



Research Article

In-house validation of a lamp kit for diagnosis of *Plasmodium*, *Plasmodium falciparum* and *Plasmodium vivax* in Vietnam

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Abstract

Vietnam announced the elimination of malaria in 25 provinces in 2019 and an advance to eliminate malaria nationwide by 2030. Quick, accurate diagnosis and prompt treatment play a very important role in malaria eradication strategies. In this study, we developed the LAMP technique to detect *Plasmodium* spp., *P. falciparum*, *P. vivax* from different kinds of sample such as whole blood, dried blood spot, etc. The advantages of this method included high specificity, fast detection time, and simple equipment use. Primer set was designed for founding on 18S rRNA gene. A positive reaction was visualised with the naked eye, using the color indicator, Malachite Green.

Introduction

Up to day, Malaria is still considered as a public health problem in tropical and subtropical regions. In 2018, there were approximately 228 million estimated cases of malaria identified globally [1]. According to a report of the national malaria prevention program, in 2019 Vietnam had 4,665 malaria parasites detected by cyanoscopy reduced 3.08% compared to 2018. Rate of parasite / 1,000 people was 0.048. The number of malaria cases gradually decreased through 10-year period from 2010 to 2019. In 2019 malaria patients decreased by 89.16% compared to 2010. Malaria parasites concentrated mainly in the Central and Central Highland provinces. The number of malaria parasites may be lower than they actually are, due to the presence of asymptomatic malaria and the low density of parasites below the detection threshold of a microscope; According to some unpublished reports in Vietnam, this rate can reach 30–50% [2]. Currently, there are three main groups of methods applied in the diagnosis of malaria parasites: Giemsa stained blood smear, rapid diagnostic test (based on immune method), Molecular biology techniques like PCR, Real time-PCR [3]. Giemsa-stained smear test is still the

gold standard in the confirmed diagnosis of malaria case [4]. This method is widely used, most popular in many countries around the world, with an average detection threshold of about 50 – 100 parasites/ μ L of blood from all *Plasmodium* spp, low test costs, performed even in laboratory conditions and field testing [1]. However, this method is difficult to detect cases with low parasite density, the testing staff must be trained, must be retrained regularly, especially in areas with low prevalence of malaria or eliminate areas where there is little opportunity for testing [5–7]. Rapid diagnostic tests (RDTs) are also widely used, especially in areas where microscopy sites have not yet been available, but RDTs still have limitations such as low parasite detection threshold, false positive rate, and long positive time [5,7,8]. The common disadvantage of these two methods is the low threshold for detection of malaria parasites [9,10]. Therefore, it is estimated that a large number of people with low-density parasite infection are not detected. According to WHO (2014), people carrying low-density parasites below detection threshold of GSB and RDTs may be responsible for contributing 20–50% of the pathogen to malaria vector and vice versa [10]. Molecular biology techniques based on DNA analysis have proven to be more sensitive and specific than microscopic



detection or rapid test (RDTs) [4]. In particular, traditional PCR techniques such as Nested-PCR, Seminested Multiplex-PCR based on the target gene of 18S rRNA are widely applied in the diagnostic studies of malaria for high sensitivity and specificity [11-13]. The detection was determined to be between 1-5 parasites / μ l of blood [13]. The Real time-PCR technique has a high sensitivity at the LOD > 0.5 KST / μ l of blood [14]. However, current DNA amplification techniques are time-consuming, complex and not field-friendly.

To eliminate malaria globally, strategic measures are needed to increase the ability to detect sources of disease. The technical limitations of current testing methods, which requires the development of a highly sensitive diagnostic kit which has a detection threshold comparable to that of DNA amplification techniques in the laboratory, but requires no expensive equipment, faster testing times, is easy for training and can be done in the field. The LAMP technique is a molecular biology technique that uses heat to amplify single strands of DNA, first described by Notomi, et al. [15]. The method has a high amplification efficiency in 15-60 minutes [15]. And then Han ET, et al. [16], developed LAMP to detection of four *Plasmodium* species for clinical diagnosis [16]. Imai Kazuo, et al. (2017) reported the novel diagnostic method for malaria using LAMP and MinION™ nanopore sequencer [17]. Jaymin C, et al. (2013) developed the RealAmp technique, a LAMP technique combined with real-time isotherms to quickly detect malaria parasites. This study also built a primer pair to detect *P. vivax* by RealAmp method [18]. Compared with other molecular methods such as PCR, real time PCR ..., LAMP has the advantage of having similar accuracy, simple implementation, especially short execution time and can detect results by naked eye [11,19-22]. Therefore, LAMP is often used to create quick diagnostic kits which are widely popular in the world. LAMP has been utilized to detect malaria parasites mainly using the conventional 18S ribosomal RNA (18S rRNA) gene as the target sequence [6,12,16,23-27]. In this study, we have developed and evaluated the LAMP technique for the detection of *Plasmodium* genus, *Plasmodium falciparum* and *Plasmodium vivax* from whole blood and dry blood spot.

Methods

Samples

All of samples used for positive control purposes were Whole Blood (WB) and Dry Blood Spot (DBS) from human infected with *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*. The samples were collected from human Gia Lai province, Vietnam. There were confirmed from WHO EQA program. There used DNA from *Plasmodium* as positive control and DNA from other parasites was also extracted to determine the specificity.

The use of the human and the collection of samples were approved by the bioethics committee of National Institute of Malariology, Parasitology and Entomology (NIMPE).

DNA extraction protocol

Using the QIAamp DNA microkit of Qiagen (Germany)

extracted total DNA of *Plasmodium* species from WB and DBS samples. We followed the extraction procedure is performed according to the manufacturer's instructions.

LAMP primer design

Primers used for the LAMP assay were designed based on a highly conserved region of *Plasmodium* genus, *Plasmodium falciparum*, *Plasmodium vivax* genome. Genbank sequences (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table 1) including the 18S rRNA gene were tested in silico through BLAST searches and alignment analysis using the Clutal W module of MEGA 7 software, 521 base pairs, 437bp and 562 bp consensus sequences were selected for the design of specific primers for *Plasmodium* genus, *Plasmodium falciparum*, *Plasmodium vivax* using Primer Explore V5 (<http://primerexplorer.jp/lampv5e/index.html>).

Table 1: Genbank accession numbers for the different *Plasmodium* spp isolates included in the alignment.

No	Isolates	Genbank accession number
1	<i>Plasmodium falciparum</i>	MF155937
2	<i>Plasmodium vivax</i>	U83877
3	<i>Plasmodium malariae</i>	AF488000
4	<i>Plasmodium ovale</i>	L48987
5	<i>Plasmodium knowlesi</i>	DQ530263

Optimization of the assay

PCR assay: The outer primers of the LAMP primer designed in this study (the F3 and B3 primers) were tested for their specificity in PCR assay. DNA from WB and DBS which comprised of *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* were used as the DNA templates for the specificity test. A standard PCR, using 0.5 μ M of primers F3 and B3 and 2 μ L DNA, was carried out in the total volume of 20 μ L using HotstarTaq Plus Master Mix kit (Qiagen, Germany). The annealing temperature is 60°C for 30 seconds. PCR products were analysed by electrophoresis in 2% agarose TBE. All reactions were carried out in triplicate.

LAMP assay: There was set up testing different temperatures, MgSO₄ concentrations, reaction time, visually detected dye concentrations. LAMP reaction mixtures (25 μ L) contained 1x Isothermal Amplification buffer (New England Biolabs, UK), MgSO₄ (4, 6 or 8 mM) (New England Biolabs, UK), 1.4mM of each dNTPs (Qiagen, Germany), 5pmol of each F3 and B3 primers, 40pmol of each FIB and BIP primers, 20pmol of each LF and LB primers, and 8U of Bst 2.0 DAN polymerase (New England Biolabs, UK) with 5 μ L of DNA. Different temperatures were tested using a thermocycler (PCR gradient Nesux GX1, Eppendorf) set from 56°C to 65°C for 40 and 60 minutes and then 80°C for 5 minutes. Amplifications were visually detected by adding Green Malachite at 0.012%, 0.008%, 0.004% and 0.001%. Light blue was observed in successful LAMP reactions and colourless in negative reactions. LAMP reaction products were also analyzed by electrophoresis in 2% TBE agarose. All experiments were completed in all three times.

Preparation of *Plasmodium* genus, *P. falciparum*, *P. vivax* plasmid DNA templates

For the detection and evaluation limits of estimate sensitivity, plasmids containing target regions of the rRNA 18S gene were established for each species and calculated the copy number. The limit of detection (LOD) of LAMP assay was determined by using a ten-fold serial dilution of recombinant plasmid encompassing the target region of the 18S rRNA gene were constructed for all *Plasmodium* species, *P. falciparum* and *P. vivax* (from 10⁻⁶ ng/μl to 10⁻¹¹ ng/μL). The final dilutions were calculated following the formula as mentioned bellow for the corresponding copy number of the 18S rRNA gene as a single copy gene.

The specific PCR primers for cloning were designed based on a highly specific region of the 18S rRNA genes of *Plasmodium* genus, *P. falciparum*, *P. vivax*. Then, genomic DNA was subsequently amplified by PCR, which produced the product 521, 437, 562 bp in size corresponding to *Plasmodium* genus, *P. falciparum*, *P. vivax*. After that, the PCR product was purified and cloned into pUC19 vector (Invitrogen, USA). Concentrations of recombinant plasmid DNA were measured with a Nanophotometer (IMPLEN, Germany), and corresponding copy numbers were calculated as mole multiplies of Avogadro's number using an online program (<http://cels.uri.edu/gsc/cndna.html>), following the formula as the number of copies by amount × 6.022 × 10²³/length × 10⁹ × 650.

Clinical sample

A total of 200 DBS samples were collected from Gia Lai provinces, Vietnam (between 2017-2018), All these clinical samples were evaluated by LAMP assay comparing with qPCR as gold standard test. The percentage of sensitivity, specificity was calculated in format of 2×2 cross-tabulation Table.

LAMP in-house methode	Gold standard test†	
	Positive	Negative
Positive	TP	FP
Negative	FN	TN
Total	TP + FN	FP + TN

† TP = true positive, FP = false positive, TN = true negative, FN = false negative.

$$\text{Sensitivity (\%)} = \frac{TP}{TP+FN} \times 100$$

$$\text{Specipicity (\%)} = \frac{TN}{FP+TN} \times 100$$

In silico comparisons of LAMP primer sets showed a homology of 100% with *Plasmodium* genus, *P. falciparum* and *P. vivax*. The PCR specificity test using F3 and B3 primers of LAMP Primer amplified DNA from *P. vivax*, *P. falciparum*, *P. malariae*, *P. ovale*, and *P. knowlesi* with an amplicon length from 196 to 199bp for *Plasmodium* genus, 221bp for *P. falciparum* and 217bp for *P. vivax* (visualized by gel electrophoresis and UV detection). With *P. falciparum* and *P. vivax* no cross reactivity was observed with any of the tested simian malaria parasite species or with the malaria-negative human DNA control (Figure 1) Table 2.

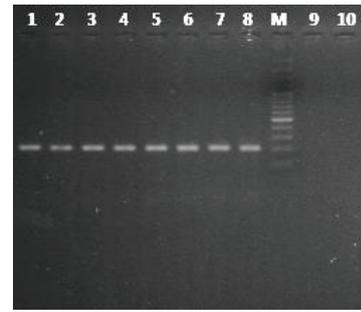


Figure 1a: PCR products using F3-B3 primers of Plasmodium genus: 1-2: *P. falciparum*; 3-4: *P. vivax*; 5-6: *P. malariae*; 7: *P. ovale*; 8: *P. knowlesi*.

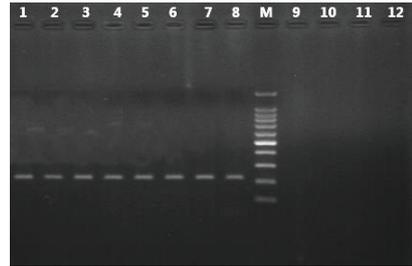


Figure 1b: PCR products using F3-B3 primers of *P. falciparum*: 1-8: *P. falciparum*; 9: *P. vivax*; 10: *P. malariae*; 11: *P. ovale*; 12: *P. knowlesi*.



Figure 1c: PCR products using F3-B3 primers of *P. vivax*: 1-7: *P. vivax* ; 8: *P. falciparum* ; 9: *P. malariae*; 10: *P. ovale*; 11: *P. knowlesi*.

Table 2: Nucleotide sequences of the LAMP primers targeting the 18sRNA gene.

Primer sets	Primer name	Sequence
Genus (PAN-LAMP)	F3	GGCGCGTAAATTACCCAA
	B3	AGCGTTTTAACTGCAACAA
	FIP	CCATCATTCCAATTACAAAGCCAGA-AGAAGAGAGGAGTAGTGACAAG
	BIP	AACCTTCCCAAACGCTCAATTGGA-TTTTAATATACGCTATTGGAGCT
	LB	GCAGCCGCGGTAATTCC
	<i>P. falciparum</i> (Pf-LAMP)	F3
B3		AATAGAGGAAGCGTATTAAGC
FIP		CGCTATTGGAGCTGGAATTACC-AGAGTAACAATTGGAGGGC
BIP		GTTGCAGTTAAAACGCTCGTAGTT-GAAAAGCTAAAATAGTTCGCCCT
LF		GCTGCTGGCACCAGACT
<i>P. vivax</i> (Pv- LAMP)		F3
	B3	AAACAGTATGAAAAGCGAACT
	FIP	CCGCTAATTAGCAGGTTAAGATCTC-TTCGTGAATATGATTTGTCTGG
	BIP	TACGTGGGACTGAATTCGGT-GGAAAGGAAAACGTTTACG

The data revealed that the reaction temperatures at 60°C (*Plasmodium* genus) and 63°C (*P. falciparum* and *P. vivax*) and the concentration of MgSO₄ 8mM had highest LAMP amplification efficiency (Figure 2).

The optimum concentration of MG dye indicated that 0.004% MG was suitable for discrimination of the results as light blue and colourless in positive and negative reactions, respectively (Figure 3).

To determine the lower detection limit, 10-fold serial dilutions of each plasmid DNA were amplified. The detection limit for LAMP was 2,97 x 10⁻² copies for *P. falciparum* and 2,85 copies for *P. vivax*, respectively. The amplification products were visualised on an agarose gel as a ladder of multiple bands. (Figure 4).

Although microscopic examination by Giemsa microscopy has been identified as the gold standard for the diagnosis of malaria parasites. But the detection threshold of this technique is from 20 to 100 parasites/1µl of blood; much higher than the detection threshold of LAMP technique which is about 2-3 parasites/µl of blood; If we compare these two techniques directly, the sensitivity will be very low such as Table 3.

We therefore compared the results of the LAMP test with the qPCR technique, which has similar sensitivity and specificity Tables 4-6.

Total 95 positive samples and 105 negative samples base on qPCR were used. Of the 95 positives, 45 were positive for *P. falciparum*, 43 were *P. vivax* positive and seven were positive for remaining *Plasmodium spp.* The sensitivity was 97.89%, 97.78% and 97.67% for PAN-LAMP, Pf-LAMP and Pv-LAMP assay, respectively. The Specific was 97.14%, 98.06% and 97.45% for PAN-LAMP, Pf-LAMP and Pv-LAMP assay, respectively.

In 2019, Vietnam announced the elimination of malaria in 25 provinces, including 16 provinces in the north, 1 province

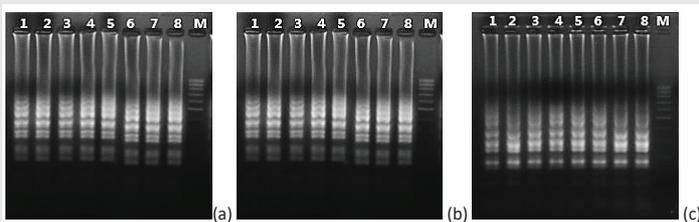


Figure 2: LAMP product at 63°C and MgSO₄ 8mM of Plasmodium genus (a), *P. falciparum* (b) and *P. vivax* (c).

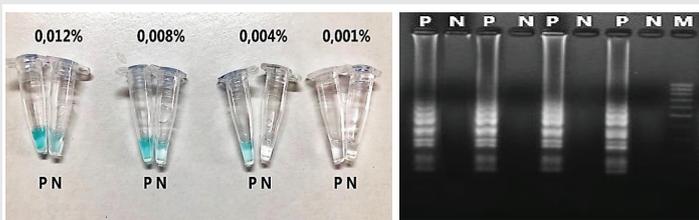


Figure 3: MG concentration tests at 0.012%, 0.008%, 0.004% and 0.001% at 63°C for 60 min. P lane and N lane represent positive and negative reactions, respectively.

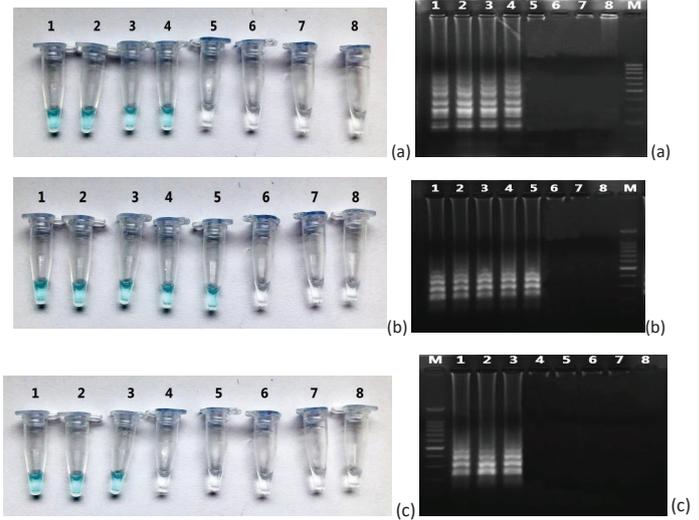


Figure 4: Detection limit of LAMP-MG assay of Plasmodium genus (a); *P. falciparum* (b); *P. vivax* (c). 1-6: 10⁻⁶ to 10⁻¹¹ ng/µl; 7-8: negative control. Detection limit of LAMP-2.0% AGE of Plasmodium genus (a); *P. falciparum* (b); *P. vivax* (c). 1-6: 10⁻⁶ to 10⁻¹¹ ng/µl; 7-8: negative control.

Table 3: Sensitivity and specificity of LAMP detect Plasmodium compared with GIEMSA stain.

PAN-LAMP	GIEMSA	
	Positive	Negative
Positive	58	105
Negative	37	0
Total	95	105
	Se: 61.05% (CI: 50.50% - 70.89%)	Sp: 100% (CI: 96.55 - 100%)

Table 4: Sensitivity and specificity of LAMP detect Plasmodium.

PAN-LAMP	qPCR	
	Positive	Negative
Positive	93	3
Negative	2	102
Total	95	105
	Se: 97.89% (CI: 92.60% - 99.74%)	Sp: 97.18% (CI: 91.88% - 99.41%)

Table 5: Sensitivity and specificity of LAMP detect *P. Falciparum*.

Pf-LAMP	qPCR	
	Positive	Negative
Positive	44	3
Negative	1	152
Total	45	155
	Se: 97.78% (CI: 88.23% - 99.94%)	Sp: 98.06% (CI: 94.45% - 99.60%)

Table 6: Sensitivity and specificity of LAMP detect *P. Vivax*.

Pv-LAMP	qPCR	
	Positive	Negative
Positive	42	4
Negative	1	153
Total	43	157
	Se: 97.67% (CI: 87.71% - 99.94%)	Sp: 97.45% 93.61% - 99.30%

in the central and 8 provinces in the south. Vietnam's goal is to be recognized as a country eliminating malaria by 2030 [28]. To achieve this goal, the National Malaria Control



Program of Vietnam has been implementing many appropriate strategies such as strengthening monitoring, detection, diagnosis and timely treatment. Every year in Viet Nam, the health system conducts tests of more than 2.2 million blood smears, nearly 500,000 rapid diagnostic tests for malaria [28]. The disadvantage of these two techniques is the low detection threshold. Therefore, it is estimated that a large number of people with low-density parasites are not detected. In areas where seasonal malaria is transmitted, people infected with low-density parasites below the detection threshold of blood smear and RDTs are both a regular source of transmission, but also a source of parasite storage through a low infectious season to a high infectious season [28]. Interventions with investigation and treatment to reducing malaria transmission have limited to rely on the detection of antigens using RDTs to identify asymptomatic cases of malaria patients.

Therefore, developing a test with high sensitivity, specificity and easy to do in the field is essential. Numerous studies have demonstrated that the LAMP technique is a highly specific, sensitive, short reaction time and allows the discovery of visual amplification products simply by observing turbidity, fluorescent dye or pH indicator dye. Herein, we have developed and evaluated the LAMP technique for the detection of *Plasmodium* genus, *Plasmodium falciparum* and *Plasmodium vivax* from whole blood and dry blood spot based on the 18S rRNA gene.

We tested our assay specificity with all 5 species *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* with satisfying results. However, our assay did not test another nonhuman malaria species. The nucleotide sequence analysis using the BLAST showed that no nonhuman malaria species were related to *P. falciparum* and *P. vivax* primer sequences in this study (data not shown).

LAMP assay was carried out for 60 minutes and observed with the naked eye based on the color change in the tubes after amplification. In this study, we used MG as a color indicator dye. The color change of Malachite Green (cationic form) depends on the pH of the solution (pH <2: yellow, pH = 3-9: blue, pH > 10: colorless). Absorption wavelength for MG is 621 nm. In the LAMP-MG assay, positive and negative samples that are easily distinguished by the naked eye with light blue and colorless, respectively. Using of MG as pH-sensitive indicator dye for visual end-point assessment of LAMP products in various infections caused by bacteria [29], protozoa [13,15], and malaria [31] have been reported. Adding MG to LAMP buffer before conducting the reaction did not affect the activity of Bst DNA polymerase, while eliminating the risk of contamination between samples. The optimum concentration of MG used throughout this study was at 0.004%, making positive and negative perfectly differentiated. The higher the MG concentration leads to an increase in false positive, the lower the MG concentration will not be able to distinguish between positive and negative samples. The blue color in the positive tubes still remains in color after the reaction for up to 6 weeks, at room temperature. Time after 6 weeks was not investigated in this study.

Recently, Loopamp™ MALARIA Pan/Pf Detection Kit (Eiken Chemical) has become commercially available for the diagnosis of malaria by the LAMP method. This LAMP kit has good specificity and a lower detection limit of 25 parasites/μL [13,31-34]. The detection limit of our LAMP methods for all *Plasmodium* species, *P. falciparum* and *P. vivax* was 2.89×10^{-1} , 2.97×10^{-2} and 2.85 copies/reaction, respectively (5μL of sample DNA could be loaded per reaction). Therefore, the theoretical detection limit of the LAMP assay was equivalent to that of the previously described Loopamp™ MALARIA Pan/Pf and P.v Detection Kit.

The sensitivity and specificity of LAMP assay were evaluated with 200 clinical samples and used qPCR as a gold standard. The results show that the sensitivity and specificity of all assay are over 97%, proving that the LAMP assay in this study has high sensitivity and specificity such as PCR and qPCR. However, PCR and real-time PCR methods require expensive equipments such as thermal cyclers or real-time PCR systems for accurate temperature control, expensive cost and difficult to apply in the field.

Conclusion

LAMP is a molecular method with high sensitivity and specificity. LAMP products are visible to the naked eye after the addition of the MG color indicator, which allows LAMP to be easily deployed in the field in medical facilities.

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