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Reliable interpretation and long-term stability using SYBR™ safe fluorescent assay for loop-mediated isothermal amplification (LAMP) detection of *Leishmania* spp.

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Abstract. Leishmaniasis, a vector-borne disease caused by *Leishmania*, is the second leading mortality after malaria. Continuously increasing cases of cutaneous and visceral leishmaniasis (CL/VL) have been documented in Thailand. Recently, loop-mediated isothermal amplification (LAMP) based on malachite green (MG) colorimetric assay that detects *Leishmania* DNA was developed to facilitate epidemiological studies of leishmaniasis in affected areas. However, ambiguous reading interpretation sometimes occurred using the MG-LAMP assay. In this study, the efficiency and effectiveness of the SYBR™ Safe fluorescent assay for LAMP detection of *Leishmania siamensis* (MON-324) and *Leishmania martiniquensis* (MON-229) were compared under two different light sources, i.e., blue light and ultraviolet light transilluminators. Regarding the SYBR™-LAMP assay, the detection limit of DNA of both *L. siamensis* and *L. martiniquensis* was 10^3 parasites/mL. The assay exhibited consistency and reproducibility without requiring any post-reaction preparations. The dye is generally available, affordable and safe while reliable interpretation can be easily visualized under both blue light and ultraviolet light transilluminators. Using buffy coat of VL patients, the SYBR™-LAMP offers an alternative method for screening samples with high sensitivity and specificity. This cost effective SYBR™ Safe fluorescent assay is simple to use without ambiguous evaluation which could provide another suitable choice of a standard LAMP assay in molecular laboratories as well as further development in field studies.

INTRODUCTION

Leishmaniasis is a vector-borne disease caused by an intracellular protozoan of the genus *Leishmania*, which is globally recognized as the second leading infectious disease causing high mortality and morbidity (WHO 2010). Its three main clinical manifestations include cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) (David and Craft 2009; Reithinger *et al.*, 2007). Autochthonous CL and VL caused by *Leishmania siamensis* and *Leishmania martiniquensis* are considered emerging diseases in Thailand (Chiewchanvit *et al.*, 2015; Leelayoova *et al.*, 2013). Since the first case report in 1996, the prevalence of leishmaniasis has been continuously increasing in Thailand, especially among patients with HIV/AIDS (Bualert *et al.*, 2012; Chiewchanvit *et al.*, 2015; Chusri *et al.*, 2012; Maharom *et al.*, 2008; Manomat *et al.*, 2017; Phumee *et al.*, 2013; Suankratay *et al.*, 2010). Serological methods including the Immunofluorescence Antibody Test (IFAT), Enzyme Linked Immunosorbent Assay (ELISA), immuno-

blotting (Blot) and Direct Agglutination Test (DAT), have been widely used to diagnose VL. However, among patients with HIV and *Leishmania* coinfection, decreases in sensitivities of these serological methods to detect antibodies against *Leishmania* have been reported (Cota *et al.*, 2012). Comparatively high sensitivity and specificity of Polymerase Chain Reaction (PCR) has been shown to detect *Leishmania* DNA in asymptomatic and symptomatic *Leishmania* infections (Abbasi *et al.*, 2013; Manomat *et al.*, 2017; Sriworarat *et al.*, 2015). However, PCR methods require molecular equipment as well as several steps of preparation making them unsuitable for field work studies. Thus, a simplified method is required for *Leishmania* amplicon detection under limited resources and environments (Nzelu *et al.*, 2014).

Recently, loop-mediated isothermal amplification or LAMP method using several complex primers and a strand-displacement polymerase using simple laboratory equipments has been widely used to screen *Leishmania* infection (Adams *et al.*, 2010; Sriworarat *et al.*, 2015; Tiwananthagorn *et al.*, 2015) as well as other vector-borne pathogens (Lucchi *et al.*, 2016). LAMP products can be visualized and measured using several assays including turbidity, fluorescence and color (Sriworarat *et al.*, 2015). Malachite green (MG) has recently been developed and validated to detect *Leishmania* spp. as a colorimetric assay (Nzelu *et al.*, 2014; Sriworarat *et al.*, 2015; Tiwananthagorn *et al.*, 2017). However, ambiguous reading interpretation sometimes occurred between a very pale blue and transparency for the MG-LAMP assay. Comparatively, fluorescence measurement, e.g., SYBR Green I (Tsai *et al.*, 2009), calcein (Tomita *et al.*, 2008) and propidium iodide (Hill *et al.*, 2008) require UV illumination to reveal the LAMP products. Among these, SYBR Green I, a costly fluorescent dye requiring post-reaction preparation, has been widely used for the fluorescence-LAMP assay. In contrast, the FDR reagent (Eiken), a good choice of fluorescent dye, can be added in a pre-reaction to avoid post-amplification preparation step which is commonly a contamination prone step in the LAMP assay (Adams *et al.*, 2010). Different detection devices have been developed and applied to correct the ambiguous evaluation such as real-time turbidimeter (Illumigene, Meridian Bioscience) or real-time PCR. In addition, microfluidic chip-based detection has been recently applied with LAMP that enabled quantitative detection upon hybridization to target amplicons (Fang *et al.*, 2010; Patterson *et al.*, 2013). However, the use of expensive and specialized equipment reduces the versatility and limits the use of LAMP (Nzelu *et al.*, 2014). SYBR™ Safe, a low-cost non-mutagenicity fluorescent dye with environmental safety and available in all molecular laboratories, was used as an alternative dye indicator for the LAMP assay in this study. We demonstrated the efficiency and effectiveness of the SYBR™ Safe fluorescent LAMP assay to detect DNA of *Leishmania* without the need of post-reaction preparation. Ambiguous evaluation of positive or negative results was eliminated due to the clarity of reaction. This SYBR™ Safe-LAMP reaction could be easily recognized both under blue light (BL) and ultraviolet light (UV) transilluminators which are available in all molecular laboratories.

MATERIALS AND METHODS

Leishmania parasites and blood sample preparation

L. siamensis (MON-324; MHOM/TH/2010/TR) and *L. martiniquensis* (MON-229; MHOM/TH/2011/PG) promastigotes were harvested from an axenic culture in Schneider's *Drosophila* medium (Sigma, USA) supplemented with 20% heat inactivated fetal bovine serum (GE Healthcare, UK), 100 IU of penicillin-streptomycin (GE Healthcare, UK) and 100 µg/mL gentamicin (GPO, Thailand) at 25°C. A total of 10^7 promastigotes were washed three times with phosphate buffered saline (PBS). Then DNA was extracted using the Illustra™ Tissue and Cells Genomic Prep

Mini Spin Kit (GE Healthcare, UK). For clinical samples, whole blood of eligible participants >18 years old and attending an HIV clinic, ChiangRai Prachanukroh Hospital, ChiangRai Province was centrifuged at 900 × *g* for 10 minutes to separate the buffy coat. DNA was extracted from 200 µL of buffy coat sample using the Gen UP™ gDNA Kit (Biotech, Germany). The extracted DNA was eluted to 100 µL final volumes and stored at –20°C until used.

PCR assay and sequence analysis

PCR amplicons of the ITS1 region of the ribosomal RNA gene of *Leishmania* was amplified using the MJ Mini™ thermal cycler (BioRad, USA) as described by Manomat *et al.*, 2017. DNA of *L. martiniquensis* promastigotes (MHOM/MQ/92/MAR1) was used as the positive control. The results were visualized by Molecular Imager® Gel Doc™ XR+ System with Imager Lab™ 3.0 (BioRad, USA). Positive PCR products were sent to U2Bio Co. Ltd., South Korea for DNA sequencing. The sequences were validated using BioEdit Version 7.0.1 and confirmed using multiple-alignment with reference *Leishmania* strains retrieved from the GenBank.

Loop-mediated isothermal amplification (LAMP)

The LAMP assay was performed in 25 µL of reaction mixture described by Sriworarat *et al.* (2015). The primers were designed for pan-leishmania detection based on 18S ribosomal RNA gene using PrimerExplorer, Version 4 Software (http://primerexplorer.jp/elamp4.0.0 /index.html) (Sriworarat *et al.*, 2015). The LAMP primers are shown in Table 1. In brief, each reaction mixture consisted of 1× Isothermal Amplification Buffer (New England Biolabs, USA), 8 mM $MgSO_4$, 0.8 M Betaine (Sigma-Aldrich, USA), 1.4 mM dNTP, 40 pmol of each inner primer (FIP and BIP), 40 pmol of each outer primer (F3 and B3), 8 U of *Bst* 2.0 WarmStart® DNA polymerase (New England Biolabs, USA) and 2 µL of template DNA. In addition, 0.008% MG (Malachite Green) (Sigma-Aldrich, USA) was added to each reaction mixture as the colorimetric indicator (Sriworarat *et al.*, 2015), whereas 1× SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, USA) was used to replace MG as the fluorescent indicator. A negative (water) sample or no template control (NTC) was included in all LAMP amplifications. The mixture was incubated at 65°C for 75 min in a heating block and then heated at 80°C for 10 min to inactivate the reaction. To evaluate the effect of temperature on LAMP amplification, the reaction mixture was parallel performed at different temperatures, i.e., 4°C, 37°C and 65°C for 75 min. LAMP positive DNA products were kept at 4°C for one month, then observed to determine the stability of the LAMP-dye-complex. Regarding limits of detection, ten-fold serial dilutions of *L. siamensis* and *L. martiniquensis* from 10^6 to 10^2 parasites/mL were used as template DNA. Eleven *Leishmania* DNA positive and 22 negative clinical samples (1:2) were nonrandomly selected and used to evaluate the sensitivity and specificity of the SYBR™ Safe-LAMP assay.

LAMP amplicons visualization

At the end of incubation, the LAMP amplicons of target DNA were analyzed and confirmed based on direct visual inspection of the reaction tubes. For the MG colorimetric indicator, a positive

Table 1. LAMP primers sequences used in this study

Primer name	Sequence (5´ to 3´)
F3	CGAAAGCTTTGAGGTTACAGTCT
B3	CAAACAAATCACTCCACCGAC
FIP	GTCAAATTAAACCGCACGCTCCACGGGGGAGTACGTTCGCAA
BIP	TCAACACGGGGAACTTTACCAGATCACCACCATTCAGGGAATCGA

amplification showed as light blue, whereas the reaction mixture became colorless in the absence of amplification as described previously (Sriworarat *et al.*, 2015). For the SYBR™ Safe indicator, a positive amplification showed brilliant glowing fluorescence, whereas nonglowing fluorescence was observed in the absence of amplification under either a blue light (BL) or ultraviolet light (UV) trans-illuminator. Regarding validation of LAMP amplification, 5 µL of reaction mixture was electrophoresed on 2.5% agarose gel and visualized using an UV transilluminator. The light intensity of each sample was measured using the Molecular Imager® Gel Doc™ XR+ System with Imager Lab™ 3.0 (BioRad, USA).

Sensitivity and specificity of the LAMP assay

To study the sensitivity and specificity of the LAMP assay, the test was evaluated using two groups of human DNA extracted samples of buffy coat from an affected area of VL; one group positive for *Leishmania* infection (n = 11) and one noninfected group (n = 22). Diagnosis of VL infection was confirmed when amastigotes were seen in Giemsa-stained smears or when using the buffy coat. Additionally, PCR targeting the internal transcribed spacer 1 region was also positive for *Leishmania* DNA.

Statistical analysis

The sensitivity and specificity of the SYBR™ Safe-LAMP assay was evaluated using PCR results for validation. The strength of the agreement between SYBR™ Safe-LAMP and PCR assays was assessed using the kappa statistical test and P-value <0.05 was considered statistically significant. The analysis was performed using STATA, Version SE14 (Stata Corporation, College Station, TX, USA).

Ethics statement

The study was approved by the Ethics Committee of the Royal Thai Army Medical Department (IRBRTA 1650/2560).

RESULTS

LAMP successfully amplified 18S ribosomal RNA gene of *L. siamensis*, Ls, and *L. martiniquensis*, Lm (Fig. 1). Using MG colorimetric measurement, successful amplification was associated with a light blue color, whereas transparency demon-strated failure of amplification, as described by Sriworarat *et al.*, 2015 (Fig. 1A). For the SYBR™ Safe fluorescent assay using BL and UV detection, brilliant glowing fluorescence indicated the presence of LAMP amplicon-

Figure 1. LAMP detection of *L. siamensis* and *L. martiniquensis* by colorimetric and fluorescent assays. (A) Malachite green colorimetric detection, MG. (B) Blue light SYBR™ Safe detection, BL. (C) Ultraviolet SYBR™ Safe detection, UV. (D) Gel electrophoresis detection. Abbreviations: 100 bp marker (M), *L. siamensis* (Ls), *L. martiniquensis* (Lm), no template control (NTC).

SYBR™ Safe dye-complex but non-glowing fluorescence represented the absence of amplification (Figs. 1B and 1C). The amplification of LAMP products was then validated using gel electrophoresis, exhibiting a mixture of stem-loop DNAs with various stem lengths (Fig. 1D) (Notomi *et al.*, 2000). The primers showed no occurrence of cross amplification with human' genomic DNA where identical interpretation of LAMP specificity was observed in MG colorimetric as well as SYBR™ Safe fluorescent assays.

The presence of LAMP amplification was obviously visible and detectable by the glowing fluorescence of SYBR™ Safe indicator and repeated consistency of amplification could be clearly achieved in both *L. siamensis* and *L. martiniquensis*. The measurement of fluorescent intensity (Int) values demonstrated that the presence of LAMP amplicons was double those absent (presence; mean ± s.e. = 1,544.6 ± 37.0, n = 21, absence; 726.5 ± 15.0, n = 6, respectively). BL and UV showed no different intensity of fluorescent emission detecting *L. siamensis* and *L. martiniquensis*. Nevertheless, UV image interpreted a comparatively distinctive translucent color regarding no amplification (NTC). Consequently, a valid agreement of evaluation between the presence and absence of LAMP amplification could be easily interpreted and achieved by non-laboratory observers. Although colorimetric and fluorescent assays could detect *L. siamensis* and *L. martiniquensis*, colorimetric instability, particularly the fading color of MG, was observed after overnight preservation at 4°C in this study.

Positive reactions of LAMP amplification were still present after incubation at 65°C, whereas no amplification at 4°C and 37°C was observed, suggesting *Bst* 2.0 WarmStart® DNA polymerase could be activated neither at room temperature nor under 37°C. LAMP products consistently emitted glowing fluorescence after 30 days suggesting a high stability of the LAMP-SYBR™ Safe dye-complex (Fig. 2). The detection limit of the SYBR™ Safe-LAMP assay was 10^3 parasites/mL by the naked eye under both BL and UV transilluminators, whereas LAMP amplification of 10^2 parasites/mL could be detected using only gel electrophoresis (Fig. 3).

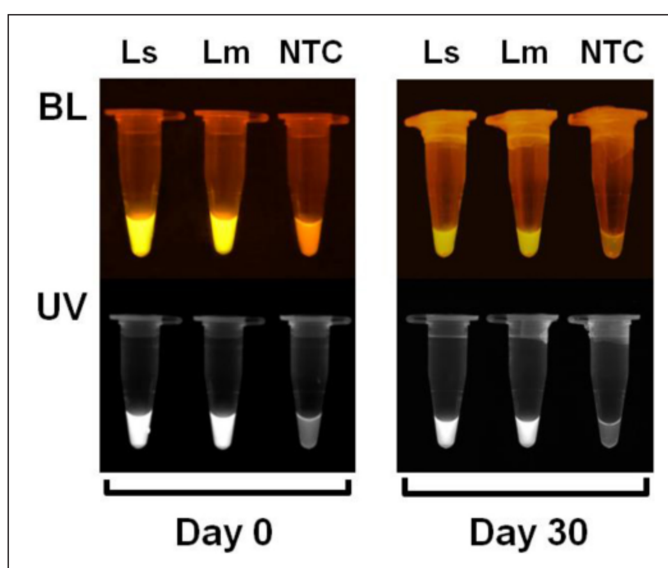

Figure 2. Sensitivity and stability of SYBR™ Safe fluorescent assay at days 0 and 30 detected by blue light (BL) and ultraviolet (UV). Abbreviations: *L. siamensis* (Ls), *L. martiniquensis* (Lm), no template control (NTC).

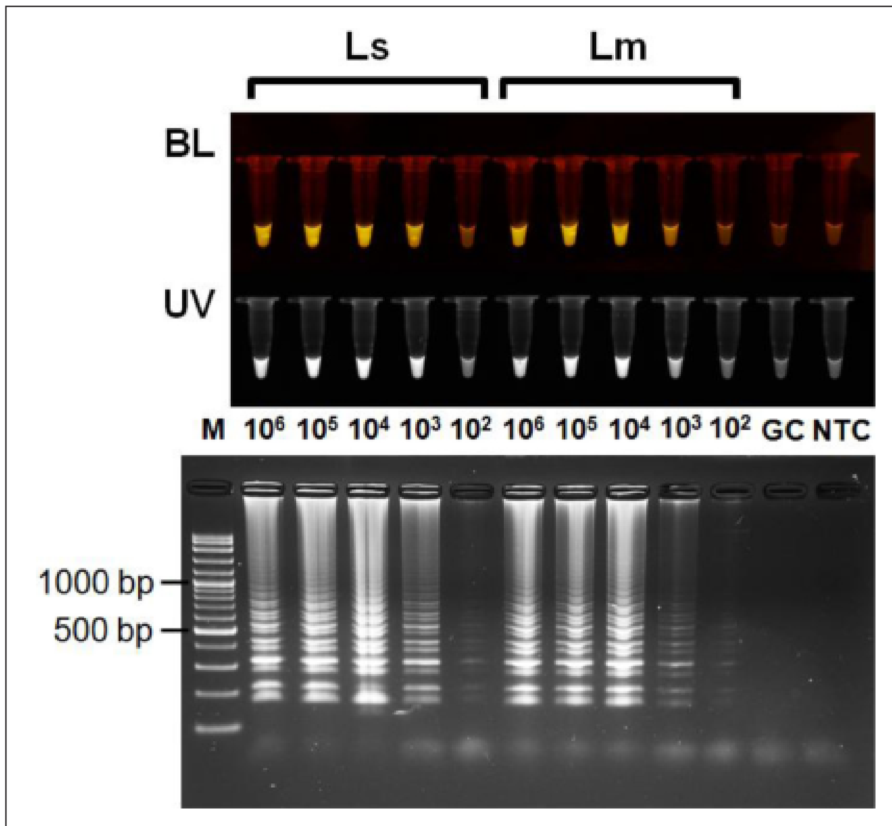

Figure 3. Detection sensitivity of SYBR™ Safe assay to detect *L. siamensis* and *L. martiniquensis* (tenfold serial dilution; 10^6–10^2 parasites/mL) using blue light (BL), ultraviolet (UV) and gel electrophoresis. Abbreviations of variable names: 100 bp marker (M), *L. siamensis* (Ls), *L. martiniquensis* (Lm), genomic DNA (GC), no template control (NTC).

Determination of sensitivity and specificity of the SYBR™ Safe-LAMP assay using extracted DNA from buffy coat samples of positive VL cases and non-VL cases showed a sensitivity of 90.9% (10/11; 95% confidence interval [CI], 58.7 to 99.8%) and specificity of 100% (22/22; 95% CI, 84.6 to 100%). Positive predictive value (PPV) and negative predictive value (NPV) were 100% (10/10; 95% CI, 69.2 to 100%) and 95.7% (22/23; 95% CI, 78.1 to 99.9%), respectively. The SYBR™ Safe-LAMP assay showed significant results that were in almost perfect agreement when compared with PCR (P-value <0.001, Kappa = 0.93).

DISCUSSION

The detection of LAMP amplicons visualizing by the SYBR™ Safe fluorescent indicator under either BL or UV trans-illuminators demonstrated equivalent and apparently brilliant glowing fluorescence of LAMP positive results for *L. siamensis* and *L. martiniquensis*. The difference of fluorescent intensity between the presence and absence of LAMP amplicons (2 times higher) could be validated with solid agreement of LAMP interpretation by non-laboratory observers. No difference was observed between MG colorimetric and

SYBR^{TM} Safe fluorescent assays in detecting *L. siamensis* and *L. martiniquensis* even though uncertain evaluation of MG color was sometimes observed due to an unclear cut in color between light blue versus transparency suggesting an ambiguous decision. The detection sensitivity of the SYBR^{TM} Safe-LAMP assay was limited to 10^3 parasites/mL similar to that reported by Mikita *et al.* (2014) and Sriworarat *et al.* (2015). Unlike electrophoresis where the different sized amplicons of LAMP stem-loop DNA formed densely distinct bands, using 10^2 parasites/mL, the evaluation of SYBR^{TM} Safe-LAMP assay detected dispersible LAMP amplicon-SYBR^{TM} Safe dye-complex in reaction tubes that could result in scatter emission causing unnoticeable fluorescence. Moreover, background fluorescence in the negative control was observed; thus, false evaluation could have occurred by the naked eye. The SYBR^{TM} Safe LAMP assay exhibited consistency and reproducibility even though different light sources, BL and UV, were used for visualization. Although BL and UV presented equivalent fluorescence, the use of UV is comparatively more efficient and effective to detect LAMP amplicons of *L. siamensis* and *L. martiniquensis* according to clear interpretation and lower fluorescence background between the presence and absence of LAMP products. The use of optimal light wavelength of BL trans-illuminator that matches the exciting stage of the SYBR^{TM} Safe dye should eliminate the strengthening fluorescence background in the negative reaction tube and produce a clearer interpretation between the presence and absence in very low LAMP amplicons. Not only could SYBR^{TM} Safe fluorescent assays be applied to detect *L. martiniquensis* and *L. siamensis*, but Sriworarat *et al.* (2015) also showed that LAMP primers developed for a pan-leishmania detection could amplify *L. aethiopica*, *L. braziliensis*, *L. donovani*, and *L. tropica*. Additionally, the stability of the LAMP-SYBR^{TM} Safe dye-complex makes the assay suitable for field storage and re-evaluation by well-trained laboratory personnel in epidemiological studies.

The kappa statistical test indicated that the level of agreement between PCR and LAMP-SYBR^{TM} Safe assays showed diagnostic accuracy between the two assays. The SYBR^{TM} Safe assay yielded high sensitivity and specificity to detect *Leishmania* DNA in clinical samples when compared with PCR results. In this study, we used *Leishmania* DNA based on column purification. However, the LAMP primers could be used to detect *Leishmania* DNA in different extraction methods as well as different types of clinical samples, i.e., crude boiling of blood, saliva and tissue biopsy (Sriworarat *et al.*, 2015). Unfortunately, the specificity of the assay using mixed parasitic samples was not tested in the study. Nevertheless, the LAMP primers did not cross-react with DNA of *Trichomonas vaginalis* and *Giardia duodenalis* but with those of *Trypanosoma* sp., a closely related protozoan, which have different clinical presentations but have never been reported in humans in Thailand. Thus, the cross-reaction would not lead to misdiagnosis (Sriworarat *et al.*, 2015). The results demonstrated that the SYBR^{TM} Safe-LAMP assay could be a suitable method to diagnose *Leishmania* infection in molecular laboratories as well as to facilitate epidemiological studies of leishmaniasis in Thailand.

Recently, the LAMP method based on MG colorimetric assay was developed to facilitate epidemiological studies of leishmaniasis in Thailand (Sriworarat *et al.*, 2015). MG-LAMP revealed its user-friendly diagnostic tool without the need of expensive equipment. However, ambiguous reading interpretation sometimes occurred between a very pale blue and transparency (Nzelu *et al.*, 2014; Sriworarat *et al.*, 2015), especially, along with the presence of a low amount of amplicons together with an unoptimized MG concentration (Lucchi *et al.*, 2016). Therefore, well-trained laboratory personnel as well as an optimum reaction mixture of the MG-LAMP assay are highly recommended to eliminate any false

negative outcomes. Fluorescent indicators have not usually been chosen for LAMP detection assays due to their high cost and the need to add dyes during post-amplification reaction (Adams *et al.*, 2010; Hill *et al.*, 2008; Tomita *et al.*, 2008; Tsai *et al.*, 2009). To avoid post-amplification handling and limit contamination prone steps, pre-adding dyes such as FDR (Adams *et al.*, 2010) as well as SYBR™ Safe are appropriate fluorescent dyes, whereas ethidium bromide (EtBr) has been excluded due to its carcinogenic effects. In this study, we used an alternative indicator, SYBR™ Safe DNA Gel Stain to detect the LAMP amplicons. A distinct difference of fluorescence detection by the naked eye between presence and absence of LAMP amplification reaction made the assay more feasible and reliable. In contrast to previous fluorescent measurements that require costly dyes (Adams *et al.*, 2010; Hill *et al.*, 2008; Tomita *et al.*, 2008; Tsai *et al.*, 2009), the SYBR™ Safe dye is cost effective; equivalent to $0.0046/reaction, and available in all molecular laboratories. Moreover, the dye could be directly added to the pre-reaction due to its non-inhibitory effect to reduce the chance of contamination when performing the assay. Other advantages are its high sensitivity and affinity for nucleic acids including fluorescence enhancements upon binding.

Because SYBR™ Safe-LAMP assay uses fluorescence to interpret the presence and absence of LAMP amplicons, thus, fluorescent light sources such as BL and UV transilluminators are needed for reaction evaluation. To solve the limitation of using the reading light source in fieldwork, a light weight BL transilluminator (<1 kg), which consumes less energy and has durable long life emission LED, is now suitable for carrying in the field environment. The LED BL transilluminator would well facilitate the use of the SYBR™ Safe-LAMP assay in epidemiological studies in the future. Currently, a highly reliable LAMP detecting instrument (Illumigene, Meridian Bioscience) has been developed and is commercially available to detect a broad spectrum of pathogens based on turbidity of magnesium pyrophosphate that the LAMP assay can quantify in one step. Nevertheless, multiplex genes could not be detected by LAMP assay based on turbidity, colorimetric and fluorescent assays due to the nonspecific association of the dyes and magnesium pyrophosphate to LAMP amplicons. Multiplex gene detection using LAMP assay may be possible if the different fluorescent dye labeling LAMP primers was specifically synthesized (Gadkar *et al.*, 2018) or if a specific colorimetric LAMP microfluidic chip has been developed (Yuan *et al.*, 2018) in which expensive instruments are needed and forestall its use in field settings. Thus, these instruments could limit the wide use of this technique making the LAMP assay not simple and affordable as a field-friendly diagnostic in point-of-care settings, especially in developing countries where resources are limited. On the other hand, the SYBR™ Safe assay requires simple inexpensive equipment and is easy to be delivered to end-users.

In conclusion, this study demonstrated a cheap and reliable interpretation of the SYBR™ Safe-LAMP assay with longterm stability to detect the 18S ribosomal RNA gene of *L. siamensis* and *L. martiniquensis* in blood samples. Multiple contamination was not found due to no post-reaction preparation. The use of the SYBR™ Safe indicator could reduce the risk of ambiguous results and facilitate laboratory personnel to simply evaluating LAMP amplification reaction.

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