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 ºC 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.

Keywords: *Leishmania*, LAMP, FTA, malachite green, Peru

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., 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 25ºC. 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 *L*. (*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: *L*. (*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, the FTA-LAMP assay had high detection sensitivity down to a level of 0.01 parasites/µl (Fig. 1A), which agreed with our previous report using purified DNA as a template (Nzelu et al., 2014). All the positive reactions turned light blue while the negative reactions became colorless without adding any reagent after incubation. The colorimetric results were confirmed by gel electrophoresis (Fig. 1B), and there was agreement in the detection of the amplification products by both systems. Furthermore, we observed that DNA amplification could also be detected within 30 min of incubation, even down to the level of 0.01 parasites/µl (data not shown). No cross-reactivity was recorded from *Trypanosoma cruzi* (both Tulahuen and Y strains), *Trypanosoma brucei gambiense* (both IL2343 and Wellcome strains), human and dog genomic DNAs. In addition, the LAMP assay reliably yielded positive reactions from 2.0-mm-diameter punch of an FTA card spotted with cutaneous lesion-materials of the experimentally-infected mouse (data not shown). These observations indicated the robustness and efficiency of the present LAMP assay in amplification of the target DNA from FTA card templates. The structures of the LAMP amplicons were confirmed by direct sequencing.

We further evaluated the field- and clinic- applicability of the developed FTA-LAMP using 122 clinical samples, and LAMP amplicons were detected in 71/122 (58.2%) samples. Overall, the results indicated that the FTA-LAMP was as efficient as the nested PCR using an FTA card as a template (Kato et al., 2010). The accuracy of the LAMP amplicons was confirmed by direct sequencing.

Particularly important is the fact that in this study LAMP could reliably amplify *Leishmania* DNA directly from FTA card templates. Studies have shown that PCR has lower sensitivity for

amplification of DNA on FTA cards compared to LAMP (Kuboki et al., 2003); it is suggested to be due to PCR inhibitors in blood components, which does not affect the *Bst* DNA polymerase used in the LAMP assay. Recently, we showed that LAMP can amplify *Leishmania* DNA from crude sand fly templates (Nzelu et al., 2014), and previous reports have also shown superior tolerance of LAMP for biological substances (Poon et al., 2006; Kaneko et al., 2007). To our knowledge, this is the first time tissue materials spotted on FTA cards have been used for diagnosis of leishmaniasis by LAMP assay. Generally, the use of FTA card as a DNA template is critical, especially when parasite density is very low; the probability of a punch containing a parasite DNA is very low since parasite DNA is not evenly spread across the FTA matrix, but localized in areas where parasites in the tissue materials are fixed. In addition to assay sensitivity, to obtain a successful detection, careful attention is necessary during sample collection to avoid spotting of diluted or blood-containing materials onto FTA cards, which reduces the density of parasites on the cards.

The closed colorimetric MG detection system is highly sensitive, which enables visual discrimination of results by the naked eye without the aid of any specialized instrument and UV illuminator or ordinary light (Nzelu et al., 2014). Pre-addition of MG eliminates the openings of tubes and completely avoids contamination problems. Collectively, the use of FTA cards for direct sample collection and storage without the need for a cold chain, while MG dye which does not require freezing for storage (Nzelu et al., 2014) coupled with the LAMP reagents reported to be stable at 25 and 37 ºC for 30 days (Thekisoe et al., 2009), indicates the assay's potential as a field diagnostic tool deployable in developing countries. Furthermore, since the described LAMP assay is not species-specific, as the primers can amplify both Old and New World *Leishmania*

219 species (Nzelu et al., 2014), the developed universal-LAMP assay allows for the screening and
220 detection of multiple species of the genus *Leishmania* in endemic areas.

221 In conclusion, this study provided a rapid, simple and highly sensitive colorimetric LAMP
222 assay combined with an FTA card as a direct sampling tool for diagnosis of leishmaniasis. The
223 established LAMP assay is in line with the recent global trend in seeking rapid, point-of-care tests
224 for the control of infectious diseases. The simplicity, rapidity and sensitivity of the LAMP assay
225 make it an ideal routine diagnostic tool that will strongly support the cooperation of patients and
226 clinicians in endemic areas. With such advantages, the established FTA-LAMP assay will be a
227 good molecular tool for active screening and diagnosis of leishmaniasis in endemic areas.

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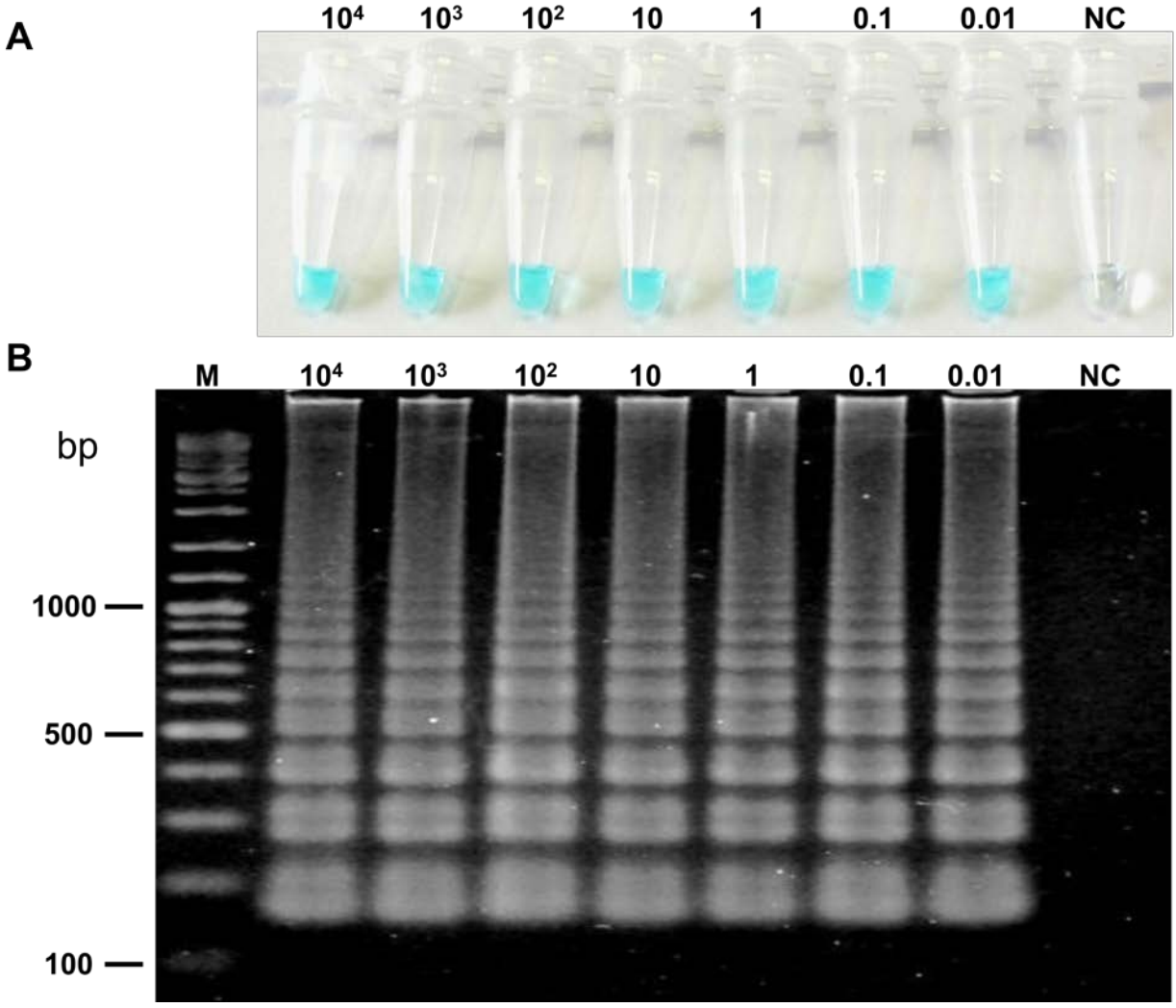
FIGURE LEGEND

FIG. 1. Colorimetric FTA-LAMP detection sensitivity. Genome DNAs from 10-fold serially diluted *Leishmania* (*Leishmania*) *mexicana* (MNYC/BZ/1962/M379) applied directly onto FTA cards were used as templates. (A) Malachite green based FTA-LAMP sensitivity reaction products inspected by the naked eye without a UV illuminator or ordinary light immediately after incubation. Light blue: positive reactions; colorless: negative reaction. (B) Sensitivity of the FTA-LAMP studied by agarose gel electrophoresis. bp: base pair; M: gene ruler; NC: negative control.


A
10⁴
10³
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1
0.1
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NC
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10⁴
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1
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0.01
NC
bp
1000
500
100

Fig. 1