JCM
Journals.ASM.org

Loop-Mediated Isothermal Amplification Assay for Rapid Diagnosis of Malaria Infections in an Area of Endemicity in Thailand

Jetsumon Sattabongkot,[a] Takafumi Tsuboi,[b] Eun-Taek Han,[c] Sirasate Bantuchai,[a] Sureemas Buates[d]

Mahidol Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand[a]; Proteo-Science Center and Venture Business Laboratory, Ehime University, Matsuyama, Ehime, Japan[b]; Department of Medical Environmental Biology and Tropical Medicine, Kangwon National University School of Medicine, Chuncheon, Gangwon-do, Republic of Korea[c]; Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand[d]

The loop-mediated isothermal amplification (LAMP) method, developed by our group for diagnosis of four human malaria parasites, was evaluated on a large scale at a remote clinic in Thailand where malaria is endemic. A total of 899 febrile patients were analyzed in this study. LAMP was first evaluated in 219 patients, and the result was compared to those of two histidine-rich protein (HRP)-2 rapid diagnostic tests (RDTs) and microscopy as a gold standard. LAMP DNA extraction was conducted by a simple boiling method, and the test results were assessed visually. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 95.7%, 100%, 100%, and 98%, respectively, for LAMP and 98.6%, 98%, 95.8%, and 99.3%, respectively, for RDTs. Since RDT-positive results were based on one out of two RDTs, the sensitivity of RDTs was slightly higher than that of LAMP. However, LAMP tended to be more specific than RDTs. LAMP next was evaluated in 680 patients, and the result was compared to that of microscopy as a gold standard. Sensitivity, specificity, PPV, NPV, and diagnostic accuracy of LAMP were 88.9%, 96.9%, 92.2%, 95.5%, and 94.6%, respectively. Nested PCR was used to confirm the discrepant results. Malaria LAMP in a remote clinic in Thailand achieved an acceptable result, indicating that LAMP malaria diagnosis is feasible in a field setting with limited technical resources. Additionally, the rapid boiling method for extracting DNA from dried blood spots proved to be simple, fast, and suitable for use in the field.

According to the 2011 World Health Organization (WHO) report, there are 106 countries or regions where malaria is endemic, and up to half of the world's population is at risk for acquiring malaria infection (1). Both malaria morbidity and mortality cases declined from 225 million cases and 781,000 deaths in 2009 to 216 million cases and 655,000 deaths in 2010 (1). While the decrease in both cases and deaths is notable, the potential threat of a malaria epidemic in a low-transmission status is of great concern, as the majority of the asymptomatic carriers are untreated (2). Asymptomatic infections serve as reservoirs for local malaria transmission and as a risk factor for symptomatic attacks (3–7). A prerequisite in the effort to eliminate malaria from an area in which it is endemic is to identify asymptomatic carriers for treatment (8, 9). Thus, a rapid and accurate diagnosis, as well as prompt and effective treatment, is critical for malaria elimination strategies in those regions.

Microscopic examination of thin and/or thick blood smears is the most commonly used diagnostic method for malaria detection in the field, and it still remains the gold standard (10). This method is inexpensive and effective, since it can provide quantitative data and can identify species when performed correctly. However, it is laborious and time-consuming. Misdiagnosis of the infection species is common in cases of low parasitemia or if performed by inexperienced personnel. Recently, lateral-flow-based malaria rapid diagnostic tests (RDTs) have become available. RDTs are most commonly based on using specific antibodies to detect malaria antigens such as histidine-rich protein 2 (HRP-2) and lactate dehydrogenase (LDH). Although RDTs are simple and quick, they can only identify *Plasmodium falciparum*- and *P. vivax*-specific antigens and panmalarial antigens, and their sensitivity and specificity are lower for non-*falciparum* species (11). Moreover, RDTs for HRP-2 should not be used to monitor response to therapy, since this antigen does not clear from blood for up to 30 days after treatment (12). RDT-based *P. vivax* detection poses difficulties due to low parasitemia observed in vivax patients and instability of LDH at higher temperatures.

Therefore, a good alternative for malaria diagnosis may be a nucleic acid-based molecular method, since it can correctly differentiate all human *Plasmodium* species and detect low levels of parasitemia. Nested PCR and real-time quantitative PCR have also been developed. These methods have high sensitivity, with a detection limit of 1 to 5 parasites/μl of blood (which is below the threshold of microscopy and RDTs), and enable greater specificity for mixed infections. However, they have not been implemented in field clinics in most areas where malaria is endemic due to requirements for relatively expensive equipment and advanced training (13–17).

Loop-mediated isothermal amplification (LAMP), a high-performance method for detecting DNA, is a practical alternative. This technique is relatively simple and field adaptable (18). Unlike PCR, LAMP uses simpler equipment and is less time-consuming. LAMP has the potential to be used as a molecular diagnostic tool for point-of-care (POC) testing in both developing and developed countries. It has already been used for the diagnosis of various infectious diseases, including malaria (19–24). LAMP amplifies and detects target DNA in a single isothermal step using a *Bacillus*

Received 26 November 2013 **Returned for modification** 20 December 2013
Accepted 14 February 2014
Published ahead of print 26 February 2014
Editor: P. H. Gilligan
Address correspondence to Sureemas Buates, sbuates@hotmail.com.

doi:10.1128/JCM.03313-13

stearothermophilus DNA polymerase with strand displacement activity and a set of four specifically designed primers that recognize a total of six distinct regions of the target DNA (18, 25). The autocycling strand displacement DNA synthesis amplifies a few DNA copies to 10^9 copies in less than an hour. The amplified products consisting of a series of stem-loop DNA structures of various lengths can be detected simply by visual inspection of the turbidity of magnesium pyrophosphate, a by-product of DNA synthesis which is produced in proportion to the amount of amplified DNA (26). In addition, real-time detection can be performed using a Loopamp real-time turbidimeter. Moreover, LAMP reactions can be accelerated by using two extra primers, known as loop primers, which can shorten the amplification time by around one-third to one-half (27).

A genus- and species-specific LAMP diagnostic method that was recently developed by our group has been applied to malaria diagnosis at a field clinic in an area where malaria is endemic in Thailand (28). Using heat-treated clinical samples, LAMP demonstrated sensitivity and specificity of 98.3% and 100%, respectively, compared to the gold standard of microscopy (28). In this study, we further evaluated the feasibility of our species-specific LAMP diagnostic method for malaria field diagnosis on a large scale in a resource-limited setting. The LAMP performance was compared to that of RDT using conventional microscopy as a gold standard.

MATERIALS AND METHODS

Ethics, study site, and population. Participation consent forms were provided by the Mahidol University ethical committee, Mahidol University, Bangkok, Thailand, and signed consent was obtained from patients and/or legal guardians. Patient care was not influenced by LAMP results. The study was conducted from May 2008 to May 2011 in Maesot district, Tak province, northwestern Thailand at a walk-in malaria clinic, a government-sponsored health facility, where malaria diagnosis is based on microscopy. In this region, *P. falciparum* and *P. vivax* cause the great majority of malaria infections, but *P. ovale* and *P. malariae* have occasionally been detected (28). Study selection criteria were the following: walk-in patients aged 15 years or older, documented presence of febrile illness, and absence of evidence of severe illness. At enrollment, the patient's history of previous malaria infection, age, sex, blood pressure, respiratory rate, hematocrit, blood group, and axillary temperature were recorded.

Sample size and blood sample collection. Sample size calculations were based on an assumption of microscopy-confirmed prevalence of 10% among symptomatic patients and LAMP sensitivity for *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* compared to expert microscopy of 95% (95% confidence interval [CI], 90% to 99%) and specificity of 95% (95% CI, 90% to 99%), which required 994 participants. A target of 1,017 participants was set. A total of 1,017 blood samples were collected by finger prick using new sterilized lancets. A portion of the finger prick blood was used directly for thick and thin blood smear preparations and RDTs. The other portion was collected using 3 pieces of filter paper (dimensions, 0.5 cm by 4.5 cm). Finger-prick blood was absorbed 1 to 3 cm from the bottom of the strips (approximately 10 to 30 μl of blood volume). Strips were kept at room temperature and used for LAMP assays. The RDT and LAMP tests were carried out on site and interpreted by independent researchers blinded to the origin of the specimens and the microscopic results.

Conventional microscopy. Thick and thin blood films were prepared by clinical staff and stained with 10% Giemsa for 10 min. A thick blood film was examined immediately under a light microscope (1,000× magnification) according to routine standard operating procedures for malaria diagnosis by clinic staff to identify malaria parasites for use in case management. The patient received regular treatment if the blood smear was positive for malaria. The initial thick film was classified as negative if no parasites were found after 500 white blood cells were counted. The result from the thick blood film was confirmed by an expert microscopist from Bangkok (expertise level 1 or 2 following the WHO competency assessment protocol; www.searo.who.int/LinkFiles/Malaria_MalariaMicroscopyManual.pdf). A thin blood film was prepared and used to determine the species of malaria parasites. To clarify any discrepancies in results, blood films were reexamined by a second expert microscopist. During the course of this study, all microscopist were blinded to the results of LAMP, previous microscopy, and RDT (rapid diagnostic test). Parasitemia from a thin blood film was defined as the number of parasites detected per 10,000 red blood cells (RBCs) and was calculated by assuming an RBC count of 5×10^6 RBCs/μl of blood (29).

DNA extraction. Two different DNA extraction methods were evaluated: the saponin/Chelex method (30) and the simple heating method. For the saponin/Chelex method, the blood filter was cut and placed in a 1.5-ml microcentrifuge tube containing 1.0 ml of 0.5% saponin–phosphate-buffered saline (PBS). The tube was inverted 2 to 3 times and incubated at room temperature (RT) for 4 h or more. The brownish-red solution was removed and replaced with 1 ml of saponin-PBS. The tube was inverted and incubated at 4°C for 15 to 30 min. Fifty μl of 20% Chelex-100 (Bio-Rad, Richmond, CA, USA) was fully suspended by vortexing in 150 μl of double-distilled water (DDW) and heated to 100°C. After aspiration of the saponin-PBS, 200 μl of hot Chelex was added, vortexed at the highest speed for 30 s, and boiled at 100°C for 5 min. The tube was vortexed for 30 s and spun at 10,000 × *g* for 2 min. The supernatant containing DNA was collected into a new clean tube, and 5 μl was used immediately for each LAMP reaction or kept at −20°C for up to 10 months.

For the simple heating method, a blood filter was cut into small pieces, placed in a 1.5-ml microcentrifuge tube containing 150 μl of distilled water, and boiled at 100°C for 5 min. The blood sample was centrifuged at 2,046 × *g* for 3 min, after which 100 μl of the supernatant containing DNA was collected, and 5 μl was used immediately for each LAMP reaction.

LAMP conditions. The LAMP primer sets, as previously described by Han et al., were used in this study to amplify the gene coding for the 18S rRNA (21). These primers were specific for the *Plasmodium* genus, i.e., *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). Each 25-μl reaction mixture contained 1 μl of each forward inner primer (FIP) and backward inner primer (BIP) (1.6 μM for *P. falciparum* and *P. ovale* and 2.4 μM for *P. vivax* and *P. malariae*), 1 μl of 0.2 μM each forward outer primer (F3) and backward outer primer (B3), 1 μl of 0.8 μM each loop primer forward (LPF) and loop primer backward (LPB), 12.5 μl of 2× reaction mix, 1 μl of *B. stearothermophilus* DNA polymerase, 5 μl of DNA sample, and 0.5 μl of RNase- and DNase-free water. The LAMP reaction mixture was incubated in a water bath at 60°C for 90 min. For result confirmation, the LAMP reaction was placed into a Loopamp real-time turbidimeter (RT-320C; Eiken Chemical Co., Tokyo, Japan) for 90 min at 60°C, followed by 2 min at 80°C to inactivate the enzyme and halt amplification. Positive and negative controls were included in each run.

Analysis of LAMP products. A successful LAMP reaction results in large amounts of amplified DNA and turbidity of insoluble magnesium pyrophosphate, a by-product of DNA synthesis produced in proportion to the amount of amplified DNA. The turbidity can be observed by the naked eye. A LAMP reaction was considered positive for *Plasmodium* spp. and for four *Plasmodium* species DNA if an obvious increase in the turbidity was observed by the naked eye compared to the negative control. Results were read by two researchers blinded to the origin of the specimens and the microscopic results. Results were considered valid if turbidity was present in the positive control and absent from the negative control.

RDT. Each blood sample was also tested by RDT diagnostic kits as a reference rapid assay for malaria. The RDT rapid diagnostic kit, the Care-

Start malaria pLDH/HRP2 combo (Pf/Pv) kit, and the CareStart malaria PF/VOM combo kit (Access Bio, Inc., NJ, USA) were purchased from a local distributor based on the recommendation of the Thai Ministry of Public Health and based on good performance as documented by the WHO/Foundation for Innovative New Diagnostics (FIND) malaria RDT evaluation program. The CareStart malaria pLDH/HRP2 combo (Pf/Pv) kit is based on the detection of histidine-rich protein-2 (Pf HRP-2) antigen produced by *P. falciparum* trophozoites and young gametocytes and lactate dehydrogenase (pLDH) produced by both sexual and asexual parasitic stages of *P. vivax*. The CareStart malaria PF/VOM combo kit is based on the detection of *P. falciparum*-specific histidine-rich protein-2 (Pf HRP-2) antigens and *Plasmodium*-specific LDH antigens or a panmalaria antigen of *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*. The assays were performed in parallel according to the manufacturer's instructions using a drop of whole blood from a finger prick, and the results were observed after 20 min by the naked eye.

Nested PCR. For nested PCR, the species-specific nucleotide sequences of the 18S rRNA genes of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* were amplified as described previously (21). This assay was performed off site and was used to verify the discrepant results.

Statistical analysis. Specimens were classified as true positive, true negative, false positive, or false negative for each test under evaluation compared to the microscopic examination as a gold standard. The clinical sensitivity, specificity, positive and negative predictive values (PPV and NPV), agreement, diagnostic accuracy, and 95% CI of the *Plasmodium* LAMP and RDT diagnostic kit were calculated. Sensitivity was calculated as (number of true positives)/(number of true positives + number of false negatives) × 100, and specificity was calculated as (number of true negatives)/(number of true negatives + number of false positives) × 100. The PPV was calculated as (number of true positives)/(number of true positives + number of false positives) × 100, and the NPV was calculated as (number of true negatives)/(number of true negatives + number of false negatives) × 100. The accuracy was calculated as (number of true positives + number of true negatives)/(total number of patients) × 100. The degree of agreement between two diagnostic tests was measured by the concordance response rate (percentage of responses with both positive or both negative results). Data were analyzed using SAS software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Blood samples were collected with informed consent from 1,017 febrile participants, 683 (67.2%) of which were males, and 334 (32.8%) females. At the beginning of this study, two different DNA template preparation methods (saponin/Chelex and simple heating) were used to evaluate the LAMP assay. Results from the initial data demonstrated that there was no significant difference between LAMP results obtained by different DNA template preparation methods (data not shown). This indicated that the simple heating method proved efficient for use. Therefore, the simple boiling method for DNA extraction was used for the remainder of our study.

During the beginning of this present study, it was found that LAMP assays with the simple heating DNA extraction method failed. This is probably due to the instability of DNA samples, which were kept on ice for 4 to 5 h after the blood filters were heated before being used in the LAMP assay. This indicated that the DNA from heat-treated samples should be used immediately after extraction. As a result of this failure, samples and data from 118 febrile patients were excluded from analysis.

Therefore, of the 1,017 total enrolled patients, data were evaluated from 899 patients, where 595 (66.2%) were males and 304 (33.8%) were females. All patients self-reported a history of fever. The age of patients ranged between 15 and 79 years. All participants were screened for *Plasmodium* infection. Microscopic examination revealed malaria parasites in the blood smears of 263 (29.3%) patients (7,075 ± 6,300 parasites/μl [means ± standard deviations {SD}]; range, 14 to 42,140 parasites/μl). Of these, 61 patients (23.2%) were positive for *P. falciparum* infection (154 to 41,062 parasites/μl), 194 (73.8%) with *P. vivax* infection (140 to 42,140 parasites/μl), 1 (0.4%) with *P. malariae* infection (714 parasites/μl), 1 (0.4%) with *P. ovalae* infection (4,200 parasites/μl), and 6 (2.3%) with mixed *P. falciparum* and *P. vivax* infection (280 to 14,014 parasites/μl). The remaining 636 (70.7%) samples were negative.

TABLE 1 Detailed comparison of microscopy, RDT, and LAMP for malaria parasite detection and species identification

Parasite(s) detected by microscopy, RDT, and LAMP[a] (no. of samples; n = 219 blood samples)		
Microscopy	LAMP	RDT
P. falciparum (16)	*P. falciparum* (14), **negative**[b] (2)	*P. falciparum* (16)
P. falciparum + *P. vivax* (1)	*P. falciparum* + *P. vivax* (1)	*P. falciparum* + *P. vivax* (1)
P. vivax (52)	*P. vivax* (51), **negative**[c] (1)	*P. vivax* (52)
P. malariae (1)	*P. malariae* (1)	**Negative**[d] (1)
Negative (149)	Negative (149)	Negative (146), ***P. falciparum***[e] (2), ***P. vivax***[f] (1)

[a] Each row displays results obtained from identical blood samples. Discordant results between LAMP and RDT are shown in boldface.
[b] Positive for *P. falciparum* by nested PCR.
[c] Positive for *P. vivax* by nested PCR.
[d] The RDT can detect only *P. falciparum* and *P. vivax*, and this sample was positive for *P. malariae* by nested PCR.
[e] Negative for *P. falciparum* by nested PCR.
[f] Negative for *P. vivax* by nested PCR.

LAMP performance compared to RDTs and microscopy. Of 899 blood samples, 219 were analyzed by LAMP, RDTs, and microscopy as a gold standard. The LAMP assay, RDTs, and microscopy were performed on site. Two different RDT kits, the CareStart Malaria pLDH/HRP2 Combo (Pf/Pv) and CareStart Malaria PF/VOM Combo kits, were used in this study. These RDT kits detect only *P. falciparum* and *P. vivax*. The detailed comparison of LAMP, RDTs, and microscopy for diagnosis of four human-infecting *Plasmodium* species is depicted in Tables 1 and 2.

Table 1 shows that of the 219 patients examined, 70 (32%) were positive by microscopy (7,750 ± 6,550 parasites/μl; range, 168 to 42,140 parasites/μl); 16 (22.9%) had *P. falciparum* infection (1,666 to 14,028 parasites/μl), 52 (74.3%) had *P. vivax* infection (168 to 42,140 parasites/μl), 1 patient (1.4%) had mixed *P. falciparum* and *P. vivax* infection (14,014 parasites/μl), and 1 patient (1.4%) had *P. malariae* infection (714 parasites/μl). The remaining 149 patients were negative for malaria parasites.

Table 2 shows that 70/219 (32%) patients tested positive by microscopy for *Plasmodium* infection. LAMP identified malaria patients in 67/70 (95.7% sensitivity; 95% CI, 89.7 to 101.8%) microscopy-positive samples. None of the 149 samples that were negative by microscopy was positive by LAMP (100% specificity; 95% CI, 100 to 100%). LAMP for malaria diagnosis had PPV and NPV of 100% (95% CI, 100 to 100%) and 98% (95% CI, 95.2 to 100.8%), respectively. The diagnostic accuracy and agreement between LAMP and microscopy results were 98.6% and 96.8%, re-

TABLE 2 Comparison of LAMP and RDTs to microscopy for malaria diagnosis ($n = 219$ blood samples)

Method and result	No. of results for microscopy		Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	Diagnostic accuracy (%)	Agreement (%)
	Positive	Negative						
Microscopy								
Positive	70	0						
Negative	0	149	100	100	100	100	100	100
LAMP								
Positive	67	0						
Negative	3	149	95.7 (89.7–101.8)	100 (100–100)	100 (100–100)	98 (95.2–100.8)	98.6	96.8
RDTs								
Positive	69	3						
Negative	1	146	98.6 (95.0–102.1)	98.0 (95.1–100.9)	95.8 (89.9–101.7)	99.3 (97.6–101)	98.2	95.8

spectively. For RDTs, both kits produced comparable results, except for one microscopically *P. falciparum* positive sample. This sample was positive by pLDH/HRP2 RDT but negative by PF/VOM RDT. Since the RDT-positive result was based on one out of two RDTs, this sample was considered positive. The RDT was positive for *P. falciparum* and *P. vivax* in 69/70 (98.6% sensitivity; 95% CI, 95 to 102.1%) microscopically positive samples. Of the 149 microscopically negative samples, 146 were negative by RDT (98% specificity; 95% CI, 95.1 to 100.9%). RDT for malaria diagnosis showed 95.8% PPV (95% CI, 89.9 to 101.7%) and 99.3% NPV (95% CI, 97.6 to 101%). The diagnostic accuracy and agreement between RDTs and microscopy results were 98.2% and 95.8%, respectively. Overall, both LAMP and RDTs yielded results very similar to those of microscopy. The exceptions were three nonconcordant results (1.4%) for LAMP, and these were two cases for *P. falciparum* and one case for *P. vivax* and were later shown to be positive by nested PCR (used as a confirmation method for the discrepant results and performed off site by a researcher at a laboratory in Bangkok). There were four discordant results (1.8%) for RDTs, and these were of *P. falciparum*, *P. vivax*, and *P. malariae* infection. Seven samples (3.2%) had discordant results between LAMP and RDTs. It has to be noted that RDT kits used in this study detected only *P. falciparum* and *P. vivax*; therefore, one microscopically *P. malariae*-positive sample was negative by RDT. Three samples negative by LAMP and microscopy but positive for *P. falciparum* and *P. vivax* by RDTs were later shown to be negative by nested PCR. Since the RDT-positive result was based on one out of two RDTs, the sensitivity and NPV were slightly higher than those of LAMP. However, LAMP was prone to having greater specificity, PPV, and agreement than RDTs.

LAMP performance compared with microscopy. Since LAMP showed comparable results with RDTs, the remainder of the 680 blood samples was analyzed by LAMP and microscopy as a gold standard. Out of 680 patients examined, 193 (28.4%) were positive by microscopy (6,850 ± 6,200 parasites/μl; range, 140 to 41,062 parasites/μl); 45 (23.3%) had *P. falciparum* infection (154 to 41,062 parasites/μl), 142 (73.6%) had *P. vivax* infection (140 to 32,060 parasites/μl), 5 (2.6%) had mixed *P. falciparum* and *P. vivax* infection (280 to 14,000 parasites/μl), and 1 patient (0.5%) had *P. ovale* infection (4,200 parasites/μl). The remaining 487 patients were negative for malaria parasites by microscopy.

After analysis, there were 46 discordant results (6.8%) composed of 8 of 45 samples positive for *P. falciparum* by microscopy, 17 of 142 samples positive for *P. vivax*, and 21 of 487 negative samples. The discrepancies between LAMP and microscopy results were later confirmed by nested PCR, which showed results consistent with those of microscopy. However, out of 21 cases negative by microscopy but positive by LAMP, 6 were later shown to be positive by nested PCR (3 positives for *P. falciparum* and 3 positives for *P. vivax*). This indicated that microscopy gave underestimated results. To reflect the most accurate data, the results are shown after the discrepancy analysis as shown in Table 3.

Table 4 shows that microscopy was positive for malaria infection in 199/680 (29.3%) patients. LAMP detected malaria patients in 177/199 samples positive by microscopy (88.9% sensitivity; 95% CI, 83.4 to 94.5%). Of 481 microscopy-negative samples, 466 were negative by LAMP (96.9% specificity; 95% CI, 94.9 to 98.9%). PPV and NPV of LAMP for malaria diagnosis were 92.2% (95% CI, 87.3 to 97%) and 95.5% (95% CI, 93.1 to 97.8%), respectively. The diagnostic accuracy and agreement between

TABLE 3 Detailed comparison of microscopy and LAMP for malaria parasite detection and species identification ()

Parasite(s) detected by microscopy and LAMP[a] (no. of samples; $n = 680$ blood samples)	
Microscopy	LAMP
P. falciparum (48)	*P. falciparum* (40), **negative**[b] (8)
P. falciparum + *P. vivax* (5)	*P. falciparum* + *P. vivax* (5)
P. vivax (145)	*P. vivax* (128), ***P. falciparum* + *P. vivax*** (1),[c] **genus specific**[c] (2), **negative**[c] (14)
P. ovale (1)	*P. ovale* (1)
Negative (481)	Negative (466), **genus specific**[d] (7), ***P. falciparum***[e] (1), ***P. falciparum* + *P. vivax*** (1),[f] ***P. vivax***[g] (4), ***P. malariae***[h] (2)

[a] Each row displays results obtained from identical blood samples. Discordant results between microscopy and LAMP are shown in boldface.
[b] Positive for *P. falciparum* by nested PCR.
[c] Positive for *P. vivax* by nested PCR.
[d] Negative results by nested PCR.
[e] Negative for *P. falciparum* by nested PCR.
[f] Negative for *P. falciparum* + *P. vivax* by nested PCR.
[g] Negative for *P. vivax* by nested PCR.
[h] Negative for *P. malariae* by nested PCR.

TABLE 4 Comparison of LAMP to microscopy for malaria diagnosis (n = 680 blood samples)

Method and result	No. of results for microscopy: Positive	No. of results for microscopy: Negative	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	Diagnostic accuracy (%)	Agreement (%)
Microscopy								
Positive	199	0						
Negative	0	481	100	100	100	100	100	100
LAMP								
Positive	177	15						
Negative	22	466	88.9 (83.4–94.5)	96.9 (94.9–98.9)	92.2 (87.3–97)	95.5 (93.1–97.8)	94.6	86.7

LAMP and microscopy results were 94.6% and 86.7%, respectively.

We showed the diagnostic sensitivity and specificity of LAMP toward *P. falciparum* and *P. vivax* infections in a total of 899 blood samples. Sensitivity and specificity of LAMP were 84.4% and 98.6%, respectively, for *P. falciparum* and 92.4% and 98.3%, respectively, for *P. vivax*. Sensitivity and specificity of LAMP cannot be calculated for *P. malariae* and *P. ovale* due to the scarcity of infections of these two species (only one sample for each species).

DISCUSSION

Our group developed a genus- and species-specific LAMP diagnostic method that achieved accuracy comparable to that of nested PCR in a reference laboratory setting (21). The method had initially been applied in malaria diagnosis on a small scale at a field clinic in an area in Thailand where malaria is endemic by using a simple boiling method for DNA extraction to simplify template preparation (28). This preliminary study demonstrated that LAMP showed sensitivity, specificity, PPV, and NPV comparable to those of microscopy, a reference method.

In this study, we further evaluated the feasibility of our genus- and species-specific LAMP diagnostic method for malaria field diagnosis in a resource-limited setting on a large scale. To our knowledge, our study is the first to demonstrate the reliable detection of malaria by LAMP diagnosis in a remote clinical setting on a large scale. All of the methods (LAMP, RDTs, and microscopy) used in the present study were performed on site, with the exception of nested PCR. The procedure of DNA extraction is one of the critical steps for nucleic acid amplification. Therefore, we also further evaluated the efficacy of the simple boiling method for template DNA preparation by comparing it to the saponin/Chelex method, the reference method. These two methods showed comparable results, indicating that the simple boiling method for DNA extraction was efficient as a conventional method and could be used for template DNA preparation in the field. Blood compositions, such as hemoglobin and IgG/IgM, which can interfere with the performance of PCR, showed no effect on LAMP performance (31, 32). The use of the simple boiling method for template preparation provides a good alternative to conventional (expensive and labor-intensive) DNA extraction methods, which might not always be possible in the field. In this study, we were able to successfully use heat-treated samples for LAMP amplification, similar to results reported by Sirichaisinthop et al., Lucchi et al., and Tao et al. (28, 33, 34). As such, the rapid boiling method for extracting DNA from filter paper is favorable for LAMP. This method is cheap, simple, fast, and suitable for use in the field. However, it has to be noted that the DNA from heat-treated samples could not be left on ice for a long time (4 to 5 h). It should be used immediately after extraction, as the degradation of the template DNA could occur. The cause of this DNA degradation is unclear. It may be due to the heat-resistant enzymes from white blood cells within whole blood.

In order to evaluate our LAMP assay, we first compared it to the RDTs and microscopy as a standard method. Overall, both LAMP and RDTs yielded results very similar to those of microscopy. However, the results showed that LAMP was prone to having greater specificity, PPV, and agreement than RDTs. We then compared the LAMP assay to microscopy, which revealed that LAMP showed acceptable results. Upon combination of the results from both experiments, false-negative LAMP results occurred in 25 samples, with parasitemia ranging from 154 to 14,252 parasites/μl, which is within the detectable level of LAMP. Therefore, there may be other reasons why these samples were negative for *Plasmodium* infections by LAMP, such as a lower efficiency of template DNA preparation of these specimens. It should be noted that the saponin/Chelex method was used for template DNA preparation for nested PCR, a reference method, in a reference laboratory. After the discrepancy analysis, the apparent false-positive LAMP results also occurred in 15 samples; this would pose a problem for surveillance in very-low-transmission settings or for drug efficacy monitoring. However, it is unclear whether these are truly false-positive results; at these low parasite densities, chance discrepancies are expected due to stochastic processes, and false-positive results may represent detection of a very-low-density parasitemia that were undetected by the nested PCR. We previously showed that the detection limit of LAMP was 10 copies/reaction mix (25 μl) of the target 18S rRNA genes for *P. malariae* and *P. ovale* and 100 copies/reaction mix (25 μl) for the *P. falciparum* and *P. vivax* (21). It has been demonstrated that the copy number of the rRNA gene in *P. falciparum* and *P. vivax* is approximately 7 copies per genome (35). Therefore, based on the *P. falciparum* and *P. vivax* rRNA copy number, the detection limit of our LAMP was approximately 1.43 parasites/25 μl for *P. malariae* and *P. ovale* and 14.3 parasites/25 μl for the *P. falciparum* and *P. vivax*. The detection limit of nested PCR and a microscopic examination by an experienced microscopist is approximately 5 and 50 parasites/μl, respectively (29). Since the detection limit of LAMP is far better than that of nested PCR and microscopy, it is possible that all of the false-positive LAMP results were due to a

very-low-density malaria parasitemia that could not be detected by the nested PCR and microscopy.

LAMP results reported here demonstrated comparable sensitivity to the recent study on the performance of a new loop-mediated isothermal amplification kit, which showed 90% of the LAMP assay's sensitivity compared to 3-well nested PCR (36). The previous study used mitochondrial *P. falciparum*-specific primer sets to study 272 outpatient blood samples and used two methods for DNA preparation, a Loopamp pure DNA extraction kit and a boil-and-spin method. The LAMP result was read visually by fluorescence under a blue light-emitting diode light (37). The process of DNA isolation in both of these methods is quite complicated and is not suitable for use in the field, and a device for product detection would still be needed.

The LAMP study by Tao et al. used mitochondrial *P. vivax*-specific primer sets to study 89 blood samples and utilized a simple boiling method for DNA extraction. The visualization for the LAMP product was done by the naked eye using a microcrystalline wax dye capsule containing the DNA fluorescence dye SYBR green I (34). That study showed 98.3% sensitivity and 100% specificity of LAMP results compared to microscopy. However, a visualized step after amplification is complicated, since LAMP reaction tubes have to be transferred to a general PCR machine to melt the wax dye capsule and to release SYBR green I into the reaction mixture. The use of the PCR machine makes this technique unsuitable for use in the field.

The LAMP study by Surabattula et al. used our LAMP primers to study 52 febrile patients on site and demonstrated 95% sensitivity and 93.3% specificity compared to microscopy (38). That study used a simple DNA extraction procedure; however, the LAMP tube scanner is still required for fluorescence detection of the amplified product. One of the limitations of this present study is that the diagnostic efficacy of our LAMP assay could only be calculated for *P. falciparum* and *P. vivax*. Although our LAMP method can be used to diagnose all four human malaria parasites (21), attempts were made only for *P. falciparum* and *P. vivax* due to the scarcity of *P. malariae* and *P. ovale* infections. However, our LAMP assay can be used for the detection of all human-infecting malaria parasites to monitor and evaluate of malaria control programs in the field. Moreover, our LAMP method also can be used as a confirmatory assay for malaria infections in place of a conventional PCR-based assay.

Overall, compared to previous studies described above, our LAMP assay exhibited acceptable sensitivity and specificity profiles compared to microscopy. The utility of various reference tests, including different PCR-based assays, or conventional microscopy obviously influences the sensitivity and specificity profile obtained. Moreover, differences in the parasite densities of the samples used in the various studies clearly influence the sensitivities and specificities, which could explain the variations observed across these studies.

The use of any diagnostic test for point-of-care and field use will depend, among other things, on whether it is economical and simple to perform without compromising its sensitivity and specificity. In summary, this study has shown that the LAMP method is a potentially field-usable molecular tool for the diagnosis of malaria. LAMP can provide an alternative method to conventional microscopy when the experience of the operator is limited in resource-limited field setting. Moreover, LAMP can also be used as an alternative to a standard PCR-based analysis for field use in clinical and operational programs.

The rapid boiling method for extracting DNA from dried blood spots is simple, fast, and suitable, and it can be used as an alternative to conventional DNA isolation methods in the field. This method can be further improved to make it more efficient, for example, by leaving the dried blood spot to stand in DDW for 5 min before boiling. The detection of the LAMP product turbidity by the naked eye can also be enhanced by briefly spinning down the LAMP product before observing it.

ACKNOWLEDGMENTS

We thank the staff of the Mahidol Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, for technical support and the staff of the malaria clinic in Mae Sot District, Tak Province, Thailand, for their support in blood sample collection and microscopic examination. We also acknowledge Malla Rao for his suggestion on an experimental design.

This study was supported by grant number 1R01AI075588-01 from the National Institute of Allergy And Infectious Diseases.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

REFERENCES

1. **WHO.** 2011. Global malaria programme. World malaria report 2011. World Health Organization, Geneva, Switzerland.
2. **Lee PW, Liu CT, do Rosario VE, de Sousa B, Rampao HS, Shaio MF.** 2010. Potential threat of malaria epidemics in a low transmission area, as exemplified by Sao Tome and Principe. Malar. J. **9:**264. http://dx.doi.org/10.1186/1475-2875-9-264.
3. **von Seidlein L, Drakeley C, Greenwood B, Walraven G, Targett G.** 2001. Risk factors for gametocyte carriage in Gambian children. Am. J. Trop. Med. Hyg. **65:**523–527.
4. **Bousema JT, Gouagna LC, Drakeley CJ, Meutstege AM, Okech BA, Akim IN, Beier JC, Githure JI, Sauerwein RW.** 2004. *Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya. Malar. J. **3:**18. http://dx.doi.org/10.1186/1475-2875-3-18.
5. **Njama-Meya D, Kamya MR, Dorsey G.** 2004. Asymptomatic parasitaemia as a risk factor for symptomatic malaria in a cohort of Ugandan children. Trop. Med. Int. Health **9:**862–868. http://dx.doi.org/10.1111/j.1365-3156.2004.01277.x.
6. **Karl S, Gurarie D, Zimmerman PA, King CH, St Pierre TG, Davis TME.** 2011. A submicroscopic gametocyte reservoir can sustain malaria transmission. PLoS One **6:**e20805. http://dx.doi.org/10.1371/journal.pone.0020805.
7. **Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM, Joshi H.** 2012. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malar. J. **11:**29. http://dx.doi.org/10.1186/1475-2875-11-29.
8. **Perkins MD, Bell DR.** 2008. Working without a blindfold: the critical role of diagnostics in malaria control. Malar. J. **7**(Supp 1)**:**S5. http://dx.doi.org/10.1186/1475-2875-7-S1-S5.
9. **Bisoffi Z, Gobbi F, Angheben A, den Ende JV.** 2009. The role of rapid diagnostic tests in managing malaria. PLoS Med. **6:**e1000063. http://dx.doi.org/10.1371/journal.pmed.1000063.
10. **Coleman RE, Sattabongkot J, Promstaporm S, Maneechai N, Tippayachai B, Kengluecha A, Rachapaew N, Zollner G, Miller RS, Vaughan JA, Thimasarn K, Khuntirat B.** 2006. Comparison of PCR and microscopy for the detection of asymptomatic malaria in a *Plasmodium falciparum/vivax* endemic area in Thailand. Malar. J. **5:**121. http://dx.doi.org/10.1186/1475-2875-5-121.
11. **Murray CK, Gasser RA, Jr, Magill AJ, Miller RS.** 2008. Update on rapid diagnostic testing for malaria. Clin. Microbiol. Rev. **21:**97–110. http://dx.doi.org/10.1128/CMR.00035-07.
12. **McMorrow ML, Aidoo M, Kachur SP.** 2011. Malaria rapid diagnostic tests in elimination settings–can they find the last parasite? Clin. Microbiol. Infect. **17:**1624–1631. http://dx.doi.org/10.1111/j.1469-0691.2011.03639.x.

13. **Snounou G, Viryakosol S, Zhu XP, Jarra W, Pinheiro L, Rosario VE, Thaithong S.** 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol. Biochem. Parasitol. **61:**315–320. http://dx.doi.org/10.1016/0166-6851(93)90077-B.
14. **Kimura K, Kaneko O, Liu Q, Zhou M, Kawamoto F, Wataya Y, Otani S, Yamaguchi Y, Tanabe K.** 1997. Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. Parasitol. Int. **46:**91–95. http://dx.doi.org/10.1016/S1383-5769(97)00013-5.
15. **Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA.** 1999. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am. J. Trop. Med. Hyg. **60:**687–692.
16. **Hanscheid T, Grobusch MP.** 2002. How useful is PCR in the diagnosis of malaria? Trends Parasitol. **18:**395–398. http://dx.doi.org/10.1016/S1471-4922(02)02348-6.
17. **Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, Medici MC, Arcangeletti MC, Snounou G, Dettori G, Chezzi C.** 2004. Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. J. Clin. Microbiol. **42:**1214–1219. http://dx.doi.org/10.1128/JCM.42.3.1214-1219.2004.
18. **Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T.** 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. **28:**E63. http://dx.doi.org/10.1093/nar/28.12.e63.
19. **Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W, Tangpukdee N, Yuen KY, Guan Y, Looareesuwan S, Peiris JS.** 2006. Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clin. Chem. **52:**303–306. http://dx.doi.org/10.1373/clinchem.2005.057901.
20. **Boehme CC, Nabeta P, Henostroza G, Raqib R, Rahim Z, Gerhardt M, Sanga E, Hoelscher M, Notomi T, Hase T, Perkins MD.** 2007. Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. J. Clin. Microbiol. **45:**1936–1940. http://dx.doi.org/10.1128/JCM.02352-06.
21. **Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J, Iriko H, Jin L, Takeo S, Tsuboi T.** 2007. Detection of four *Plasmodium* species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. J. Clin. Microbiol. **45:**2521–2528. http://dx.doi.org/10.1128/JCM.02117-06.
22. **Njiru ZK, Mikosza AS, Matovu E, Enyaru JC, Ouma JO, Kibona SN, Thompson RC, Ndung'u JM.** 2008. African trypanosomiasis: sensitive and rapid detection of the sub-genus Trypanozoon by loop-mediated isothermal amplification (LAMP) of parasite DNA. Int. J. Parasitol. **38:**589–599. http://dx.doi.org/10.1016/j.ijpara.2007.09.006.
23. **Aonuma H, Yoshimura A, Perera N, Shinzawa N, Bando H, Oshiro S, Nelson B, Fukumoto S, Kanuka H.** 2009. Loop-mediated isothermal amplification applied to filarial parasites detection in the mosquito vectors *Dirofilaria immitis* as a study model. Parasit. Vectors **2:**15. http://dx.doi.org/10.1186/1756-3305-2-15.
24. **Polley SD, Mori Y, Watson J, Perkins MD, González IJ, Notomi T, Chiodini PL, Sutherland CJ.** 2010. Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification. J. Clin. Microbiol. **48:**2866–2871. http://dx.doi.org/10.1128/JCM.00355-10.
25. **Nagamine K, Watanabe K, Ohtsuka K, Hase T, Notomi T.** 2001. Loop-mediated isothermal amplification reaction using a nondenatured template. Clin. Chem. **47:**1742–1743.
26. **Mori Y, Nagamine K, Tomita N, Notomi T.** 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. **289:**150–154. http://dx.doi.org/10.1006/bbrc.2001.5921.
27. **Nagamine K, Hase T, Notomi T.** 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell Probes **16:**223–229. http://dx.doi.org/10.1006/mcpr.2002.0415.
28. **Sirichaisinthop J, Buates S, Watanabe R, Han ET, Suktawonjaroenpon W, Krasaesub S, Takeo S, Tsuboi T, Sattabongkot J.** 2011. Evaluation of loop-mediated isothermal amplification (LAMP) for malaria diagnosis in a field setting. Am. J. Trop. Med. Hyg. **85:**594–596. http://dx.doi.org/10.4269/ajtmh.2011.10-0676.
29. **Moody A.** 2002. Rapid diagnostic tests for malaria parasites. Clin. Microbiol. Rev. **15:**66–78. http://dx.doi.org/10.1128/CMR.15.1.66-78.2002.
30. **Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE.** 1995. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am. J. Trop. Med. Hyg. **52:**565–568.
31. **Al-Soud WA, Jönsson LJ, Rådstrom P.** 2000. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. J. Clin. Microbiol. **38:**345–350.
32. **Al-Soud WA, Rådstro MP.** 2001. Purification and characterization of PCR-inhibitory components in blood cells. J. Clin. Microbiol. **39:**485–493. http://dx.doi.org/10.1128/JCM.39.2.485-493.2001.
33. **Lucchi NW, Demas A, Narayanan J, Sumari D, Kabanywanyi A, Kachur SP, Barnwell JW, Udhayakumar V.** 2010. Real-time fluorescence loop mediated isothermal amplification for the diagnosis of malaria. PLoS One **5:**e13733. http://dx.doi.org/10.1371/journal.pone.0013733.
34. **Tao ZY, Zhou HY, Xia H, Xu S, Zhu HW, Culleton RL, Han ET, Lu F, Fang Q, Gu YP, Liu YB, Zhu GD, Wang WM, Li JL, Cao J, Gao Q.** 2011. Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of *Plasmodium vivax* infection. Parasit. Vectors **4:**115. http://dx.doi.org/10.1186/1756-3305-4-115.
35. **Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, Crabtree J, Angiuoli SV, Merino EF, Amedeo P, Cheng Q, Coulson RM, Crabb BS, Del Portillo HA, Essien K, Feldblyum TV, Fernandez-Becerra C, Gilson PR, Gueye AH, Guo X, Kang'a S, Kooij TW, Korsinczky M, Meyer EV, Nene V, Paulsen I, White O, Ralph SA, Ren Q, Sargeant TJ, Salzberg SL, Stoeckert CJ, Sullivan SA, Yamamoto MM, Hoffman SL, Wortman JR, Gardner MJ, Galinski MR, Barnwell JW, Fraser-Liggett CM.** 2008. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. Nature **455:**757–763. http://dx.doi.org/10.1038/nature07327.
36. **Hopkins H, González IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, Agaba B, Kyabayinze DJ, Sutherland CJ, Perkins MD, Bell D.** 2013. Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. J. Infect. Dis. **208:**645–652. http://dx.doi.org/10.1093/infdis/jit184.
37. **Polley SD, González IJ, Mohamed D, Daly R, Bowers K, Watson J, Mewse E, Armstrong M, Gray C, Perkins MD, Bell D, Kanda H, Tomita N, Kubota Y, Mori Y, Chiodini PL, Sutherland CJ.** 2013. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. J. Infect. Dis. **208:**637–644. http://dx.doi.org/10.1093/infdis/jit183.
38. **Surabattula R, Vejandla MP, Mallepaddi PC, Faulstich K, Polavarapu R.** 2013. Simple, rapid, inexpensive platform for the diagnosis of malaria by loop mediated isothermal amplification (LAMP). Exp. Parasitol. **134:**333–340. http://dx.doi.org/10.1016/j.exppara.2013.03.031.