Development of Loop-Mediated Isothermal Amplification–Based Lateral Flow Device Method for the Detection of Malaria

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Abstract. The present study aims to develop a method for rapid diagnosis of malaria using loop-mediated isothermal amplification (LAMP) combined with a lateral flow device (LFD). By adding the biotin-labeled and fluorescein amidite-labeled loop primers to the LAMP reaction solution, the end product can be visualized on a LFD. The entire procedure takes approximately 42 minutes to complete, LAMP assay exhibited high sensitivity, as the detection limit was 0.01 pg/μL for all five *Plasmodium* species. It was demonstrated that all *Plasmodium knowlesi* ($N = 90$) and *Plasmodium vivax* ($N = 56$) were positively amplified by LAMP-LFD assay, whereas healthy donor samples ($N = 8$) were negative. However, not all mixed infections were positive, and other infected nonmalaria samples were negative. Loop-mediated isothermal amplification-LFD represents a robust approach with potential suitability for use in resource-constrained laboratories. We believe that LAMP-LFD has a potential to be developed as point-of-care diagnostic tool in future.

Malaria is a mosquito-borne disease caused by *Plasmodium* spp., a genus of unicellular parasites. Accurate assays for early diagnosis of malaria play an important role in the elimination of malaria infections among the global population. Most malaria cases in 2016 were in the World Health Organization (WHO) African Region (90%), followed by the WHO South-East Asia Region (7%), and the WHO Eastern Mediterranean Region (2%). India carried 80% of the global malaria burden among 91 countries reporting indigenous malaria cases in 2016.¹ Most malaria cases are reported from rural areas due to poverty, low health consciousness and disease control, and poor transport facilities. In early years, human malaria was thought to be caused by *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium falciparum*. However, *Plasmodium knowlesi* has now emerged as the fifth human malaria parasite since a large number of human infections were reported in Kapit Division, Sarawak, Malaysia in 2004. Since then, cases have been reported throughout other Southeast Asian countries, such as Thailand, Indonesia, Singapore, Philippines, Vietnam, Cambodia, and Myanmar.²

Traditionally, Giemsa-stained microscopy examination is used as the laboratory diagnosis tool for early detection of malaria. However, substantial expertise is required for interpretation of smears, especially in samples with low parasitemia. Besides, rapid diagnostic test (RDT) kits based on examination of parasite antigens in blood are also used as a laboratory diagnosis tool. Nevertheless, both diagnostic tools are lacking clinical and analytical sensitivity.

To improve the efficiency of malaria diagnosis techniques, molecular approaches, such as polymerase chain reaction (PCR), are used to diagnose malaria. Polymerase chain reaction provides an accurate result because of the gene-specific primers involved. Lately, conventional PCR has been modified and developed into real-time PCR, nested PCR, reverse transcriptase PCR, and multiplex PCR. However, PCR-based methods cannot be widely used for diagnosis because

of the costly equipment, PCR reagents, laborious operation, and long reaction time required. Loop-mediated isothermal amplification provides an alternative nucleic acid amplification method used for the detection of malaria parasites. This method is principally based on the characteristic of *Bacillus stearothermophilus* (*Bst*) DNA polymerase enzyme, which possesses high autocycling strand displacement reaction.³ Loop-mediated isothermal amplification involves four primers (forward inner primer [FIP], backward inner primer [BIP], F3, and B3) identified at six distinct regions on the targeted gene. However, only the inner primers (FIP and BIP) are used to synthesize the new DNA strand. To shorten amplification duration, two loop primers (forward-loop primer [FLP] and backward loop primer [BLP]) are incorporated into the reaction mixture.⁴ The end product consists of stem-loop DNA structures with multiple inverted repeats of the target regions. The final result is evaluated by inspection of turbidity derived from precipitation of magnesium ions from the reaction.

By contrast to PCR, LAMP does not require a high-temperature denaturation step because this technique makes use of *Bst* polymerase, which has built-in strand displacement capabilities. The entire amplification reaction is performed at an isothermal temperature (65°C). With the addition of loop primers into the reaction, the sensitivity of LAMP reaction is typically increased. This creates an advantage for developing a point-of-care (POC) LAMP for malaria detection. The present study aims to develop a LAMP-based lateral flow device (LAMP-LFD) detection technique to efficiently and rapidly diagnose malaria infection. When LAMP end product is loaded into the specifically designed lateral flow dipstick, the reaction begins to move with the addition of running assay buffer. A significant band on the dipstick can be visualized within 2 minutes. Loop-mediated isothermal amplification-LFD for the detection of *P. falciparum* and *P. vivax* has been reported previously⁵ but this is the first work demonstrating visualization of LAMP results using a lateral flow dipstick for the detection of *P. knowlesi*.

To conduct the LAMP-LFD assay, dried blood samples were obtained from the University of Malaya Medical Center, Sarawak State Health Department; National Institute of Malaria Research (Ranchi, Jharkhand); Indian Council of Medical Research institutions; and MNR Medical College

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(Sangareddy). DNA was extracted from dried blood spots (DBS) using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany). All samples were previously examined under a microscope and diagnosed using the Genomix Malaria Pf/Pv Ag Rapid Test Kit and Genomix Malaria Pf/pan Ag Rapid Test Kit (Genomix Molecular Diagnostics Pvt. Ltd., Hyderabad, India) 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 healthy donor and non-*Plasmodium* species samples.

The LAMP assay was performed using the primers listed in Table 1. The *P. knowlesi* small subunit ribosomal RNA (18S rRNA) primers used in this study were designed using the Primer-Explorer V3 software (Eiken Chemical Co., Ltd., Tokyo, Japan) described by Lau et al.⁶ Meanwhile, the *P. falciparum* and *P. vivax* 18S rRNA and *Plasmodium* genus-specific primers used in this study were adapted from Han et al.⁷ End-point detection of the amplification product was performed by lateral flow technology requiring a specifically designed loop primer. The Loop-F primers were fluorescein-5,6-isothiocyanate labeled at the 5' end and Loop-B primers were biotin labeled at the 5' end.

Loop-mediated isothermal amplification assay was performed following the manufacturer's instructions. Briefly, a 25 µL reaction mixture consisting of 1.6 µM FIP and BIP primer, 0.8 µM Loop-F and Loop-B primer, 0.2 µM F3 and B3 primer, 12.5 µL 2 × reaction mixture (provided in the kit), 1 µL *Bst* DNA polymerase enzyme (provided in the kit), 0.7 µL sterile distilled water, 1 µL fluorescent detection reagent (provided in the kit), and 2 µL of template DNA. The entire amplification process was conducted at 65°C in a loop amp real-time turbidimeter (LA-320; Teramecs, Co., Eiken Chemical Ltd., Tokyo, Japan). The LAMP-LFD was performed in separate reaction tubes for each *Plasmodium* species (*P. knowlesi*,

P. falciparum, and *P. vivax*). Following the completion of the reaction process, end products (20 µL) and assay running buffer (100 µL) were loaded on the well of the lateral flow dipstick (Genomix Molecular Diagnostics Pvt. Ltd.). Amplified samples exhibited colored lines at both control (C) and test (T) positions, denoting a positive sample, whereas the un-amplified samples exhibited lines at the 'C' position only, which denotes a negative sample (Figure 1A).

In this study, 290 samples were tested using the genus- and species-specific LAMP method. These samples included from 90 *P. knowlesi*, 49 *P. falciparum*, 56 *P. vivax*, 15 mixed infections of *P. falciparum* and *P. vivax*, 60 nonmalaria infected human samples, one *Toxoplasma gondii*, one *Sarcocystis* spp., and eight healthy donor samples. The results indicate that all 90 *P. knowlesi* and *P. vivax* samples were positively amplified by LAMP-LFD assay. However, one *P. falciparum* and one mixed-infection samples was not amplified, possibly because of low parasitemia or DNA degradation. Although LAMP-LFD was negative for the two cases of *P. falciparum* infections, its sensitivity for *P. vivax* remains high. Of 60 nonmalaria-infected human samples, two samples were detected to be positive for *P. vivax*. The remaining eight healthy donor samples were negatively amplified.

To determine the detection limit of the developed LAMP-LFD assay in this study, DNA templates used in this assay were sourced from the Malaria Research (MR4) Center. Species included in this study were *P. knowlesi* (MRA-456G), *P. falciparum* (MRA-102G), *P. vivax* (MRA-178), *P. ovale* (MRA-179), and *P. malariae* (MRA-180). A 10-fold serial dilution of the stock (100 to 0.01 pg/µL) was prepared using sterile distilled water. Two microliters of each diluted DNA solutions were used as the template. In the present study, the detection limit of all five *Plasmodium* spp. was 0.01 pg/µL. The DNA template from each serial dilution was tested in duplicate and repeated twice to ensure the accuracy of the result. Figure 2 presents the detection limits for all five *Plasmodium* spp. using the LAMP-LFD assay developed in this study. Cassettes 1, 2, 3, 4,

TABLE 1
Primers used in this study

<i>Plasmodium</i> genus/species	Primer	Sequence (5'-3')
<i>Plasmodium</i> genus	FIP	TCGAACCTCTAATTCCTGTTACCTATCAGCTTTTGATGTTAGGGT
	BIP	CGGAGAGGGAGCCTGAGAAATAGAATTGGGTAAATTCACGC
	FLP	(F)CGTCATAGCCATGTTAGGCC
	BLP	(B)AGCTACCACATCTAAGGAAGGCAG
	F3	GTATCAATCGAGTTTCTGACC
	B3	CTTGTCACCTACCTCTCTTCT
<i>Plasmodium knowlesi</i>	FIP	GTTGTTGCCTTAACTTCCTTGTGTTCTTGATTGTAAAGCTTCTTAGAGG
	BIP	TGATGTCCTTAGATGAACCTAGGCTTTGCAAGCAGCTAAAATCGT
	FLP	(F)TAGACACACATCGTT
	BLP	(B)GCACGCGTGCTACACT
	F3	CCATCTATTTCTTTTTGCGTATG
	B3	CAGTGGAGGAAAAGTACGAA
<i>Plasmodium vivax</i>	FIP	CTATTGGAGCTGGAATTACCGCTCCCAAACTCAATTGGAGG
	BIP	AATTGTTGCAGTTAAACGCTCGTAAGCTAGAAGCGTTGCT
	FLP	(F)GCTGCTGGCACCAGACTT
	BLP	(B)AGTTGAATTTCAAAGAATCG
	F3	GGAATGATGGGAATTTAAACCT
	B3	ACGAAGTATCAGTTATGTGGAT
<i>Plasmodium falciparum</i>	FIP	AGCTGGAATTACCGCGGCTGGGTTCTAGAGAAACAATTGG
	BIP	TGTTGCAGTTAAACGTTCTAGCCCCAACCCAGTTTAAATGAAAC
	FLP	(F)GCACCAGACTTGCCCT
	BLP	(B)TTGAATATTAAAGAA
	F3	TGTAATTGGAATGATAGGAATTTA
	B3	GAAAACCTTATTTGAACAAAGC

Features: B = biotin; F = FITC.

FIGURE 1. (A). Detection of Loop-mediated isothermal amplification (LAMP) amplified product on lateral flow dipstick. Cassette 1 and 2 represent the negatively and positively amplified samples, respectively by LAMP-Lateral Flow Device (LAMP-LFD) assay. (B). Determination of the specificity test for LAMP-LFD assay. Representative image of specificity test for LAMP-LFD on genomic DNA extracted from patient samples. Cassette 1: *Plasmodium falciparum*; 2: *Plasmodium vivax*; 3: Healthy donor; 4: *Plasmodium knowlesi*; 5: *Toxoplasma gondii*; 6: *Sarcocystis* spp.; T: *Plasmodium* genus-specific or *Plasmodium* species-specific 18S rRNA gene detection; C: Control line. This figure appears in color at www.ajtmh.org.

and 5 present LAMP-LFD assay images using the stock dilutions (100 to 0.01 pg/ μ L) as templates.

The specificity of LAMP-LFD was tested using DNA templates from *P. knowlesi*, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and non-*Plasmodium* parasites, such as *T. gondii* and *Sarcocystis* spp. Figure 1B indicates that amplified products of all *Plasmodium* spp. have the capability to bind at the “C” and “T” positions of LAMP-LFD cassettes, whereas the amplified products of non-*Plasmodium* parasites such as *T. gondii* and *Sarcocystis* spp. bound to the “C” position only. Cassettes 1, 2, 3, 4, 5, and 6 indicate LAMP-LFD using *P. falciparum*, *P. vivax*, healthy donor, *P. knowlesi*, *T. gondii*, and *Sarcocystis* spp. as templates. In further experiments, DNA extracted from eight healthy donors was used as a template in the LAMP-LFD reaction. The LAMP-LFD did not detect any of the negative DNA samples.

FIGURE 2. Representative image of determination for the detection limit of loop-mediated isothermal amplification-lateral flow device (LAMP-LFD) assay. A 10-fold serial dilution of the stock (100 to 0.01 pg/ μ L *Plasmodium knowlesi* plasmid) was performed with sterile distilled water. Cassettes 1, 2, 3, 4, and 5 indicate LAMP-LFD assay using 100, 10, 1, 0.1, and 0.01 pg/ μ L of plasmid as template. T: *Plasmodium* genus-specific or *Plasmodium* species-specific 18S rRNA gene detection; C: Control line. This figure appears in color at www.ajtmh.org.

The LAMP-LFD has a minimum detection limit (0.01 pg/ μ L) that surpasses the analytical sensitivity of microscopy examination (200 p/ μ L) and RDTs, such as the BinaxNow Malaria kit (> 100 p/ μ L). Thus, the LAMP-LFD is either more sensitive or equivalent in the detection of *Plasmodium* spp. than other isothermal diagnostic methods such as the thermophilic helicase-dependent amplification assay.⁸ By using 18S rRNA as the target gene, Zhang et al.⁹ managed to develop a LAMP assay to specifically detect *P. falciparum*, with five parasites/ μ L as the lower detection limit. Moreover, Imai et al.¹⁰ also reported a LAMP assay targeting the 18S rRNA gene of all five human *Plasmodium* species, including two *P. ovale* subspecies (*P. falciparum*, *P. vivax*, *P. ovale wallikeri*, *P. ovale curtisi*, *P. knowlesi*, and *P. malariae*) and managed to achieve 10–100 copies of plasmid DNA as the lowest detection limit. Both studies support the 18S rRNA gene as a suitable target gene candidate in the development of LAMP assays.

Loop-mediated Isothermal Amplification has gained popularity among many researchers and laboratory technologists because of its rapidness, cost-effectiveness, and suitability for on-site diagnosis. Considering its advantages, a LAMP kit, Eiken Loopamp™ MALARIA Pan Detection Kit (Eiken Chemical Co., Ltd., Tokyo, Japan), has been developed and put on the market. In 2017, Piera et al. reported that the limit of detection for using this kit for both *P. knowlesi* and *P. vivax* was two parasites/ μ L.¹¹ Moreover, LAMP was further developed into real-time LAMP (RealAmp) method, and the limit of detection for *P. vivax* was 125 parasites/ μ L by using LAMP primers previously published by Han et al.^{7,12}

Endpoint assessment of the end product can be monitored via the addition of colorimetric agents, fluorescent agents, turbidimeters, and gel electrophoresis. Most LAMP end products were assessed using the turbidity of reaction due to the accumulation of pyrophosphate ion by-products in the solution. These ions react with Mg²⁺ ions to form an insoluble end product magnesium pyrophosphate that can be detected by colorimetric assay. For example, by adding the hydroxyl naphthol blue dye to a reaction, the color for a positive reaction changes from ultra violet to sky blue, whereas calcein resulted in a color change from orange to yellow-green. However, in our newly developed LAMP-LFD assay, results can be obtained by loading the end product into a dipstick. Without the need for extra incubation time, results can be read within 2 minutes, wherein a positive reaction is indicated if a visible line appears at both the “T” and “C” position. This instrument-free detection is particularly suited to POC use.

In this study, a lateral flow test device was used to detect amplified products following the completion of a LAMP assay. The test strip contains a nitrocellulose membrane coated with T and C lines. The dipstick consists of a sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad. The sample pad is used for sample loading, whereas the conjugate pad holds the conjugate matrix containing gold nanoparticles conjugated with reactants. The nitrocellulose membrane is coated with captured components specific to detection components. The complete setup of test strips is kept in a dipstick with a well for sample loading and detector window labeled with “T” and “C” lines. The primers were specifically designed with additional of biotin and FAM-labels, which enable the end product to bind to the respective control and test lines. In the presence of biotin- and FAM-labeled primers, LAMP synthesized numerous biotin- and FAM-attached end products. The end products were detected by LFD through biotin/

streptavidin reaction (biotin on the end product and streptavidin on the gold nanoparticles) and immunoreactions (FAM on the end product and anti-FAM on the LFD test line). The accumulation of gold nanoparticles yielded a visible red line on the LFD. Thus, both control and test lines appear for positive samples. However, no reaction occurs at test line for negative samples. Several advantages of the LFD are observed, including stability at room temperature, ease of use, and reduced analysis time.

Loop-mediated isothermal amplification-LFD assay represents an ideal method for the rapid detection of malaria in comparison with conventional PCR methods. The LAMP-LFD assay does not require a lengthy amplification time and expensive equipment. Also, results are read easily without the need for trained personnel. Although the RDT kit is a well-known rapid diagnosis method widely used since its introduction in 1994, several drawbacks of RDTs test kit are apparent. For example, most kits only recognize *P. falciparum*- and *P. vivax*-specific antigens and panmalarial antigens. Also, histidine-rich protein 2 should be avoided as the target gene because of this antigen not clearing from a patient's blood following 30 days of treatment, which can result in false-positive results. Moreover, RDT-based *P. vivax* detection kits are less sensitive due to the instability of lactate dehydrogenase at higher temperatures, and it is unable to detect *P. vivax* patients with low parasitemia.¹³

Recently, the use of LAMP-LFD in the detection of *P. falciparum* and *P. vivax* was developed. Yongkiettrakul et al.⁵ reported that LAMP-LFD assays exhibited a 10-fold higher detection limit than conventional PCR by using genomic DNA samples from *P. falciparum* and *P. vivax*. However, LAMP-LFD assays indicated a similar detection limit to PCR by using plasmid DNA carrying malaria *dhfr-ts* genes. To date, using LAMP-LFD for the detection of *P. knowlesi*, *P. ovale*, and *P. malariae* has not been reported elsewhere. Nonetheless, LAMP-LFD has been developed in other fields of medical importance such as food biotechnology and agrobiotechnology. Lalle et al.¹⁴ managed to detect *T. gondii* oocysts down to 25 oocysts/50 g in ready-to-eat baby lettuce. Wang et al.¹⁵ reported a multiplex LAMP-LFD for the simultaneous detection of *Staphylococcus aureus* and *Enterococcus faecalis* by using FITC- and digoxigenin (Dig)- modified primers in the LAMP assay. Moreover, Foo et al.¹⁶ reported a multiplex LAMP-LFD for the detection of pathogenic *Entamoeba histolytica* and non-pathogenic *Entamoeba* spp.

Despite numerous advantages of LAMP, cross-contamination remains a major drawback of LAMP. To prevent cross-contamination, different sets of pipettes and work areas were designated for template preparation, reaction mixture preparation, and DNA amplification. All experiments were performed in duplicate at least twice. Loop-mediated isothermal amplification also involved a tedious primer design procedure due to either four or six primers being included in each reaction. False-negative results may be generated due to the inhibitory effect arising from blood, anticoagulants from the tube, and residuals from lysis buffer used during DNA extraction. These inhibitory factors have an influence on enzymatic amplification by directly binding to the enzymes or hindering the binding of cofactors to the enzyme.¹⁷ To improve the results, uniform sampling and handling methods are recommended. Blood samples can be collected in the same type of tubes. Thus, the samples involved in this study were prepared from DBS on filter paper.

The DBS method was widely used since it was first introduced by Robert Guthrie in 1963.¹⁸ The DBS method does

not require a large amount of storage space, and filter papers are easily transported due to room temperature being sufficient. Dried blood spots is also less invasive to patients, as blood samples are easily collected from a finger prick.

Though LAMP-LFD represents a fast and simple diagnostic method, this method is unfortunately restricted to detecting a single or two target genes simultaneously. For the wider application of LFD devices, a multiplex LAMP-LFD in malaria is expected to be developed in future.

In conclusion, the LAMP-LFD assay developed here provides a robust diagnostic method for the detection of malaria that is more advanced than currently available DNA diagnostic methods. Because of the fact that LAMP-LFD requires minimal infrastructure and the results are easily interpreted by untrained personnel, we believe that, with further improvements, this approach could be deployed as an effective POC device for the easy detection of asymptomatic and low density malaria parasites.

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