers, covering 219 bp, while FIP and BIP were internal primers, composed of F1c/F2 and B1c/B2, respectively. LoopF and LoopB were also designed to enhance the specificity. The internal primers were purified to HPLC grade, and the FIP and LoopF primers were labeled with FITC and biotin at the 5' end.

Figure 1 Schematic representation for nucleotide sequence alignment of 12 *Plasmodium* sp. based on *18S rRNA* gene and F3, B3, FIP and BIP sequences used in the *Pk*LAMP assay

Table 1 The nucleotide sequences of LAMP primers

Primers	Sequence (5'–3')	Length (bp)
FIP	***FITC***–TAAAAGAACACATAATAGCTCCACATAA CTGATGCCTCCG	40
BIP	TAAGATTCATCTATTAATCAAAGTAACAAGATCATCA	37
F3	GCAACGCTTCTAGCTATA	18
B3	CTGTAGTATTCAAACGCAC	19
LF	***Biotin***–GCGCACAGTCGATACGC	17
LB	AATTCGCTTCGGCATAAT	18

Plasmid construction

The PCR amplicon was amplified using the F3 and B3 primers, purified, and then ligated into the pGEM-T easy vector (Promega, Madison, WI). The resulting recombinant plasmid was transformed into *E. coli* JM109 competent cells (Promega, Madison, WI) using the heat-shock method. The transformed solution was shaken at 37 °C for 2 hours and then streaked onto an LB agar plate containing 100 µg/mL of ampicillin. The plate was incubated at 37 °C overnight. Positive clones were verified using the colony PCR method after randomly picking 15-20 colonies. The recombinant plasmids were purified with a GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA). The concentration of plasmid was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) based on UV absorption at 260 nm. The plasmid copy number per microliter was calculated using the following formula: copy number/µL= [(6.02×(10^{23})×(ng of plasmid per microliter)]/[(vector (bp)+insert (bp)) ×660×10^{9}]. Based on the initial plasmid concentration of 69.75 ng/µL, the copy number was determined as 2x10^{10} copies/µL.

Lateral flow assay

The lateral flow device in this study comprised a sample pad, which absorbs the sample, and a conjugate pad housing a gold nanoparticle coated with streptavidin that binds to the biotin-labeled *Pk*LAMP product. Anti-FITC and bovine serum albumin (BSA) conjugated with biotin were immobilized at the test line and control line, respectively (Figure 2). Five microliters of *Pk*LAMP product, comprising FITC and biotin-labeled LAMP products corresponding to the FIP and LF primers, respectively, were loaded into the well of a lateral flow cassette, followed by the addition of 2 drops of running buffer (approximately 100 µL), with results observed within 5 minutes. The biotin-labeled *Pk*LAMP amplicon formed a complex with streptavidin on the conjugate pad, which then moved through the membrane, resulting in labeled-FITC binding to anti-FITC coated on the test line and a color change. In case of amplification, any remaining streptavidin conjugated with the gold nanoparticles bound to BSA-biotin on the control line. The red-colored control line must be present to accurately interpret the result.

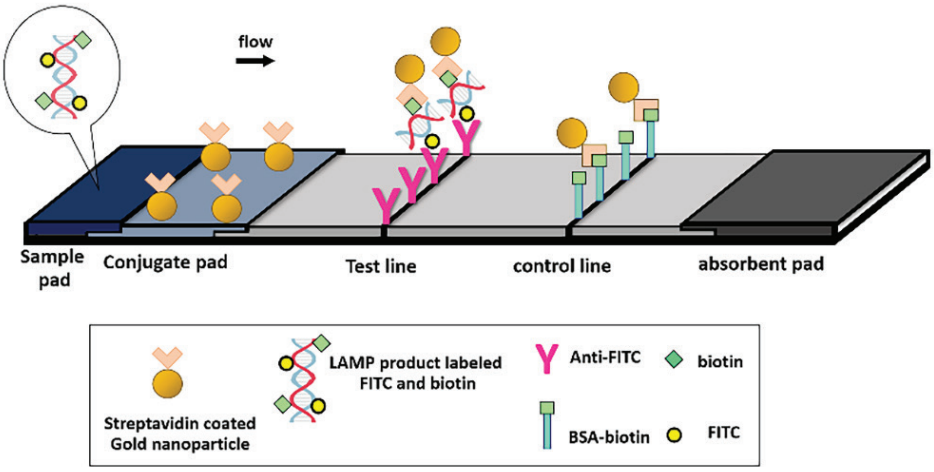


Figure 2 Schematic diagram of *Pk*LAMP-FLA products on a lateral flow test

*Pk*LAMP optimization

The *Pk*LAMP method was optimized using 1 µL of *P. knowlesi 18S rRNA* plasmid (10^5 copies/µL) as a template in a total reaction volume of 25 µL. The reaction mixture consisted of 1X amplification buffer, 3.5 mM dNTPs, 1.6 µM inner primers, 0.2 µM outer primers, and 8 U of *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs, MA, USA). Different concentrations of 4, 6, and 8 mM $MgSO_4$ and 0.4 and 0.8 µM loop primers were tested while varying the temperature and time at 60, 63, and 65 °C for 60 minutes. The reaction was stopped by heating it at 80 °C for 10 minutes, and the LAMP products were analyzed by electrophoresis in 2% agarose gel.

Specificity and sensitivity of *Pk*LAMP–LFA detection

The specificity of the *Pk*LAMP primers was confirmed using the DNA of *P. knowlesi* and other *Plasmodium* species, including 1 sample of *P. malariae*, 5 samples of *P. vivax*, 3 samples of *P. falciparum*, 1 sample of *P. inui*, 1 sample of *P. cynomolgi*, 1 sample of *P. coatneyi*, and 2 samples of human DNA. All parasite DNA was species–confirmed by sequencing analysis. For the sensitivity detection of *Pk*LAMP–LFA, 2 different types of samples were used. First, the plasmid carrying the *18S rRNA* gene was serially diluted 10–fold using nuclease–free water, varying from 10^5 to 10^{-3} copies/µL. Second, the plasmid was spiked using whole blood, treated with a lysis buffer (400 nM NaCl, 40 mM Tris pH 6.5, 0.45% SDS)[26], and then boiled at 95 °C for 5 minutes in a water bath, followed by centrifugation at 10,000 g for 1 minute. The supernatant was pipetted into a new 1.5 mL centrifuge tube using a boil–and–spin protocol as previously described[26]. All plasmid templates (1 µL/reaction) and DNA templates (3 µL/reaction) were tested by the *Pk*LAMP–LFA method. The amplified products were analyzed using both agarose gel electrophoresis and lateral flow assay (5 µL). The primers F3 and B3 were used to compare the sensitivity of the developed method with conventional PCR. A total volume of 25 µL was used in the PCR reaction, including 1X buffer, 2.5 mM $MgSO_4$, 200 mM of dNTPs, 200 nM of each primer, 0.4U of *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, California, USA), and 1 µL of the diluted plasmid. The PCR temperature cycling conditions were as follows: initial denaturation at 95 °C for 5 minutes, 35 cycles of 95 °C for 1 minute, 58 °C for 1 minute, and 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes. The Limit of Detection (LOD) was determined by a probit regression analysis curve using MedCalc software. The LOD was obtained from 6 replicates of plasmid sample serial dilutions ranging from 10^5 to 10^{-3} copies/µL and from 3 replicates of serial dilutions from plasmid spiked in whole blood samples.

Evaluation of clinical samples

All DNA samples, whether from the 31 dried blood spots or 20 whole bloods, were extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For dried blood samples, a single spot with a blood volume of approximately 50–80 µL and a diameter of 1.5–2 cm was used for DNA extraction. For whole blood samples, 200 µL of blood was subjected to DNA extraction following the manufacturer's protocol. The DNA concentration from each sample was measured using a nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) based on UV absorption at 260 nm. The DNA concentration of dried blood samples ranged from 3.19 ng/µL to 93.74 ng/µL, with a mean value of 13.4 ng/µL and a standard deviation of 15.75 ng/µL. For the whole blood samples, DNA concentration varied from 3.23 ng/µL to 49.2 ng/µL, with a mean value of 14.36 ng/µL and a standard deviation of 12.75 ng/µL. To evaluate the practicality of *Pk*LAMP in clinical samples and direct blood testing, 37 purified DNA samples, comprising

31 from dried blood spots and 6 from whole blood, were tested using an extraction kit. To detect *P. knowlesi* DNA in the samples, 14 of the 20 samples were treated with a lysis buffer (mentioned above) to lyse the red blood cells, followed by the boil-and-spin method to extract the DNA.

The samples were then confirmed as positive for malaria species detection using a nested PCR assay targeting the mitochondrial *cytochrome b* (*Cytb*) gene[27]. First round of PCR was performed in a 20 µL reaction containing 1X PCR buffer (Invitrogen Life Technologies, CA), 3 mM $MgSO_4$, 250 µM dNTPs, 150 nM of each primer, 1 U of *Taq* DNA polymerase, and 2 µL of DNA template. The thermal cycling conditions for the first round included an initial denaturation at 94 °C for 4 minutes, followed by 25 cycles of 94 °C for 40 seconds, 50 °C for 30 seconds, and 72 °C for 30 seconds, with a final extension at 72 °C for 5 minutes. The second round of PCR was conducted using the same cycling parameters for 35 cycles. Amplification products were analyzed by agarose gel electrophoresis. Positive control DNA for *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* was obtained from clinically confirmed mono-infection samples and validated by sequencing.

For *Pk*LAMP-LFA assay, 3 microliters of each sample were tested. The amplification products were visualized using gel electrophoresis, and a portion of the products (5 µL) was subjected to lateral flow assay. If none of the malaria species were amplified, the original sample was retested by increasing the DNA concentration using the speed vacuum method. This provided a larger quantity of DNA for the PCR reaction, thereby potentially improving the amplification results[28].

The accuracy of the *Pk*LAMP-LFA assay in diagnosing *P. knowlesi* in clinical samples was evaluated by comparison with the nested PCR, which served as the reference standard. The results were categorized as true positive (TP), true negative (TN), false positive (FP), or false negative (FN). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated at the 95% confidence interval (95% CI) using Medcalc® statistical software. Sensitivity was calculated as TP/(TP+FN)×100, with specificity TN/(TN+FP)×100, PPV TP/(TP+FP)×100, and NPV TN/(TN+FN)×100.

Results

Optimization of the *Pk*LAMP conditions

The *Pk*LAMP assay was tested with different components and conditions, including concentrations, durations, and temperatures, to optimize the reaction system for *P. knowlesi* detection based on the *18S rRNA* gene. In this study, the optimized reaction system for *Pk*LAMP assay included 2.5 µL of 10X isothermal amplification buffer, 8 mM of $MgSO_4$, 1.4 mM of dNTPs, 1.6 µM of each FIP and BIP, 0.8 µM of each LF and LB, 0.2 µM of each F3 and B3, and 8 units of *Bst* 2.0 WarmStart DNA polymerase (New England BioLabs Inc.) with nuclease-free water added to bring the total reaction volume to 25 µL per reaction. The *Pk*LAMP amplification reactions were carried out at 65 °C for 60 minutes, and the enzyme activity was stopped at 80 °C for 10 minutes. Various components and conditions were optimized to improve the ability of the *Pk*LAMP-LFA assay to detect *P. knowlesi*.

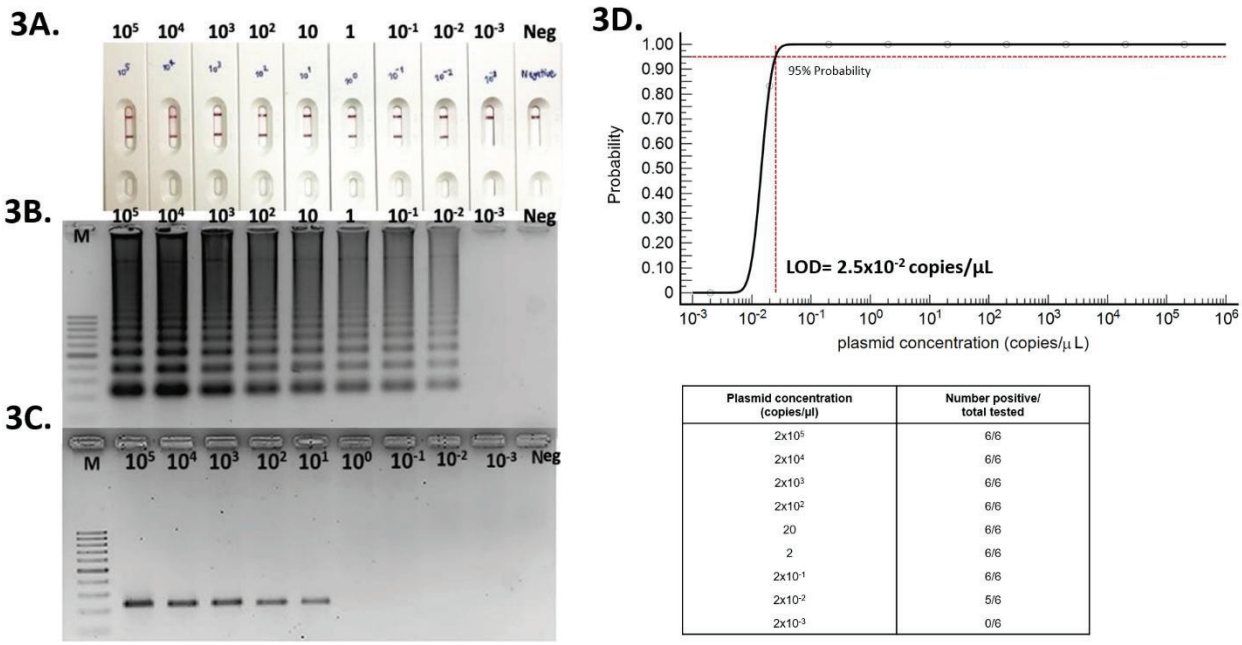
Analytical specificity and sensitivity of *Pk*LAMP-LFA assay

The accuracy of *Pk*LAMP-LFA primers for detecting *P. knowlesi* was validated against various *Plasmodium* sp., including *P. knowlesi*, *P. malariae*, *P. vivax*, *P. falciparum*, *P. inui*, *P. cynomolgi*, and *P. coatneyi*, as well as human DNA. The assay accurately detected *P. knowlesi* without producing any false positives with other human and non-human primate *Plasmodium* species (Supplementary Figure 1). The LOD of the *Pk*LAMP-LFA assay with plasmid at 95%

probability was 2.5x10^{-2} copies/μL, compared with the PCR assay giving 1,000-fold higher sensitivity, with an LOD of 10 copies/μL (Figure 3). However, for the spiked whole blood with plasmid extracted using the boil-and-spin method, *P. knowlesi* was detected at 0.87 copy/μL (Figure 4). This result showed lower sensitivity in plasmid *P. knowlesi* detection compared to plasmid diluted with nuclease-free water by a factor of approximately 300, possibly due to the high temperature during boiling that denatured the plasmid DNA, causing the double-stranded molecule to break apart into single strands. The centrifugation process also degraded plasmid DNA, resulting in the formation of smaller, fragmented DNA molecules that were not properly amplified by downstream methods, leading to lower sensitivity[29].

Validation of clinical samples by *Pk*LAMP-LFA assay

A total of 51 malaria samples were analyzed, comprising 31 dried blood spot samples and 20 whole blood samples. Microscopic evaluation determined that 20 whole blood samples (39.2%) were positive for *P. knowlesi* mono-infections, 23 dried blood spot samples (45.1%)



Plasmid concentration (copies/μl)	Number positive/ total tested
$2x10^5$	6/6
$2x10^4$	6/6
$2x10^3$	6/6
$2x10^2$	6/6
20	6/6
2	6/6
$2x10^{-1}$	6/6
$2x10^{-2}$	5/6
$2x10^{-3}$	0/6

Figure 3 The limit of detection (LOD) of PkLAMP-LFA assay with varying concentrations of plasmid. 3A) Detection of PkLAMP products by lateral flow assay, and 3B) Visualization of PkLAMP products on 2% agarose gel electrophoresis, and 3C) Comparison of PkLAMP products and PCR results, respectively. 3D) Estimated LOD using probit regression analysis. The data in the table show the number of positive samples/total analyzed samples. 3A) – 3C): Serially diluted P. knowlesi plasmid DNA. M: 100 bp DNA ladder, lanes 1-8 represented 105 to 10-3 copies/μL, Neg; non-template control.

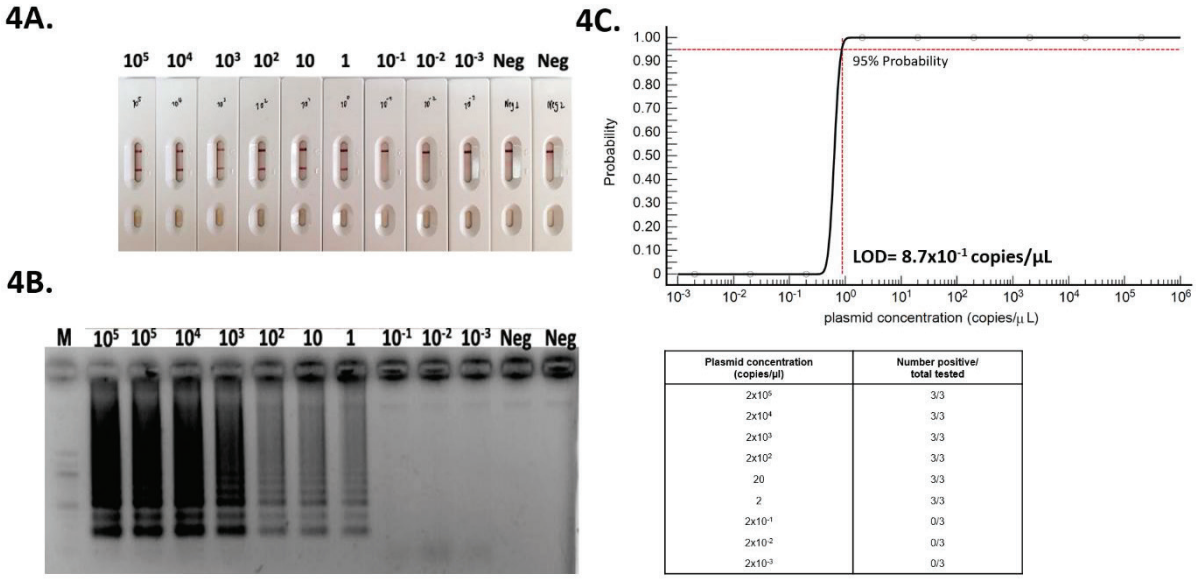


Figure 4 The limit of detection (LOD) of the boil–and–spin extraction method for whole blood spiked with serially diluted plasmid DNA (10–fold dilution). 4A) Detection of PkLAMP product by lateral flow assay, and 4B) Visualization of PkLAMP products on 2% agarose gel electrophoresis. 4C) Estimated LOD using probit regression analysis. The data in the table show the number of positive samples/total analyzed samples. M; 100 bp DNA ladder, lanes 1 and 2 contained 105 copies/µL of P. knowlesi plasmid DNA. Lanes 3–10 display a range from 104 to 10–3 copies/µL of P. knowlesi plasmid DNA. Neg; non–template control.

were reported as either *P. knowlesi* or *P. knowlesi* and/or *P. malariae* infections, and 8 dried blood samples (15.7%) were positive for *P. malariae* and *P. vivax* human malaria parasites. The nested PCR results showed that 48 out of 51 (94.1%) samples were positive for *P. knowlesi* detection, and 2 of these were positive for a mix of *P. knowlesi* and *P. malariae*. For the remaining 3 samples, 2 were positive for *P. malariae*, and 1 sample was not amplified. The *Pk*LAMP–LFA method accurately detected *P. knowlesi* in 46 out of 48 clinical isolates, while only 2 dried blood spot samples were not amplified (Table 2, Supplementary Figure 2), possibly due to factors such as ambient storage temperature, parasitemia, and length of storage[30]. The *Pk*LAMP–LFA assay showed a diagnostic sensitivity of 95.83% compared to *Pk*–nested PCR at 100%. The PPV of the *Pk*LAMP–LFA assay was 100%, while the NPV was 60% and the accuracy was 95.9%.

Table 2 Comparison of *P. knowlesi* diagnostic results between *Pk*LAMP–LFA and nested PCR

	Nested PCR for *P. knowlesi*		
***Pk*LAMP–FLA**	**Positive**	**Negative**	**Total**
Positive	46	0	46
Negative	2	3	5
Total	48	3	51

Discussion

Microscopic examination is widely considered the most accurate method for malaria diagnosis, but misdiagnosis remains common, particularly with *P. knowlesi*. Patients with *P. knowlesi* infection have demonstrated effective responses to antimalarial treatment[31]; however, delayed treatment in cases of high parasite infection can result in severe consequences, leading to complications and death[32]. The sensitivity of microscopy in diagnosing malaria varies between 50–500 parasites/μL, depending on the level of expertise and training of the microscopist. Rapid diagnostic tests (RDTs) have a lower sensitivity and can only detect parasitemia at less than 100 parasites/μL for 4 *Plasmodium* species[33]. Because of their limited sensitivity, RDTs are not considered sufficient for clinical diagnosis[34,35]. Other molecular methods, such as nested polymerase chain reaction (PCR) tests, are highly sensitive and can detect *P. knowlesi* parasitemia at as low as 10 parasites/μL[36]. However, PCR tests require a significant amount of laboratory resources and are also time-consuming. This study developed a LAMP technique to detect *P. knowlesi* with high sensitivity and specificity, based on beta-tubulin and AMA-1 genes capable of detecting 100 and 10 copies/μL, respectively[37,38]. The LAMP assay had a specificity of only 30% due to cross-reactivity with *P. vivax* mitochondrial genes[39], despite having an LOD of 0.2 parasites/μL. The Eiken LoopampTM Malaria Pan Detection Kit was developed to improve clinical malaria diagnosis. This has higher sensitivity than RDTs but cannot identify the *P. knowlesi* species. Detection of *P. knowlesi* using a real-time turbidimeter is expensive and not practical for field applications[40–42]. To address this limitation, LAMP was combined with lateral flow devices to create an easy-to-use diagnostic tool with high sensitivity, which has been used to detect malaria species and improve product visualization[43,44]. This tool has been used to detect *P. knowlesi* parasite plasmids with a minimum detection limit of 0.01 pg/μL[45].

In this study, the *Pk*LAMP–LFA assay demonstrated a lower limit of detection of $2.5x10^{-2}$ copies/μL or $5x10^{-8}$ pg/μL, for both purified and spiked plasmids, as 1,000-fold higher than conventional PCR. Using *Bst* 2.0 WarmStart DNA polymerase provided robust amplification and stability at room temperature, allowing for a convenient reaction setup that does not raise concerns about the enzyme during transportation and storage, making it suitable for application in field conditions. The LAMP assay is well known for its tolerance to inhibitors commonly present in biological samples, enabling efficient amplification of target DNA from minimally processed specimens. In this study, a lysis buffer—easily prepared in the laboratory—was used in combination with the boil-and-spin method to simplify sample preparation. The LAMP–LFA technique, which is robust against impurities such as hemoglobin in whole blood, was successfully validated for the specific detection of *P. knowlesi* without the need for commercial DNA purification kits. This simplified approach reduces both the cost and complexity of DNA extraction, making it more practical for field-based studies and point-of-care applications. Whole blood spiked with the plasmid, followed by extraction using the boil-and-spin method, resulted in detection at 0.87 copy/μL, indicating a lower sensitivity for plasmid *P. knowlesi* detection compared to plasmid diluted 300-fold with nuclease-free water. This was due to the high temperature during boiling, which denatured the plasmid DNA, causing the double-stranded molecule to break apart into single strands. The physical process of centrifugation may also damage or degrade plasmid DNA, which can negatively impact downstream amplification methods such as LAMP[30]. The *Pk*LAMP–LFA technique successfully detected *P. knowlesi* infection from whole blood samples processed using a lysis solution in combination with the boil-and-spin method, achieving a detection threshold as low as 3.23 ng/μL of DNA. However, there were 2 samples, which initially had DNA concentrations of 6.2 ng/μL and 7.1

ng/μL as measured by Nanodrop, that were retested. After concentrating the DNA using a speed vacuum, the DNA yield increased by 2–3 times. This study lacked raw parasitemia data for these samples, limiting our ability to correlate DNA concentration with parasite load, which is an important factor that could have strengthened the interpretation of our results. The *Pk*LAMP-LFA assay was determined as an effective and user-friendly diagnostic tool for detecting *P. knowlesi* in clinical samples. The *Pk*LAMP-LFA assay exhibited a high level of reliability, as indicated by 95.9% accuracy and a relatively low rate of false results. Various factors, such as sample quality, storage conditions, and parasite densities, can affect the results of the diagnostic tests. In this study, microscopy showed a lower accuracy of 80.4% compared to nested PCR, mainly because of misidentification. Our results suggest that the *Pk*LAMP-LFA assay demonstrates comparable sensitivity and accuracy to the nested PCR, offering superior performance to microscopy. However, some discrepancies were observed between the results of *Pk*LAMP-LFA assay and their nested PCR. These inconsistencies may be attributed to the nature of the clinical specimens, which consisted of dried blood spots collected and stored over a five-year period. Sample degradation over time could have affected DNA quality and, consequently, assay performance. Additionally, the relatively low negative predictive value (NPV) observed in this study is acknowledged as a limitation. To more accurately evaluate the diagnostic performance of the *Pk*LAMP-LFA assay, a larger and more diverse set of clinical samples is required. Future studies should also incorporate a control group of uninfected individuals to improve diagnostic accuracy and enhance the robustness and generalizability of the validation process.

Furthermore, the good sensitivity and specificity of the *Pk*LAMP-LFA method demonstrate its potential support and applicability for field-based detection of *P. knowlesi*. As little as 200 μL of whole blood, processed using an extraction solution followed by the boil-and-spin method, is sufficient for DNA extraction. This simplified approach offers advantages in terms of cost-effectiveness and reduced turnaround time, with an estimated cost of approximately 150 THB per reaction and a total turnaround time of 90 minutes from sample preparation to result visualization.

This expanded evaluation is crucial for enhancing the method's accuracy and reliability, which are prerequisites for its eventual deployment as a practical tool for malaria diagnosis. The use of the boil-and-spin method for blood DNA extraction, along with the lateral flow strip test for result visualization, was shown to be a suitable tool for field studies. *Pk*LAMP-LFA assay has the potential for adaptation to portable and handheld devices, facilitating point-of-care testing. This adaptability will empower healthcare workers in the field to conduct immediate diagnosis, resulting in faster treatment initiation and reducing the burden on centralized healthcare facilities.

Conclusion

A diagnostic tool, *Pk*LAMP-LFA, was developed by combining the LAMP method with lateral flow chromatography. This assay targets the *18S rRNA* gene of *P. knowlesi* and can be performed in 1 hour using a heat block or water bath, with simple DNA extraction without the need for complicated extraction procedures or an amplification machine. Findings indicate that the *Pk*LAMP-LFA assay demonstrated high reliability, showing sensitivity comparable to that of nested PCR, and displayed greater accuracy than microscopy. The use of lateral flow chromatography makes the *Pk*LAMP-LFA assay easy to use and suitable for field studies, providing potential improvements for point-of-care malaria diagnostics.

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Conflict of interest

The authors declare that there are no competing interests.

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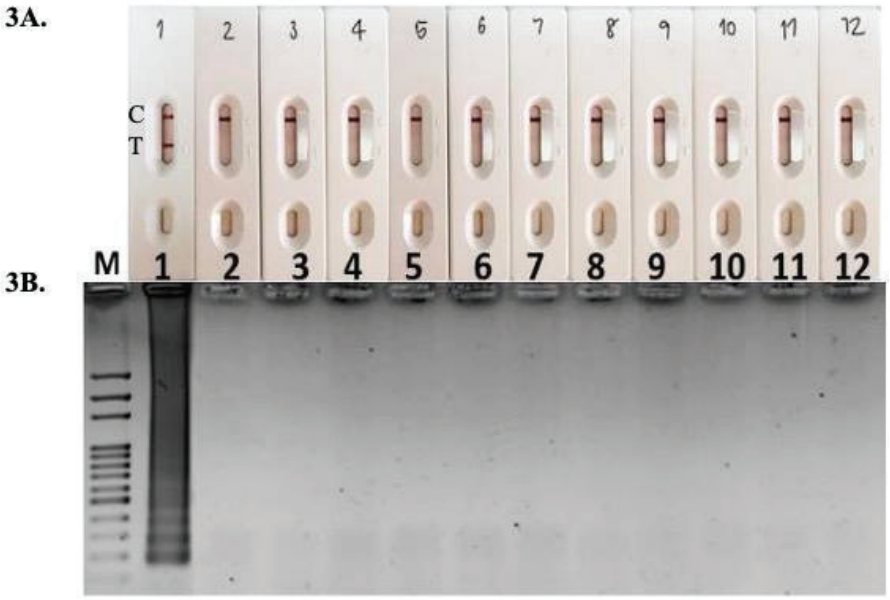
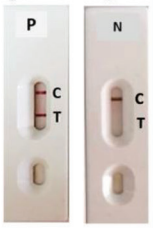
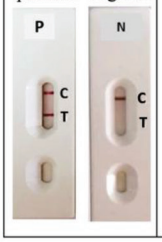
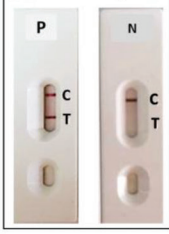
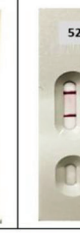


A) Detection of *Pk*LAMP products by lateral flow assay, and B) visualization of compatible *Pk*LAMP bands using agarose gel electrophoresis. Genomic DNA of *P. knowlesi* was used as a positive control in lane 1, *P. malariae;* lane 2, two of *P. vivax*; lane 3 and 4, two of *P. falciparum;* lane 5 and 6, *P. inui;* lane 7, *P. cynomolgi;* lane 8, *P. coatneyi*; lane 9, human DNA; lane 10 and two of non-template control; lane 11 and 12. M; 100 bp DNA ladder

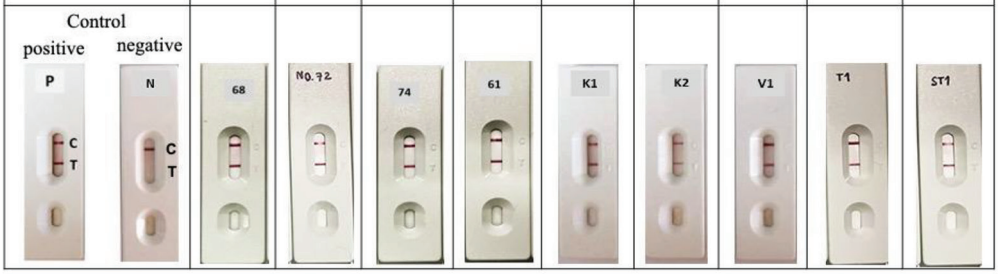
Supplementary Figure 1 The validation of the specificity of the *Pk*LAMP-LFA assay designed for *P. knowlesi* using lateral flow assay and agarose gel electrophoresis

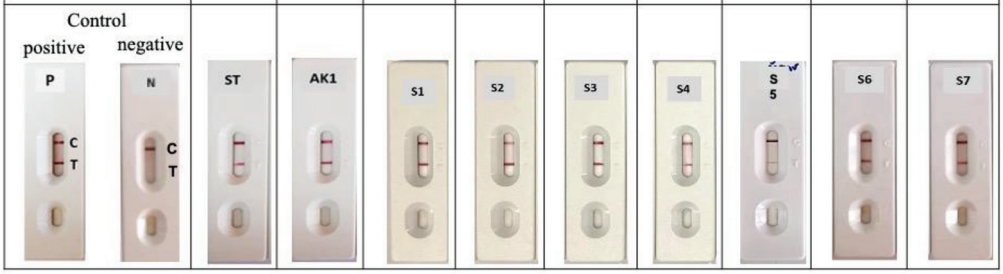
Sample no.	1	2	3	4	5	6	7	8	9
Microscopy	Pv	Pm	Pm/Pk	Pm/Pk	Pm/Pk	Pm	Pm	Pm/Pk	Pm/Pk
Nested PCR	Pk	Pk	Pk	Pk	Neg	Pm/Pk	Pk	Pk	Pk
*Pk*LAMP-LFA	Neg	Pk	Pk	Pk	Neg	Pk	Pk	Pk	Pk
Control positive negative									

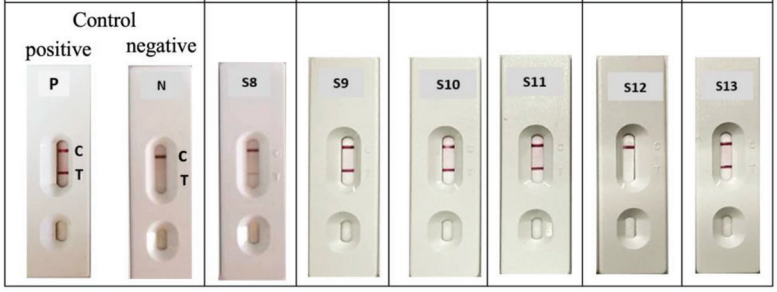
Sample no.	10	11	12	13	14	15	16	17	18
Microscopy	Pm/Pk	Pm/Pk	Pm/Pk	Pm	Pm/Pk	Pv	Pv	Pm/Pk	Pm/Pk
Nested PCR	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk
*Pk*LAMP-LFA	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk
Control positive negative									

Sample no.	19	20	21	22	23	24	25	26	27
Microscopy	Pm/Pk	Pv	Pm/Pk	Pm/Pk	Pm/Pk	Pm/Pk	Pm/Pk	Pm/Pk	Pm/Pk
Nested PCR	Pm/Pk	Pm	Pk	Pk	Pk	Pk	Pk	Pk	Pk
*Pk*LAMP-LFA	Neg	Neg	Pk	Pk	Pk	Pk	Pk	Pk	Pk
Control positive negative									

Supplementary Figure 2 Validation of clinical samples using the developed *Pk*LAMP method combined with lateral flow assay

Sample no.	28	29	30	31	32	33	34	35	36
Microscopy	Pm/Pk	Pm/Pk	Pm/Pk	Pm/Pk	Pk	Pk	Pk	Pk	Pk
Nested PCR	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk
*Pk*LAMP-LFA	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk
									

Sample no.	37	38	39	40	41	42	43	44	45
Microscopy	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk
Nested PCR	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk
*Pk*LAMP-LFA	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk	Pk
									

Sample no.	46	47	48	49	50	51
Microscopy	Pk	Pk	Pk	Pk	Pk	Pk
Nested PCR	Pk	Pk	Pk	Pk	Pm	Pk
*Pk*LAMP-LFA	Pk	Pk	Pk	Pk	Neg	Pk
						

Supplementary Figure 2 (continued)