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RESEARCH PAPERS

Real-Time PCR Assay for detection and quantification of *Leishmania*: standardization, positive control, validation, and intra-laboratory assay

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Highlights

- In this work is presented product and process for the diagnosis and quantification of *Leishmania* parasite load by PCR and qPCR
- The method sensitivity was determined as 0.01 parasites/µL
- The method has 100% concordance among human participants in the intra-laboratory validation study

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KEYWORDS
Real-time PCR;
Parasite load;
Leishmania.

Abstract: his study aimed to develop a method to investigate PCR sensitivity for diagnosis and ensure reproducibility for parasite load quantification in tissues based on qPCR. In the first step, genes were selected to quantify the parasite load; then, a standard was developed to quantify the concentration of different *Leishmania* species. These tools were evaluated in intra-laboratory assays. The sensitivity was determined as 0.01 parasites/µL, and the method was reproducible with 100% concordance among human participants in the intra-laboratory validation study. Furthermore, the results demonstrated the specificity of the method in detecting the genus *Leishmania* without showing cross-reaction with *Trypanosoma cruzi* or human DNA.

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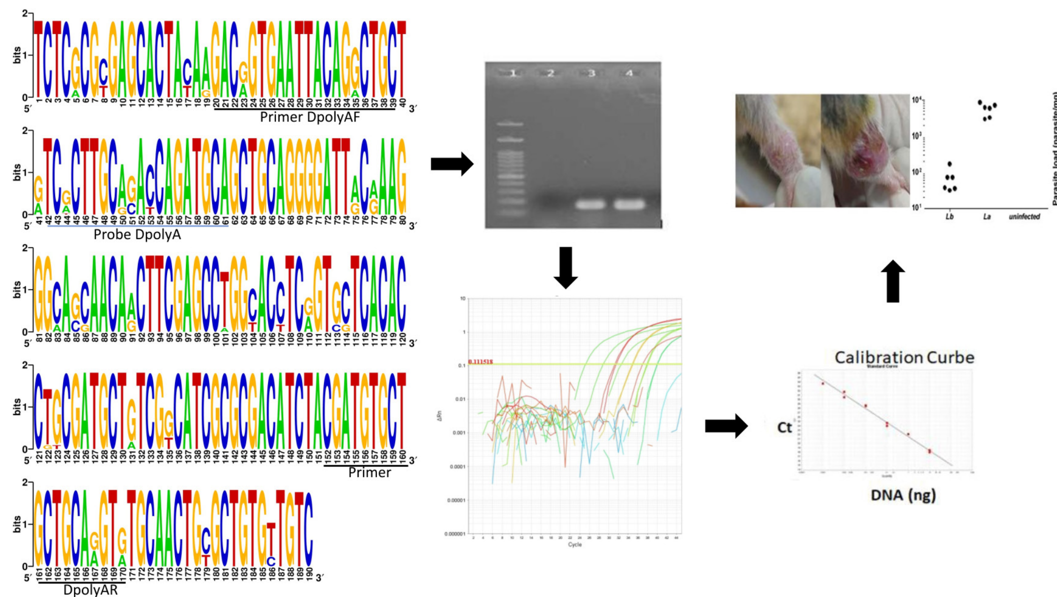
Graphical Abstract



Introduction

Leishmaniasis is a complex of diseases caused by parasites of the genus *Leishmania* (Ross, 1903). Around 21 species of this genus can infect humans and cause different clinical manifestations. Leishmaniases are classified according to their clinical manifestations into a spectrum of diseases, including cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and post-kala-azar dermal leishmaniasis (PKDL). Among these, visceral leishmaniasis (VL) can prove fatal without effective diagnosis and treatment. In this scenario, several ideas for human vaccines have been developed, including live vaccines with attenuated parasites, various *Leishmania* protein subunits, fusion proteins, and others. The current course of treatment is based on chemotherapy using a small number of drugs. However, these drugs have shown significant drawbacks, e.g., high cost and toxicity, challenging routes of administration, and low efficacy in endemic areas (Baneth & Solano-Gallego, 2022; Ostolin et al., 2022; Tandon et al., 2023; Wu et al., 2020). On the other hand, diagnosing leishmaniasis (VL, CL, and MCL) is challenging due to the wide spectrum of clinical manifestations and parasite diversity. From this perspective, several methods have been developed and tested to reach a diagnostic solution and obtain precise and accurate results. Parasitological tests include direct parasite search, culturing, and histopathological examination. Immunological exams, in turn, include the indirect immunofluorescence assay, the enzyme-linked immunosorbent assay (ELISA), and the Montenegro skin test (MST) (Brito et al., 2020; Kumari et al., 2022). However, the specificity, sensitivity, and reproducibility of methodologies for diagnosing leishmaniasis depend on several factors, including the technical knowledge of the personnel responsible for performing the tests (staff training), the quality of the equipment and reagents, the use of quality controls, intrinsic characteristics of the method, the clinical samples, the lesions' evolution time, the clinical forms, and the *Leishmania* species involved in the disease. Studies have shown that molecular biology techniques, particularly polymerase chain reaction (PCR), have better accuracy than immunological and parasitological exams. Real-time PCR (qPCR) has been applied to quantitatively analyze protozoa and help diagnose and monitor therapy response (Akhoundi et al., 2017; Kumari et al., 2021). The targets commonly used in the molecular diagnosis of leishmaniasis are ribosomal DNA in special internal transcribed spacer (ITS), heat-shock proteins (HSPs), glucose-6-phosphate dehydrogenase (G6PD), mannose phosphate isomerase genes (MPI), kinetoplast DNA (kDNA), and cytochrome oxidase (CO) (Araujo-Pereira et al., 2018; Castilho et al., 2003; Fraga et al., 2010; Schönian et al., 2003; Tabbabi et al., 2020).

Another point to consider is that a rapid and effective diagnosis is essential to identify the active disease and monitor patients after treatment. Moreover, an accurate epidemiological diagnosis avoids cross-reactivity with other diseases, which helps decrease disease severity by anticipating the specific therapy. However, the current methods have limited accuracy, requiring the development of new tests and diagnostic strategies. In research laboratories, PCR has been proposed as an alternative tool for quantifying *Leishmania* sp. due to its higher sensitivity than traditional parasitological techniques. However, this technique has disadvantages due to its time-consuming nature, the high risk of false positive results due to carry-over contamination or unspecific PCR products, and the difficulty in performing quantitative analyses (Brito et al., 2020; Filgueira et al., 2020; Kumari et al., 2022; Thomaz et al., 2021). From this perspective, this study aimed to develop and validate a methodology to detect and quantify the parasite load of *Leishmania* spp. by real-time PCR (qPCR) that includes a positive control, a standard, a primer, and probes for use in diagnosis and throughout disease development.

Material and methods

Strains and DNA collection

Strains of *Leishmania* spp. and *Trypanosoma cruzi* were provided by the culture collection of the Bioprocess Engineering and Biotechnology Department of UFPR, whereas ANILAB LTDA provided the Syrian hamster. The DNA extraction and qPCR reagents were of molecular grade and DNAse- and RNAse-free. Promastigotes of the reference strains *Leishmania braziliensis* (MHOM/BR/1975/M2903) and *Leishmania amazonensis* (MOM/BR/1970/BH46) were grown at 24 °C in biphasic brain–heart infusion broth (BHIB) (Sigma, St. Louis, MO, USA), which was supplemented with 10% rabbit blood. *Trypanosoma cruzi* (Y strain) was grown at 27 ºC in RPMI medium (Sigma), which was supplemented with 10% inactivated fetal bovine serum (FBS, Sigma).

Primer and probe design

Known sequences of the two targets for *Leishmania* spp., one for a protein-coding gene (DNA polymerase A) and another for a non-coding region of kinetoplast DNA (zXM_001563712.2, AF231100.1), were selected for this procedure. Sequences of each gene were aligned using the software Clustal X (Larkin et al., 2007). The alignments were enriched by additional sequencing of more strains from known sequences of *Leishmania* (Supplementary Material), and then scanned for regions of high intraspecies sequence conservation to be used for designing primers and probes. Primers were designed using the Primer-BLAST software (Ye et al., 2012). For the marker catalytic subunit of DNA polymerase A (DNApolyA), primers (DpolyAF 5'-ACGGTGAATTACAGGCTGCT-3'; DpolyAR: 5'-ATACTTGCAGCAGCACATCG-3') were designed to amplify a 150-bp fragment. A TaqMan® hydrolysis probe specific for the DNApolyA marker was designed (FAM 5'-TCACTTGCACATCAGATGCA-3' BHQ1). For the kinetoplast DNA marker (kDNA), primers (KNPLF 5'-CCTATTTTACACCAACCCCC-3'; KNPLR: 5'-ACATGATACTTCCCCGCTAC -3') were designed to amplify a 245 bp fragment. A TaqMan® hydrolysis probe specific for kDNA (FAM 5'- CATCTCCAACCACTAACAGGCTTCAGCCA-3' BHQ1) was designed and synthesized by Macrogen (Macrogen Inc., Seoul, Korea). All primers and probes were designed *de novo*, and the analytical specificity was assessed by BLAST (Basic Local Alignment Search Tool; NCBI). The *in-silico* analysis was performed by a BLAST search on the amplicon (200 bp) generated by the DNApolyA and kDNA primers.

Positive control construction

Linear plasmids with inserts of the target sequences were used as positive controls and standards. The plasmid was constructed for each marker by inserting the test-specific DNA sequence into plasmid DNA (pTOP Blunt V2), synthesized by Macrogen (Macrogen Inc., Seoul, Korea), through which two plasmids (pDNApolyA and pkDNA) were generated. The transformation of *Escherichia coli* with the plasmids (pDNApolyA and pkDNA) consisted of two steps—first, the $CaCl_2$.$MgCl_2$ method was employed to prepare competent cells of *E. coli* (Sambrook & Russell, 2001), which were suspended in a fresh, ice-cold 100 mM $CaCl_2$ solution. Then, the modified heat shock method was employed to insert the plasmids into the bacteria (Froger & Hall, 2007; Sambrook & Russell, 2001). Next, 1 ng of plasmid DNA was mixed with 200 µL of freshly prepared competent cells and incubated on ice. After 30 min, heat shock was applied for 60 s at 42 °C, followed by instant transfer to the ice for 2 min and the addition of 800 µL of Super Optimal Broth with Catabolite repression (SOC). Finally, the cells were incubated at 37 °C for two hours, after which 100 µL aliquots were spread on nutrient agar plates containing 50 µg/mL of ampicillin. One colony per plasmid was used to inoculate Luria-Bertani (LB) broth supplemented with 50 µg/mL of ampicillin. After incubation at 37 ºC for 18 h, plasmid isolation was performed using the manual alkaline lysis method (Green & Sambrook, 2016). After purification, the plasmid DNA was digested with *Hind*III (Invitrogen) following the manufacturer's recommendation and stored at -20 ºC.

Construction of standard curves for parasite load

The plasmid concentration was measured using a NanoVue™ UV/Visible Spectrophotometer (GE Healthcare), and the corresponding copy number (CN) was calculated according to Fu et al. (2009) using the Equation 1:

$$CN = \frac{\left[\left(6.02 \times 10^{23} copy / mol\right) \times DNA\,amount\left(\mathrm{g}\right)\right]}{\left[DNA\,length\left(bp\right) \times 660(\mathrm{g} / mol / bp\right]} \qquad (1)$$

A ten-fold serial plasmid dilution (pDNApolyA or pkDNA) was performed starting from 10^4 to 10^{-1} copies/µL, which was used to construct the standard curves. In addition, threshold cycle (Ct) values in each dilution were measured in duplicate and plotted against the logarithm of their initial template copy numbers. Finally, the correlation coefficient (R^2) of each test was determined.

Design of experiments (DoE)

A complete factorial experiment was created to standardize the qPCR conditions using the Minitab© Statistical Software V. 18.1 (2017 Minitab, Inc.) with central points included (Table 1). The qPCR was performed using StepOne™ Real-Time PCR System (Applied Biosystems). The reaction parameters of DNA load (ng), primer concentration (nM), and probe concentration (nM) were optimized within the levels presented in Table 1. PCR cycling conditions for DNApolyA were 95 °C for 10 min and then 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. PCR cycling conditions for kDNA were 95 °C for 10 min and then 40 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. Samples were analyzed in duplicate along with the extraction of negative controls and standard curves, and at least three non-template negative controls were included in each plate.

Experimental infection for parasite load evaluation

Five groups of Syrian hamsters were selected for experimental infection. The first, second, and third groups

Table 1. Real and coded values of the independent variables from the full factorial experimental design (2^3) for optimization of the real-time PCR.

Coded parameters			Real value parameters		
DNA (ng)	Primer (pmol/µL)	Probe (nM)	DNA (ng)	Primer (pmol/µL)	Probe (nM)
+1	-1	+1	50	0.5	0.50
0	0	0	40	1	0.25
-1	-1	-1	30	0.5	0.50
+1	+1	+1	50	1.5	0.50
-1	+1	+1	30	1.5	0.50
+1	+1	-1	50	1.5	0.125
+1	-1	-1	50	0.5	0.125
-1	+1	-1	30	1.5	0.125
0	0	0	40	1	0.25
-1	-1	-1	30	0.5	0.125
0	0	0	40	1	0.25

were infected with 10^6 promastigotes of *L. braziliensis*. The fourth group was infected with 10^6 promastigotes of *L. amazonensis*. The fifth group (uninfected) consisted of uninfected animals. The animals of the first group were sacrificed after 75 days post-infection, the second group after 120 days, and the last two groups after 140 days, after which spleen and skin samples from the paws were collected from these groups.

DNA extraction

The extraction started with 50 mg of skin samples obtained from the experimental infection of the Syrian hamsters, which was homogenized with a pestle and mortar and passed through a syringe with a needle, followed by incubation with tissue digestion buffer (containing 100 mM NaCl, 10 mM Tris pH 8.4, 25 mM EDTA, 0.5% SDS, and 0.6 mg/mL of proteinase K) at 55 ºC for 12 h. After phenol/chloroform/isoamyl alcohol extraction, DNA was precipitated in the presence of 1/2 volume of 7.5 M ammonium acetate with 2.5 volumes of absolute ethanol, spooled out, washed in 70% ethanol, briefly air-dried, dissolved in 50 µL of 10 mM Tris pH 8.0, 1 mM EDTA, and stored at -20 ºC. DNA extraction began with 200 µL of culture sample (*L. braziliensis*, *L. amazonensis*, and *T. cruzi*) and 300 µL of human peripheral blood. The procedure was completed using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's recommendation, eluted in 50 µL of elution buffer, and stored at -20 ºC.

Validation of the method

The proposed method was validated in the following points: first, DNA, parasite and parasite diluted in human peripheral blood were used; second, the effectiveness of the chosen method with real samples of animals infected with *L. braziliensis* and *L. amazonensis* was evaluated. The third and last point was an intra-laboratory assay with a mixture of the types of samples used in the previous phases. A ten-fold dilution series was prepared for each plasmid DNA starting from 1×10^5 to1×10^{-1} copies/µL and used as a template in the real-time PCR assays under optimized conditions to determine the amplification, efficiency, and reproducibility. The limit of detection (LoD) was determined by PROBIT regression analysis at a 95% confidence level, when six replicates of each plasmid DNA dilution were assayed per run. A ten-fold dilution series in human peripheral blood was prepared for two species of *Leishmania* starting from 1×10^5 to 1×10^{-1} parasites/mL and included human peripheral blood and *T. cruzi* as negative samples to test the specificity of the methods (DNA extraction and qPCR).

The assay was validated with hamster skin samples of animals experimentally infected with *Leishmania* to assess the diagnostic test performance in tissue. In addition, six samples from animals infected with *L. amazonensis*, six samples from animals infected with *L. braziliensis*, and six samples from uninfected animals, collected after 140 days post-infection, were used in the experiment.

An intra-laboratory study was designed to determine the assay's sensitivity, specificity, and concordance. Two panels were set up, the first consisting of DNA samples and the second of tissue samples, and were distributed to five members of the laboratory staff who usually performed molecular biology testing using commercial reagents. The first panel included water (sample 1) and *T. cruzi* (sample 3) as negatives samples, two samples constructed from a culture of *L. amazonensis* and *L. braziliensis* diluted in human peripheral blood (10–1000 parasite/mL sample 2 and sample 4 respectively), and DNA samples of sample 5 (water) sample 6 (*T. cruzi*) and sample 7 (*L. amazonensis*, or *L. braziliensis*). The second panel was constructed to monitor the evolution of leishmaniasis infection in an animal model. It consisted of spleen and skin samples isolated from animals infected with *L. braziliensis* at different disease

stages. The samples were distributed according to the following scheme: five skin tissue samples from animals infected 75 days before (sample 1), five skin (sample 2) and spleen (sample 3) tissue samples from animals infected 120 days before, and five skin (sample 4) and spleen (sample 5) tissue samples from uninfected animals. All tissue samples were previously homogenized by grinding.

Statistical analyses

Results were presented as means ± standard deviation (SD), and DoE data were analyzed using the Minitab© Statistical Software V. 18.1 (2017 Minitab, Inc.). Sensitivity, specificity, concordance, relative accuracy, and limit of detection using Probit test results were determined using MedCalc (Windows, 15.0v).

Ethical issues

All applicable international, national, and institutional guidelines for the care and use of animals were followed. The present study was approved by the Committee on the Ethical Handling of Research Animals of the Federal University of Paraná (CEUA/BIO-UFPR), Curitiba, Parana, Brazil (Process n. 101328/2015-69).

Results

Real-time PCR assay standardization

Primer design and qPCR standardization for *Leishmania* sp. were performed after sequence alignment. The regions chosen for the markers, corresponding to the kinetoplast DNA (kDNA) and DNA polymerase A (DNApolyA) catalytic subunit (Figure 1a), showed low diversity, and no alignments with other sequences from microorganisms or other mammalian species were found using BLASTN. *In vitro* testing of the primers was done using DNA samples from *L. amazonensis, L. braziliensis,* and *T. cruzi,* because they belong to the same family. Results demonstrated that the only samples amplified contained DNA from different *Leishmania* species (Figure 1b); the same test was performed for kDNA and showed the same results (data not shown). Figure 1c depicts the outcomes of the optimization of DNApolyA qPCR master mix conditions. The effects of three variables on the response variable and their combined effects were assessed. The white background variables that did not affect the response variable demonstrated that there is no interaction between the three variables evaluated, and that lower primer and probe concentrations provided better results. The optimized conditions for DNApolyA consisted of 1x TaqMan™ Universal

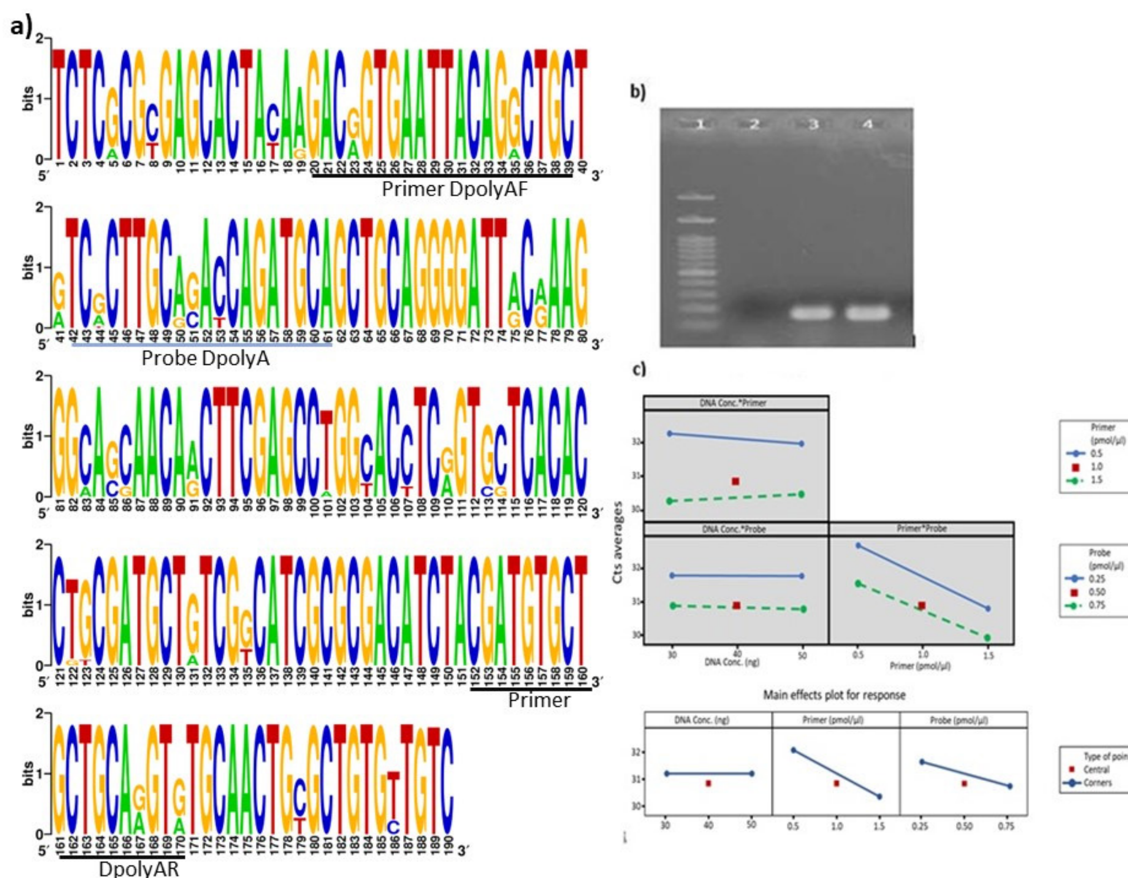


Figure 1. Primer design and DNApolyA optimization based on qPCR for parasite quantification a) Multisequence alignment based on 13 sequences homologous to *Leishmania braziliensis* DNApolyA using NCBI Blast (for details see Table S1); b) DNApolyA primers used to amplify a 150-bp product specifically. Product visualized with ethidium bromide staining in 1% agarose gel run. 1). 100 bp DNA ladder; 2) *Trypanosoma cruzi*; 3) *Leishmania braziliensis*; 4). *L. amazonensis*; c) Influence of the tested variables.

PCR Master Mix (Applied Biosystems), 0.5 nM of each primer, 0.125 nM of the probe, and 50 ng of the sample DNA (2 µL) in a final volume of 10 µL. The PCR cycling conditions were 95 °C for 10 min and then 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The PCR conditions for kDNA consisted of 1x TaqMan™ Universal PCR Master Mix (Applied Biosystems), 0.5 nM of each primer, 0.125 nM of the probe, and 50 ng of the sample DNA (2 µL) in a final volume of 10 µL. The PCR cycling conditions were 95 °C for 10 min and then 40 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s.

Validation of the method

Lower limit of detection (LoD)

With the kDNA marker, it was possible to amplify the six points of the standard curve. However, for the *L. braziliensis* and *L. amazonensis* samples, the points corresponding to 0.1 copies/µL DNA were not amplified (Figure 2a, 2d). With the DNApolyA marker, it was possible to amplify four points of the standard curve using the plasmid (pDNApolyA), and only three points of the *L. braziliensis* and *L. amazonensis* samples; the points corresponding to 1 and 0.1 copies/µL DNA were not amplified (Figure 2b, 2d). There were differences in the quantification of *Leishmania* per sample when using the standard curves created with each plasmid. For example, the sample with 1000 parasites per mL resulted, for *L. brazilienesis* and *L. amazonensis,* in 88.07 and 236.55 parasites/mL, respectively, when using the kDNA method, generating a distortion corresponding to approximately three times the number of parasites (Figure 2a). On the other hand, when using the DNApolyA method (Figure 2b), the values of 764.64 and 844 parasites/mL were obtained for *L. brazilienesis* and *L. amazonensis*, respectively. It was observed that the DNApolyA method provided a result closer to the initial number of parasites in the sample, thus being a more accurate method for parasite quantification.

In order to define the LoD, the results of the different replicates are shown in Table 2. For the kDNA marker, it was possible to amplify all points of the standard curve using the plasmid (pKDNA), but not in all samples of each dilution. Thus, the PROBIT method had to be used to determine the LoD, which was 8.34 copies/µL at a probability level of 0.95 (p=0.0075). When using the DNApolyA marker, it

Table 2. Detection limit evaluation for pkDNA and pDNApolyA plasmids.

Copies/uL	Samples detected	
	pkDNA	pDNApolyA
10000	6	6
1000	6	6
100	6	6
10	6	5
1	3	0
0.1	1	0

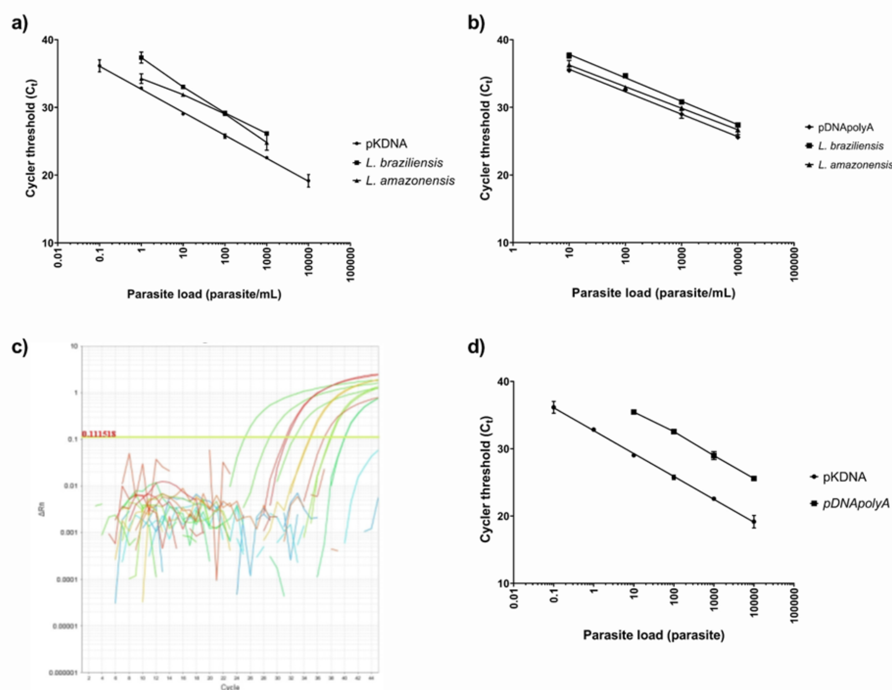


Figure 2. Standard curves generated with the two markers compared for *L. amazonensis* or *L. braziliensis*. (a) Standard kDNA marker curves generated from the linear region of each amplification. The amplification efficiency of each primer set was determined using the following equation: efficiency (E) =1−10(−1/slope), where kDNA E = 96.84% and R^2 = 0.9949; (b) Standard curves for the DNApolyA marker, E = 93.07% and R^2 = 0. 9934; (c) Curve of amplifications generated after the amplification of pDNA with the different markers; (d) comparison of the standard curves for the two qPCR methods used to detect *Leishmania* DNA.

was possible to amplify four points on the standard curve (pDNApolyA); the LoD was 12.40 copies/μL at a probability level of 0.95 (p= 0.0094). In addition, the specificity of the marker with human and *T. cruzi* DNA was tested, but no amplification was detected.

Validation of qPCR by experimental infection

The assay accurately measured a parasite load in skin samples for different infection agents of cutaneous leishmaniasis. Reactions with DNA from uninfected paws, included as negative controls, did not amplify. As a result, no false positive was detected. *Leishmania* resulted in an acute parasitemic phase easily detectable by qPCR under infection by different *Leishmania* species, especially the infection with *L. amazonensis* after 140 days post-infection. In addition, 100% of the samples from animals infected with *Leishmania* were quantified despite differences in the symptoms caused by different *Leishmania* species, with no false negatives. Figure 3c represents the determination of parasite load in skin samples from infected and uninfected animals. For the samples evaluated, the samples from *L. amazonensis*, the one with the highest parasite load ($5.75X10^3$), and *L. braziliensis* with a parasite load of ($7.01X10^1$), were consistent with the size of the lesion in the legs of the hamsters.

Intra-laboratory test

The participants of the intra-laboratory test reported not obtaining amplification in culture samples 1 and 3, corresponding to *T. cruzi* and water samples. These results indicate no cross-contamination during the extraction and preparation of reagents for qPCR, and there were no unspecific amplifications. This scenario is supported by the results obtained with DNA samples 1 and 2, which corresponded to *T. cruzi* and water, respectively. For culture samples 2 (1000 parasites/mL) and 4 (10 parasites/mL), the participants could detect the presence of the parasite at both high and low levels of parasite concentration. When the results were reported, there were variations between what was reported for high levels of *L. amazonensis* (836.78 parasites/mL) and *L. braziliensis* (638.39 parasites/mL) and low levels of *L. amazonensis* (4.36 parasites/mL) and *L. braziliensis* (4.51 parasites/mL). On the other hand, there were DNA samples that showed a parasite load of 114.83 parasites/mL for *L. braziliensis* and 127.64 parasites/mL for *L. amazonensis*.

The qPCR results were expressed in 1 mL equivalent of the parasite, as shown in Table 3.

With the data obtained (Table 4), it was possible to determine the concordance between the results observed and the nominal values of the samples with p-values <0.05 ($p=8.45x10^{-18}$). With regard to the relative sensitivity, the test of the proportion of true positives revealed that the method developed is sensitive, with a p-value >0.05 (p=1). Furthermore, relative specificity with the true negative ratio test determined that the method is specific, with a p-value >0.05 (p=1). Finally, the concordance test determined the relative accuracy, showing that the method is accurate, with a p-value >0.05 (p=1). From the results of the second panel, it was found that, in 5/5 of the skin samples, *Leishmania* DNA was detected with a parasite load between 89.43 and 184.34 parasites/mg. In the next group, with samples from 120 days post-infection, *Leishmania* DNA was detected in both spleen ($6.34x10^3$ to $9.19x10^6$ parasites/mg) and skin samples ($3.23x10^2$ to $1.33x10^5$ parasites/mg). In the group of negatives, all samples were free of *Leishmania* DNA.

Discussion

In research laboratories, PCR has been proposed as an alternative tool for the direct diagnosis and quantification of *Leishmania* sp. as a more sensitive method than traditional parasitological techniques (Antinori et al., 2009; Bensoussan et al., 2006; Ceccarelli et al., 2014; Torpiano & Pace, 2015). The parasitological smear has disadvantages since it is time-consuming and shows a high risk of false positive results due to carry-over contamination (Piron et al., 2007). In this context, qPCR is emerging as an alternative tool for monitoring the parasite load in experimental *Leishmania* sp. infections. However, a standardized qPCR protocol needs to be explicitly optimized for animal models. Instead, protocols are usually developed for DNA isolated from a known number of parasites/mL as the standard for quantification (Mota et al., 2022). In this study, two types of markers were used for parasite load quantification. The first, kDNA, is

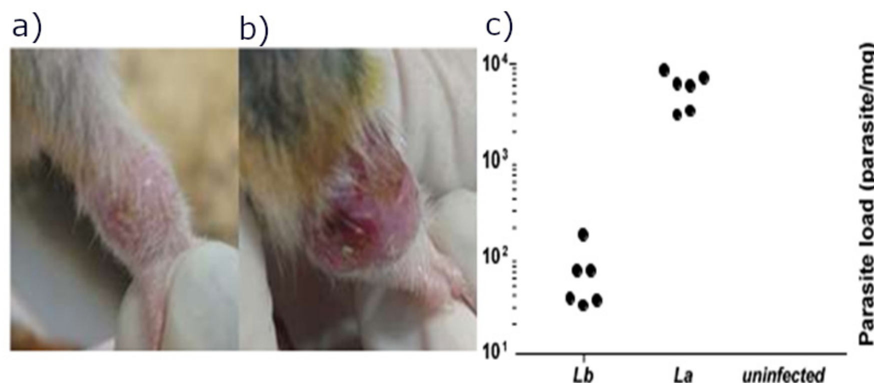


Figure 3. Determination of parasite load in skin samples from animals with experimental infection. The photograph shows the hamster's hind leg infected with different species of *Leishmania*. a) Animal infected with *L. braziliensis* (Lb). b) Animal infected with *L. amazonensis* (La). c) Parasite load of the samples.

Table 3. Reproducibility of the parasite load (parasite in the sample) quantified from intra-laboratory tests.

Participant	Sample 1	Sample 2	Sample 3	Sample 4
From culture (parasite/mL)				
1*	0	1251.88	0	3.78
2+	0	1308.56	0	10.64
3+	0	171.16	0	2.28
4+	0	435.45	0	1.35
5*	0	421.68	0	4.94
From DNA (ng/uL)				
	Sample 5	Sample 6	Sample 7	
1*	0	0	170.60	
2+	0	0	106.39	
3+	0	0	134.96	
4+	0	0	103.15	
5*	0	0	84.69	
From tissue (parasite/mg)				
	Sample 1	Sample 2	Sample 3	Sample 4
1+	0	89.43	$3.23x10^2$	$6.34x10^3$
2+	0	126.32	$4.57X10^3$	$7.31x10^3$
3+	0	170.21	$3.24X10^3$	$2.33x10^4$
4+	0	100.27	$1.33x10^5$	$1.84x10^5$
5+	0	184.34	$2.03X10^4$	$9.19x10^6$
	Sample 5			
1+	0			
2+	0			
3+	0			
4+	0			
5+	0			

*Samples from L. amazonensis; + Samples from L. braziliensis.

Table 4. Result of intra-laboratory tests.

Participants	Really Positive	False-Positive	False-Negative	Really Negative
1	6	0	0	6
2	6	0	0	6
3	6	0	0	6
4	6	0	0	6
5	6	0	0	6
Total	30	0	0	30

traditionally the most frequently used target for detecting and identifying *Leishmania* species due to its multicopy nature (the number of copies differs between species) and for showing high sensitivity (Jara et al., 2013). However, for quantifying the parasite load, the problem with the kDNA marker's performance is due to a different relative abundance

between species and for being particularly unstable in terms of copy numbers in the lifecycle stages of the parasite, making it difficult to determine the parasite load of a sample without prior knowledge of which species caused the disease (Ceccarelli et al., 2017; Mary et al., 2004; Simpson et al., 2015; Weirather et al., 2011). On the other hand, a marker of unique copy per genome was selected, which was less sensitive about kDNA but comparable between different species of *Leishmania*, allowing parasite load quantification and not requiring a process of correlation between marker copy per genome and the number of parasites. While not subject to changes in copy number across lifecycle stages, a single copy gene protocol can still be applied for diagnosis and evaluation of treatment effectiveness in patient or animal models for vaccine effectiveness evaluation.

The preparation of standards is required for a qPCR reaction. Also, genomic DNA from the target is usually employed, including a DNA extraction process to determine the number of copies using the whole genome size of reference strains. However, this estimation may cause inaccurate quantification since the whole genome size might vary between strains. The development of pDNApolyA allowed us to have a stable size, speed up the quantification process of the parasite load, and achieve higher precision, making qPCR reactions comparable among essays. Standardized negative controls were produced, and a novel pDNApolyA was developed for the proposed qPCR methodology to later standardize and validate the qPCR technique in an animal model. Likewise, the validated approach can be used to measure the parasite load in vaccinated animals experimentally infected with *Leishmania* and verify whether the quantification of *Leishmania* sp. DNA would reflect the sensitivity and number of live parasites in the sample.

The method's evaluation allowed us to determine the sensitivity, which was 0.01 parasites per µL. In addition, the method proved to be reproducible since it allowed the samples to be classified as positive or negative. The great advantage of qPCR is its application to monitor parasites in tissue samples (skin and spleen) with high sensitivity. However, despite technological advances in the last few years, qPCR is far from being used as a routine technique for diagnosing or quantifying parasites due to the costs of reagents and equipment. Another point to consider is the correct sample type selection and the interval selection for diagnosis or parasite load quantification.

In this study, samples obtained at different time intervals after infection were used, which showed that, if the type of sample is poorly selected or the time intervals to be evaluated are not well chosen, false negative results can be generated if only one type of sample is taken as a basis for diagnosis. Results such as those were reported for spleen samples from animals infected 75 days before. In summary, in this study, a qPCR methodology was developed with a standard base on a gene of single copy per genome, showing good sensitivity and reproducibility. This allowed us to quantify samples of different *Leishmania* species obtained from animal tissues.

Conflict of interests

The authors declare no conflicts of interest.

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Supplementary Material

Supplementary material accompanies this paper.

Sequences used for the design of primer and probe DNA polymerase

Sequences used for the design of primer and probe for kinetoplast

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