mechanisms, including activating chemotaxis, increasing vascular permeability, propagating the inflammatory cytokine cascade, and stimulating macrophage action and smooth muscle cell migration *(12, 13)*. For example, COX-2 and its prostaglandin E_2 production have been shown to induce production of the proinflammatory cytokine interleukin-6 *(13)*, an important cytokine that stimulates CRP production in the liver. Thus, COX-2 may promote acute inflammatory processes after AMI.

Statins may decrease CRP concentrations and have effects on inflammation, plaque stabilization, and improvement of endothelial function in acute coronary syndrome. Our findings confirm that atorvastatin can decrease CRP concentrations in early-stage AMI. However, atorvastatin can inhibit COX-2 expression, which is closely correlated with CRP. This finding suggests that atorvastatin might have antiinflammatory effects at least partly through the COX-2 pathway. Hernandez-Presa et al. *(14)* also found that atorvastatin decreased COX-2 expression in a rabbit model of atherosclerosis and in cultured vascular smooth muscle cells. The mechanism underlying this effect of atorvastatin is probably related to inhibition of nuclear factor-κB activity secondary to a decrease in isoprenylation of proteins involved in intracellular signal transduction necessary for their correct function, because COX-2 is controlled by this transcription factor, and it has been confirmed that statins can directly decrease nuclear factor-κB activity *(15)*.

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DOI: 10.1373/clinchem.2005.057893

Sensitive and Inexpensive Molecular Test for Falciparum Malaria: Detecting *Plasmodium falciparum* DNA Directly from Heat-Treated Blood by Loop-Mediated Isothermal Amplification, *Leo L.M. Poon,[1*] Bonnie W.Y. Wong,[1] Edmund H.T. Ma,[1] Kwok H. Chan,[1] Larry M.C. Chow,[2] Wimal Abeyewickreme,[3] Noppadon Tangpukdee,[4] Kwok Y. Yuen,[1] Yi Guan,[1] Sornchai Looareesuwan,[4] and J.S. Malik Peiris[1]* ([1] Department of Microbiology, The University of Hong Kong, Hong Kong SAR; [2] Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR; [3] Department of Parasitology and Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya, Sri Lanka; [4] Department of Clinical Tropical medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; * address correspondence to this author at: Department of Microbiology, University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong SAR; fax 852-2855-1241, e-mail llmpoon@hkucc.hku.hk)

Background: **Malaria is one of the most important parasitic infections in humans. A sensitive diagnostic test for malaria that could be applied at the community level could be useful in programs to control the disease. The aim of the present work was to develop a simple, inexpensive molecular test for *Plasmodium falciparum*.**
Methods: **Blood was collected from controls (n = 100) and from patients diagnosed with falciparum malaria infection (n = 102), who were recruited to the study. Heat-treated blood samples were tested by a loop-mediated isothermal amplification (LAMP) assay for *P. falciparum*. Results were interpreted by a turbidity meter in real time or visually at the end of the assay. To evaluate the assay, DNA from these samples was purified and tested by PCR. Results from the LAMP and PCR assays were compared.**
Results: **The LAMP assay detected *P. falciparum* directly from heat-treated blood. The quantitative data from the assay correlated to the parasite counts obtained by blood-film microscopic analyses. When we used the PCR assay as the comparison method, the sensitivity**

and specificity of the LAMP assay were 95% and 99%, respectively.

Conclusions: **Unlike PCR, the LAMP assay does not require purified DNA for efficient DNA amplification, thereby reducing the cost and turnaround time for *P. falciparum* diagnosis. The assay requires only basic instruments, and assay positivity can be verified by visual inspection.**

The recently published World Malaria Report *(1)* highlighted the importance of reliable diagnostic tests to guide prompt and effective therapy. Early confirmation of the diagnosis is important because the clinical features of malaria overlap with those of many other infections *(2)*. Of the 4 *Plasmodium* species that produce human disease, *P. falciparum* causes most of the severe and fatal cases *(3)*. Blood-film microscopy remains the mainstay of malaria diagnosis *(4)*, but it is subjective, and the availability of personnel with the necessary expertise may be suboptimal in areas where the disease is under partial control *(2, 4, 5)*. Furthermore, in areas nonendemic for malaria, increased air travel requires rapid exclusion of a diagnosis of malaria *(6)*. PCR provides such an alternative to microscopies, but it is expensive.

In this proof-of-concept study, we describe a simple and inexpensive molecular test to detect the highly conserved 18S ribosomal RNA gene of *P. falciparum* by use of loop-mediated isothermal amplification (LAMP) technology *(7, 8)* (Fig. 1A). This assay does not cross-react with DNA from *P. vivax*, *P. malariae*, or *P. ovale*, or with human DNA (see below and Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol52/issue2/).

We retrospectively studied packed erythrocyte samples from patients (n = 102) admitted to the Bangkok Hospital for Tropical Diseases, Thailand, with *P. falciparum* infection. The diagnosis of these patients was made by standard malaria microscopy at the time of admission. Samples were collected with informed consent, and the study was approved by the ethics committees of the Faculty of Tropical Medicine, Mahidol University (Bangkok). We studied 100 samples from healthy volunteer donors in the same population as controls. The blood samples were tested by the PCR and LAMP tests at The University of Hong Kong. Each of the molecular tests was carried out and interpreted by independent researchers blinded to the origin of the specimens and the laboratory results. Samples with discrepant results in the 2 tests were retested.

To reduce the cost and turnaround time of the LAMP test, we investigated the feasibility of testing without previous DNA extraction *(9)*. We heated 50 μL of each specimen at 99 °C for 10 min and tested 2 μL of the supernatant directly by the LAMP assay. Results of these reactions were recorded, separately, by direct visual inspection and by a real-time turbidity meter. When we used control erythrocyte samples with added plasmid DNA containing the targeted sequence as calibrators, samples containing ≥10 copies of the plasmid DNA were consistently positive in the assay (see Fig. 2 in the online Data Supplement).

Using the real-time turbidity meter, we found that of 102 blood samples that scored positive in the microscopic test, 96 were positive in the LAMP test (Fig. 1B). To validate these findings, we purified DNA from all blood samples, using DNA extraction columns (Qiagen), and tested them with a PCR assay for *P. falciparum* (Fig. 1B). Interestingly, 3 of the 6 false-negative samples in the LAMP assay were also negative in the PCR assay. These 3 samples were subsequently found to be positive in the PCR for *P. malariae (10)*, indicating that these samples were misdiagnosed by the microscopic test. By contrast, 99 of 100 human control samples were negative in the LAMP assay. The single false-positive sample in the LAMP assay was also positive in the PCR assay for *P. falciparum*. This positive sample may have been isolated from an individual with an asymptomatic *P. falciparum* infection, as subclinical infection is common in endemic regions *(11)*. The overall results indicated that the LAMP assay is highly specific.

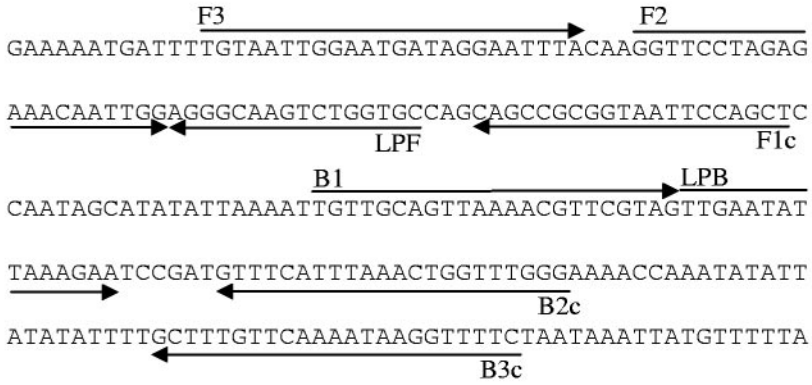
Because both molecular tests in our study identified some cases misdiagnosed by microscopic evaluation and because PCR assays can be more specific and sensitive than routine microscopic assays in optimized conditions *(4)*, we used our PCR assay as the comparison method to assess the performance of the LAMP assay. Of the 101 samples positive in the PCR assay, 96 were positive in the LAMP test (reading by turbidity meter), whereas 5 were negative in the LAMP assay. Of 101 samples negative in the PCR assays, 100 were negative in the LAMP assay. By these criteria, the sensitivity and specificity of the LAMP assay were 95% and 99%, respectively.

We also tested some of the heat-treated samples directly in the PCR assay. Of 13 heat-treated blood samples that were positive in LAMP, 7 were negative in the PCR assay. These observations agreed with previous findings that inhibitors (e.g., heme) in blood could severely affect the amplification of DNA in PCR assays *(12)*. We do not know, however, why these PCR inhibitors have little impact on the LAMP reactions. As DNA polymerases have different susceptibilities to PCR inhibitors *(12)*, we suspect that the *Bacillus stearothermophilus* (*Bst*) DNA polymerase used in the LAMP reactions is more resistant.

One of the attractive features of the LAMP test is its ability to generate a large amount of white precipitate of magnesium pyrophosphate in a positive reaction (Fig. 1B). This allows easy identification of a positive reaction via visual inspection *(13)*. To evaluate the feasibility and consistency of interpreting the LAMP reaction with the naked eye, an untrained staff member who had no prior knowledge of the study examined the above LAMP reactions. Reactions were classified as positive, negative, or ambiguous based on the turbidity of the reaction. Of 202 samples examined, the results were consistent with those deduced from the real-time turbidity meter, with

(A)

REGION	PRIMER SEQUENCE (5' to 3')
F1P (F1C+F2)	AGCTGGAATTACCGCGGCTGGGTTCCTAGAGAAACAATTGG
B1P (B1+B2C)	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATGAAAC
F3	TGTAATTGGAATGATAGGAATTTA
B3C	GAAAACCTTATTTTGAACAAAGC
LPF	GCACCAGACTTGCCCT
LPB	TTGAATATTAAAGAA



(B)

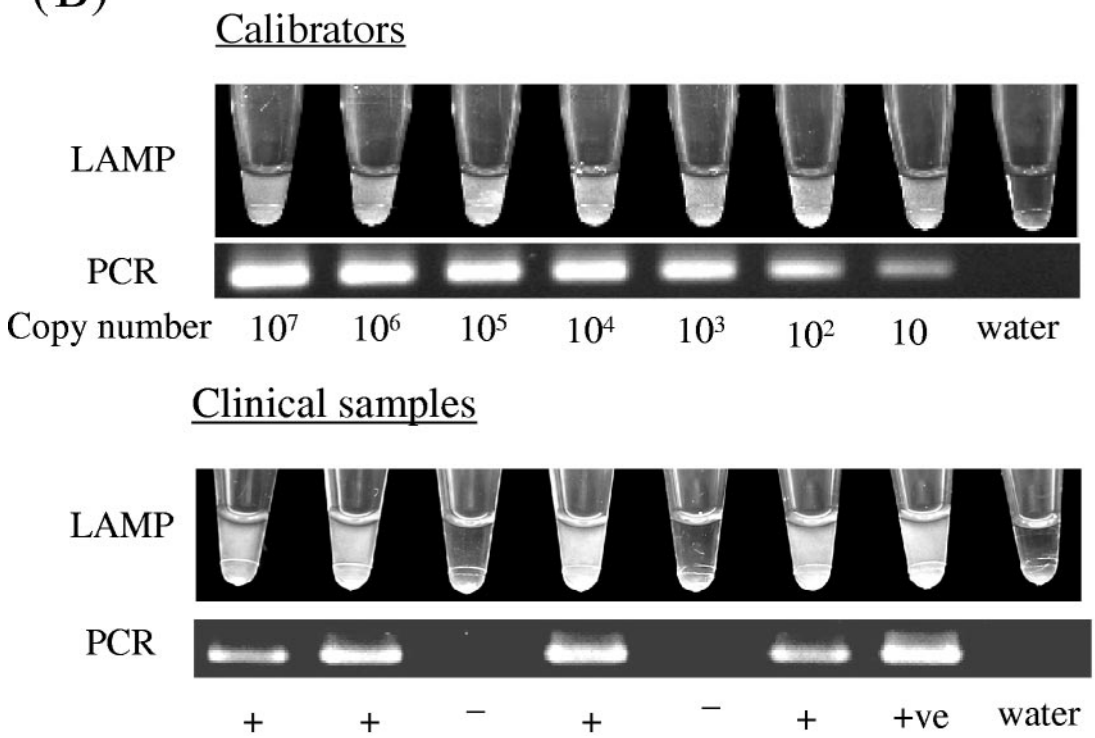


Fig. 1. Detection of 18S ribosomal RNA gene of *P. falciparum*.

(*A*), sequences of primers F1P, B1P, F3, B3, LPF, and LPB used in the LAMP assay. Locations of primer-binding regions in the reference sequence (GenBank accession no. M19173.1) are indicated by *arrows*. Only the positive sense of the reference sequence is shown. In a typical LAMP reaction, 2 μL of supernatant of a centrifuged heat-treated sample was mixed with 12.5 μL of 2× reaction buffer [40 mmol/L Tris-HCl (pH 8.8), 20 mmol/L KCl, 16 mmol/L $MgSO_4$, 20 mmol/L $(NH_4)_2SO_4$, 2 mL/L Tween 20, 1.6 mol/L betaine, and 2.8 mmol/L each of the deoxynucleotide triphosphates], 1 μL of *Bst* DNA polymerase (New England Biolabs), and 9.5 μL of primer mixture containing 40 pmol of primers FIP and BIP, 20 pmol of primers LPF and LPR, and 5 pmol of primers F3 and B3. Reaction mixtures were incubated and analyzed at 60 °C for 120 min in a real-time turbidity meter (LA-200; Treamecs). (*B*), detection of *P. falciparum* in positive controls and clinical samples by LAMP and PCR assays. After incubation, turbidities of the LAMP reactions were inspected visually. For the PCR assay, DNA from 50 μL of sample was extracted with the QIAamp DNA Mini Kit (Qiagen), and 2 μL of purified DNA was amplified by primers F3 and B3 in a 25-μL PCR reaction for 40 cycles. The copy numbers of plasmid DNA used in the calibrators are indicated. +, positive specimen; −, negative specimen; *+ve*, positive control; *water*, negative control.

the exception of 4 samples that were classified as ambiguous (see Table 1 in the online Data Supplement).

The limit of detection of a microscopic examination by an experienced microscopist is ~50 parasites/μL *(2, 4)*. To investigate whether the LAMP assay was more sensitive than a microscopic test, we serially diluted 10 of our clinical specimens with parasite counts ranging from 72 to 46 890 parasites/μL and tested them by the LAMP assay. The positive end-point of each of the diluted samples was correlated with the parasite count. For those samples with high concentrations of parasites, positive signals could be observed in reactions with 1000-fold–diluted samples (see file Supplementary Data 1 in the online Data Supplement). In one case, a reaction containing 4.46 parasites was positive. By contrast, of samples with relatively low concentrations of parasites, only those reactions with less diluted samples were positive in the LAMP assay. We used the microscopic parasite counts of these samples as references and estimated that the minimal parasite count in blood for a positive reaction was ~6 parasites/μL (see file Supplementary Data 1 in the online Data Supplement). We also compared the quantitative data generated from the real-time LAMP assay with the parasite counts. Of 96 specimens positive in the LAMP reaction, parasite count data from 83 specimens were available for analysis. The number of parasites estimated in the LAMP assay correlated positively with the parasite count deduced from the microscopic study (Pearson correlation analysis, $r = 0.642$; $P < 0.001$; see Fig. 3 in the online Data Supplement). These results indicated that, with the real-time turbidity meter, the LAMP assay can be used as an objective method to determine the parasite counts in blood samples.

To test the reactivity of the LAMP assay with strains found in other regions, we tested blood samples from 21 patients with *P. falciparum* or *P. vivax* infections in Ragama, Sri Lanka. The LAMP and PCR tests produced identical results, with 10 samples positive for *P. falciparum.* One of these 10 was originally considered to be *P. vivax* by the microscopic method. This microscopically misdiagnosed case was negative in a PCR test for *P. vivax (10)*, indicating that there was no coinfection of *P. falciparum* and *P. vivax* in that patient. These results further confirm the specificity of the LAMP assay.

The LAMP test for detection of *P. falciparum* is simple; it does not require thermal cyclers, expensive reagents (or time-consuming steps) for DNA purification, or downstream processing for amplicon detection (see Fig. 4 in the online Data Supplement). The DNA amplification step in the assay is highly robust *(7, 8, 14)*. The technician "hands-on" time for the assay is estimated to be one third that of a manual PCR test. In resource-limited situations, rather than using a real-time turbidity meter, one might perform this test with only a water bath. More importantly, the interpretation of results from this closed-tube test does not require highly experienced staff. We estimate the running cost for a LAMP test would be less than €0.25 (see file Supplementary Data 2 in the online Data Supplement). These features make the LAMP assay an option for the molecular diagnosis of *P. falciparum* even in basic healthcare settings. In this proof-of-concept study, we were able to recruit only microscopically positive retrospective samples for the study. Further work in evaluating the performance of this test in bedside or clinical situations is needed.

Part of this work was supported by The Research Grant Council of Hong Kong (HKU 7343/04M) and Seed Funding from The University of Hong Kong (Seed Funding). We thank colleagues from the Faculty of Tropical Medicine at Mahidol University and CH Hapuarachchi, University of Kelaniya, for sample recruitments. We also thank W.C. Shiu and P. Lui for technical advice on statistical analysis, and O.T.Y. Li for examining the LAMP reactions. The sponsors of the study had no role in study design, data collection, analysis, interpretation, or in the writing of this report.

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Previously published online at DOI: 10.1373/clinchem.2005.057901