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A rapid molecular diagnosis of cutaneous leishmaniasis by colorimetric malachite green-loop-mediated isothermal amplification (LAMP) combined with an FTA card as a direct sampling tool

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ABSTRACT

Leishmaniasis remains one of the world's most neglected diseases, and early detection of the infectious agent, especially in developing countries, will require a simple and rapid test. In this study, we established a quick, one-step, single-tube, highly sensitive loop-mediated isothermal amplification (LAMP) assay for rapid detection of *Leishmania* DNA from tissue materials spotted on an FTA card. An FTA-LAMP with pre-added malachite green was performed at 64 °C for 60 min using a heating block and/or water bath and DNA amplification was detected immediately after incubation. The LAMP assay had high detection sensitivity down to a level of 0.01 parasites per μl. The field- and clinic-applicability of the colorimetric FTA-LAMP assay was demonstrated with 122 clinical samples collected from patients suspected of having cutaneous leishmaniasis in Peru, from which 71 positives were detected. The LAMP assay in combination with an FTA card described here is rapid and sensitive, as well as simple to perform, and has great potential usefulness for diagnosis and surveillance of leishmaniasis in endemic areas.

1. Introduction

Leishmaniasis is a wide spectrum of diseases caused by an intracellular protozoan parasite of the genus *Leishmania*, transmitted by the bite of an infected female sand fly. *Leishmania* infection can result in three main clinically distinct manifestations in the human host; cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). A critical component towards an understanding of the epidemiology and proper control/treatment of leishmaniasis is early and accurate diagnosis.

Conventionally, leishmaniasis is diagnosed by microscopic examination of skin smear/biopsy samples or aspirates from lesions for CL and MCL, and splenic or bone marrow aspirates for VL (Reithinger and Dujardin, 2007; De Vries et al., 2015). Despite high specificity, these methods are insensitive, invasive, and also require technical expertise (Reithinger and Dujardin, 2007; De Vries et al.,

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2015). Molecular approaches such as polymerase chain reaction (PCR) assays have been employed in the diagnosis of leishmaniasis (Reithinger and Dujardin, 2007; De Ruiter et al., 2014; De Vries et al., 2015); however, the need for expensive specialized equipment, the long time to result and lack of field applicability have greatly hindered the integration of these techniques into the diagnostic algorithm in endemic areas.

Recently, a rapid and simplified molecular technique, the loop-mediated isothermal amplification (LAMP) has been shown to be an effective tool in detection of human pathogenic infectious agents (Notomi et al., 2000; Mori et al., 2012; Dhama et al., 2014). The technique has been applied in the detection of *Leishmania* using purified DNA extracted from patient's materials (Takagi et al., 2009; Adams et al., 2010; Khan et al., 2012) or swab boiled samples from CL model mice (Direct Boil-LAMP method; Mikita et al., 2014). However, the efficiency of the reported Direct Boil-LAMP method as a rapid diagnostic tool for CL remains to be demonstrated with clinical samples. Furthermore, the Foundation for Innovative New Diagnostics (FIND) has also devoted its effort towards reducing the burden of visceral leishmaniasis through innovative LAMP technique (http://www.finddiagnostics.org/). Despite the progress made with LAMP in diagnosis of leishmaniasis, a simple and efficient procedure for field and clinic sample collection and storage without the need for liquid handling and refrigerant/cold storage, is necessary. Flinders Technology Associates (FTA) cards (Whatman) lyse spotted cells and pathogens, and protect the nucleic acids from oxidation, nucleases and UV damage at room temperature for long storage. Studies have shown the utility of FTA cards for nested PCR analyses in epidemiological studies of leishmaniasis (Kato et al., 2010, 2011). However, the potential usefulness of FTA cards as a direct sampling tool for diagnosis of leishmaniasis by LAMP assay has not yet been well-explored. Therefore, this study reports the establishment of a quick, one-step, and single-tube, sensitive colorimetric malachite green (MG) based LAMP in combination with an FTA card for the detection of *Leishmania* DNA from patients' cutaneous lesion-materials.

2. Materials and methods

2.1. Parasites and template preparation

A WHO reference strain of *Leishmania* (*Leishmania*) *major* (MHOM/SU/1973/5ASKH) was cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Cansera International, Etobicoke Ontario, Canada), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 25 °C. Parasites were harvested in the log phase and suspended in phosphate–buffered saline (PBS), counted using a Neubauer counting chamber (Hirschmann, Germany), and then 10^6 to 1 parasites were prepared from the cultures. Each set of 10^6 to 1 parasites was applied to FTA cards (Whatman, Newton Center, MA, USA), and the coded cards were allowed to air dry and stored at room temperature. Ten to eleven months later, 2.0-mm-diameter discs were punched from each FTA card using a Harris micro-punch tool (Whatman) and washed twice with an FTA purification reagent (Whatman) and once with distilled water. The discs were air-dried and used directly as the DNA template for the LAMP assay. For the 1 parasite level, LAMP assay was repeated with different single punch in order to achieve amplification since parasite DNA is localized on the FTA card matrix. In addition to the analytical sensitivity of the FTA-LAMP using live parasites, 10-fold serial dilutions of purified *Leishmania* (*L.*) *mexicana* (MNYC/BZ/1962/M379) DNA (equivalent to 10^4 to 0.01 parasites) were individually applied to 2.0-mm-diameter pre-punched FTA cards and washed, air-dried, and used as templates to verify the detection sensitivity of the LAMP. The specificity of the LAMP assay was assessed against *Leishmania*-related human pathogenic *Trypanosoma* parasites using DNA prepared from *Trypanosoma cruzi* (both Tulahuen and Y strains) and *Trypanosoma brucei gambiense* (both IL2343 and Wellcome strains), as well as human and dog genomic DNAs.

Furthermore, to test the reliability of the LAMP assay in the amplification of *Leishmania* DNA on an FTA card, tissue materials were aspirated from skin lesion of a mouse experimentally infected with *L.* (*L.*) *major* and spotted onto an FTA card. Discs of 2.0-mm-diameter were punched out from the sample areas, washed, air-dried, and directly used as a template for the LAMP assay. The experiment was conducted following the guidelines of the Ethics Committee on Animal Experimentation of Hokkaido University (approval number: 13-0139).

2.2. Clinical samples

A total of 122 samples previously collected from patients with CL and MCL who visited the rural health centers at 15 Departments: Piura, Amazonas, Loreto, Lambayeque, Cajamarca, La Libertad, San Martin, Ancash, Lima, Pasco, Junin, Ayacucho, Apurimac, Cusco and Madre de Dios in Peru, for the diagnosis and treatment of leishmaniasis, analyzed by nested PCR (Kato et al., 2010) were used for the developed FTA-LAMP evaluation under the approval of the research ethics committee of Hokkaido University (license number: vet26-4). Briefly, the tissue materials were taken by aspirating or scraping the active edge of the lesions of a patient by local physicians and well-trained laboratory technicians and spotted onto an FTA card, coded, air dried and enclosed in self-sealing bag and stored at room temperature (Kato et al., 2010).

2.3. FTA-loop-mediated isothermal amplification assay

The LAMP assay was carried out as previously described (Nzelu et al., 2014). The primer sequences based on *Leishmania* 18S rRNA gene were forward inner primer (FIP—Le.rRNA), 5′ -TACTGCCAGTGAAGGCATTGGTGGCAACCATCGTCGTGAG-3′; backward inner primer (BIP—Le.rRNA) 5′-TGCGAAAGCCGGCTTGTTCCCATCACCAGCTGATAGGGC-3′; forward outer primer (F3—Le.rRNA) 5′-GGGTGTTCTCCACTCCAGA-3′; backward outer primer (B3—Le.rRNA), 5′-CCATGGCAGTCCACTACAC-3′ (Nzelu et al., 2014). One punch of an FTA card from sample areas was used as a template for the LAMP assay. The mixture was incubated at 64 °C for 60 or 30 min in a heating block and then heated at 80 °C for 5 min to terminate the reaction. A positive control (DNA from a reference stain: *Leishmania* (*V.*) *braziliensis*—MHOM/BR/1975/M2904) and a negative (water) sample were included in each LAMP run. The LAMP assay using one punch of an FTA card template per sample was repeated not more than twice for samples negative in the first LAMP assay in order to achieve gene amplification.

2.4. Sequencing

To confirm that the LAMP products had the target sequence, direct sequencing of the LAMP amplicons was performed. The LAMP products were purified using a PCR purification kit (NIPPON Genetics, Tokyo, Japan) and then sequenced with a FIP-Le.rRNA primer (Nzelu et al., 2014) using a BigDye Terminator version 3.1 Cycle-Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

3. Results and discussion

The LAMP assay could detect all the *L.* (*L.*) *major* (10^6 to 1 parasites) levels from 2.0-mm-diameter FTA cards templates. No amplification was detected in the negative controls. Additionally,