

LONDON  
SCHOOL of  
HYGIENE  
& TROPICAL  
MEDICINE



Hopkins, H; Gonzalez, IJ; Polley, SD; Angutoko, P; Ategeka, J; Asimwe, 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. *The Journal of infectious diseases*, 208 (4). pp. 645-652. ISSN 0022-1899  
DOI: <https://doi.org/10.1093/infdis/jit184>

Downloaded from: <http://researchonline.lshtm.ac.uk/1229419/>

DOI: [10.1093/infdis/jit184](https://doi.org/10.1093/infdis/jit184)

#### Usage Guidelines

Please refer to usage guidelines at <http://researchonline.lshtm.ac.uk/policies.html> or alternatively contact [researchonline@lshtm.ac.uk](mailto:researchonline@lshtm.ac.uk).

Available under license: <http://creativecommons.org/licenses/by-nc-nd/2.5/>

# 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

Heidi Hopkins,<sup>1,3</sup> Iveth J. González,<sup>3</sup> Spencer D. Polley,<sup>4</sup> Patrick Angutoko,<sup>1</sup> John Ategeka,<sup>1</sup> Caroline Asimwe,<sup>1</sup> Bosco Agaba,<sup>2</sup> Daniel J. Kyabayinze,<sup>1</sup> Colin J. Sutherland,<sup>4,5,6</sup> Mark D. Perkins,<sup>3</sup> and David Bell<sup>3</sup>

<sup>1</sup>Foundation for Innovative New Diagnostics and <sup>2</sup>Malaria Control Programme, Uganda Ministry of Health, Kampala, Uganda; <sup>3</sup>Foundation for Innovative New Diagnostics, Geneva, Switzerland; <sup>4</sup>Department of Clinical Parasitology, Hospital for Tropical Diseases, University London Colleges NHS Foundation Trust, <sup>5</sup>HPA Malaria Reference Laboratory, and <sup>6</sup>Department of Immunology and Infection, London School of Hygiene and Tropical Medicine, United Kingdom

(See the major article by Polley et al on pages 637–44.)

**Background.** Current malaria diagnostic tests, including microscopy and antigen-detecting rapid tests, cannot reliably detect low-density infections. Molecular methods such as polymerase chain reaction (PCR) are highly sensitive but remain too complex for field deployment. A new commercial molecular assay based on loop-mediated isothermal amplification (LAMP) was assessed for field use.

**Methods.** Malaria LAMP (Eiken Chemical, Japan) was evaluated for samples from 272 outpatients at a rural Ugandan clinic and compared with expert microscopy, nested PCR, and quantitative PCR (qPCR). Two technicians performed the assay after 3 days of training, using 2 alternative blood sample-preparation methods and visual interpretation of results by fluorescence assay.

**Results.** Compared with 3-well nested PCR, the sensitivity of both LAMP and single-well nested PCR was 90%; the microscopy sensitivity was 51%. For samples with a *Plasmodium falciparum* qPCR titer of  $\geq 2$  parasites/ $\mu\text{L}$ , LAMP sensitivity was 97.8% (95% confidence interval, 93.7%–99.5%). Most false-negative LAMP results involved samples with parasitemia levels detectable by 3-well nested PCR but very low or undetectable by qPCR.

**Conclusions.** Malaria LAMP in a remote Ugandan clinic achieved sensitivity similar to that of single-well nested PCR in a United Kingdom reference laboratory. LAMP dramatically lowers the detection threshold achievable in malaria-endemic settings, providing a new tool for diagnosis, surveillance, and screening in elimination strategies.

**Keywords.** malaria; diagnosis; LAMP; loop-mediated isothermal amplification; sensitivity and specificity; PCR; polymerase chain reaction; molecular diagnosis; DNA; *Plasmodium falciparum*; Uganda; Africa.

Accurate detection of malaria is of increasing importance as the malaria prevalence declines across much of

its range [1, 2], with surveillance and screening becoming increasingly important in program management [3–5].

Received 11 February 2013; accepted 13 February 2013; electronically published 30 April 2013.

Presented in part: BioMed Central Challenges in Malaria Research Conference, Basel, Switzerland, October 2012 [abstract O30]; Royal Society of Tropical Medicine and Hygiene Biennial Meeting, Coventry, United Kingdom, September 2012; Rwanda Malaria Forum, Kigali, Rwanda, September 2012.

Correspondence: Heidi Hopkins, MD, Malaria & Acute Febrile Syndrome Program, Foundation for Innovative New Diagnostics, av de Budé 16, Geneva 1202, Switzerland (heidi.hopkins@finddiagnostics.org).

**The Journal of Infectious Diseases** 2013;208:645–52

© The Author 2013. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (<http://creativecommons.org/licenses/by-nc-nd/3.0/>), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work properly cited. For commercial re-use, please contact [journals.permissions@oup.com](mailto:journals.permissions@oup.com).

DOI: 10.1093/infdis/jit184

Microscopy and antigen-detecting rapid diagnostic tests (RDTs), when performed correctly, provide accurate diagnosis for case management [6] but cannot reliably detect lower-density parasitemia that may occur in asymptomatic individuals, who then represent reservoirs of infection. Such low-density infections with *Plasmodium* species are widely documented [7–12] and may contribute to transmission [13–15]. To eliminate malaria and prevent reintroduction, sustaining the capacity to detect such infections will be critical. The development of field-ready assays that can detect foci of infection in a way timely enough to enable treatment is therefore recognized as a major priority for malaria elimination [3, 16, 17]. Such highly sensitive assays could also benefit antimalarial drug efficacy monitoring, vaccine studies, and screening of vulnerable populations, such as pregnant women, in which low-density infections may have significant clinical consequences [18–20].

Polymerase chain reaction (PCR) detects parasite DNA, can identify infections below the threshold of detection for microscopy and RDTs, and is commonly considered the gold standard to detect malaria infection. However, PCR requires sophisticated laboratory infrastructure and advanced training, making it challenging and costly to implement in most malaria-endemic areas. Although PCR is used in some cases for focal screening and treatment strategies [11, 21], its restriction to central reference laboratories, often far from the sampled population, greatly limits its usefulness.

Loop-mediated isothermal amplification (LAMP) may offer a practical alternative. Like PCR, LAMP is a molecular technique that amplifies nucleic acids but uses simpler equipment and is less time intensive. A prototype LAMP assay designed for use in resource-constrained settings was developed through a public/private collaboration between the Foundation for Innovative New Diagnostics, Switzerland, and Eiken Chemical Co., Ltd., Japan. The assay forms the basis for a CE-marked product commercially released in July 2012 as the Loopamp Malaria Pan/Pf Detection Kit, list number LMC562 (Eiken Chemical). It is consistent with a recent description of “an ideal LAMP detection format” [22], and the kit consists of vacuum-dried reagents stable at ambient temperature. The assay’s primers target *Plasmodium* genus or *P. falciparum*-specific sequences. Assay performance includes either of 2 simple blood-processing methods, a 40-minute reaction time in a closed amplification unit, and a visual readout. The result is essentially qualitative. The relative simplicity and robustness of LAMP opens the potential for sustainable nucleic acid amplification in laboratories and near-patient locations in malaria-endemic countries. An evaluation of the LAMP test kit in a United Kingdom reference laboratory, reported in the article by Polley et al in this issue of the *Journal*, showed that LAMP sensitivity approximated that of nested PCR [23]. This article presents results of the same kit evaluated in a rural clinic in Uganda.

## METHODS

### Ethics and Protocol

All participants, or their parents or guardians, provided written informed consent. LAMP results did not influence care of patients. The study protocol was approved by the Uganda National Council of Science and Technology (UNCST; reference HS160) before participant recruitment and sample collection began (Supplementary Materials). The study and report follow STARD (Standards for the Reporting of Diagnostic Accuracy Studies) guidelines (Supplementary Materials).

### Study Site and Population

The study was conducted from November 2010 to February 2011 in Tororo District, eastern Uganda, at Nagongera Health Center, a government-sponsored health facility where malaria diagnosis is based on microscopy. A portable generator was used to ensure a stable electrical current for the LAMP incubator and other study equipment. The entomological inoculation rate in this area in 2001–2002 was estimated to be 562 infective bites per person per year [24]. A 2008 study in this area found that 31% of symptomatic patients aged  $\geq 5$  years tested positive by expert microscopy (H. Hopkins et al, unpublished data), whereas a year earlier 39% were found positive by a combination of microscopy and PCR [25]. *P. falciparum* accounts for the great majority of malaria cases ( $\geq 94\%$ ) in this region [26–28].

Study selection criteria included patient age of  $\geq 5$  years, documented presence of fever or self-reported history of fever within the previous 24 hours, and absence of evidence of severe illness.

### Sample Size and Sample Collection

Sample size calculations were based on the assumption of a microscopy-confirmed prevalence of malaria of 30% among symptomatic patients and a LAMP sensitivity of 95% (95% confidence interval [CI], 90%–98%) and specificity of 80% (95% CI, 75%–85%) for *P. falciparum*, compared with expert microscopy; calculations revealed that 351 participants were required. A target of 380 participants was set. Over 18 days, consecutive outpatients referred to the laboratory for malaria testing by clinic staff according to the usual standard of care were asked to participate in the study. Study personnel were not involved in the decision to refer patients to the laboratory or in any decision regarding clinical management. At enrollment, the patient’s age, sex, and axillary temperature were recorded; a 2–3-mL venous blood sample was collected in a tube containing ethylenediaminetetraacetic acid (EDTA) for preparation of blood smears and determination of white blood cell count; and a 2-mL heparinized sample was collected and stored at ambient temperature until LAMP was performed the same day. Four hundred microliters of the EDTA sample was stored for up to 4 days at 4°C before freezing at  $-80^{\circ}\text{C}$ .

## LAMP

The LAMP assay used in this study is based on a published mitochondrial *P. falciparum*-specific primer set [29]; the LAMP kits consisted of plastic reaction tubes containing thermostable vacuum-dried reagents that were specific for amplification of *P. falciparum* DNA. A successful reaction results in large quantities of DNA and insoluble magnesium orthophosphate, a byproduct of amplification that can be detected by fluorescence assay (using calcein as an indicator) or by turbidimetry [29]; this study used only fluorescence reading.

After blood sample processing by one of 2 methods described below, the assay was performed according to the manufacturer's instructions by 2 laboratory technologists with 3 days of training in the test procedure. In short, 25  $\mu\text{L}$  of processed blood was added to reaction tubes, and reagents were reconstituted by shaking the tubes. The tubes were placed into the LF-160 LAMP incubator (Eiken), which accommodates 16 tubes per run; each run consisted of incubation for 40 minutes at 65°C, followed by incubation for 5 minutes at 80°C to inactivate the enzyme and halt amplification. Reaction tubes were then read in the fluorescence visualization unit under blue light-emitting diode light (integral to the LF-160 incubator). Positive and negative controls were included in each run. The test kits and controls, sealed at the time of manufacture in moisture-proof packaging, had been stored at room temperature in Uganda for 4 weeks before use. Storage temperature was monitored but not controlled and remained at  $\leq 30^\circ\text{C}$ .

A LAMP reaction was considered positive for *P. falciparum* DNA if an obvious increase in fluorescence was observed, compared with the negative control. Results were read by a single laboratory technologist and were considered valid if fluorescence was present in the positive control and absent in the negative control. LAMP results were interpreted by study staff; the interpreter of each result was blinded to LAMP results for the same sample using the alternative sample-preparation method and to health facility microscopy results. In the case of vague or indeterminate LAMP results, the second technologist confirmed the reading in real time.

Two sample-preparation methods to extract DNA from patient blood were used for this study: a commercially available kit designed specifically for LAMP (Loopamp PURE DNA Extraction Kit) and a boil and spin method. As described in the accompanying article by Polley et al [23], the PURE device is a series of interlocking plastic components providing a closed system for preparation of a 35- $\mu\text{L}$  blood aliquot without centrifugation or micropipetting. For the boil and spin method, 60  $\mu\text{L}$  of blood was added to 60  $\mu\text{L}$  of extraction solution (400 mM NaCl, 40 mM Tris pH 6.5, and 0.4% sodium dodecyl sulfate) in an Eppendorf tube, heated for 5 minutes at 95°C with a water bath, and centrifuged at 10 000  $\times g$  for 1 minute; 25  $\mu\text{L}$  of the supernatant was pipetted into a dilution tube containing 287.5  $\mu\text{L}$  of molecular-grade water (Qiagen, Germany);

and 25  $\mu\text{L}$  of the resulting solution was transferred into a reaction tube for use in the LAMP assay. Complete standard operating procedures for the LAMP assay, PURE, and boil and spin method are online (available at: [http://www.finddiagnostics.org/programs/malaria-afs/lamp/standard\\_procedures/index.html](http://www.finddiagnostics.org/programs/malaria-afs/lamp/standard_procedures/index.html)). Estimates of sample inputs for LAMP and PCR are as follows: 25  $\mu\text{L}$  of PURE extract contains *P. falciparum* DNA equivalent to the number of parasite genomes in 2.8  $\mu\text{L}$  of the original blood sample (confirmed experimentally by S. Polley [unpublished data]), and 2  $\mu\text{L}$  of boil and spin extract (prior to 12.5  $\times$  dilution) contains *P. falciparum* DNA equivalent to 1.6  $\mu\text{L}$  of the original blood sample (assuming 80% recovery of DNA). The volume of Qiagen DNA extract used for PCR is equivalent to 10  $\mu\text{L}$  of the original blood sample (assuming 100% recovery).

## Microscopy

Blood smears were stained with 3% Giemsa for 35 minutes and mounted with a coverslip and DPX. Leukocyte count was measured on site, using a KX-21 hematology analyzer (Sysmex, Tokyo, Japan) or a HemoCue point-of-care instrument (Quest Diagnostics, Ängelholm, Sweden), to allow accurate calculation of parasite density. One thick smear was prepared and read immediately by health facility staff for use in case management. A second thick smear and a thin smear were prepared by study staff and read by 2 expert microscopists (expertise level 1 or 2, following the World Health Organization [WHO] competency assessment protocol [30]) after completion of study enrollment. Thick smears were read for parasite density, and thin smears were read for parasite species. Smears with discrepant results were reread by a third expert reader. All microscopists were blinded to results of LAMP and previous microscopy.

## PCR

Nested PCR provided the reference standard for assessment of LAMP performance. Whole blood samples stored at  $-80^\circ\text{C}$  were shipped from Uganda on dry ice to the Hospital for Tropical Diseases in London, United Kingdom, and analyzed by PCR to confirm the presence or absence of parasite DNA. PCR was performed according to standardized protocols and published methods [31, 32], as detailed in the accompanying article by Polley et al [23]. Results were independently scored by 2 investigators blinded to microscopy and LAMP results.

After nested PCR was performed for *P. falciparum* with a single well for each sample, nested PCR was repeated in 3 wells for samples that tested negative in the first run, 3 random positive samples, and the within-run negative controls, increasing the overall sensitivity of the reference standard. A sample was rescored as testing positive if any of the replicate PCR reactions yielded an amplicon of the correct size.

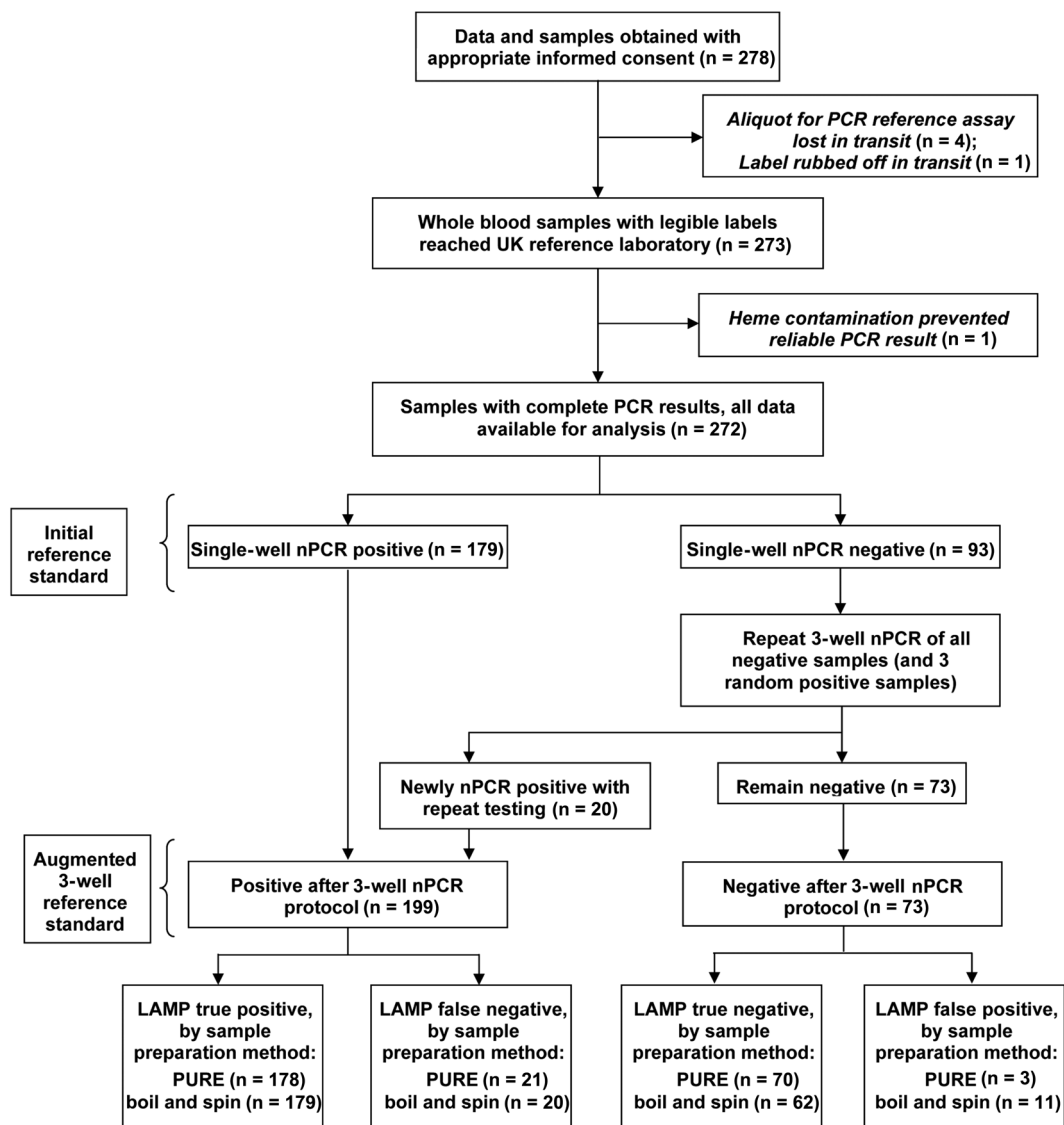
All samples positive for *P. falciparum* DNA by nested PCR underwent real-time quantitative PCR (qPCR) analysis. Two microliters of Qiagen-extracted DNA was used in a multiplex

real-time PCR to detect *P. falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, and both subspecies of *Plasmodium ovale* [33] as described in the accompanying article [23]. To determine the *P. falciparum* density, a dilution series of the WHO international standard for *P. falciparum* DNA was included in each run [33], together with DNA from a clinical *P. falciparum* sample confirmed by microscopy to contain 5000 parasites/ $\mu$ L. This qPCR assay provided a limit of detection of 1 parasite/ $\mu$ L. All *P. falciparum* samples that were positive by nested PCR but

negative by qPCR were therefore assumed to have parasite densities of  $<1$  parasite/ $\mu$ L.

#### Data Management and Statistical Analysis

LAMP and microscopy data were double entered into an EpiInfo, version 6.04, database (Centers for Disease Control and Prevention, Atlanta, GA). PCR data were entered in Microsoft Office Excel 2007. Data were analyzed with Stata, version 9, (Stata, College Station, TX), and exact binomial CIs were calculated.



**Figure 1.** Flow diagram of the study. The diagram summarizes results of the loop-mediated isothermal amplification (LAMP) malaria assay for *Plasmodium falciparum* at a remote clinical site in Uganda, using 2 alternative simple sample-preparation methods (PURE device and boil and spin), and results of 2 reference standards: single-well nested polymerase chain reaction (nPCR) and 3-well nPCR. The number of samples positive by nPCR increased by 20 after inclusion of 3-well nPCR results. Results of expert microscopy and of quantitative PCR (qPCR) to determine *P. falciparum* titer and presence of various *Plasmodium* species are presented in the text. Most false-negative LAMP results occurred in samples with parasite titers that were very low or undetectable by qPCR (Table 3); no sample with a titer of  $\geq 1$  parasite/ $\mu$ L was missed by both LAMP methods. False-positive LAMP results may represent errors in sample-preparation technique or detection of low-density parasitemias that were undetected by nPCR because of stochastic effects in sampling.

**Table 1. Characteristics of 272 Study Subjects**

Characteristic	Value
Age, y	
Median	23
Range	5–81
Interquartile range	14–32
Female sex	173 (63.6)
Self-reported history of fever	272 (100)
Measured elevated temperature <sup>a</sup>	22/268 <sup>a</sup> (8.2) <sup>b</sup>

Data are for 272 subjects, unless otherwise indicated.

<sup>a</sup> Defined as an axillary temperature of  $\geq 37.2^{\circ}\text{C}$ ; data were missing for 4 participants. Three participants (1.1%) had an axillary temperature of  $\geq 38.0^{\circ}\text{C}$ .

## RESULTS

During the study, errors were noted in the informed consent process, and on consultation with UNCST, samples and data from 197 participants with possible consent errors were excluded from analysis. Data were therefore evaluated from 272 patients referred for malaria testing at Nagongera Health Center in eastern Uganda (Figure 1). Table 1 shows characteristics of the study population. Fifty-two participants (19.1%) were aged 5–10 years, 69 (25.4%) were aged 11–20 years, and 151 (55.1%) were aged  $\geq 21$  years. All self-reported a history of fever, while 8.2% had an elevated axillary temperature (defined as an axillary temperature of  $\geq 37.2^{\circ}$ ).

### PCR Results

Single-well nested PCR found 179 samples (65.8%) to be positive for *P. falciparum* DNA. The number of positive samples increased to 199 (73.2%) after 3-well nested PCR was performed (Figure 1). Of the additional 20 samples that tested positive by 3-well PCR, the qPCR-determined *P. falciparum* density was  $<1$  parasite/ $\mu\text{L}$  in 9 and undetectable in 11. Of all 199 samples that tested positive by nested PCR, parasite DNA was detectable by qPCR in 178. Of these 178 samples, the qPCR assay identified 121 (68.0%) as *P. falciparum* mono-infections; 38 (21.4%) as *P. falciparum* and *P. malariae* mixed infections; 10 (5.6%) as *P. falciparum*, *P. malariae*, and *P. ovale* mixed infections; 8 (4.5%) as *P. falciparum* and *P. ovale* mixed infections; and 1 (0.6%) as *P. ovale* mono-infection. Since the sample identified by qPCR as a *P. ovale* mono-infection was found to contain *P. falciparum* DNA by nested PCR, it was most likely a mixed infection containing a level of *P. falciparum* that was below the detection threshold of qPCR. For the 176 infections with qPCR-detectable *P. falciparum* DNA, the median titer by qPCR was 34 parasites/ $\mu\text{L}$  (range,  $<1$ –259 442 parasites/ $\mu\text{L}$ ).

### LAMP Performance, Compared With Reference Standards

Of the 272 total samples, 181 (66.5%) prepared with the PURE device and 190 (69.9%) prepared by the boil and spin method

**Table 2. Loop-Mediated Isothermal Amplification (LAMP) Sensitivity, Compared With 3-Well Nested Polymerase Chain Reaction (nPCR), Stratified by Quantitative PCR (qPCR)-Determined Parasite Density**

<i>P. falciparum</i> Density, <sup>a</sup> Preparation Method	Subjects, Proportion <sup>b</sup>	Sensitivity, % (95% CI)
$<1$ parasite/ $\mu\text{L}$		
PURE	33/50 <sup>c,d</sup>	66.0 (51.2–78.8)
Boil and spin	33/50 <sup>c,d</sup>	66.0 (51.2–78.8)
$\geq 1$ parasite/ $\mu\text{L}$		
PURE	145/149	97.3 (93.3–99.3)
Boil and spin	146/149	98.0 (94.2–99.6)
$\geq 2$ parasites/ $\mu\text{L}$		
PURE	134/137	97.8 (93.7–99.5)
Boil and spin	134/137	97.8 (93.7–99.5)
$\geq 5$ parasites/ $\mu\text{L}$ <sup>e</sup>		
PURE	119/122	97.5 (93.0–99.5)
Boil and spin	119/122	97.5 (93.0–99.5)

Abbreviations: CI, confidence interval; *P. falciparum*, *Plasmodium falciparum*.

<sup>a</sup> Determined by qPCR.

<sup>b</sup> Data are no. of samples positive by LAMP/no. of samples positive by the gold standard of nPCR.

<sup>c</sup> Includes 23 samples with parasites detected by nPCR with 3-well assay but not detectable by multiplex real-time qPCR. These results are therefore assumed to represent very-low-density infections below the limit of detection of the qPCR assay (ie, 1 parasite/ $\mu\text{L}$  for *P. falciparum*).

<sup>d</sup> Of the 50 samples positive by 3-well nPCR and with a *P. falciparum* titer of  $<1$  parasite/ $\mu\text{L}$  by qPCR, 29 (58%) had parasites detected by LAMP with both PURE and boil and spin sample-preparation methods, 4 each (8%) had parasites detected only with either PURE or boil and spin, and 13 (26%) did not have parasites detected by LAMP with either sample-preparation method.

<sup>e</sup> Five parasites per microliter is the lower limit of detection targeted in LAMP product specifications.

were positive by LAMP. Agreement between PURE and boil and spin results was 91% ( $\kappa=0.79$ ).

Compared with single-well nested PCR, LAMP performed on samples prepared with PURE had a sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 93.3%, 85.0%, 92.3%, and 86.8%, respectively. For samples prepared by boil and spin, results were 93.9%, 76.3%, 88.4%, and 86.6%, respectively. When 3-well nested PCR was used as the gold standard, sensitivity, specificity, PPV, and NPV were 89.5%, 95.9%, 98.3%, and 76.9%, respectively, for PURE and 90.0%, 84.9%, 94.2%, and 75.6%, respectively, for boil and spin. For all samples, when compared with 3-well nested PCR, the LAMP assay's sensitivity (with PURE, 89.5% [178/199]; with boil and spin, 90.0% [179/199]) approximated that of single-well nested PCR (90.0% [179/199]). When analysis was restricted to samples with a *P. falciparum* titer of  $\geq 1$  parasite/ $\mu\text{L}$  by qPCR, the sensitivity of LAMP with PURE, compared with 3-well nested PCR, increased to 97.3% (95% CI, 93.3%–99.3%), and the sensitivity of LAMP with boil and spin increased to 98.0% (95% CI, 94.2%–99.6%; Table 2). When the

**Table 3. Characteristics of Samples With a *Plasmodium falciparum* Titer of >1 Parasite/μL for Which Loop-Mediated Isothermal Amplification Results Were False-Negative When Compared With the Nested Polymerase Chain Reaction (nPCR) Reference Standard**

Preparation Method, Sample Identification no.	Expert Microscopy		qPCR	
	Species	Parasite Density, Parasites/μL	Species	<i>P. falciparum</i> Titer, Parasites/μL
<b>PURE</b>				
33	<i>P. falciparum</i>	39.5	<i>P. falciparum</i> , <i>P. malariae</i>	9.5
501	Negative	. . .	<i>P. falciparum</i> , <i>P. malariae</i> , <i>P. ovale</i>	1.2
571	<i>P. falciparum</i>	563	<i>P. falciparum</i>	678
581	<i>P. falciparum</i>	743	<i>P. falciparum</i> , <i>P. malariae</i> , <i>P. ovale</i>	1458
<b>Boil and spin</b>				
7	<i>P. falciparum</i>	24	<i>P. falciparum</i>	7.4
251	<i>P. falciparum</i>	1327	<i>P. falciparum</i> , <i>P. malariae</i>	529
562	<i>P. falciparum</i>	126	<i>P. falciparum</i> , <i>P. malariae</i>	250

Abbreviations: qPCR, quantitative PCR; *P. malariae*, *Plasmodium malariae*; *P. ovale*, *Plasmodium ovale*.

threshold was raised to a *P. falciparum* titer of  $\geq 2$  parasites/μL, the sensitivity of LAMP with both preparation methods was 97.8% (95% CI, 93.7%–99.5%), compared with 3-well nested PCR.

As shown in Table 3, most false-negative LAMP results occurred in samples with parasite titers that were very low or undetectable by qPCR. Parasites in 4 samples with a higher parasite density were missed by LAMP with PURE, and parasites in 3 were missed by LAMP with boil and spin; these were different samples, and no sample with a titer of  $\geq 1$  parasite/μL tested negative by both methods (Table 3). The sample mentioned earlier, with *P. ovale* mono-infection detected by qPCR (and *P. falciparum* detected by nested PCR), was negative by *P. falciparum* LAMP, using both preparation methods. Twelve samples were negative by 3-well nested PCR (and by microscopy) but positive by LAMP with PURE alone (n = 1), boil and spin alone (n = 9), or both methods (n = 2; Figure 1).

Expert microscopy detected parasites in 102 samples (37.5%), with a median density of 196 parasites/μL (range, 11–147 032 parasites/μL). For the 102 microscopy-positive samples, the median qPCR titer was 176 parasites/μL (range, 0–259 442 parasites/μL). Most (97 of 102) were identified by microscopy as *P. falciparum* mono-infections, while 2 were reported as mixed *P. falciparum* and *P. malariae* infections, 2 as *P. malariae* mono-infections, and 1 as *P. ovale* mono-infection.

LAMP achieved far higher sensitivity than microscopy but had a lower specificity, using PCR as a reference standard. This is consistent with the threshold of detection being close to that of the reference standard. Compared with single-well nested PCR, the sensitivity, specificity, PPV, and NPV of microscopy were 56.4%, 98.9%, 99.0%, and 54.2%, respectively. With 3-well PCR as the gold standard, these measures were 51.3%, 100%, 100%, and 42.9%, respectively. One sample negative by LAMP with both preparation methods was positive by microscopy;

this sample was also negative by single-well nested PCR and qPCR but positive by 3-well nested PCR, suggesting a very-low-density infection. Three samples that were positive by microscopy (and PCR) were negative by LAMP with PURE, while 3 different samples were negative by boil and spin (Table 3).

## DISCUSSION

The malaria LAMP kit used in this study achieved accuracy comparable to that of nested PCR in a United Kingdom reference laboratory setting [23]. The study reported here demonstrates similar sensitivity when performed in a remote clinic in a malaria-endemic area. LAMP results for blood samples prepared with 2 simple methods and read visually by fluorescence under a blue light-emitting diode light far exceeded the sensitivity of expert microscopy and compared well with a reference standard of 3-well nested PCR, with very high sensitivity for infections of  $\geq 2$  parasites/μL. LAMP results were available within 60–90 minutes of starting sample processing. The results were achieved by technicians who had no previous training in molecular diagnostic techniques and just 3 days of training in LAMP procedures. Sample collection, preparation, and testing were performed on 1 countertop in a small room at the health center. The closed amplification and reading system reduces the likelihood of workspace amplicon contamination.

Several evaluations of malaria LAMP assays have reported detection of cultured parasites or field samples transported to research laboratories [35–38]; one study evaluated another LAMP assay in a field clinic in Thailand, comparing results with microscopy [39]. To our knowledge, the current study is the first to demonstrate the reliable detection of parasites to a very low threshold in a remote clinical setting and the first to use field-stable reagents. The assay demonstrated a detection

threshold equivalent to that of single-well nested PCR, when compared to 3-well nested PCR. Performance of both LAMP and single-well nested PCR declined in samples containing <1 parasite/ $\mu$ L.

A small number of false-negative results occurred with samples containing parasite densities well above the limit of detection of expert microscopy. This occurred with both sample-preparation methods but on different samples, suggesting a failure in the sample-preparation steps rather than failure of recognition by the LAMP primer sets. Further investigation could identify and minimize preparation errors. Some apparent false-positive LAMP results also occurred; this would pose a problem for surveillance in settings of very low transmission or for drug-efficacy monitoring. However, it is unclear from this study and from the parallel United Kingdom reference laboratory study [23] whether these are really false-positive results. At these low parasite densities, chance discrepancies are expected because of stochastic processes, and false-positive results may represent detection of very-low-density parasitemias that were undetected by the nested PCR reference standard. False-positive results were not seen during assay development when testing against parasite-negative samples from areas where malaria is not endemic, using the sample-preparation methods described here [23].

The ability to detect very-low-density malaria parasitemia in remote settings raises opportunities and new strategic questions for malaria management and elimination. In many regions of endemicity, including southern Africa and Southeast Asia, malaria is now a relatively uncommon disease, and asymptomatic low-density infections pose a significant public health challenge. In these areas, hotspots of higher transmission can form foci for wider reintroduction [40]. Strategies of focal screening and treatment to identify and eliminate such parasite reservoirs have been hampered by a lack of a highly sensitive diagnostic test close to the site of sample collection [16]. This study demonstrates that LAMP detects parasites with an accuracy similar to that of PCR in a remote setting, removing the main logistical barrier to implementation of focal screening and treatment strategies. It will be important to assess the LAMP assay's performance in a variety of malaria-endemic settings to determine the robustness of the platform.

The relative simplicity of the LAMP procedure and the low infrastructure costs open a range of other research and monitoring opportunities by bringing molecular-level parasite detection within reach of national malaria control programs. Although highly sensitive parasite detection may have limited usefulness for routine fever case management in malaria-endemic areas and the per-sample cost of LAMP remains well above that of microscopy or RDT, LAMP may have specific applications in antenatal screening, screening in antimalarial drug efficacy trials, and resistance monitoring programs, vaccine trials, and evaluations of other diagnostic tools [18–20]. Costs

may be further reduced by pooled screening [41, 42]. In higher-resource countries, use of this LAMP assay in the diagnosis of malaria in febrile returned travelers offers advantages of speed and/or accuracy over current methods [23].

In conclusion, this malaria LAMP assay dramatically lowers the detection threshold that can be achieved in routine malaria-endemic settings. Future product improvements should include scaling up throughput and lowering costs to address screening requirements in malaria-endemic settings. As seen with the recent introduction of malaria RDTs, introduction of novel diagnostic methods can open new avenues for malaria control and elimination, as well as new challenges in identifying where such capacity is best used.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Acknowledgments.** We thank the study participants and staff at the Nsongera Health Center IV in Tororo District, Uganda; Samana Schwank, Ajay Kumar Thirumala, Adoke Yeka, and Christopher Eruaga, for logistical assistance and advice; Maxwell Kilama, for his diligence in quality-control reading of blood smears; and Jean Nsekera and Nora Champouillon, for administrative support.

**Financial support.** This work was supported by the Foundation for Innovative New Diagnostics, through grants from the Bill and Melinda Gates Foundation; the Ministry of Foreign Affairs of the Government of the Netherlands; and the United Kingdom Department for International Development.

**Potential conflicts of interest.** H. H., I. J. G., P. A., J. A., C. A., D. J. K., M. D. P., and D. B. are employees of the Foundation for Innovative New Diagnostics, a codeveloper of the malaria LAMP assay and the funding organization. S. P. was supported by a project grant from the Foundation for Innovative New Diagnostics (to C. S.). B. A. certifies no potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

## References

1. WHO. World malaria report, 2011. Geneva: World Health Organization, 2012.
2. Feachem RG, Phillips AA, Hwang J, et al. Shrinking the malaria map: progress and prospects. *Lancet* 2010; 376:1566–78.
3. Moonen B, Cohen JM, Snow RW, et al. Operational strategies to achieve and maintain malaria elimination. *Lancet* 2010; 376:1592–603.
4. Hsiang MS, Hwang J, Kunene S, et al. Surveillance for malaria elimination in Swaziland: a national cross-sectional study using pooled PCR and serology. *PLoS One* 2012; 7:e29550.
5. Mueller I, Slutsker L, Tanner M. Estimating the burden of malaria: the need for improved surveillance. *PLoS Med* 2011; 8:e1001144.
6. WHO. Guidelines for the treatment of malaria. 2nd ed. Geneva: World Health Organization, 2010.



7. Roper C, Elhassan IM, Hviid L, et al. Detection of very low level *Plasmodium falciparum* infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan. *Am J Trop Med Hyg* **1996**; 54:325–31.
8. Zakeri S, Najafabadi S, Zare A, Djajid N. Detection of malaria parasites by nested PCR in south-eastern, Iran: Evidence of highly mixed infections in Chahbahar district. *Malar J* **2002**; 1:2.
9. Toma H, Kobayashi J, Vannachone B, et al. A field study on malaria prevalence in southeastern Laos by polymerase chain reaction assay. *Am J Trop Med Hyg* **2001**; 64:257–61.
10. Alves FP, Durlacher RR, Menezes MJ, Krieger H, Silva LH, Camargo EP. High prevalence of asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections in native Amazonian populations. *Am J Trop Med Hyg* **2002**; 66:641–8.
11. Steenkeste N, Incardona S, Chy S, et al. Towards high-throughput molecular detection of *Plasmodium*: new approaches and molecular markers. *Malar J* **2009**; 8:86.
12. Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. *J Infect Dis* **2009**; 200:1509–17.
13. Schneider P, Bousema JT, Gouagna LC, et al. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg* **2007**; 76:470–4.
14. Ouedraogo AL, Bousema T, Schneider P, et al. Substantial contribution of submicroscopic *Plasmodium falciparum* gametocyte carriage to the infectious reservoir in an area of seasonal transmission. *PLoS One* **2009**; 4:e8410.
15. Manjurano A, Okell L, Lukindo T, et al. Association of sub-microscopic malaria parasite carriage with transmission intensity in north-eastern Tanzania. *Malar J* **2011**; 10:370.
16. malERA Consultative Group on Diagnoses and Diagnostics. A research agenda for malaria eradication: diagnoses and diagnostics. *PLoS Med* **2011**; 8:e1000396.
17. Hoyer S, Nguon S, Kim S, et al. Focused screening and treatment (FSAT): a PCR-based strategy to detect malaria parasite carriers and contain drug resistant *P. falciparum*, Pailin, Cambodia. *PLoS One* **2012**; 7:e45797.
18. Imoukhuede EB, Andrews L, Milligan P, et al. Low-level malaria infections detected by a sensitive polymerase chain reaction assay and use of this technique in the evaluation of malaria vaccines in an endemic area. *Am J Trop Med Hyg* **2007**; 76:486–93.
19. Campos IM, Uribe ML, Cuesta C, Franco-Gallego A, Carmona-Fonseca J, Maestre A. Diagnosis of gestational, congenital, and placental malaria in Colombia: comparison of the efficacy of microscopy, nested polymerase chain reaction, and histopathology. *Am J Trop Med Hyg* **2011**; 84:929–35.
20. Nosten F, McGready R, Mutabingwa T. Case management of malaria in pregnancy. *Lancet Infect Dis* **2007**; 7:118–25.
21. Rogawski ET, Congpuong K, Sudathip P, et al. Active case detection with pooled real-time PCR to eliminate malaria in Trat province, Thailand. *Am J Trop Med Hyg* **2012**; 86:789–91.
22. Njiru ZK. Loop-mediated isothermal amplification technology: towards point of care diagnostics. *PLoS Negl Trop Dis* **2012**; 6:e1572.
23. Polley SD, González IJ, Mohamed D, et al. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. *J Infect Dis* **2013**. In this issue.
24. Okello PE, Van Bortel W, Byaruhanga AM, et al. Variation in malaria transmission intensity in seven sites throughout Uganda. *Am J Trop Med Hyg* **2006**; 75:219–25.
25. Hopkins H, Bebell L, Kambale W, Dokomajilar C, Rosenthal PJ, Dorsey G. Rapid diagnostic tests for malaria at sites of varying transmission intensity in Uganda. *J Infect Dis* **2008**; 197:510–8.
26. Clark TD, Njama-Meya D, Nzarusaba B, et al. Incidence of malaria and efficacy of combination antimalarial therapies over 4 years in an urban cohort of Ugandan children. *PLoS One* **2010**; 5:e11759.
27. Nsoby SL, Parikh S, Kironde F, et al. Molecular evaluation of the natural history of asymptomatic parasitemia in Ugandan children. *J Infect Dis* **2004**; 189:2220–6.
28. Pullan RL, Bukirwa H, Staedke SG, Snow RW, Brooker S. *Plasmodium* infection and its risk factors in eastern Uganda. *Malar J* **2010**; 9:2.
29. Polley SD, Mori Y, Watson J, et al. Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification. *J Clin Microbiol* **2010**; 48:2866–71.
30. WHO. Malaria microscopy quality assurance manual. Version 1. February 2009. [http://www.who.int/malaria/publications/atoz/mmicroscopy\\_qam/en/](http://www.who.int/malaria/publications/atoz/mmicroscopy_qam/en/), AND [http://www.who.int/malaria/publications/malaria\\_microscopy\\_QA\\_manual.pdf](http://www.who.int/malaria/publications/malaria_microscopy_QA_manual.pdf). Accessed 10 May 2013.
31. Polley SD, Sutherland CJ, Regan F, Hassan M, Chiodini PL. Increased sensitivity for detecting malaria parasites in human umbilical cord blood using scaled-up DNA preparation. *Malar J* **2012**; 11:62.
32. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol* **1993**; 58:283–92.
33. Shokoples SE, Ndao M, Kowalewska-Grochowska K, Yanow SK. Multiplexed real-time PCR assay for discrimination of *Plasmodium* species with improved sensitivity for mixed infections. *J Clin Microbiol* **2009**; 47:975–80.
34. Padley DJ, Heath AB, Sutherland C, Chiodini PL, Baylis SA. Collaborative Study Group. Establishment of the 1st World Health Organization International Standard for *Plasmodium falciparum* DNA for nucleic acid amplification technique (NAT)-based assays. *Malar J* **2008**; 7:139.
35. Han ET, Watanabe R, Sattabongkot J, et al. Detection of four *Plasmodium* species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. *J Clin Microbiol* **2007**; 45:2521–8.
36. Lucchi NW, Demas A, Narayanan J, et al. Real-time fluorescence loop mediated isothermal amplification for the diagnosis of malaria. *PLoS One* **2010**; 5:e13733.
37. Paris DH, Imwong M, Faiz AM, et al. Loop-mediated isothermal PCR (LAMP) for the diagnosis of falciparum malaria. *Am J Trop Med Hyg* **2007**; 77:972–6.
38. Poschl B, Waneesorn J, Thekisoe O, Chutipongvivate S, Karanis P. Comparative diagnosis of malaria infections by microscopy, nested PCR, and LAMP in northern Thailand. *Am J Trop Med Hyg* **2010**; 83:56–60.
39. Sirichaisinthop J, Buates S, Watanabe R, et al. Evaluation of loop-mediated isothermal amplification (LAMP) for malaria diagnosis in a field setting. *Am J Trop Med Hyg* **2011**; 85:594–6.
40. Bousema T, Griffin JT, Sauerwein RW, et al. Hitting hotspots: spatial targeting of malaria for control and elimination. *PLoS Med* **2012**; 9:e1001165.
41. Hsiang MS, Lin M, Dokomajilar C, et al. PCR-based pooling of dried blood spots for detection of malaria parasites: optimization and application to a cohort of Ugandan children. *J Clin Microbiol* **2010**; 48:3539–43.
42. Taylor SM, Juliano JJ, Trottman PA, et al. High-throughput pooling and real-time PCR-based strategy for malaria detection. *J Clin Microbiol* **2010**; 48:512–9.
43. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* **2000**; 28:E63.
44. Eiken Chemical Co., Ltd. Eiken genome site LAMP method. <http://loopamp.eiken.co.jp/e/>. Accessed 10 May 2013.
45. Francois P, Tangomo M, Hibbs J, et al. Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunol Med Microbiol* **2011**; 62:41–8.
46. Poon LL, Wong BW, Ma EH, et al. Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clin Chem* **2006**; 52:303–6.