

## AMPLIFICATION OF HUMAN DNA BY PRIMERS TARGETED TO *LEISHMANIA* KINETOPLAST DNA AND POST-GENOME CONSIDERATIONS IN THE DETECTION OF PARASITES BY A POLYMERASE CHAIN REACTION

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**Abstract.** We evaluated the *Leishmania Viannia*-specific primers B1-B2 to detect *Leishmania* in normal skin and peripheral blood monocytes of patients with active cutaneous leishmaniasis. Southern blotting and sequencing of polymerase chain reaction (PCR) products confirmed the specificity of kinetoplast DNA (kDNA) amplification from tissue fluid from healthy skin, whereas the PCR with monocytes also amplified a human sequence of a size similar (718 basepairs) to the expected kDNA product (750 basepairs), resulting in false-positive results. Although B1 was not homologous to any human DNA sequence, B2 showed homology to a human chromosome 2 intergenic region (AC010878) at positions 35,881–36,599, which are spaced 718 nucleotides apart. Amplification of the human artifact from monocyte DNA was confirmed using the primer B2 alone. Examination of other primers reported for the PCR of kDNA from various species of *Leishmania* showed that six of seven were homologous to human DNA sequences. These findings underscore the importance of exploiting sequencing, bioinformatics, and DNA probes to refine molecular amplification techniques and to validate the performance of primers when used for new applications.

### INTRODUCTION

Parasites of the genus *Leishmania* currently rank second only to malaria in the World Health Organization (WHO) list of protozoan diseases causing highest human death rates, (WHO Fact Sheet No. 116, 2000; WHO Report, 1999 [http://www.who.org]) with estimates of 12 million people infected and up to 2 million new cases annually.<sup>1,2</sup> Cutaneous leishmaniasis (CL) is the most widespread form of leishmaniasis, causing 1–200 primary skin lesions that often self-heal within a few months, but from which parasites can disseminate to the nasopharyngeal mucosa and cause the disfiguring and debilitating secondary lesions typical of mucocutaneous leishmaniasis (WHO Report, 2002 [http://www.who.org]). Early diagnosis of CL is therefore important, and during the past decade polymerase chain reaction (PCR) approaches have been developed as sensitive alternatives<sup>3–6</sup> to augment existing diagnostic procedures based largely upon microscopic analysis of lesion smears, culture of lesion biopsies and aspirates, and/or clinical scores with or without the leishmanin skin test.<sup>4</sup>

Several molecular targets for a diagnostic PCR have been identified in *Leishmania* including minicircle kinetoplast DNA (kDNA), the miniexon (spliced leader RNA) gene, ribosomal DNA, and the glucose-6-phosphate dehydrogenase gene, among others.<sup>3–9</sup> Kinetoplast DNA represents an ideal PCR target since minicircles are present at very high copy numbers (10,000 per parasite) and contain both conserved and variable regions that allow differentiation between *Leishmania* species.

Molecular amplification methods developed for diagnosis are also applicable to epidemiologic studies and to the understanding of natural history of infection.<sup>10</sup> Sample characteristics influence the outcome of the amplification of nucleotide sequences, particularly the quantity and ratio of target to non-target DNA. Examination of tissues or sites that are not adversely affected or where organisms may persist in an inactive or latent state in small numbers raises new issues of specificity and sensitivity.

High specificities and sensitivities have been reported for several primers amplifying either specific kDNA sections or entire kDNA minicircles from a number of biopsy sample

types.<sup>3,6–8</sup> Nonetheless, during our validation of kDNA amplification using B1-B2 primers<sup>3</sup> to detect *Leishmania Viannia* infections in blood monocytes and tissue fluid from healthy skin (as part of an epidemiologic study to be published elsewhere), we identified co-amplification of a human DNA fragment of a size similar to the 750-basepair (bp) kDNA product in monocyte samples from a healthy control subject and a high proportion of CL patients. These findings prompted a re-examination of the target specificity of B1–B2 and other primers used previously for a PCR of kDNA by exploiting genomic data and bioinformatics tools that were largely unavailable in earlier studies.

### MATERIALS AND METHODS

**Participants.** Individuals enrolled in the study were diagnosed with acute CL (less than three months evolution) at Centro Internacional de Entrenamiento e Investigaciones Médicas clinics in Cali or Tumaco, Colombia on the basis of clinical scores as well as parasitologic culture from lesion biopsies and microscopic analysis of lesion smears as previously described.<sup>6,11</sup> All clinical procedures used in this project were performed in strict accordance with the guidelines of both institutional and national ethics committees, and all patients enrolled provided written informed consent.

**Clinical samples.** *Aspirates.* A single dermal tissue fluid sample (50–100  $\mu$ L) was obtained by aspiration from healthy skin sites located at a standard distance  $\geq$  10 cm from cutaneous lesions of 93 CL patients.<sup>12</sup>

*Monocytes.* A 10-mL sample of peripheral blood was collected from 75 CL patients by venipuncture into two sterile Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ) containing 7.2 mg of EDTA. Monocytes were separated from 5-mL samples of whole blood using the Nycoprep system (Gibco-BRL, Gaithersburg, MD) following the protocol of the manufacturer.

**Extraction of DNA.** *Control DNA from Leishmania (Viannia) panamensis.* Positive control (total) DNA was prepared from promastigotes of *L. V. panamensis* strain MHOM/CO/86/1166, which was grown in Schneiders' *Drosophila* medium at 26°C for 3 days. Routinely,  $1 \times 10^6$  promastigotes

were suspended in 100  $\mu$ L of lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA) and heated at 95°C for 30 minutes. Extracts were centrifuged (10,000  $\times g$  at 4°C for 10 minutes) and the supernatant fractions containing total DNA were collected for the PCR.

**Tissue fluid aspirates.** Skin aspirates were centrifuged (10,000  $\times g$  at 4°C for 10 minutes) and the resulting cell pellets were lysed by incubation in 30  $\mu$ L of lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 mg/mL of proteinase K) at 65°C for 2 hours, with subsequent heating at 100°C for 30 minutes to denature the enzyme. Cell debris and proteins were removed by centrifugation (10,000  $\times g$  at 4°C for 10 minutes) and the supernatant fraction containing the DNA used for the PCR.

**Monocytes.** Extraction of DNA from 200  $\mu$ L of human monocytes was performed using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA) according to the protocol of the manufacturer. Comparison of the average number of mononuclear cells (200  $\times 10^3$ ) separated from 5-mL blood samples by the Nycoprep system with the typical yield of DNA from these samples (40  $\mu$ g) enabled us to calculate that the amount of human monocyte DNA used in these assays (0.6  $\mu$ g) corresponded to approximately 3  $\times 10^3$  monocytes.

**Polymerase chain reaction amplification of *L. Viannia* kDNA. B1-B2 primers.** The PCR was initially performed using the oligonucleotide primers B1 (5'-GGG GTT GGT GTA ATA TAG TGG-3') and B2 (5'-CTA ATT GTG CAC GGG GAG G-3'), which were previously reported to specifically amplify the entire 750-bp minicircle kDNA of *L. Viannia* species.<sup>3,6</sup> For all clinical samples, the PCR was performed using aliquots of both undiluted DNA and with DNA diluted 1:10 in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to minimize the possibility of inhibition by sample contaminants. The PCR amplification was conducted using a PTC-100 thermocycler (MJ Research Inc., Waltham, MA) following the thermal profile previously used.<sup>6</sup> Aliquots (10  $\mu$ L) of amplified PCR mixtures were subjected to electrophoresis on 1% agarose gels using 0.5 $\times$  TBE running buffer (0.045 M Tris-borate, 1 mM EDTA), and PCR product bands were visualized by staining with ethidium bromide (0.4  $\mu$ g/mL). Duplicate samples of all PCRs were subjected to electrophoresis without staining with ethidium bromide, and subsequently analyzed by Southern hybridization. The lower limit of detection of the PCR method was determined by titration using serial dilutions of *L. panamensis* total DNA as the target. The specificity of the B1-B2 primers was tested by a PCR of negative control monocyte DNA from a healthy individual with no previous exposure in areas of CL transmission, and by determining the ability to detect serial dilutions of total *Leishmania* DNA in the presence of negative control monocyte DNA.

**Design of a new primer (LV) and PCR using LV-B1.** A novel primer designated LV (5'-ATTTTGAACGGGTT-TCTG-3') was developed as a replacement for B2. The composition of PCR mixtures was as previously described.<sup>6</sup> The thermal profile was composed of 35 cycles at 95°C for 5 minutes, 92°C for 1 minute, 60.5°C for 40 seconds, and 72°C for 30 seconds.

**Southern hybridization and chemiluminescent detection.** Following PCR amplification and electrophoretic separation on 1% agarose gels as indicated earlier in this report, Southern blotting of PCR was performed using standard procedures.<sup>12</sup> Hybridization was performed via chemilumines-

cent detection using the Alka-Phos direct labeling and detection system with CDP-Star (Amersham-Pharmacia Biotech, Little Chalfant, Buckinghamshire, United Kingdom) according to the protocol of the manufacturer. The 750-bp PCR product band amplified from *L. V. panamensis* kDNA was purified after electrophoresis from agarose gel slices using the Wizard PCR Preps kit (Promega, Madison, WI), labeled with alkaline phosphatase, and used to probe blots of PCR products at 65°C.

**Dot-blot hybridization and chemiluminescent detection.** Aliquots (25  $\mu$ L) of PCRs were denatured by the addition of an equal volume of 0.5 M NaOH, 1.5 M NaCl, incubated for 10 minutes at room temperature, and neutralized by adding 50  $\mu$ L of 2 M sodium acetate, pH 8.0, and incubating for 10 minutes on ice. The DNA was blotted onto a nylon N+ membrane using the Minifold I (Schleicher & Schuell, Keene, NH) essentially as previously described.<sup>6</sup> Following cross-linking by treatment with ultraviolet light, the PCR products were probed with the 750-bp kDNA product using the Alka-Phos system, with all hybridization solutions and temperatures as described earlier in this report for Southern blotting. The PCR product used as probe was first checked by sequencing.

**Subcloning and DNA sequencing of PCR products.** The identities of PCR products obtained using primers B1-B2 and LV-B1 were determined by DNA sequencing. The PCR products were extracted after electrophoresis from agarose gels and purified using the Wizard-Plus Mini-Preps kit (Promega), ligated into the Promega pGEM-T-easy vector following the protocol of the manufacturer, and cloned DNAs were transformed into *Escherichia coli* DH5 $\alpha$  cells. Purified clones were checked for inserts by restriction analysis and sequenced on both strands through collaboration with the University of Glasgow (Glasgow, United Kingdom). Sequencing was conducted on the ~750-bp PCR products generated using primer B1-B2 from four groups: 1) *L. panamensis* total DNA (positive control) that was later used as a probe; 2) a healthy skin aspirate sample positive by both PCR and hybridization (true positive); 3) monocyte samples positive by PCR but negative by hybridization (false positive); and 4) a monocyte sample positive by PCR and hybridization (true positive). The corresponding products of groups 1, 2, and 4 amplified by the LV-B1 primers were also sequenced.

The 750-bp product bands amplified from monocyte samples using primer B1-B2 were expected to comprise a mixture of kDNA and human DNA due to the cross-annealing observed during the PCR standardization process (see Results). To distinguish kDNA clones from human DNA prior to sequencing, transformed bacterial colonies were screened for kDNA inserts via transfer to a nylon N+ membrane by replica plating following standard protocols.<sup>12</sup> Representative clones containing the *Leishmania* kDNA insert and putative human DNA were isolated and sequenced as described earlier in this report.

**Blast searches and sequence alignments.** The PCR products were identified by screening against all nucleotide sequences in the National Center for Biotechnology Information (NCBI) (Bethesda, MD) genomic database (Genbank; <http://www.ncbi.nih.gov>) using the BLAST-n program.<sup>13</sup> Primer annealing sites within the database sequences were determined by sequence alignment using the ClustalW program (<http://www.ebi.ac.uk>).<sup>14</sup>

## RESULTS

**Standardization of the B1-B2 PCR and cross-amplification of monocyte DNA.** Initial standardization of the B1-B2 PCR using serial dilutions of total DNA from *L. panamensis* showed amplification of the characteristic 750-bp minicircle band (Figure 1) and determined the lower limit of detection to be 30 fg of DNA (corresponding to  $3 \times 10^{-3}$  parasites/ $\mu$ L) (Figure 1, lane 10), a value consistent with the 10-fg limit previously reported.<sup>3,6</sup> This titration also illustrated inhibition of the PCR at very high DNA sample concentrations (Figure 1, lane 1).

The PCR of human monocyte DNA samples containing from 0.06 to 0.6  $\mu$ g of DNA from a healthy control subject with no previous exposure to CL-endemic areas showed amplification of a band of a size similar to the standard 750-bp kDNA product (Figure 2A, lane 1). This apparent cross-amplification of human DNA was supported by Southern blotting, which clearly showed that neither of the PCR products from control monocyte DNA hybridized with the *L. panamensis* kDNA probe (Figure 2B, lane 1), and was subsequently confirmed by sequencing of the ~750-bp product. The PCR of serial dilutions of *L. panamensis* DNA mixed with a fixed quantity of monocyte DNA (0.6  $\mu$ g) from the healthy donor showed amplification of the kDNA band (as confirmed by hybridization with the kDNA probe) at DNA concentrations corresponding to as little as  $9 \times 10^{-3}$  parasites/ $\mu$ L (Figure 2B, lane 5). However, it is important to note that at lower ratios of parasite:monocyte DNA, the amplification of human DNA-derived PCR products increased (Figure 2, lane 5).

**The B1-B2 PCR, dot-blotting, and Southern hybridization of clinical samples.** Dot-blotting was tested as a means to improve detection of kDNA. However, the lack of separation of DNA products in the PCR mixtures, combined with significant hybridization of the kDNA probe with primer dimers, interfered with the discriminatory detection of kDNA, resulting in low specificity and frequent false-positive results (as confirmed by Southern hybridization) in both monocyte and aspirate samples.

In contrast, due to the electrophoretic separation of individual PCR products prior to hybridization, Southern blotting proved invaluable in identifying kDNA amplified by the B1-B2 PCR of monocyte samples. Southern analyses improved specificity, showing cross-amplification of the non-hybridizing human band at the expected size (false-positive PCR results) in 25 (33.3%) of 75 monocyte samples evaluated. Figure 3

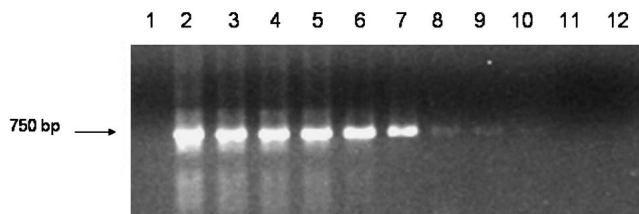


FIGURE 1. Sensitivity titration curve to estimate the sensitivity of B1-B2 polymerase chain reaction (PCR) using serial dilutions of authentic *Leishmania panamensis* total DNA. Lanes 1–11 show PCR products generated from the following amounts of DNA: Lane 1, 12  $\mu$ g; lane 2, 3  $\mu$ g; lane 3, 0.3  $\mu$ g; lane 4, 30 ng; lane 5, 3 ng; lane 6, 0.3 ng; lane 7, 30 pg; lane 8, 3 pg; lane 9, 30 fg; lane 10, 3 fg; lane 11, 0.3 fg. Lane 12, PCR control without DNA. bp = basepairs.

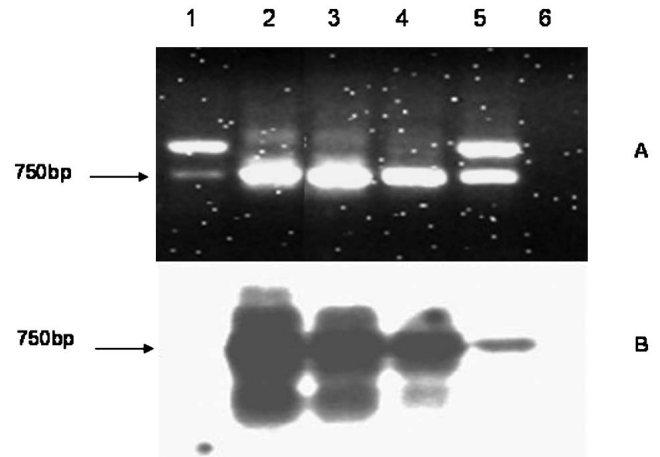


FIGURE 2. Effect of the quantity of *Leishmania* target DNA on cross-amplification of human monocyte DNA by the B1-B2 primers. Shown are polymerase chain reaction (PCR) products obtained over a range of  $9 \times 10^3$  to 0.009 *Leishmania panamensis* promastigotes and a constant quantity of human monocyte DNA (0.6  $\mu$ g) equivalent to 3,000 monocytes obtained from a healthy donor. **A**, Agarose electrophoresis of PCR products. **B**, Southern hybridization analysis of the products using the cloned *L. panamensis* minicircle kinetoplast DNA probe. Lane 1, monocyte DNA; lane 2, monocyte DNA plus *L. panamensis* DNA equivalent to  $9 \times 10^3$  parasites; lane 3, monocyte DNA plus *L. panamensis* DNA equivalent to 90 parasites; lane 4, monocyte DNA plus *L. panamensis* DNA equivalent to 0.9 parasites; lane 5, monocyte DNA plus *L. panamensis* DNA equivalent to  $9 \times 10^{-3}$  parasites; lane 6, PCR control without DNA. Bp = basepairs.

shows representative PCR results for nine of the 75 monocyte samples. Although an ~750-bp band was amplified by the PCR in clinical samples (Figure 3A, lanes 4–9), hybridization showed that only lanes 4 and 8 were true-positive samples, lanes 5, 6, 7, and 9 were false-positive samples, and lanes 3, 10, and 11 were true-negative samples (Figure 3B). The larger band amplified in most samples also corresponds to a human DNA product (Figure 3, lane 12). Southern hybridization also improved the biologic sensitivity of detection, enabling the identification of kDNA in seven monocyte samples negative by PCR (false-negative PCR results).

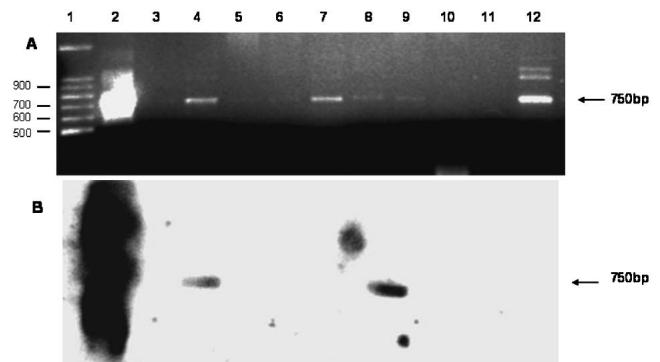


FIGURE 3. Distinction of true-positive and false-positive amplification products using the B1-B2 polymerase chain reaction (PCR) and Southern hybridization of monocyte DNA samples from patients with cutaneous leishmaniasis. **A**, Agarose electrophoresis of PCR products. **B**, Southern blot analysis of products. Lane 1, molecular mass marker (100-basepair [bp] DNA ladder; Promega); lane 2, positive control (1  $\mu$ g of DNA); lanes 3–11, PCR products from monocyte DNA of different patients; lane 12, monocyte DNA (0.6  $\mu$ g) obtained from a healthy control donor.

The PCR of kDNA in healthy skin and scar aspirates was specific with no false-positive results detected by Southern blotting. Biologic sensitivity was also improved by Southern blotting, enabling the detection of seven positive samples in addition to the four detected by the PCR alone (and confirmed by Southern blotting) among 11 positive aspirates of 93 samples evaluated. Figure 4 shows representative PCR and Southern hybridization results for six aspirate samples. This figure shows one true-positive product (lane 6) and one false-negative product (lane 7) that were confirmed as positive by hybridization (Figure 4B, lane 7). In addition, in Figure 4B, lanes 5 and 6 show lower bands. These bands correspond to hybridization of primer dimers with the probe.

**Sequencing of DNA of cloned B1-B2 PCR products.** Specific amplification of the 750-bp minicircle kDNA in positive controls (containing parasite DNA only) and in selected aspirate samples positive by PCR and hybridization was confirmed by DNA sequencing of the respective cloned PCR products and sequence alignment. In all cases, 90–100% nucleotide identities were observed with minicircle sequences from *L. Viannia* species (GenBank Accession numbers LPU19811 [U19811], LBU19807 [U19807], and LEIMINIC [M87316]).

Sequencing showed that the actual size of putative human PCR product amplified from healthy monocytes is 718 base-pairs, which, on a 1% agarose gel, is difficult to distinguish from the authentic 750-bp kDNA product. The 718-bp product showed 100% nucleotide identity with a 718-bp section of intergenic region of human chromosome 2 (nucleotides 35,881–36,599 of BAC clone RPII-230E20; NCBI Accession no. AC010878), but was not homologous with any reported *Leishmania* DNA sequence. This human DNA sequence was also identified in randomly selected false-positive PCRs from patient monocyte samples. Notably, even in monocyte samples positive by PCR and hybridization, prod-

uct sequencing showed a mixture of kDNA and the human DNA artifact.

Furthermore, primer B2 exhibited 73.7% and 68.4% identities with nucleotides 35,881–35,999, and 36,599–36,581, respectively, of the coding and non-coding strands of the human sequence AC010878. In addition, the 3'-end of B2 was 100% identical with the first 8 nucleotides of the coding strand (nucleotides 35,891–35,999) and the first 11 nucleotides of the non-coding strand (nucleotides 36,581–36,591) (Table 1), which strongly indicates that cross-annealing of primer B2 was responsible for amplification of monocyte DNA. We have confirmed this hypothesis experimentally by demonstrating that amplification of the 718-bp human artifact from monocyte DNA of a healthy subject occurs using primer B2 alone. In addition, when we replaced primer B2 with the new LV primer designed in this study on the basis of a highly conserved region of minicircle kDNA found in species of the *L. Viannia* subgenus, the cross-amplification disappeared (Figure 5, lane 9). Furthermore, genomic database searches confirmed that the primer LV target sequence is unique to *L. Viannia* species, while B2 showed high homology with several other human sequences, notably an inter-repeat region of chromosome 2 (BAC clone RPII-230E20, GenBank AC092591) (Table 1). Primer B1 was not homologous to the human 718-bp product, and database screening showed exclusive specificity of this primer for kDNA of *L. Viannia* and New World *Trypanosoma* species (Table 1).

**Polymerase chain reaction using primer pair LV-B1.** To avoid amplification of human DNA from monocyte samples, primer LV was designed on the basis of a highly conserved region of minicircle kDNA found in all four species of the *L. Viannia* subgenus. Genomic database searches confirmed that the LV target sequence is unique to *L. Viannia* species. The PCR was performed using primer LV in combination with the B1 primer,<sup>3,6</sup> which also specifically targets *L. Viannia* and New World trypanosome kDNA. Preliminary evaluation indicated that the PCR using the primer pair LV-B1 was an order of magnitude more sensitive than primer pair B1-B2, with a lower limit of detection of 3 fg of kDNA (Figure 5). This level of sensitivity was increased an additional 10-fold (to 0.3 fg) by Southern hybridization of the PCR products. Critically, primer pair LV-B1 did not cross-amplify any product from 0.06 to 0.6  $\mu$ g of healthy control monocyte DNA (Figure 5, lane 9) or from representative CL patient samples that gave false-positive results in the B1-B2 PCR, thus improving the PCR specificity to 100%.

**BLAST searches of primers reported for PCR of *Leishmania* kDNA.** The striking identity of the B2 primer with human DNA prompted us to perform database screening with other primers reported previously as specific for *Leishmania* kDNA to determine potential homologies with host sequences. Of the seven primers analyzed, only one was completely specific for *Leishmania* kDNA; the remainder showed between 80% and 100% identity with human (and/or mouse) DNA sequences (Table 1).

## DISCUSSION

Several previous studies have used primers B1 and B2 to amplify *Leishmania* minicircle kDNA from different types of clinical samples.<sup>3,6,15</sup> The PCR with these primers has been

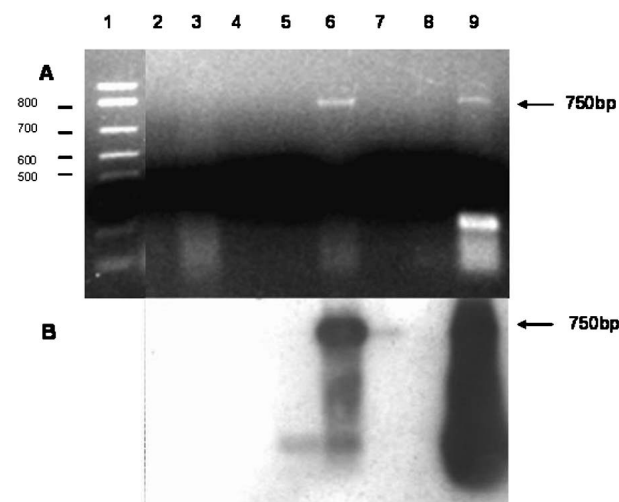


FIGURE 4. Sensitivity of the B1-B2 polymerase chain reaction (PCR) using Southern hybridization of skin aspirate DNA samples from patients with cutaneous leishmaniasis. **A**, Agarose electrophoresis of PCR products. **B**, Southern hybridization analysis of the products. Lane 1, molecular mass marker (1-kb DNA ladder); lanes 2–7, DNA samples from healthy skin aspirates of different CL patients; lane 8, negative PCR control (minus DNA); lane 9, positive control (10 ng of DNA). bp = basepairs.

TABLE 1  
Homologies of PCR primers reported for *Leishmania* kDNA.

PCR primer	Homologue: species, description (GenBank Accession Number)	Nucleotide identity % (nt/nt)
LV	<i>L. Viannia</i> kDNA, (AY366071, LBU19807, LEIMINIC)	100 (20/20)
B1 <sup>3</sup>	<i>L. major/L. donovani</i> kDNA, (PBLH3/AF399822)	100 (20/20)
	<i>L. Viannia</i> kDNA, (AY366071, LBU19807, LEIMINIC)	100 (21/21)
B2 <sup>3</sup>	<i>T. cruzi</i> kDNA, (AF241157)	100 (21/21)
	<i>L. Viannia</i> kDNA, (AY366071, LBU19807, LEIMINIC)	100 (19/19)
B3 <sup>3</sup>	<i>Homo sapiens</i> Chr 2 inter-repeat region, (AC092591)	84 (16/19)
	<i>H. sapiens</i> Chr 2 intergenic region, (AC010878)	74(+)(14/19), 68(-)(13/19)
	<i>M. musculus</i> _Chr 11, (AL670399)	84 (16/19)
	<i>L. Viannia</i> kDNA, (AY366071, LBU19807, LEIMINIC)	100 (20/20)
	<i>M. musculus</i> _Chr 8 intergenic region (AC127240)	90 (18/20)
13A <sup>8</sup>	<i>H. sapiens</i> Chr 2 repeat region "ERVL", (AC105397)	80 (16/20)
	<i>L. mexicana/L. infantum</i> kDNA, (AY145437)	100 (18/18)
13B <sup>8</sup>	<i>H. sapiens</i> Chr 12 inter-repeat region, (AC026765)	94 (17/18)
	<i>L. mexicana/L. major</i> kDNA, (AY145437/PBLH3)	100 (21/21)
13Y <sup>8</sup>	<i>M. musculus</i> _Chr 8 intergenic region, (AC122370)	76 (17/21)
	<i>L. donovani</i> kDNA, (LDKINMINI)	100 (17/17)
13Z <sup>8</sup>	<i>M. musculus</i> _Chr 7 inter-repeat region, (AC123047)	100 (17/17)
	<i>H. sapiens</i> Chr 16 CpG island UBE21, (AE006466.1)	100 (17/17)
	<i>L. mexicana</i> kDNA, (AY145437)	100 (22/22)

\* nt = nucleotides; *L.* = *Leishmania*; *T.* = *Trypanosoma*; *H.* = *Homo*; chr = chromosome; *M.* = *Mus*.

considered a highly sensitive and specific method. However, in this study, we have demonstrated that the B2 primer can cross-amplify human DNA in samples such as monocytes that contain substantial quantities of host DNA and/or high ratios of host:parasite DNA, leading to false-positive results. Overall, the standardization data indicated that although the detection of kDNA via the B1-B2 PCR was feasible in monocyte samples, verification of the identity of PCR products by Southern hybridization was essential due to the cross-amplification of human DNA.

In addition to identifying the human PCR product and putative annealing site of the B2 primer, we have exploited bioinformatics tools as such as BLAST searches and sequence alignment to establish that several other primers reported for the PCR of kDNA also exhibit significant homologies not only with human DNA sequences but also with other important host organisms in the study of leishmaniasis such as

mouse (*Mus musculus*), as well as other protozoan parasites including *Trypanosoma* spp., all of the latter that could generate false-positive results.

Although dot-blot hybridization has occasionally been used to improve the sensitivity of detection of kDNA following PCR,<sup>6</sup> this technique was not applied in the present study since hybridization of the kDNA probe with primer dimers resulted in frequent false-positive results. These observations emphasize the need to exclude the possibility of hybridization with primer dimers in dot-blot analyses or to design probes that hybridize with sequences internal to the annealing sequences of the primers. Conversely, Southern hybridization considerably improved the biologic specificity and sensitivity of kDNA detection compared with the PCR and dot-blotting. If Southern blotting is taken as the gold standard, the PCR alone is less sensitive and less specific. Thus, the biologic sensitivity and specificity of kDNA detection by the B1-B2 PCR in aspirates and monocyte samples were calculated as a percentage by comparison with the results obtained via Southern hybridization, which was taken as the reference gold standard in this study. The sensitivity of the PCR = the number of positive samples by PCR/the number of positive samples by Southern hybridization × 100%, and the specificity of the PCR = the number of negative samples by both the PCR and hybridization/the number of negative samples by hybridization × 100%.

Among 75 monocyte samples evaluated in this work, 33% (25 of 75) were false positive by the PCR and 9% (7 of 75) were false negative. In contrast, PCR amplification of 93 tissue fluid samples generated no false-positive products, presumably due to the low levels of host DNA present relative to kDNA target, but did result in 7.5% (7 of 93) false-negative results. The few previous studies on *Leishmania* using a PCR in combination with Southern hybridization have also demonstrated substantial improvements in sensitivity.<sup>16,17</sup> Southern hybridization has been reported to detect PCR products containing as little as 10 fg of *Leishmania* DNA diluted in a 10 million-fold excess of human DNA,<sup>16</sup> and to increase the sen-

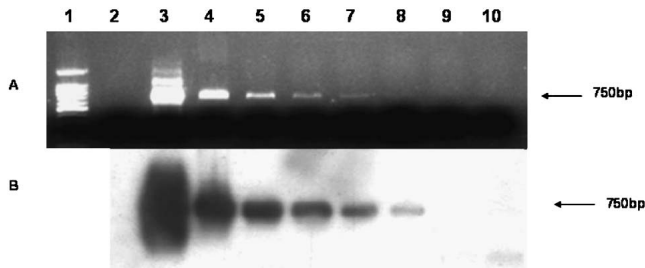


FIGURE 5. Titration curve to estimate threshold of detection of target kinetoplast DNA by the LV-B1 polymerase chain reaction (PCR) and Southern hybridization using serial dilutions of *Leishmania panamensis* total DNA. **A**, Agarose electrophoresis of PCR products. **B**, Southern hybridization analysis of the products. Lane 1, molecular mass marker (1-kb DNA ladder); lanes 2–8, PCR products generated from the following amounts of *L. panamensis* DNA: 3 µg, 3 ng, 3 pg, 0.3 pg, 30 fg, 3 fg, and 0.3 fg; lane 9, monocyte DNA (0.6 µg) from a healthy control donor; lane 10, negative PCR control (minus DNA). The lower limit of detection was 3 fg of DNA in the PCR and 0.3 fg in the Southern hybridization. bp = basepairs.

sitivity of the PCR of patient blood samples by 2–3 orders of magnitude.<sup>17</sup> However, the need to use Southern hybridization to confirm the specificity of the PCR has not been previously identified. Southern hybridization also augments the sensitivity of the PCR in detecting other microbial pathogens, as in the case of *Clostridium* spp., where hybridization increased sensitivity by a factor of 10<sup>3</sup> compared with the PCR.<sup>18</sup>

Previous studies using the B1 and B2 primers were performed with the standardizations and controls deemed necessary based on potential sources of false-positive or false-negative results. The potential presence of homologous sequences in human DNA as a source of false-positive results was controlled by extraction or dilution of the sample or a human DNA control. The homologous sequence found in this study was not amplified from the monocytes of every individual; therefore, the use of a single human DNA sample would probably not detect this source of misleading results. Our findings do not invalidate previous results, but they do qualify the interpretation of studies conducted using monocytes, leukocytes, or whole blood. Most previous studies focused on clinical samples such as aspirates or biopsies of active lesions, in which the ratio of parasite:human DNA is higher than in monocyte fractions. Our results for amplification of skin aspirates using primer pair B1-B2 substantiate the specificity of this PCR for *Leishmania* kDNA in such samples. Thus, the possibility that prior results with tissue aspirates represent cross-amplification is low.

The specificity considerations underscored by our findings are currently more easily addressed by bioinformatics strategies. Indeed, the design of microbial PCR primers and molecular probes should routinely involve screening against genomic databases to detect and avoid homologies that could lead to cross-hybridization with DNA of the host organism. Nevertheless, the existence of sequence polymorphisms in some individuals could still result in homologies; in fact, not all patient samples showed the amplification product from monocytes, suggesting that the complete B2 annealing sequence is not found in the genome of all individuals.

The development of PCR primers with unique specificity for the target microbial sequence has the added advantage of reducing the required stringency of annealing conditions, thus facilitating greater sensitivity. Furthermore, standardization of PCR products could be improved by Southern hybridization since this technique can detect not only false-positive results but also false-negative results. Using the approaches presented in this report, we successfully designed a novel primer (LV) that did not show homology with human DNA for use in combination with the B1 primer. The LV-B1 PCR showed a higher biologic specificity than the B1-B2 primer pair. The diagnostic efficiency of the novel primers remains to be determined.

The findings of this study reaffirm the power and precision offered by current bioinformatics tools and genome databases, and demonstrated the important gains in resolution when applied to the design and interpretation of molecular detection strategies for microbial pathogens.

Received June 18, 2004. Accepted for publication October 24, 2004.

Acknowledgments: We thank M. Ximena Varona, Horacio Cadena and Dr. Claudia Posso (Centro Internacional de Entrenamiento e Investigaciones Médicas) for provision of samples and clinical data,

and Dr. Gareth D. Westrop and Professor Graham H. Coombs (University of Glasgow, Glasgow, United Kingdom) and Carolina J. Ramirez (Centro Internacional de Entrenamiento e Investigaciones Médicas) for facilitating the sequencing of the PCR products. We are also grateful to Dr. Douglas C. Barker (Medical Research Council, University of Cambridge, Cambridge, United Kingdom) for his long-standing collaborative research association in the area of *Leishmania* molecular diagnostics.

Financial support: This work was supported by program grant no. 059056/Z/99/Z from the Wellcome Trust (United Kingdom) and grant no. 162-2002 from the Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología Francisco José de Caldas.

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