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# A Highly Sensitive and Specific Conventional Molecular Diagnosis for *Leishmania infantum chagasi* Based on a Single Copy Gene

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## Abstract

Leishmaniasis are zoonotic diseases caused by a protozoa from *Trypanosoma* family and of the genus *Leishmania*, being transmitted by sandfly vectors. *Leishmania* genus comprise 30 species, including 20 species able to cause disease with different clinical manifestations in humans, ranging from asymptomatic, cutaneous and mucocutaneous lesions, to the severe visceral form. According to the World Health Organization, visceral leishmaniasis, caused by *Leishmania infantum chagasi* in the Americas, is the most severe form of the disease, and is lethal if not treated. Brazil is part of the group of countries with the highest prevalence of this disease, concentrating 90% of the cases registered in Latin America. A rapid and accurate diagnostic method is of great importance to detect and treat specifically *L. i. chagasi* in the American continent where others trypanosomiasis circulate, making a specific diagnostic difficult, due to cross reaction. In this work, a new conventional molecular diagnostic method was developed, based on the single copy *L. infantum* chitinase encoding gene, which presented high specificity and showed increased sensitivity when compared to the method based on the gene encoding the internal transcribed spacer 1 of the *Leishmania* rRNA (rDNA ITS-1) to diagnose *L. i. chagasi* on human clinical samples.

**Keywords:** *Leishmania infantum chagasi;* Molecular diagnose; Visceral leishmaniasis; Chitinase; rDNA ITS-1; Single copy gene

## Introduction

The genus *Leishmania*, an important group of parasites transmitted by the bite of infected phlebotomine sandflies to different species of lizards and mammals, including humans [1], is the causative agent of a complex disease called leishmaniasis [2]. *Leishmania* genus comprise 30 species, including 20 species able to cause disease with different clinical manifestations in humans, ranging from asymptomatic, cutaneous and mucocutaneous lesions, to the severe visceral form.

Visceral leishmaniasis is a neglected tropical disease with an estimated 200.000-400.000 new cases and 20.000-40.000 [3] deaths annually, and it is thus a serious public health problem [4]. Visceral leishmaniasis is caused by the protozoan parasites *Leishmania (L.) donovani*, in India, Bangladesh, Nepal and Pakistan; *L. (L.) infantum*, responsible for zoonotic leishmaniasis in the Europe and Africa; and *L. (L.) infantum chagasi*, which shows high genetic similarity with *L. (L.) infantum,* and has been correlated with zoonotic leishmaniasis in different countries of the Americas (New World) [5-7].

In the New World, Brazil is the country that accounts for the highest number (90%) of visceral leishmaniasis cases, which are distributed through five regions, in 21 States. Based on data of the World Health Organization (WHO), approximately 4200 to 6300 new cases are reported per year [4,8,9]. The expansion of visceral leishmaniasis in Latin America, including Brazil, follow the dispersion of *Lutzomya longipalpis*, its major vector, which is getting adapted to periurban and urban locations [10-12].

Unless treated, visceral leishmaniasis is fatal, and in order to decrease the risks of *L. i. chagasi* transmission in Brazil, besides the control of the reservoir and vector agents, the strategy of Brazilian Ministry of Health Visceral Leishmaniasis Control Program includes the necessity of an early and accurate diagnosis to improve treatment. Circulation of *Trypanosoma cruzi* and others *Leishmania* species in America are important drawbacks for *L. i. chagasi* diagnosis. Classically, parasite detection is performed by microscopic examination of amastigotes in Giemsa stained white blood cells obtained by bone marrow, lymph node and spleen aspiration, which are painful, invasive and risky procedures, principally in children. Accuracy of this method depends on the tissue examined, the quality of reagents used and the technician identification ability [13]. Serological methods to diagnose *L .i. chagasi* present several shortcomings including cross reaction with different *Leishmania* species and also with others American endemic trypanosomiasis [14].

Two other diagnostic methods used, correspond to culture isolation and nucleic acid based detection techniques, such as PCR, directly from biological specimens of patients infected with *L. i. chagasi*. Culture isolation is the gold standard, however is less sensitive and time consuming when compared to nucleic acid detection. Molecular targets for PCR comprise amplification of gene fragments encoding the small subunit of ribosomal RNA (SSU rDNA) [15], the internal transcribed spacer of ribosomal DNA (ITS) [16,17], sequences corresponding to the kinetoplast (kDNA) [18], the mini-exon [19], the gene encoding the heat shock protein HSP70 [20], etc. However, for identification of the

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infecting *Leishmania* species, the obtained PCR products are subjected to post-PCR handling, as nested PCR [21,22], sequencing and detection of size polymorphism by restriction analysis [23-25]. Real time PCR can also be used to held identification of *Leishmania* specie [26]. Disadvantages of nucleic acid detection techniques encompass its high operational cost, complexity, cross reactivity with other pathogenic agents, such as species of *Trypanosoma* [27], and necessity for post-PCR analysis. On the other hand, a high sensitivity and specificity can be obtained with a conventional PCR depending on the molecular target employed. Thus, in this work we developed an accurate *L. i. chagasi* molecular approach based on conventional PCR targeting a highly conserved and specific *Leishmania sp* single copy chitinase encoding gene, for detection of parasites in bone marrow and peripheral blood from adults and children.

## Methods

### Patients

Thirty one adults and thirteen children consecutive patients, with clinical signs of visceral leishmaniasis, presented at the hematology-oncology ambulatory of Marilia Hemocenter, from February 2008 to November 2008, were included in this study. Peripheral blood and bone marrow samples were collected for hematologic and parasitological examination. After laboratorial required exams, the remaining human samples that would be discarded, were submitted to DNA extraction. Samples of bone marrow and peripheral blood were obtained from 12 adult patients; bone marrow and peripheral blood were available for 12 and 7 adult patients, respectively. From children, only bone marrow was obtainable. The protocol used is in agreement with the Helsinki Declaration and was approved by the Ethical Committee in Human Research from Marilia Medical School.

### *Leishmania sp* parasites

*Leishmania* reference species *L. (L). Mexicana*, *L. (L.) donovani*, *L. (V.) guyanensis*, *L. (V.) braziliensis*, *L. (L.) tropica*, *L. (L.) amazonensis*, *L. (L.) major*, *L. hetigi*, *L. equatoriensis*, *L. (V.) lainsoni*, *L. colombiensis*, *L. (V.) naiffi*, *L. (V.) shawi*, used in the study were obtained from the Reference Laboratory for *Leishmania* typing of the Research Laboratory of *Leishmaniasis* of Instituto Oswaldo Cruz, Oswaldo Cruz Foundation, Ministry of Health. Reference *Leishmania* species were maintained in culture medium M199 or LIT according to the species requirement and exponential growing parasites were used for DNA extraction.

### DNA extraction and gene target oligonucleotide design

DNA from human samples and parasites cultures were extracted by employment of DNeasy® Blood and Tissue Kit (QIAGEN®, Valencia, CA, USA) under manufacturer's instructions. Quality of DNA was verified by visualization of a high molecular weight fragment in 1% agarose gel electrophoresis and the amount of DNA was measured by comparing the band intensity with the Low Mass Ladder standard from Invitrogen/Thermo Fischer Scientific, Waltham, Massachusetts, USA.

For the design of oligonucleotides a survey of all sequences (complete and partial) encoding the chitinase enzyme gene from *Leishmania* species available in databases, *L. donovani* (AF009354.1, AY518255.1, AY518247.1, AY518245.1, AY518243.1, AY518241.1, AY518239.1, AY518237.1, AY518235.1, AY518233.1, AY518231.1, AY518232.1, AY518256.1, AY518254.1, AY518252.1, AY518246.1 ,AY518244.1, AY518242.1, AY518240.1, AY518238.1, AY518236.1, AY518234.1, AY518248.1), *L. major* (XM_001682106.1, AY518229.1, AY518227.1, AY518225.1, AY518228.1, AY518226.1, AY518224.1), *L. infantum* (XM_001464530.1, AY518250.1, AY518251.1, AY518249.1), *L. tropica* (AY518230.1, AY518258.1), *L. mexicana* (AY572789.1), *L. braziliensis* (XM_001563632.1), and *L. amazonensis* (XM_001682106.1), were subjected to multiple alignments by the Clustal X program v.2.1 [28,29]. To obtain a graphical representation of the patterns present in the alignment, and to display more accurate description of sequence similarity, the online free available program WebLogo 3 was employed [30]. Thus, considering the conserved regions in the same *Leishmania* species and between groups of species with the same clinical presentation, specific oligonucleotides to amplify a *L. i. chagasi* chitinase encoding a fragment of 999 bp (LCQuitFow 5' GCTGCCTGAGCGCTCTACT 3' and LCQuitRev 5' CTCCCTCGACCCATGTTG 3') were designed after *in silico* evaluation using the tools: Primer3Plus [31], primer-Blast [32] and MFOLD [33]. The specificity and sensitivity of the designed oligonucleotides were experimentally determined by varying PCR reaction and cycling conditions with the enzyme GoTaq® Hot Start Polymerase (Promega ™, Madison, WI, USA). PCR was standardized using DNA of *Trypanosoma cruzi*, *Homo sapiens*, and *Leishmania* species *L. (L). Mexicana*, *L. (L.) donovani*, *L. (V.) guyanensis*, *L. (V.) braziliensis*, *L. (L.) tropica*, *L. (L.) amazonensis*, *L. (L.) major*, *L. hertigi*, *L. equatoriensis*, *L. (V.) lainsoni*, *L. colombiensis*, *L. (V.) naiffi*, and *L. (V.) shawi*.

### *L. i. chagasi* molecular diagnosis on human samples

50-100ng of DNA from human peripheral blood and bone marrow were subjected to *L. i. chagasi* chitinase 999 bp PCR diagnosis with the enzyme GoTaq® Hot Start Polymerase (Promega ™, Madison, WI, USA), according to standard manufacturer conditions, and one cycle of 94°C 3 minutes, 40 cycles of 94°C 1 minute, 62°C 30 seconds, and 72°C 45 seconds, final extension of 72°C 7 minutes.

As control of human genomic DNA quality, a pair of oligonucleotides designed to amplify a 438 pb fragment encoding the first exon of human β-globin gene (–globinFow, 5 'ATGGTGCATCTGACTCCT 3', and β-globinRev 5' GTTCTCAGGATCCACGTGCAG 3') was used. To compare the sensitivity and specificity of molecular diagnostic using primers corresponding to *the L. i. chagasi* chitinase encoding gene, all clinical human samples were subjected to molecular diagnosis with oligonucleotides LITS-R and L5.8S, which amplify a 320 pb fragment of the gene encoding the *Leishmania* species rRNA intergenic region 1 (rDNA ITS-1) [16]. The PCR products stained with Gel Red™ (Biotium®, Hayward, CA, USA), were analyzed by electrophoresis in 1% agarose gel.

The *L. i. chagasi* chitinase amplicons were purified on GFX™ columns of PCR DNA and Gel Band Purification kits (GE Healthcare, Buckinghamshire, UK), and submitted to sequencing reactions using ABI PRISM ® BigDye™ Terminator-kit (Perkin-Elmer® - Applied Biosystems®, Foster, CA, USA) according to manufacturer's specifications, and resolved in automatic sequencer ABI PRISM®-310 Genetic Analyzer (Perkin-Elmer® - Applied Biosystems®, Foster, CA, USA).

## Results

Initially, the specificity and sensitivity of the *L. i. chagasi* diagnostic method based on the 999 bp PCR fragment corresponding to the chitinase gene were determined on DNA control samples including *Homo sapiens*, *Trypanosoma cruzi*, and thirteen *Leishmania* species as described in material and methods. Maximum PCR accuracy was obtained with 62°C of annealing temperature, and the smallest amount of DNA necessary to detect the parasite corresponded to two specimens of *L. infantum*, *L. i. chagasi, L. donovani* or *L. tropica*. The chitinase diagnostic method enabled distinction of *L. i. chagasi* from all others *Leishmania* species circulating in

America, and also from *T. cruzi*. In the Old World, *L. donovani, L. tropica and L. infantum* can be distinguished from *L. major*.

After standardization of *L.i.chagasi* chitinase PCR assay, DNA obtained from bone marrow of 24 adult and 13 children, and 19 peripheral blood samples of adult patients with clinical symptoms of visceral leishmaniasis were subjected to chitinase molecular diagnosis. For comparative studies, the same amount of DNA from these clinical samples were also used in PCR reactions with primers corresponding to a fragment of 320 pb encoding the *Leishmania sp* ITS-1 rRNA gene under the conditions described by Schonian et al., 2003 [16].

From 13 children bone marrow samples, 10 were *Leishmania sp* positive by means of both molecular diagnostic tests, with a 100% of concordance to parasitological exam, indicating its high specificity and sensitivity. From 24 bone marrow adult samples, 17 were positive for chitinase PCR while 8 were positive for rDNA ITS-1 PCR; and from 19 peripheral blood samples, 15 were positive for chitinase PCR and 7 were positive for rDNA ITS-1 PCR. PCR results with both target genes were concurrent in peripheral blood and bone marrow clinical samples. The specificity of the chitinase PCR assay was confirmed by sequencing of amplicons obtained from all samples.

Both, chitinase and ITS-1 molecular diagnostic methods presented the same sensitivity in samples obtained from children. In this case, the advantage of the chitinase molecular diagnosis is the direct evidence of *L. i. chagasi* by PCR product analysis through electrophoresis on agarose gel, without post-PCR amplicon manipulation by sequence or restriction analysis. However, in adult samples, chitinase PCR diagnosis presented a higher sensitivity (approximately 50%) than rDNA ITS-1 PCR diagnosis. From 14 adult clinical samples positive for *L. i. chagasi* through chitinase PCR diagnosis, five were negative in parasitological exam. All 999 bp chitinase amplicons were sequenced and confirmed the homology to the *L. infantum* chitinase encoding gene, corroborating the increased sensitivity of the chitinase PCR diagnosis compared to parasitological exam.

## Discussion

In Latin America, the visceral leishmaniasis caused by *L. i. chagasi* is expanding, causing mortality and morbidity in adult and children populations [34]. Brazil accounts for the majority of cases and early diagnosis is essential to treat the disease and to manage *L. i. chagasi* dispersion through control of insect vector and canine reservoir [35]. The parasitological and culture methods are the gold standards for diagnosis of visceral leishmaniasis [36,37], presenting high specificity, however a variable sensitivity since distribution of parasites in different tissues is not homogeneous [38].

The most commonly used tests in humans and animals for the diagnosis of visceral leishmaniasis are the serological techniques, with the employment of different antigens, including the most recent described recombinant antigen rk39 [39]. Serological tests of choice include the enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFAT) [40], both frequently used in epidemiological surveys [41]. These methods show high sensitivity, nevertheless, there are problems with *Leishmania* species discrimination and cross reaction with other *Trypanosoma* species [42]. Also, in immunodeficient individuals, as in HIV and *Leishmania sp* co-infected patients, sensitivity of the serological test is also compromised [43].

After the development of molecular detection techniques in the 80's decade, PCR is being used for the diagnosis of infectious agents, including leishmaniasis [44]. Depending on the gene target selected for amplification, the number of genomic encoding target copies, PCR can be a fast, sensitive and specific method applied to a variety of clinical samples [45]. There are several nucleic acid detection methods described to diagnose *L. i. chagasi* on clinical samples of human and dogs [46]. Although PCR shows high specificity and sensitivity, it is best used in epidemiological studies than as a routine diagnostic method because it is complex and the operational cost is still high to be used on a large scale, due to necessity of post-PCR manipulation to distinguish the *Leishmania* infecting specie [47-49]. Furthermore, PCR has been widely used to study the function of many genes, establish the parasite/host relationship and to investigate possible chemotherapy targets [38].

In this scenario, in this work we described an accurate *L. i. chagasi* PCR diagnosis based on the use of the single copy and highly conserved species-specific chitinase encoding gene [50]. Comparative investigation showed a higher sensitivity of the chitinase molecular method than the PCR diagnosis founded on the multiple copy gene encoding the rDNA ITS-1. Probably, the *Leishmania sp* chitinase encoding gene is located in a most accessible genomic topological region, with less possibility of secondary structure formation during the PCR reaction, when compared to the rDNA *locus*. Also, due to the *Leishmania sp* chitinase encoding gene specificity, detection of *L. i. chagasi* presented high accuracy and absence of cross reaction with others *Leishmania* species and *T. cruzi*.

Accordingly, the chitinase conventional PCR approach described here could be used in routine American visceral leishmaniasis diagnosis in bone marrow and peripheral blood clinical samples, being particularly useful to immunocompromised patients and children.

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