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Original Article

Development and Assessment of Loop-Mediated Isothermal Amplification (LAMP) Assay for the Diagnosis of Human Visceral Leishmaniasis in Iran

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Abstract

Background: Parasitological methods for the diagnosis of Visceral leishmaniasis (VL) require invasive procedures, so serological and molecular approaches have been developed but are not generally applicable in the field. We evaluated a loop mediated isothermal amplification (LAMP) assay using blood from VL patients and compared it to nested PCR.

Methods: Forty-seven subjects with clinical features (fever, hepatosplenomegaly and anemia) were confirmed positive for VL by the direct agglutination test (DAT) at titers >3200. Forty DAT negative individuals from non-endemic areas with no clinical signs or symptoms of VL served as controls. A LAMP assay was performed using a set of six primers targeting *Leishmania infantum* kinetoplast DNA (kDNA) minicircle gene under isothermal (64 °C) conditions. For nested PCR we used primers targeting the kDNA minicircle gene.

Results: The LAMP assay provided a detection limit of 1 parasite in 1 ml of peripheral blood and detected *L. infantum* DNA in 44 of 47 DAT-confirmed VL cases, with diagnostic sensitivity of 93.6% (95% CI). No *L. infantum* DNA was amplified in controls, indicating a specificity of 100%. The nested PCR yielded sensitivity of 96% (95% CI) and a specificity of 100% (95% CI).

Conclusion: The LAMP assay gave results similar to those of nested PCR but in a shorter time. The LAMP method is simple; requires no sophisticated equipment; has a short reaction time; and results, indicated by turbidity of the reaction mixture, are observable with the naked eye.

Introduction

Visceral leishmaniasis (VL) is a major public health problem in Iran, with endemic areas including Ardabil and East Azerbaijan in the northwest and the provinces of Fars and Bushehr in the south (1). The disease is frequently fatal if untreated, with a worldwide incidence of almost 500 000 and 50 000 deaths annually (2). Early definitive diagnosis of VL is of crucial to the initiation of therapy to prevent severe complications. The parasitological techniques conducted on bone marrow aspirates that are generally used for detection and identification of VL lack sensitivity (microscopy) or are time consuming (culture). They require invasive procedures and are difficult to repeat for patient follow-up. Serological methods such as the Direct Agglutination Test (DAT) have been used successfully for the diagnosis of VL (3-4). Direct Agglutination is appropriate for the detection of VL in immunocompetent patients when large numbers of specific antibodies are present but has a low sensitivity in immunocompromized patients, including HIV-infected, and is of limited value in evaluating treatment effectiveness, since the antibodies remain detectable for an extended time after clinical cure (5-6). Antigen detection in urine through the latex agglutination test (KAtex) has been suggested as a tool for diagnosis of VL, but several studies have reported poor to moderate sensitivity (7-10). Molecular methods based on DNA amplification, such as nested PCR and real-time quantitative PCR, have been developed and evaluated as potential tools for rapid and sensitive detection of leishmaniasis (11-13). To avoid invasive procedures, peripheral blood and buffy coat is often used. Reported sensitivity levels of PCR with blood have ranged from 70% to 96% (11, 14-16). However, cost and the necessity for sophisticated laboratory equipment and skilled technicians render this technology inappropriate for routine diagnosis in hospital laboratories and under field conditions in areas where

VL is endemic. More recently, a rapid, simple, and sensitive technique called loop-mediated isothermal amplification (LAMP) was developed (17). The assay has been used for detection of bacterial, viral, protozoan, and fungal diseases (18-21), and used successfully for the diagnosis of several parasitic infections (22-24).

The goal of this study was to use the kinetoplast DNA (kDNA) region to design LAMP primers for detection of *Leishmania infantum* DNA in human peripheral blood samples and compare the results to those of nested PCR. This is the first report of the LAMP assay to detect *L. infantum* DNA in human peripheral blood samples in Iran.

Materials and Methods

Ethical approval

This study was reviewed and approved by the Tehran University of Medical Sciences Ethics Committee and all subjects provided written informed consent.

Patients and controls

Peripheral blood samples were obtained from 47 patients at the Tehran University of Medical Sciences Hospitals (Children's Medical Centers, Aliasghar Child Hospital, Bahrami Children's Hospital) with confirmed VL. The patients presented characteristic clinical symptoms of fever, hepatosplenomegaly, and anemia consistent with infection in bone marrow aspirate detected by microscopy (n=22) and serology (DAT \geq 3200). Thirty individuals from non-VL endemic areas with no clinical signs or symptoms of VL and negative DAT results, as well as 10 patients exhibiting non-VL infectious diseases (cutaneous leishmaniasis, malaria, tuberculosis, toxoplasmosis, hepatitis, herpes virus) constituted controls.

Microscopy

Bone marrow samples were obtained from VL patients and smears were prepared on glass slides, dried, fixed in methanol, stained

with Giemsa in PBS, and examined microscopically at 1000 x for *Leishmania* sp.

Serology

Leishmania antibodies were detected by DAT (1, 3). DAT antigens were prepared from *L. infantum* promastigotes at the Protozoology Unit of the School of Public Health at Tehran University of Medical Sciences. Sera were diluted with 0.9% saline containing 0.8% mercaptoethanol. Initially, for screening purposes, two dilutions of 1: 800 and 1: 3200 were made and tested. If both of these two dilutions were positive, the samples with titers 1: 800 were diluted further to give end-point titers of 1: 102400. Negative control wells (antigen only) and known negative and positive controls were tested in each plate daily. Fifty μ l of antigen was added to wells containing 50 μ l of diluted serum and incubated overnight. The cut-off value was defined as the highest dilution at which agglutination was visible as a blue dot compared to negative control wells, which had clear blue dots. Specific *Leishmania* antibodies \geq 1:3200 were considered positive.

DNA preparation

The DNA template for the nested PCR and LAMP assay was extracted from the buffy coat layer obtained from 2-5 ml whole peripheral blood collected in EDTA using the QIAamp DNA Blood mini kit (Qiagen, Courtaboeuf, France) according to manufacturer's instructions. Following centrifugation and washing, DNA was eluted from the spin column in 100 μ l elution buffer and stored at -20 °C before use in the PCR-based assay. The concentration and purity of extracted DNA were assessed by the ratio of the optical density at 260 and 280 nm. Five- μ l and 2 μ l portions of the extracted DNA were used for nested PCR amplification and LAMP assay, respectively.

Nested PCR-based Assay

For nested PCR, the specific nucleotide sequences of variable segments of kDNA minicircles from *L. infantum* were amplified as described by Noyes et al. with slight modifica-

tion (25). Each 25 μ l first-round reaction mixture contained 5 μ l template DNA, 0.2 mM each dNTPs (Roche, Penzberg, Germany), 1.5 mM MgCl₂, 1.0 U Taq polymerase, 50 mM Tris-HCl (pH 7.6), and 20 pmol of each of CSB1XR and CSB2XF primers (first-round primer). The DNA amplification was carried out under the following conditions: 95 °C for 5 min; 35 cycles of 30 s at 95 °C, 1 min at 59 °C, and 45 min at 72 °C; and a final extension of 10 min at 72 °C. The first PCR product was diluted 10-fold in ultrapure water. One μ l was used as a template for the second amplification, under the same conditions and reaction mixture as the first round, except that LiR and 13Z (second-round primer) were used as the primers. The amplified products of the second round were visualized in 2% agarose gels stained with ethidium bromide. The DNA purified from promastigote cultures of the reference strain of *L. infantum* (MHOM/TN/80/IPT1) were amplified in each PCR experiment and used as positive control. Negative controls (the products of PCR in which ultrapure water replaced the template DNA) were also run. An internal control (β -glubulin housekeeping gene) was used to ensure that negative results were genuine and did not reflect PCR inhibition, DNA loading, sample degradation, or absence of human cells.

Loop-mediated isothermal amplification (LAMP)

A set of six oligonucleotide primers were used for the LAMP assay, targeting eight regions in the sequence of *L. infantum* kDNA (GenBank accession no. Z35271). The outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), and loop forward (LF) and backward (LB) primers were designed using the online Primer Explorer V4 software (Eiken Chemical Co. Ltd., Tokyo, Japan-<http://primerexplorer.jp/elamp4.0.0/index.html>). kDNA-specific LAMP primers were designed according to the general criteria de-

scribed by Notomi et al. (17). The location and nucleotide sequences of all primers are shown in Fig. 1 and Table 1, respectively. The LAMP reaction contained the following components: 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, 20 mM Tris-HCl (pH 8.8), 10mM KCl, 10 mM (NH₄)₂ SO₄, 8 mM MgSO₄, 0.8 M betaine (Sigma-Aldrich), 2mM each of deoxynucleo-

side triphosphate, 0.1% TritonX-100 (Roche Applied Science), 8 units Bst DNA Polymerase, Large Fragment (New England Biolabs) and 2 µl extracted nucleic acid in a total volume of 25 µl. The reaction was incubated at 64 °C for 90 min in a heat block for amplification and held at 90 °C for 2 min to inactivate the reaction.

Table 1: Primer sets used for amplification of kDNA genes in LAMP assay for *L. infantum*

Primer	Type	Length mer	Sequence (5'-3')
FIP(F1C+F2)	Forward Inner primer	38	GTGGTCTGGGTAGTGGCTT-GGTT-GGATTGGCCTGAAAC
BIP(B1C+B2)	Backward Inner primer	36	TGCGCCTTGGACTTTCGG-TAATGTTGGGCACATGCT
F3 (FOP)	Forward outer primer	19	TGGTTGAAAATTGGCTCTC
B3 (BOP)	Backward outer primer	22	GATTGAGTTGGATTCAATTGACG
FLP	Forward Loop primer	20	CTGGGTCTGGAGGCTTTATT
BLP	Backward Loop primer	22	GGCTTTTGTGGGATTTTGTG

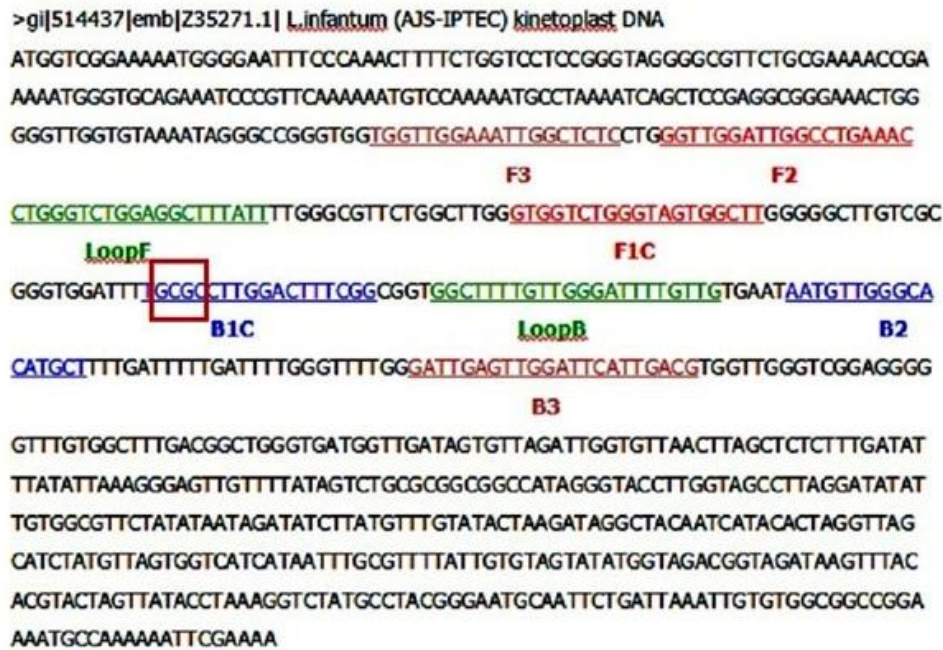


Fig. 1: Location and sequence of LAMP targets and priming sites in the *L. infantum* reference sequence (GenBank accession no. Z35271). Forward outer primer (FOP) and backward outer primer (BOP) are respectively homologous to the sequences of F3 and B3 regions of the target. Forward inner primer (FIP) contains the sense sequence of F2 region at the 3' end directly linked to the F1c (complementary sequence to F1 region) at the 5' end, and backward inner primer (BIP) contains the sense sequence of B2 region at the 3' end directly linked to the B1c (complementary sequence to B1 region) at the 5' end. Forward loop primer (FLP) and backward loop primer (BLP), respectively, contain the sequence complementary to a part of sequence between F1 and F2 and between B1 and B2 regions. The red box indicates the Hha I cutting site

Determination of analytical sensitivity and specificity of LAMP assay

The sensitivity of the LAMP assay was determined by spiking a defined number of cultured promastigotes of *L. infantum* (MHOM/TN/80/IPT1) into a negative *Leishmania* blood sample. Ten-fold serial dilutions ranging from 100 000 to 1 parasites per ml blood were used. After extraction of nucleic acid, the dilutions were amplified by both LAMP and nested PCR, and products were visualized by 2% agarose gel electrophoresis. The species specificity of the LAMP primers was determined by testing against DNA of *L. tropica* and *L. major* and non-leishmanial infections of malaria, toxoplasmosis, tuberculosis, and HBV, HCV, HSV, and CMV.

Clinical sensitivity and specificity

The clinical sensitivity and specificity of the LAMP assay were calculated using 47 whole-blood samples and DAT as the reference standard. Due to the agreement between the DAT and BM results, DAT was considered the gold standard. Sensitivity was calculated as the (number of true positives)/(number of true positives+number of false negatives), and specificity was calculated as (number of true negatives)/(number of true negatives+number of false positives). Sensitivity of LAMP and nested PCR were compared with McNemar's test using SPSS16 software.

Results

Detection and confirmation of LAMP products

The LAMP reaction causes turbidity, observable with the naked eye, in the reaction tube proportional to the amount of amplified DNA, which is linked to the production of insoluble salt of magnesium pyrophosphate (Fig. 2A). Positive reactions turned green upon addition of SYBR Green I, while negative reactions remained orange (Fig. 2B). For further confirmation, the LAMP products were

subjected to electrophoresis on 2% agarose gel containing 0.5 µg/ml ethidium bromide and visualized under UV light. A positive result was defined as the appearance of a typical ladder-like pattern on the gel (Fig. 3).

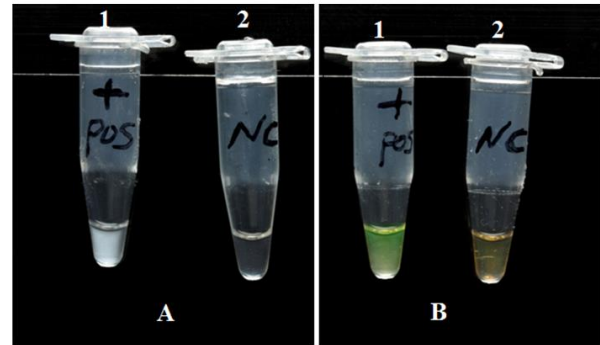


Fig. 2: Visual endpoint detection method of LAMP reaction. **(A)** Assessment of LAMP reaction by turbidity. 1A, with template DNA (positive); 2A, without template (negative). **(B)** Detection of LAMP reaction by fluorescence using SYBR Green I. 1B, with template (positive, color of reaction mixture changes from original orange to bright green); 2B, without template (negative, color of reaction mixture remains unchanged)

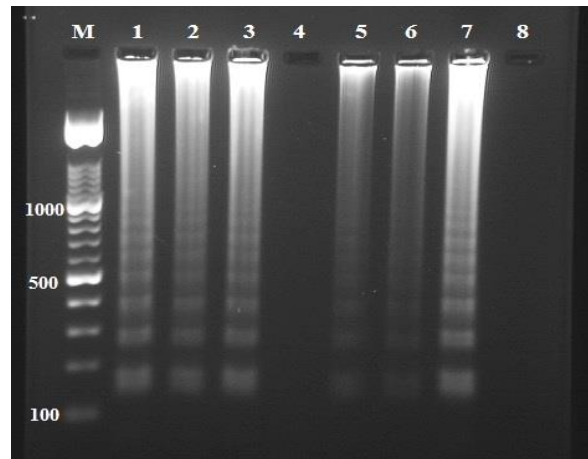


Fig. 3: Detection of *L. infantum* DNA by loop-mediated isothermal amplification (LAMP) . Lane M, 100 bp DNA ladder marker (Roche Applied Science); lanes 1–3, 5 and 6, DNA sample from each patient; lane 7, *L. infantum* positive control, lanes 4 and 8, DNA sample from a healthy human and PCR grade water (negative control), respectively

This pattern is due to the formation of an assembly of stem-loop DNAs with cauliflower-like structures of various stem lengths. The accuracy of the LAMP reaction was confirmed by digestion with restriction enzyme Hha I to ensure that the LAMP products exhibited the corresponding sequence of the kDNA gene of *L. infantum*. The size of fragments produced by digestion was in agreement with that predicted from the expected DNA structures: 76 and 121 bp (Fig. 4).

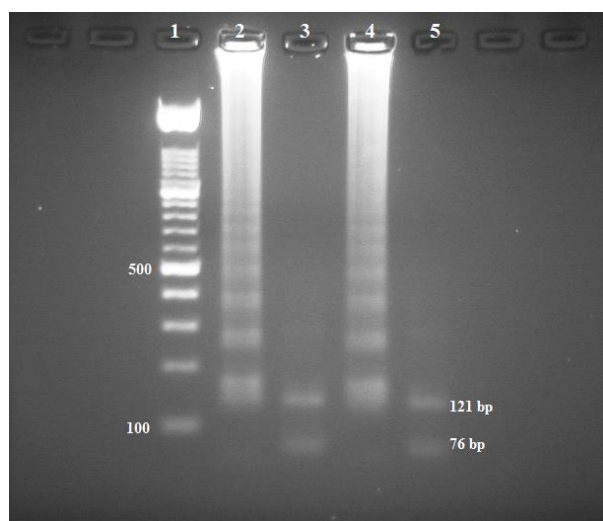


Fig. 4: Restriction enzyme analysis of the LAMP products amplified from the kDNA gene. The digestion products were run on a 3% agarose gel. Lane 1, 100 bp DNA ladder marker (Roche Applied Science); lanes 2 and 4 LAMP reactions performed on the DNA extracted from the clinical sample and *L. infantum* (MHOM/TN/80/IPT1), respectively; lanes 3 and 5, LAMP product of lanes 2 and 4, respectively, after digestion with Hha I (121- and 76-bp bands were expected)

Analytical sensitivity and specificity of LAMP assay

To estimate the sensitivity, serially diluted blood samples containing 1 to 100 000 *L. infantum* promastigotes per ml blood were examined. The detection limit of both LAMP and nested PCR was 1 parasite in a 1 ml blood (Fig. 5). To evaluate the specificity of the LAMP assay, reactions were also conducted for am-

plification of other *Leishmania* parasites as well as non-leishmanial infections. Non-target DNA was not amplified, showing the LAMP assay to be specific for detection of *L. infantum*. Results were similar to those obtained with nested PCR (data not shown).

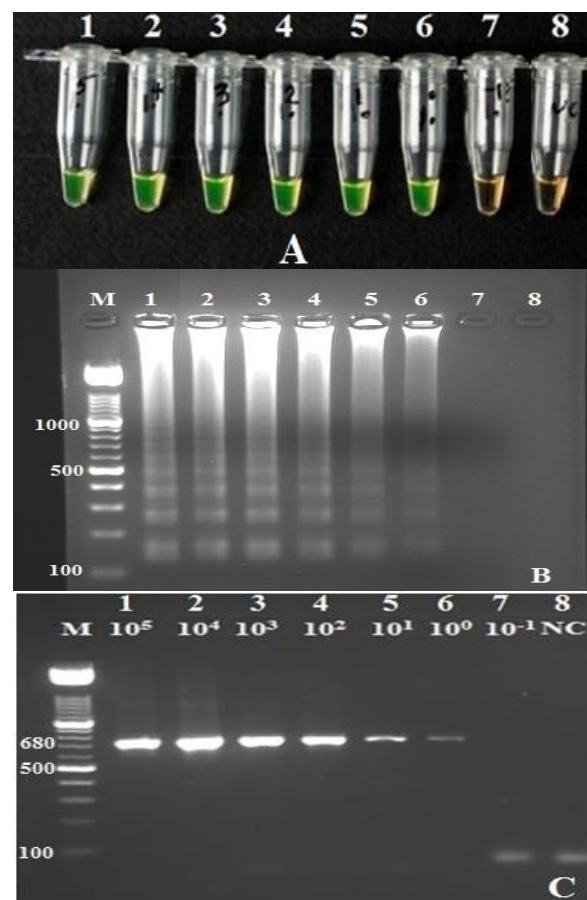


Fig. 5: Comparison of sensitivity of loop-mediated isothermal amplification (LAMP) to nested PCR. Lane M, 100 bp DNA ladder marker (Roche Applied Science); tubes and lanes 1-7, reactions contained 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , 10, and 10^{-1} cultured promastigote of *L. infantum* per 1 ml blood, respectively; lane 8, negative control (PCR grade water). **(A)** Detection limit of the LAMP reaction based on color change in SYBR Green I by the naked eye under visible light: The color of positive reactions (tubes 1-6) became bright green while the color of the negative reactions (tubes 7-8) remained unchanged (orange). **(B)** Detection limit of the LAMP reaction using electrophoresis of 2 μ l of each reaction mixture in 2% agarose gel fol-

lowed by ethidium bromide staining: lanes 1–6 showed the typical ladder-shaped pattern of a positive reaction. (C) Sensitivity of the nested PCR and producing a 680-basepair fragment

Detection of *L. infantum* DNA from blood samples from patients with VL

The LAMP products were examined in light and electrophoresed in agarose gels, and similar positive results were observed. Among 47 confirmed VL cases, 45 were positive with nested PCR and all controls were negative, resulting in a sensitivity of 96% (95% CI) and

specificity of 100% (95% CI). Forty-four of 47 VL cases were positive with LAMP, and all controls gave negative results, yielding a sensitivity of 93.6% (95% CI) and a specificity of 100% (95% CI). All samples positive by LAMP were also positive by nested PCR (Table 2). We compared the sensitivity and specificity of the LAMP assay with that of nested PCR using the McNemar test and found no significant difference ($P=0.31$). As all the controls were negative by both methods, the McNemar test was not performed for specificity.

Table 2: Comparison of loop-mediated isothermal amplification (LAMP) to nested PCR for diagnosis of *L. infantum*

Diagnostic assay	n (%) with Nested PCR			
		Positive	Negative	Total n. (%)
n (%) with LAMP PCR	Positive	42(89.3)	2(4.3)	44(93.6)
	Negative	3(6.4)	0(0)	3(6.4)
	Total n (%)	45(95.7)	2(4.3)	47(100)

Discussion

Accurate and sensitive procedures for diagnosis of VL are required for development of an effective program of control and treatment to reduce the morbidity and mortality rate. The LAMP assay reported by Notomi et al. (16) is a low cost, simple, and rapid DNA amplification technique based on a unique primer design. This technique has been used to detect DNA of protozoan's such as several African trypanosome species (18, 26) as well as *Malaria* (27), *Giardia* (28), *Cryptosporidium* (29), and tuberculosis (30). In this study, we developed a LAMP assay capable of detecting a single parasite in 1 ml blood, which corresponds to 0.025 parasites per reaction. This high sensitivity was achieved through the presence of 10 000 copies per cell (31-32). The specificity of LAMP is generally high, as it uses six primers for the recognition of eight distinct regions on the target DNA sequences (17). However, the LAMP assay will need further investigation with more samples from other disease-

endemic areas. In this study, peripheral blood samples collected from patients with confirmed VL ($n=47$) and from healthy individuals ($n=30$) were analyzed using LAMP and nested PCR assays simultaneously. Of 47 patients, 44 (93.6%) were positive by LAMP and 45 (95.7%) were positive by nested PCR. These results showed efficacy of the LAMP assay to be comparable to nested PCR, which is widely used for the diagnosis of leishmaniasis. Takagi et al. (22) reported 8 of 10 (80%) confirmed VL patients to be positive for parasite DNA with LAMP. Adam et al. (21) in a study of 30 confirmed VL patients, found diagnostic sensitivity of 83% and specificity of 98%. A recent LAMP-based assay for the detection of *Leishmania* DNA in buffy coat of blood from 75 confirmed VL patients reported sensitivity of 90.7% and specificity of 83% (33). In our study, we found a higher sensitivity of 93.6% and specificity of 100%. The LAMP developed in the present study demonstrated sensitivity and specificity similar to that of nested PCR for the detection of *L. infantum* parasites.

Although the sensitivity level of 93.6% falls slightly below the WHO requirement of >95% for an acceptable test, there are several advantages of the LAMP assay. Diagnosis by LAMP assay does not require a complicated thermal cycler, a turbidimeter, or skilled technicians. The assay is performed under isothermal conditions using a simple incubator such as a heat block or water bath to provide a constant temperature of 60-65 °C, making it more practical and economical than nested PCR. The time requirement for the procedure is also an advantage. Only 1.5 h was needed to perform the LAMP assay compared to 5 h for nested PCR. In addition, the LAMP assay is interpreted by observing turbidity due to magnesium pyrophosphate accumulation as a by-product of DNA amplification, which reduces time and cost compared with conventional PCR analysis. In all reactions, positive samples from negative samples simply, by the turbidity of the reaction mixtures. Alternatively, inspection for amplification can be performed with the naked eye by using SYBR green I, which turns green in the presence of amplified DNA. A major drawback of this assay is a high risk of production of secondary LAMP products. This is probably caused by the extremely high efficacy of the reaction. The risk can be reduced by changing gloves between assays and conducting preparation of the reagents and the detection of the products in separate areas.

Conclusion

LAMP is an assay that, for the first time, enables a molecular diagnosis of *L. infantum* where there are minimal facilities or under field conditions. The technique presents sensitivity and specificity similar to that of nested PCR, does not require sophisticated equipment, and is simpler and more cost-effective than nested PCR. To assess the on-site performance of LAMP for rapid diagnosis of VL

in the field further evaluation of this technique is necessary.

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