A Real-Time Polymerase Chain Reaction Assay for the Identification and Quantification of American Leishmania Species on the Basis of Glucose-6-Phosphate Dehydrogenase

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Abstract. A real-time polymerase chain reaction (PCR) test was developed on the basis of the Leishmania glucose-6-phosphate dehydrogenase locus that enables identification and quantification of parasites. Using two independent pairs of primers in SYBR-Green assays, the test identified etiologic agents of cutaneous leishmaniasis belonging to both subgenera, Leishmania (Viannia) and Leishmania (Leishmania) in the Americas. Furthermore, use of TaqMan probes enables distinction between L. (V.) braziliensis or L. (V.) peruviana from the other L. (Viannia) species. All assays were negative with DNA of related trypanosomatids, humans, and mice. The parasite burden was estimated by normalizing the number of organisms per total amount of DNA in the sample or per host glyceraldehyde-3-phosphate dehydrogenase copies. The real-time PCR assay for L. (Leishmania) subgenus showed a good linear correlation with quantification on the basis of a limiting dilution assay in experimentally infected mice. The test successfully identifies and quantifies Leishmania in human biopsy specimens and represents a new tool to study leishmaniasis.

INTRODUCTION

Protozoan parasites of the genus Leishmania1–3 have two hosts in their life cycle. In phlebotomine sand flies, promastigote forms develop in the intestinal lumen, and in the vertebrate host, amastigote forms are found in macrophages. Infection is acquired through the bite of the sand fly and in humans (who are normally accidental hosts in Americas) the infection can result in diseases known as leishmaniasis. Clinical forms of leishmaniasis are numerous, depending on the species of Leishmania and the genetic background of the patient. These forms are broadly classified as cutaneous, borderline disseminated cutaneous, diffuse cutaneous, mucocutaneous, and visceral or post-kala-azar dermal. Twenty-two species of Leishmania have been reported in human infections.5–6 These parasites are found in more than 80 countries in tropical, subtropical, and temperate regions. They are important emerging/re-emerging zoonoses,5 and according to the World Health Organization,6 an estimated 1.5–2 million new cases occur annually.

In South America, these organisms are grouped into two subgenera, Leishmania (Leishmania) and L. (Viannia), a classification initially based on the mode of development of promastigotes in the sand fly gut7 but recently confirmed by molecular methods.9,10 Although there is no direct correlation between a specific clinical form and the causative species,6 some associations are found. For example, Leishmania (V.) braziliensis is responsible for the most morbid and disfiguring form of the disease known as espundia or mucocutaneous leishmaniasis.6,11 This species is found between latitudes 19°N and 29°S.7 Other L. (Viannia) species, such as L. (V.) panamensis, L. (V.) guyanensis, and L. (V.) shawi are the common causes of cutaneous leishmaniasis in the rain forests of Central America and Amazonia.

Quantification of viable Leishmania in host tissues has been generally evaluated by methods that rely on the capacity of tissue amastigotes to differentiate into promastigotes in culture media.12–15 There is one quantification protocol based on a polymerase chain reaction (PCR),16 but it has not been compared with other methods, such as a competitive PCR method that was used to evaluate parasite burden.17 The most commonly used protocol for parasite quantification is the limiting dilution assay (LDA).13,14 This assay is arduous, time-consuming, and depends on sterile conditions and highly trained personnel. These characteristics nearly exclude the possibility of it being used for routine examinations of biopsy specimens collected under field conditions from sylvatic animals and patients.

With the advances in PCR technology during the past decade, specifically real-time PCR and analysis of kinetics of amplicon formation, it is now possible to quantify the initial number of template molecules.18–21 Real-time PCR assays are being successfully used for diagnosis of viral infections22 and toxoplasmosis.23 Real-time PCR assays have been described for Leishmania detection24 based on the DNA polymerase gene of L. (L.) infantum;25 the glucose phosphate isomerase (GPI) gene,26 kinetoplast DNA (kDNA),27,28 and ribosomal DNA (rDNA).29 However, most of these tests are not species specific. Some real-time PCR assays differentiate between groups of Leishmania,30,31 such as parasites of the L. donovani complex, and the subgenus L. (Viannia) and other species of the subgenus L. (Leishmania). More recently, one of these assays was used to diagnose human cases but the individual species were identified by sequencing of the cytochrome b gene.31 These methods are already being used in clinical studies32,33 and with experimental infections.25,34,35

To our knowledge, there are no publications describing a real-time PCR assay that can distinguish Leishmania (Viannia) organisms at species level. The existing literature points to an urgent need for new probes that either alone or in association with those already described will identify different Leishmania (Viannia) species and quantify the parasites.

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In this report, we describe real-time PCR assays based on the glucose-6-phosphate dehydrogenase (G6PD) gene that detect and quantify Leishmania species associated with cutaneous and mucocutaneous leishmaniases in the Americas. Using either non-specific sequence methodology (SYBR-Green) or sequence-specific amplicon detection (TaqMan probes), assays detected and quantified Leishmania DNA. Furthermore, they differentiated L. (V.) braziliensis from other L. (Viannia) species and from those of L. (Leishmania). In addition, an assay based on mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPD) was developed to quantify mammalian DNA, which enabled the number of parasites to be expressed per host GAPD copies.

MATERIALS AND METHODS

Organisms and cell culture. Promastigotes of all Leishmania species listed in Table 1 were grown at 25°C in M199 medium (GIBCO-BRL, Gaithersburg, MD) containing 10% fetal bovine serum. Trypanosoma cruzi and Crithidia fasciculata were grown at 28°C in liver infusion medium.37 C1A.R2 cells were grown in RPMI 1640 medium (GIBCO-BRL) at 37°C in an atmosphere of 5% CO₂.

Mice and experimental infection. Six BALB/c mice were infected in the hind footpad with 2 × 10⁶ stationary growth phase promastigotes of L. (L.) amazonensis (MHOM/BR/1973/M2269). The parasite burdens were determined by methods using the ELIDA program.13,14,38 Mice were killed by overexposure to CO₂, which complied with the current recommendations for animal use of the Colégio Brasileiro de Experimentação Animal de Brazil and the Comissão de Ética em Experimentação Animal of the Instituto de Ciências Biomédicas (Universidade de São Paulo).

Patient biopsies. Biopsies were taken with a punch from patients from Rondônia State in the northwestern region of Brazil and preserved in NET buffer (0.15 M NaCl, 50 mM EDTA, 0.1 M Tris-HCl, pH 7.5) at room temperature during transportation to the city of São Paulo. The material was washed three times with 1× phosphate-buffered saline (PBS) (7 mM Na₂HPO₄, 26 mM NaH₂PO₄, 130 mM NaCl) and then processed as described for total DNA. The Institutional Ethical Commission reviewed and approved the study.

Purification and analysis of nucleic acids. DNA from cultured cells, control human DNA, or biopsy specimens was purified using the sodium dodecyl sulfate/protease K/phenol extraction method.39 Mouse tissue DNA was extracted by a modified protocol. Each tissue homogenate was prepared using surgical instruments extensively washed with soap, treated with bleach, extensively washed, and baked at 200°C for 2 hours prior to use. The homogenates were suspended in 1× PBS and incubated with two volumes of trypanocyte lysis buffer (0.15 M ammonium chloride, 0.1 mM EDTA, 10 mM potassium bicarbonate, pH 7.3) for five minutes. After adding 3.3 volumes of 1× PBS, the homogenate was centrifuged and the DNA was extracted as described above from the pellet. The extracted DNA was precipitated with ethanol and dissolved in 10–50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA), quantified by measuring absorbance at 260 nm, and adjusted to a concentration of 10 ng/µL (this solution was then diluted to perform the reactions). The nucleic acids were fractionated by agarose gel electrophoresis 1× TAE buffer (40 mM Tris-acetate, 2 mM EDTA).

### Table 1

Organisms used and reactions in the glucose-6-phosphate dehydrogenase (G6PD) real-time polymerase chain reaction assay

<table>
<thead>
<tr>
<th>Organism*</th>
<th>Reference†</th>
<th>G6PD real-time polymerase chain reaction assay‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L</td>
</tr>
<tr>
<td>L. (V.) braziliensis</td>
<td>MHOM/BR/1975/M2903$</td>
<td>–</td>
</tr>
<tr>
<td>L. (V.) braziliensis</td>
<td>MHOM/BR/1994/631†</td>
<td>–</td>
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<tr>
<td>L. (V.) braziliensis</td>
<td>MHOM/BR/1984/648§</td>
<td>–</td>
</tr>
<tr>
<td>L. (V.) braziliensis</td>
<td>MHOM/BR/1994/M15140¶</td>
<td>–</td>
</tr>
<tr>
<td>L. (V.) braziliensis</td>
<td>MHOM/BR/1994/M15176†</td>
<td>–</td>
</tr>
<tr>
<td>L. (V.) peruviana</td>
<td>MHOM/PE/1984/Lc39</td>
<td>–</td>
</tr>
<tr>
<td>L. (V.) guyanensis</td>
<td>MHOM/BR/1975/M4147¶</td>
<td>–</td>
</tr>
<tr>
<td>L. (V.) panamensis</td>
<td>MHOM/PA/1971/L94</td>
<td>–</td>
</tr>
<tr>
<td>L. (V.) lainsoni</td>
<td>MHOM/BR/1981/M6426§</td>
<td>–</td>
</tr>
<tr>
<td>L. (V.) utingensis</td>
<td>ITUB/BR/1977/M4964¶</td>
<td>–</td>
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<td>L. (L.) amazonensis</td>
<td>MHOM/BR/1973/M2269§</td>
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<td>L. (L.) amazonensis</td>
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<td>C. fasciculata</td>
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<td>T. cruzi</td>
<td>Y strain§</td>
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<td>Human C1A.R2 cells#</td>
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<tr>
<td>Male human DNA††</td>
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<td>–</td>
</tr>
<tr>
<td>BALB/c mice#</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>C57B/6 mice#</td>
<td>–</td>
<td>ND</td>
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* L. = Leishmania; V. = Viannia; S. = SaoPauloLeishmania; C. = Crithidia; T. = Trypanosoma
† Isolates are indicated as described by the World Health Organization (1984).
‡ L and VS are the reactivity on SYBR-Green assays for cutaneous L. (Leishmania) species and for L. (Viannia) species, respectively; and nbra and bra are the reactivity of G6PD-VIC-nbra and G6PD-FAM-bra probes, respectively, on the L. (Viannia) TaqMan assay (see Table 2 for more details about the primers and Materials and Methods for details about the probes). ND = not determined.
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¶ Instituto Evandro Chagas, Belém, Pará, Brazil.
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** The year that this strain was isolated is not known.
†† Applied Biosystems, Foster City, CA.
Quantified DNA standards. The quantified standards are PCR products, containing the respective amplicon, cloned in PCR2.1-TOPO (Invitrogen, Carlsbad, CA) with the TOPO TA Cloning® Kit (Invitrogen) according to the manufacturer’s instructions. For the Leishmania G6PD assays, the product was the 5’ end of G6PD mRNA obtained by reverse transcription–PCR as previously described. For the mammalian GAPD assay, the amplicon was amplified with the external primers GAPD-EF (5’-CCA GAA CAT CAT CCC TGC-3’) and GAPD-ER (5’-GGT GCT AGT AGC CCA-3’) as adapted from the original description with annealing at 55°C and extension for 30 seconds.

Real-time PCR. The real time PCR was conducted in an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) in a volume of 50 μL. Either SYBR-Green® PCR Master Mix or TaqMan® Universal Master Mix (both from Applied Biosystems) were used. Oligonucleotides were used at concentrations of 50 nM for both reactions, and probes were used at concentrations of 200 nM for the TaqMan assays. The thermal profile was an initial activation of DNA polymerase at 95°C for 10 minutes, followed by cycles of 95°C for 15 seconds. Annealing temperatures and extension times are specified for each assay in Table 2. The LDA method was compared with the L SYBR-Green assay (normalized by the M SYBR-Green assay) that was conducted in an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad Laboratories) with oligonucleotides at concentrations of 500 nM. The thermal profile was an initial activation of DNA polymerase 95°C for 3 minutes, followed by cycles of 95°C for 30 seconds and extension for 30 seconds at 60°C or 63°C for the L. m. and L. m. assays, respectively. The efficiency of each assay was calculated by the formula E = 10−1/slope − 1, where the slope is the linear coefficient obtained from the linear regression of the standard curve.

Sequence analysis. The BLASTs program was used to check the nucleotide sequence similarity with other proteins in GenBank. Sequences were aligned with BioEdit and Clustal-W. The primers were designed with Primer Express Software version 2.0 (Applied Biosystems) and OligoTech version 1.0 (Oligo Etc. Incand and Oligo Therapeutics Inc., Wilsonville, OR). Real-time PCR data were acquired and analyzed in the ABI Prism 7000 SDS (Applied Biosystems).

RESULTS

Nucleotide sequence of G6PD in the genus Leishmania. We isolated a fragment of the 5’ end of G6PD mRNA sequence of L. (L.) infantum and L. (L.) chagasi (Figure 1A) using the same strategy previously used to characterize the G6PD sequence of Leishmania (nucleotide sequence is available in GenBank database under accession numbers DQ212793 and DQ212794). By comparing the G6PD sequence of L. (L.) amazonensis, L. (L.) mexicana, L. (L.) chagasi, L. (L.) infantum, L. (L.) braziliensis, L. (L.) guyanensis, L. (L.) panamensis, L. (L.) lainsoni, L. (L.) shawi, and L. (L.) naiff, we designed oligonucleotides that in SYBR-Green assays selectively amplify L. (Viannia) parasite DNA (G6PD-LVF and G6PD-LVR, V assay) or cutaneous L. (Leishmania) DNA (G6PD-LLF and G6PD-LLR, L assay). We also designed two labeled probes to be used together in the V assay to distinguish L. (L.) braziliensis (G6PD-FAM-bra) from the other L. (Viannia) species (G6PD-VIC-bra) in a TaqMan assay (VT assay). The position of each oligonucleotide and probe is shown in Figure 1A and their sequences are shown in Table 2.

Subgenus specific assays. The L SYBR-Green assay (annealing at 58°C and extension for 1 minute; Table 2) successfully generated amplification products from L. (L.) amazonensis, L. (L.) mexicana, and L. (L.) pifanoi. Results of the assay were negative for DNA from phylogenetically related L. (L.) infantum, all L. (Viannia) species tested, L. (Sauroleishmania) adleri, C. fasciculata, T. cruzi, humans, and mice (Table 1).

DNA templates from all L. (Viannia) subgenus species tested produced amplification products in the V SYBR-Green assay (annealing at 65°C and extension for 30 seconds; Table 2). Results of the same assay were negative with DNA from L. (L.) amazonensis, L. (L.) mexicana, L. (L.) pifanoi, L. (L.) infantum, L. (L.) adleri, C. fasciculata, T. cruzi, humans, and mice (Table 1).

For the L and V SYBR-Green assays, melting curve analysis showed a single peak indicating its specificity, which was confirmed by fractionation in an agarose gel. The melting temperature (Tm) for the L SYBR-Green assay was 79.8°C ± 0.2°C, and although the Tm for L. (L.) braziliensis and L. (L.) peruviana in the V assay was slightly lower than for other L. (Viannia) species, it cannot be used in their distinction be-

---

Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Tm, °C</th>
<th>Ori</th>
<th>Assay†</th>
<th>A/C1</th>
<th>E§</th>
<th>L4</th>
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<tr>
<td>G6PD-LLF</td>
<td>CTTGTTGCTCCGCTGCTAC</td>
<td>55.5</td>
<td>For</td>
<td>L</td>
<td>58°C</td>
<td>1 m</td>
<td>101</td>
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<td>G6PD-LLR</td>
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<td>56.2</td>
<td>Rev</td>
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<td>45</td>
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<tr>
<td>G6PD-LVF</td>
<td>TTAGTACAGTTGACATGTTAAG</td>
<td>55.7</td>
<td>For</td>
<td>VS</td>
<td>65°C</td>
<td>30 s</td>
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<tr>
<td>G6PD-LVR</td>
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<td>56.5</td>
<td>Rev</td>
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<td>GAPD-F</td>
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<td>For</td>
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<td>GAPD-R</td>
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<td>G6PD-FAM-bra</td>
<td>TGGCCTTCAAGGATGCTCATC</td>
<td>67.6</td>
<td>For</td>
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<td>30 s</td>
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<td>68.2</td>
<td>For</td>
<td></td>
<td>50</td>
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</table>

* Tm = melting temperature; Ori = orientation of primer; G6PD = glucose-6-phosphate dehydrogenase; GAPD = glyceraldehydes-3-phosphate dehydrogenase. For = forward; Rev = reverse.
† Assay name.
‡ Assay annealing temperature (°C) and number of cycles.
§ Assay extension time in minutes (m) or seconds (s).
¶ Length of the PCR product in basepairs.
# Used with oligonucleotides from pair VS.
cause inter-assays variations were detected (80.15°C ± 0.06°C for \(L. (V.)\) brianziliensis M2903, 80.05°C ± 0.06°C for \(L. (V.)\) peruviania, 80.3°C ± 0.14°C for \(L. (V.)\) guyanensis, 80.55°C ± 0.06°C for \(L. (V.)\) panamensis, 80.5°C ± 0.12°C for \(L. (V.)\) lainsoni, and 81°C ± 0.12°C for \(L. (V.)\) utingensis).

Identification of \(L. (V.)\) brianziliensis in \(L. (V.)\) subgenus. In the VT TaqMan assay (annealing at 63°C and extension for 30 seconds; Table 2), the \(G6PD\)-FAM-bra probe showed a positive reaction with DNA of all \(L. (V.)\) brianziliensis strains tested and with \(L. (V.)\) peruviania and a negative
reaction with DNA of all other *L.* (*Viannia*) species. Conversely, the *G6PD*-VIC-nbra probe showed a negative reaction with DNA of all *L.* (*V.*) *braziliensis* strains tested and with *L.* (*V.*) *peruviana*, and a positive reaction with all other *L.* (*Viannia*) species. Both probes showed negative reactions with DNA from *L.* (*L.*) *amazonensis*, *L.* (*L.*) *mexicana*, *L.* (*L.*) *pifanoi*, *L.* (*L.*) *infantum*, *L.* (*S.*) *adleri*, *C.* *fasciculata*, *T.* *cruzi*, humans, and mice (Table 1).

**Leishmania quantification assays.** Plasmids carrying the 5′ end of the *G6PD* gene of *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis* were used in serial dilutions as standards in the VT TaqMan assay (Figure 2A and B) or in the V SYBR-Green Assay (Figure 2C). Plasmids with the 5′ end of the *G6PD* gene from *L.* (*L.*) *amazonensis* were used as a standard in L SYBR-Green assay (Figure 2D). The standard curves for the assays (threshold cycle versus logarithm of the initial amplicon copy number) showed a good linear correlation in the range of $2 \times 10^5$ to $2 \times 10^1$ *G6PD* copies. The efficiencies for the VT TaqMan assay were 0.886 and 0.957 for *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis*, re-

![Figure 2](image_url)

**Figure 2.** Sensitivity of the VT, L, V, and M assays. Standard curves (threshold cycle versus logarithmic of template copy number) of the VT TaqMan assay for glucose-6-phosphate dehydrogenase (*g6pd*)-FAM-bra probe (A) or for *g6pd*-VIC-nbra probe (B), and of the V (C), L (D), and M (F) SYBR-Green assays were obtained by using dilution of plasmids containing the respective amplicons. The *G6PD* assays used as a template 10-fold serial dilutions representing $2 \times 10^5$ to $2 \times 10^1$ plasmid copies per reaction (in duplicate), and the *GAPD* assay used as a template 10-fold serial dilutions from $2 \times 10^6$ to $2 \times 10^2$ plasmids copies per reaction. The amplicons were cloned from *Leishmania* (*Viannia*) *braziliensis* M2903, *L.* (*V.*) *guyanensis* M4147 and *L.* (*Leishmania*) *amazonensis* M2269 DNA for the *G6PD* assays and from human male DNA (Applied Biosystems, Foster City, CA) for the *GAPD* assay, respectively (see Materials and Methods for details). Determination of the interference of human DNA (200 ng extracted from C1A.R2 cells) on the V SYBR-Green assay (C) used as template $2 \times 10^5$ to $2 \times 10^1$ plasmids copies (containing the *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis* amplicons; the average of both assays is shown). Determination of interference of different amounts of human DNA (extracted from C1A.R2 cells) on the V SYBR-Green assay (E) used as template $2 \times 10^5$ plasmid copies (containing the *L.* (*V.*) *braziliensis* amplicon).
spectively, and the efficiency for the L SYBR-Green assay was 0.504.

A small level of interference was observed that was proportional to the amount of non-specific DNA (Figure 2E). This interference was probably caused by changes in the baseline fluorescence generated by the addition of more DNA. However, the presence of up to 200 ng of non-specific human DNA (extracted from C1A.R2 cells) did not significantly interfere in the quantification of the copy number per reaction in the range of 2 × 10^7 to 2 × 10^8 G6PD copies (Figure 2C).

We also designed two primers in conserved regions the GAPD gene from different mammals (Figure 1B) that were used together in a SYBR-Green assay (GAPD-F and GAPD-R, M assay; Table 2). The M SYBR-Green assay (annealing at 63°C and extension for 30 seconds) showed a positive result with human and mouse DNA and a negative result with L. (L.) amazonensis or L. (V.) guyanensis DNA. It is noteworthy that pseudogenes were also amplified. This was evaluated from the melting curve analysis and also from the sequence of the PCR products. This is a common problem with oligonucleotides that target this gene. Recently, a set of oligonucleotides were designed that is specific for human GAPD by exploiting sequences outside of the open reading frame in an intron-exon boundary. Unfortunately, because our goal was to have an assay to amplify mammalian DNA from different potential reservoirs as well as humans, these oligonucleotides cannot be used. However, as long as the number of pseudogenes is relatively constant in the population being studied, it should not interfere in the analysis.

We amplified and cloned the GAPD amplicon obtained with external primers GAPD-EF and GAPD-ER from human DNA (Applied Biosystems). The M SYBR-Green assay containing between 2 × 10^7 and 2 × 10^5 plasmid copies showed a good linear correlation (Figure 2F). This assay enables normalization of the number of parasites in each sample by the number of mammalian host GAPD copies.

Initial attempts to detect Leishmania DNA in tissue homogenates of BALB/c mice were unsuccessful. We verified the presence of DNA polymerase inhibitors by adding plasmids containing the cloned amplicon to the samples. Such effects were not observed previously with DNA extracted from cultured human cells. To circumvent this problem, we performed the reactions with a maximum of 20 ng of total DNA per well, and at least three serial dilutions (1:4) were analyzed (i.e., 2, 0.4 and 0.08 ng/reaction). We evaluated the efficiency of parasite quantification assay by comparing it to the LDA method. Six mice infected with L. (L.) amazonensis were killed at different time points after infection. The parasite burden was determined by both LDA and G6PD-based real-time PCR assays. In the latter assay, DNA was extracted from undiluted tissue homogenate and then tested with the L SYBR-Green assay.

Some samples showed amplification patterns, which indicated DNA inhibitors (concentrated solutions appeared to have fewer or about the same number of parasites and mammalian DNA as diluted samples). Also, in some samples, the level of parasite DNA seemed to be diluted below our detection limit. To guide the analyses, we defined two objective parameters, namely, the empirically dilution factor (ODF), which is the copy number in a concentrated solution divided by the copy number in its diluted solution, and the theoretical dilution factor (TDF), which is the theoretical dilution (i.e., in a 1:4 dilution the theoretical dilution factor is 5). To analyze inhibition in concentrated samples, we used three requirements: 1) a sample should be excluded if the ODF:TDF ratio is less than 0.5; 2) in a dilution series after the first dilution satisfies the first requirement, subsequent dilutions are included in the analysis; and 3) samples with no parasites are excluded.

We normalized the parasite number by the amount of DNA used (Figure 3A) or by the mammalian GAPD copy number obtained by the M SYBR-Green assay (Figure 3B). Both curves showed a good linear correlation when plotted against the LDA quantification (R^2 = 0.975 and 0.938, respectively). This re-enforces the use of real-time PCR technology to estimate parasite burden in tissues, as previously conducted by targeting the DNA polymerase gene. However, it is essential to determine the presence of DNA polymerase inhibitors for an accurate quantification. In this respect, a secondary GAPD M assay functions as control for the presence of inhibitors in the amplification reactions.

The results of normalizing the copy number of Leishmania by either the total amount of DNA or by the copy number of the mammalian GAPD gene were similar (Figure 3). From 2 to 21,100 parasites in 2 ng of total DNA could be detected. The M assay is not necessary for quantification, but when

![Figure 3](image-url)
there is a restriction on the amount of DNA, it becomes extremely useful to confirm that the sample is negative. The M assay adds precision to the assay because total DNA in the dilutions cannot be readily verified for accuracy because of the low concentrations involved. Furthermore, because the selected region in the GAPD gene for the M assay is highly conserved, it can be used with any sylvatic Leishmania reservoir DNA. However, we strongly recommend that the ratio of GAPD amplicons per nanogram of DNA be evaluated. Even in areas where both L. (V.) braziliensis and other L. (Viannia) species are sympatric, it will be possible to study the incidence and distribution without having to isolate the parasite.

Use of G6PD-based real-time PCR with DNA of human biopsy samples. As expected, normalization by GAPD copy number for the BALB/c mice was linear for an isogenic population. However, this is not the case in a human population. Consequently, we determined the ratio of GAPD copies per nanogram of total DNA from 3 different healthy donors with differences less than 25% (Figure 4A).

We used the V TaqMan and M SYBR-Green assays in DNA samples from 12 patients. Eleven of these patients were positive for Leishmania by G6PD-based PCR40; one patient was negative by PCR, although positively diagnosed for leishmaniasis on clinical grounds (Table 3). The DNA polymerase inhibition was an issue in human biopsy specimens because some samples had to be diluted to 8 pg per reaction to enable amplification. Samples that are positive for L. (V.) braziliensis by the G6PD-based PCR are also positive for the G6PD-FAM-bra probe. Furthermore, samples that are positive for L. (Viannia) non-braziliensis species are positive with the G6PD-VIC-nbra probe (Figure 4B). The number of parasites varied from $51 \times 10^3$ to $8.36 \times 10^3$ per $10^5$ mammalian GAPD copies. The sample from the patient initially negative by PCR was positive using the G6PD-FAM-bra probe, with a level of 88.7 parasites detected per $10^5$ mammalian GAPD copies. Thus, the TaqMan assay is more sensitive than the previously described G6PD PCR-based method40 and also can generate an estimate of parasite load that could be useful for monitoring or assessing the course of disease and response to treatment.

DISCUSSION

Identification of species of Leishmania using classic methods, such as zymodemes10,47 or monoclonal antibodies,48 generally requires isolation of the parasite. The only exception to this is in the case of sand fly infections, where parasites have been successfully identified49 in smears made directly from the fly. Isolation procedures are especially difficult under field conditions or in laboratories in disease-endemic areas where technical resources are poor. Cultures are liable to be contaminated and some strains grow poorly in axenic medium.50 During the past two decades, methods based on DNA technology have been developed that enable parasite identification in DNA extracted from host tissues, thus eliminating the necessity of isolation. Distinct loci of kinetoplast (mitochondrion genome) and nuclear DNA have been used as targets for their identification. Among these loci are kDNA, rDNA, mini-exon, β-tubulin, gp63, randomly amplified polymorphic DNA fragments, repetitive sequences, microsatellites, and subtelomeric sequences.51–54 Methods with these loci were initially restricted to the use of kDNA hybridization.

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**Figure 4.** Number of mammalian glyceraldehydes-3-phosphate dehydrogenase (GAPD) copies per nanogram of total human DNA from three subjects (A) and ratio of Leishmania per number of mammalian GAPDH copies in human biopsy samples of patients from Rondônia (B). In the latter, the number of parasites normalized by the mammalian GAPD copies was obtained through the VT and M assay, respectively. Each sample was quantified in triplicate for both assays in different concentrations of template DNA (5, 1, and 0.2 ng, but some samples had to be diluted to 40 and 8 pg). Samples positive for g6pd-FAM-bra are shown in the hatched bars and samples positive for g6pd-VIC-nbra are shown in the filled bars. The negative sample for Leishmania using the G6PD-polymerase chain reaction test40 gave a positive result in the VT TaqMan assay (indicated by an asterisk), this sample and the ones indicated by ** had the parasite burden estimated from just one dilution. Error bars indicate the standard deviation.
probes. After development of PCR assays, they were developed using kDNA and rDNA as targets. The PCR methods have been shown to have high levels of sensitivity but their specificity varies according to the target sequence. The ability to distinguish among members of the *L. (Viannia)* subgenus has been elusive.

We describe a real-time PCR assay that focuses on identification and quantification of different *L. (Viannia)* species. On the basis of *G6PD* sequences, we designed two pairs of primers that can be used in SYBR-Green assays to specifically identify parasites in the Americas belonging to either the subgenera *L. (Viannia)* or *L. (Leishmania)*. The specificity of the assays was tested with human and mouse DNA and with DNA of organisms that are phylogenetically close to *Leishmania*, such as *T. cruzi* (sympathic in many areas) and *Leishmania* (*S.* adleri) (not pathogenic to humans and belongs to the same genus but a different subgenus).

The real-time PCR showed that *L. (V.) peruviana* was recognized by the *G6PD-FAM*-bra probe. This result is not surprising because both species are phylogenetically similar and only a few differences have been reported between *L. (V.) peruviana* and *L. (V.) braziliensis*. However, because the geographic distribution of *L. (V.) peruviana* is restricted, this should not represent a serious problem overall in terms of parasite identification. The TaqMan assay probes *G6PD-FAM*-bra and *G6PD-VIC*-bra were designed using our previously described nucleotide differences. These probes retained their specificities within the real-time PCR assay, and this is more sensitive than the former assay, detecting even one parasite in a reaction.

The presently reported assays have several advantages over other PCR-based assays. The time needed to analyze a given sample is shorter because there is no need to electrophoretically separate the products. Apart from this, the assay detects the amplicon, in a closed vial, thus minimizing the risk of laboratory contamination with the amplified products. Therefore, there is little training and overall technical expertise required to perform the assay and interpret the results. The methodology is thus more suitable for routine diagnostic laboratories because the risk of false-positive results is minimized. Furthermore, these systems are constantly being improved and have been coupled to robotic DNA extraction that would make analysis easier.

The present work and that of others that target DNA pol or GPI have single copy genes as targets. This allows for quantification because two copies of the target will correspond to one parasite. When kDNA is the target sequence, the assay detected a difference in the threshold cycle for the same number of parasites of different species, a result consistent with the known variation in the number of copies of specific kDNA sequences per cell in different species.

Although any amplification assay based on a single-copy target is less sensitive than the one based on multi-copy targets, the method presented in this report was effective for identification and quantification of parasites in human biopsy samples. The assay identified and estimated as few as 10 parasites in 0.2 ng of total DNA from a human biopsy sample. Although there was no obvious relationship between estimation of parasite numbers and the specific *Leishmania* species found in tissues from human lesions, the number of *L. (V.) braziliensis* parasites varied significantly. This observation does not support the general accepted idea that this species is always present in small numbers in lesions. Further studies using a larger number of samples will provide important information on the relationship between clinical symptoms and parasite load. The test will also be useful in analyzing responses to treatment and was done recently for cases of visceral leishmaniasis. However, for cutaneous and mucocutaneous diseases, other clinical samples would be needed (peripheral blood mononuclear cells or lesion aspirates). In addition, the assay can be used to guide therapeutic prognosis. This aspect is important because different *L. (Viannia)* species show different responses to treatments (miltefosine versus pentavalent antimonials). Quantification and identification of parasites in immunologic studies will help in understanding the mosaic of clinical manifestations.

The ability to identify *L. (V.) braziliensis* in material collected in disease-endemic areas using simple methods of preservation is important for both clinical and epidemiologic studies. The new assay described in this report can detect, quan-
tify, and distinguish parasites of the subgenera L. (Viannia) and L. (V.) braziliensis that are the principal causes of cutaneous and mucocutaneous leishmaniasis in humans throughout the Americas. Besides its clinical applications, the test will help resolve many of the crucial questions related to reservoir hosts. For instance, determining the numbers of parasites in the skin and blood of different hosts will help to assess their relative importance as reservoirs. Also, determining levels of infection in naturally infected sand flies might shed light on the importance of different species in transmission. Similarly, studying seasonal variations in the availability of Leishmania in hosts from different habitats throughout the year will provide important insights into the epidemiology and feasibility of control measures.

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