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Analytical Performance of a Loop-Mediated Isothermal Amplification Assay for *Leishmania* DNA Detection in Sandflies and Direct Smears of Patients with Cutaneous Leishmaniasis

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Abstract. Loop-mediated isothermal amplification (LAMP) is ideal for the detection of *Leishmania* DNA as it is a quick and easy-to-perform test that does not require complex or sophisticated equipment or infrastructure. However, the application of this technique in the detection of *Leishmania* DNA has not been comprehensively analyzed to date (analytical validation). Our objective was to evaluate the sensitivity and analytical specificity (anticipated reportable range [ARR], the limit of detection [LoD], and accuracy) of LAMP targeting the 18S rRNA gene in the diagnosis of six New World *Leishmania* species. We then applied the validated LAMP assay across 50 samples of sandflies and 50 direct smears from a recent outbreak of cutaneous leishmaniasis in Colombia to determine its diagnostic performance. The LAMP assay exclusively amplified the DNA of *Leishmania* spp., and an ARR of between 1×10^{4} and 1×10^{-2} equivalent parasites/mL was determined. An LoD of 1×10^{-2} equivalent parasites/mL was established and there was no statistically significant variation in terms of accuracy. Finally, a sensitivity of 100% in direct smears and sandflies samples was calculated and a specificity of 90.9% for direct smears using microscopy as reference and 96.8% for sandflies using real-time polymerase chain reaction as reference were determined. To our knowledge, this is the first attempt to analytically validate a LAMP test to detect *Leishmania* DNA, which showed good diagnostic potential from sandflies and direct smear samples.

INTRODUCTION

Leishmaniasis encompasses a group of neglected tropical diseases caused by parasites of the genus *Leishmania* that are transmitted by the bite of female sandflies from the Phlebotominae subfamily.[1] The spectrum of diseases can be classified into three clinical manifestations according to the associated signs and symptoms (cutaneous, mucocutaneous, and visceral leishmaniasis). Cutaneous leishmaniasis (CL) is considered the most common manifestation of the disease in the New World.[2] The disease is prevalent in 98 countries, with around 350 million people estimated to be at risk and around 12 million cases, with an annual incidence of 0.7–1.2 million cases of CL.[2,3] In the New World, leishmaniasis is endemic in many areas of North, Central, and South America, thus constituting a major public health problem.[2,4,5]

Molecular tools have been used for the diagnosis of leishmaniasis, owing to their high levels of sensitivity (40–92%) and specificity (57.1–100%).[6,7] However, the requirement for complex equipment and infrastructure means that molecular diagnostic techniques are not suitable for use in remote areas or in the search for active cases (field work). In 2000, Notomi et al.[8] designed a new technique known as loop-mediated isothermal amplification (LAMP). This method is characterized by its ability to amplify large amounts of DNA from a few copies in an average time of 30–60 minutes.[9] The reaction occurs under isothermal conditions without the need for thermal cyclers.[8] It has a high degree of sensitivity owing to the design and quantity of the primers used (i.e., two external primers, two internal primers, and two first loop primers).[10] Some studies have demonstrated the use of LAMP against extracts from macerated sandflies[8,11] and several alternatives exist for the visualization of the results including turbidity, fluorescence, and/or color changes.[8–10,12–14]

To date, LAMP has been used for the diagnosis of bacterial,[15] fungal,[16,17] and viral[18,19] infections, as well as parasitic infections such as those caused by *Trypanosoma brucei*,[20] *Plasmodium falciparum*,[21] and *Trypanosoma cruzi*.[22] For the diagnosis of leishmaniasis, LAMP has been used with the implementation of primers targeting the small ribosomal subunit (18S rRNA gene),[10,11,23] kinetoplast DNA (kDNA) for the detection of Old World *Leishmania* species,[24–26] and the Internal Transcribed Spacer 1 (ITS-1).[12] Loop-mediated isothermal amplification has also been used in the detection of parasites in their insect vectors, as in the case of *Dirofilaria immitis* detected in *Aedes aegypti*,[27] and *T. cruzi* and *Trypanosoma rangeli*.[28] Importantly, there has only been one report of the analytical validation of a LAMP assay for *T. cruzi*.[22]

Although LAMP platform-based tests targeting different markers (most frequently 18S rRNA gene) have shown to be efficient for the detection of *Leishmania* in biological samples, a comprehensive assessment of the sensitivity and analytical specificity of these tests to elucidate their diagnostic potential is lacking, especially in endemic regions and where several species coexist.[29] Therefore, the objective of this study was to determine, for the first time in *Leishmania*, the analytical specificity (exclusivity and inclusivity) and sensitivity (anticipated reportable range [ARR], limit of detection [LoD], and accuracy) of the LAMP assay for detecting 18S rRNA gene to evaluate its performance against sandflies and direct smears of CL lesions from a recent outbreak of CL in Colombia.

METHODS

Ethical statement. This project has a certificate of approval from the ethics committee of the National University of Colombia, number 002-010-15, issued on February 12, 2015. The patients included in this study signed a written informed consent.

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Reference strains. Promastigote cultures of the major *Leishmania* reference strains frequently associated with CL and mucocutaneous leishmaniasis in Colombia were donated by the International Center for Medical Research and Training that has a preexisting collection of *Leishmania* species (MHOM/BR/75/M2903 *Leishmania braziliensis*, MHOM/PA/71/LS94 *Leishmania panamensis*, MHOM/BR/75/M4147 *Leishmania guyanensis*, MHOM/TN/80/IPT1 *Leishmania infantum*, IFLA/BR/67/PH8 *Leishmania amazonensis* and MHOM/BZ/82/BEL21 *Leishmania mexicana*). These strains were cloned and maintained in Novy, Nicolle, and McNeal media or Schneider medium supplemented with 20% fetal bovine serum (Microgen, Bogotá, Colombia).

DNA extraction and serial dilutions. DNA extraction was performed according to the instructions of the High Pure PCR Template Preparation kit (Roche® Ref. 11796828001, Basel, Switzerland) from a stock that contained 1×10^5 parasite equivalents/mL (An average of 100 ng/μL was measured using Nanodrop equipment). DNA obtained from each reference strain was subsequently used to perform serial dilutions from 1×10^4 to 1×10^{-2} parasite equivalents/mL to determine the analytical performance of the LAMP test.

Loop-mediated isothermal amplification assays. Primers targeting the 18S rRNA gene, reported by Nzelu et al.[23] (Table 1), were used for the implementation of the LAMP test. The assays were performed at a final volume of 25 μL consisting of 40 pM of each internal primer forward inner primer and back inner primer, 15 pM of each external primer (F3 and B3), 2 × the Loopamp DNA Amplification reaction mix from Eiken® (Ref. LMP205), 8 U of *Bst* DNA polymerase (Eiken, Tokyo, Japan), 0.004% malachite green, and 2 μL of DNA. The mixture was incubated at 63°C for 60 minutes, with a final step at 80°C for 5 minutes for inactivation of the enzyme in a dry heating block (Labnet, Edison, NJ). At the end of the reaction, the amplifications were confirmed by visual inspection (light blue color in positive reactions and colorless in negative reactions). Then, to reconfirm the results, 2 μL of each LAMP product were subjected to 2% agarose electrophoresis plus the addition of Syber Safe (Invitrogen, Carlsbad, CA) for staining.

Loop-mediated isothermal amplification analytical specificity. Analytical specificity was evaluated in terms of the selectivity of the LAMP assay to distinguish blank from non-blank samples. This was analyzed on the basis of the following.

Inclusivity. This describes the ability of the assay to detect the diversity of blank DNA, which in this case included six New World *Leishmania* species associated with CL. We performed LAMP on the DNA of six *Leishmania* reference strains within a single day using the conditions described earlier.

Exclusivity. This describes the ability of the assay to provide a negative result when closely related but non-target sample sources are tested. In this case, we selected microorganisms that were phylogenetically related to *Leishmania* and also those associated with differential diagnoses of CL. DNA of parasites belonging to the order Kinetoplastida and obtained from a biological supply vendor (ATCC: The Global Bioresource Center) (ATCC PRA-330 *T. cruzi* and ATCC 30032 *T. rangeli*) and eight microorganisms with differential diagnoses of CL (ATCC 25923 *Staphylococcus aureus*, ATCC 12344 *Streptococcus pyogenes*, ATCC 26033 *Histoplasma capsulatum*, ATCC 27294 *Mycobacterium tuberculosis*, ATCC 26329 *Sporothrix schenckii*, and ATCC 18827 *Fonsecaea pedrosoi*) were subjected to the LAMP assay within a single day.

Loop-mediated isothermal amplification analytical sensitivity. The analytical selectivity of the assay involved measuring the degree of error that can exist within specified limits, using the following parameters.

Anticipated reportable range. The ARR refers to the range of concentrations over which an analyte can be determined with an adequate level of confidence and accuracy. To achieve this, seven serial dilutions (1×10^4 to 1×10^{-2} parasite equivalents/mL) of each DNA of the six *Leishmania* species were subjected to the LAMP assay. With the values obtained, consensus tables of the results were constructed.

Limit of detection. The LoD was calculated as the lowest dilution providing 95% positive results, as established by the National Committee for Clinical Laboratory Standards.[30] Five serial dilutions of each DNA of the six *Leishmania* species were subjected to LAMP. The amplification of each dilution was performed with eight replicates over five consecutive days. The LoD was determined using Probit Regression software (Probit Minitab 15 software, College Station, PA).

Accuracy. Intra-assay reproducibility was assessed in terms of the accuracy of each test. A dilution above and below the LoD for each DNA of the six *Leishmania* species was evaluated in triplicate for 10 days (one run per day) under the same conditions. With the values obtained, consensus tables of the results were constructed.

Evaluation of LAMP in biological samples (sandflies and direct smears from skin lesions of patients with CL). Two sets of biological samples were used to evaluate the performance of the test. The sets of samples were selected by convenience due to the lack of studies expressing values of sensitivity and specificity for LAMP tests in *Leishmania.* The first set consisted of 50 direct smears, collected during an outbreak of suspected CL cases that occurred in the municipality of El Chaparral in the Department of Tolima during 2003 and 2004, that had previously been tested by microscopy. The second set consisted of 50 pools of samples of female sandflies corresponding to the following: 30 pools of *Psychodopygus panamensis*, 12 pools of *Micropygomyia cayennensis*, and eight pools of *Lutzomyia gomezi*, which were captured in Valledupar, Cesar, in northeastern Colombia. The sandfly pools were subjected to DNA extraction using the ZR Tissue & Insect DNA Microprep kit (Zymo Research® Ref. D6016, Irving, CA) eluting in 50 μL. The direct smears of skin

TABLE 1

Sequences of LAMP primers used to target the 18S rRNA gene

Target	Label	Sequence 5′–3′
18S rRNA gene	F3	GGGTGTTCTCCACTCCAGA
	B3	CCATGGCAGTCCACTACAC
	FIP	TACTGCCAGTGAAGGCATTGGTGGCAACCATCGTCGTGAG
	BIP	TGCGAAAGCCGGCTTGTTCCCATCACCAGCTGATAGGGC

LAMP = loop-mediated isothermal amplification.

lesions that were fixed to glass slides, stained with Giemsa, and analyzed by microscopy for reference, were immersed in Xilol for 2 seconds to remove any remaining immersion oil. Subsequently, 200 µL of lysis buffer contained in the High Pure PCR Template Preparation kit were added and the entire slide was scraped. Then, the contents were transferred to a clean Eppendorf tube with the aid of a micropipette, and DNA was purified using the protocol described by the manufacturer eluting in 100 µL. The DNA was then subjected to real-time PCR (qPCR) and LAMP. Finally, we compared the operative capabilities of microscopy, qPCR, and LAMP.

Amplification using qPCR. To compare the LAMP assay with qPCR, DNA was obtained from the direct smears, and-pools of sandflies were subjected to qPCR amplification, which is considered the optimal methodology for molecular diagnosis in many cases. This test was implemented using the primers R223-TCCCATCGCAACCTCGGTT and R333-AAAGCGGGCGCGGTGCTG,[31] which target the same molecular marker as the LAMP assay (18S rRNA gene). The master mix, at a final volume of 12 µL, contained 5.0 µL of Fast SYBR Green (Ref. 4385370; Applied Biosystems, Foster City, CA), 0.6 µL of each primer (5 pM), and 2 µL of DNA. The thermal profile comprised 50°C for 2 minutes followed by 40 cycles of 95°C for 10 minutes, then 95°C for 45 seconds, and 60°C for 15 seconds. The qPCR was executed using a 7,500 Fast Real-Time PCR System (Applied Biosystems).

Statistical analyses. The rate of detection of positive samples for each test and each sample type (sandflies or direct smears) was reported in terms of the frequency and the corresponding 95% confidence interval (95% CI). The agreement between the tests (LAMP, qPCR, and microscopy in the case of the smears) was determined by calculating the concordance percentages and was supported by the Kappa coefficients with 95% CI. The operative capabilities of the LAMP test were determined through simple calculations of the sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively), and the likelihood ratio (LR), using microscopy and qPCR as reference tests for the smears, and qPCR only as a reference test for the sandfly samples. These tests were considered appropriate references because they are the only tests currently available for diagnosis in practice, in contexts similar to those in this study.

RESULTS

The implementation of the LAMP platform using primers directed to the 18S rRNA gene of *Leishmania* revealed adequate levels of amplification under the conditions described. The main parameters used to evaluate the analytical performance of the test are described in the following paragraphs.

Inclusivity and exclusivity. When analyzing the DNA from the six *Leishmania* strains using the LAMP assay, a light blue color was observed in the reaction tubes of all of the samples. The assay, therefore, appears to be inclusive when amplifying all *Leishmania* DNAs (Figure 1). Regarding exclusivity, the technique was found to be unique for *Leishmania* amplification with the 18S rRNA gene, as no color was observed in any of the reaction tubes containing DNAs from samples with a differential diagnosis (Figure 2).

Anticipated reportable range, LoD, and accuracy. Consensus results were obtained for each dilution of each parameter evaluated (ARR, LoD, and accuracy). The ARR for the LAMP test was evaluated for dilutions 1×10^4 to 1×10^{-2} equivalent parasites/mL of DNA extracted from the six *Leishmania* species (Figure 3A). It was determined that the LoD was up to the 1×10^{-2} dilution (Figure 3B). These results were consistent with the ARR. The results obtained for more than 10 days of analysis indicated no statistically significant differences and thereby high levels of accuracy (Figure 3C). The findings were concordant with those observed for the ARR and LoD.

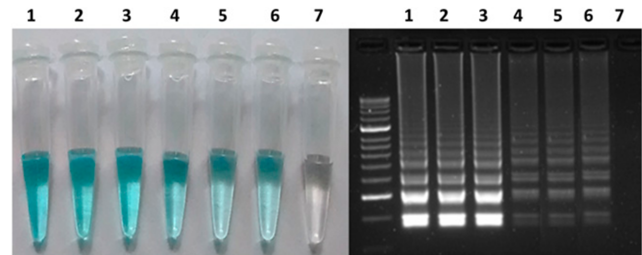

FIGURE 1. Inclusivity of the loop-mediated isothermal amplification test directed to the 18S rRNA gene. 1 = *Leishmania amazonensis*; 2 = *Leishmania braziliensis*; 3 = *Leishmania guyanensis*; 4 = *Leishmania panamensis*; 5 = *Leishmania mexicana*; 6 = *Leishmania infantum*; 7 = negative control. This figure appears in color at www.ajtmh.org.

***Leishmania* DNA detection from biological samples.** To analyze the ability of the tests to detect *Leishmania* DNA from biological samples, the tests were first applied to a set of 50 CL smears. Previously, by traditional microscopic diagnosis, 72.0% positivity was detected for *Leishmania* DNA using this set of smears (n = 36; 95% CI: 57.5–83.8). This frequency of infection was lower than that detected by molecular tests, which in the case of qPCR was 78.0% (n = 39; 95% CI: 64.0–88.5) and 80.0% for the LAMP test (n = 40; 95% CI: 60.3–90.0). The second set of biological samples corresponded to 50 pools of sandflies, for which an infection rate of 40.0% was detected (n = 20; 95% CI: 26.4–54.8) by the same two molecular tests (qPCR and LAMP) (Supplemental Table 1). The results obtained for these two sets of samples are presented in Table 2A. The evaluated tests showed high concordance (> 90% in all cases), with high kappa coefficients. The results of comparisons between the tests are shown in Table 2B.

The operative capabilities of the LAMP test were determined using the techniques currently used to detect infection in each of the biological sample sources (smears and pools) evaluated as reference tests. The LAMP test was found to display 100% sensitivity for the detection of *Leishmania* DNA for the different sample sources. Although the sensitivity

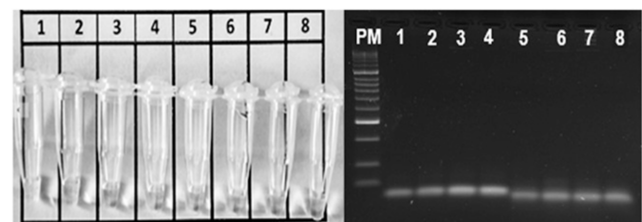

FIGURE 2. Exclusivity of the loop-mediated isothermal amplification test directed to the 18S rRNA gene. 1 = *Trypanosoma cruzi*; 2 = *Trypanosoma rangeli*; 3 = *Staphylococcus aureus*; 4 = *Streptococcus pyogenes*; 5 = *Histoplasma capsulatum*; 6 = *Mycobacterium tuberculosis*; 7 = *Sporothrix schenckii*; 8 = *Fonsecaea pedrosoi*.

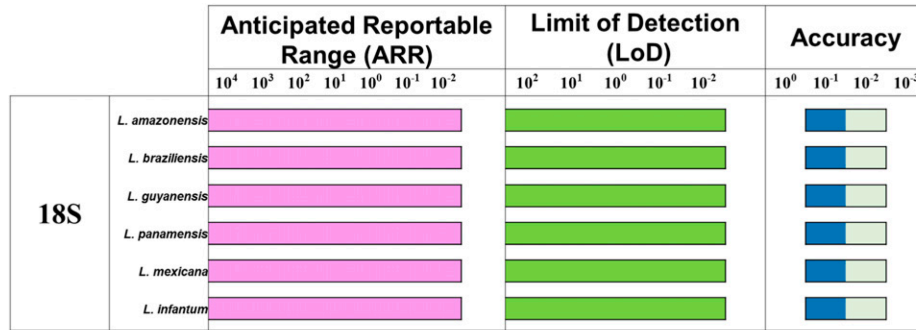

FIGURE 3. Analytical sensitivity for loop-mediated isothermal amplification. For each concentration, a positive result is determined by the presence of a light blue color in the reaction tube. (**A**) Anticipated reportable range determined from seven serial dilutions analyzed; (**B**) Limit of detection (LoD) as a consensus of five serial dilutions and (**C**) accuracy, including dilution above and below the LoD (box with lighter color). This figure appears in color at www.ajtmh.org.

was lower (71.4%) compared with that of microscopy, the values remained greater than 90% for both types of samples when compared with qPCR. In agreement with these findings, the NPVs were 100% in all cases and the PPVs were higher than 90%. In the case of the LR (useful for predicting risk in clinical practice), the LR (+) were higher than 3.0 and the LR (–) were not quantifiable because in none of the cases was a positive result obtained for the reference and a negative result obtained for the LAMP test. The results of the operative capabilities of the LAMP test versus the reference test used are described in Table 3.

DISCUSSION

Routine diagnosis of leishmaniasis has been developed based on the direct demonstration of the causative agent from lesions and/or aspirates depending on the clinical manifestation.[32] However, these procedures are invasive and often uncomfortable for the patient.[33,34] In the case of CL, microscopy remains the "gold standard" in primary health care centers in endemic regions. This test is characterized by its high specificity (100%), ease to perform, and low cost. However, it can present low and variable levels of sensitivity (between 40% and 74.4%) depending on the number and dispersion of parasites in the sample, the sampling procedure, and the ability and expertise of the person preparing and reading the sample smear.[35–39] The disadvantages presented by parasitological tests prompted the search for molecular techniques that would be effective for the diagnosis and surveillance of CL.

Several molecular tests have been used to improve the diagnosis of CL[40,41]; however, PCR has been found to offer high sensitivity and specificity compared with traditional parasitological methods[37] and can detect parasite DNA in a variety of clinical samples,[6,38] including direct smears,[42–44] Flinders Technology Associates filter paper,[45] and swabs from the lesion.[46] In many cases, the efficacy of PCR depends on the DNA extraction process, the number of copies of the molecular marker selected, and the type of PCR used (PCR-restriction fragment length polymorphism, conventional PCR, or qPCR).[47,48] According to the literature, the molecular markers predominantly used to detect parasite DNA by conventional PCR and qPCR are kDNA with a sensitivity of 88.2–97% and a specificity of 57.1–87%,[49–51] the 70 kDa heat shock protein 70 with a 90–95% sensitivity and a specificity of 95–100%,[4,52] transcribed ITS-1 with 40–91% sensitivity and 96% specificity[33,53] and finally the 18S rRNA gene, for which the results were similar to those of kDNA.[10] However, the requirement for complex equipment and infrastructure demonstrate that these tests are not an option as diagnostic tools in remote areas or in the search of active cases (field work). In 2000, Notomi and collaborators designed LAMP, which is characterized as an isothermic technique with a fast reaction time and high sensitivity that offers several alternatives for the visualization of results including turbidity, fluorescence, and/or color changes.[8–10,12–14] In 2016, Mondal et al.[54] reported the use of a recombinant polymerase amplification assay for the detection of *Leishmania donovani*; this technique offers a shorter reaction time compared with LAMP. Despite advances in the development of new diagnostic techniques, studies often only report certain aspects relating to the sensitivity and specificity of these techniques. A comprehensive evaluation of the analytical performance of these tests and the molecular markers used has not been carried out to date.

TABLE 2
Comparison of the detection of *Leishmania* DNA by a range of tests

		Smears				Sandflies	
		Microscopy		qPCR		qPCR	
A.		Positive, *n* (%)	Negative, *n* (%)	Positive, *n* (%)	Negative, *n* (%)	Positive, *n* (%)	Negative, *n* (%)
LAMP test	Positive	36 (72.0)	4 (8.0)	39 (78.0)	1 (2.0)	20 (40.0)	0
	Negative	0	10 (20.0)	0	10 (20.0)	0	30 (60.0)
B.	Concordance (%)	92.0		98.0		100	
	Kappa index	0.7826		0.9398		1.000	
	95% confidence interval	(0.5832–0.9821)		(0.8231–1.000)		(1.000–1.000)	

qPCR = real-time polymerase chain reaction.

TABLE 3
Operative capabilities of the LAMP test compared with microscopy and qPCR

	Smears		Sandflies
Reference test	Microscopy % (95% CI)	qPCR % (95% CI)	qPCR % (95% CI)
Sensitivity	100 (98.6–100.0)	100 (98.7–100.0)	100 (97.5–100.0)
Specificity	71.4 (44.2–98.7)	90.9 (69.4–100.0)	96.8 (88.9–100.0)
PPV	90.0 (79.5–100.0)	97.5 (91.4–100.0)	95.2 (83.8–100.0)
NPV	100.0 (95.0–100.0)	100.0 (95.0–100.0)	100.0 (98.3–100.0)
LR (+)	3.5 (1.5–8.0)	11.0 (1.7–71.3)	31.0 (4.5–213.8)
LR (–)	–	–	–

LAMP = loop-mediated isothermal amplification; LR = likelihood ratio; NPV = negative predictive value; PPV = positive predictive value; qPCR = real-time polymerase chain reaction. The estimator could not be calculated because one of the fields contained no data during the dispersion analysis.

Evaluation of the analytical performance is vital to determine the relative analytical specificity and sensitivity of the various molecular methods. Such evaluations have been performed for qPCR and LAMP in Chagas disease[22,55,56] and conventional PCR and qPCR in toxoplasmosis,[57] but not for the LAMP assay in leishmaniasis.

Herein, we evaluated the analytical sensitivity and specificity of a LAMP test targeting the 18S rRNA gene in six *Leishmania* species circulating in Colombia and determined the operational capabilities of the technique in direct smears from patients with presumptive CL and in sandflies. Our study was the first to include microorganisms with differential diagnoses of CL and two parasites of the order Kinetoplastida to evaluate the exclusivity of the technique. Our findings confirmed the exclusivity of the LAMP assay (Figure 2). Nzelu et al.[23] included trypanosome DNA of anurans in their assays and found no cross-reactivity with these parasites. Contrary to the findings of Adams et al.[10] who used DNA from differentially diagnosed microorganisms for visceral leishmaniasis and CL (*P. falciparum*, *Escherichia coli*, *S. aureus*, *M. tuberculosis,* and *Mycobacterium leprae*) and *T. brucei* and *T. cruzi* DNA, and found cross-reactivity with these last two parasites using PCR. In our study, we also found that the assay was inclusive for at least the six *Leishmania* species tested (Figure 1). However, future studies including a larger range of human infective *Leishmania* species are needed to confirm the inclusivity of this test. To our knowledge, this is the first time that a large number of microorganisms have been used to determine the exclusivity of the LAMP test, showing its tremendous potential for field implementation in the diagnosis of CL.

We also determined the ARR of the test over a range of dilutions (1×10^4 to 1×10^{-2} equivalent parasites/mL) that were appropriate to test the LoD. The LoD was established to be 1×10^{-2} equivalent parasites/mL (Figure 2), similar to that reported in the Nzelu study using serial dilutions of *L. mexicana*.[23,40] Our results differed from those reported by Adams et al.[10] with a LoD of 1×10^2 parasites/mL for *L. donovani* and Sriworarat et al.[14] with a LoD of 1×10^3 parasites/mL for *Leishmania siamensis* in whole blood samples. In addition, a report by Tiwananthagorn et al.[58] concluded that a PCR-kDNA assay was more sensitive than PCR-ITS and LAMP with the 18S rRNA gene.[42] Therefore, a comprehensive study to evaluate these parameters in a vast set of *Leishmania* species causing CL in the New World is needed.

Finally, we evaluated the diagnostic performance of the LAMP test using DNA from sandflies and, for the first time, DNA extracted from direct smear slides that had previously been stained with Giemsa and diagnosed by microscopy. Comparison of the results of the LAMP test with other tests, considered as reference tests in this study, revealed high concordance (> 90%, with kappa coefficients > 0.78) and no cases in which a positive test result was obtained for the reference test that was then found to be negative for the LAMP test (Table 2). This may indicate the high analytical sensitivity of the LAMP test for the detection of *Leishmania* DNA. Analysis of the operative capabilities of this technique revealed 100% sensitivity and NPVs, and greater than 90% specificity and PPVs, with the exception of comparisons with microscopy where the sensitivity was reduced to 71.4% (Table 3). These findings may be related to the fact that different types of techniques are being compared, which together with the sensitivity and analytical specificity data for the LAMP assay, indicate that the cases of non-concordance between the tests may correspond to failures in baseline tests and not limitations of the LAMP assay. However, field studies should be considered where the diagnostic performance of the technique is evaluated over a representative number of samples. Positive samples were obtained for LAMP from *P. panamensis* DNA, a sandfly known to be a vector of parasites of the subgenus *Viannia* in the country,[59,60] further indicating that LAMP could be of potential use for the entomological surveillance of CL. Our results clearly depict the potential use of this LAMP assay in remote or rural areas, especially owing to the possibility of amplifying DNA from smears collected on slides that had previously been stained with Giemsa, indicating the potential to conduct retrospective studies and plausibly identify infective species. Future studies need to standardize a method of DNA extraction that could be conducted in the field, which is a current limitation for the implementation of a LAMP test or other type of molecular test such as a recombinant polymerase amplification assay. In terms of cost effectiveness, Adams et al.[10] performed a comparison of LAMP versus qPCR and PCR-RFLP. Loop-mediated isothermal amplification using the Eiken fluorescent reagent was estimated to cost approximately $3.5 per reaction, compared with $12 for qPCR and $2.5 for PCR. The use of malachite green to visualize the results, as reported in the current study, may reduce the cost per reaction considerably. These findings differ from our previous results that revealed better analytical performance for LAMP than PCR-based tests.[61]

In conclusion, LAMP is a valuable diagnostic tool that offers advantages compared with qPCR and microscopy such as a LoD of 1×10^{-2} parasites/mL, exclusivity for *Leishmania* amplification, lower cost, and ease to perform and interpret. One of the most important advantages is its ability to detect *Leishmania* DNA from direct smears and sandflies, which indicate its potential application in primary care centers and in the diagnostic and entomological surveillance of leishmaniasis in endemic countries. Field

trials will confirm the potential of this LAMP assay for the timely diagnosis of leishmaniasis in communities vulnerable to infection.

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