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Simple, rapid, inexpensive platform for the diagnosis of malaria by loop mediated isothermal amplification (LAMP)

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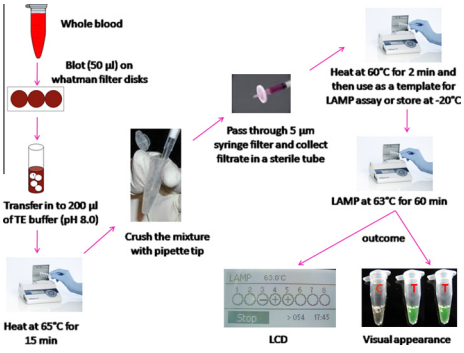
HIGHLIGHTS

- Improved LAMP assay for the diagnosis of Malaria in a resource limited settings.
- We developed a simple platform for the diagnosis of malaria by genus- and species-specific LAMP and successfully used a simple DNA extraction method.
- LAMP-Tube scanner method is capable of detecting the two most common species of malaria parasites within 30 min and it's proved to be sensitive, reliable and feasible.
- Analogous results were obtained for LAMP-Tube scanner versus traditional microscopic method and LAMP-Thermocycler.

GRAPHICAL ABSTRACT



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ABSTRACT

We attempted to improve the loop-mediated isothermal amplification (LAMP) method for malaria diagnosis by using a simple DNA extraction procedure, and a portable device performing both the amplification and detection of LAMP in one platform. Additionally, the device served as a heating block for the DNA preparation. We refer this method as LAMP-Tube scanner, and evaluated using 209 microscopically positive malaria samples and compared them to RDTs and LAMP-Thermocycler. Two most common human infecting *Plasmodium* species were detected. The LAMP-Tube scanner method is found to be simple and allowed real-time detection of DNA amplification. The time to amplification varied but was closely less than 60 min. Sensitivity and specificity of LAMP-Tube scanner in detecting *Plasmodium falciparum* were 95% and 93.3%, compared to microscopy and 98.3% and 100% respectively, compared to standard LAMP-Thermocycler. In addition, it showed a detection limit of 10 and 40 copies of the parasitemia for *Plasmodium vivax* and *P. falciparum*. Accordingly, in comparison to the results obtained by microscopy, the LAMP-Tube scanner had a less divergence in sensitivity and specificity, and yielded results similar to those of LAMP-Thermocycler. This method has the great potential as a field usable molecular tool for the diagnosis of malaria and is an alternative to conventional PCR-based diagnostic methods for field use.

1. Introduction

Malaria is one of the most important tropical infectious diseases. About 350–500 million clinical episodes of malaria occur

Abbreviations: RDT, rapid diagnostic test; *Pf*, *Plasmodium falciparum*; *Pv*, *Plasmodium vivax*; NTC, no template control.

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each year, and the disease is responsible for more than 1 million deaths annually (WHO, 2008). The range of areas inhabited by malaria-carrying mosquitoes is expanding due to global climate change (Patz and Olson, 2006). Thus, there is a chance of recovery of malaria, not only as an outcome of the increasing number of imported cases, but also as an effect of the rise of habitats apt for malaria-carrying mosquitoes. Therefore, the hasty diagnosis of this disease is extremely important.

For the past 100 years, malaria has been diagnosed by microscopic examination of Giemsa, Wright, or Field stained blood films (Bruce-Chwatt, 1987; Warhurs and Williams, 1996; White and Silamut, 1989). However, it is well documented that microscopy has limitations: it is time-consuming, and misdiagnosis of the infecting species is common if the microscopist lacks experience and/or when the parasitemia is low (Kain et al., 1998; Milne et al., 1994; Singh et al., 1999; Snounou et al., 1993a). More recently, serological diagnostic methods and new rapid diagnostic tests have become available, which are most commonly based on the detection of parasite antigens such as histidine-rich protein (HRP)-2 and lactate dehydrogenase (LDH) in an easy-to-use dipstick or a lateral flow format (Beadle et al., 1994; Makler et al., 1998). The advantages offered by these methods, such as a result can obtain within half an hour by unskilled technicians, are tempered by a few limitations (Moody, 2002). RDT methods do not offer improved sensitivity over microscopy, the sensitivity decreases as parasitemias fall below 100 parasites/μL (Mills et al., 1999). False positive results are observed, particularly after treatment, as the parasite antigens detected can remain in the circulation following parasite clearance. Moreover, the majority of the commercial RDTs detect HRP-2, which is only expressed by *P. falciparum*; however, not by other species and therefore this test offers specific diagnosis of falciparum malaria. Most of the non-HRP-2 based tests (LDH and aldolase) are usually pan-species test that allows for the speciation of *P. falciparum* and/or non-falciparum species when used in combination with HRP-2 based tests.

Several PCR based assays have also been developed for the detection and identification of malaria parasites. Most often based on genus- or species-specific sequences of the parasites rRNA gene (Singh et al., 1999; Kawamoto et al., 1996; Snounou et al., 1993b). PCR-based assays have various advantages over microscopy and RDTs: they are highly specific and sensitive (Kain et al., 1998; Snounou et al., 1993b; Zhong and Kain, 1999), and as few as five parasites per microliter of blood can be detected (Makler et al., 1998). Regrettably, the current PCR-based methods are beyond the capacity of most malaria-endemic countries as they need expensive inspections, sophisticated laboratory and training that make these techniques expensive and practically challenging to implement in the field or resource limited settings. The recently developed loop-mediated isothermal amplification method is a relatively simple and field-adaptable technique (Notomi et al., 2000). This method does not require a thermocycler or sophisticated training. It has the potential to be used as a molecular diagnostic tool for point-of-care (POC) testing in both developing and developed countries. LAMP has been used for the detection of several infectious diseases such as severe acute respiratory syndrome (Hong et al., 2004), West Nile virus (Parida et al., 2004), avian influenza virus (Imai et al., 2006), norovirus (Fukuda et al., 2006) and *Legionella* bacteria (Annaka et al., 2003).

Lately, the LAMP assay for malaria parasites has already been reported by Poon et al., 2006; Han et al., 2007; Paris et al., 2007; Yamamura et al., 2009. However, LAMP assays can be used in the resource limited areas, but there is a need for electricity to power equipment such as thermocycler, water bath, centrifuge etc., second, they require a complex procedure for the sample preparation. Although highly specific and sensitive, the time and resources needed are restricting it from being used in tribal areas. Therefore, there is a requirement for a minimal DNA preparation and field-usable instrument that can allow a faster and objective readout for the diagnosis of malaria in resource-limited settings. Here, we report a simple DNA extraction procedure and the use of a mini portable device in which both amplification and real-time detection units are combined into a single unit for LAMP assay. We regard this method as LAMP-Tube scanner.

2. Materials and methods

2.1. Study site and sample collection

Clinically related work in this study was performed at Genomix Molecular Diagnostics Pvt. Ltd., (Hyderabad, India) and the portable device connected work carried out at QIAGEN Lake Constance GmbH (formerly ESE-GmbH, Stockach, Germany). For field diagnosis of malaria, 52 febrile patients were analyzed on-site by LAMP-Tube scanner method. Microscopy, LAMP-Thermocycler and RDTs were performed off-site for the same samples. For the laboratory detection of malaria, a total of 128 blood samples that were positive for *P. falciparum* and 40 samples that were positive for *P. vivax* and 15 samples that were negative for malaria parasites by microscopy and RDTs collected from the clinical diagnostic centers and medical institutes throughout the Andhra Pradesh, southern province of India, where *P. falciparum* and *P. vivax* are the common most parasites transmitted. Patients samples previously diagnosed with malaria and treated with antimalarials were excluded. All blood samples and samples saved as blood spots on filter paper were stored at −20 °C. Purified *P. falciparum* DNA (3D7) was provided by Dr. Udhayakumar Venkatachalam (CDC, Atlanta, USA).

2.2. Microscopy

Thick and thin blood films were stained according to the method described by Field (White and Silamut, 1989) and examined by an experienced microscopist. Parasitemia was assessed either per 1000 erythrocytes in the thin film or at low parasitemias per 200 white blood cells in the thick film. In case of a putative negative film, 500 WBC were assessed to confirm the absence of parasites. The number of parasites/μL was determined by counting the total number of RBCs/μL.

2.3. Rapid diagnostic test

The blood samples were also analyzed with the Malaria test kits (Genomix Molecular Diagnostics Pvt. Ltd., Hyderabad, India), based on immunological detection of the *P. falciparum* specific histidine-rich protein 2 (PfHRP2) and *P. vivax* specific lactate dehydrogenase (pLDH). The assays were performed in parallel according to the manufacturer's instructions using a drop of EDTA anticoagulated whole blood and the results were observed after 15 min by the naked eye.

2.4. Processing of samples and DNA extraction for LAMP

The DNA template used for LAMP-Thermocycler (LAMP performed using a thermocycler, Bio-Rad, USA) was prepared from 200 μL of whole blood using the QIAamp DNA Blood Mini kit (QIAGEN, Hilden). For the LAMP-Tube scanner (LAMP performed using a tube scanner, QIAGEN Lake Constance GmbH, Germany), a second simple and cheap method of DNA extraction was used and described in this work (Fig. 5). Briefly, 50 μL of human blood was directly blotted onto Whatman filter paper disks and air-dried at room temperature for 5 min. Blotted disks were heated in 200 μL of TE buffer (pH 8.0) at 65 °C for 15 min followed by crushing the