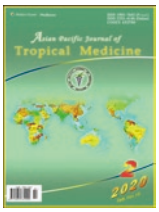

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A PCR and RFLP-based molecular diagnostic algorithm for visceral leishmaniasis

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ABSTRACT

Objective: To determine an algorithm for molecular diagnosis of visceral leishmaniasis (VL) by kinetoplast DNA (kDNA) (RV1/RV2) and internal transcriber spacer (ITS1) (LITSR/L5.8S) polymerase chain reaction (PCR), complemented by ITS1 PCR restriction fragment length polymorphism (RFLP), using peripheral blood or bone marrow aspirate from patients with suspected VL.

Methods: Biological samples were submitted to the gold standard for the diagnosis of VL and molecular diagnosis represented by ITS1 PCR, kDNA PCR, and ITS1 PCR RFLP. The samples were obtained from seven groups: group Ⅰ, 82 samples from patients with confirmed VL; group Ⅱ, 16 samples from patients under treatment for VL; group Ⅲ, 14 samples from dogs with canine visceral leishmaniasis (CVL); group Ⅳ, a pool of six experimentally infected sandflies (*Lutzomya longipalpis*); group Ⅴ, 18 samples from patients with confirmed tegumentary leishmaniasis (TL) and groups Ⅵ and Ⅶ were from control groups without VL.

Results: The following gold standard and molecular examination results were obtained for each of the seven groups: group Ⅰ: parasitologic and immunochromatographic tests showed a sensitivity of 76.3% (61 of 80) and 68.8% (55 of 80), respectively, and a sensitivity of 97.6% (80 of 82) and 92.7% (76 of 82) by ITS1 and kDNA PCR, respectively. After ITS1 PCR RFLP (Hae Ⅲ) analysis of the 80 positive samples, 52.5% (42 of 80) generated three fragments of 180, 70, and 50 bp, corresponding to the pattern of *Leishmania infantum infantum*; group Ⅱ: negative for the parasitologic methods and positive for IrK39 (100%, 16 of 16), presented 12.5% (2 of 16) of positivity by ITS1 PCR and 25.0% (4 of 16) by kDNA PCR; group Ⅲ: positive in the parasitologic and serologic tests (100%, 14 of 14), presented 85.7%(12 of 14) of positivity by ITS1 PCR and kDNA PCR. ITS1 PCR RFLP showed that 83.3% (10 of 12) of the canine samples contained parasites with profiles similar to *L. infantum*; group Ⅳ presented amplifications by ITS1 PCR and kDNA PCR. ITS1 PCR products were analyzed by RFLP, generating a profile similar to that of *L. infantum*; group Ⅴ: positive in the parasitologic examination (100%, 18 of 18), presented 72.2% (13 of 18) of the samples by ITS1 PCR positive. A total of 69.2% (9 of 13) showed profiles corresponding to a *Viannia* complex by ITS1 PCR RFLP; and group Ⅵ and group Ⅶ were negative by ITS1 and kDNA molecular tests. Comparing the molecular results with the parasitologic and serologic diagnosis from group Ⅰ, almost perfect agreement was found (κ both>0.80, P<0.001). ITS1 and RV1/RV2 PCR detected 90.2% (74 of 82) of the samples. Two samples positive by RV1/RV2 were negative by LITSR/L5.8S, and six samples positive by LITSR/L5.8S were negative by RV1/RV2. Therefore, these two systems complemented each other; they diagnosed 100% of the samples as belonging to the *Leishmania* genus.

Conclusions: We suggest an algorithm for the molecular diagnosis of VL, which must consider previous parasitologic and serologic (immunochromatographic) diagnoses, and should combine kDNA and ITS1 to determine the *Leishmania* subgenus using RFLP as a complement method to define the *L. infantum* species.

KEYWORDS: *Leishmania infantum*; Molecular diagnosis; Visceral leishmaniasis; PCR; RFLP

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1. Introduction

According to the World Health Organization (WHO), 300 000 cases of visceral leishmaniasis (VL) are reported worldwide annually with 40 000-50 000 deaths[1]. In the Americas, 90% of cases occur in Brazil, and *Leishmania (L.) infantum* (*donovani* complex) is the causative agent of VL[2]. Accurate and rapid diagnosis of VL is necessary, because it can be lethal. Parasitologic and serologic methods (gold standard) are used for the laboratory diagnosis of VL; however, they have low sensitivity, especially the serologic methods in immunocompromised patients. Polymerase chain reaction (PCR) is more sensitive[3,4] and specific[3,5] than these routine methods[1,6]. In addition, it is possible to identify the species of *Leishmania* using different targets and methodologies. In some situations, identification of *Leishmania* is crucial, especially for patients coinfected with *Leishmania* and HIV[2,7,8], because the dermotropic species (*L. braziliensis* and *L. amazonensis*) can cause visceral lesions[9-11]. On the other hand, cutaneous and mucosal leishmaniasis can be caused by *L. infantum*[8,12] in both immunocompetent and immunosuppressed patients. Therefore, this wide range of clinical presentations make identification of the causative agent necessary, and molecular biology techniques can play an important role[5,9-13]. Identification of the *Leishmania* species is also important to identify the species circulating in a given area, especially in regions where different species are present, as occurring in Brazil. Using molecular biology, various sequences from both genomic and extra-chromosomal regions have been exploited as targets of amplification by PCR[14], such as *Leishmania* kinetoplast DNA (kDNA) RV1/RV2 and *Leishmania* internal transcriber spacer DNA (ITS1 DNA) LITSR/L5.8S. PCR using kDNA primers presents high sensitivity, because kDNA is present in large numbers within the mitochondria of the parasite. ITS sequences are composed of highly conserved regions, allowing their use in PCR for diagnostic purposes, and they have polymorphic regions that can be used in restriction fragment length polymorphism (RFLP) assays to determine *Leishmania* species[15]. In addition, bone marrow aspirate (BMA) and peripheral blood (PB) can be used as sources for *L. infantum* DNA research[6]. Therefore, in order to diagnose VL and identify the *Leishmania* species causing VL, we aimed to determine an algorithm for the molecular diagnosis of VL using kDNA PCR (RV1/RV2) and ITS1 PCR (LITSR/L5.8S), complemented by ITS1 PCR RFLP, using PB or BMA from patients with suspected VL.

2. Materials and methods

2.1. Location of the study and ethical approval

After approval of the Commission for Research Projects Analysis (CAPPesq, process number 191.806/2013), the study was carried out between July 2014 and November 2016 in Instituto de Medicina Tropical da Universidade de São Paulo (Tropical Medicine Institute), São Paulo, Brazil.

2.2. Biological samples

The biological samples used in this study were obtained from seven groups as follows. Group Ⅰ: 82 samples (PB and/or BMA) were obtained from patients attending the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP) and at the Universidade Federal do Mato Grosso do Sul (UFMS) with VL confirmed by clinical, epidemiologic, parasitologic, and/or serologic examinations performed by the immunochromatographic rapid test (IrK39). Group Ⅱ: 16 samples from patients under treatment for VL, confirmed by clinical, epidemiologic, parasitologic, and/or serologic examination (data extracted from medical records). Group Ⅲ: 14 BMA samples from dogs with canine visceral leishmaniasis (CVL) confirmed by clinical, parasitologic, and serologic examination (data provided by veterinarians). Group Ⅳ: a pool of six experimentally infected sandflies (*Lutzomya longipalpis*) fed with blood from dogs with CVL. Group Ⅴ: 18 samples from patients with confirmed tegumentary leishmaniasis (TL) who attended the HCFMUSP complex. Diagnoses were confirmed by clinical manifestations associated with parasitologic visualization of parasites on skin scrapings or skin biopsy samples (data extracted from medical records). Two control groups were included: Group Ⅵ comprised 30 samples from healthy blood donors from HCFMUSP provided by the Departamento de Biologia Molecular, Fundação Pró-Sangue/Hemocentro de São Paulo (Certificate of Ethics Presentation, 39278514.8.0000.0065). Group Ⅶ comprised 47 samples (PB and/or BMA) from patients with signs and symptoms suggestive of VL and/or from areas endemic for leishmaniasis (data extracted from medical records) who had other diagnosed diseases.

2.3. Reference strains

The reference strains of *Leishmania* spp. used as PCR positive controls were: *Leishmania (Leishmania) infantum* (MHOM/BR/72/strain46), *L. (L.) amazonensis* (MHOM/BR/1973/M2269), *L. (L.) donovani* (MHOM/IN/80/DD8), *L. (L.) major* (MHOM/1L/80/Friedlin), *Leishmania (Viannia) braziliensis* (MHOM/BR/75/M2903), *L. (V.) guyanensis* (MHOM/BR/1975/M4147), *L. (V.) shawi* (MHOM/BR/2001/M19672), *L. (V.) lainsoni* (MHOM/BR/81/M6426), and *L. (V.) naiffi* (MDAS/BR/79/M5533).

2.4. DNA samples from different pathogens

To ensure amplification specificity, DNA samples from different

pathogens were tested: *Trypanosoma (T.) cruzi* (5 samples from patients and Y strain); *T. brucei*; *Mycobacterium (M.) tuberculosis*; *Toxoplasma gondii* (3 different strains); *Plasmodium falciparum*; *Histoplasma capsulatum*, and *Schistosoma (S.) mansoni*.

2.5. Gold standard methods for laboratory diagnosis of human VL

Parasitology and/or serology are the gold standard methods for the laboratory diagnosis of human VL (HVL), recommended by Ministério da Saúde do Brasil (Health Ministry of Brazil). These methods are used to define cases of leishmaniasis. Parasitology is based on microscopy examination of stained smears or cultures prepared from BMA or PB buffy coat samples. Serology is based on ELISA using PB samples or IrK39 using BMA or PB samples.

2.5.1. Parasitology (stained smear technique)

Smears from BMA and/or from PB buffy coat samples were prepared using 5 μL of sample stained with panotic dye (Newprov, Pinhais, Brazil) and analyzed by microscopy (1 000× magnification).

2.5.2. Parasitology (culture technique)

Forty microliters of BMA and/or PB buffy coat were transferred into tubes containing Novy-MacNeal-Nicolle/brain heart infusion medium (DIFCO, Detroit, MI, USA). Aliquots of 10 μL were obtained from the cultures and analyzed by microscopy (400× magnification) once a week, for 30 d.

2.5.3. Serology (IrK39 test)

Whole blood, plasma, or BMA was analyzed using IrK39-IT LEISH (Bio-Rad/DiaMed, Cressier, Switzerland) according to the manufacturer's pre-established protocols.

2.6. Gold standard method for laboratory diagnosis of CVL

Canine samples were analyzed by parasitologic and serologic examination (ELISA and rapid immunochromatographic dual-path platform tests- Biomanguinhos/FIOCRUZ, Rio de Janeiro, Brazil) to diagnose CVL. These parasitologic and serologic tests are the gold standard for the laboratory diagnosis of CVL. The tests were carried out at the Instituto Adolfo Lutz (Adolfo Lutz Institute), São Paulo, Brazil.

2.7. Gold standard method for laboratory diagnosis of TL

Visualization of parasites on skin scrapings or skin biopsy samples (data extracted from medical records) from 18 patients with TL were carried out at HCFMUSP.

2.8. Molecular techniques performed for groups Ⅰ-Ⅶ

Samples from BMA or PB (groups Ⅰ, Ⅱ, Ⅲ, Ⅵ, and Ⅶ), macerated from a pool of sandflies (group Ⅳ), and from biopsy samples (group Ⅴ) were subjected to PCR. Genomic DNA was extracted from 200 μL of PB or BMA using a QIAamp DNA blood kit (QIAGEN, Hilden, Germany) and a QIAamp DNA tissue kit (QIAGEN), according to the manufacturer's recommendations. The concentration of the DNA samples was analyzed in a NanoDrop 1 000 spectrophotometer (Thermo Fisher, Boston, MA, USA) and set at 200 ng by PCR. Filter tips with physical barriers were used to minimize the risk of PCR carry over, such as the use of separate work areas (reagent, extraction, and amplification room). All DNA samples from groups Ⅰ-Ⅱ and Ⅴ-Ⅶ were subjected to PCR of the constitutive human *beta-actin* gene (B1 and B2) to evaluate that this constitutive protein has not been affected in the tested samples, ensuring the quality of the samples and the inexistence of inhibitors[16].

The procedures for ITS1 PCR (LITSR/L5.8S) and kDNA PCR (RV1/RV2) are described in Table 1[17,18]. Products of PCR were visualized on ethidium bromide-stained 2% agarose gels (Agargen, Madrid, Spain) examined on a transilluminator (Alpha Innotech, San Leandro, CA, USA). Twenty microliters of ITS1 PCR products were

Table 1. Description of target sequences of ITS1-PCR (LITSR/L5.8S) and kDNA-PCR (RV1/RV2), primers, amplified fragments (bp), references and PCR conditions.

Target sequence	Primer sequence (5'-3')	Amplified fragment (base pairs)	Reference	PCR conditions		
				Reagents concentration	No. of cicles	Cycles
Internal transcriber spacer 1 of ribossomal RNA of *Leishmania* spp (ITS1)	LITSR: CTGGATCATTTT CCGATG L5.8S: TGATACCACTTA TCGCACTT	320	El Tai *et al*, 2000[17]	1×Buffer 0.2 mM of dNTP's 4 mM of $MgCl_2$ 400 nM of each primer 2 U of Taq DNA Polimerase	40	Initial denaturation: 95 ℃, 360 sec; Denaturation: 95 ℃, 20 sec; Annealing: 53 ℃, 30 sec; Extension: 72 ℃, 60sec; Final extension: 72 ℃, 360 sec.
Kinetoplast DNA of *Leishmania* spp (kDNA)	RV1: CTTTTCTGGTCC CGCGGGTAGG RV2: CCACCTGGCCTA TTTTACACCA	145	Ravel *et al*, 1995[18]	1×Buffer 0.2 mM of dNTP's 1 mM of $MgCl_2$ 0.25 μM of each primer 1.5 U of Taq DNA Polimerase	30	Initial denaturation: 95 ℃, 300 sec; Denaturation: 95 ℃, 30 sec; Annealing: 60 ℃, 30 sec; Extension 72 ℃, 30 sec; Final extension: 72 ℃, 300 sec.

digested with 1 U of the restriction enzyme HaeⅢ in 1× buffer (Fermentas, Burlington, ON, Canada), following the manufacturer's instructions. Restriction fragments were visualized on ethidium bromide-stained MetaPhor agarose gels (Lonza Rockland Inc., Rockland, Maine, USA) examined on a transilluminator (Alpha Innotech).

2.9. Sequencing

Ten ITS1 PCR amplicons from samples belonging to group Ⅰ (VL patients), 2 samples from dogs with confirmed CVL and *L. (L.) infantum* strain (MHOM/BR/81/M6445), used as a reference, were sequenced. Sequencing reactions were performed on the ABI PRISM 3500 genetic analyzer platform (Thermo Fisher) using the BigDye terminator cycle sequencing kit (Applied Biosystems, Waltham, MA, USA). Then, the electropherograms (sequences), the positive control *L. (L.) infantum* strain (MHOM/BR/81/M6445), and the reference sequence from the region of interest (KF985171.1) retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank) were manually edited using the BioEDIT sequence alignment editor. The alignment of sequences was examined using the codon code aligner and compared using the basic local alignment search tool (BLAST) sequence analysis tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Sequencher 4.1.4 program (Genes Code Corporation, Ann Arbor, MI, USA).

Data analysis was performed to determine the sensitivity, specificity, positive and negative predictive values, and efficiency of ITS1 PCR and kDNA PCR. The kappa index was used to evaluate the proportion of agreement, in addition to that expected by randomness, between the two molecular tests and the gold standard examinations, adopted as reference in the present study (parasitologic tests on BMA and PB samples, IrK39, and clinical and epidemiologic data). The confidence interval was 95% based on the estimated standard kappa error of samples and their *Z* score. A *P* value <0.05 was considered significant. The STATA program, version 13.0 (Stata Corp LP, College Station, TX, USA) was used for the statistical analyses.

3. Results

3.1. Gold standard methods for laboratory diagnosis of HVL, CVL, and TL

According to Table 2, panotic-stained smears and cultures were positive in 76.3% (61 of 80) and 27.3% (12 of 44) of the samples from group Ⅰ (82 samples of HVL), respectively. A total of 68.8% (55 of 80) of these samples from group Ⅰ were positive by the IrK39 test (Table 2), and 27.3% (15 of 55) of the samples positive by IrK39 were from patients coinfected with *Leishmania* and HIV. Among the samples those were negative by IrK39, 40.0% (10 of 25) were from patients coinfected with *Leishmania* and HIV. Groups Ⅱ, Ⅲ, and Ⅴ presented the following results for the gold standard methods specific to each group (Table 2): 16 patients (group Ⅱ) under treatment for HVL were negative for the parasitologic methods and positive for Ir39 (100%, 16 of 16); 14 dogs (group Ⅲ) were positive in the parasitologic and serologic tests (100%, 14 of 14); and 18 patients (group Ⅴ) with TL were positive in the parasitologic examination (100%, 18 of 18).

3.2. Molecular techniques for groups Ⅰ–Ⅶ

All samples from groups Ⅰ-Ⅱ, Ⅴ-Ⅶ successfully amplified the 520 base pair (bp) fragment from the *beta-actin* gene, which ensured the absence of amplification inhibitors; this constitutive protein was unaffected in all samples tested.

ITS1 (LITSR/L5.8S) and kDNA (RV1/RV2) generated fragments of 320 bp (Figure 1) and 145 bp (Figure 2), respectively. They were positive in 97.6% (80 of 82) and 92.7% (76 of 82) of the samples from group Ⅰ (82 patients with HVL), respectively (Table 2).

Table 2. Results of the gold standard exams and molecular techniques of the 7 groups.

Groups	Lab diagnosis for HVL (GS)									Lab diagnosis for CVL (GS)			Lab diagnosis for TL (GS)		Molecular diagnosis			
	Parasitological (BMA/buffy coat of PB)						Serological			Serological			Parasitological (skin scraping/biopsies)					
	Smear			Culture			IrK39			DPP and ELISA			Smear and culture		kDNA		ITS1	
	Pos	Neg	Mis	Pos	Neg	Mis	Pos	Neg	Mis	Pos	Neg	Mis	Pos	Neg	Pos	Neg	Pos	Neg
Group Ⅰ	61	19	2	12	32	38	55	25	2	-	-	-	-	-	76	6	80	2
Group Ⅱ	0	16	0	0	16	0	16	0	0	-	-	-	-	-	4	12	2	14
Group Ⅲ	14	0	0	-	-	-	-	-	-	14	0	0	-	-	12	2	12	2
Group Ⅳ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Pool	0	Pool	0
Group Ⅴ	-	-	-	-	-	-	-	-	-	-	-	-	18	0	-	-	13	5
Group Ⅵ	-	-	-	-	-	-	0	30	0	-	-	-	-	-	0	30	0	30
Group Ⅶ	0	47	0	0	47	0	0	47	0	-	-	-	-	-	0	47	0	47

Group Ⅰ: 82 patients with HVL; Group Ⅱ: 16 patients with HVL in treatment; Group Ⅲ: 14 dogs with CVL; Group Ⅳ: 1 pool containing 6 sandflies; Group Ⅴ: 18 patients with TL; Group Ⅵ: (CG) 30 blood donors; Group Ⅶ: (CG) 47 from other diseases; GS: Gold standard; HVL: human visceral leishmaniasis; CVL: canine visceral leishmaniasis; TL: tegumentary leishmaniasis; CG: control group; BMA bone marrow aspirates; PB: peripheral blood; Pos: positive; Neg: negative; Mis: missing; -: Not done.

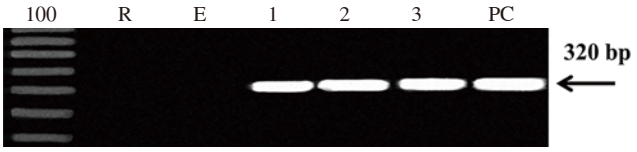

Figure 1. Agarose gel electrophoresis (2%) of ITS1-PCR (320 base pairs) from positive samples of patients with visceral leishmaniasis (VL). (100) 100 bp DNA ladder. (R) Negative control of reagents room. (E) Negative control of extraction room. Lanes 1, 2 and 3-samples of patients with VL. (PC) *L. (L.) infantum* positive control.

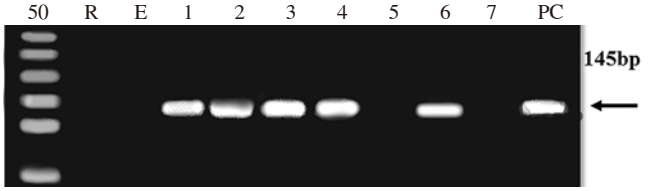

Figure 2. Agarose gel electrophoresis (2%) of kDNA(RV1-RV2)-PCR (145 bp) from positive samples of patients with visceral leishmaniasis (VL). (50) 50 bp DNA ladder. (R) Negative control of reagents room. (E) Negative control of extraction room. Lanes 1, 2, 3 and 4 samples of patients with VL. Lanes 5 and 7-samples of patients with VL but negative in kDNA (RV1-RV2). (PC) *L. (L.) infantum* positive control.

After ITS1 PCR RFLP (Hae Ⅲ) analysis of the 80 positive samples, 52.5% (42 of 80) generated three fragments of 180, 70, and 50 bp, corresponding to the pattern of *L. (L.) infantum* (Figure 3).

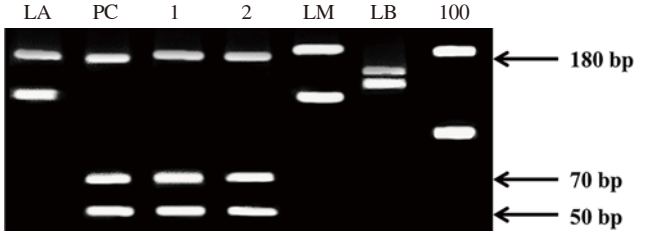

Figure 3. Metaphor agarose gel electrophoresis (4%) of ITS1-PCR-RFLP for the evaluation of profiles obtained from samples of patients with visceral leishmaniasis (VL). (100) 100 bp DNA ladder. (LA) *L. (L.) amazonensis* (190 and 140 bp), (PC) *L.(L.) infantum* (180, 70 and 50 bp). Lanes 1 and 2-samples from patients with VL. (LM) *L. (L.) major* (200, 140 bp). (LB) *L. (V.) braziliensis* (160, 150 bp).

Of the 16 samples analyzed from group Ⅱ (VL patients under treatment), 37.5% (6 of 16) presented positive results for at least one of the molecular tests. ITS1 PCR detected 12.5% (2 of 16) of the samples from group Ⅱ, and these 2 samples generated no profiles by ITS1 PCR RFLP, because the 320 bp fragment remained unrestricted. Of the 16 samples, 25.0% (4 of 16) were positive by kDNA PCR (RV1/RV2).

ITS1 PCR and kDNA PCR diagnosed 85.7% (12 of 14) from group Ⅲ (CVL). According to the results obtained by kDNA, these 12 samples belonged to *Leishmania* subgenus. ITS1 PCR RFLP showed that 83.3% (10 of 12) of the canine samples contained parasites with profiles similar to *L. infantum*.

Pool samples of DNA from six infected sandflies (group Ⅳ) were tested by ITS1 PCR and kDNA PCR, and both systems yielded amplifications (Table 2). ITS1 PCR products were analyzed by RFLP, generating a profile similar to that of *L. infantum*.

In group Ⅴ (patients with TL), 72.2% (13 of 18) of the samples analyzed by ITS1 PCR were positive (Table 2) and 69.2% (9 of 13) showed profiles corresponding to a *Viannia* complex by ITS1 PCR RFLP.

Regarding the specificity of the ITS1 PCR, there was no amplification with DNA samples from other pathogens, whereas kDNA PCR amplified DNA from *S. mansoni*. When kDNA PCR was carried out with DNA from reference strains, *L. (L.) amazonensis* and *L. (L.) infantum* were amplified. These two species belong to *Leishmania* subgenus.

Thirty samples from group Ⅵ (blood donors) and 47 from group Ⅶ (other diseases) were negative by ITS1 and kDNA molecular tests (Table 2). As shown in Table 3, when PCR results were compared with the gold standard for HVL, near perfect agreement was observed, with a kappa index >0.80 and a *P* value <0.001.

For samples from patients with confirmed HVL, 90.2% (74 of 82) showed agreement between ITS1 and kDNA PCR. There was disagreement in 9.8% (8 of 82) of the samples; two were negative by ITS1 PCR and positive by kDNA PCR, and six were positive by ITS1 PCR and negative by kDNA PCR. Five of these six samples presented a profile corresponding to *L. (L.) infantum* by ITS1 PCR RFLP.

Table 3. Comparative analysis of molecular diagnosis by ITS1-PCR and kDNA-PCR using samples from confirmed cases of visceral leishmaniasis (Group Ⅰ) and samples from healthy individuals (Group Ⅵ).

Molecular tests		Gold standard			Kappa index (95% *CI*)	*P* value
		Positive (*n*=82)	Negative (*n*=30)	Total (*n*=112)		
kDNA-PCR (RV1/RV2)	Positive	76	0	76	0.871 (0.687-1.000)	<0.001
	Negative	6	30	36		
ITS1-PCR (LITSR/L5.8S)	Positive	80	0	80	0.955 (0.771-1.000)	<0.001
	Negative	2	30	32		

Gold standard: clinical manifestations, epidemiological information and parasitological and/or immunocromatographic (IrK39) techniques results.

3.3. Sequencing

It was possible to analyze the results of nine of 12 samples sequenced (eight from group Ⅰ and one canine sample with CVL from group Ⅳ) and 100% (9 of 9) showed 99% similarity with the reference sequence (KF985171.1).

3.4. The proposed algorithm

Based on our findings (parasitologic, immunologic, and molecular tests), an algorithm is proposed for processing samples until the species is defined (Figure 4). According to our proposal, the parasitologic and immunochromatographic diagnoses should be considered before the molecular tests, and kDNA PCR, for the determination of *Leishmania* subgenus, should be done after a negative ITS1 PCR. A positive ITS1 PCR must be followed by ITS1 PCR RFLP to define the agent causing VL.

4. Discussion

A case of VL is confirmed when, even before clinical suspicion; a positive laboratory diagnosis is demonstrated by parasitologic or serologic tests (indirect immunofluorescence ELISA, or

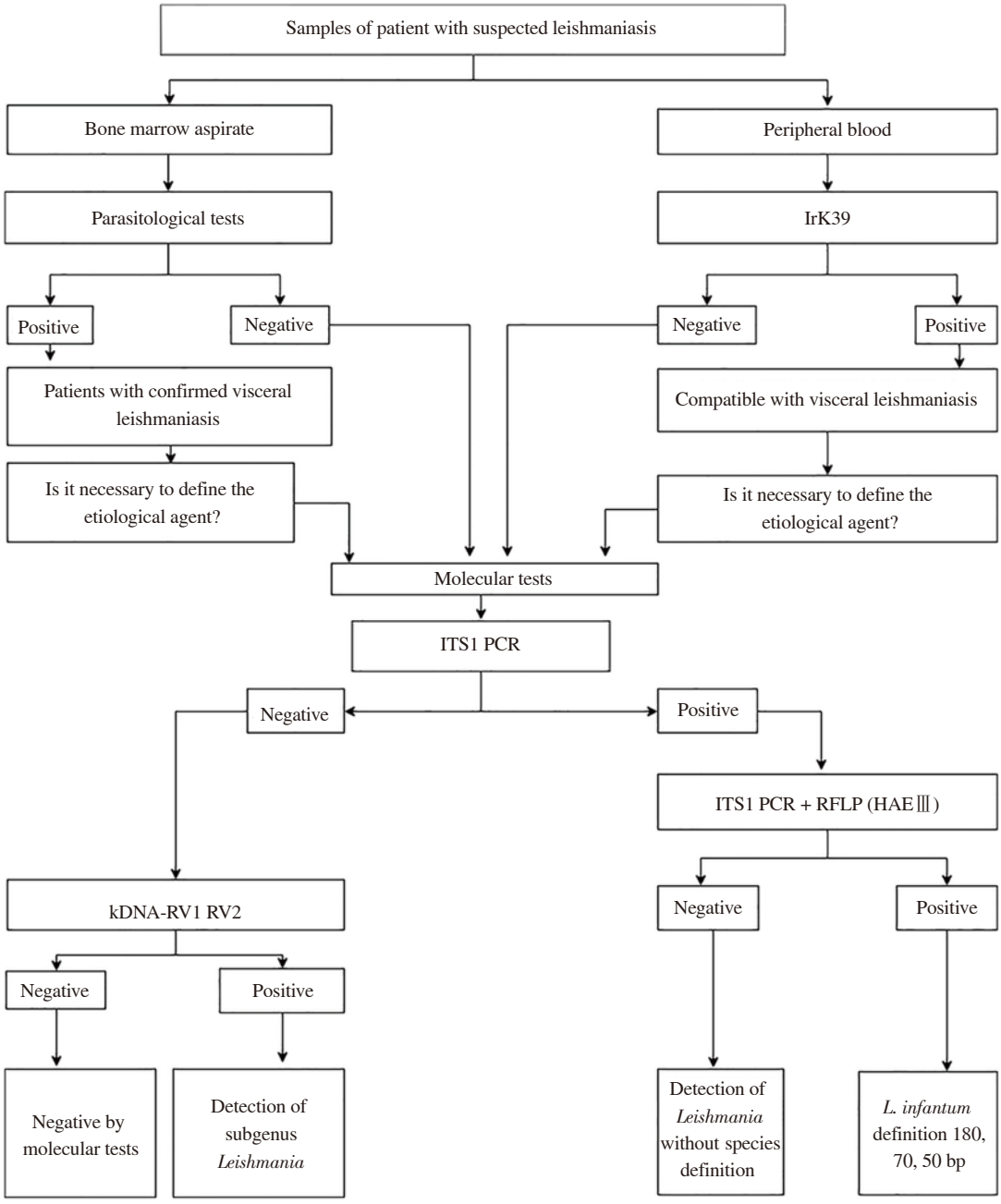

Figure 4. Algorithm proposed for processing samples at the species level from patients with suspected VL. VL=visceral leishmaniasis; Irk39= immunochromatographic test rk39; BM=bone marrow; PB=peripheral blood; PT=parasitological tests; NEG=Negative; POS=Positive; bp=base pairs.

immunochromatographic tests using recombinant antigens)[14]. In our study, 76.3% (61 of 80) of patients with VL (group Ⅰ) were positive by parasitologic examination of smears from BMA/PB stained with Panotic dye. Koltas *et al*.[3] reported that 20.8% (10 of 48) and 80.0% (8 of 10) of smears and cultures of BMA from children with clinical suspicion of VL were positive, respectively. Sensitivities of 98.0%, 87.9%, and 72.7%, respectively, were obtained by kDNA PCR, smears, and cultures of samples of patients with TL in a study by Rasti *et al*[4]. Although parasitologic tests demonstrate the presence of the parasite in the samples, they can not define the causative agent of VL, because it is not possible to distinguish the parasite by microscopic analysis in both techniques[3,19].

Several studies report high sensitivity and specificity of IrK39[20]. Although the test is considered specific for the diagnosis of VL[21], there are reports of false-positive results[22,23]. IrK39 was positive in 68.8% (55 of 80) of the samples from group Ⅰ, which comprises patients with confirmed VL. The samples that were positive for IrK39 included some patients coinfected with *Leishmania* and HIV, and 60.0% (15 of 25) of these samples were positive in IrK39. This result corroborates findings from other authors in which IrK39 had decreased sensitivity due to the presence of immunodeficiency and achieved 45% sensitivity[21] .

Although VL is usually caused by *L. (L.) infantum* in Brazil, it is important to identify the species responsible for the VL to allow suitable treatment. It is also necessary to identify species in epidemiologic surveys and to define the species responsible for atypical symptoms in patients with *Leishmania*/HIV coinfection or even in immunocompetent patients[3,24]. Using molecular approaches, such as real-time PCR or conventional PCR, it is possible to identify the species involved in the leishmaniasis infection. The sensitivity of real-time PCR is superior to conventional PCR, in addition to presenting other advantages[24,25]; however it is an expensive technology. Because leishmaniasis is a neglected disease, this technology is not available to the public health system in Brazil, which explains our choice of conventional PCR. With conventional PCR, depending on the target chosen in DNA, and/or performing RFLP, it is possible to define the species of the agent responsible for VL[25]. Therefore, ITS1 sequences were chosen because they are composed of highly conserved regions, allowing their use in PCR for diagnostic purposes, and they have polymorphic regions that can be used in RFLP assays to determine the species. Also, having kDNA as a target and using the RV1/RV2 primer pair, it is possible to demonstrate the *Leishmania* subgenus present in the infection. When analyzing the sensitivity of the PCR with the primer pair (LITSR and L5.8S) target in the ITS1 region of the DNA, the sensitivity was superior (97.6%) to that of kDNA (92.7%) when tested in samples from patients with VL (group Ⅰ); however, this difference was not significant. These sensitivities (ITS1 and kDNA) were higher than the sensitivity for parasitology (76.3%) and IrK39 (68.8%) for samples from patients with VL. On the other hand, in a study with 431 blood donors from the state of Ceará (Brazil), ELISA detected more positive samples (13.2%, 57 of 431) than kDNA PCR (K20/K22) (4.6%, 20 of 431)[26]. Khan *et al*.[14] reported sensitivities of 98.4% (60 of 61) and 96.7% (59 of 61) with IrK39 and ITS1, respectively.

In contrast to our findings with ITS1, Beldi *et al*.[27] found low sensitivity (63.9%, 23 of 36) in samples from patients with VL in Algeria; however, the authors used smears of BMA to obtain the DNA of the parasite. Koltas *et al*.[3] reported a sensitivity of 90% (9 of 10) with ITS1 in samples from patients with VL.

Regarding specificity, ITS1 did not amplify any DNA in pathogen samples (*T. cruzi, T. brucei, M. tuberculosis, Toxoplasma gondii, Plasmodium falciparum, Histoplasma capsulatum, and S. mansoni*) in our study. Ozerdem *et al*.[28] also reported 100% specificity with ITS1. Some studies have tested ITS1 with strains of *M. tuberculosis, M. leprae, S. mansoni, Wuchereria bancrofti,* and *T. cruzi*, and also without non-specific amplification, demonstrating the importance of this target in terms of analytical specificity[28,29]. In our findings, kDNA (RV1/RV2) was considered acceptable to define the *Leishmania* subgenus, because there was amplification of DNA from a reference strain of *L. (L.) amazonensis*, which belongs to the same subgenus of *L. (L.) infantum*. Solcà *et al*.[30] demonstrated the amplification of kDNA (RV1/RV2) for *L. (L) major* and *L. (L.) amazonensis*. Therefore, amplification of the subgenus *Leishmania* using RV1/RV2 primers was demonstrated in these studies.

Multilocus enzyme electrophoresis is the gold standard technique for the identification of *Leishmania* species. However, it is an expensive and laborious method that requires culturing of the parasite before its execution[8]. We have proposed an algorithm for the molecular diagnosis of VL-specific species using the primer pair LITSR/L5.8S (ITS1 PCR) to identify the species. Thus, we used RFLP as an alternative to multilocus enzyme electrophoresis, with restriction enzyme Hae Ⅲ, on the products of ITS1 PCR. The ITS1 PCR RFLP technique is widely used in studies involving leishmaniasis in both the Old and New World[19,25,31]. It is known that the causative agent found in the American continent is *L. (L.) infantum*, which belongs to a *donovani* complex[2]. Of the 80 positive samples that amplified in the ITS1-PCR, 52.5% (42 of 80) demonstrated a profile similar to that of *L. (L.) infantum* by ITS1 PCR RFLP. However, ITS1 PCR diagnosed 85.7% of the canine samples (12 of 14) from group Ⅲ, and ITS1 PCR RFLP showed that 83.3% (10 of 12) of dog samples contained parasites with profiles similar to *L. (L.) infantum*. The distinction between species of the *Viannia* and *Leishmania* subgenus was very clear, even without the use of high-resolution gel. But the different sensitivities obtained using ITS1 PCR RFLP between humans (52.5%, 42 of 80 in group Ⅰ) and dogs (83.3%, 10 of 12 in group Ⅲ) can be related to the low parasitic load in humans[31], explaining the lack of species

definition using ITS1 PCR RFLP in 48.8% (39 of 80) of our positive human VL samples (group Ⅰ).

In the study by Hijjawi *et al*.[32], ITS1 PCR RFLP was able to identify the species responsible for TL in Jordan in 28 of the 30 positive samples in ITS1 PCR. Monroy-Ostria *et al*[19], when testing skin lesions in patients from Mexico with ITS1 PCR RFLP (Hae Ⅲ), obtained different restriction profiles for the species *L. (L.) mexicana*, *L. (L.) amazonensis*, and a third profile that grouped the species *L. (V.) panamensis, L. (V.) guianensis*, and *L. (V.) braziliensis*.

Of the 18 samples belonging to group Ⅴ (patients with TL), 72.2% (13 of 18) were positive in ITS1. The 13 positive samples showed an electrophoretic profile similar to that found for *L. (V.) braziliensis*, *L. (V.) lainsoni*, *L. (V.) shawi*, and *L. (V.) guyanensis*. Amro *et al*.[33] have described possible inhibition or failure of ITS1 amplification in samples from TL patients in the Old World.

These two systems (ITS1 and kDNA) complemented each other in our study; they diagnosed 100% of the samples belonging to the *Leishmania* genus. In addition, kDNA defined *Leishmania* subgenus in 92.7% of the samples and more specifically, *L. (L.) infantum* was identified by ITS1 PCR RFLP in 52.5% (42 of 80) of positive samples. Based on these findings, we suggest an algorithm for the molecular diagnosis of VL, which must first consider the parasitologic and immunochromatographic diagnosis. This molecular diagnosis can combine two PCR target systems: ITS1 and kDNA. ITS1 complemented with RFLP can define the *L. (L.) infantum* species, corroborating the IrK39 findings (donovani complex). Also, for samples with negative results of ITS1, kDNA target (RV1/RV2) should be used to determine, at least, the *Leishmania* subgenus.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Authors’ contributions

N.S.G. and L.M.A.B. were involved in the design of the study, the literature search, sample collection, laboratory diagnosis, data acquisition, data analysis and interpretation, statistical analysis, writing, editing, and review of the manuscript. L.M.A.B. was also involved in the concept of the study, was responsible for obtaining funding, and supervising the project. M.S.L. was involved in sample collection, laboratory diagnosis, data acquisition, data analysis and interpretation, editing some figures, and contributing to the final version of the manuscript. V.L.C. was involved in the laboratory diagnosis, data acquisition, and manuscript editing. J.A.L.L. was involved in selecting and recruiting the patients, sample collection, clinical analysis, data acquisition, and preparation and review of the manuscript. T.S.O. was involved in clinical and data analysis and preparation and review of the manuscript. All authors read and approved the final manuscript.

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