Development of High-Sensitivity Loop-Mediated Isothermal Amplification (LAMP) for Detection of Low Density Parasitaemia for Malaria Elimination

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***Abstract*—** Asymptomatic malaria infections pose a challenge for global malaria elimination. There is a need for assays that detect sub-patent malaria infections that are missed by common diagnostic tests. This study developed and validated an in-house LAMP assay that is cost effective, simple, rapid, and suitable for low resource settings. The Methanol method and the Tris-EDTA (TE) methods were compared to identify the most effective method for DNA extraction from dried blood spots (DBS) for LAMP testing. SYBR Green I and calcein were compared to determine the most effective method for reading results of the LAMP assay. For DNA extraction, the methanol method was found to be more effective (87% sensitivity) than the TE method (35% sensitivity). Both assays showed 100% specificity. For reading the results, SYBR Green I (ratio 1:4 with nuclease free water) or 250 μM calcein (with 500 μM $MnCl_2$) were the optimal concentrations for detection of the LAMP results.

***Keywords*—** LAMP, malaria, Calcein, dried blood spots, SYBR Green I, DNA extraction**.**

I. Introduction

Malaria caused by *Plasmodium* parasites remains a serious infectious disease, with the greatest burden of disease in the Africa, Asia-Pacific and South America. According to the World Health Organisation (WHO), it was estimated that there are 3.2 billion people at risk of malaria worldwide with approximately 216 million cases and 445,000 deaths in 2016 [1]. To achieve the goal of malaria eradiation, WHO has set a target to reduce the mortality rate and new cases by 90% and eliminating malaria in at least 35 countries by the year 2030 [1]. This will require that all populations at risk can access methods for malaria prevention, diagnosis and treatment. Furthermore, surveillance programs will need to be strengthened and will be the foundation of malaria elimination strategies [2]. Therefore, there is a need for high sensitivity, rapid and cost-effective methods for detection of *Plasmodium* parasites.

The most common methods of malaria detection include rapid diagnostic tests (RDTs) and blood-film microscopy. RDTs are relatively quick (~20 minutes), need minimal training and do not require other equipment. The limit of detection for RDTs is ~100 parasites/μl [3-5]. Blood-film light microscopy is also a quick and cost-effective method; however it requires some equipment and staff training. When performed by highly trained staff, the limit of detection of microscopy is ~50 parasites/μl [3].

In a laboratory setting, nucleic amplification methods such as polymerase chain reaction (PCR) are also used as they have higher sensitivity and specificity, can detect mixed infections at the level of 0.5-5 parasites/μl, and can be used quantify the density of parasites [3]. However, PCR can take 2-3 hours to perform, is expensive, requires sophisticated equipment, and well trained staff. Therefore, it is not suitable for field-deployment or high-throughput processing that is required to screen large populations [6]. Loop-mediated Isothermal Amplification (LAMP) is a nucleic amplification method that is rapid, simple, sensitive and specific. LAMP amplifies DNA isothermally so does not require a thermocycler, and results can be achieved in one hour. The amplified LAMP products can be seen by the naked eye under UV light, and highly trained personnel are not necessary [7]. A commercial LAMP kit for malaria is available but it is very expensive (at ~US$16 per test), therefore there is a need for an alternative LAMP assay to be developed.

This project will focus on an in-house LAMP assay for the detection of *P. falciparum* that is simple and cheap enough to be deployed in low resource settings in areas such as villages in Vietnam. The focus of this study is optimizing DNA extraction from DBS, and determining the most effective method and conditions for visualising the results of the LAMP assay. This assay would be useful for identifying low-density malaria infections that cannot be detected by the currently used diagnostics.

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II. Methodology

A. Sample preparation

Plasmodium falciparum (3D7 strain) was cultured using established methods [8]. Cultured parasites were then mixed with uninfected whole blood to make a final parasite densities with a 10-fold titration: 1% (50,000 parasites/µl), 0.1% (5,000 parasites/µl), 0.01% (500 parasites/µl), 0.001% (50 parasites/µl), and 0.0001% (5 parasites/µl). A 0% (no parasite) control was also prepared. For each sample, three DBS (50µl spots) were made on 3MM chromatography paper (GE Healthcare Life Sciences, United Kingdom) and dried at room temperature for three hours, and then stored in plastic zip lock bags with desiccant at room temperature until used for experiments. Twelve replicates were assessed in each experiment.

B. DNA extraction methods

The Tris-EDTA method and the methanol method are two simple methods of DNA extraction that were evaluated for use in with the LAMP assay. Three punches of the DBS (3.0 mm diameter punch) were used for each extraction. Three 3.0 mm diameter punches represents 16.6µl of whole blood.

*Tris-EDTA method***:** Three punches of DBS were put into 1.5ml tubes (Eppendorf, Hamburg, Germany). Then, 100µl of TE buffer (10 mM Tris base Tris- HCl, pH 8.0) were added to tubes and incubated at 65°C for 15 minutes, then 97 °C for 15 minutes on a heat-block (Ratek, Australia). Supernatant was removed and kept at room temperature for immediate use or stored at −20°C [9].

Methanol method: Three punches of the DBS were put into 1.5ml tubes (Eppendorf, Hamburg, Germany). Then 125µl of methanol was added and incubated at room temperature for 15 minutes. The methanol was removed, and 60µl of nuclease free water (NFW) was added. The tubes were then heated at 97°C for 15 minutes on a heat-block (Ratek, Australia). Supernatant was removed and kept at room temperature for immediate use or stored at −20°C [9].

C. LAMP assay

LAMP primers that target the conserved region of the 18S rRNA gene of *P. falciparum* (GenBank: M19173.1) (Figure 1) [7] were synthesised (GeneWorks, Australia) (Table 1). The components of LAMP reaction and detection by SYBR green I and calcein can be found in Table 2. LAMP assays were incubated in a thermal cycler (Applied Biosystems, USA) at 65^0C for 60 minutes followed by 90^0C for 2 minutes to terminate the reaction. Positive and negative controls were included in all tests. The positive control was a sample of 4% parasitaemia extracted by QIAamp DNA minikit (Qiagen, Germany) following the manufacturer's instructions. The negative controls used nuclease-free water instead of DNA template.

D. LAMP detection

LAMP results were determined visually (UV and white light) with the aid of colour changing dyes. Two dyes were optimized and their efficiency in accurately detecting amplified DNA was compared.

TABLE I
PRIMERS TARGET 18S RRNA GENES IN LAMP AMPLIFICATION

Primers	Sequence (5' - 3')
FIP (F1c-F2)	AGCTGGAATTACCGCGGCTGGGTTCCTAGAGA AACAATTGG
BIP (B1B2c)	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCA GTTTAAATGAAAC
F3	TGTAATTGGAATGATAGGAATTTA
B3c	GAAAACCTTATTTTGAACAAAGC
LPF	GCACCAGACTTGCCCT
LPB	TTGAATATTAAAGAA

FIP=Forward Inner Primer, BIP=Backward Inner Primer,
F3=Forward Outer Primer, B3c=Backward Outer Primer,
LPF= Loop Primer Forward, LPB=Loop Primer Backward

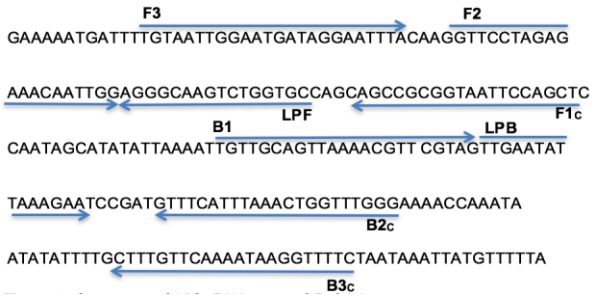

Fig. 1 Sequence of 18S rRNA gene of *P. falciparum.*

SYBR Green I: SYBR Green I (10,000x) was made up to a ratio of 1:4 (with nuclease free water) to final stock is 2500x. Then different volumes of SYBR green: 0.25 µl, 0.5 µl, 1 µl and 2 µl were added to each reaction tube after amplification. SYBR Green must be added after amplification as it inhibits the *Bst* polymerase. Colour and fluorescence change was visually observed and compared across all concentrations.

Calcein: Concentrations of calcein ranging from 500 µM to 62.5 µM (final concentration) were titrated against concentrations of $Mncl_2$ ranging from 125 µM to 100 µM to identify the appropriate concentration of calcein and $Mncl_2$ to detect amplification.

TABLE II
THE COMPONENTS OF EACH LAMP

Component	Amount per reaction if detected by SYBR Green	Amount per reaction if detected by calcein
Amplification Buffer	1x	1x
FIP	1.6 µM	1.6 µM
BIP	1.6 µM	1.6 µM
F3	0.2 µM	0.2 µM
B3	0.2 µM	0.2 µM
LF	0.8 µM	0.8 µM
LB	0.8 µM	0.8 µM
Bst Polymerase	8 units	8 Unit
dNTPs	1.4 mM	1.4 mM
$MgS0_4$	2 mM	8 mM
NFW	13.8 µl	7 µl
Calcein	-	250 µM
$MnCl_2$	-	500 µM
SYBR Green I(ratio 1:4 with NFW)	1 µl	-
DNA template	2 µl	2 µl
TOTAL	25 µl	25 µl

III. Results

A. Assessment of Tris-EDTA (TE) and methanol methods for DNA extraction

The optimal method of DNA extraction was assessed for detection of low density parasitaemia (e.g. 0.5 parasites/µl) in the LAMP assay. The emphasis was to use low-cost simple methods. The limit of detection for the methanol method was 5 parasites/µl while the TE method was not as effective, with a limit of detection of 500 parasites/µl (Figure 2). Therefore, at 0.5 parasites/µl, all positive samples appeared negative with the TE method, but it was possible to detect 3/12 positive samples with the methanol method. The overall sensitivity for the methanol method was also higher (87%) compared to the TE method (35%). The overall specificity for both methods was 100% (Table 3).

TABLE III
COMPARISION OF TE METHOD AND METHANOL METHOD

	Tris-EDTA		Methanol	
	Absent	Present	Absent	Present
Test positive	0	25	0	63
Test negative	12	59	12	9
Sensitivity	35%		87%	
Specificity	100%		100%	

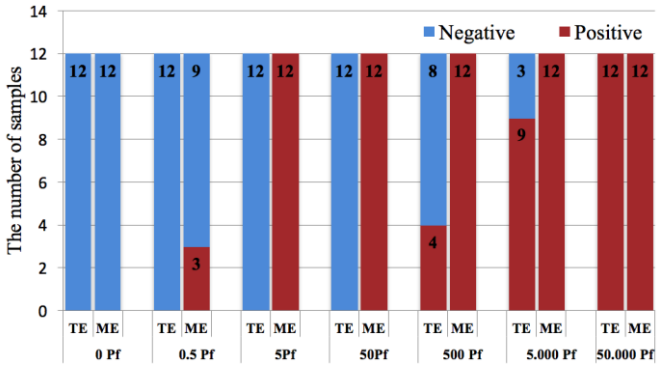

Fig.2 LAMP results for TE and methanol method at a range of different parasite densities in 1µl blood.
TE= Tris-EDTA, ME= Methanol, Pf= *Plasmodium falciparum*

B. Optimisation of SYBR Green visualising LAMP results

The results of the LAMP reaction need to be detected visually, and one option for detection is using SYBR Green I. It is important to determine the optimal amount of SYBR green I that provides a clear distinction between positive and negative samples, under white light and UV light. SYBR Green must be added after LAMP amplification as it inhibits the *Bst* polymerase. Four different volumes (0.25µl, 0.5µl, 1µl and 2µl) of SYBR Green I (2500x) were added to completed LAMP assays. The results suggest that 1µl SYBR green I was optimal for differentiating positive and negative samples under both white light and UV light (Figure 4).

C. Optimisation of calcein for detection LAMP results.

Similarly, Calcein is another method for visualising the results of LAMP results but it can be added before amplification. It was important to determine the optimal concentration of calcein that can differentiate between positive and negative samples. Four concentrations of calcein were evaluated (62.5, 125, 250 and 500 µM). The results showed that 250 µM Calcein (with 500 µM of $MnCl_2$) was the optimal concentration for detecting the LAMP result under white light and UV light. (Figure 5).

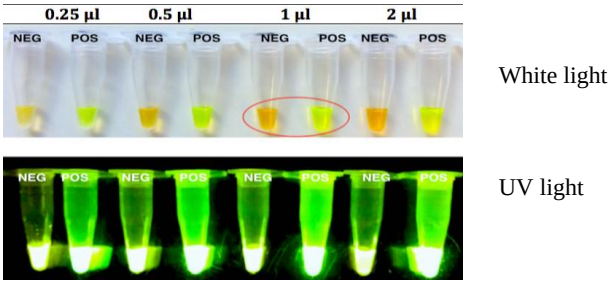

Fig. 4 LAMP results under white light and UV light using SYBR green I

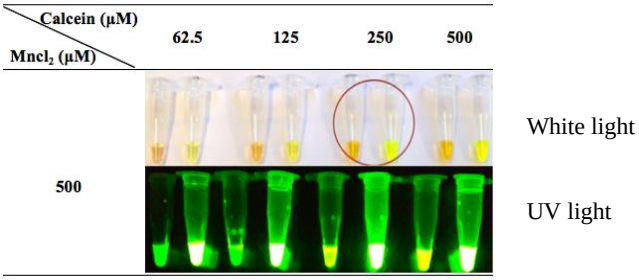

Fig. 5 LAMP results under white light and UV light using calcein

IV. Discussion

There is a need to develop and optimise high-sensitivity methods for malaria diagnosis. The in-house LAMP assay described is specific for *P. falciparum*, and it is cost-effective ($0.40), rapid and has high sensitivity. The most effective method for DNA extraction from DBS was assessed. The results show that methanol method was more effective than TE method in DNA extraction for LAMP assay. The sensitivity and specificity of the two methods were 87% and 100% (methanol method); 35% and 100% (TE method) respectively. Furthermore, it was important to determine the most effective dyes and amounts of each to detect the results of the LAMP assay. The results show that 1µl SYBR Green I (added after amplification) or 250 µM calcein (with 500 µM of $MnCl_2$) (added before amplification) were most appropriate

Dried blood spots are a field-friendly method for collecting samples for LAMP testing. Our objective was to assess which method of DNA extraction was most suitable for use with the LAMP assay. In this study, the methanol method was found to be superior to TE in DNA extraction. This result was similar to that of Bereczky et al.[9] and Strøm et al.[10]. This could be because the TE method may not completely lyse red blood cells containing parasites. This would in turn effect DNA extraction as the parasites are not exposed. This effect is more predominant in samples containing low levels of parasites,

leading to more false negative results and lower sensitivity. In contrast, the methanol method was effective with a limit of detection of 5 parasites/µl. Therefore, the methanol method is a simple, fast and high-performance DNA extraction method that can be used with the in-house LAMP assay.

The other objective of this study was to determine the most effective method for detecting the amplified DNA from the LAMP assay. One option is to use SYBR Green I. SYBR Green I is a DNA intercalating dye that can bind to ssDNA and dsDNA. If it binds to ssDNA molecules, it emits a weak fluorescence signal. If it binds to double-stranded DNA, it emits a strong fluorescence signal under UV light. Under natural light, it changes from orange to green in colour if the reaction is positive, i.e. amplified DNA is present [11]. We observed that SYBR Green I at 1µl was most suitable for interpreting the LAMP results. A lower concentration of SYBR Green I made it difficult to distinguish the negative from positive by the naked eye, as the colour of reaction tubes did not change clearly from orange to green nor did it clearly fluoresce under UV light. Although these results are clear, one limitation is that SYBR Green I cannot be added to the tube before amplification because it will inhibit the reaction [12]. Therefore, it must be added after the reaction, however a large amount of amplified DNA is produced in a LAMP reaction and when tubes are opened, this DNA can contaminate the surrounding workspace. Therefore, another option for detection was also investigated.

Calcein is another option for detecting amplified DNA and visualizing the results of the LAMP reaction. Calcein is advantageous as it can be added into the reaction tube before amplification. Calcein binds to by-products of amplification in order to change colour. Manganese quenches calcein in the reaction initially and therefore calcein does not emit any fluorescence. During the LAMP amplification, the by-product pyrophosphate is produced and the binding of manganese and pyrophosphate releases calcein, allowing it to fluoresce under UV light and change colour from orange to green [13-14]. For concentrations of calcein less than 250 µM, the positive tubes do not change colour from orange to green, therefore it was difficult to interpret the results. The concentrations of calcein and $MnCl_2$ used in this study (250 µM calcein, 500 µM $MnCl_2$) were the same as used by Zhang et al. [14]. These concentrations can now be used for further validation of the in-house LAMP assay.

This study shows clear evidence of an effective in-house LAMP assay that is able to detect parasitaemia down to 5 parasites/µl, and the results can be clearly detected visually. The limitations of this study include only developing an in-house assay for *P. falciparum* and not including other species such as *P. vivax* that are also prevalent in malaria elimination settings. This will be addressed in future studies that develop and validate a LAMP assay for other malaria species. Furthermore, this study used samples from laboratory cultured samples, and not clinical samples. This will be addressed in further research, using dried blood samples from a cross-sectional study conducted in Vietnam to assess the the effectiveness of LAMP in a pre-elimination setting.

V. Conclusion

In summary, an in-house LAMP assay was used to determine the sensitivity of simple DNA extraction methods such as the methanol and TE methods. The methanol method was found to be more sensitive than the TE method. The detection of the LAMP assay was also improved by optimising the conditions of SYBR Green I and Calcein for detecting amplified products. It is best to use the non-inhibiting dye calcein over the DNA intercalating SYBR Green, as no post-amplification processing is required and limiting the chance of cross contamination. Future studies will use this in-house LAMP assay to test dried blood spots collected in a cross-sectional study in Vietnam.

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