Field Evaluation of a Real-Time Fluorescence Loop-Mediated Isothermal Amplification Assay, RealAmp, for the Diagnosis of Malaria in Thailand and India

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(See the editorial commentary by Hsiang, Greenhouse, and Rosenthal on pages 1167–9.)

Background. To eliminate malaria, surveillance for submicroscopic infections is needed. Molecular methods can detect submicroscopic infections but have not hitherto been amenable to implementation in surveillance programs. A portable loop-mediated isothermal amplification assay called RealAmp was assessed in 2 areas of low malaria transmission.

Methods. RealAmp was evaluated in 141 patients from health clinics in India (passive surveillance) and in 127 asymptomatic persons in Thailand (active surveillance). The diagnostic validity, precision, and predictive value of RealAmp were determined using polymerase chain reaction (PCR) as the reference method. A pilot study of RealAmp was performed on samples from patients presenting at a Thai health center.

Results. A total of 96 and 7 positive cases were detected in India and Thailand, respectively, via PCR. In comparison with nested PCR, the sensitivity and specificity of RealAmp in India were 94.8% (95% confidence interval [CI], 88.3%–98.3%) and 100% (95% CI, 92.1%–100%), respectively, with correct identification of all 5 Plasmodium vivax cases. In Thailand, compared with pooled real-time PCR, RealAmp demonstrated 100% sensitivity (95% CI, 59.0%–100%) and 96.7% specificity (95% CI, 91.7%–99.1%). Testing at the health center demonstrated RealAmp’s potential to serve as a point-of-care test with results available in 30–75 minutes.

Conclusion. RealAmp was comparable to PCR in detecting malaria parasites and shows promise as a tool to detect submicroscopic infections in malaria control and elimination programs worldwide.

Keywords. malaria; Plasmodium falciparum; Plasmodium vivax; diagnosis; loop-mediated isothermal amplification; LAMP; RealAmp; sensitivity; specificity; positive predictive value; low-transmission; surveillance; elimination.

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Since 2000, significant progress has been made in the global control of malaria. As a result, 39 countries are currently working towards malaria elimination [1–4]. Strategies to interrupt malaria transmission include prompt identification and treatment of asymptomatic infections, the majority of which contain low level densities of Plasmodium parasites and are undetectable by microscopy or rapid diagnostic tests (RDTs) [5–9]. These submicroscopic infections represent a reservoir of infection...
that can currently only be identified reliably by polymerase chain reaction (PCR) [7, 10, 11]. However, PCR is time and resource intensive, and it is not easily transferable to field operations [9, 12]. Hence, there is a clear need for field-amenable diagnostic tools that can rapidly and accurately detect submicroscopic infections [2, 9, 13].

The loop-mediated isothermal amplification (LAMP) technique is a relatively new method for the rapid amplification and detection of DNA [14, 15]. It has been used successfully for detection of malaria parasites and other infectious pathogens [16, 17] and for diagnosis of malaria in returning travelers [18]. Recently, a commercial LAMP assay was shown to have high sensitivity (93.3%) in detecting falciparum malaria among febrile patients in Uganda [19]. However, it achieved only 85% specificity. In areas with low malaria prevalence (where elimination is being considered), such a test would yield more false-positive cases than true-positive cases. Furthermore, for LAMP-based assays to be considered for elimination programs, they need to be field-tested in non-African settings, where Plasmodium vivax infections often predominate [11, 20].

RealAmp is a version of the LAMP technique that is housed in a portable instrument in which the generation of fluorophores can be followed in real time to monitor the amplification of DNA, similar to real-time PCR. This leads to the advantage of an objective determination of fluorescence above a defined threshold, as opposed to reliance on a visual assessment of turbidity or fluorescence, commonly used for other LAMP-based assays. In a study involving laboratory-cultured parasites, a RealAmp method for Plasmodium spp. amplification of 18S ribosomal RNA (rRNA) gene target has been shown to detect as few as 1–10 parasites/µL [21]. A P. vivax RealAmp assay has recently been validated, as well [22]. However, there have been no field studies evaluating RealAmp as a diagnostic tool for malaria. Here, we present the results of a study demonstrating sensitive and specific detection of malaria parasites by RealAmp in 2 areas of low malaria transmission in India and Thailand.

**METHODS**

**Ethics Statement**

Informed consent was obtained from all study participants or from their parents or guardians. All personal identifiers were removed from the study database, and only the clinic staff at the study sites was able to link test results to patient records. Any patients testing positive through standard-of-care diagnostic methods were treated appropriately according to national guidelines. The study was approved by the ethical review boards of University of North Carolina and the Indian Council of Medical Research in India. Active surveillance is a routine component of the national malaria control program in Thailand and was not subject to additional approval as human subjects research.

**Study Design and Population**

The study was conducted in India and Thailand. India experiences the highest burden of malaria outside of Africa [4]. Thailand and its neighboring countries in the Greater Mekong Subregion experience low malaria transmission but have been known as the epicenter for emergence of malaria drug resistance [20, 23]. Both Plasmodium falciparum and P. vivax contribute to the malaria burden in these countries [4]. Study sites within countries were representative of the different surveillance systems in place as part of each country’s national malaria control program. Passive surveillance to detect clinical malaria cases in local health clinics is part of routine efforts by India’s malaria control program to reduce malaria-related morbidity and mortality. Active surveillance (ie, active and proactive case detection) is a key component of Thailand’s malaria control program, especially in areas that experience high rates of imported malaria due to human migration from the neighboring countries of Myanmar and Cambodia [24]. The study was conducted in agreement with the criteria laid out by the standard for the reporting of diagnostic accuracy studies (Figure 1A and 1B) [25].

In India, between January 2010 and February 2012, febrile patients were screened for Plasmodium parasites through microscopy at local health clinics in Balaghat and Jabalpur districts, Madhya Pradesh, and selected through simple random sampling to be included in the study. Study selection criteria included presence of fever and current residence in the study area. Dried spots of blood obtained by finger prick were collected from microscopy-positive and microscopy-negative participants for nested PCR and RealAmp testing. At the time of enrollment, each patient’s age and sex were recorded.

In June 2012, 3 small agricultural villages in Sai Yok district, Kanchanaburi Province, located in western Thailand near the Thai-Myanmar border, were selected for a proactive case detection survey. Study selection criteria included no signs or clinical symptoms of malaria and current residence in the study area. Healthy individuals who met these criteria were randomly selected to be tested for Plasmodium parasites. A set of 2 thick and thin microscopy slides were prepared using blood specimens obtained by finger prick from each participant. Approximately 30 µL of blood was also collected for dried blood spot testing by pooled real-time PCR and RealAmp analyses. Each participant’s age, sex, occupation, and migrant status were recorded, along with their location at the time of enrollment.

In addition to proactive case detection in the villages, pilot testing was done to determine the feasibility of RealAmp as a point-of-care diagnostic test. Blood specimens from 12 febrile patients presenting on the day of the survey to the local malaria clinic were tested for Plasmodium parasites by local microscopy and RealAmp on site and later by pooled real-time PCR and RealAmp at the reference laboratory, located in Bangkok.
DNA Extraction

In India, genomic DNA (gDNA) was extracted at the reference laboratory in Jabalpur by soaking the dried blood spots in 200 µL of TE buffer (10 mM Tris and 0.1 M ethylenediaminetetraacetic acid) for 2–3 hours, as previously described. The blood spots were then incubated at 50°C for 15 minutes and crushed with pipette tips. They were incubated for a second time at 97°C for 15 minutes and then centrifuged at 8944 g for 2 minutes. The supernatant was aspirated and used as template DNA [26]. In Thailand, gDNA was extracted from dried blood spots at the Bureau of Vector Borne Disease reference laboratory in Bangkok as previously described [27], using 20% Chelex-100
(Bio-Rad, Richmond, CA). All samples extracted from dried blood spots at both sites were sealed securely and stored at −20°C. For on-site RealAmp testing, gDNA was extracted from whole blood, using the boil and spin method, as described previously [21].

**RealAmp Assay**

All samples were tested for *Plasmodium* species by means of the RealAmp assay, using 18S ribosomal RNA (rRNA)–specific primers, as described in detail previously [21]. Briefly, 12.5-µL reactions containing 2X in-house buffer (40 mM Tris-HCl [pH 8.8], 20 mM KCl, 16 mM MgSO4, 20 mM (NH4)SO4, 0.2% Tween-20, 0.8 M betaine, and 2.8 mM of dNTPs each), 0.25 µL of a 1:100 dilution of SYBR green, 8 units of Bst DNA polymerase (New England Biolabs, Ipswich, MA), genus-specific *Plasmodium* primers, and 2 µL of template gDNA were prepared. Amplifications were performed at 64°C for 90 minutes, with the ESE-Quant Tube scanner (Qiagen, Venlo, Netherlands) collecting fluorescence signals at 1-minute intervals. Positivity was determined by an amplification plot 3 standard deviations (SDs) from the baseline fluorescence, with a slope >30 mV/minute. A predetermined cutoff of 60 minutes was used; specimens with amplification emerging after the cutoff were considered negative for *Plasmodium* species. All samples were run twice, and if 2 runs were discordant, the sample was run a third time, and a criterion of 2 concordant results was used to determine the RealAmp result. In India, in addition to the genus-specific RealAmp assay, the recently developed *P. vivax* RealAmp assay was field-tested, as well [22]. All samples that tested positive by the RealAmp assay for *Plasmodium* species were then tested for *P. vivax* by the RealAmp assay for *P. vivax*. In Thailand, a RealAmp kit, which combined all reagents for the assay except for the Bst polymerase, was used for the first time. The kit was prepared at the Centers for Disease Control and Prevention (CDC) laboratory by premixing genus-specific primers, fluorescent dye, and buffer components for 300 reactions, and aliquots for 100 reactions were prepared and stored at 4°C. The goal of the kit was to reduce the number of steps and preparation time for healthcare personnel and limit the opportunities for contamination.

**Microscopy and PCR**

Thick and thin smears were examined by local microscopists at both field sites. Smears were stained for detection of malaria parasites by using Jaswant Singh Bhattacharji stain in India [28] and Giemsa stain in Thailand. In Thailand, all slides were also reviewed by an expert microscopist at the Bureau of Vector Borne Disease laboratory in Bangkok. All slides at both field sites were examined according to the current World Health Organization guidelines [29].

Nested PCR and pooled real-time PCR targeting the 18S *Plasmodium* rRNA gene were used as reference standards in India and Thailand, respectively. These PCR methods were the established reference diagnostic methods used by the respective national or state malaria control laboratories. In India, nested PCR was performed as previously described [30]. Briefly, the first round of PCR used genus-specific primers and 5 µL of gDNA to amplify the parasite DNA in positive samples. PCR products from the primary reaction were then diluted 1:10, and a 4–6-µL aliquot was used as a template DNA for the second-round PCR, in which species-specific primers for *P. falciparum* and *P. vivax* were used to identify the infecting *Plasmodium* species. All secondary PCR products were visualized on a 1% agarose gel. In Thailand, pooled real-time PCR was performed as described previously [31]. Briefly, pools of 4 samples were prepared by mixing 2 µL of gDNA per sample and were tested by a genus-specific real-time PCR assay, using 5 µL of pooled gDNA. Then, individual samples of positive pools were amplified using a second-round real-time PCR to identify infecting species, using probes specific to detect *P. falciparum, P. vivax, Plasmodium malariae,* and *Plasmodium ovale,* using 5 µL of gDNA. All reactions were performed in duplicate on the Bio-Rad CFX96 Real-Time PCR system.

**Data Analysis**

The sensitivity and specificity of RealAmp and of local and/or expert microscopy were calculated using nested PCR as the reference standard in India and pooled real-time PCR as the reference standard in Thailand [32]. To assess the degree of agreement between 2 diagnostic tests, κ coefficients were calculated between the reference and index test [33]. The precision of the estimates was determined by calculating exact 95% confidence intervals (CIs) for each test statistic.

Since the positive predictive values (PPVs) and negative predictive values (NPVs) of a diagnostic tool are population specific and influenced by prevalence, separate PPV and NPV estimates were calculated for the study population in Thailand only. Additionally, to examine the usefulness of RealAmp outside the study population, PPV and NPV were calculated for a wide range of malaria prevalences at different specificities [32]. Data were entered into Microsoft Excel 2010 (Microsoft, Redmond, WA) and analyzed in SAS, version 9.2.2 (SAS, Cary, NC).

**RESULTS**

**Study Populations**

Specimens from 268 persons in the 2 countries were tested by microscopy, reference molecular tests (nested or pooled real-time PCR), and RealAmp. In India, between 2010 and 2012, the slide-positivity rate (SPR) among symptomatic patients in the study area varied from 10.9% [34] to 65.7% [35]. 141 patients (96 microscopy positive and 45 microscopy negative) were randomly selected to be included in the analysis (Table 1 and Figure 1). The median age of study participants was 8 years (range, 0.5–60 years), and 38% (54) were female. The median
parasite density of microscopy-positive persons was 1560 parasites/µL (range, 80–2040 parasites/µL; Table 1). In Thailand, 127 healthy individuals who participated in a proactive case detection survey in June 2012 near the Thai-Myanmar border were included in the analysis. The malaria prevalence in this region is estimated to be 1.7%–6.6% [36, 37]. The median age of survey participants was 24 years (range, 1–75 years) and 48% (61) were female. The majority of participants (78.7% [100]) were migrants from Myanmar, and 63.8% (81) were agricultural workers (Table 1).

**PCR Results**

In India, among the 141 febrile patients, nested PCR identified 96 cases of infection: 91 involved *P. falciparum*, and 5 involved *P. vivax*, with no mixed infections (Table 2). These findings were 100% concordant with microscopy results. In Thailand, among the 127 healthy individuals, pooled real-time PCR identified infections in 7 (5.5%): 5 involved *P. vivax*, 1 involved *P. malariae*, and 1 was mixed (i.e., it involved *P. vivax* and *P. falciparum*; Table 2). Of these 7 PCR-positive cases, local and expert microscopists were able to identify 3 and 4 infected cases, respectively (Table 2). The median parasite density among the 4 cases identified by expert microscopists was very low (4.5 parasites/µL [range, 3–7 parasites/µL]; Table 1). In both sites, there were no differences in demographic characteristics (i.e., age, sex, occupation, and migrant status) between PCR-positive and PCR-negative cases.

**RealAmp Sensitivity and Specificity**

Nested or real-time PCR results were used as the reference standards to assess the *Plasmodium* spp. RealAmp assay performance (Table 2). In India, RealAmp correctly detected 91 of 96 nested PCR–positive samples as positive and 45 of 45 nested PCR–negative samples as negative. Thus, compared with nested PCR, RealAmp had a sensitivity of 94.79% (95% CI, 88.26%–98.29%) and a specificity of 100% (95% CI, 92.13%–100%). Overall agreement between nested PCR and RealAmp was high (κ = 0.92; 95% CI, .85–.99). The median time to amplification for the 96 positive samples was 20 minutes. In contrast, amplification by nested PCR required approximately 2 hours. Further, the *P. vivax* RealAmp assay correctly identified all 5 *P. vivax* infections.

In Thailand, RealAmp correctly detected 7 of 7 real-time PCR–positive samples as positive and 116 of 120 real-time PCR–negative samples as negative. Thus, when pooled real-time PCR was used as the reference standard, RealAmp had a sensitivity of 100% (95% CI, 95.04%–100%) and a specificity of 96.7% (95% CI, 91.69%–99.08%). In comparison, local microscopy and expert microscopy were much less sensitive but slightly more specific when compared to pooled real-time PCR (Table 2).

The actual specificity of the assay in Thailand could have been higher. There were 4 samples that tested positive by RealAmp but negative by pooled real-time PCR in Thailand and were classified as false positives. After the PCR results were known, all slides from the pools of these subjects were reexamined by the expert microscopist, and the 4 samples were

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**Table 1. Characteristics of Study Subjects in India and Thailand**

<table>
<thead>
<tr>
<th>Country, Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>India (n = 141)</strong></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>8</td>
</tr>
<tr>
<td>Female sex</td>
<td>54 (38.3)</td>
</tr>
<tr>
<td>Microscopy positive</td>
<td>96 (68.1)</td>
</tr>
<tr>
<td>Parasite density, parasites/µL</td>
<td>1560</td>
</tr>
<tr>
<td><strong>Thailand (n = 127)</strong></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>24</td>
</tr>
<tr>
<td>Female sex</td>
<td>61 (48)</td>
</tr>
<tr>
<td>Migrant</td>
<td>100 (78.7)</td>
</tr>
<tr>
<td>Agricultural worker</td>
<td>81 (63.8)</td>
</tr>
<tr>
<td>Microscopy positive</td>
<td>4 (3.1)</td>
</tr>
<tr>
<td>Parasite density, parasites/µL</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Data are median value or no. (%) of patients.

**Table 2. Diagnostic Accuracy of RealAmp and Microscopy, Compared With Reference Polymerase Chain Reaction (PCR) Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive, No.</th>
<th>Negative, No.</th>
<th>Sensitivity, % (95% CIa)</th>
<th>Specificity, % (95% CIa)</th>
<th>κ (95% CIa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>India (n = 141)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nested PCR</td>
<td>96</td>
<td>45</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>RealAmpb</td>
<td>91</td>
<td>45</td>
<td>94.8 (88.3–98.3)</td>
<td>100 (92.1–100)</td>
<td>0.92 (.85–.99)</td>
</tr>
<tr>
<td><strong>Thailand (n = 127)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled real-time PCR</td>
<td>7</td>
<td>120</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Local microscopy</td>
<td>3</td>
<td>120</td>
<td>42.86 (.01–.81.6)</td>
<td>100 (96.7–100)</td>
<td>0.59 (.22–.95)</td>
</tr>
<tr>
<td>Expert microscopy</td>
<td>4</td>
<td>117</td>
<td>57.14 (18.4–90.1)</td>
<td>97.5 (92.9–99.5)</td>
<td>0.55 (.22–.87)</td>
</tr>
<tr>
<td>RealAmpc</td>
<td>7</td>
<td>116</td>
<td>100 (59.0–100)</td>
<td>96.7 (91.7–99.1)</td>
<td>0.76 (.54–.99)</td>
</tr>
</tbody>
</table>

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a Exact 95% confidence intervals (CIs).

b Genus-specific RealAmp assay.
reclassified as having positive results, which would have made the specificity 100%. However, to eschew bias, the lower, the more conservative specificity was chosen.

There was a high degree of agreement between RealAmp and real-time PCR ($\kappa = 0.76$; 95% CI, .54–.99), whereas moderate agreement was observed between both local and expert microscopy and pooled PCR ($\kappa = 0.59$ [95% CI, .22–.95] and $\kappa = 0.55$ [95% CI, .22–.87], respectively; Table 2). The median time to amplification for the 7 positive samples was 25.7 minutes, shorter than the time required for amplification by pooled real-time PCR (approximately 62 minutes).

**RealAmp as Point-of-Care Testing**

We performed a pilot study of RealAmp by using freshly obtained whole blood specimens from 12 individuals who presented with fever to the local health clinic in Thailand. Samples from these 12 individuals were tested by RealAmp on site and again by real-time PCR at the reference laboratory in Bangkok. The on-site RealAmp analysis detected 4 cases of malaria parasite infections. Local and expert microscopy detected infections in only 2 subjects, both of whom were RealAmp positive. Subsequent testing confirmed that the 4 RealAmp-positive samples were positive by reference real-time PCR, as well, and that the 8 RealAmp-negative samples were also real-time PCR negative. Thus, findings of RealAmp analyses of samples from these 12 patients were 100% accurate. The median time to amplification among the positive Thai clinical samples was 19 minutes. Combined with the approximately 15 minutes required for DNA extraction by the boil and spin method, RealAmp results were thus available within 30–75 minutes.

**PPV and NPV, Based on Local Malaria Prevalence**

The PPV and NPV of RealAmp in Thailand were 63.6% and 100%, respectively. PPV and NPV could not be determined for samples from India since the samples were selected on the basis of infection status. The effects of malaria prevalence on the PPV are shown in Figure 2. At low malaria prevalences, the effects of specificity become marked. For example, at a malaria prevalence of 2.5% and the lower observed specificity (ie, 97%), RealAmp would be predicted to have a PPV of 43.5% and a NPV of 100%. However, when the specificity is only 85% [19], the PPV is only 13.8%.

**DISCUSSION**

The results presented here extend previous laboratory studies and show RealAmp’s usefulness in detecting malaria in 2 field sites where both *P. falciparum* and *P. vivax* are endemic [21, 22]. Compared with 2 different reference molecular tests (nested and real-time PCR), RealAmp had sensitivities of 95% and 100%, respectively, and specificities of 100% and 97%, respectively, in India and Thailand (Table 2). RealAmp was found to be applicable to direct testing of samples in the field. The high sensitivity reported here could translate into a screening test with high PPV and NPV in areas of low malaria prevalence where attempts are being made to eliminate this disease.

Several screening and intervention approaches have been proposed as ways to achieve malaria elimination. They include mass screening and treatment, in which entire populations in a given area are screened, and focal screening and treatment (FSaT), in which targeted populations are screened [20, 38, 39]. Recently in Cambodia, FSaT was used to contain and eliminate malaria parasites. However, lack of a point-of-care field molecular test was cited as a logistical challenge, as it took an average of 8 days to receive PCR diagnostic results from a central reference laboratory [39]. While current efforts entail the use of microscopy or RDTs, the use of a molecular test such as RealAmp would enable the detection and treatment of many infected individuals whose parasitemias are too low to be detected by RDTs or microscopy and who represent important potential reservoirs [6, 7, 11, 39].

There are several features of RealAmp that make it a potential tool for field use in malaria control and elimination programs. First, it has a much higher specificity than previously reported methods [19], which cuts down dramatically on the frequency of false-positive results (Figure 2). Second, it has demonstrated efficacy in detecting and identifying *P. vivax* infections, which often predominate areas approaching malaria elimination, such as Southeast Asia and Latin America [11, 22, 40]. Third, the RealAmp tube scanner is compact (height, 74 mm; width, 178 mm; and depth, 188 mm) and portable, can be powered by an external Li-Ion rechargeable battery, and is less resource intensive than standard PCR tests. It is also equipped with a small LCD screen, which can display results without the need of a computer. The cost of the tube scanner (approximately $6300) is comparable to that of PCR thermocyclers ($3000–$8000), but the cost of running per sample is cheaper on the RealAmp platform when in-house buffer is used, compared...
with nested PCR ($2.66 vs $3.67) [21]. Training is required to use the machine and the boil and spin DNA extraction method, but it can be performed by technicians after appropriate training in a rural health clinic or field site. Fourth, the RealAmp kit format used in this study reduced preparation time for the assay, and there is further scope for improving the kit for easy operation in the field. The buffer mix from the kit appeared to be stable throughout the study period, and no drop-off in its efficiency to amplify DNA under isothermal conditions was observed.

The study is limited in that, first, different PCR methods were used as reference standards at each site, because each site had validated a different technique. However, one of the goals of the study was to compare RealAmp’s performance against the established reference diagnostic methods. Second, the study populations in the 2 study sites were different. All patients were asymptomatic in Thailand, as would be expected for active surveillance. In contrast, all patients were febrile in India, as would be expected for passive surveillance conducted in health facilities. Accordingly, there were no asymptomatic infections in our India site. Further studies in both active and passive surveillance settings are needed to confirm this assay’s usefulness.

Future studies should investigate how well RealAmp performs in the hands of nonexpert personnel. Even though the current prototype RealAmp tube scanner can only test 8 samples at a time, it provides an objective reading of test results that can be monitored in real time. Further, it might be possible to decrease cost and increase throughput by using a pooling strategy [31] and designing other formats of the tube scanner that support testing of a greater number of samples. Overall, this study adds to the increasing evidence that LAMP-based assays are viable methods for detecting low-level malaria parasite infections in the field and could become valuable tools for aiding global malaria control and elimination programs.

Notes

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