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Sensitive, Specific, and Rapid Detection of *Leishmania donovani* DNA by Loop-Mediated Isothermal Amplification

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Abstract. We have applied a loop-mediated isothermal amplification (LAMP) technique to detect *Leishmania donovani* DNA. The LAMP technique detected 1 fg of *L. donovani* DNA, which was 10-fold more sensitive than a conventional polymerase chain reaction (PCR). All nested PCR–positive blood samples from visceral leishmaniasis patients were positive with the LAMP technique, and DNA samples from *L. infantum*, *L. major*, *L. mexicana*, *L. tropica*, *L. braziliensis*, *Plasmodium falciparum*, and healthy humans were negative with the LAMP technique. The advantages of the LAMP method are its shorter reaction time, a lack of requirement of sophisticated equipment, and visual judgment of positivity based on the turbidity of reaction mixture. Our LAMP technique can be a better alternative to a conventional PCR, especially under field conditions.

INTRODUCTION

Visceral leishmaniasis (VL) is caused by an intracellular protozoan parasite of *Leishmania donovani* complex, and considered as one of the most neglected diseases.[1] Ninety percent of VL cases occur in just five countries: Bangladesh, India, Nepal, Sudan, and Brazil.[2] There are an estimated 500,000 new cases of VL and more than 50,000 deaths from the disease each year.[3] The definitive diagnosis of VL or post-kala-azar dermal leishmaniasis cases is essential for providing individual treatments and understanding the disease epidemiology. A definitive diagnosis is usually made by detecting parasites in aspirates from the spleen or other tissues (such as bone marrow, lymph nodes, or skin), parasite DNA in the tissue and blood samples, and, with lesser specificity, parasite antigen or antibody in blood or urine.[4–6] Although the demonstration of parasites is most specific, the techniques are invasive and require skilled personnel and proper facilities, and the sensitivity of bone marrow aspirates has been reported to be variable.[4,7]

In the past decade, polymerase chain reaction (PCR)–based techniques have been used more in the diagnosis of leishmaniasis.[8,9] To avoid invasive procedures, peripheral blood is often used, and the reported sensitivity of PCR with blood ranged from 70% to 96%.[4,8–10] However, PCR requires a well-established laboratory and equipment such as a thermal cycler and a system to detect and analyze amplicons.

More recently, loop-mediated isothermal amplification (LAMP) was developed as a novel method to amplify DNA with rapidity and high specificity under an isothermal condition.[11,12] The method consists of incubating a mixture of a target gene, four different primers, *Bst* DNA polymerase, and substrates for 1 hour at 60–65°C by using basic equipment such as a heat block or water bath. Moreover, because LAMP reactions cause turbidity in the reaction mixture proportional to the amount of amplified DNA, identification of positive or negative results is easily to make visually.[13] A recent report that LAMP successfully amplified DNA of *Plasmodium falciparum* directly from heat-treated blood suggests further simplification and cost savings of the method.[14]

In the present study, we report a highly sensitive and specific LAMP assay to detect *L. donovani* kinetoplast DNA and its application to blood samples from patients with VL. The LAMP assay was compared with conventional and nested PCRs.

MATERIALS AND METHODS

Parasite DNA. Promastigotes of *L. donovani* strain DD8, isolated from a patient in Bangladesh, were used in this study.[15] Promastigotes of *L.* (*L.*) *mexicana* (MNYC/BZ/62/M379), *L.* (*L.*) *major* (MHOM/SU/73/5ASKH), *L.* (*L.*) *infantum* (MHOM/TN/80/I-PT1), *L.* (*L.*) *tropica* (MHOM/SI/74/K-27), *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2904), and schizont/trophozoite–rich cultured *Plasmodium falciparum* NF54 strain (kind donation by Professor Takafumi Tsuboi, Ehime University, Ehime, Japan) were also used. DNA of each species was extracted by the phenol extraction method.

Clinical samples. Venous blood samples were collected from 10 patients with VL confirmed by spleen biopsy at Rajshahi Medical College Hospital in Bangladesh. The blood (1 mL) was mixed with an equal volume of AL buffer (Qiagen, Valencia, CA) and kept at room temperature until it was processed at Aichi Medical University, Japan, within 1 month. DNA was extracted by using the QIAamp DNA Blood Midi Kit (Qiagen) according to the manufacturer's protocol.

This study was reviewed and approved by the Ethical Committee of Aichi Medical University School of Medicine, Japan, and the Ethical Review Committee of the Bangladesh Medical Research Council. Blood samples were collected after obtaining informed consent.

Loop-mediated isothermal amplification. The LAMP reaction was carried out according to the original reports described by Notomi and others[11] and Nagamine and others.[12] A set of four primers was designed specific for *L. donovani* kinetoplast minicircle DNA (GenBank accession no. Y11401) using Primer Explorer software (http://primerexplorer.jp/). It was not possible to design all four primers for the conserved

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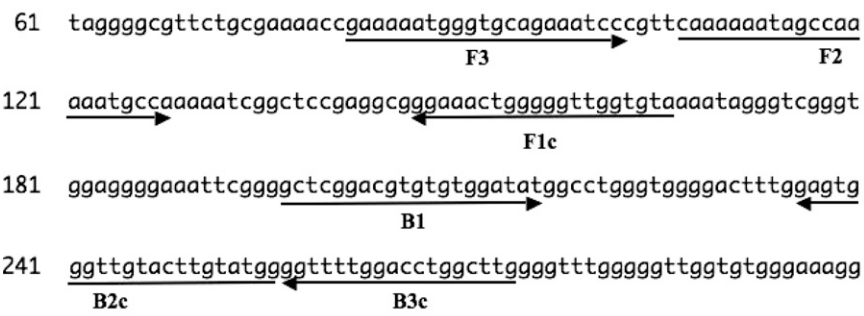
A



B

Name of primers	Sequence
FIP	5′-tacaccaacccccagtttcc-caaaaaatagccaaaaatgcca-3′
BIP	5′-gctcggacgtgtgtggatat-ccatacaagtacaacccactc-3′
F3	5′-gaaaaatgggtgcagaaatcc-3′
B3c	5′-caagccaggtccaaaacc-3′

FIGURE 1. Primer set used for amplification of *Leishmania donovani* kinetoplast minicircle DNA by the loop-mediated isothermal amplification technique **A**, Locations of the primer sequences. **B**, Names and sequences of four primers. Primer FIP consists of F1 complementary sequence and F2 direct sequence. Primer BIP consists of B1 direct sequence and B2 complementary sequence.

region because of its short length; thus, two primers were designed for the variable region. The locations of targeted sequences are shown in Figure 1A.

The LAMP reaction was performed in 25 μL of reaction mixture containing 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3c primers, 1.4 mM of each deoxynucleoside triphosphate, 0.8 M betaine, 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% TritonX-100, 8 units of *Bst* DNA polymerase large Fragment (New England Biolabs, Ipswich, MA), and 2 μL of sample DNA. The mixture was incubated at 65°C for 50 minutes in a heat block. As the LAMP reaction progresses, the reaction by-product (pyrophosphate ions) binds to magnesium ions and forms a white precipitate of magnesium pyrophosphate, making the reaction fluid turbid. After incubation, the turbidity was inspected visually. For further confirmation, 3 μL of the LAMP products was subjected to electrophoresis with a 100-basepair DNA ladder (New England Biolabs) on a 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide (5 μg/mL).

Conventional PCR and nested PCR. The conventional PCR was performed according to the report of Salotra and others.[16] The primers used had the sequences 5′-AAATCG GCTCCGAGGCGGGAAAC-3′ and 5′-GGTACACTCTAT CAGTAGCAC-3′. The reaction mixture in a total volume of 50 μL contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer, and 1.25 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The products were analyzed by electrophoresis on a 2% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA). The gels were stained with ethidium bromide and photographed under ultraviolet illumination.

To augment the sensitivity and specificity, a nested PCR was performed according to the report of Sreenivas and others.[17] The primers used had the sequences 5′-TCGGAC GTGTGTGGATATGGC-3′ and 5′-CCFATAATATAGTAT CTCCCG-3′. These primers amplified a 385-basepair fragment internal to the 592-basepair product of the first PCR. The nested PCR used 1 μL of the diluted (1:10) product from the first PCR under the same conditions as the first PCR, except for the primers.

RESULTS

Sensitivity of the LAMP. A set of oligonucleotide primers designed for LAMP reaction in *L. donovani* kinetoplast DNA amplified the targeted sequences (Figure 1). To estimate the sensitivity, serially diluted *L. donovani* DNA samples containing 100 pg to 1 fg were examined. Within these concentrations, the amplification product was detected in all samples (Figure 2A and B). Conversely, with conventional PCR, the 1-fg sample was too faint to be judged positive or negative (Figure 2C, lane 6). The nested PCR showed a positive reaction in 100-pg to 1-fg samples (Figure 2D). To assess further the sensitivity of the LAMP, 20–25 aliquots of 1-fg and 100-pg DNA samples were tested. With 1 fg, 20 (80%) of 25 aliquots were positive. With 100 pg, 1 (5%) of 20 were positive. In a similar experiment using a conventional PCR, 1 (7%) of 15 test results was positive with 1 fg of parasite DNA. The nested PCR amplified 11 (55%) of 20 samples with 1 fg, but none of 20 with 100 pg. These results indicated that our LAMP was more sensitive than the nested PCR.

Specificity of the LAMP. To evaluate the specificity of the LAMP reaction, DNA samples from five other *Leishmania* species (*L. infantum*, *L. major*, *L. mexicana*, *L. tropica*, and *L. braziliensis*) were examined. When 100 ng of DNA was used for each *Leishmania* sample, the amplification product was not detected (Figure 3A). The same result was obtained with the nested PCR (Figure 3C). Several amplification products were detected with the conventional PCR, but they had unexpected sizes (Figure 3B, lanes 2–6). DNA from *P. falciparum* NF45 strain (100 ng) or human genomic DNA samples (500 ng each) from 11 healthy persons were also examined by LAMP; all showed negative results. These results showed that the LAMP was specific for detection of *L. donovani*.

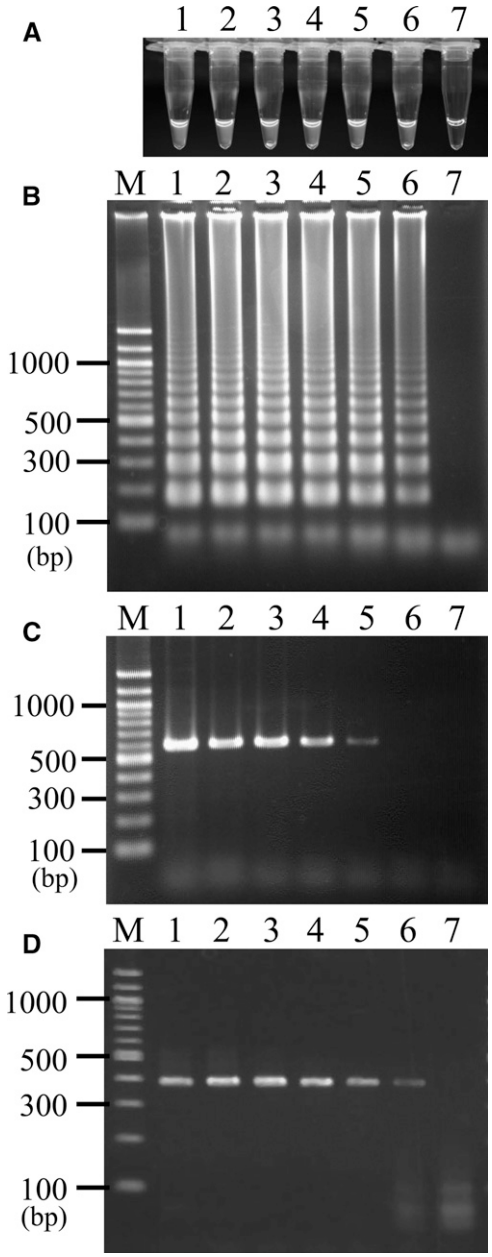


FIGURE 2. Sensitivity of loop-mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR) for detection of *Leishmania donovani* DNA. **A**, Turbidity of reaction fluid produced by LAMP. **B**, Agarose gel electrophoresis of LAMP products, **C**, Conventional PCR producing a 592-basepair fragment. **D**, Nested PCR producing a 385-basepair fragment. Lane M, 100-basepair ladder; lanes 1, 2, 3, 4, 5, and 6, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg of DNA, respectively; lane 7, water (negative control).

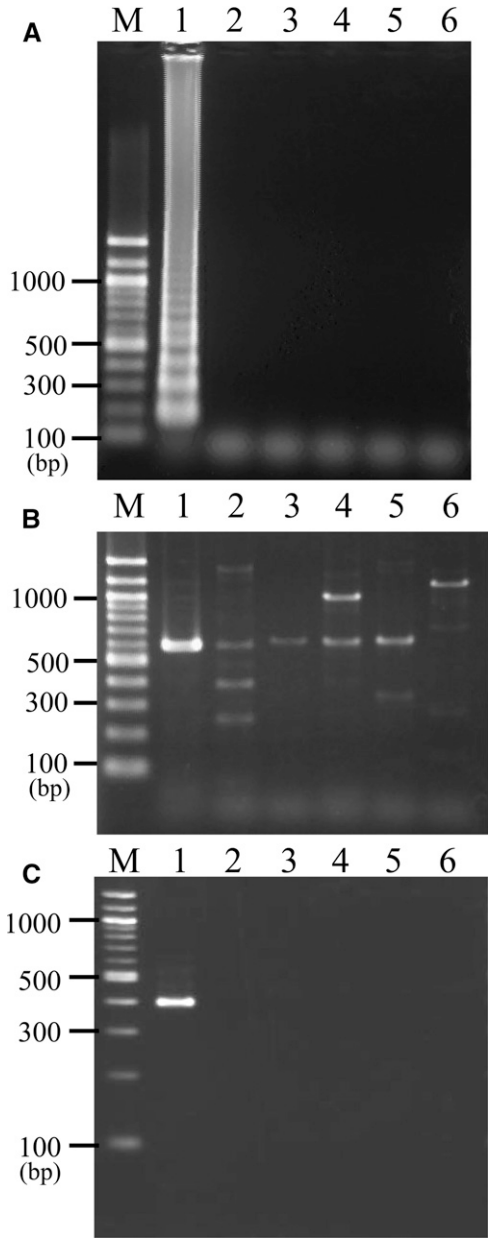


FIGURE 3. Specificity of loop-mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR) for detection of *Leishmania donovani* DNA studied by agarose gel electrophoresis. **A**, LAMP. **B**, Conventional PCR. **C**, Nested PCR. Lane M, 100-basepair DNA ladder; lane 1, *L. donovani*; lane 2, *L. infantum*; lane 3, *L. major*; lane 4, *L. mexicana*; lane 5, *L. tropica*; lane 6, *L. braziliensis*.

Detection of *L. donovani* DNA in blood samples from patients with VL. Blood samples from 10 parasitologically confirmed patients with VL were examined by LAMP, conventional PCR, and nested PCR. Eight of 10 samples showed positive results with the LAMP (Figure 4A) and nested PCR (Figure 4C). Seven of the eight samples with positive results also showed positive results with the conventional PCR (Figure 4B). The DNA fragments seen in lanes 2 and N (negative control) at approximately 600 basepairs (Figure 4B) were nonspecific products, which was confirmed by DNA sequencing. These results indicated that the LAMP was as sensitive as the nested PCR for detecting *L. donovani* in blood samples.

DISCUSSION

Detection of DNA of a pathogenic agent by PCR is a definitive breakthrough in diagnosis. The LAMP assay reported by Notomi and others[11] is a simple and rapid DNA amplification technique based on a unique primer design and does not require a denaturation step during amplification. The new technique was applied to detect DNAs of protozoans such as *Trypanosoma* species and *P. falciparum*.[14,18,19] In this study, we developed a LAMP assay that could detect 1 fg of *L. donovani*

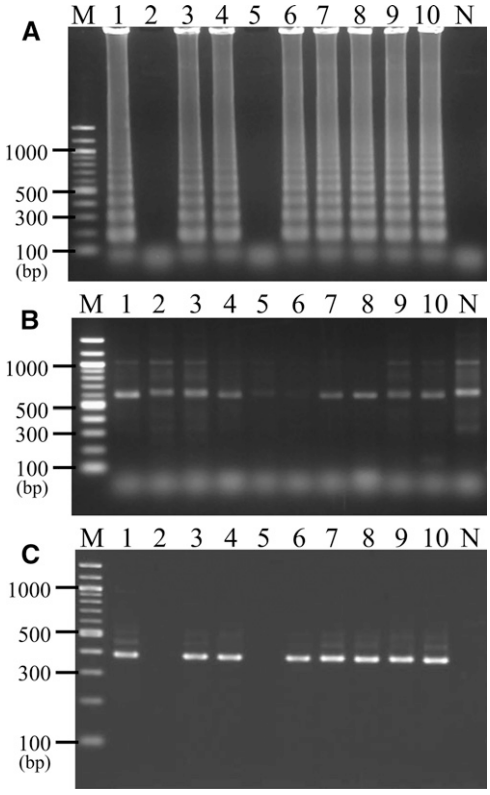


FIGURE 4. Diagnosis by loop-mediated isothermal amplification (LAMP), conventional polymerase chain reaction (PCR), and nested PCR with blood samples from confirmed patients with visceral leishmaniasis. **A**, LAMP. **B**, Conventional PCR. **C**, Nested PCR. Lane M, 100-basepair DNA ladder; Lanes 1–10, DNA sample from each patient; Lane N, DNA sample from a healthy human (negative control).

DNA from cultured promastigotes. The 1-fg amount is equivalent to approximately 0.1 parasites.[16] This high sensitivity could be achieved because of the presence of kinetoplast minicircle DNA (thousands of copies per cell).[20]

The specificity of LAMP is generally high because it uses four primers that recognize six locations on sample DNA.[11] In our study, 100 ng of DNA from each of five other species of *Leishmania* (*L. mexicana*, *L. major*, *L. infantum*, *L. tropica*, and *L. braziliensis*), 100 ng of *P. falciparum* DNA, or 500 ng of human genomic DNA all showed negative results in the LAMP assay. We designed FIP and F3 primers specific for the conserved region and BIP and B3c primers specific for the variable region of kinetoplast minicircle DNA.[16,21,22] Specificity for these regions might contribute further to the high specificity of the LAMP assay. However, our LAMP assay will need further confirmation with more clinical samples from different disease-endemic areas, particularly in relation to differences in *L. donovani* strains. The negative results of our PCR with *L. infantum* are in contrast to those of another report,[16] even though the same primer set was used (Figure 3B). Different strains of *L. infantum* used in the two studies might have some relevance to the differences observed.

Among 10 blood samples from human patients with VL confirmed by spleen biopsy, 8 (80%, 95% confidence interval = 55–100%) were positive by LAMP, and the same result was obtained by nested PCR. Although the sensitivity was not high, the present study showed comparable efficiency of the LAMP to the nested PCR, which has been generally accepted to have much higher sensitivity than the conventional PCR.[17,23] Unfortunately, there is no report that evaluated the nested PCR with blood samples for the diagnosis of *L. donovani* infection.[8]

As for the sensitivity of the conventional PCR with blood for the diagnosis of VL, a range of 68.8% to 100% was reported in 24 papers.[8] The discrepancy was suugested to be caused by differences in the volume of blood used,[24] the stage of disease,[24,25] parasite species/strains,[24] and past treatment.[24] In addition, sensitivity could be influenced by immunologic status and age of the hosts and endemicity levels of their habitats.[8]

The presence of PCR inhibitors in a sample was also reported,[26] and the use of blood samples spotted on a filter paper might negatively influence results.[16,27] In this regard, Kaneko and others.[28] reported that the sensitivity of LAMP was less affected by contamination with serum, plasma, or other inhibitory components in clinical samples of DNA samples than the sensitivity of the PCR.

We have developed a sensitive, urine-based, enzyme-linked immunosorbent assay (ELISA) suitable for the diagnosis of VL in the field in Bangladesh.[6] The LAMP could be applied to ELISA-positive urine samples to identify active infection. With a high sensitivity, LAMP would also be useful in detecting *L. donovani* DNA in skin lesions of patients suspected of having post-kala-azar dermal leishmaniasis.

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