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# Loop-Mediated Isothermal PCR (LAMP) for the Diagnosis of Falciparum Malaria

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*Abstract.* A recently described loop-mediated isothermal polymerase chain reaction (LAMP) for molecular detection of *Plasmodium falciparum* was compared with microscopy, PfHRP2-based rapid diagnostic test (RDT), and nested polymerase chain reaction (PCR) as the "gold standard" in 115 Bangladeshi in-patients with fever. DNA extraction for LAMP was conducted by conventional methods or simple heating of the sample; test results were either assessed visually or by gel electrophoresis. Conventional DNA extraction followed by gel electrophoresis had the highest agreement with the reference method (81.7%, $\kappa = 0.64$), with a sensitivity (95% CI) of 76.1% (68.3–83.9%), comparable to RDT and microscopy, but a specificity of 89.6% (84.0–95.2%) compared with 100% for RDT and microscopy. DNA extraction by heat treatment deteriorated specificity to unacceptable levels. LAMP enables molecular diagnosis of falciparum malaria in settings with limited technical resources but will need further optimization. The results are in contrast with a higher accuracy reported in an earlier study comparing LAMP with a non-validated PCR method.

## INTRODUCTION

An accurate parasite-based diagnosis of malaria is essential for proper treatment of the individual patient. Reliably excluding the diagnosis is equally valuable, because this will guide the clinician to consider an alternative diagnosis, which can be life saving.[1] A correct diagnosis is also important for public health, because avoiding inappropriate antimalarial treatment will reduce costs and helps prevent the spread of drug resistance. Traditionally laboratory diagnosis has relied on the identification of parasites in a peripheral blood film using either Giemsa, Wright, or Field stain.[2,3] Microscopy is an accurate tool but requires well-trained staff, particularly for detecting low levels of parasitemia. In experienced hands, the limit of detection is ~50 parasites/μL.[4] More recently, the malaria rapid diagnostic tests (RDT) have become available, which are most commonly based on the detection of the *Plasmodium falciparum*–specific antigens histidine-rich protein 2 (PfHRP2) or parasite specific lactate dehydrogenase (pLDH) in an easy to use dipstick or lateral flow format. Their limit of detection is ~50–100 parasites/μL[5,6]; the specificity of the PfHRP2 based test is compromised by the persistence of the antigen in the blood after parasite clearance, especially in the presence of gametocytemia.[7,8] Nested polymerase chain reaction (PCR) using *Plasmodium* genus–specific primers for the initial PCR amplification and either genus- or species-specific primers for the secondary amplifications can be considered as the most sensitive technique, able to detect parasitemias as low as 1–5 parasites/μL, as well as being highly specific.[9,10] The method is laborious and requires high technical experience but is the most sensitive and specific test and can serve as the "gold standard" in the evaluation of other diagnostic tests.[11]

Recently a new, simple, and inexpensive molecular test to detect the highly conserved 18S ribosomal RNA gene of *P. falciparum* by use of a loop-mediated isothermal amplification PCR (LAMP) was described.[12,13] This method has the potential to combine the high sensitivity of a molecular diagnostic test with the possibility of performing the test under field conditions with limited technical resources. The LAMP method uses a robust DNA polymerase with low sensitivity to inhibitors and a set of three primer pairs that produce a specific double hairpin DNA template, which is amplified and concatenated with very high efficiency, leading to DNA concentrations close to 1 μg/μL within 90 minutes. The technique has been applied successfully as a diagnostic tool for several viral diseases.[14–17] In patients with dengue, for example, very low copy numbers can be detected, even outperforming the sensitivity of real-time PCR.[17] We evaluated the accuracy of this method in falciparum malaria and compared it with microscopy and a PfHRP2-based RDT, using nested PCR as the gold standard.

## MATERIALS AND METHODS

**Patients and setting.** The study was performed at Chittagong Medical College Hospital (CMCH), Chittagong, Bangladesh, from June to July 2006.

Consecutive admitted patients with a clinical suspicion of falciparum malaria were included in the study. Patients previously diagnosed with malaria and treated with antimalarials within the week before admission were excluded. The screening was done as part of a study on the role of levamisole as adjuvant therapy in falciparum malaria, the results of which will be published separately. All patients signed an informed consent before enrollment. Ethical approval for this study was obtained from the Bangladesh Medical Research Council and the Oxford Tropical Medicine Research Ethical Committee (OXTREC). CMCH is a tertiary referral center with a catchment population of ~15 million people. Malaria transmission is low in this area, with a seasonal peak from May until August during the rainy season.

**Blood collection.** A 1-mL EDTA anticoagulated blood sample (TekLab, Durham, UK) was taken from the antecubital vein on admission of the patient. Microscopy and RDT were performed on site, and the remainder of the blood sample was stored in liquid nitrogen until further processing. Nested PCR and LAMP were performed in the Faculty of Tropical Medicine, Mahidol University (Bangkok, Thailand).

**Microscopy.** Thick and thin blood films were stained according to the method described by Field.[2] The slides were

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read by an experienced microscopist who was blinded for the results of the other diagnostic tests. Parasitemia was assessed either per 1,000 erythrocytes in the thin film or at low parasitemias per 200 white blood cells (WBC) in the thick film. In case of a putative negative film, 500 WBC were assessed to confirm the absence of parasites.

**Rapid diagnostic test.** The PfHRP2 antigen-based lateral flow test (Paracheck; Orchid Biosystems, Goa, India) was used according to the manufacturer's instructions, using a drop of EDTA anticoagulated whole blood. Test results were read after 15 minutes.

**Preparation of the DNA template.** The template DNA used for nested PCR and LAMP (LAMP-DNA-extract) was prepared from 200 μL of frozen whole EDTA blood using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), providing elutes of 50 μL of template DNA. Two microliters was used per sample, corresponding to ~8 μL of the original blood sample. For the LAMP assay, a second simple and cheap method of DNA extraction[13] was used by freezing 50 μL of blood (cell lysis), followed by heating for 3 minutes at 99°C and then centrifugation at 13,000 rpm for 3 minutes (LAMP-heat). In contrast to a previous report, we heated the specimens for 3 minutes instead of 10 minutes at 99°C. Two microliters of the resulting supernatant was used as template for the LAMP assay.

**Nested PCR assay.** This assay targets the *Plasmodium* small subunit ribosomal RNA (*ssrRNA*) gene, resulting in a first 620-bp and a secondary 240-bp amplicon. The oligonucleotide primers used for the nested PCR assay were identical to those previously published.[10] The reaction mixture for the first PCR step consisted of 2 μL of DNA template, 250 nmol/L of each primer (*rPLU 1* and *rPLU 5*), 4 mmol/L $MgCl_2$ (Boehringer Mannheim, Indianapolis, IN), PCR buffer (50 mmol/L KCl, 10 mmol/L *Tris*-HCl), 200 μmol/L of each deoxynucleoside triphosphate, 1.25 units of *Taq* DNA polymerase (Boehringer Mannheim), and water to a final volume of 50 μL. Primary amplification conditions were 94°C for 4 minutes; 35 cycles at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minutes; and final extension at 72°C for 4 minutes. Two microliters of the first amplification product was used as DNA template for each of the 20-μL secondary amplifications. The conditions and concentrations of the secondary amplification were identical to those of the primary, except for the annealing temperature of 58°C and the amount of *Taq* Polymerase being 0.5 units. The PCR products of the secondary amplification round were analyzed by gel electrophoresis and stained with ethidium bromide.

**LAMP assay.** All LAMP PCR reactions were performed with the Loopamp DNA Amplification Kit (Eiken Chemical Co., Tokyo, Japan); the primer set and methods used were previously described.[13] The reaction consisted of 7 μL of primers (FIP and BIP 40 pmol, Loop-F and Loop-B 20 pmol, F3 and B3 5 pmol), 12.5 μL of reaction mixture (40 mmol/L Tris-HCl, 20 mmol/L KCl, 16 mmol/L $MgSO_4$, 20 mmol/L $NH_4SO_4$, 0.2% Tween 20, 1.6 mol/L betaine, deoxynucleotide triphosphates 2.8 mmol/L each), 1 μL *Bst* DNA polymerase, 2 μL template DNA, and distilled water to a total volume of 25 μL. The reaction mixture was incubated in a hot water bath at 65°C for 120 minutes.[13]

**Endpoint assessment.** Turbidity of the reaction mixture, which is based on the precipitation of magnesium pyrophosphate as a by-product of the reaction,[18] was scored as a positive test. Four independent people, who were masked to all malaria diagnostic test results, assessed turbidity as turbid or not turbid. The tubes were spun down (10,000 rpm for 3 minutes) to produce a whitish pellet if precipitate was present. The same four interpreters performed a second round of endpoint assessment pellet or no pellet, again masked to previous results, and a fifth person collected the scores. A positive test result was defined as follows: each individual positive reading scored one point; thus, a maximum of four points for turbidity and four for pellet formation was possible. If a sample collected five points or more, the test was considered positive; in case the outcome was equivocal with a score of four points ($N = 2$), the pellet endpoint result was decisive because it was more discriminatory. Besides the assessment of turbidity by the naked eye, the tubes were also analyzed by gel electrophoresis to assess if the LAMP DNA product was present. All reactions were analyzed by 2.0% (wt/vol) agarose gel by electrophoresis in Tris-Acetate-EDTA (TAE) buffer stained with ethidium bromide (BioRad, San Diego, CA), and positive results were defined by the appearance of typical ladder-bands of various sizes.

**UV fluorescence detection.** During evaluation of endpoints in the pilot phase of this study, all reactions with heat-treated blood sample templates showed some level of positive fluorescence when using SYBR-green intercalating dye, including those clearly LAMP negative as shown by negative turbidity, pellet formation, and gel electrophoresis. Even blood samples from healthy controls showed some level of pre-LAMP fluorescence when using commercial DNA extraction methods, clearly showing that UV fluorescence detection would provide false-positive results. For this reason, the use of intercalating dyes to determine the assay endpoint was discontinued.

**Statistical analysis.** All statistical analyses were performed using Stata SE Version 9 Software (2005). Specificity, sensitivity, and positive and negative predictive values of the different diagnostic tests were compared with nested PCR results as the gold standard. The interobserver variation in assessing turbidity "by eye" in the LAMP assay was expressed as a κ-value. Comparison of specificity and sensitivity of the LAMP with microscopy was calculated by comparison of equivalence of proportions of the studied methods.

## RESULTS

Of a total of 127 consecutive patients with a clinical suspicion of malaria, 115 patients were included in this study. Twelve patients were excluded because of prior diagnosis and treatment at the referring health facility.

Nested PCR, which was used as the reference method, was positive for *P. falciparum* in 67 (58.3%) patients. Both microscopy and the PfHRP2-based RDT did not produce any false-positive results, translating into a specificity and positive predictive value of 100% (Table 1). The sensitivity (95% CI) of the RDT was comparable with microscopy: 77.6% (70.0–85.2%) versus 73.1% (65.0–81.2%; $P = 0.55$; Table 2). The negative predictive value (95% CI) of the RDT was 76.2% (68.4–84.0%).

The LAMP assay proved easy to conduct, although accurate prevention of contamination appeared essential. Gloves were changed between every PCR assay, strict separation in preparation of reagents and processing of DNA template was respected, and post-PCR work was done in a separate room.

TABLE 1
Agreement, sensitivity, specificity, PPV, and NPV of various tests for malaria diagnosis versus gold standard PCR

| | Percent agreement with nPCR (κ) | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) |
|---|---|---|---|---|---|
| LAMP-DNA-extract | 81.7% (0.65) | 76.1 (68.3–83.9) | 89.6 (84.0–95.2) | 91.1 (85.9–96.3) | 72.9 (64.8–81.0) |
| LAMP-heat-gel | 79.1% (0.58) | 76.1 (68.3–83.9) | 83.3 (76.5–90.1) | 86.4 (80.2–92.7) | 71.4 (63.2–79.7) |
| LAMP-heat-eye | 70.4% (0.38) | 79.1 (71.1–86.5) | 58.3 (49.3–67.3) | 72.6 (64.5–80.8) | 66.7 (58.1–75.3) |
| RDT | 87.0% (0.74) | 77.6 (70.0–85.2) | 100 (100, 100) | 100 (100, 100) | 76.2 (68.4–84.0) |
| Microscopy | 84.4% (0.69) | 73.1 (65.0–81.2) | 100 (100, 100) | 100 (100, 100) | 72.7 (64.6–80.9) |

Kappa statistic (range 1 to –1) measures the observed percentage of agreement between tests: (No. positive by both tests + No. negative by both tests)/*N*, against what might be expected by chance.

LAMP-DNA-extract, DNA template with commercial DNA extraction method and gel electrophoresis; LAMP-heat-gel, heat-treated sample and gel electrophoresis; LAMP-heat-eye, heat-treated sample and visual interpretation of endpoint; PPV, positive predictive value; NPV, negative predictive value; RDT, rapid diagnostic test.

In this study, we evaluated the LAMP technique using two different DNA template preparation methods and three different endpoints, leading to three practical modalities for assessing a positive test result.

The highest agreement with nested PCR (nPCR) was achieved with the LAMP-DNA-extract modality (81.7%, $\kappa = 0.64$). Both assays using gel electrophoresis as the read-out method (LAMP-DNA-extract and LAMP heat-gel) showed a similar level of agreement with nPCR (Table 1). The sensitivities of all three LAMP assays were comparable to the RDT. There was a trend that the sensitivity was better than microscopy (Table 2), but this was not significant. Specificity of the LAMP-DNA-extract method was superior to the LAMP-heat-eye and LAMP-heat-gel methods (Table 1) but remained inferior compared with RDT or microscopy ($P = 0.02$; Table 2). However, the negative predictive value of the LAMP-DNA-extract was similar to microscopy ($P = 0.99$), whereas the positive predictive value for both assays was significantly higher for microscopy ($P = 0.03$; Table 2).

The LAMP method most suitable for deployment in the field would use heat treatment to obtain template DNA and visual assessment of turbidity and pellet formation after centrifugation as endpoints (LAMP-heat-eye). Even though this method showed a sensitivity of 79.1%, its specificity was 58.3%. When, instead of a visual assessment of turbidity, the DNA product was analyzed by agarose gel electrophoresis, the specificity of the LAMP in heat-treated samples (LAMP-heat-gel) rose to 83.3%. The visual interpretation of turbidity in the test tube seemed to be slightly less prone to interobserver variability than pellet formation, with κ values between the four observers of 0.88 and 0.82, respectively. The κ statistic (range, 1 to –1) measures the observed percentage of agreement between tests against what might be expected by chance, ranging from $< 0$ = poor agreement to 1 = perfect agreement.[19]

## DISCUSSION

The recently described LAMP technique has the potential of combining the accuracy of a molecular technique with the low costs and low technical requirements of the antigen-based tests. To perform the test, only a water bath and sterile pipetting techniques are needed. The costs for the assay are even lower than the currently available RDTs; one LAMP assay for malaria diagnosis costs between US$0.4 and US$0.7.[13]

Evaluation of new diagnostic tests in malaria has the advantage of the availability of a highly sensitive and specific gold standard, which is nested PCR.[11] In this study, we compared three different LAMP modalities, a PfHRP2-based RDT, and microscopy to nested PCR as the reference method. Both microscopy and RDT showed specificity and positive predictive values of 100%. The combined results for the best LAMP modality, LAMP-DNA-extract, showed no significant difference in sensitivity, with a lower PPV than microscopy and RDT and reasonable specificity and NPV. The basic LAMP-heat-eye modality showed the highest incidence of false-positive results.

A previous study on this technique using the same primer sets as in this study reported a higher specificity and sensitivity than in our results.[13] This can be explained in part by the use of a real-time turbidimeter. However, an important source of error in the previous study could have been the use of a not formally validated PCR method as the gold standard for comparison. Because this PCR was based on the same primer set as used in the LAMP test, this could have resulted in an overestimation of the quality of the LAMP assay.

The results of this study showed that the performance of

TABLE 2
Comparison of the three LAMP modalities with microscopy for falciparum malaria in a referral hospital in Chittagong, Bangladesh

| | Percent difference (95% CI) | *P* value |
|---|---|---|
| Sensitivity | | |
| LAMP-DNA-extract vs. microscopy | 3.0 (–11.7 to 17.7) | 0.69 |
| LAMP-heat-gel vs. microscopy | 3.0 (–11.7 to 17.7) | 0.69 |
| LAMP-heat-eye vs. microscopy | 6.0 (–84.3 to 20.4) | 0.42 |
| RDT vs. microscopy | 4.5 (–10.1 to 19.0) | 0.55 |
| Specificity | | |
| LAMP-DNA-extract vs. microscopy | –10.4 (–19.1 to –1.8) | 0.02 |
| LAMP-heat-gel vs. microscopy | –16.7 (–27.2 to –6.1) | 0.003 |
| LAMP-heat-eye vs. microscopy | –41.7 (–55.6 to –27.7) | < 0.001 |
| RDT vs. microscopy | 0 (0.0–0.0) | 1.00 |
| PPV | | |
| LAMP-DNA-extract vs. microscopy | –8.9 (–16.4 to –1.5) | 0.03 |
| LAMP-heat-gel vs. microscopy | –13.6 (–22.3 to –4.8) | 0.01 |
| LAMP-heat-eye vs. microscopy | –27.4 (–37.6 to –17.2) | 0.001 |
| RDT vs. microscopy | 0 (0.0–0.0) | 1.00 |
| NPV | | |
| LAMP-DNA-extract vs. microscopy | 0.2 (–15.47 to 15.8) | 0.98 |
| LAMP-heat-gel vs. microscopy | –1.3 (–17.3 to 14.7) | 0.87 |
| LAMP-heat-eye vs. microscopy | –6.1 (–23.9 to 11.8) | 0.50 |
| RDT vs. microscopy | 3.5 (–11.57 to 18.5) | 0.65 |

nPCR was used as reference test. The *P* value concerns the difference with the nested PCR.

LAMP-DNA-extract, DNA template with commercial DNA extraction method and gel electrophoresis; LAMP-heat-gel, heat-treated sample and gel electrophoresis; LAMP-heat-eye, heat-treated sample and visual interpretation of endpoint; PPV, positive predictive value; NPV, negative predictive value; RDT, rapid diagnostic test.

the test in its current form needs further optimization; specificity and sensitivity need to be improved. Accuracy is currently insufficient to promote its use as a diagnostic or research tool because the test currently shows unacceptably high rates of both false positives and false negatives. A low rate of false-negative results is particularly important to clinicians using diagnostic tests, because failing to treat a true case of falciparum malaria may result in progression to severe disease and death.

Possibilities for improvement of the LAMP assay include the definition of an unequivocal post-PCR endpoint; the use of turbidity and pellet formation are subject to individual variation in interpretation. Turbidity is caused by the formation of magnesium-pyrophosphate as a side product of the amplification reaction.[18] Modification of this end product through labeling or chemical conversion into a colored product could make interpretation of the test more reliable. Heat-treated samples may contain cellular debris or have a yellowish appearance if the sample is not centrifuged properly; this can mimic pellet formation or turbidity, although this can easily be distinguished with increased experience. The use of a real-time turbidimeter to detect the exponential increase of turbidity can effectively reduce misjudgment of positive endpoint turbidity by the naked eye and thus improves accuracy. Unfortunately, these devices are not currently commercially available.

Therefore, although the use of simple heat-treated samples as a source for DNA template, together with visual interpretation of turbidity for assessment of test positivity, has the advantage that it can be deployed in a resource limited setting, in its current form, it shows an unacceptably high rate of false-positive results. The amplification efficiency of the LAMP method is extremely high because all reactions are conducted at the same temperature optimal for the enzyme, with no time lost by thermal changes as in conventional PCR. The LAMP method is capable of synthesizing 20 μg of specific DNA for 25 μL of reaction mixture within 60 minutes.[18] Even though this method is a closed single-tube assay, contamination of work areas can easily occur if a tube is opened post-PCR, because large amounts of DNA (close to 1 μg/μL) are present within the tube. Avoidance of contamination should have priority when conducting the assay, which can be obtained by changing gloves between every assay and strict spatial separation of reagents' preparation and performance of the test.

In conclusion, LAMP is a promising new technique, which for the first time enables a molecular diagnosis of falciparum malaria in a setting with limited resources. However, in contrast to the findings in a more favorable earlier report, the LAMP assay currently lacks sufficient accuracy. Development of improved methods for visualization of the LAMP end product or a derived product (magnesium pyrophosphate) will be important.

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