Am. J. Trop. Med. Hyg., 97(6), 2017, pp. 1777–1781
doi:10.4269/ajtmh.17-0225
Copyright © 2017 by The American Society of Tropical Medicine and Hygiene

Performance of Loop-Mediated Isothermal Amplification for the Identification of Submicroscopic *Plasmodium falciparum* Infection in Uganda

Shereen Katrak,[1]* Maxwell Murphy,[1] Patience Nayebare,[2] John Rek,[2] Mary Smith,[3] Emmanuel Arinaitwe,[2,4] Joaniter I. Nankabirwa,[2,5] Moses Kamya,[2,5] Grant Dorsey,[1] Philip Rosenthal,[1] and Bryan Greenhouse[1]

[1]*Department of Medicine, University of California San Francisco, San Francisco, California;* [2]*Infectious Diseases Research Collaboration, Kampala, Uganda;* [3]*Chicago Medical School, Rosalind Franklin University, North Chicago, Illinois;* [4]*London School of Hygiene and Tropical Medicine, London, United Kingdom;* [5]*School of Medicine, Makerere University College of Health Sciences, Kampala, Uganda*

Abstract. Accurately identifying and targeting the human reservoir of malaria parasitemia is critical for malaria control, and requires a reliable and sensitive diagnostic method. Loop-mediated isothermal amplification (LAMP) is increasingly used to diagnose submicroscopic parasitemia. Although most published studies report the sensitivity of LAMP compared with nested polymerase chain reaction (PCR) as ≥ 80%, they have failed to use a consistent, sensitive diagnostic as a comparator. We used cross-sectional samples from children and adults in Tororo, Uganda, a region with high but declining transmission due to indoor residual spraying, to characterize the sensitivity and specificity of pan-*Plasmodium* LAMP for detecting submicroscopic infections. We compared LAMP results targeting a mitochondrial DNA sequence conserved in all *Plasmodium* species, performed on DNA extracted from dried blood spots, to those of a gold standard quantitative PCR assay targeting the *var* gene acidic terminal sequence of *Plasmodium falciparum* (*var*ATS qPCR), performed on DNA extracted from 200 μL of whole blood. Using LAMP and *var*ATS qPCR increased the detection of parasitemia 2- to 5-fold, compared with microscopy. Among microscopy-negative samples, the sensitivity of LAMP was 81.5% for detecting infection ≥ 1 parasites/μL. However, low density infections were common, and LAMP failed to identify more than half of all infections diagnosed by *var*ATS qPCR, performing with an overall sensitivity of 44.7% for detecting submicroscopic infections ≥ 0.01 parasites/μL. Thus, although the LAMP assay is more sensitive than microscopy, it missed a significant portion of the submicroscopic reservoir. These findings have important implications for malaria control, particularly in settings where low-density infections predominate.

INTRODUCTION

Ambitious goals for malaria control and elimination highlight the need for a reliable, sensitive, and cost-effective method of detecting malaria infection, including the large proportion of asymptomatic and low-density infections that are not detected by microscopy. Loop-mediated isothermal amplification (LAMP) is a simple method for diagnosing submicroscopic malaria parasitemia, and it has been validated in both high- and low-transmission settings.[1–4] DNA can be extracted from dried blood spots (DBSs), reagents specific for amplification of *Plasmodium* species mitochondrial DNA are vacuum dried into premade kits, and nucleic acid amplification can be performed without a thermal cycler, making LAMP more accessible than polymerase chain reaction (PCR) in field settings.

Previous studies have reported LAMP sensitivity for the detection of *Plasmodium* species infection ranging from 76% to 98%, compared with other nucleic acid amplification-based techniques,[1–5] with a lower limit of detection of 1–5 parasites/μL.[6] Studies have generally used DNA extracted from DBS, and have included comparisons of pan-*Plasmodium* LAMP, targeting a mitochondrial DNA sequence that is conserved in all *Plasmodium* species,[7] to either nested or real-time PCR targeting the cytochrome *b* gene (Cyt b) of the four major human *Plasmodium* species, or to nested PCR targeting the *Plasmodium falciparum* 18S ribosomal RNA (rRNA) gene, with a range of sensitivities mostly > 80%.[1,2,8–10] Unfortunately, the lack of a consistent comparator limits our ability to define the operating characteristics of LAMP, and by extension, to precisely define the parasite reservoir at a particular location. This is problematic for areas where low-density infections, including infections that are missed by microscopy or immunochromatographic rapid diagnostic tests, make significant contributions to transmission.[11,12] Nested PCR is quickly being replaced as the standard diagnostic for identifying submicroscopic infections, and more sensitive DNA-based methodologies using larger sample volumes and improved targets have uncovered a pool of ultralow-density infections, with parasite densities < 1 parasite/μL.[13] The availability of a quantitative PCR method targeting the *var* gene acidic terminal sequence of *P. falciparum* (*var*ATS qPCR) offers the opportunity for a "gold standard" comparator for LAMP. The reported lower limit of detection of the *var*ATS qPCR assay is 0.03–0.15 parasites/μL blood, 10-fold more sensitive than standard 18S rRNA qPCR.[14] In this study, we used samples from repeated cross-sectional surveys in an Ugandan cohort to compare a field-applicable LAMP method, pan-*Plasmodium* LAMP, performed using DNA extracted from DBSs, to a highly sensitive *var*ATS qPCR assay, performed on 200 μL of whole blood. LAMP is currently a widely used diagnostic for identifying submicroscopic parasitemia. We were interested in the performance of LAMP, as used in real-world field settings, for diagnosing malaria infection in patients who were microscopy negative.

MATERIALS AND METHODS

The study took place in Nagongera subcounty, Tororo District, Uganda, near the Kenyan border. In this area, malaria transmission has been high and perennial,[15] with annual entomological inoculation rates of > 300 person/year in 2011–2012,[16] but transmission intensity declined dramatically

* Address correspondence to Shereen Katrak, Department of Medicine, University of California San Francisco, 513 Parnassus Avenue, Room S380, Box 0654, San Francisco, CA 94143. E-mail: shereen.katrak@ucsf.edu

after initiation of indoor residual spraying (IRS) of insecticides in December 2014.[17] Samples for this study were obtained from repeated cross-sectional surveys in a cohort of children (age 0.5–10 years) and adults (age ≥ 18) between May and December 2015, coinciding with a second and third round of IRS that took place in the district June to July 2015 and November to December 2015. Cohort enrollment, cross-sectional surveys, and clinical care have been described previously.[16,18] Briefly, participants presented to the clinic for routine visits every 90 days, meaning most participants had two routine visits in the 8-month study period. At each routine visit, a blood smear, DBS, and 200 μL aliquot of blood were collected. *Var*ATS qPCR from whole blood was performed on all samples. LAMP from DBS was performed on samples from patients who were microscopy negative. Participants were invited to visit the clinic any time they were ill, and microscopy was performed when there was a reported or documented fever. Patients with temperature greater than 38°C and positive microscopy were diagnosed with clinical malaria, and treated with standard dosing of artemether-lumefantrine. Participants with asymptomatic parasitemia were not provided antimalarial therapy, in accordance with local standards.

Thick smears were prepared with 2% Giemsa and microscopy was performed by experienced laboratory technicians. Slides were double read by two microscopists; a third microscopist resolved discrepancies. Microscopy was not used for *Plasmodium* species determination. DBS specimens were prepared by spotting approximately 30 μL of blood onto filter paper, drying completely, and storing at room temperature. DNA was extracted for LAMP using Chelex, as previously described,[19] yielding 200 μL of DNA extraction product. LAMP was performed using Eiken Loopamp™ Malaria Pan Detection Kit (Eiken Chemical, Tokyo, Japan) reaction tubes and 15 μL of extracted DNA, per manufacturer's guidelines. The LAMP primer set targets a mitochondrial DNA sequence that is conserved in all four major human malaria species.[7] Although we were primarily interested in *P. falciparum* infections, which make up the majority of infections in our setting, we used pan-*Plasmodium* LAMP kits (Eiken Chemical) because they are readily available for purchase and widely used worldwide. LAMP reactions were assessed based on visual detection of fluorescence under an ultraviolet lamp. Each batch of 48 LAMP reactions included three controls with known *P. falciparum* densities (10, 1, and 0 parasite/μL), as well as one positive and one negative control from the Eiken kit.

For our gold standard comparator, DNA was extracted from 200 μL of whole blood samples and controls using QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany), yielding 200 μL of DNA extraction product. Molecular detection and quantification of *P. falciparum* was performed with quantitative PCR targeting the *var*ATS region on 5 μL of extraction product, using previously published methods.[14] Parasite density was quantified by comparison to a standard curve of cultured parasites. Cultured W2 parasites were synchronized to ring stage, with initial density determined by flow cytometry, then normalized to a maximum concentration of 10^4 parasites/μL, and serially diluted in whole blood from 10^4 to 10^{-2} parasites/μL. All samples and controls were quantified in duplicate and averaged, with samples having a parasite density greater than 10^{-1} parasites/μL and a C_t coefficient of variation > 0.05 repeated. For all samples that were LAMP positive and *var*ATS qPCR negative, nested PCR amplifying the Cyt b, using 5 μL template DNA was performed using previously published methods.[20] Species determination was based on AluI restriction digestion of the amplified Cyt b, as previously described.[21] All qPCR was done on an Applied Biosystems (Foster City, CA) StepOnePlus Real-Time PCR System.

Data were analyzed using STATA (version 13; STATA Corp., College Station, TX). Malaria incidence was defined as the number of episodes of clinical malaria (temperature > 38°C and patent parasitemia, as previously defined in this cohort)[16] detected by passive surveillance per person-year of observation. Parasite prevalence was calculated as follows: 1) microscopic parasitemia, defined as the proportion of routine visits with a positive microscopy, with or without fever, 2) microscopic or LAMP parasitemia, defined as the proportion of routine visits with either positive microscopy or a positive LAMP reaction, and 3) microscopic or *var*ATS parasitemia, defined as the proportion of routine visits with either positive microscopy or positive *var*ATS qPCR. Distribution of parasite densities refers to the proportion of samples with parasite density by *var*ATS qPCR in a given range, among all samples with *var*ATS qPCR results ≥ 0.01 parasites/μL. Mean parasite densities for different risk categories were estimated by calculating the geometric mean of all samples in a given category with a *var*ATS qPCR result ≥ 0.01 parasites/μL. The sensitivity and specificity of LAMP for detecting submicroscopic infection was determined based on results of 554 samples that were microscopy negative, in which both LAMP and *var*ATS qPCR testing was performed.

RESULTS

Study participants, parasite prevalence, and parasite densities. Characteristics of study participants are presented in Table 1. During the 8 months of observation, 232 child and 78 adult participants made 675 routine (every 90 days) visits to the study clinic. As expected, the majority of episodes of malaria diagnosed during the study period, either at or between routine visits, occurred in children, with an incidence rate of 0.24 episodes/person-year in children and 0.02 episodes/person-year in adults. Using microscopy, the

TABLE 1
Study characteristics

	Children	Adult
Number of participants	232	78
Mean age, years (SD)	6.5 (2.6)	42.0 (12.7)
Number female (%)	106 (46)	71 (91)
Number of routine visits	492	183
Incidence of malaria during study period (episodes per person year)	0.24	0.02
Malaria parasite prevalence		
By microscopy	21.7%	7.7%
By microscopy or LAMP	40.7%	26.8%
By microscopy or *var*ATS qPCR	51.2%	42.1%
Distribution of parasite densities by *var*ATS qPCR*		
Parasite density ≥ 0.01 to < 0.1	25.5%	31.2%
Parasite density ≥ 0.1 to < 1	12.8%	18.1%
Parasite density ≥ 1	61.7%	50.7%

LAMP = loop-mediated isothermal amplification; qPCR = quantitative polymerase chain reaction; SD = standard deviation; *var*ATS = *var* gene acidic terminal sequence of *Plasmodium falciparum*.
* Parasite density in parasites/μL.

prevalence of parasitemia at routine visits was 21.7% in children and 7.7% in adults. When microscopy was used in conjunction with either LAMP or *var*ATS qPCR, parasite prevalence was 2- to 5-fold higher than with microscopy alone, with a prevalence of 40.7% or 51.2% in children, and 26.8% or 42.1% in adults, respectively ($P < 0.01$ for all pairwise comparisons within age groups). When prevalence based on LAMP or *var*ATS qPCR was directly compared, differences were more pronounced among adults than children, with the use of *var*ATS qPCR increasing prevalence by > 50% in adults, compared with 26% in children. The distribution of parasite densities by *var*ATS qPCR differed between adults and children. Among patients who were *var*ATS qPCR positive, adults were more likely to have a parasite density < 1 parasite/μL, compared with children ($P = 0.04$). However, roughly 40% of children with detectable parasitemia had a parasite density < 1 parasite/μL, indicating that in this setting of declining transmission, low parasite density infections were common in both adults and children.

Parasite prevalence based on varATS qPCR, stratified by LAMP and microscopy results. Results from all 675 routine visits were stratified by LAMP and microscopy results, allowing for determination of parasite prevalence by the *var*ATS qPCR assay among different risk groups (Table 2). Parasite prevalence was 27.0% among LAMP-negative individuals, with a geometric mean parasite density in these individuals of 0.11 parasites/μL. Among individuals that were LAMP positive but microscopy negative, parasite prevalence by *var*ATS qPCR was 72.7%, with a mean parasite density of 5.70 parasites/μL. Thirty-five samples, from 30 children and 5 adults, were LAMP positive but *var*ATS qPCR negative. Because *var*ATS qPCR is specific for *P. falciparum* infection, these samples were tested for the presence of another *Plasmodium* species using Cyt b nested PCR. Of these 35 samples, 15 (43%) were positive for *P. ovale*, three (9%) were positive for *P. falciparum*, three (9%) had mixed infections with *P. falciparum* plus either *P. vivax* or *P. malariae*, one (2%) was positive by Cyt b but failed speciation due to insufficient DNA, and 13 (37%) were Cyt b negative. Among samples that were microscopy positive, parasite prevalence was > 90% by *var*ATS qPCR. Cyt b PCR was also performed on three samples with microscopic parasite densities above 1,000 parasite/μL but negative *var*ATS qPCR, and yielded 1/3 samples positive for *P. falciparum*, 1/3 with mixed *P. falciparum* and *P. vivax* infection, and 1/3 Cyt b negative.

Sensitivity of LAMP assay. Of 675 visits at which *var*ATS qPCR was performed, 554 had negative microscopy, and thus had associated LAMP tests allowing for direct comparison of LAMP and *var*ATS qPCR (Table 3). The 15 infections previously determined to be *P. ovale* were excluded from this comparison. Among all microscopy-negative patients with parasitemia detectable by *var*ATS qPCR (limit of detection ≥ 0.01 parasites/μL), LAMP had a sensitivity of only 44.7%, with a specificity of 94.0%. For the lowest parasite densities detected by *var*ATS qPCR (0.01–0.1 parasites/μL), LAMP detected approximately one in 10 infections. Compared with *var*ATS qPCR reactions with parasite density ≥ 1 parasites/μL among microscopy-negative patients, LAMP performed with a sensitivity of 81.5%. Sensitivity was not affected by recent treatment of malaria, and was less than 50% overall in both children and adults.

DISCUSSION

We compared a field-applicable method for diagnosing submicroscopic parasitemia to a more sensitive gold standard, using repeated cross-sectional surveys from a cohort in Uganda. Consistent with our prior findings,[18] LAMP increased the detection of parasitemia, compared with microscopy, by more than 2-fold in both children and adults. However, our results indicate that LAMP may miss a substantial portion of the parasite reservoir, particularly when considering asymptomatic, ultralow-density infections. In our study, low-density infections were common in both children and adults, and LAMP detected less than half of all submicroscopic infections identified by *var*ATS qPCR.

Similar to results from previous reports,[1–3,9] in our study LAMP performed with ≥ 80% sensitivity and specificity, compared with qPCR, when using a parasite density of ≥ 1 parasite/μL as the diagnostic threshold. However, when considering the lower limit of detection of the *var*ATS qPCR assay, and thus including ultralow-density infections, LAMP had only 45% sensitivity. This finding may be particularly relevant in low-burden areas, where submicroscopic infections make up a majority of the parasite reservoir.[22] Notably in our study, which took place in an area of high but declining

TABLE 2

Prevalence of parasitemia and mean parasite density, stratified by LAMP or microscopy result

	Prevalence by *var*ATS qPCR	Geometric mean parasite density* (parasites/μL) (95% CI)
LAMP and microscopy result		
LAMP (–) Microscopy (–)	115/426 (27.0%)	0.11 (0.07–0.17)
LAMP (+) Microscopy (–)	93/128 (72.7%)	5.70 (3.00–10.8)
Microscopy (+) Density by LM < 1,000	70/76 (92.1%)	181.7 (95.8–344.7)
Microscopy (+) Density by LM ≥ 1,000	42/45 (93.3%)	3884.0 (2,562.4–5,887.2)

CI = confidence interval; LAMP = loop-mediated isothermal amplification; qPCR = qunantitative polymerase chain reaction; *var*ATS = *var* gene acidic terminal sequence of *Plasmodium falciparum*.

* Densities of < 0.01 parasites/μL were considered zero and excluded from estimation of geometric mean.

TABLE 3

Operating characteristics of LAMP, compared with *var*ATS qPCR, in microscopy-negative patients*

	Sensitivity (%)	Specificity (%)
Stratified by parasite density (parasites/μL)†		
Parasite density, all samples ≥ 0.01	93/208 (44.7)	311/331 (94.0)
Parasite density ≥ 0.01 to < 0.1	9/83 (10.8)	–
Parasite density ≥ 0.1 to < 1	18/44 (40.9)	–
Parasite density ≥ 1	66/81 (81.5)	–
Stratified by recent malaria		
No recent malaria episode	80/181 (44.2)	302/322 (94.0)
Malaria episode in past 30 days	13/27 (48.2)	9/9 (100)
Stratified by age		
Child	63/145 (43.5)	210/225 (93.3)
Adult	30/63 (47.6)	101/106 (95.3)

* Sensitivity and specificity calculated in microscopy-negative patients, excluding those with non-*falciparum* infections ($N = 539$).

† Parasite density in parasites/μL.

transmission, more than a third of children and nearly half of adults with detectable infection had a parasite density < 1 parasite/μL.

When *var*ATS qPCR results were stratified based on LAMP, more than a quarter of LAMP-negative samples had detectable parasitemia by *var*ATS qPCR, and these infections had a mean parasite density of < 1 parasite/μL. The use of DNA extracted from a relatively large volume of whole blood, coupled with an extremely sensitive qPCR assay, allowed us to detect infections that would otherwise have been missed. Although the majority of infections missed by LAMP in this study were ultralow density, these findings may have important implications for malaria transmission. At present, there is debate as to what degree submicroscopic infections contribute to malaria transmission; this contribution is probably mitigated whether the setting is high or low burden, and by whether the infection is predominately asexual or sexual parasitemia. Submicroscopic gametocyte carriage is clearly relevant to transmission, with evidence from mosquito-feeding studies suggesting that individuals can infect mosquitos in the absence of microscopically detectable gametocytes[23,24]; however, individuals with asexual parasitemia and no gametocytes detectable by nucleic acid sequence-based amplification are also capable of infecting mosquitoes.[12] In a low-endemicity setting, submicroscopic gametocyte infections were recently shown to be relatively unimportant to malaria transmission[25]; however, in higher endemicity settings, submicroscopic infection may account for a large proportion of the transmission cycle.[11,12] Thus, identifying carriers of low-density infection, which can persist for months,[26] may be important when programmatic goals shift from reducing morbidity and mortality to controlling malaria transmission and moving toward malaria elimination.

We identified 35 samples that were positive by LAMP, but negative by *var*ATS qPCR. When nested Cyt b PCR was performed, the majority of these samples were positive for *P. ovale* or mixed infection with *P. falciparum* plus either *P. vivax* or *P. malariae*, adding to existing evidence that non-*falciparum* species play a role in the epidemiology of this region.[27–30] However, more than a third of these samples were Cyt b negative. Previous studies have also reported LAMP-positive samples with infections that were not identified by PCR,[2,3] which may be due to the large number of primers per target in LAMP and increased likelihood of primer–primer interactions, leading to false-positive results.

It is notable that the cross-sectional surveys described here took place in the context of a major IRS campaign, which led to a marked reduction in transmission intensity.[17] Although spraying with the carbamate bendiocarb took place in the district 6 months before sample collection, and again during the course of these cross-sectional surveys, with malaria incidence dropping dramatically, we describe a significant submicroscopic reservoir during and immediately after IRS. A high proportion of infections were of low density, with one in 10 participants having extremely low-density infections of 0.01–0.1 parasite/μL. Although further studies are needed to better characterize this reservoir, our results suggest that in addition to impacts on malaria morbidity and slide positivity in Uganda,[31,32] IRS may shift the distribution of parasite densities to lower density infections. We hypothesize that in a high endemicity setting experiencing a rapid decline in transmission, a greater proportion of infections detected may be low density, due to controlled infections in a population that retains natural immunity.

A better understanding of the epidemiology of malaria parasitemia is critical to best allocating resources and assessing the impact of malaria control efforts. LAMP offers markedly improved diagnostic sensitivity compared with microscopy and available rapid diagnostic tests, and it is usable in field settings, but it may miss a significant portion of the submicroscopic reservoir. We found that ultralow-density infections were common in both children and adults in a high-transmission setting that recently underwent an IRS campaign, and that LAMP missed approximately half of detectable submicroscopic infections. As malaria control improves and we move toward elimination in traditionally high-burden areas, it is important to remember that all infections, including those missed by current diagnostics, are potential drivers of transmission. Our findings highlight the need to consider the limitations of our diagnostic tests in the setting of declining transmission.

Received March 20, 2017. Accepted for publication May 31, 2017.

Published online October 9, 2017.

Note: Supplemental figure appears at www.ajtmh.org.

Authors' addresses: Shereen Katrak, Maxwell Murphy, Grant Dorsey, Philip Rosenthal, and Bryan Greenhouse, Department of Medicine, University of California, San Francisco, CA, E-mails: shereen.katrak@ucsf.edu, maxwell.murphy@ucsf.edu, grant.dorsey@ucsf.edu, philip.rosenthal@ucsf.edu, and bryan.greenhouse@ucsf.edu. Patience Nayebare, John Rek, and Emmanuel Arinaitwe, Infectious Diseases Research Collaboration, Kampala, Uganda, E-mails: pnayebare@ymail.com, jrek@idrc-uganda.org, and earinaitwe@idrc-uganda.org. Mary Smith, Rosalind Franklin University of Medicine and Science, Chicago Medical School, North Chicago, IL, E-mail: marydsmith37@yahoo.com. Joaniter I. Nankabirwa and Moses Kamya, Makerere University College of Health Sciences, Kampala, Uganda, and Infectious Disease Research Collaboration, Kampala, Uganda, E-mails: jnankabirwa@yahoo.co.uk and mkamya@idrc-uganda.org.

REFERENCES

1. Aydin-Schmidt B, Xu W, González IJ, Polley SD, Bell D, Shakely D, Msellem MI, Björkman A, Mårtensson A, 2014. Loop mediated isothermal amplification (LAMP) accurately detects malaria DNA from filter paper blood samples of low density parasitaemias. *PLoS One 9:* e103905–e103909.
2. Cook J et al., 2015. Loop-mediated isothermal amplification (LAMP) for point-of-care detection of asymptomatic low-density malaria parasite carriers in Zanzibar. *Malar J 14:* 43.
3. Hopkins H et al., 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.
4. Vallejo AF, Martínez NL, González IJ, Arévalo-Herrera M, Herrera S, 2015. Evaluation of the loop mediated isothermal DNA amplification (LAMP) kit for malaria diagnosis in *P. vivax* endemic settings of Colombia. *PLoS Negl Trop Dis 9:* e3453.
5. Paris DH, Imwong M, Faiz AM, Hasan M, Yunus EB, Silamut K, Lee SJ, Day NPJ, Dondorp AM, 2007. Loop-mediated isothermal PCR (LAMP) for the diagnosis of falciparum malaria. *Am J Trop Med Hyg 77:* 972–976.
6. Oriero EC, Jacobs J, Van geertruyden J-P, Nwakanma D, D'Alessandro U, 2015. Molecular-based isothermal tests for field diagnosis of malaria and their potential contribution to malaria elimination. *J Antimicrob Chemother 70:* 2–13.
7. 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.
8. Han E-T, 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.
9. Polley SD et al., 2013. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. *J Infect Dis 208:* 637–644.
10. Pöschl B, Waneesorn J, Thekisoe O, Chutipongvivate S, Panagiotis K, 2010. Comparative diagnosis of malaria infections by microscopy, nested PCR, and LAMP in northern Thailand. *Am J Trop Med Hyg 83:* 56–60.
11. Gaye A, Bousema T, Libasse G, Ndiath MO, Konaté L, Jawara M, Faye O, Sokhna C, 2015. Infectiousness of the human population to Anopheles arabiensis by direct skin feeding in an area hypoendemic for malaria in Senegal. *Am J Trop Med Hyg 92:* 648–652.
12. Ouédraogo AL et al., 2015. Dynamics of the human infectious reservoir for malaria determined by mosquito feeding assays and ultrasensitive malaria diagnosis in Burkina Faso. *J Infect Dis 213:* jiv370–jiv399.
13. Imwong M et al., 2015. The epidemiology of subclinical malaria infections in South-East Asia: findings from cross-sectional surveys in Thailand–Myanmar border areas, Cambodia, and Vietnam. *Malar J 14:* 381.
14. Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I, 2015. Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. *PLoS Med 12:* e1001788.
15. Okello PE, Van Bortel W, Byaruhanga AM, Correwyn A, Roelants P, Talisuna A, D'Alessandro U, Coosemans M, 2006. Variation in malaria transmission intensity in seven sites throughout Uganda. *Am J Trop Med Hyg 75:* 219–225.
16. Kamya MR et al., 2015. Malaria transmission, infection, and disease at three sites with varied transmission intensity in Uganda: implications for malaria control. *Am J Trop Med Hyg 92:* 903–912.
17. Katureebe A et al., 2016. Measures of malaria burden after long-lasting insecticidal net distribution and indoor residual spraying at three sites in Uganda: a prospective observational study. *PLoS Med 13:* e1002167.
18. Rek J et al., 2016. Characterizing microscopic and submicroscopic malaria parasitaemia at three sites with varied transmission intensity in Uganda. *Malar J 15:* 470.
19. 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.
20. Schwartz A, Baidjoe A, Rosenthal PJ, Dorsey G, Bousema T, Greenhouse B, 2015. The effect of storage and extraction methods on amplification of *Plasmodium falciparum* DNA from dried blood spots. *Am J Trop Med Hyg 92:* 922–925.
21. Hsiang MS, Lin M, Dokomajilar C, Kemere J, Pilcher CD, Dorsey G, Greenhouse B, 2010. PCR-based pooling of dried blood spots for detection of malaria parasites: optimization and application to a cohort of Ugandan children. *J Clin Microbiol 48:* 3539–3543.
22. Wu L, van den Hoogen LL, Slater H, Walker PGT, Ghani AC, Drakeley CJ, Okell LC, 2015. Comparison of diagnostics for the detection of asymptomatic *Plasmodium falciparum* infections to inform control and elimination strategies. *Nature 528:* S86–S93.
23. Bonnet S, Gouagna LC, Paul RE, Safeukui I, Meunier JY, Boudin C, 2003. Estimation of malaria transmission from humans to mosquitoes in two neighbouring villages in south Cameroon: evaluation and comparison of several indices. *Trans R Soc Trop Med Hyg 97:* 53–59.
24. Bousema T et al., 2012. Mosquito feeding assays to determine the infectiousness of naturally infected *Plasmodium falciparum* gametocyte carriers. *PLoS One 7:* e42821.
25. Lin JT et al., 2016. Microscopic *Plasmodium falciparum* gametocytemia and infectivity to mosquitoes in Cambodia. *J Infect Dis 213:* 1491–1494.
26. Felger I, Maire M, Bretscher MT, Falk N, Tiaden A, Sama W, Beck H-P, Owusu-Agyei S, Smith TA, 2012. The dynamics of natural *Plasmodium falciparum* infections. *PLoS One 7:* e45542.
27. Roh ME, Oyet C, Orikiriza P, Wade M, Kiwanuka GN, Mwanga-Amumpaire J, Parikh S, Boum Y II, 2016. Asymptomatic *Plasmodium* infections in children in low malaria transmission setting, southwestern Uganda. *Emerg Infect Dis 22:* 1494–1498.
28. Daniels RF et al., 2017. Evidence of non-*Plasmodium falciparum* malaria infection in Kédougou, Sénégal. *Malar J 16:* 9.
29. Miller RH, Obuya CO, Wanja EW, Ogutu B, Waitumbi J, Luckhart S, Stewart VA, 2015. Characterization of *Plasmodium ovale* curtisi and *P. ovale* wallikeri in western Kenya utilizing a novel species-specific real-time PCR assay. *PLoS Negl Trop Dis 9:* e0003469.
30. Williams J et al., 2016. Non-falciparum malaria infections in pregnant women in West Africa. *Malar J 15:* 53.
31. Steinhardt LC et al., 2013. The effect of indoor residual spraying on malaria and anemia in a high-transmission area of northern Uganda. *Am J Trop Med Hyg 88:* 855–861.
32. Tukei BB, Beke A, Lamadrid-Figueroa H, 2017. Assessing the effect of indoor residual spraying (IRS) on malaria morbidity in northern Uganda: a before and after study. *Malar J 16:* 4.