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# Development of loop-mediated isothermal amplification (LAMP) for simple detection of *Leishmania* infection

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## Abstract

**Background:** Leishmaniasis is a neglected tropical disease that is caused by an obligate intracellular protozoan of the genus *Leishmania*. Recently, an increasing number of autochthonous leishmaniasis cases caused by *L. martiniquensis* and the novel species *L. siamensis* have been described in Thailand, rendering an accurate diagnosis of this disease critical. However, only a few laboratories are capable of diagnosing leishmaniasis in Thailand. To expand leishmaniasis diagnostic capabilities, we developed a simple colorimetric loop-mediated isothermal amplification (LAMP) technique for the direct detection of *Leishmania* DNA.

**Methods:** LAMP was performed for 75 min using four primers targeting the conserved region of the 18S ribosomal RNA gene, and the DNA indicator used was malachite green (MG). To simulate crude samples, cultured promastigotes of *L. siamensis* were mixed with blood or saliva. Also, clinical samples (blood, saliva, and tissue biopsies) were obtained from patients with cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). All samples were boiled for 10 min and introduced directly into the LAMP reaction mixture without DNA purification.

**Results:** The use of MG resulted in an unambiguous differentiation of positive and negative controls. For *L. siamensis*, the detection limit was 10<sup>3</sup> parasites/mL or 2.5 parasites/tube. Saliva, tissue biopsies, and whole blood were indicative of active *Leishmania* infection, and their direct usages did not adversely affect the detection limit. In addition, this LAMP assay could detect DNA from multiple *Leishmania* species other than *L. siamensis* and *L. martiniquensis*, including *L. aethiopica*, *L. braziliensis*, *L. donovani* and *L. tropica*.

**Conclusions:** The simplicity and sensitivity of LAMP in detecting active *Leishmania* infection could enable the rapid diagnosis of leishmaniasis, thereby facilitating the survey and control of leishmaniasis in Thailand. However, our limited number of samples warranted a further validation with a larger cohort of patients before this assay could be deployed.

**Keywords:** *Leishmania martiniquensis*, *L. siamensis*, LAMP, Malachite green, Diagnosis

## Background

Causing more than 50,000 deaths annually [1], leishmaniasis is one of the most debilitating poverty-related diseases and presents a severe threat to socioeconomic development. This disease is caused by more than 20 species of the obligate intracellular protozoa *Leishmania* [2]. These are transmitted to humans through the bites of female sand flies [1]. Upon infection, three main

clinical forms can be recognized: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). CL, the most common form, is characterized by the presence of various ulcerative lesions, which lead to disfiguring and/or disabling scars [3, 4]. Consequently, patients with CL often live in obscurity [5], thereby preventing expeditious treatment and increasing the probability of transmission. MCL is described by a severe destruction of mucosal regions (nose, mouth, and throat) [4]. VL is an infection of the internal organs that is characterized by prolonged fever, anemia, hepatosplenomegaly, and weight loss. VL is fatal if left untreated [6].

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Since the first case report in 1996 [7], indigenous leishmaniasis has been increasingly prevalent in Thailand, especially in HIV patients. Case reports include both VL [7–12] and CL [10, 11, 13–15] and are concentrated in the northern and southern part of Thailand. In contrast to other regions in Asia, most cases in Thailand are caused by either *L. martiniquensis* or *L. siamensis* [12, 16]. As early detection is one of the most important aspects of disease containment, the need for a robust and rapid diagnostic method has never been higher. To summarize, currently available methods can be divided into three groups: parasitological methods, serological methods, and molecular methods, each of which presents various advantages and disadvantages.

Parasitological methods, which include microscopy and parasite culturing, have been considered the gold standard in diagnosing leishmaniasis. In Thailand, however, only a handful of laboratories could culture parasites. Also, serological diagnostic methods with comparable sensitivity to parasitological methods have been developed; however, most of them (enzyme-linked immunosorbent assay (ELISA) [17, 18], immunofluorescence antibody test (IFA) [19], and western blotting [20]) require sophisticated instruments, limiting their usages in healthcare environments. Moreover, these serological techniques have never been evaluated for the diagnosis of leishmaniasis in Thailand.

Molecular methods with great sensitivities and specificities have also been developed to diagnose leishmaniasis. One of the most classical techniques, polymerase chain reaction (PCR), is widely used [21–24]. However, the requirements for expensive equipment, DNA purification, and gel visualization have forestalled its utilization in field settings.

In 2000, Notomi et al. [25] developed the loop-mediated isothermal amplification (LAMP) method. In short, this method uses several complex primers and a strand-displacement polymerase to achieve amplification. Though conceptually challenging, LAMP has several advantages over PCR from a field diagnostics point of view. 1) The reaction proceeds isothermally, thereby obviating the need for expensive thermal cyclers [25]. 2) Crude DNA extracts can be used directly without purification [25, 26]. 3) The products can be detected visually using multiple parameters, including turbidity, fluorescence, and color.

Nevertheless, turbidity is challenging to discern and is unstable over time. Fluorescence measurement requires costly dyes (SYBR Green I [27], calcein [28], propidium iodide [29]) and is technically inconvenient due to its requirement for UV illumination. Due to their inhibitory effects, these dyes must be introduced post-reaction, increasing contamination risks. Colorimetric measurements are among the most straightforward of all of the detection methods. Several dyes have been reported to

be useful, such as hydroxynaphthol blue (HNB) [30] and malachite green (MG) [31]. HNB requires the operator to distinguish between blue and violet, which can be ambiguous. In contrast, MG only requires distinction between blue and transparency.

LAMP has also been applied for the detection of *L. infantum*, both in dogs and humans [32, 33]. Pan-leishmania LAMP is also reported by Karani et al., Mikita et al. and Nzelu et al. [26, 31, 34]. However, *Leishmania*-specific colorimetric LAMP from clinical samples has never been described. Therefore, in this study, we developed a LAMP method using MG to detect *Leishmania* DNA from crude clinical samples. These data can be useful for the deployment of LAMP in field settings and can further enable detailed surveying of *L. siamensis* and *L. martiniquensis* in Thailand.

## Methods

### Primer design

To develop a pan-leishmania assay, we chose the highly conserved 18S ribosomal RNA gene, as in previous pan-leishmania assays [26, 31, 34]. A consensus sequence was made from nine different *Leishmania* species, including *L. tropica* [GenBank: KF041809.1], *L. martiniquensis* [KJ467218.1], *L. mexicana* [KF041806.1], *L. hertigi* [KF041804.1], *L. donovani* [KF041801.1], *L. chagasi* (syn. *L. infantum*) [KF041797.1], *L. infantum* [KF302752.1], *L. amazonensis* [KF302746.1], and *L. enriettii* [KF041798.1]. However, *L. siamensis* was not included in this process due to the absence of its sequences on GenBank. The consensus sequence was imported into the PrimerExplorer version 4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>), and primers were designed to avoid any mutations that were presented. To ensure optimality, primers with the lowest change in Gibb's free energy ( $\Delta G$ ) for dimer formation and the highest change in hybridization  $\Delta G$  were chosen. The primers' thermodynamic properties were further validated using the OligoCalc software (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). A final verification of specificity was performed using Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis against human DNA and any other organisms included in the differential diagnosis of leishmaniasis. The resulting primers are shown in Table 1 and Fig. 1, and were synthesized by BioDesign Co., Ltd, Pathumthani, Thailand.

### Quantitative polymerase chain reaction

The 25  $\mu$ l reaction mixture contained 12.5  $\mu$ l of 2 $\times$  reaction mix from the SuperScript<sup>®</sup> III Platinum<sup>®</sup> SYBR<sup>®</sup> Green One-Step qRT-PCR Kit (Life Technologies, USA), 0.08  $\mu$ M of F3 primer, 0.08  $\mu$ M of B3 primers, and 1 U of BIOTAQ<sup>™</sup> DNA Polymerase (Bioline, Germany). The reaction was performed using the CFX96<sup>™</sup> real-time PCR system (Bio-Rad



DNA using the Invisorb® Spin Tissue Mini Kit (Stratec Biomedical AG, Germany), and the other was boiled at 100 °C for 10 min, as described previously [26].

To simulate infected blood, 100 µl dilutions of *L. siamensis* in human blood were made as described above and divided into two portions. The first portion was added with Triton X-100 to a final concentration of 1 %, and boiled at 100 °C for 10 min, which resulted in a coagulum. Next, 50 µl of ddH<sub>2</sub>O was added, and the coagulum was broken up by vigorous agitation with a pipette tip. The second portion was subjected to DNA extraction using the Invisorb® Spin Blood DNA Mini Kit (Stratec Biomedical AG, Germany); 2.5 µl of each resultant was directly introduced to both qPCR and LAMP.

### Clinical samples

Peripheral blood and saliva were obtained from two patients.

Patient 1 was initially reported by Phumee et al. [35]. In short, the patient was a 49-year-old man who was HIV positive and presented with multiple nodules on his body. Microscopy and cell culturing revealed the presence of *Leishmania* parasites in the nodules, and molecular analysis confirmed *L. martiniquensis* infection [16]. He was successfully treated with amphotericin B and itraconazole. Blood, saliva, and tissue biopsy were obtained. The blood was treated in the same manner as described above, while the saliva and tissue biopsy (drenched in 1× PBS) were boiled at 100 °C for 10 min. 2.5 µl of the supernatant were used as the template.

Patient 2 was described by Chusri et al. [10]. He was a 30-year-old man who was HIV positive. Similar to Patient 1, he had multiple papules on his skin, but this patient also had internal organ involvements. *Leishmania*

parasites were microscopically confirmed to be infiltrating the bone marrow and ulcers. Further molecular data confirmed this *Leishmania* species to be *L. martiniquensis* [16]. He was also successfully treated with amphotericin B and itraconazole. Blood, saliva, and bone marrow biopsy were obtained. The saliva and blood was treated as described earlier, and the bone marrow biopsy was treated in the same manner as the blood.

### Ethical statement

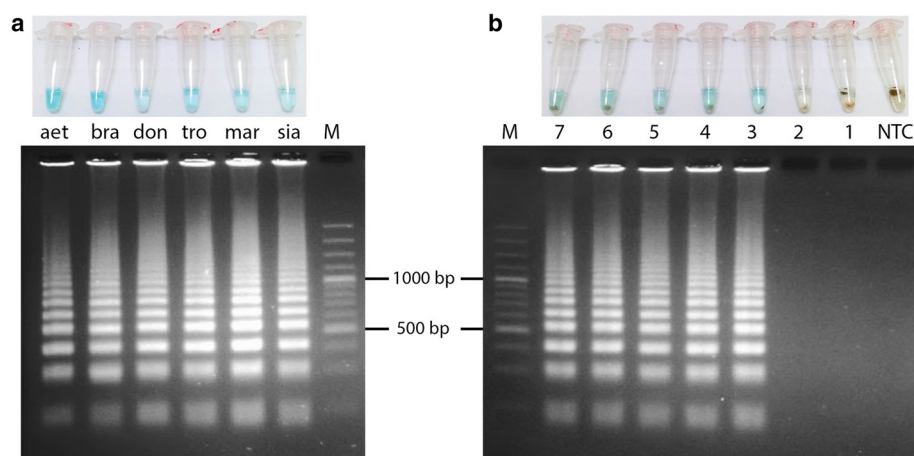
This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University (COA No. 725/2013).

### Results

Both qPCR and LAMP successfully amplified regions that were specified by the newly designed primers. For LAMP, successful amplification was associated with MG's characteristic light blue color, whereas failed amplification was associated with transparency (Fig. 2). Gel electrophoresis of the LAMP products also exhibited their characteristic "mixture of stem-loop DNAs with various stem lengths, and cauliflower-like structures with multiple loops" [25], which further confirmed their successful amplifications.

The detection limits (log<sub>10</sub> parasites/ml) of *L. siamensis* for LAMP, as defined by the appearance of its light blue color, and qPCR, as defined by fluorescence above the C<sub>t</sub>, are shown in Table 2.

For qPCR, our results indicated that the direct use of the samples was possible but was associated with increases in the detection limits. However, whole blood could not be used, due to the total inhibition of *Taq* polymerase.



**Fig. 2** Detection of LAMP Products by MG-based Colorimetric Changes and Gel Electrophoresis. **a** LAMP was able to detect multiple species of *Leishmania*. (aet = *L. aethiopicus*; bra = *L. braziliensis*; don = *L. donovani*; tro = *L. tropica*; mar = *L. martiniquensis*; sia = *L. siamensis*) **b** LAMP could detect *L. siamensis* in the presence of whole blood. Ten-fold dilutions of *L. siamensis* in whole blood (log parasites/ml) were lysed, boiled, and subjected to LAMP. The black precipitates are coagulated blood



**Table 2** Detection limits of LAMP and qPCR under various conditions (log parasites/ml) (F\* = Fail to amplify)

Diluent	Method	LAMP	qPCR
1X PBS	Boiled	3	5
	Extracted	3	4
Saliva	Boiled	3	4
	Extracted	4	4
Whole blood	Boiled	3	F*
	Extracted	4	5

In the case of LAMP, which is more tolerant of PCR inhibitors, removal of the extraction process allowed a lower detection limit of  $10^3$  parasites/ml across all samples. Crude samples did not affect the properties of MG as all of the positive samples displayed MG's characteristic color. The use of whole blood did shift the color toward a greenish tone, whereas the negative samples were yellow in color (Fig. 2). However, an excessive amount of whole blood or the presence of uncoagulated blood could prevent the discrimination between positive and negative results, as hemoglobin absorption spectrum overlapped that of MG.

Clinical samples also yielded useful information in microscopically diagnosed patients. Patient 1, who had CL, had detectable *Leishmania* DNA in his saliva and tissue biopsy. Patient 2, in contrast, who had CL and VL, had the DNA presented in his bone marrow, blood, and saliva (Fig. 3).

## Discussion

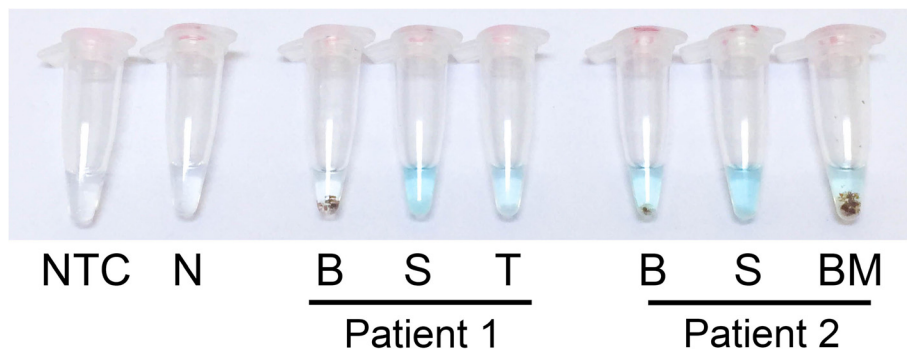
With an increasing number of cases, leishmaniasis is now an emerging infectious disease in Thailand. However, current diagnostic methods require experienced personnel, advanced facilities, and a large amount of time. Thus, simplification is now critical to bring diagnostics to point-of-care settings. Therefore, we developed the LAMP method to complement leishmaniasis

diagnostic process and to facilitate epidemiological studies of leishmaniasis in Thailand.

Molecular techniques have exploited multiple genes to detect *Leishmania*, most of which are high-copy-number genes, including cysteine protease B [36], *gp63* [37], internal transcribed spacer 1 (ITS1) [38], 18S ribosomal RNA [26, 31], and minicircle kinetoplast DNA [24]. As expected, comparative studies have shown that the minicircles, which have the highest copy number, yield the highest sensitivity [39, 40]. However, minicircles are highly variable and due to LAMP's requirement of six conserved regions, it is not feasible to design pan-leishmania LAMP primers that target minicircles. In our approach, we chose the 18S ribosomal RNA gene, which represents a balanced trade-off between copy number and variability. As Karani et al., Mikita et al. and Nzelu et al. recently reported, LAMP has been shown to detect most species of *Leishmania* [26, 31, 34]. Here, we also experimentally confirmed the detection of *L. martiniquensis* and *L. siamensis*. Our detection limit was comparable to that of Mikita et al. ( $10^3$  parasites/ml), but was 10-fold higher than that of Nzelu et al. ( $10^2$  parasites/ml) [26, 31].

We detected *Leishmania* DNA in the blood and bone marrow, and in the tissue and saliva of the VL and CL patient, respectively. Our findings agreed with others that *Leishmania* DNA can be found in multiple noninvasive sources, including saliva (CL, VL) [10, 12, 41], skin swabs (CL) [26] and peripheral blood (VL) [42, 43]. We recommend the use of multiple DNA sources to reduce the probability of false negatives.

Our results also suggested that LAMP could be used to detect *Leishmania* DNA from crude clinical samples without compromising the detection limit. Furthermore, the use of crude samples even lowered the detection limit by 10-fold ( $10^3$  parasites/ml), but the same could not be said with qPCR, which increased this limit by 10-fold. We suspected that in the case of LAMP, the removal of inhibitors did not compensate for the loss of



**Fig. 3** LAMP Could Be Used Directly with Clinical Samples. Crude clinical samples were directly introduced into the LAMP reaction after being subjected to heating. (NTC = no template control; N = healthy patient control; B = blood; S = saliva; T = tissue biopsy; BM = bone marrow)

DNA from the extraction process, as in the case of qPCR. These results are in concordance with other studies that used boiled samples in LAMP [26, 44, 45]. In addition, of all of the DNA preparation methods compared, Sun et al. [46] reported that simple boiling results in the highest sensitivity.

Furthermore, the use of MG greatly facilitates the interpretation of results as it is highly discernable and consistent [31]. Because MG is inexpensive and can be stored at room temperature, this dye could tremendously increase the applicability of LAMP in the field.

Earlier attempts to couple direct blood samples with a colorimetric detection method were unsuccessful, due to the intense color of hemoglobin. To solve this problem, we induced the precipitation of the blood using its own coagulation system. We initially lysed the blood and its accompanying parasites with Triton X-100 and then promoted coagulation by boiling the sample. After boiling the sample, the coagulum was pulverized using a pipette tip. From this approach, we could clearly distinguish the results using MG, even with 2.5  $\mu$ l of whole blood.

Because LAMP can be performed isothermally using a simple heat block, stable electricity is not required. Recently, using phase-change material, LaBarre et al. [47] developed a stable heat block that did not require electricity. Microfluidics lab-on-a-chip for LAMP has also been developed and has enabled highly multiplexed reactions and further simplification [48]. Moreover, Tanner et al. [49] reported that the LAMP reaction mixture can be left at 37 °C for two hours without compromising the detection limit. LAMP mixtures have also been lyophilized with a reported storage time at room temperature of at least seven months [50].

Nevertheless, because of the low prevalence of leishmaniasis in Thailand, we could only obtain a small number of microscopically diagnosed patients. Future work in a statistically significant group of patients is required to warrant the performance of this method.

Also, LAMP is particularly prone to contamination due to the large amount of DNA that it can generate, and its capability to amplify minute amounts of DNA. During this study, we nevertheless encountered multiple contamination issues. Therefore, we recommend that post-amplification reaction mixtures should not be opened due to aerosolization risks, and that all proper precautions be taken [51]. To prevent false positive results, no template control and healthy patient control should also be used. We also tested our assay specificity with *T. vaginalis* and *G. lamblia* DNA, with satisfying results. However, our assay did cross-react with DNA from *Trypanosoma* sp., a closely related protozoon (data not shown). Nevertheless, as each protozoon has a distinct set of clinical presentations, this cross-reaction would not impose a significant risk of misdiagnosis.

## Conclusion

Sensitive molecular techniques to diagnose leishmaniasis have been introduced; nevertheless, due to their complexities, they have not yet been in widespread use. However, as our results demonstrated, LAMP could be used with unpurified clinical samples without compromising sensitivity and specificity, and its results could be unambiguously interpreted using MG.

With the advent of LAMP, molecular techniques can now be seamlessly integrated with field diagnostics. We anticipate that this combination will be crucial for surveying and controlling leishmaniasis in Thailand. However, further evaluation with a large cohort of patients will be required before the assay can be confidently deployed.

## Abbreviations

CL: Cutaneous leishmaniasis; DAT: Direct agglutination test; ELISA: Enzyme-linked immunosorbent assay; ICT: Immunochromatographic test; IFA: Indirect fluorescence antibody; LAMP: Loop-mediated isothermal amplification; MCL: Mucocutaneous leishmaniasis; MG: Malachite green; qPCR: Quantitative polymerase chain reaction; VL: Visceral leishmaniasis.

## Competing interests

We declare that we have no competing interests.

## Authors' contributions

Designed the experiments, conducted molecular laboratory work, and wrote the manuscript: CS, AP, and PS. Sample Collection: CS, AP, MM, SL, and PS. All authors read and approved the final manuscript.

## Authors' information

Mr. Chaichontat Sriworarat is a student at Bangkok Christian College.

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