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Artículo original

A PCR assay for the identification of *Leishmania* species of the *Viannia* subgenus

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***Abstract*:** We have identified a novel DNA sequence of 500 bp (β500-DNA) on the *Leishmania (Viannia)* subgenus, located in the intergenic region of one of the loci of the β-tubulin gene family. The sequence analysis showed that this sequence has no homology to any other sequence described so far, including the β-tubulin gene. We improved a specific β500-PCR assay, which generated a PCR product of 375 bp for total genomic DNA from *Leishmania* strains belonging to the *L. (Viannia)* subgenus. In contrast, no amplification was found when using genomic DNA from species of *L. (Leishmania)* subgenus or other organisms. Under our PCR conditions, the lower detection limit was 1 fg when a purified DNA clone (pLgβ4), which contains one copy of the β500-DNA sequence, was used. The β500-DNA PCR assay confirmed the preliminary diagnosis of cutaneous leishmaniasis in clinical samples in which the Montenegro skin test was positive and parasite cultures were negative. The analytical specificity and the sensitivity of the PCR assay provide a tool for epidemiological studies of the disease.

Keywords: *Leishmania*, leishmaniasis, diagnostic, restriction fragments length polymorphism, polymerase chain reaction, β-tubulin gene.

Un ensayo de PCR para identificar especies de *Leishmania* del subgénero *Viannia*

***Resumen*:** En este trabajo identificamos una nueva secuencia de DNA de 500 pb (β500-DNA) en *Leishmania* del subgénero *Leishmania (Viannia)*, localizada en la región intergénica de uno de los loci de la familia de los genes de la β tubulina. El análisis de secuencia mostró que β500 no tiene homología con ninguna otra secuencia previamente descrita, incluido el gen de la β tubulina. Nosotros implementamos un ensayo de PCR específico para β500, β500-PCR, que genera un producto de PCR de 375 pb a partir del DNA genómico de cepas de *Leishmania* pertenecientes al subgénero *L. (Viannia)*. No hubo amplificación alguna cuando se utilizó el DNA genómico de especies del subgénero *L. (Leishmania)* o el de otros organismos. En las condiciones establecidas, utilizando DNA purificado del clon pLgβ4, que contiene una copia de la secuencia de DNA de β500, el límite de detección más bajo fue de 1 fentogramo. El ensayo β500-PCR confirmó el diagnóstico preliminar de leishmaniasis cutánea en muestras clínicas de pacientes positivas a la prueba de Montenegro y negativas en el cultivo de parásitos. La especificidad y sensibilidad analítica del ensayo de PCR proporciona una herramienta para estudios epidemiológicos de la enfermedad.

Palabras clave: *Leishmania*, leishmaniasis, diagnóstico, polimorfismo de la longitud de los fragmentos de restricción, reacción en cadena de la polimerasa, gen de la β-tubulina.

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Introduction

Leishmania is the causal agent of the disease known as leishmaniasis. The species of the *Leishmania* genus are grouped in two subgenera: *L. (Leishmania)* and *L. (Viannia)* [1]. Those species of the *L. (Leishmania)* subgenus cause in humans the cutaneous and visceral forms of the disease, in both the New and the Old World, whereas the *L. (Viannia)* subgenus species are autochthonous of America, and responsible for cutaneous and mucocutaneous leishmaniasis. Several molecular markers have been used to identify *Leishmania* [2-5]. For instance, the development of the PCR-based typing methods has provided a new set of markers to be used in the molecular identification of *Leishmania* and epidemiological studies of the disease [6]. Thus, PCR assays with higher sensitivity and specificity

have been developed to detect *Leishmania* species of both subgenera, using as targets the unusual kinetoplast DNA (kDNA) [2], or particular nuclear sequences such as the mini-exon [3], the internal transcribed spacer region (ITS) of the ribosomal RNA [7], the small subunit of the rRNA [8], hsp70 genes [9], and the single copy gene of the glucose-6-phosphate dehydrogenase [10], among others.

The β-tubulin multigene family of *Leishmania* showed sufficient polymorphism to discriminate between the *Leishmania* subgenera [11]. Upstream of the β-tubulin genes, we have identified a sequence of 500 bp, β500-DNA, which showed specificity to the *L. (Viannia)* subgenus [11]. In this study we evaluated the analytical sensitivity, specificity and reliability of a β500-PCR assay. The PCR assay was highly sensitive and detects the parasites on of patient´s biopsies with typical cutaneous lesions.

Materials and methods

Parasites: The *Leishmania* strains used in this study, many of them WHO reference strains, are described and listed in table 1. For all analyses, promastigotes were grown at room temperature in Schneider's *Drosophila* medium (SIGMA) supplemented with 10% heat-inactivated foetal calf serum and 40 µg/ml chloramphenicol [12].

Clinical samples: A total of 12 clinical samples (biopsies) were collected from two different endemic areas. A first group was obtained from three patients of the Urama village, Carabobo State, Venezuela, an area where an active focus of leishmaniasis due to *L. (V.) braziliensis* was identified [13]. These patients were referred to the Servicio de Parasitología of the Universidad de Carabobo, Carabobo State, Venezuela, to complete diagnosis and treatment. A second group of nine samples was collected in different regions of Colombia, where the majority of lesions are caused by *L. (V.) panamensis* or *L. (V.) braziliensis*; treatment and studies of these patients are under the PECET (Program of Study and Control of Tropical Disease), Universidad de Antioquia, Colombia. The selected patients showed a suggestive clinical diagnosis of leishmaniasis and Montenegro skin test was positive in the majority of them. A fragment of biopsy of approximately 1-2 mm^3 was collected in each case in a 0.5 mL sterile tube and frozen at -80 °C, and the parasites were detected by at least one method (Table 2).

Cell fractionation and genomic DNA preparation: Total genomic DNA from *Leishmania* promastigotes (Table 1) was isolated and purified as described before [14]. DNA purification from biopsies was carried out by two different methods. The Venezuelan samples were placed in lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM NaCl, 1% SDS) and Proteinase K (SIGMA) was added to a final concentration of 40 µg/ml. The samples were incubated at 56 °C for 1 h, and then at 90 °C for 30 min, followed by two phenol extractions and ethanol precipitation. The precipitate was dissolved in 10 µL water and appropriate volumes (2-5 µL) were taken for the PCR reaction [2,15]. The DNA from

Table 1. Designation of *Leishmania* species and other organisms.

Subgenus	Species[a]	Strain[b] designation	Abbreviation	Origin
Leishmania (Leishmania)	*mexicana*	MHOM/BZ/82/BEL21	BEL21	Belize
		MNYC/BZ/62/M379	M379	Belize
	amazonensis	IFLA/BR/67/PH8	PH8C5[c]	Brazil
	major	MHOM/SU/59/P	P	USSR
	tropica	MHOM/00/88/RTC	RTC	Iran
	donovani	MHOM/IN/80/DD8	DD8	India
Leishmania (Viannia)	*braziliensis*	MHOM/BR/75/M2903	M2903	Brazil
	guyanensis	MHOM/BR/75/M4147	M4147	Brazil
	panamensis	MHOM/PA/71/LS94	LS94	Panamá
		MHOM/PA/86/UA113[d]	UA113	Colombia
		MHOM/PA/88/UA318[d]	UA318	Colombia
		MHOM/PA/89/UA352[d]	UA352	Colombia
Other Kinetoplastida:				
Trypanosoma cruzi			EP	
Crithidia fasciculata			ATCC12858	
Leptomonas samueli				

[a]The nomenclature of *Leishmania* proposed by Lainson and Shaw [1]. [b]The majority of these strains have been designated as reference strains by WHO. [c]This is a cloned derivative of *L. (L.) amazonensis* stock PH8. [d]These strains have been identified by isoenzyme analysis as *panamensis* by PECET, Universidad de Antioquia, Colombia.

Table 2. Parasitological, serological and PCR assay of clinical samples collected in endemic leishmaniasis area of Venezuela and Colombia

Patient [1]	Origin[2]	Age (y)	Sex[3]	MST[4] (mm)	Culture	Strain identification	PCR assay[5]	
							B_1/B_2	A_2/A_{10}
Venezuela								
UC75a	Urama	52	M	+	-	NT	NT	+
UC79	Urama	44	M	+	-	NT	NT	+
UC116	Urama	37	F	+	-	NT	NT	+
Colombia								
W3440	Santander	24	M	NT	+	*panamensis*	-	+
W3482	Sucre	22	M	-	-	NT	-	+
W3488	Antioquia	24	M	+	-	NT	-	+
W3490	Antioquia	19	M	+	+	*panamensis*	+	+
W3501	Chocó	16	F	+	-	NT	+	+
W3507	Caquetá	20	M	+	+	*panamensis*	+	+
W3519	Antioquia	45	M	+	+	*panamensis*	-	+
W3522	Antioquia	24	F	+	+	*panamensis*	+	+
W3528	Antioquia	75	F	+	+	*panamensis*	+	+

[1]UC and W are the sample code of Universidad de Carabobo and the PECET, Universidad de Antioquia, Colombia, respectively. [2]Urama is a village in the Carabobo State, Venezuela. Antioquia, Sucre, Chocó, Caquetá and Santander are Departments of Colombia. [3]F, female; M, male. [4]MST, Montenegro Skin Test. [5]B_1/B_2, specific kDNA-PCR assay to some *Leishmania* species of the *L. (Viannia)* subgenus [2]. NT, not tested; (+), positive reaction; (-), negative reaction.

Colombian samples was extracted and purified with a DNA extraction kit (QIAmp Tissue kit QIAGEN, Chatsworth, CA) following the manufacturer's instructions; samples were submitted to ethanol precipitation and resuspended in distilled sterile water for further use.

Southern blot analysis: Total genomic DNA was digested with Pst I restriction endonuclease (Promega) under the conditions suggested by the suppliers, fractionated by electrophoresis on 1% agarose gel, Southern transferred onto a Gene Screen Plus membrane (DuPont), and hybridised with the 32P-labelled probes. The *Leishmania* β500-DNA sequence (pLgβ500) and the recombinant plasmid pLgβtub1 [11], which contain the coding region of the β-tubulin gene, were used as probes; both probes were random primer labelled using the multiprime DNA labelling kit from Amersham Biosciences [16].

Hybridisation: DNA containing filters were prehybridised and hybridised as previously described [17]. After hybridisation, the filters were washed for one hour under conditions of medium stringency (2X SSC, 0.1% SDS) at 65 °C with a minimum of four changes of buffer. The filters were exposed for autoradiography at -80 °C during 24-48 h.

Sequencing of β500-DNA: Sequencing of the β500-DNA fragment (pLgβ500) was conducted using the forward and reverse universal primers of the pUC18 vector, according to standard procedures. The sequence was analysed by conventional methodologies and examined for homology to other sequences in the GenBank databases. The accession numbers to the β-tubulin gene and the β500-DNA sequences of *L. (V.) guyanensis,* assigned by Gene Bank were DQ836297 and AY151193.1, respectively.

PCR assay: PCR was performed in a final volume of 25 µL containing PCR-Mastermix (Promega) cocktail, 50 pmol of primers A_2 (5'-GACACGCGCTTGCGCACTCGT-3') and A_{10} (5'-CCCCCTGCCTCGCCTGC-3'), and 5.0 ng of total purified genomic DNA. The amount of DNA from biopsies was not determined. The PCR reaction was performed in a MJ Research PTC-200 thermocycler, comprising 5 min preincubation at 95 °C, followed by 40 cycles of 1 min at 95 °C, 1 min at 55 °C (low stringency) or 60 °C (high stringency), and 2 min at 72 °C, with a final extension at 72 °C for 10 min. The product was analysed by electrophoresis on 1.0 or 1.5% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). In some cases, to improve a multiplex amplification, we included a second set of primers designed from the coding region of the β-tubulin gene, Tub1 (5'-ATGCGTGAGATCGTTTCC-3') and Tub6 (5'-GGCGGCCTGCATCAT-3'), which generated a 900 bp fragment at 55 ºC or 60 °C annealing temperature.

Sensitive evaluation: The recombinant plasmid pLgβ4 previously described [11], which contains one copy of the β500-DNA, was used to evaluate the analytical sensitivity

of the β500-PCR assay. After evaluation of the A_{260}, we prepared a stock solution of the recombinant plasmid at a concentration of 1.0 ng/mL (1000 pg/mL). In order to evaluate the minimal amount of purified DNA clone which amplified the β500-DNA sequence, serial dilutions from the stock solution were carried out until amounts of 100, 10, 1, 0.1, 0.01, and 0.001 pg of DNA were reached, which were used to run the PCR assay under the high stringency conditions described above.

Results

Homology relationship between the β500 sequence and the β-tubulin gene: We performed a Southern blot analysis using genomic DNA from WHO *Leishmania* reference strains and Colombian field isolates digested with the Pst I endonuclease and the pLgβtub1 or pLgβ500 as probes. The pLgβtub1 probe, at medium stringency conditions of hybridisation, recognized different fragments in all *Leishmania* strains tested; comparison of the fragment patterns showed differences between species of the *Leishmania* and *Vianna* subgenera (Figure 1A). In addition, the patterns of the *L. (Viannia)* subgenus species were not identical among them, whereas under the same conditions, the β500-DNA probe showed one unique faint band around 2.30 kbp region in species belonging to the *L. (Leishmania)* subgenus (lanes 1 and 2), suggesting partial homology with Lgβtub1 sequence. In contrast, those species of the *L. (Viannia)* subgenus (lanes 3 to 8) showed one strong signal around 500 bp (Figure 1B).

Independent experiments to confirm the analytical specificity of the novel *Leishmania* β500-DNA sequence were carried out using genomic DNA-Pst I of different organisms and high stringency conditions of hybridisation (Figure 1C). A positive signal with the probe was found only in the genus *Leishmania*, strictly with those species belonging to the *L. (Viannia)* subgenus (lanes 3 and 4). In contrast, no homology with the β500-DNA probe was found in species of either the *L. (Leishmania)* subgenus or other genera of the Kinetoplastida order. Similar results were found with other restriction enzymes, such as Bam HI, Eco RI, Eco RII and Pvu II (data not shown). The results suggested a lack of homology between the β500-DNA and the coding region of the β-tubulin gene.

Analtical specificity of the nuclear β500-DNA sequence. PCR analysis: The complete sequence of the β500-DNA from *L. (V.) guyanensis* (Gene Bank Accession number: AY151193.1) was obtained. Comparison of the sequence analysis with the databank showed that the β500 sequence has apparently no identity with other sequences, including the β-tubulin gene, previously described in *Leishmania* or other organisms.

In order to ascertain the analytical specificity of the β500-DNA PCR amplification, other *Leishmania* strains belonging to the *L. (Leishmania)* subgenus were examined under low stringency conditions for PCR assay and

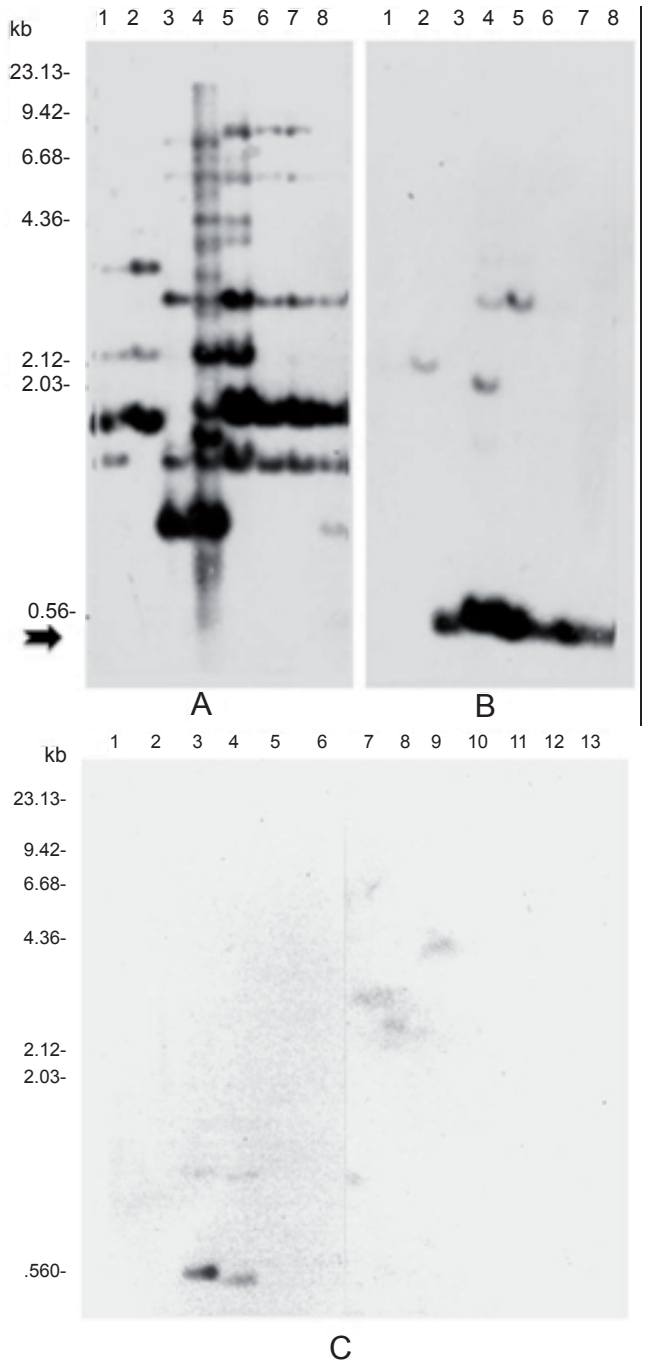

Figure 1. Homology between the β-tubulin gene and the β500 sequence. Genomic DNA from different *Leishmania* strains representative of both subgenera, *Leishmania* and *Viannia* respectively, was digested with Pst I, fractionated on a 1% agarose gel, and after bidirectional transfer the membranes were hybridised independently at medium stringency conditions, to the ^{32}P-pLgβtub1 (A) and ^{32}P-pLgβ500 (B) probes. To evaluate the analytical specificity of the β500-DNA sequence (C) an independent experiment was carried out at high stringency conditions. Panel (A) and (B), the *L. (Leishmania)* subgenus is represented by the species: lane 1, *amazonensis* PH8; and lane 2, *mexicana* BEL21; the species of the *L. (Viannia)* subgenus: lane 3, *guyanensis* M4147; lane 4, *braziliensis* M2903; and lane 5, *panamensis* LS94. Other *Leishmania* isolates are represented by the Colombian strains: lane 6, UA352; lane 7, UA113; and lane 8, UA318. Panel (C), the *L. (Leishmania)* subgenus species: lane 1, *amazonensis* PH8C5; lane 2, *mexicana* BEL 21; lane 5, *major* P; and lane 6, *tropica*; the *L. (Viannia)* subgenus species include: lane 3, *braziliensis* M2903; and lane 4, *guyanensis* M4147. Other Kinetoplastida: lane 7, *Trypanosoma cruzi* EP; lane 8, *Crithidia fasciculate*; and lane 9, *Leptomonas samueli*. Other organisms: lane 10, *Plasmodium falciparum*; lane 11, yeast; lane 12, human; and lane 13, sugar cane. Size markers in all cases correspond to Hind III-fragments of λ-DNA. The arrow in (B) indicates the position of the β500-DNA sequence.

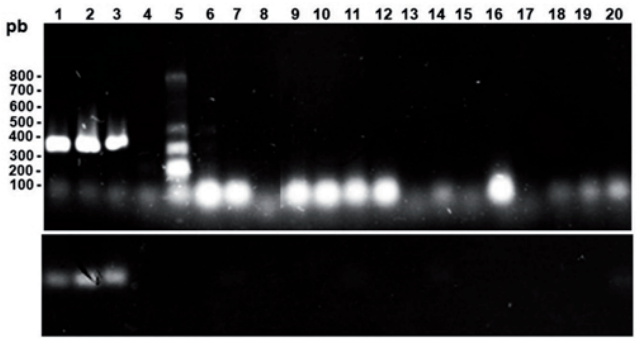

Figure 2. Analytical specificity of the β500-DNA amplification. Five nanograms of total genomic DNA of different organisms including different species representative of both *Leishmania* subgenera was subjected to the amplification of the β500 sequence using the oligonucleotide set A_2/A_{10}. The PCR reaction was carried out at 55 °C as annealing temperatures during 35 cycles. The PCR product were resolved on a 1.5% agarose gel and visualised by ethidium bromide (A). After Southern blot of the gel, the filters were hybridised to ^{32}P-β500 at medium stringency condition (B). The species of the *L. (Viannia)* subgenus include: lane 1, *braziliensis* M2903; lane 2, *panamensis* LS94; lane 3, *guyanensis* M4147. The *L. (Leishmania)* subgenus is represented by the New World *Leishmania* species: lane 4, *mexicana M379*; lane 5; *amazonensis* AZV; lane 6, *mexicana* M7326; lane 7, *pifanoi* LL1; lane 8, *mexicana* BEL 21; lane 20, *garnhami* HM76; and the Old Word *Leishmania* species: lane 9, *donovani* DD8; lane 10, *major* P; lane 11, *tropica*. Other Kinetoplastida include: lane 12, *Trypanosoma evansi*; lane 13, *T. rangeli*; lane 14, *T. cruzi*; lane 15, *T. brucei*; lane 16, *Leptomonas samueli*; lane 17, *Crithidia fasciculata*; lane 18, *Endotrypanum spp*. Other organisms are represented by: lane 19, *Plasmodium falciparum*; lane 20, *S. cervisiae*. Molecular size markers are PM2 phage DNA-Hae III fragments.

hybridisation with the ^{32}P-β500-DNA probe (Figure 2). There was a PCR product with the expected size, 375 bp, and homology to the probe when DNA from species of the *L. (Viannia)* subgenus was used in PCR amplification (lanes 1 to 3). In contrast, the Venezuelan isolates AZV (lane 5), and *L. (L.) mexicana* M379 (lane 4), and M7326 (lane 6) belonging to the *L. (Leishmania)* subgenus, generated non-specific amplification, with no homology to the probe. The *Leishmania (Viannia)* specific primers did not yield any PCR products when were used, under identical conditions. DNA from neither the New World nor the Old World *Leishmania* belonging to this subgenus (lane 7, 8, 9, 10 and 11), or other Kinetoplastida such as *Trypanosoma evansi* (lane 12), *T. rangeli* (lane 13), *T. cruzi* (lane 14), *T. brucei* (lane 15), *L. samueli* (lane 16), *C. fasciculate* (lane 17), *Endotrypanum sp*. (lane 18), or from other organisms such as *Plasmodium sp*. (lane 19) and *Saccharomyces cerevisiae* (lane 20), did not generate specific amplification either. After Southern transfer of the PCR products and hybridisation at medium stringency with the ^{32}P-β500-DNA (Figure 2B), we demonstrated the specific homology of the products generated from the genomic DNA of species of the *L. (Viannia)* subgenus (lanes 1 to 3).

We evaluated the sensitivity of the β500-DNA PCR assay at high stringency (60 °C), using a serial dilution of the clone pLgβ4, which contains one copy of the β500-DNA. The lowest amount of DNA reliably amplified was 1.0 fg (Figure 3). The results suggest a high specificity and sensitivity to the β500-PCR assay.

The β500-PCR assay in clinical samples: The majority of clinical samples (10/12) from Venezuelan and Colombian patients were positive to the Montenegro skin test (Table 2). The β500-PCR assay at 60 °C yielded the expected diagnostic 375 bp fragment in all samples (Figure 4). The positive results obtained with the Colombian biopsy from Sucre Department (lane 1) are noteworthy, since the patient was negative to the Montenegro test and the characterisation by a kDNA-PCR assay specific to the *L. (Viannia)* subgenus was unsuccessful. Similar results were found with three other samples, two from the Antioquia Department (lane 2 and 6) and one from the Santander Department (lane 10), for which the *L. (Viannia)* subgenus kDNA assay was negative. The primer combinations A_2/A_{10} and Tub1/Tub6

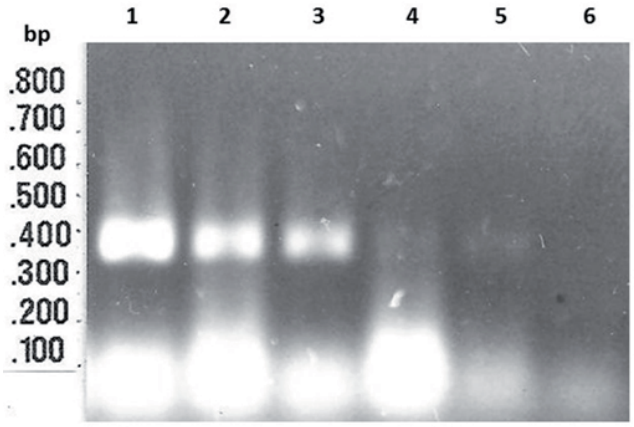

Figure 3. Analytical sensitivity of the β500-PCR assay. Tenfold dilutions of pLgβ4 DNA, started with an initial amount of 1 ng/ml (1000 pg/ml), was amplified using the primers A_2/A_{10}, and an annealing temperature of 60 °C for 40 cycles. The PCR products were resolved onto a 1.5% agarose gel and visualized by ethidium bromide. The amounts of DNA and *Leishmania* species are indicated in picograms (1 ng = 10^3 pg; 1 pg = 10^3 fg). Lanes 1, 10.0; lane 2, 1.0; lane 3, 0.1; lane 4, 0.01; lane 5, 0.001; lane 6, no DNA (negative control). The concentration of the original sample was determined by estimation of the A_{260} nm and quantification in agarose gel. Molecular size markers correspond to 100 bp DNA ladder. pg: picogram; fg: fentogram.

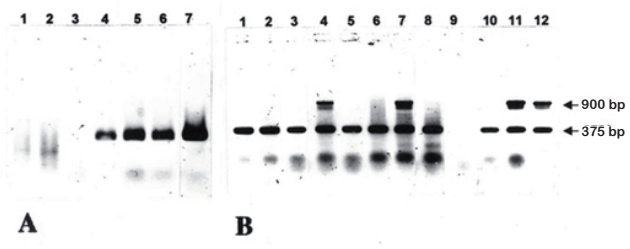

Figure 4. The β500-DNA PCR assay in clinical samples. A. Clinical specimens were obtained from skin biopsies of three Venezuelan patients diagnosed by the Montenegro skin test. Crude DNA was prepared from biopsies as described in materials and methods. The PCR reaction and the products evaluation were performed as Fig. 3. A. Lane 1, *L. (L.) donovani* DD8; lane 2, *L. (L.) mexicana* M379; lane 3, no DNA; lane 4, UC75a; lane 5, UC79; lane 6, UC116; lane 7, pLgβ4. B. The Colombian biopsies included: lane 1, W3482; lane 2, W3488; lane 3, W3490; lane 4, W3501; lane 5, W3507; lane 6, W3519; lane 7, W3522; lane 8, W3528; and lane10, W3440; lane 9, no DNA; lane 11, *L. (V.) braziliensis* M2903; and lane 12, *L. (V.) panamensis* LS94. The primers Tub1/Tub6, which generated a 900 bp fragment of the internal coding region of the β-tubulin gene, was included in the samples of the lanes 4, 7, 11 and 12.

included in some cases (lanes 4, 7, 11 and 12) generated the expected PCR products, suggesting that Tub1/Tub6 may be used when negative assays for the β500-DNA sequence are obtained.

Discussion

In this report we present a PCR assay using the β500 sequence as target; the results showed that this assay was specific, reliable and sensitive for the detection of *Leishmania* species of the *L. (Viannia)* subgenus, particularly in clinical samples. The β500 sequence is located in the intergenic spacer of the β-tubulin gene [11]. A comparison analysis of the sequence of the β500-DNA with sequences from the Gene Bank database showed that this sequence has apparently no identity with other sequences previously described in *Leishmania* or other organisms; comparison of β500 with the β-tubulin gene sequences did not show significant identity. Nevertheles, a particular motif of five to ten nucleotide sequences has partial homology with the coding region of the β-tubulin gene, which could explain the faint bands at high molecular weight found in the Southern blot analysis at medium stringency conditions of hybridisations. An interesting result was the presence of the β500-DNA sequence in *Leishmania* strains recently isolated, such as the Colombian isolates UA113, UA318 and UA352, suggesting that this sequence is not a product of strain selection due to the continuous culture of the reference strains.

The β500-PCR assay was capable of detecting a DNA amount as small as less as one parasite has [18]. PCR methods for other targets, which identify and distinguish between the two *Leishmania* subgenera, have been described; however, comparative studies related with sensitive and reproducibility is scarse. The range of analytical sensitivity of our assay was comparable to that demonstrated in PCR assays using kinetoplast DNA [2, 19, 20] or nuclear DNA repeats [21] as targets. Our assay is highly reproducible and effective in identifying *L. (V.) braziliensis* and *L. (V.) panamensis* from cutaneous lesions where a low number of parasites are expected. We confirmed the preliminary diagnosis of cutaneous leishmaniasis in clinical samples of Venezuelan and Colombian patients, in which the Montenegro skin test was positive; the diagnostic PCR product of the *L. (Viannia)* subgenus was present in all samples. The positive result obtained with the Colombian biopsy taken from Sucre Department was remarkable, since the patient was negative to the Montenegro test and parasite identification by a kDNA-PCR assay was unsuccessful [2]. Due to the extreme analytical sensitivity of our method and the kDNA assay, comparison of the two methods, with similar samples and under the same conditions, are necessary in order to establish differences in analytical sensitivity and reproducibility.

The β500-PCR assay was independent from protocols used for DNA extraction from clinical samples; however, DNA yield was improved with the QUIAGEN kit. We predict a high potential of this assay for use in epidemiological studies; the β500-PCR assay is straightforward, reproducible, highly sensitive and very reliable in the identification of *Leishmania* species belonging to the *L. (Viannia)* subgenus. Even though oligonucleotide primers $A2/A_{10}$ have highly conserved regions in the β500-DNA sequences among species of the *L. (Viannia)* subgenus, improvements on the sequencing of β500-DNA in other species of the *L. (Viannia)* subgenus would give us the possibility to establish identity among them. Combine more than one PCR primer in a multiplex reaction, should allow identification of the parasites at species level also.

We demonstrated the simultaneous amplification of the β-tubulin gene fragment (900 bp) and the specific amplification of the β500-DNA sequence (375 bp), through a PCR assay using purified DNA from biopsies. Since the β-tubulin gene is highly conserved and the gene is present in all *Leishmania* species, its amplification facilitates the interpretation of negative results for the β500-DNA. The follow-up of patients testing for the presence of parasites after treatment will be important in terms of improving the assay, similarly to the procedure followed in the case of visceral leishmaniasis [22, 23].

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