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# Development of a loop-mediated isothermal amplification method for rapid mass-screening of sand flies for *Leishmania* infection

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ABSTRACT

Entomological monitoring of *Leishmania* infection in leishmaniasis endemic areas offers epidemiologic advantages for predicting the risk and expansion of the disease, as well as evaluation of the effectiveness of control programs. In this study, we developed a highly sensitive loop-mediated isothermal amplification (LAMP) method for the mass screening of sand flies for *Leishmania* infection based on the 18S rRNA gene. The LAMP technique could detect 0.01 parasites, which was more sensitive than classical PCR. The method was robust and could amplify the target DNA within 1 h from a crude sand fly template without DNA purification. Amplicon detection could be accomplished by the newly developed colorimetric malachite green (MG)—mediated naked eye visualization. Pre-addition of MG to the LAMP reaction solution did not inhibit amplification efficiency. The field applicability of the colorimetric MG-based LAMP assay was demonstrated with 397 field-caught samples from the endemic areas of Ecuador and eight positive sand flies were detected. The robustness, superior sensitivity, and ability to produce better visual discriminatory reaction products than existing LAMP fluorescence and turbidity assays indicated the field potential usefulness of this new method for surveillance and epidemiological studies of leishmaniasis in developing countries.



## 1. Introduction

Leishmaniasis is a vector-borne parasitic disease caused by an obligate intracellular protozoan of the genus *Leishmania*. It is one of the most neglected diseases worldwide, distributed especially in tropical and subtropical areas, and it has strong and complex associations with poverty (Alvar et al., 2006). *Leishmania* parasites cause three forms of leishmaniases (cutaneous, mucocutaneous and visceral leishmaniasis) depending on the infecting species. The protozoan is transmitted by the bite of an infected female sand fly of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Munstermann, 2004; Kato et al., 2010). The spread of leishmaniasis largely depends on the distribution of sand fly vectors. Approximately 800 sand fly species have been described, but only a few are medically important (Munstermann, 2004; Kato et al., 2010).

The monitoring of natural *Leishmania* infection in sand fly populations is an important epidemiological parameter for predicting the risk and prevalence of disease, the estimation of which depends on the reliable identification of infected sand flies. Estimation of *Leishmania* infection rates in the vector could serve as an indicator of a change in transmission intensity at a given endemic area. Such studies have been conducted either by dissecting individual insects and detecting the parasites under a microscope, or polymerase chain reaction (PCR)-based techniques to detect *Leishmania* DNA in sand flies (Kato et al., 2005). The former is laborious and time-consuming due to the low *Leishmania* infection rate in sand flies in most endemic areas (Aransay et al., 2000; Kato

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et al., 2005). The latter method has been proved to have both high sensitivity and specificity; however, the PCR methods require specialized instruments and take several hours, which make their use in resource-limited countries and under field conditions unfeasible. There is therefore a need for a simplified method of amplification and amplicon detection that can complement the available surveillance tools and generate information on the distribution or expansion of the disease.

As an alternative, the loop-mediated isothermal amplification (LAMP) technique may provide an answer to vector monitoring needs. This technique has been proven to be an accurate, rapid and simple method, which amplifies the target nucleic acid under isothermal conditions (Notomi et al., 2000). Recently, wide applicability of LAMP in the detection of parasitic protozoa such as *Babesia*, *Plasmodium*, *Leishmania* and *Trypanosoma* in clinical samples has been demonstrated (Ikadai et al., 2004; Poon et al., 2006; Njiru et al., 2008; Takagi et al., 2009; Thekisoe et al., 2007; Laohasinnarong et al., 2011). Studies have also shown the application of LAMP to survey vectors of infectious diseases (Aonuma et al., 2009; Thekisoe et al., 2010; Nakao et al., 2010); however, these studies used purified DNA as a template for the LAMP assays. The use of a DNA extraction kit and the time-consuming DNA purification process in template preparation reduces the utility of LAMP technique as a surveillance tool for mass screening of infected vectors with pathogens in endemic regions. To date, no reports have been available on the use of the LAMP method for detection of *Leishmania*-infected sand flies. Considering these points, we developed and validated a novel LAMP assay for mass screening of sand fly vectors for *Leishmania* infection. In this study, a newly developed LAMP method for rapid detection of the *Leishmania* DNA within sand fly vectors is described, in addition to the use of a non-fluorescent cationic dye, namely, malachite green (MG) for the first time as a simpler colorimetric assay for the detection of LAMP reactions. The present method was evaluated with field-captured sand fly samples in order to demonstrate its efficacy and reliability as an important potential tool in assessing *Leishmania* infection and/or transmission intensity in endemic areas of Ecuador.

## 2. Materials and methods

### 2.1. Sand fly collection

Wild sand flies were collected in March, July and August 2012 in Andean areas of Ecuador; Alausi, Chanchan and Huigra (Province of Chimborazo), where Andean-type cutaneous leishmaniasis caused by *Leishmania* (*Leishmania*) *mexicana* is endemic (Kato et al., 2005, 2008). Sand flies were caught using protected human bait, CDC light traps and Shannon traps. The collected sand flies were dissected and then identified at the species level mainly based on the morphology of their spermathecae (Young and Duncan, 1994). These flies were examined for *Leishmania* infection by microscopy. To validate the newly developed LAMP method, sand fly collections were also made at the same areas using the capture methods mentioned above. These flies were fixed in absolute ethanol and stored at room temperature.

### 2.2. DNA preparation

DNA samples used in this study were prepared from the following *Leishmania* reference strains: *Leishmania* (*Leishmania*) *major*-like (MHOM/EC/1988/PT-115), *L.* (*L.*) *mexicana* (MNYC/BZ/1962/M379), *Leishmania* (*Leishmania*) *amazonensis* (MHOM/BR/1973/M2269), *Leishmania* (*Viannia*) *guyanensis* (MHOM/BR/1975/M4147), *Leishmania* (*Viannia*) *braziliensis* (MHOM/BR/1975/M2904) and *Leishmania* (*Viannia*) *panamensis* (MHOM/PA/1971/LS94) in the New World, and *L.* (*L.*) *major* (MHOM/SU/1973/5ASKH), *Leishmania* (*Leishmania*) *tropica* (MHOM/SU/1958/strainOD), *Leishmania* (*Leishmania*) *infantum* (MHOM/ES/1993/PM1), *Leishmania* (*Leishmania*) *donovani* (MHOM/SU/1962/2S-25M-C2) in the Old World. DNA from *Leishmania*-related parasites capable of infecting sand flies, such as *Endotrypanum* (Kato et al., 2007) and anuran *Trypanosoma* species (Ferreira et al., 2008), and also DNA from the sand flies *Phlebotomus papatasi* and *Lutzomyia ayacuchensis*, were included to evaluate the present LAMP primer specificity.

For sand fly crude DNA extraction, ethanol-fixed sand fly samples were individually lysed in 50 μl of DNA extraction buffer [150 mM NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] in the presence of proteinase K (100 μg/ml) without homogenization. The samples were incubated overnight at 37 °C. After 25 μl of distilled water was added, the samples were preheated at 95 °C for 5 min, and 1 μl was directly used as a template for the LAMP method.

**Table 1**
Nucleotide sequences for the *Leishmania* 18S rRNA LAMP primers.

| Name of primer | Sequence (5′-3′) |
|---|---|
| F3-Le.rRNA | GGGTGTTCTCCACTCCAGA |
| B3-Le.rRNA | CCATGGCAGTCCACTACAC |
| FIP-Le.rRNA | TACTGCCAGTGAAGGCATTGGTGGCAACCATCGTCGTGAG |
| BIP-Le.rRNA | TGCGAAAGCCGGCTTGTTCCCATCACCAGCTGATAGGGC |

### 2.3. Polymerase chain reaction amplification

Conventional PCR was performed with previously described primers specific for *Leishmania* minicircle kinetoplast DNA (mkDNA) (Kato et al., 2005, 2007). The primer sequences used for amplification were 5′-CTRGGGGTTGGTGTAAAATAG-3′ (L.MC-1S) and 5′-TWTGAACGGGRTTTCTG-3′ (L.MC-1R) (Kato et al., 2005). The reaction was carried out in a volume of 15 μl using a pair primers (0.4 μM each) and 2× Ampli Taq Gold PCR Master Mix (Applied Biosystems, NJ, USA). After an initial denaturation at 95 °C for 10 min, PCR amplification was performed with 30 cycles of denaturation (95 °C, for 1 min), annealing (55 °C, for 1 min), and polymerization (72 °C, 1 min), followed by a final extension at 72 °C for 10 min. The PCR products were resolved by agarose gel electrophoresis.

To identify sand fly by PCR-restriction fragment length polymorphism (PCR-RFLP), PCR amplification with *Lutzomyia* 18S ribosomal RNA (rRNA) gene-specific primers and subsequent digestion with the *Afa* I enzyme were performed as previously described (Kato et al., 2007, 2008).

### 2.4. Loop-mediated isothermal amplification method

Sets of forward and backward external primers (F3 and B3) and forward and backward internal primers (FIP and BIP) were designed using PrimerExplorer version 4.0 software (http://primerexplorer.jp/elamp4.0.0) based on the conserved region of the *Leishmania* 18S rRNA gene. We considered this region to be appropriate because of its conservation across *Leishmania* species. Selection of primers used required a number of preliminary LAMP experiments for optimization. Selected LAMP primers are shown in Table 1. The LAMP assay was conducted in 15 μl of a reaction mixture consisting of a 1.6 μM concentration of each inner primer (FIP and BIP), a 0.2 μM concentration of each outer primer (F3 and B3), 1× reaction mix (Eiken, Tochigi, Japan), 8 U *Bst* DNA polymerase (Eiken), 0.004% malachite green (MG) dye (dissolved in distilled water), and 1 μl of template DNA. The mixture