

## Optimization and Testing of LAMP Assay for Diagnosis of Malaria

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Since the launch of the Roll Back Malaria Initiative by WHO in 1998, and particularly in the past few years, malaria control has been intensified in endemic countries. As a consequence malaria is going to be eliminated in 34 countries in near future. The low levels of parasitaemia during the elimination phase possess a challenge to early diagnosis and prompt treatment of the disease at individual as well as community level because of low efficacy of microscopy and currently available RDTs. To tackle this situation loop-mediated isothermal amplification (LAMP) of nucleic acids seems to be a promising technique. We have optimized a LAMP assay using a new proportion of primer mix and DNA extracted by heat treatment that can detect *P falciparum*, *P vivax* and *P malariae* in naked eye by color distinction. The sensitivity and specificity of this assay is similar to the PCR assay and the detection limit of parasite is significantly high than the microscopy. The test can be performed by a technician at CHC level hospital in developing countries like India.

**Keywords:** LAMP, malaria, optimization, *P falciparum*, *P vivax*, *P malariae*

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Malaria is one of the three major infectious diseases in the world. Of the 106 countries that had ongoing malaria transmission in 2000, reported data in 66 were found to be sufficiently complete and consistent to reliably assess trends between 2000 and 2013. Based on an assessment of trends in reported malaria cases, a total of 64 countries are on track to meet the Millennium Development Goal target of reversing the incidence of malaria. In 2013, two countries reported zero indigenous cases for the first time (Azerbaijan and Sri Lanka), and ten others succeeded in maintaining zero cases (Argentina, Armenia, Iraq, Georgia, Kyrgyzstan, Morocco, Oman, Paraguay, Turkmenistan and Uzbekistan). Another four countries reported fewer than 10 local cases annually (Algeria, Cabo Verde, Costa Rica and El Salvador)<sup>1</sup>. Similarly India has more than halved the number of malaria cases, down from 2 million to 882 000 in 2013 and advancing

towards elimination<sup>2</sup>. However the low levels of parasitaemia during the elimination phase possess a challenge to early diagnosis and prompt treatment of the disease, which has been accepted as one of the key strategies for Roll Back Malaria (RBM) program, at individual as well as community level because of low efficacy of microscopy and currently available RDTs. Alternatively polymerase chain reaction (PCR) has been used because of its higher sensitivity and specificity. But the technique requires extensive training, high end resources and time. To overcome this situation loop-mediated isothermal amplification (LAMP) of nucleic acids seems to be a promising technique, since it has the ability to amplify DNA into mass quantities at an isothermal temperature between 60-65°C within 60-100 minutes and fluorescence detection using Syber Safe I make it an ideal assay. We had undertaken this study to optimize and evaluate the utility of the LAMP assay for diagnosis of malaria in developing countries like India.

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## METHODS

DNA was extracted from 57 microscopically malaria positive blood samples (P falciparum: 25, P vivax: 25 and P malariae: 7) and 15 from microscopically malaria negative blood samples by phenol-chloroform and alcohol precipitation method<sup>3</sup> as well as boiling method<sup>4</sup> from whole blood to be tested. With the purified DNA, nested PCR was conducted to detect malaria parasites using species specific primers as described by Snounou et al. (1993)<sup>5</sup>. The PCR was run for 35 cycles of denaturation (95 °C for 30 second), annealing (57°C for P falciparum/56°C for P vivax / 52°C for P malariae for 40 second) and extension (68°C for 1minute) to amplify targeted DNA. The final product was then subjected to electrophoresis in 2% agarose gel containing ethidium bromide (0.5 µg/ml) to read out the results. Expected bands for P falciparum was located at 918 bp, for P vivax at 714 bp and for P malariae at 513bp.

The same 57 purified DNA malaria positive/ 15 malaria negative samples and DNA extracted by boiling method from whole blood were used for LAMP assay. The LAMP assay was performed using Loopamp DNA amplification kits (Eiken Chemical Co Ltd, Tokyo) and species specific primer sets as described by Han et al (2007)<sup>6</sup>. Our optimized master mixture (25 µl) contains 12.5µl of 2x reaction mix [ 40mM Tris-HCl (pH 8.8), 20 mM KCl, 16mM MgSO<sub>4</sub>, 20mM(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2% Tween20, 1.6M betaine and 2.8mM each dNTPs], 1 µl of Bst DNA polymerase (1U), primer mix (1µl) [ 50 pmol each of the inner primers (HPLC purified FIP and BIP), 5 pmol each of the outer primers(F3 and B3) and 25 pmol each of loop primers (FLP and BLP), 3-4 µl of DNA sample, 1 µl of fluorescent detection reagent(FD) and 6.5 µl of distilled water (DW). The LAMP were performed at 63°C for 60 min for P. falciparum, P. vivax, and P.malariae. The sample was judged "positive" when the reaction mixture was colored bright green whereas "negative" when colored orange.

## RESULTS

After PCR amplification of purified DNA and running the gel, all 15 negative samples and 57

positive samples matched the microscopy result. PCR is 100% compared to microscopy. But LAMP assay could detect P falciparum in 24 of 25 (sensitivity: 96%, 95% CI: 79.65-99.90%), P vivax in 23 of 25 (sensitivity: 92.00%, 95% CI: 73.97 to 99.02%) and P malariae in 5 of 7 (sensitivity: 71.43%, 95% CI: 29.04% to 96.33%) microscopically positive samples. No false positive results were detected by LAMP or nested PCR among 15 blood samples from healthy individuals (a specificity of 100% for both) The minimum detection limit of LAMP was 5 parasites/ µl compared to 2 parasites/ µl by PCR and 20 parasites/ µl by microscopy in case of P falciparum, 10 parasites/ µl compared to 2 parasites/ µl by PCR and 20 parasites/ µl by microscopy in case of P vivax and 20 parasites/ µl compared to 2 parasites/ µl by PCR and 30 parasites/ µl by microscopy in case of P malariae. For DNA extraction using boiling method LAMP showed high sensitivity (P falciparum 92%, P vivax 92% and P malariae 71.4%) compared to PCR (P falciparum 88%, P vivax 88% and P malariae 57.14%) while specificity remaining same (100%).

## DISCUSSION

Loop-mediated isothermal amplification (LAMP) is a novel molecular technology that amplifies the previously determined genes and can be used to detect any pathogen. Poon and others (2005)<sup>7</sup> were the first to design the loop primers for the detection of P falciparum by LAMP assay. They have claimed 95% sensitivity and 99% specificity for the test based on the results of a small laboratory study conducted in Hong Kong. Subsequently Paris et al (2007)<sup>8</sup> observed only ~ 75% sensitivity and ~83% specificity with same sets of loop primer in Bangladesh in a hospital based study. In 2007 Han and others from Japan and Thailand have made an attempt to design the loop primers for all 4 species of human Plasmodium and claim a very high sensitivity (98.5%) and specificity (94.3%). These studies warrants further optimization of the LAMP assay in clinical and field conditions. Because evaluation of new diagnostic tests in malaria has the advantage of availability of a highly sensitive and specific gold standard, which is nested PCR (Banoo et al 2006)

In our study, we have used for the first time the primer mix (FIP-BIP, FLP- BLP and F3- B3)

in a proportion of 10:5:1 and compared two modality of LAMP and nested PCR with microscopy as reference method. During the present study though the overall sensitivity and specificity of LAMP-purified DNA modality was low compared nested PCR but LAMP-boiling DNA modality showed higher sensitivity and specificity than the PCR. Our observation is at par with most other studies conducted in other parts of the globe<sup>6, 8, 10, 11</sup>. However the detection limits of our test using the primer mix of 10:5:1 was significantly high compared to others<sup>11</sup>. Further optimization may improve the performance of the test. With regard to costing the current cost of each test using commercial kit is higher (Rs 650.00/US\$ 10.8) than nested PCR (Rs 195.00/ US\$ 3.2) and hence further studies are required to make the test cost effective.

The LAMP test is simple and DNA extraction by boiling method is highly robust. The test only needs a mini water bath, mini centrifuge and auto pipettes. More importantly the interpretation of the results does not require any experienced staff. The technician “hands-on” time for the assay is estimated to be three hours. From our study we have observed that training imparted for one day to 10 technicians who have no knowledge on PCR or LAMP could perform the test and generated identical results as experts on LAMP assay. Such type of result demonstrating easy training and good result was reported by Cook et al (2015)<sup>12</sup> in a study conducted in Zanzibar. These features make the LAMP assay an option for the molecular diagnosis of malaria even in basic health care settings in countries like India.

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