Parasitology

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Identification of *Leishmania infantum* in blood donors from endemic regions for visceral leishmaniasis

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Research Article

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Abstract

Visceral leishmaniasis is an endemic protozoonosis observed in over 60 countries, with over 500 000 new cases recorded annually. Although the diagnostic procedure of its symptomatic forms is well established, for asymptomatic patients, who represent about 85% of those infected, there is no consensus on the best method for its identification. Recent studies have presented molecular techniques as viable identification methods, with good sensitivity and specificity indices in asymptomatic individuals. Therefore, we aimed to use molecular methods to assess their effectiveness in identifying the presence of asymptomatic infection by *Leishmania infantum* (*L. infantum*) individuals from endemic regions of Brazil. Screening was performed by real-time polymerase chain reaction (qPCR) and confirmed by sequencing the cytochrome B gene. Of the 127 samples [from 608 blood donors who had participated in a previous study, of which 34 were positive by the enzyme-linked immunosorbent assay (ELISA) rK39] tested by qPCR, 31 (24.4%) were positive. In the sequencing of 10 qPCR-positive samples, five were identified as *L. infantum*. Complimentary samples of the ELISA rK39 and conventional PCR showed only reasonable and low agreement with qPCR, respectively. The qPCR confirmed the presence of infection in five of the 10 sequenced samples, ELISA confirmed three, and the conventional PCR confirmed none.

Introduction

Visceral leishmaniasis (VL) is an endemic protozoonosis found in four continents (Europa, Asia, Africa and America). However, approximately 90% of the cases are concentrated in Asia, Africa and Latin America (Alvar *et al.*, 2012). In 2018, more than 95% of new cases appeared in 10 countries: China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan, Sudan and Brazil (WHO, 2020). The disease can be severe and fatal, especially in immunocompromised individuals (Mpaka *et al.*, 2009; Menezes *et al.*, 2016). Despite the severity of symptomatic cases, approximately 85% of individuals remain asymptomatic, harbour the parasite for decades, and become a source of infection and spread of the disease, especially in endemic areas (Michel *et al.*, 2011; Jimenez-Marco *et al.*, 2012).

Asymptomatic individuals, especially those from endemic areas, show an immune response (production of specific antibodies or cellular) against *Leishmania infantum* (*L. infantum*) or presence of parasitic DNA in the peripheral blood, but remain healthy, without clinical presentation of the characteristic symptoms of the disease (Alvar *et al.*, 2020).

Studies have reported that the prevalence of asymptomatic infection among blood donors varies between 5 and 15% in different regions of Brazil, one of the seven countries with the highest prevalence of this endemic disease (Luz *et al.*, 1997; Urias *et al.*, 2009; França *et al.*, 2013; Ferreira-Silva *et al.*, 2018). Moreover, the survival and infectious capacity of *L. infantum* in stored blood components and transmission records, where transfusion of blood components is the only possible means of transmission, increases the need for measures to ensure maximum safety in transfusions, especially in endemic regions (Grogl *et al.*, 1993; Cummins *et al.*, 1995).

The diagnosis of symptomatic VL has appropriately been established and is based on clinical manifestations and epidemiological and laboratory data. However, there is still no consensus on the best method for identification of the asymptomatic form, which hinders the collection of accurate epidemiological data and limits the control of the disease. Several studies have shown that various serological techniques have low sensitivity and specificity, in addition to low agreement (Romero *et al.*, 2009; Silva *et al.*, 2011, 2013; Ferreira-Silva *et al.*, 2018). Recent studies have presented molecular techniques as viable alternatives with better indices of sensitivity and specificity in asymptomatic individuals (Sudarshan and Sundar, 2014; Kaushal *et al.*, 2017; Galluzzi *et al.*, 2018).

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In Brazil, laboratory diagnosis of VL is performed using commercial serological tests in most health services, and molecular diagnosis is practically restricted to research centres. Therefore, the present study aimed to use molecular methods to assess their effectiveness in the identification of the presence of asymptomatic infection by *L. infantum* in individuals from endemic regions of Brazil; it also compared the results with those found by other techniques.

Materials and methods

Participants

This study included 127 individuals eligible for blood donation, with no previous history of VL, selected from 608 blood donors who had participated in a previous study (Ferreira-Silva *et al.*, 2018). All 127 were selected from four cities in Brazil where the disease is endemic: Fortaleza and Sobral/Ceará (CE), Teresina/Piauí (PI) and Montes Claros/Minas Gerais (MG). We used 34 of the 37 positive samples by enzyme-linked immunosorbent assay (ELISA), which presented complete epidemiological data, and 93 negative samples were selected at random. All the participants signed a consent form to participate in the study. This study was approved by the Human Research Ethics Committee of the Federal University of Triângulo Mineiro and Hemominas Foundation (Protocol 2104 and 332, respectively.)

ELISA rK39 and conventional polymerase chain reaction (PCR)

ELISA rK39 (Kalazar Detect ELISA InBios® Seattle, Washington, USA) was performed following the manufacturer's instructions, and conventional PCR was conducted as described by Ferreira-Silva *et al.* (2018).

Quantitative real-time polymerase chain reaction (qPCR)

DNA extraction from the blood samples was performed using Qiagen's DNA Mini Kit from Qiagen (Hilden, Germany) following the manufacturer's recommended method. The 127 samples were subjected to qPCR to identify *Leishmania* spp., with primers described by Nicolas *et al.* (2002). The reaction consisted of 1XSYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA), 10 pM of each primer, 50 ng of DNA and ultrapure water for a final volume of 20 μL. Amplification was performed at an initial holding temperature of 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 60 °C for 1 min and melting curve analysis in 1 °C increments from 60 to 95 °C. Each assay included an internal control of human β-actin in each sample. The database was analysed using Applied Biosystems 7500 Real-Time PCR Software v2.3 (Thermo Fisher Scientific, Waltham, MA, USA). DNA from *Trypanosoma cruzi* (*T. cruzi*) was used to demonstrate the absence of cross-reactivity of the selected target, as described by Nicolas *et al.* (2002).

PCR amplification of mitochondrial cytochrome B gene and sequencing technique

The presence of a cytochrome B (CytB) gene fragment was determined by sequencing 10 positive samples in qPCR. The amplification reaction was carried out in a 50 μL volume container containing 1x buffer supplied with Taq polymerase (Promega, USA), 100 mM dNTPs, 50 pM of each primer for CytB, 1.25 U of GoTaq polymerase (Promega) and 30 ng of DNA. Electrophoresis was performed on an agarose gel (1%) stained with 1 × GelRed™ (Biotium, USA) and visualized on a UV transilluminator. The PCR products were precipitated with 70% alcohol and sequenced on ACTGene (Molecular Analysis Company, Brazil). The obtained sequences were then submitted to the nucleotides' basic local alignment search tool (BLASTn) for a similarity search with *Leishmania* sequences deposited in GenBank databases (https://www.ncbi.nlm.nih.gov/genbank).

Table 1. Results of the analysis of κ agreement between ELISA and qPCR and conventional PCR and qPCR for the diagnosis of asymptomatic visceral leishmaniasis

	qPCR			
ELISA	Positives	Negatives	Total	κ
Positives	14	20	34	
Negatives	17	76	93	0.23
Total	31	96	127	
PCR				
Positives	5	3	8	
Negatives	26	93	119	0.17
Total	31	96	127	

ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; PCR, polymerase chain reaction.

Statistical analyses

The results were analysed in SPSS 21 using descriptive statistics and estimates of prevalence. The concordance of the tests was verified using κ coefficients (Miot, 2016).

To edit the sequencing components, SeaView 4.5.2 was used, and alignment was performed by ClustalW 1.8 with the following sequences: *L. infantum* (KX061911; MF344874; HQ908261), *Leishmania donovani* (AB095957), *Leishmania amazonensis* (MF344893; MF344892; MF344890), *Leishmania equatoriensis* (AB434686), *Leishmania braziliensis* (MF344881; MF344879; MF344878) as the outgroup and *Trypanosoma erneyi* (JN040956) (Luyo-Acero *et al.*, 2009; Gómez-Hernández *et al.*, 2017). A maximum composite likelihood analysis, using Tamura's three parameters, was performed using MEGA 7.0. To evaluate the robustness of the phylogenetic analysis, we used 1000 bootstraps (Kumar *et al.*, 2016). All sequences obtained in this work were deposited in GenBank (IDs: MH979699–MH979703).

Results

Characteristics of participants

Of the 127 participants, 4.7% were from Fortaleza, 35.4% from Sobral, 25.3% from Teresina and 34.6% from Montes Claros; their average age was 28.05 ± 8.52 years, and 63% were males.

Leishmania *spp. identification by qPCR and comparison with ELISA rK39 and conventional PCR*

Of the 127 samples analysed by qPCR, 31 (24.4%) were positive for *Leishmania* spp. The agreement indexes between qPCR and ELISA rK39 and the conventional PCR are presented in Table 1. Comparing ELISA with qPCR, we obtained a κ index of 0.23, showing reasonable agreement between the tests. However, comparing PCR with qPCR, we had a κ index of 0.17, which shows low agreement.

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Table 2. Comparative results between the techniques used in the 10 samples sequenced to identify infection by *Leishmania infantum*

Sample	qPCR	ELISA	PCR	Sequencing	Query cover (%)	*E*-value	Identity (%)	Accession
SO115	Positive	Negative	Negative	Satisfactory	100	0	97.89	KX061917
SO94	Positive	Negative	Negative	Satisfactory	100	0	100	MH979700
SO114	Positive	Negative	Negative	Unsatisfactory	–	–	–	–
TE62	Positive	Positive	Positive	Unsatisfactory	–	–	–	–
SO12	Positive	Positive	Negative	Satisfactory	100	0	97.72	KX061917
MCO82	Positive	Positive	Negative	Satisfactory	100	0	96.26	BK010877
MCO106	Positive	Positive	Negative	Satisfactory	99	0	99.12	KX061917
FO65	Positive	Positive	Negative	Unsatisfactory	–	–	–	–
FO89	Positive	Positive	Positive	Unsatisfactory	–	–	–	–
T E1	Positive	Negative	Negative	Unsatisfactory	–	–	–	–
[a]DNA 1	Negative	–	–	–	–	–	–	–
[a]DNA 2	Negative	–	–	–	–	–	–	–

ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; PCR, polymerase chain reaction.
[a]DNA samples from *T. cruzi* were inserted in the qPCR to detect possible cross-reactivity.

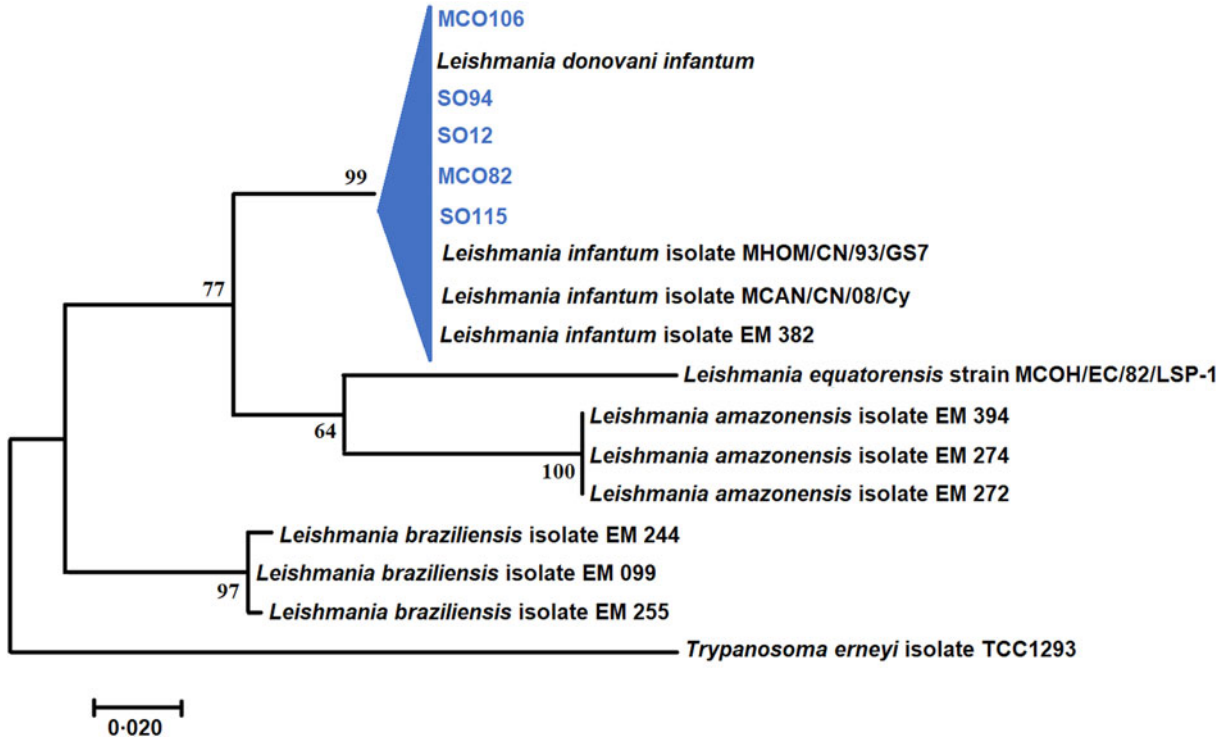

Fig. 1. Molecular phylogenetic analysis by the maximum-likelihood (ML) method of partial CytB sequence. Taxons were grouped by the percentage generated by the bootstrap. The analysis involved 17 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated. There were a total of 617 positions in the final dataset.

Confirmation of L. infantum *infection by direct sequencing and cross-reactivity exclusion*

Gene sequencing to identify *L. infantum* was then carried out in 10 samples positive by qPCR: four from Sobral, two from Fortaleza, two from Teresina and two from Montes Claros. Of these, five samples (three from Sobral and two from Montes Clear) showed positive and satisfactory results with coverage of 95–100%, enabling the identification of *L. infantum* (Table 2). These samples were grouped with reference samples of *L. infantum* by the maximum likelihood of the phylogenetic tree constructed based on the sequences of the CytB gene (Fig. 1). Additionally, two DNA samples from *T. cruzi* were tested and inserted in the qPCR to detect possible cross-reactivity. The results demonstrated the non-amplification of these samples with the primers used (Table 2).

Discussion

The severity of VL and the possible risk of transfusion transmission by contaminated blood components justify the search and implementation of measures to ensure the security of recipients. However, the laboratory diagnosis of asymptomatic patients

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remains challenging, highlighting the need for a solution and evaluation of the different tools available (Pereira *et al.*, 2014).

To find a test that could better identify these individuals, as there is still no universally accepted gold standard for such cases (Alvar *et al.*, 2020), our research group chose the available modern molecular biology techniques as tools. The molecular method of qPCR was selected because it has displayed better sensitivity and specificity than serological tests and other techniques (Reithinger and Dujardin, 2007; Ruiter *et al.*, 2014; Sudarshan et al., 2014). This has been demonstrated in a wide variety of peripheral blood samples in clinical and subclinical forms (Galluzzi *et al.*, 2018), including the identification of asymptomatic infections (Sudarshan and Sundar, 2014; Kaushal *et al.*, 2017). As for the selected system in our study, although the literature has considered TaqMan™ to have high specificity, we opted for SYBR Green, a relatively more economical and frequently used system, as it is accessible and viable for use in the public health field (Pereira *et al.*, 2014).

The presence of diseases, caused by other etiologic agents with a clinical picture similar to leishmaniasis, is common in Brazil's endemic areas for VL (Reithinger and Dujardin, 2007). Considering this, for this work, we chose a set of primers specific to conserved sequences from minicircles of kinetoplastid DNA present in *Leishmania* spp. (Nicolas *et al.*, 2012).

In the present study, the positivity for the genus *Leishmania* spp. by qPCR was 24.4%. When we compared the positive samples of ELISA rK39 and conventional PCR from the previous study (Ferreira-Silva *et al.*, 2018) to the positive samples by qPCR, we observed that the positivity relation found in the current study was low, and the concordance between ELISA rK39 and qPCR was only reasonable. Similar to our findings, a study that compared the serology and qPCR of asymptomatic infection by *L. donovani*, the aetiological agent of VL in the Old World, reported low agreement between the molecular and serological tests used (Sudarshan et al., 2014). Pessoa-E-Silva *et al.* (2019) also found discordant results when comparing serology, qPCR and parasitological examination and observed low agreement between the tests in a group of asymptomatic individuals. Poor correlation between different techniques used in the diagnosis of asymptomatic *L. infantum* infection can occur because of multiple factors, ranging from the ability of each test to detect a different stage of infection (Silva *et al.*, 2011; Pessoa-E-Silva *et al.*, 2019) to a consequence of the variable presence of antibodies or parasites in the analysed samples and cross-reactivity, which can lead to false results (Rodríguez-Cortés *et al.*, 2007; Romero *et al.*, 2009).

Even if the qPCR with the SYBR Green system is considered promising for the diagnosis of asymptomatic infection by *L. infantum*, no approach can, at the moment, be regarded as a gold standard (Kaushal *et al.*, 2017; Varani *et al.*, 2017; Galluzzi *et al.*, 2018). The 'best approach' depends on factors such as assay design, the selected target region, origin of the clinical sample, cycle temperature and the DNA extraction method (Gomes *et al.*, 2007; Medeiros *et al.*, 2017).

Some genetic targets, such as 18S (18S-rRNA), gp63 and CytB, can be used for taxonomic identification of *Leishmania* parasites (Uliana *et al.*, 1991; Mauricio *et al.*, 2001; Luyo-Acero *et al.*, 2009). The clinical-epidemiological importance of the identification of *Leishmania* species has encouraged the development and improvement of new molecular tools with greater accuracy, such as sequencing. We sequenced the CytB gene to confirm the presence of the parasite, and our results showed the presence of the *L. infantum* DNA in 50% of the analysed samples. Phylogenetic analysis of CytB gene sequencing provides high specificity in the classification of *Leishmania* species (Foulet *et al.*, 2007; Ramírez *et al.*, 2016; Gómez-Hérnández *et al.*, 2017) and allows the separation of human pathogenic from non-pathogenic species (Asato *et al.*, 2009).

This two-stage strategy (qPCR and sequencing) could not be justified in other scenarios in which the species is almost always the same and where other diagnostic tools can be equally efficient (Foulet *et al.*, 2007). Our study used qPCR and sequencing to identify and confirm the presence of *L. infantum*, given the high prevalence of other forms of leishmaniasis present in Brazil. In the diagnosis of asymptomatic infection by *L. infantum*, qPCR proved to be a suitable method in the present study, mostly as we observed that positive samples (confirmed by gene sequencing) were negative by ELISA rK39 and conventional PCR. However, we face some limitations when working with sequencing. The sequencing technique is a method of sensitive molecular biology, in which a conventional PCR must be performed before performing the sequencing. The main limitation involved is its detection capacity. Low parasitic loads may not be identified by the conventional method, and for this reason, sequencing did not identify positives. Thus, it is necessary to run a qPCR before sorting samples.

The present study is the first to detect the presence of *L. infantum* by qPCR followed by DNA sequencing in blood donors from endemic areas of Brazil. The results reinforce the need to expand the discussion on the actual risks of its transfusion transmission and to introduce relevant control measures.

The molecular method used in this study allowed us to identify the presence of *L. infantum* DNA in blood donors from endemic regions for VL in Brazil. Our findings showed that qPCR is a better tool for the identification of asymptomatic infection by *L. infantum* than ELISA and conventional PCR.

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Conflict of interest. None.

Ethical standards. All the participants signed a consent form to participate in the study. This study was approved by the Human Research Ethics Committee of the Federal University of Triângulo Mineiro and Hemominas Foundation (Protocol 2104 and 332, respectively.)

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