Evaluation of SYBR green I based visual loop-mediated isothermal amplification (LAMP) assay for genus and species-specific diagnosis of malaria in *P. vivax* and *P. falciparum* endemic regions

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ABSTRACT

Background & objectives: Loop-mediated isothermal amplification (LAMP) is an emerging nucleic acid based diagnostic approach that is easily adaptable to the field settings with limited technical resources. This study was aimed to evaluate the LAMP assay for the detection and identification of *Plasmodium falciparum* and *P. vivax* infection in malaria suspected cases using genus and species-specific assay.

Methods: The 18S rRNA-based LAMP assay was evaluated for diagnosis of genus *Plasmodium*, and species-specific diagnosis of *P. falciparum* and *P. vivax*, infection employing 317 malaria suspected cases, and the results were compared with those obtained by 18S nested PCR (n-PCR). All the samples were confirmed by microscopy for the presence of *Plasmodium* parasite.

Results: The n-PCR was positive in all *Plasmodium*-infected cases (n=257; *P. falciparum*=133; *P. vivax*=124) and negative in microscopy negative cases (n=58) except for two cases which were positive for *P. vivax*, giving a sensitivity of 100% (95% CI: 97.04–100%) and a specificity of 100% (95% CI: 88.45–99.5%). Genus-specific LAMP assay missed 11 (3.2%) microscopy and n-PCR confirmed vivax malaria cases. Considering PCR results as a reference, LAMP was 100% sensitive and specific for *P. falciparum*, whereas it exhibited 95.16% sensitivity and 96.7% specificity for *P. vivax*. The n-PCR assay detected 10 mixed infection cases while species-specific LAMP detected five mixed infection cases of *P. vivax* and *P. falciparum*, which were not detected by microscopy.

Interpretation & conclusion: Genus-specific LAMP assay displayed low sensitivity. Falciparum specific LAMP assay displayed high sensitivity whereas vivax specific LAMP assay displayed low sensitivity. Failed detection of vivax cases otherwise confirmed by the n-PCR assay indicates exploitation of new targets and improved detection methods to attain 100% results for *P. vivax* detection.

Key words LAMP; malaria; molecular diagnosis; PCR; *Plasmodium falciparum*; *P. vivax*

INTRODUCTION

Malaria is one of the major public health problems of the developing countries resulting in considerable morbidity, mortality and economic loss. World Malaria Report, 2013 illustrated that 97 out of 104 malaria-endemic countries had ongoing transmission of the malaria parasite in 2013 with an estimated 3.4 billion people at risk, of which 1.2 billion are at high risk1. India’s National Vector Borne Disease Control Programme (NVBDCP) has reported about 1.0 million cases each year from 2011–152. In India, the incidence of malaria has declined over the years but it still bears the highest malaria burden in Southeast Asia, the second most affected region in the world after Africa1.

Early and accurate malaria diagnosis is essential for appropriate treatment of patients. Microscopy is the most suitable and cost-effective technique for the diagnosis of malaria in field settings. In ideal conditions, 4–20 parasites/µl can be detected in the Giemsa-stained thick blood film by a well-trained staff but in the field condition this threshold decreases to 50–100 parasites/µl. Antigen-capture immunochromatographic technology based malaria rapid diagnostic tests (RDTs) for detection of the *P. falciparum*–specific antigen histidine-rich protein 2 (*Pf*HRP2) or parasite-specific lactate dehydrogenase (pLDH) are also widely used. RDTs provide the improved speed and precision for malaria diagnosis in areas where standard laboratory diagnosis is not available. Low detection limit of >200 parasites/µl and false positive results due to the persistence of antigen after parasite clearance are major concerns with RDTs3. Further, recent reports of false
negative results by RDTs due to deletion of *PfHRP2* and *PfHRP3* genes poses problem in the detection of *P. falciparum*. The low parasitaemia in vivax patients and instability of lactate dehydrogenase at higher temperatures pose difficulty in RDT based detection of *P. vivax*.

Several nucleic acid amplification methods like nested PCR (n-PCR) and real-time quantitative PCR have been developed for the diagnosis of malaria. Compared to microscopy, these methods have higher sensitivity, with detection limit of 1–5 parasites/µl of peripheral blood, and greater specificity for mixed infection. However, the long turn around time, high cost, and availability only in well-equipped laboratories render these methods inadequate for routine diagnosis in hospital laboratories and field clinics in the areas endemic for malaria.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that relies on autocycling strand-displacement DNA synthesis performed with *Bst* DNA polymerase. The amplification products are stem-loop DNA structures with several inverted repeats of the target and structures with multiple loops. The principal merit of this method is that it does not require denaturation of the DNA template, and all the reactions can be conducted under isothermal conditions (ranging from 60 to 65°C). The method produces a large amount of amplified product, resulting in easier detection simply by the visual judgment of the turbidity or fluorescence of the reaction mixture. Several investigators have reported and commended the usefulness of LAMP methods for the rapid identification of *Plasmodium*, *Trypanosoma*, *Babesia* and *Leishmania*, etc. LAMP has relevant characteristics of a potential point of care test for diagnosis of malaria as it exhibits high sensitivity with detection limit of 0.2–5 parasite/µl and the cost of reagent is equivalent to market cost of RDTs.

First report on the use of LAMP assay for diagnosis of malaria in the clinically proven cases dates back in 2006, wherein Poon et al. reported the detection of highly conserved 18S ribosomal RNA gene of *P. falciparum*. The malaria diagnosis by LAMP does not require expensive reagents (for DNA extraction), turbidimeter, thermal cycler, or skilled technicians and can easily be adapted to the field settings with limited technical resources/support. There are many reports where the LAMP assay has been used for *Plasmodium* spp. detection in blood with high sensitivity and specificity. Recently, the assay has been found to be applicable for noninvasive detection of *P. vivax* and *P. falciparum* using saliva and urine samples. The Pan/Pf LAMP kit has shown promising results in field-based diagnosis and was found reliable for the detection of low parasitaemia from filter paper derived samples. LAMP assays based on real-time measurement of DNA amplification have been applied with high sensitivity and specificity for *Plasmodium* detection.

Although, *P. falciparum* causes the most severe form of the disease, its geographic distribution overlaps with *P. vivax* in India and therefore, it requires the LAMP assay capable for identification of both species, as the mixed infection often remains unrecognized. In the present study, the applicability of the LAMP assay was evaluated for detection and identification of *P. falciparum* and *P. vivax* in malaria suspected cases and further the results of LAMP assay were compared with those of conventional microscopy and nested PCR.

**MATERIAL & METHODS**

**Study subjects and sample collection**

A total of 317 febrile individuals suspected of having malaria, attending/referred to the clinic at the National Institute of Malaria Research (NIMR), New Delhi from July 2011 to August 2013, and at NIMR Field Unit, Raipur between the months of December 2011 and 2012 were included in the study. The sample collection and laboratory investigations were performed as outlined in Fig. 1. Finger-prick blood samples were collected in heparinized vials or on filter paper as dried blood spot and stored at 4°C until further use.

**Ethics statement**

The study was approved by the Institutional Ethics Committee of NIMR, New Delhi (Approval No.ECR/NIMR/EC/2011/40). Written informed consent was obtained from each individual or parent/guardian (in cases of minors) before enrolment in the study.

![](image.png)

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Singh et al: Rapid molecular diagnosis of malaria
Microscopy

Thick blood smears were examined under 1000x magnification by microscopists with extensive experience in the identification of malaria parasites. The number of parasites per 200 white blood cells (WBCs) were counted and the parasite density was calculated assuming a leukocyte count of 8000/ml.

DNA extraction

The DNA was extracted using Qiagen DNA isolation kit according to manufacturer’s instructions from dried filter paper spot or 0.1 ml blood.

18S rRNA n-PCR assay

For n-PCR, the species-specific nucleotide sequences of the 18S rRNA genes of P. falciparum and P. vivax were amplified as described previously with slight modifications. Nest 1 PCR amplification was performed in a 20 μl reaction mixture containing 10 pmoles of each primer (rPLU 1 TCAAAGAATAAGCCATGCAAGTGA and rPLU 2 TAACCCCTGGTTTGCTAAACTTC), 4 mM MgCl₂, PCR buffer (50 mM KCl, 10 mM Tris-HCl), 200 mM of each deoxynucleoside triphosphate, and 1.25 units of Taq DNA polymerase and 5 μl of sample DNA. For Nest 2 assay 1 μl of Nest 1 PCR product was used with 10 pmoles of respective species-specific primers: P. vivax VIV F 5′-CGCTTCTTAGCTTTAATCCACATAACTGATAC-3′; VIV R 5′-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3′ and P. falciparum FAL F 5′-TTAACCTGGTTTGGGAAAACCAAATATT-3′; FALR ACACAATGAACTCAATCATGACTACCCGTGC. The amplified products were visualized in 2% agarose gels stained with ethidium bromide.

LAMP assay and analysis of LAMP products

The genus and species-specific LAMP primer sets designed on the basis of the genus and the nucleotide sequences of the 18S rRNA genes of P. falciparum and P. vivax as described previously were used in the study (Table 1). The reaction mixture (25 μl), containing 1.6 to 2.4 mM of each primers FIP and BIP, 0.2 mM of each primers F3 and B3c, 0.8 mM of each primers LF and LPB, 2 x reaction buffer (12.5 μl), Bst DNA polymerase (1 μl), and 5 μl of DNA sample, was incubated at 60°C for 100 min and then the enzyme was inactivated at 80°C for 2 min.

To visualize and confirm the results fluorescent dye SYBR Green I was added to the completed reaction that gives fluorescent green colour in the positive reaction as it gets intercalated in the nucleic acid while the negative reaction remained orange indicating no amplification. The two individuals scored the LAMP results and the consensus of the results was considered for further analysis.

Diagnostic accuracy analysis

Specificity, sensitivity, positive and negative predictive values and diagnostic accuracy of various tests were calculated using free online diagnostic accuracy calcu-

Table 1. Primer sequences used for genus and species-specific amplification of Plasmodium parasite

<table>
<thead>
<tr>
<th>Genus/species</th>
<th>Primer</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium</td>
<td>PLU F3</td>
<td>GTATCAATCGAGTTTCTGACC</td>
</tr>
<tr>
<td></td>
<td>PLU B3</td>
<td>TCTTGCTACTACCTCTCTCT</td>
</tr>
<tr>
<td></td>
<td>PLU FIP</td>
<td>TGAAACTTACATTCCCCCGTTACCTACGTTTTTGATGTTAGGGT</td>
</tr>
<tr>
<td></td>
<td>PLU BIP</td>
<td>CGGAGAGGGAGCCTGAGAAATAGAATTTGGTAATTTACGCC</td>
</tr>
<tr>
<td></td>
<td>PLU LPF</td>
<td>CGTCATAGCAGTAGTTAGGCC</td>
</tr>
<tr>
<td></td>
<td>PLU LPB</td>
<td>AGCTACCACTACGAAAGGCAG</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>PfF3</td>
<td>TGTAATTGGAATGAGAATTTA</td>
</tr>
<tr>
<td></td>
<td>PfB3c</td>
<td>GAAAAACCTTAAAAATTGGAAACAAAGC</td>
</tr>
<tr>
<td></td>
<td>PfFIP</td>
<td>AGCTGGAATTTACCAGGCCGCTGGTTTCTAGAAGAAAATTGG</td>
</tr>
<tr>
<td></td>
<td>PfBIP</td>
<td>TGTTGCAGTTAAAAAGCTTCTGAGCCTACCAAGAAAATTGG</td>
</tr>
<tr>
<td></td>
<td>PLPF</td>
<td>GCACCGACCTGCTT</td>
</tr>
<tr>
<td></td>
<td>PLPB</td>
<td>TTGAATATTTAAAGGA</td>
</tr>
<tr>
<td>P. vivax</td>
<td>PvF3</td>
<td>GGAATGATGGGAATTTAAACCT</td>
</tr>
<tr>
<td></td>
<td>PvB3c</td>
<td>AGCAAGTATAGCCTGATTGAT</td>
</tr>
<tr>
<td></td>
<td>PvFIP</td>
<td>CTATGTGGAGCTGGAATTACCAGCTCCCAAACTCAATTTGGAGG</td>
</tr>
<tr>
<td></td>
<td>PvBIP</td>
<td>AATTGTTGCAATTTAAAAAGCTCAGTAAGGAGCGTTGCT</td>
</tr>
<tr>
<td></td>
<td>PvLPF</td>
<td>GCTGCTGGACACCAGACTT</td>
</tr>
<tr>
<td></td>
<td>PvLPB</td>
<td>AGTTGAATTTCAGAAGATCG</td>
</tr>
</tbody>
</table>
lатор (http://www.hutchon.net/Diagnostic-test.htm). The kappa (k) statistics was applied to calculate the agreement between the results observed by LAMP and microscopy vs n-PCR assay.

RESULTS

Patient profile and microscopy results

In total, 317 blood samples from febrile individuals; 220 (69.5%) males and 97 (29.5%) females were screened for Plasmodium infection by microscopy, 18S-PCR and LAMP (genus and species-specific) assays. The age range was 1 to 80 yr. The microscopic examination revealed malaria parasites in the blood smears of 257 (81%) patients. Of these, 124 (39.1%) were found positive for P. vivax (320–61,600 parasites/µl) and 133 (41.9%) were positive for P. falciparum (200–4,96,000 parasites/µl) infection, while 60 patients were microscopically negative for the parasite.

Diagnostic performance of molecular assays (18S n-PCR and LAMP assay for species-specific diagnosis) vs microscopy

The results of microscopy, n-PCR and genus and species-specific LAMP assay for 317 investigated patients are detailed in Tables 2 and 3. The microscopic examination detected 257 patients with Plasmodium infection; of these 124 were diagnosed with P. vivax and 133 were P. falciparum cases. In aggregate, 113 samples were positive for P. vivax and 127 for P. falciparum and 58 cases were negative for Plasmodium infection by all the three molecular assays (Table 2); rest of the samples exhibited discordant results. The discordant results (5.6%) were mainly P. vivax cases and mixed infections. The microscopic slides of samples exhibiting discordant results and an equal number of randomly selected slides from samples with concordant results were re-examined blindly by two independent microscopists.

The 18S n-PCR assay detected Plasmodium in all 257 (100%) microscopically positive samples. Of these, 120 were P. vivax infection and 127 were P. falciparum infection, while 10 patients were found positive for both P. vivax and P. falciparum indicating mixed infection. Genus-specific LAMP assay detected 246/257 microscopically positive samples. The test was found 96.8% sensitive and 96.67% specific for detection of Plasmodium spp. in blood samples. It failed to detect the 11/124 microscopically confirmed P. vivax infections including two cases of mixed infection detected by n-PCR. PfLAMP detected 118 samples to be infected with P. vivax while 133 samples were found positive for P. falciparum by Pf/LAMP assay and five samples were mixed infections. All the three molecular assays detected two cases of P. vivax from 60 microscopically negative patient samples.

Sensitivity and specificity of molecular assays (LAMP and n-PCR) for identifying mixed infection of Plasmodium spp.

Microscopic examination of slides did not identify any mixed infections. The n-PCR assay identified 10 cases of mixed infection of P. falciparum and P. vivax among 317 blood samples tested. Out of these 10, six cases were microscopically diagnosed as P. falciparum and remaining four as P. vivax. On the other hand, LAMP assay identified five cases of mixed infection.

Considering n-PCR as reference, the sensitivity and specificity of microscopy in identifying P. falciparum infections were 100% (k=0.974); for P. vivax infections it was 98.4 and 100%, respectively (k=0.948) and nil for mixed infection (k=0) (Table 3). The sensitivity and specificity of LAMPassay for P. falciparum identification were 100% (k=0.961), whereas for P. vivax identification it was 95.25 and 100%, respectively (k=1). The n-PCR assay based parasite detection revealed higher number of mixed infections identification compared to LAMP assay (3 vs 1.5%; k=0.658) (Table 3). The data indicates

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>Nested PCR</th>
<th>Species-specific LAMP (Pf/PvLAMP)</th>
<th>Genus-specific LAMP (Pan LAMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. falciparum (133)</td>
<td>P. falciparum (133)</td>
<td>P. falciparum (133)</td>
<td>Plasmodium spp. (133)</td>
</tr>
<tr>
<td></td>
<td>P. vivax (6)</td>
<td>P. vivax (4)</td>
<td>Negative (0)</td>
</tr>
<tr>
<td>P. vivax (124)</td>
<td>P. vivax (124)</td>
<td>P. vivax (118)</td>
<td>Plasmodium spp. (113)</td>
</tr>
<tr>
<td></td>
<td>P. falciparum (4)</td>
<td>P. falciparum (1)</td>
<td>Negative (11)</td>
</tr>
<tr>
<td>Negative (60)</td>
<td>Negative (58)</td>
<td>Negative (58)</td>
<td>Negative (58)</td>
</tr>
<tr>
<td></td>
<td>P. vivax (2)</td>
<td>P. vivax (2)</td>
<td>Plasmodium spp. (2)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate number of samples.
that molecular tests have better sensitivity to detect low-density parasitaemia of the *Plasmodium* species that often remains masked by the high density parasitaemia of another *Plasmodium* species in mixed infection.

**DISCUSSION**

Rapid, reliable and species-specific diagnostic tools are indispensable for effective control of malaria. Light microscopy-based diagnosis to date is the most effective method; however, it demands technical expertise and suffers low detection limits in field conditions. This study was performed to compare the diagnostic performance of *Plasmodium* genus-specific, *P. falciparum* and *P. vivax* specific LAMP assays in a visual format and standard species-specific n-PCR assay with the microscopic examination for *P. falciparum* and *P. vivax* specific diagnosis of malaria infection.

Genus-specific LAMP assay accurately identified *P. falciparum* cases, however, it failed to diagnose 11 confirmed cases of *P. vivax* (<5000 parasites/µl) infection. The species-specific PfLAMP assay evaluated in this study displayed high sensitivity and specificity of 100% same as in n-PCR assay using blood samples, however, low detection rate was observed for *P. vivax* infection by PvLAMP assay compared to that with n-PCR. The n-PCR assay is a two-step assay and displays detection limit of 1 parasite/µl compared to LAMP assay with a detection limit of 40 parasite/µl for *P. vivax*. High sensitivity (100%) of LAMP for *P. falciparum* was observed in this study as well as in others13, 21, whereas for *P. vivax* detection variable sensitivity of LAMP assay ranging from 36 to 100% have been reported by researchers21, 29. Researchers have suggested the use of other targets like mitochondrial genes and consensus sequence repeats to achieve high sensitivity and specificity for *Plasmodium* detection. This study adopted the visual detection method that does not have a defined cut-off limit and therefore, it is likely that six *P. vivax* cases with low parasitaemia though positive (<1000 parasites/µl, n=3; <3000 parasites/µl, n=3) have been read as false negative compared to n-PCR assay.

Using microscopy as the reference assay, standard 18S n-PCR assay detected *Plasmodium* in all 257 (100%) microscopically positive samples. Of these 120 were *P. vivax* infection and 127 were *P. falciparum* infection, while 10 patients were found positive for both *P. vivax* and *P. falciparum* (mixed infection). Similarly, PfLAMP detected 117 samples to be infected with *P. vivax* while PfLAMP detected 129 samples as *P. falciparum* and remaining five samples were mixed infections. Giemsa microscopy remains the gold standard for malaria diagnosis in resource-limited environments; however, its sensitivity and specificity compared to molecular assays is limited in identifying mixed infection. Hence, novel, rapid and simplified molecular techniques like LAMP assay should be widely used for species-specific diagnosis of malaria in endemic areas.

**CONCLUSION**

The LAMP assay could be made applicable to a resource-limited setting as a point of care diagnostic test. However, to develop it as a point of care test it needs to be simplified further by eliminating the DNA isolation step. In this study, it was observed that *Plasmodium* genus-specific LAMP assay failed to diagnose 11 (3.2%) cases of vivax malaria confirmed by n-PCR. Screening of clinical samples first by *Plasmodium* genus-specific LAMP assay and further confirmation by a species-specific assay based on endemicity of species in the area is proposed for epidemiological surveillance. However, in areas where two or more species are co-existing, screening for clini-
cal diagnosis is debatable and such situations demand a simplified assay capable of simultaneously detecting two or more Plasmodium spp.

**Conflict of interest:** None to declare.

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**REFERENCES**


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