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Real-time PCR to assess the *Leishmania* load in *Lutzomyia longipalpis* sand flies: Screening of target genes and assessment of quantitative methods

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ABSTRACT

Visceral *Leishmaniasis* is an endemic disease in Brazil caused by *Leishmania infantum chagasi* and its main vector species is the sand fly *Lutzomyia longipalpis*. Epidemiological studies have used conventional PCR techniques to measure the rate of infection of sand flies collected in the field. However, real-time PCR can detect lower parasite burdens, reducing the number of false negatives and improving the quantification of *Leishmania* parasites in the sand fly. This study compared genes with various copy numbers to detect and quantify *L. infantum chagasi* in *L. longipalpis* specimens by real-time PCR. We mixed pools of 1, 10 and 30 male sand flies with various amounts of *L. infantum chagasi*, forming groups with 50, 500, 5000 and 50,000 *Leishmania* parasites. For the amplification of *L. infantum chagasi* DNA, primers targeting kDNA, polymerase α and the 18S ribosome subunit were employed. Parasites were measured by absolute and relative quantification. PCR detection using the amplification of kDNA exhibited the greatest sensitivity among the genes tested, showing the capacity to detect the DNA equivalent of 0.004 parasites. Additionally, the relative quantification using these primers was more accurate and precise. In general, the number of sand flies used for DNA extraction did not influence *Leishmania* quantification. However, for low-copy targets, such as the polymerase α gene, lower parasite numbers in the sample produced inaccurate quantifications. Thus, qPCR measurement of *L. infantum chagasi* in *L. longipalpis* was improved by targeting high copy-number genes; amplification of high copy-number targets increased the sensitivity, accuracy and precision of DNA-based parasite enumeration.

1. Introduction

Leishmaniasis is the collective name for a number of diseases with diverse clinical manifestations that are caused by protozoan flagellates of the genus *Leishmania*. Visceral *Leishmaniasis* (VL) is the most severe form of the disease and is characterized by a chronic and potentially lethal pathology (WHO, 2004). VL is caused by *Leishmania donovani* in India and East Africa and by *L. infantum* in Europe and North Africa (Lukes et al., 2007). In the Americas, the only species reported to cause VL is *Leishmania chagasi.* However, recent studies employing enzymatic and genetic methods have indicated that the differences between strains of *L. infantum* and *L. chagasi* from different sources are so slight that some researchers do not differentiate between them (Mauricio et al., 1999, 2000; Kuhls et al., 2005).

In the wild, the natural reservoirs of VL are foxes (*Cerdocyon thous*), opossums (*Didelphis albiventris*) and rodents (*Nectomys squamipes*); in contrast, the primary urban reservoir of VL is domestic dogs (Dantas-Torres and Brandão-Filho, 2006).

In Brazil, VL is an endemic disease with frequent outbreaks, with the phlebotomine sand fly *Lutzomyia longipalpis* as its main vector (Lainson and Rangel, 2005). Due to the lack of efficacious vaccines and the small number of drugs available to treat VL, research involving the vector remains an important component of the effort to control VL (Desjeux, 2004). The natural infection rate of VL has been traditionally estimated by the microscopic identification of *Leishmania* protozoans in the gut of dissected sand flies. However, this method is laborious and exhibits low sensitivity. Molecular methods, such as polymerase chain reaction (PCR), are being used in epidemiological studies to determine the rate of VL infection in sand flies. End-point PCR studies have shown low rates of infection, ranging from 0.4% to 3.9% (Paiva et al., 2006; Oliveira-Pereira et al., 2006; Silva et al., 2008). Compared with real-time PCR (qPCR), end-point PCR is more time-consuming and presents only qualitative data. Furthermore, qPCR products are quantitated and monitored in real time. This technique has been employed uti-

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lizing samples from dogs and humans in studies examining parasite burden, host–parasite interactions and the monitoring of drug therapy (Mary et al., 2004; Mortarino et al., 2004; Rolão et al., 2004; Francino et al., 2006). Consequently, qPCR allows for greater accuracy in the determination of the natural infective dose (Ranasinghe et al., 2008).

The detection of *Leishmania* spp. can be accomplished through the PCR amplification of specific regions of kinetoplastid DNA (kDNA) and genes encoding the small (18S) ribosome subunit, DNA polymerase α, glucose 6-phosphate dehydrogenase and hexokinase (Fernandes et al., 1993; Bretagne et al., 2001; Prina et al., 2007).

However, to date, information is lacking regarding the optimization of qPCR in studies involving the detection and quantification of *L. infantum chagasi* in sand flies. Thus, questions remain regarding the best gene to amplify, the best method of quantification and whether the number of sand flies utilized for isolation affects parasite quantification and test sensitivity. Therefore, the aim of this work was to optimize the conditions for qPCR to detect *L. infantum chagasi* in *L. longipalpis* captured in the field using two methods of quantification and single- or multiple-copy templates.

2. Materials and methods

2.1. Leishmania strain and serial dilution

The *L. infantum chagasi* strain MHOM46/LC/HZ1 was cultured at 26 °C in M199 medium supplemented with 10% heat-inactivated fetal calf serum, 40 mg/mL of gentamicin and 5% male human urine. Stationary-phase promastigotes were harvested by centrifugation (2000×*g*) and washed with phosphate-buffered saline. The solutions were divided and centrifuged again (2000×*g*) to remove the supernatants. Two pellets were resuspended in the medium to enable parasite quantification using a Neubauer hemacytometer. Counts were made in triplicate for a total of six counts, with a mean ± standard deviation of $8.056 \times 10^6 \pm 0.08$ parasites per pellet. The remaining pellets were stored at −20 °C until DNA extraction; these parasites were utilized to establish standard curves for *Leishmania* gene amplifications and to prepare the experimental groups. For the experimental groups, serial dilutions were performed to obtain solutions containing 50,000, 5000, 500 and 50 parasites prior to DNA purification.

2.2. Sand flies

In 2009, male *L. longipalpis* were captured with CDC light traps in the city of Fortaleza (−03°43'02"S, 38°32'35"W), Brazil. Once captured, total phlebotomine sand flies were killed with cotton balls soaked with ethyl acetate and stored in isopropanol at −20 °C. Species and sex identification was performed as described by Young and Duncan (1994). In order to form the experimental groups, sand flies were grouped into pools of 1, 10 and 30 specimens. In addition, only one sample of 30 sand flies was used for DNA purification to construct a standard curve for *L. longipalpis* gene amplification. All samples were stored at −20 °C until DNA extraction.

2.3. Experimental groups and DNA extraction

Before DNA purification, the serial dilutions of *Leishmania* parasites described above were mixed with sand fly pools, forming groups with various quantities of insects (0, 1, 10 and 30) and promastigotes (0, 50, 500, 5000 and 50,000). DNA was extracted using the Wizard SV Genomic DNA Purification System Kit (Promega Corporation, USA). Briefly, samples were macerated with pistils, fragmenting the sand flies, and then incubated overnight at 55 °C in a digestion solution containing 145 mM proteinase K, 90 mM EDTA and 72 mM RNaseA. After the cell debris was removed by centrifugation (2000×*g*), the samples were transferred to spin columns and eluted in 100 μL of water (first elution). A second elution of 100 μL was made to calculate the total yield of extracted DNA, as described bellow. All DNA extractions were performed in triplicate; replicates were then combined into a representative pool.

To determine the total DNA yield from *Leishmania* parasites, both the first and second DNA elutions from a triplicate sample were quantified by spectrophotometry (Biophotometer, Eppendorf, Germany), summed and then divided by the number of specimens present in the sample (about eight millions of parasites). The first elution of these samples was pooled and used to construct the standard curves for qPCR analysis.

2.4. Genes and primers

Sets of primers generating genomic *Leishmania*, kinetoplastid or *L. longipalpis* DNA amplicons were designed using unique or multiple sequences retrieved from NCBI databases (Table 1). ClustalW 2 software was utilized for sequence alignment. Oligonucleotide pairs and the similarities of primer sequences with those present in nucleotide databanks were validated using PrimerBlast software. For amplification of the 18S gene, we utilized the primer pair described by Prina et al. (2007). Additionally, amplifications targeting the sand fly V-ATPase gene were used as reference in relative quantifications.

2.5. Real-time PCR

Each reaction consisted of a total volume of 10 μL, containing 5 μL 2X Fast Start Universal SYBR Green Master (Roche), 0.3 μM of primer mix (forward and reverse) and 0.5 μL template. Amplifications were performed in a Mastercycler EP Realplex^4 S (Eppendorf). Each sample was analyzed in triplicate. The PCR protocol included an initial incubation at 95 °C for 10 min, followed by 40 cycles of the amplification program at 95 °C for 15 s and 60 °C for 30 s. SYBR Green fluorescent emission (530 wavelength) was measured at the end of the elongation step.

The specificity of each reaction was ascertained after the completion of the amplification protocol by performing melt curve analysis (55–95 °C, initiating fluorescence acquisition at 55 °C and taking measurements at 10-s intervals until the temperature reached 95 °C). Negative controls were performed without DNA templates. Additionally, DNA samples from 10 sand flies or 50,000 *Leishmania* parasites were used as negative controls in reactions for the amplification of *Leishmania* or phlebotomine genes, respectively. The sizes of the PCR products were further confirmed by gel electrophoresis using standard ethidium bromide-stained 1.5% agarose gel and visualized by exposure to UV light.

2.6. Analysis of qPCR data

Threshold and threshold cycle (C_t) values were automatically determined by Realplex 2.2 software (Eppendorf) using default parameters. The C_t and melting temperature (T_m) data are expressed as the mean ± s.d. of three measurements.

To establish standard curves, serial dilutions of *L. infantum chagasi* and *L. longipalpis* DNA were subjected to qPCR amplifications. The final concentrations of parasite DNA/reaction ranged from 2.3 ng to 0.23 fg (equivalent to 40,280 to 0.004 parasites/reaction), while the sand fly DNA/reaction ranged from 14.625 ng to 1.46 pg (equivalent to 0.15 to 0.000015 sand fly/reaction). The standard curves were also used to determine the detection limit of the assays and to assess the linearity (R^2) and the efficiency (E) of the