New PCR Assay Using Glucose-6-Phosphate Dehydrogenase for Identification of *Leishmania* Species

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Glucose-6-phosphate dehydrogenase (G6PD) is one of the multilocus enzymes used to identify *Leishmania* by zymodeme analysis. The polymorphic pattern revealed by partial characterization of the gene encoding G6PD generated molecular markers useful in the identification of different *Leishmania* species by PCR. Initially degenerate oligonucleotides were designed on the basis of data on the conserved active center described for other organisms. Primers for reverse transcription-PCR experiments, designed from the nucleotide sequence of the PCR product, enabled us to characterize the 5' and 3' untranslated regions and the G6PD open reading frame of reference strains of *Leishmania* (*Viannia*) *braziliensis*, *Leishmania* (*Viannia*) *guyanensis*, *Leishmania* (*Leishmania*) *mexicana*, and *Leishmania* (*Leishmania*) *amazonensis*. Sets of paired primers were designed and used in PCR assays to discriminate between the parasites responsible for tegumentar leishmaniasis of the subgenera *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) and to distinguish *L. (Viannia) braziliensis* from others organisms of the subgenus *Leishmania* (*Viannia*). No amplification products were detected for the DNA of *Crithidia fasciculata*, *Trypanosoma cruzi*, or *Leishmania* (*Sauroleishmania*) *tarentolae* or DNA from a healthy human control. The tests proved to be specific and were sensitive enough to detect parasites in human biopsy specimens. The successful discrimination of *L. (Viannia) braziliensis* from other parasites of the subgenus *Leishmania* (*Viannia*) opens the way to epidemiological studies in areas where more than one species of the subgenus *Leishmania* (*Viannia*) exist, such as Amazonia, as well as follow-up studies after chemotherapy and assessment of clinical prognoses.

Leishmaniasis is the collective name given to a spectrum of human diseases caused by organisms belonging to the genus *Leishmania*. They represent important emerging or reemerging zoonoses (2). Of the tropical protozoan parasites causing human disease, *Leishmania* is the second most common cause of human protozoal diseases in terms of new cases and deaths, being surpassed only by *Plasmodium* (38).

Twenty-two species of *Leishmania* have been reported to cause human infections (13, 25, 45). These protozoa are widely distributed in over 80 countries situated in tropical, subtropical, and temperate regions. According to the World Health Organization (46), 1.5 million to 2 million new cases occur annually, although only 600,000 are officially reported.

The clinical forms of leishmaniasis are broadly classified as being cutaneous, diffuse cutaneous, mucocutaneous, visceral, or post-kala azar dermal. The visceral form, if untreated, is usually fatal, but in certain situations treatment may fail (15). A taxonomic division, based on the behaviors of promastigotes in the sandfly's gut, grouped the organisms of the genus *Leishmania* into two subgenera: *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*), with the latter being geographically restricted to South America (24). There is no direct correlation between a specific clinical form and the causative species (25), although classically, in the neotropics, *Leishmania* (*Leishmania*) *chagasi* is associated with visceral leishmaniasis and *Leishmania* (*Viannia*) *braziliensis* is associated with mucocutaneous lesions. So far, each identification method described in the

literature is restricted to a group of species. No one method is able to discriminate among the entire repertoire of all described species that are causative agents of leishmaniasis in biopsy material such as lesion smears or histological sections. Morphological characteristics are of very limited use, and at present, identification depends on the examination of cultured promastigotes with monoclonal antibodies (19, 27) or by use of isoenzymatic profiles (11). Methods based on kinetoplast DNA (5, 6, 17, 39, 43) or specific nuclear DNA probes in PCR-hybridization assays (4, 18, 20, 31, 32, 42) are being used to distinguish between some species in biopsy material.

Organisms belonging to the *Leishmania* (*Viannia*) subgenus are restricted to South America and are often referred to as New World *Leishmania*. Phylogenetic studies have shown that this subgenus is closer to the root of the *Leishmania* clade than the Old World parasites (30). The morphological and biochemical characteristics of this group are conserved, and there is a high degree of nucleotide sequence similarity for molecular markers such as ribosomal DNA (42) and regions of kinetoplast DNA (16, 43). On the other hand, there is a high degree of molecular divergence for nontranscribed regions (14), which means that they are unsuitable for use in the description or identification of species of this subgenus. *L. (Viannia) braziliensis* is responsible for the most morbid and disfiguring form of the disease known as espundia or mucocutaneous leishmaniasis, which can be found between latitudes 19°N and 29°S (2, 24). Other *Leishmania* (*Viannia*) species, such as *Leishmania* (*Viannia*) *guyanensis* and *Leishmania* (*Viannia*) *panamensis*, are common causes of cutaneous leishmaniasis in the rain forests of Central America and those that lie to the north of the Amazon River.

Only a few publications have described molecular probes

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capable of discriminating some *Leishmania* (*Viannia*) species (18, 26, 28, 34), and since those organisms exist as sympatric populations in some regions (25), such as Amazonia, their discrimination will be extremely helpful for epidemiological and clinical studies. The lack of extensive studies on isolates from patients or reservoirs to verify both specificity and sensitivity, with a few exceptions (3, 33), has also been a serious drawback, since these organisms have a very high degree of heterogeneity (14). In our opinion, the existing literature points to an urgent need for new probes that, either alone or in association with those already described, can be used to positively identify the different *Leishmania* (*Viannia*) species and complexes.

Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) is one of the enzymes widely used in multilocus enzyme electrophoresis to identify *Leishmania* zymodemes (45). Eight isoforms of this enzyme have been described for *Leishmania* (10). It is inferred from the results of Cupolillo et al. (12) that isoform G6PD-7, identified in 14 of 15 different *L. (Viannia) braziliensis* isolates, is almost exclusive to this species. With this in mind, we decided to determine the *G6PD* sequences of some *Leishmania* reference strains, and, using the differences to design PCR probes, we developed a new identification test. Parasites of both subgenera are discriminated, as is *L. (Viannia) braziliensis*, from others organisms of subgenus *Leishmania* (*Viannia*). The test proved to be sensitive for the detection and identification of these species in human biopsy specimens.

MATERIALS AND METHODS

Organisms. Promastigotes of all *Leishmania* species listed in Table 1 were grown at 25°C in M199 medium (Gibco-BRL) as described by Kapler et al. (23). *Trypanosoma cruzi* and *Crithidia fasciculata* were grown at 28°C in liver infusion medium (9).

Purification and analysis of nucleic acids. DNA of cultured cells or control human DNA was purified by a sodium dodecyl sulfate-proteinase K-phenol extraction method, as described by Uliana et al. (41). The nucleic acids were fractionated in agarose gel in 1× TAE (40 mM Tris-acetate and 2 mM EDTA).

Oligonucleotides, PCR, and reverse transcription (RT)-PCR. The PCRs were performed as described by Saiki et al. (36) with *Taq* DNA polymerase (Amersham Pharmacia) and MgCl₂ at a concentration of 2 mM. The hot start was performed at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, annealing for 1 min, and 72°C for the extension. The annealing temperature and the time of extension for each pair of primers are summarized in Table 2.

The positions of the degenerate oligonucleotides are indicated in Fig. 1, and the corresponding sequences are listed in Table 2. Degenerate oligonucleotides were used in a standard PCR (annealing at 50°C and extension for 1 min) with genomic DNA at the concentrations described in the Results as the template.

The RT-PCR assay for the 5' mRNA end was performed with 5 µg of total RNA and 200 U of SuperScript II reverse transcriptase (Gibco-BRL) at 42°C for 50 min in an RT reaction primed with oligonucleotide *G6PD-F4*. After heat inactivation of the reverse transcriptase, 1/10 of the reaction product was submitted to PCR (annealing at 55°C and 3 min of extension) with the sense mini-exon sequence oligonucleotide (MEDL) and *G6PD-F2* oligonucleotides (Table 2).

The RT-PCR of the 3' mRNA end was performed as described above except for changes in primers or in the quantity of the PCR template. Primer RNA-A1 was used in RT, and 1/5 of the reaction product was used as the template in the PCR (annealing at 55°C and 4 min of extension) with primers *G6PD-F1* and RNA-A. The PCR product was then diluted 1,000 times, and a new PCR with *G6PD-F3* instead of *G6PD-F1* was performed as described above.

The PCR assays used to distinguish *Leishmania* species were performed with oligonucleotides *G6PD-ISLA*, *G6PD-F2*, *G6PD-ISVA*, *G6PD-ISVC*, *G6PD-ISVB*, and *G6PD-ISVNB*, whose sequences are indicated in Fig. 1, by using the conditions specified in Table 2.

Cloning and sequencing strategies. The desired DNA fragments, which were purified from agarose gels by electroelution with polyethylene glycol (47), or

TABLE 1. Organisms used and their behaviors in the *G6PD*-based assay

Organism	Reference no. ^a	Result of <i>G6PD</i> -based PCR assay ^b			
		L	V	nb	b
<i>L. (Leishmania) amazonensis</i>	MHOM/BR/1973/M2269 ^c	+	–	–	–
<i>L. (Leishmania) mexicana</i>	MHOM/BZ/1982/BEL21 ^c	+	–	–	–
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1975/M2903 ^c	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1994/631 ^d	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1994/648 ^d	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1994/M12454 ^d	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1994/M15176 ^d	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1994/M15140 ^d	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1999/M17615 ^e	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1999/M17618 ^e	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MBOL/BR/2000/CPqAM93 ^e	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1990/IM3641 ^f	–	+	–	+
<i>L. (Viannia) braziliensis</i>	MHOM/BR/1992/IM3846M ^f	–	+	–	+
<i>L. (Viannia) guyanensis</i>	MHOM/BR/1975/M4147 ^d	–	+	+	–
<i>L. (Viannia) guyanensis</i>	MHOM/BR/1976/M4200 ^d	–	+	+	–
<i>L. (Viannia) guyanensis</i>	MHOM/BR/1992/IM3823 ^f	–	+	+	–
<i>L. (Viannia) panamensis</i>	MHOM/PA/1971/LS94 ^d	–	+	+	–
<i>L. (Viannia) shawi</i>	MCEB/BR/84/M8408 ^d	–	+	+	–
<i>L. (Viannia) lainsoni</i>	MHOM/BR/81/M6426 ^c	–	+	+	–
<i>L. (Viannia) naiffi</i>	MDAS/BR/1979/M5533 ^d	–	+	+	–
<i>L. (Viannia) naiffi</i>	MHOM/BR/1994/IM4000 ^f	–	+	+	–
<i>L. (Viannia) naiffi</i>	MHOM/BR/1987/AXEL ^f	–	+	+	–
<i>L. (Saurolleishmania) tarentolae</i>	RTAR/FR/1978/G28 ^c	–	–	–	–
<i>C. fasciculata</i>	ATCC 30267 ^c	–	–	–	–
<i>T. cruzi</i>	Y strain ^c	–	–	–	–
<i>T. cruzi</i>	CL strain ^c	–	–	–	–

^a The isolates are labeled as described by the World Health Organization (44).

^b L, V, nb, and b, fragments generated by pairs of primers specific for *Leishmania* (*Leishmania*) species, *Leishmania* (*Viannia*) species, non-*L. (Viannia) braziliensis*, *Leishmania* (*Viannia*) species, and *L. (Viannia) braziliensis* (see Table 2 for more details about the primers).

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PCR products were ligated to the appropriate sites in pUC19, pBS (Stratagene), or pGEM-T Easy Vector (Promega). The nucleotide sequences were determined automatically as described previously (37) with an ABI Prism 310 genetic analyzer (Perkin-Elmer).

Extraction of DNA from biopsy specimens. Biopsy specimens were taken with a punch from patients from Rondônia State, which is in the northwest region of Brazil, and were 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 São Paulo. The material was washed three times with 1× phosphate-buffered saline (7 mM Na₂HPO₄, 26 mM NaH₂PO₄, 130 mM NaCl) and was then processed as described above for total DNA. The study was previously approved by the Institutional Ethical Commission.

Computational analysis. The BLASTx program (1) was used to check the similarities of the nucleotide sequences with the sequences of other proteins in GenBank. The sequences were aligned with BioEdit (21), Clustal-W (40), and Esee Eye Ball (8) software. Esee Eye Ball software was also used to generate the putative amino acid sequences.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper are available in GenBank under accession numbers AY099298 to AY099305 and AY099307.

RESULTS

Characterization of *Leishmania* *G6PD*-coding sequence and its mRNA transcript. On the basis of the conserved sequences at the catalytic domain of G6PD and closer to its active residue, namely, RIDHYLGK and GIIRDVMQ, depicted by the alignment of the amino acid sequences of *Escherichia coli* (GenBank accession number AE000279), *Emericella nidulans*

TABLE 2. Oligonucleotides used in this study specific PCR conditions for each pair of primers, and fragment length of product (°C)^a

Name	<i>T_m</i>	Sequence	Ori ^b	Primer pair	A (°C) ^c	E ^d	L (bp) ^e
G6PD-P1	ND	5'-ATH GAY CAY TAY YTN GGN AAR G-3'	For	D	50	1 min	194
G6PD-P2	ND	5'-TGK TTY TGC ATN ACK TCN CKD ATD ATN CC-3'	Rev				
MEDL	51	5'-CGC TAT ATA AGT ATC AGT TTC-3'	For	5' UTR	55	1 min	1,009
G6PD-F2	47	5'-CGG TTG GCG AAG CGT GTC-3'	Rev				
G6PD-F4	65	5'-CCG ATG GTC TCC TTG AAC GTG AT-3'	Rev				
G6PD-F1	57	5'-AAC ATC ATC ACG ACA CGC TTC-3	For	3' UTR	55	3 m	2,000
G6PD-F3	55	5'-TGC AGA TCA CGT TCA AGG AG-3'	For				
RNA-A	85	5'-GAC TCG AGT CGA CAT CGA (TTT) ₅ TT-3'	Rev				
RNA-A1	51	5'-GAC TCG AGT CGA CAT CGA-3'	Rev				
ORF1	55	5'-CTC ATC TTA TTC ACC CAC CC-3'	For	ORF ^f	55	3 m	1,967
ORF2	53	5'-CCG CCT TGA CAG ACG CTG-3'	Rev				
G6PD-ISVA	59	5'-GTC GGT TAT CCT ATT CGG GTC-3'	For	V	60	30 s	336 ^g
G6PD-ISVC	59	5'-ATC ACA ATG ATG GTC AAC GCA C-3'	Rev				
G6PD-ISLA	55	5'-CTC ATC TTA TTC ACC CAC CC-3'	For	L ^h	65	1 m	903
G6PD-ISVB	53	5'-TAC TCG CCA TGT CGG AGG-3'	For	B ⁱ	60	30 s	234
G6PD-ISVNB	49	5'-TAC TCG CCA TGT CGT CG-3'	For	Nb ⁱ	60	30 s	237

^a *T_m*, melting temperature empirically calculated by the formula $T_m = [(A+T) \cdot 2 + (G+C) \cdot 4] - 5$ where (A+T) is the A+T content and (G+C) is the G+C content. ND, not determined.

^b Ori, orientation of primer (For, forward; Rev, reverse).

^c A, annealing temperature.

^d E, extension time.

^e L, Length of the product.

^f ORF, open reading frame.

^g The length was 333 bp for *L. (Viannia) braziliensis*.

^h Oligonucleotide paired with G6PD-F2.

ⁱ Oligonucleotide paired with g6p-ISVC.

(GenBank accession number P41764), *Arabidopsis thaliana* (GenBank accession number CAB52674), humans (GenBank accession number 4503845), and *Trypanosoma brucei* (GenBank accession number AJ249254), we designed two degener-

ate oligonucleotides, G6PD-P1 and G6PD-P2 (Fig. 1), for use in the PCR with *Leishmania* genomic DNA. The expected product of 194 bp (f194) was obtained for *Leishmania (Leishmania) amazonensis*, *Leishmania (Leishmania) mexicana*, *L.*

FIG. 1. Schematic positions of the oligonucleotides in *G6PD* mRNA. Grey box, *G6PD* mRNA; white box, *G6PD* open reading frame; hatched box, region amplified by the degenerate oligonucleotides (f194); black box, region used to identify *Leishmania* organisms. The oligonucleotides and their orientations are indicated by arrows: A and A1 are primers RNA-A and RNA-A1, respectively. For the others oligonucleotides, the “G6PD-” designation was dropped from the beginnings of the oligonucleotide names. The text boxes show the partial sequences in four *Leishmania* species: La, *L. (Leishmania) amazonensis* (M2269); Lm, *L. (Leishmania) mexicana* (M7326); Lg, *L. (Viannia) guyanensis* (M4147); Lb, *L. (Viannia) braziliensis* (M2903). The underlined sequences in the text boxes are the oligonucleotides sequences, whose names are provided above each box: G6PD-ISLA in *L. (Leishmania) amazonensis* and *L. (Leishmania) mexicana*, G6PD-ISVA in *L. (Viannia) guyanensis* and *L. (Viannia) braziliensis*, G6PD-ISVB in *L. (Viannia) braziliensis*, G6PD-ISVNB in *L. (Viannia) guyanensis*, and G6PD-ISVC in *L. (Viannia) guyanensis* and *L. (Viannia) braziliensis*.

FIG. 2. Agarose gel-fractionated PCR products from DNA extracted from cultured parasites. PCR assays were carried out with 200 ng of total promastigote DNA (A) or various masses of DNA (B to D). neg, negative control with no DNA in all assays. The letters on the left of each row indicate the assay performed (see Table 2): d, degenerate oligonucleotides; L, *Leishmania* (*Leishmania*) subgenus; V, *Leishmania* (*Viannia*) subgenus; b, *L. (Viannia) braziliensis*; nb, *Leishmania* (*Viannia*) organisms except *L. (Viannia) braziliensis*. (A) DNA from *C. fasciculata* (lane Cf), *T. cruzi* Y (lane TcY), *T. cruzi* Cl (lane TcCl), *L. (Leishmania) amazonensis* (M2269) (lane La), *L. (Leishmania) mexicana* (BEL21) (lane Lm), *L. (Viannia) guyanensis* (M4147) (lane Lg), *L. (Viannia) braziliensis* (M2903) (lane Lb), *L. (Viannia) shawi* (M8408) (lane Ls), and *L. (Sauroleishmania) tarentolae* (lane Lt). (B) The amplification products obtained with diluted DNA extracted from cultured promastigotes of *L. (Viannia) braziliensis* (the number above each lane indicates the mass [in nanograms]) obtained with oligonucleotides *G6PD*-ISVC and *G6PD*-ISB. (C and D) The species (as defined for panel A) or the reference isolate is indicated above each lane (see Table 1 for details).

(*Viannia*) *braziliensis*, and *L. (Viannia) guyanensis* (Fig. 2A, row d); and the fragments were cloned and sequenced. The product was also obtained for *T. cruzi*, *C. fasciculata*, and *L. (Sauroleishmania) tarentolae*. The nucleotide sequences of all 194 fragments showed high degrees of similarity to the sequences of the 194 fragments of other nontrypanosomatid *G6PD* genes deposited in GenBank. This fragment from *Leishmania* products showed 82% identity to the *Trypanosoma brucei* *G6PD* gene, which ensured the identities of the clones.

A set of four oligonucleotides (*G6PD*-F1 to *G6PD*-F4), whose sequences correspond to regions with conserved sequences of the species analyzed, was used in the RT-PCR assays as described above. Oligonucleotides *G6PD*-F2 and *G6PD*-F4 hybridized with the fragments of 1.0 kb generated with MEDL corresponding to the 5' end of mRNA. On the other hand, oligonucleotides *G6PD*-F1 and *G6PD*-F3 hybridized with RNA-A and RNA-A1 to generate major fragments of 2.1 kb corresponding to the 3' end of mRNA (data not shown).

The RT-PCR strategy allowed us to isolate the complete *G6PD*-coding mRNA sequences of *L. (Leishmania) amazonensis*, *L. (Leishmania) mexicana*, *L. (Viannia) braziliensis*, and *L. (Viannia) guyanensis* and also the 5' ends of the mRNAs of *L. (Viannia) panamensis*, *Leishmania (Viannia) lainsoni*, *Leishmania (Viannia) shawi*, and *Leishmania (Viannia) naiiffi*. The sequence data are deposited in GenBank; and the de-

duced amino acids indicated an open reading frame of 1,689 nucleotides, yielding 170 of 177 nucleotides of the 5' untranslated region (UTR) according to the species and about 1,400 nucleotides of 3' UTR.

***G6PD*-based PCR assays for specific *Leishmania* identification.** Specific oligonucleotides were designed for use in the PCR for *Leishmania* identification, as follows: *G6PD*-ISLA and *G6PD*-F2 for organisms of the *Leishmania* (*Leishmania*) subgenus and *G6PD*-ISVA and *G6PD*-ISVC (Fig. 1) for organisms of the *Leishmania* (*Viannia*) subgenus (the results are summarized in Table 1). Total DNA (200 ng, which represents about 10^7 parasites) from *L. (Leishmania) amazonensis*, *L. (Leishmania) mexicana*, *L. (Viannia) braziliensis*, *L. (Viannia) guyanensis*, *L. (Viannia) shawi*, *L. (Sauroleishmania) tarentolae*, *C. fasciculata* and *T. cruzi* was used as the template in the PCRs (annealing temperature, 65°C; extension time, 1 min) with primers *G6PD*-ISLA and *G6PD*-F2 (Fig. 2).

The subgenus-specific oligonucleotide *G6PD*-ISLA paired to *G6PD*-F2 only amplified *Leishmania* (*Leishmania*) DNA (Fig. 2A, row L). The PCR (annealing temperature, 60°C; extension time, 30 s) with primer pair *G6PD*-ISVA and *G6PD*-ISVC exclusively amplified the DNA of the *Leishmania* (*Viannia*) subgenus (Fig. 2A, row V).

Oligonucleotides specific for *L. (Viannia) braziliensis* (*G6PD*-ISVB) and *L. (Viannia) guyanensis* (*G6PD*-ISVNB) were designed on the basis of *G6PD* sequence differences. The

PCR (annealing temperature, 60°C; extension time 30 s) with primer *G6PD*-ISVB paired to the shared primer *G6PD*-ISVC amplified only *L. (Viannia) braziliensis* (Fig. 2A, row b). The PCR performed under the same conditions but with *G6PD*-ISNB paired to *G6PD*-ISVC detected *L. (Viannia) lainsoni*, *L. (Viannia) panamensis*, *L. (Viannia) naiffi*, *L. (Viannia) guyanensis*, and *L. (Viannia) shawi* (Fig. 2A, row nb, and Table 1).

DNA from other trypanosomatids, such as *L. (Sauroleishmania) tarentolae*, *C. fasciculata*, and *T. cruzi*, was positive by PCR assays with degenerate primers but negative by PCR assays with any of the *G6PD* primers whose sequences were derived from the sequences of the two *Leishmania* subgenera that infect mammals (Fig. 2A).

The large amount of DNA used to establish the specific assay conditions completely excluded the possibility of detection of nonspecific products. On the other hand, serial dilutions of DNA presented positive PCR results with DNA present in amounts up to 2 pg, which corresponds to approximately 50 parasites, when *G6PD*-ISVC paired with *G6PD*-ISB (Fig. 2B) or *G6PD*-ISNB (data not shown) was used.

Validation of *Leishmania (Viannia)* identification assays. To validate the *G6PD*-based assay for specific identification of members of the subgenus *Leishmania (Viannia)*, we increased the number of isolates evaluated in the study. We included two *L. (Viannia) braziliensis* isolates from Paraná (isolates 631 and 648), which is in the southern region of Brazil, and three isolates from Pará (isolate M12454 from Serra dos Carajás and isolates M15140 and M15176 from Paragominas), which is in the northern region of Brazil. Those isolates were recently grouped by geographic origin in a randomly amplified polymorphic DNA analysis (22). We also included two *L. (Viannia) naiffi* zymodemes (zymodemes IM4000 and AXEL) that present the same *G6PD* isoforms (isoforms 3, 4, and 6), two *L. (Viannia) guyanensis* strains (strains M4200 and IM3823), and two different *L. (Viannia) braziliensis* zymodemes (zymodemes IM3641 and IM3846 M) that present no variations in their *G6PD* isoforms (E. Cupolillo, personal communication).

DNA from all the organisms cited above was positive by PCR with the *G6PD*-derived *Leishmania (Viannia)* subgenus-specific primers (Fig. 2C and Fig. 2D, row V). The *L. (Viannia) braziliensis*-specific PCR was positive when the DNA from strains 631, 648, M12454, M15140, M15176, IM3641, and IM3846 M was used (Fig. 2C and D, rows b). PCR with the *G6PD*-ISVNB-*G6PD*-ISVC primer pair provided positive results for the two *L. (Viannia) naiffi* and *L. (Viannia) guyanensis* strains (Fig. 2C and D, rows nb). The PCRs for the *Leishmania (Leishmania)* subgenus (with oligonucleotides *G6PD*-ISLA and *G6PD*-F2) were negative for all of the organisms described above (Fig. 2D, row L, and data not shown).

Three new variants of *L. (Viannia) braziliensis* (strains M17615, M17618, and CPqAM93) that have recently been isolated from silvatic reservoirs in Pernambuco (northeast region of Brazil) and that present different isoform profiles for malic enzyme, isocitrate dehydrogenase, and phosphoglycerate mutase (7) were analyzed. The nucleotide sequence of the 5' cDNA of *G6PD* showed 100% identity to that of *L. (Viannia) braziliensis* reference strain M2903 and had the same PCR pattern as that for reference strain M2903 (data not shown).

Application of the *G6PD*-based PCR to human biopsy spec-

FIG. 3. Agarose gel-fractionated PCR products from DNA extracted from biopsy specimens. The letters on the left of each row indicate the assay performed (see Table 2). Lanes 1 to 10, DNA from processed biopsy specimens of patients from Rondônia; lane H, control DNA from a healthy human volunteer; lane Lb, DNA from *L. (Viannia) braziliensis* (positive control); lane La, DNA from *L. (Leishmania) amazonensis* (negative control); lane Lg, DNA from *L. (Viannia) guyanensis* (positive control); lane neg, negative control without DNA.

imens. The sets of primers used in the *G6PD*-based assays described above were also tested with DNA extracted from skin lesion biopsy specimens from patients from Rondônia (which is in the northwest region of Brazil) who were clinically diagnosed with dermal leishmaniasis. The expected product for the *Leishmania (Viannia)* subgenus was detected in 21 samples; 9 were positive by the PCR assay with oligonucleotides *G6PD*-ISVB and *G6PD*-ISVC, and 12 were positive by the PCR assay with oligonucleotides *G6PD*-ISVNB and *G6PD*-ISVC. None of the samples were positive in the assay for the *Leishmania (Leishmania)* subgenus. Figure 3 shows the PCR products obtained with biopsy specimens from some patients.

DISCUSSION

The strategy of using degenerate oligonucleotides coupled to RT-PCR resulted in the isolation and characterization of the *G6PD* genes of four *Leishmania* species. The conserved residues responsible for enzyme activity described in other models were detected in the deduced amino acid sequence (data not shown). Therefore, it is reasonable to infer that the sequences characterized correspond to the active enzyme-coding gene instead of a derived pseudo-gene. As expected, the same gene is found in other members of the family *Trypanosomatidae*.

The sequence differences among *Leishmania* species were exploited to design oligonucleotide sets specific for the subgenus *Leishmania (Leishmania)* (oligonucleotides *G6PD*-ISLA and *G6PD*-F2), subgenus *Leishmania (Viannia)* (oligonucleotides *G6PD*-ISVA and *G6PD*-ISVC), *L. (Viannia) braziliensis* (oligonucleotide *G6PD*-ISVB), and other species of the *Leishmania (Viannia)* subgenus excluding *L. (Viannia) braziliensis* (oligonucleotide *G6PD*-ISVNB). These oligonucleotides were combined to anchor primers in specific PCR assays to identify the organism group (Table 2). Other organisms that may occur in field samples and that could cross-react in the assay, such as *C. fasciculata*, *T. cruzi*, and even a closely related organism such as *L. (Sauroleishmania) tarentolae*, were discriminated in the tests since their DNA did not generate amplification products.

The 100% identities of the *G6PD* sequences of the World Health Organization *L. (Viannia) braziliensis* reference strain and the three *L. (Viannia) braziliensis* strains recently isolated from wild reservoirs in the northeast region of Brazil indicate that the same species-specific differences are present in strains circulating in the enzootic cycle. This is an important observation, since it validates the usefulness of the assays described here in field studies. Furthermore, the selected differences are conserved among the different *L. (Viannia) braziliensis* zymodemes and strains of different geographic origins.

The present study is the first in which a PCR assay was developed on the basis of isoenzyme data. To our knowledge, it is also the first time that a single-copy gene has been used as a target for identification. The test detected parasite DNA in biopsy specimens from human skin lesions and discriminated *L. (Viannia) braziliensis* from the other sympatric species belonging to the same subgenus. This opens the gateway for critical clinical and chemotherapeutic follow-up trials in regions of endemicity where more than one *L. (Viannia)* species is circulating. Correct identification of the etiologic agent is important since differences in the chemotherapeutic responses of the species of the two subgenera exist (29). Recently, variations in the susceptibilities of *L. (Viannia) braziliensis* and *L. (Viannia) guyanensis* (35) to pentavalent antimonial drugs were observed, indicating an immediate application of our assay. Lastly, the test does not depend on parasite isolation and cultivation, allowing the examination of small biopsy samples that can be transported at room temperature to reference laboratories many thousands of miles away from regions of endemicity. This will definitely help provide an understanding of the epidemiology of leishmaniasis in the less accessible areas of Latin America.

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