

## Short Report: Evaluation of Loop-Mediated Isothermal Amplification (LAMP) for Malaria Diagnosis in a Field Setting

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**Abstract.** We used the loop-mediated isothermal amplification (LAMP) method developed by our group for malaria diagnosis with genus-specific and species-specific primers for the four human malaria parasites at a field clinic in comparison with standard microscopy. Among 110 blood samples collected from the malaria clinic in Thailand, LAMP detected 59 of 60 samples positive by microscopy (sensitivity = 98.3%) and none of the 50 microscopy-negative samples (specificity = 100%). Negative predictive value (NPV) and positive predictive value (PPV) of LAMP were 98% and 100%, respectively. These results indicate that LAMP is an effective tool for malaria diagnosis at a field clinic in a field setting.

A rapid and accurate diagnosis of malaria parasites is a challenge in most countries to which malaria is endemic. The conventional diagnostic method for detecting malaria parasites is microscopic examination of thin and/or thick blood smears.<sup>1</sup> Although effective and inexpensive, this method is laborious and time-consuming, and its sensitivity is poor in cases of low parasitemia or if performed by inexperienced personnel. The immunochromatographic method with antibody specific for malaria antigens is rapid, but can only identify *Plasmodium falciparum*-specific antigens and panmalarial antigens, and its sensitivity and specificity is lower for species other than *P. falciparum*.<sup>2</sup>

In Thailand, malaria is caused mainly by *P. falciparum* and *P. vivax*, but *P. ovale*, *P. malariae*, and *P. knowlesi* have been occasionally detected.<sup>3,4</sup> In many malaria-endemic areas in Thailand, *P. vivax* has recently become more prevalent than *P. falciparum*.<sup>3</sup> Because of different treatments for infections with different malaria parasite species and the requirement of high cost (artemisinin-based) drugs for *P. falciparum*, there is an increasing need for accurate diagnosis that cannot be met by microscopy in malaria-endemic areas.

Nested polymerase chain reaction (nested PCR) and quantitative PCR have been developed to achieve higher sensitivity and specificity than microscopic examination.<sup>5–7</sup> However, their implementation in field clinics has been impeded by the requirement for relatively expensive equipments.<sup>8</sup>

Loop-mediated isothermal amplification (LAMP) has enabled use of a rapid, sensitive, specific, and simple method for the diagnosis of various diseases, including parasitic diseases.<sup>9</sup> The LAMP procedure uses *Bst* DNA polymerase and a set of four specifically designed primers that recognize six distinct regions of the target DNA. Amplification and detection of the target gene can be completed in one isothermal step.<sup>9,10</sup> Autocycling strand-displacement DNA synthesis continues with an accumulation of approximately 10<sup>9</sup> copies of target DNA within a period of less than one hour. The ampli-

fied products consist of a series of stem-loop DNA structures of various lengths. Simple detection can be achieved by visual inspection of the turbidity of magnesium pyrophosphate, a byproduct of DNA synthesis, which is produced in proportion to the amount of amplified DNA.<sup>11</sup> In addition, real-time detection can be performed by using a Loopamp real-time turbidimeter.<sup>12</sup>

Our group has applied LAMP for malaria diagnosis in the laboratory using frozen blood samples collected from malaria patients at a malaria clinic in Thailand. This procedure has a sensitivity and specificity comparable with that of nested PCR.<sup>12</sup> In this study, LAMP and microscopy, a gold standard, were performed at a malaria field clinic in Thailand. We report the evaluation of LAMP for malaria diagnosis at a field clinic in a field setting. The LAMP conditions were optimized for field conditions and evaluated against standard microscopy.

A total of 110 blood samples were collected from patients  $\geq 15$  years of age who came to a malaria clinic in Mae Sot District, Tak Province, in northwestern Thailand during September–December 2007 and September 2008. Only uncomplicated malaria patients were recruited for this study. After obtaining patient and/or legal guardians signed consents, blood samples were collected by finger prick using heparinized capillary tubes (2–3 of 50- $\mu$ L capillary tubes) or filter papers (1.75 cm  $\times$  0.5 cm) (without heparin). This portion of blood samples was used for LAMP assay. Another portion was used for thick and thin blood films as a routine malaria diagnosis. Thick blood smears were examined under a light microscope (1,000 $\times$  magnification) by a clinic staff to identify malaria parasites. Results for thick blood films were confirmed by an expert microscopist from Bangkok. Thin blood films were used to identify the species of malaria parasites. Parasitemia was defined as the number of parasites detected per 500 leukocytes and was calculated by assuming a leukocyte count of 8,000 cells/ $\mu$ L of blood.<sup>13</sup> The initial thick blood film was classified as negative if no parasites were found after 500 leukocytes were counted.

Blood samples collected by two methods were subjected for DNA extraction before LAMP analysis. The LAMP assay was performed and read by a researcher from Bangkok who was blinded with respect to microscopy results. LAMP was carried out as follows. For blood obtained with capillary tubes, 50  $\mu$ L of blood samples were mixed with an equal volume of

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distilled water, boiled for 5 minutes, centrifuged at  $2,046 \times g$  for 2 minutes, and 2  $\mu\text{L}$  of supernatant were used for the LAMP assay. For blood samples collected onto filter papers, a blood filter (1.75 cm  $\times$  0.5 cm) was cut into small pieces, placed in a micro-tube, mixed with 150  $\mu\text{L}$  of distilled water, boiled, and processed as described for blood samples obtained with capillary tubes.

The LAMP assay, which used primer sets specific for the genus *Plasmodium* and the four species of human malaria parasites, was conducted as previously described.<sup>12</sup> The LAMP reaction was incubated in a water bath and a Loopamp real-time turbidimeter (RT-160C; Eiken Chemical Co., Tokyo, Japan). LAMP-amplified DNA was observed by the naked eye (turbidity; water bath incubation) or by using a turbidimeter. Results for the LAMP assay and microscopic examination performed on all samples were evaluated for sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) for *Plasmodium* spp. using microscopy as the gold standard.

Evaluation of the LAMP assay was performed with finger prick blood samples collected by using heparinized capillary tubes or filter papers. LAMP-amplified DNA was assessed by naked eye by observing the turbid solution (qualitative) and by using a turbidimeter (quantitative). There were no significant differences between LAMP assay results, both by visual inspection and using a turbidimeter, for samples collected in heparinized capillary tubes or filter papers, indicating that the anti-coagulant has no effect on the LAMP reaction.

As shown in Table 1, of 110 patients examined, 60 (54.5%) were positive by microscopy and had parasitemia of 365–16,500 parasites/ $\mu\text{L}$ ; 19 patients (24.1%) had *P. falciparum* infection (parasitemia = 365–14,150 parasites/ $\mu\text{L}$ ), 39 patients (49.4%) had *P. vivax* infection (parasitemia = 500–16,500 parasites/ $\mu\text{L}$ ), 1 patient (1.3%) had *P. falciparum* infection (parasitemia = 5,500 parasites/ $\mu\text{L}$ ) and *P. vivax* infection (parasitemia = 1,950 parasites/ $\mu\text{L}$ ), and 1 patient (1.3%) had *P. vivax* infection (parasitemia = 14,500 parasites/ $\mu\text{L}$ ) and *P. ovale* infection (parasitemia = 2,000 parasites/ $\mu\text{L}$ ). The remaining 50 patients were negative for malaria parasites.

As shown in Table 2, LAMP detected malaria parasites in 59 of 60 (sensitivity = 98.3%) samples positive by microscopy, but none of 50 samples negative by microscopy were positive by LAMP (specificity = 100%). The NPV and PPV of LAMP for malaria diagnosis were 98% and 100%, respectively. There were five nonconcordant results (4.5%) composed of 1 of 19 samples positive for *P. falciparum* by microscopy, 2 of 39 for

TABLE 2  
Sensitivity, specificity, NPV, and PPV of LAMP compared with those of microscopy, a gold standard, for malaria parasite detection and species identification, Thailand\*

Variable	LAMP	
	No. positive/no. tested (%)	
Sensitivity	59/60 (98.3)	
Specificity	50/50 (100)	
NPV	50/51 (98)	
PPV	59/59 (100)	

\*NPV = negative predictive value; PPV = positive predictive value; LAMP = loop-mediated isothermal amplification. Sensitivity = (no. true positive results)/(no. true positive results plus no. false-negative results); Specificity = (no. true negative results)/(no. true negative results plus number of false-positive results); NPV = no. true negative results/(no. true negative results plus no. false-negative results); PPV = no. true positive results/(no. true positive results plus no. false-positive results).

*P. vivax*, and 2 of 2 mixed infection. These discrepancies were later confirmed by nested PCR (performed by a researcher at a laboratory in Bangkok) (Table 1). Total time required to evaluate the last 10 cases by LAMP assays after blood collection was 75 minutes compared with the maximum 10 cases within 60 minutes by microscopy.

Isothermal DNA amplification using the LAMP technology has been developed by our group for rapid detection of the four species of human malaria parasites by using frozen clinical blood samples collected from a malaria-endemic area in Thailand and transported to the laboratory for an assay.<sup>12</sup> In this study, we evaluated a LAMP assay at a malaria clinic in northwestern Thailand. Compared with conventional microscopy, the gold standard, LAMP-based diagnosis performed equally well in terms of specificity (100%) and PPV (100%), but showed a lower sensitivity (98.3%) and NPV (98%). In one sample (parasitemia = 365 parasites/ $\mu\text{L}$ ) positive for *P. falciparum* by microscopy but negative by LAMP was later shown to be negative by nested PCR.

Because the parasitemia level was within the detectable level of LAMP or nested PCR, there may be other reasons why this sample was negative for *P. falciparum* by both LAMP and nested PCR, including the use of a smaller amount of blood or a lower efficiency of DNA extraction of this sample. Of the 2 microscopic-positive *P. vivax* samples, 1 sample (parasitemia = 2,300 parasites/ $\mu\text{L}$ ) was positive only by a genus-specific LAMP and another sample (parasitemia = 16,450 parasites/ $\mu\text{L}$ ) was positive for mixed *P. falciparum* and *P. vivax* infection. Nested PCR confirmed this mixed infection result.

Of two samples positive for mixed infection by microscopy but positive only for *P. vivax* by LAMP, nested PCR confirmed the latter results. The nonconcordance between the results may be caused by multiple genotypes of parasites in natural infection,<sup>14,15</sup> indicating that new primer sets would be required to amplify regions that are universally conserved among *Plasmodium* genotypes (especially for *P. vivax*) to obtain more reliable results when using DNA amplification methods. Overall, LAMP yielded results comparable to those of microscopy. However, on the basis of confirmation of nested PCR diagnosis of malaria, LAMP may have greater specificity than microscopy.

We have demonstrated that malaria diagnosis by the LAMP method can be performed at a field clinic. The minimum equipment required is a water bath or a heat block for carrying out the LAMP reaction. However, this method needs to be validated by using a larger set of samples before it can be adopted for routine malaria diagnosis in the field. Presently,

TABLE 1

Comparison of microscopy and LAMP for malaria parasite detection and species identification, Thailand\*

Parasites detected by microscopy and LAMP (No. samples)†	
Microscopy	LAMP
<i>Plasmodium falciparum</i> (19)	<i>P. falciparum</i> (18), negative (1)‡
<i>P. falciparum</i> plus <i>P. vivax</i> (1)	<b><i>P. vivax</i> (1)§</b>
<i>P. vivax</i> (39)	<i>P. vivax</i> (37), <b><i>P. falciparum</i></b> plus <b><i>P. vivax</i> (1),¶ Genus specific (1)§</b>
<i>P. vivax</i> plus <i>P. ovale</i> (1)	<b><i>P. vivax</i> (1)§</b>
Negative (50)	Negative (50)

\*LAMP = loop-mediated isothermal amplification.

† Each row shows results obtained from identical blood samples. LAMP results that were not concordant from those of microscopy are shown in bold.

‡ Negative for *P. falciparum* by nested polymerase chain reaction.

§ Positive for *P. vivax* by nested polymerase chain reaction.

¶ Positive for *P. falciparum* and *P. vivax* by nested polymerase chain reaction.

our group is carrying on further validation for LAMP to identify specificity, sensitivity, NPV, and PPV in a larger sample size of patients.

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