Am. J. Trop. Med. Hyg., 65(5), 2001, pp. 573–582

DETECTION OF *LEISHMANIA* CAUSING VISCERAL LEISHMANIASIS IN THE OLD AND NEW WORLDS BY A POLYMERASE CHAIN REACTION ASSAY BASED ON TELOMERIC SEQUENCES

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Abstract. We present a new polymerase chain reaction assay based on telomeric sequences of *Leishmania donovani*. When this assay was used in dilutions of purified *L. donovani* DNA, a strong amplification signal was observed with 1 fg of DNA. In a specificity test that used purified DNA from Old World and New World *Leishmania*, the assay recognized all parasites isolated from patients with visceral leishmaniasis, except for 2 isolates of *Leishmania colombiensis* from Venezuela and 1 isolate from Brazil. All *Leishmania major* and *Leishmania tropica* isolates tested were negative, except for one isolate in each species. We also used the assay on fresh and archive bone marrow samples recovered from Giemsa-stained slides and from dried blood stains.

INTRODUCTION

Visceral leishmaniasis (VL) is a widespread parasitic disease caused by the species of the genus *Leishmania.* In the Old World, *L. donovani* is the main etiological agent. However, in the New World, the identity of the *Leishmania* species causing VL is still being debated.[1] Currently, leishmaniasis is endemic in 82 countries, and nearly 350 million people live in areas at risk for acquiring the infection. Official figures reveal that VL alone accounts for 75,000 deaths annually, with more than half of these occurring in Sudan and India,[2,3] including 50,000 cases of kala-azar in Bihar-India.[4] Kala-azar is usually fatal if untreated. The symptoms of fever and malaise are often confused with those of malaria, tuberculosis, and other parasitic diseases because the symptoms are insufficiently specific for early diagnosis.

Routine diagnosis for VL consists of direct microscopy on samples from spleen, lymph node, or bone marrow, or by cell culture. Microscopy, although simple and cheap, may be risky and of limited sensitivity.[5] On the other hand, detection of parasites by cell culture is a time-consuming and labor-intensive process. An urgent need exists for the development and evaluation of more sensitive and specific methods.

Here, we report the development of a polymerase chain reaction (PCR) assay that is based on telomeric sequences of *L. donovani* and its application to the diagnosis of this parasite in bone marrow and blood samples of patients with VL.

MATERIALS AND METHODS

Parasite and culture conditions. Old World *Leishmania* strains used for the present work are shown in Table 1 and were provided by Drs. K. P. Chang, Chicago School of Medicine, and K. Stuart, Seattle Biomedical Research Institute. New World strains came from the sources indicated in Table 2. Confirmatory identification of nonreference strains was performed by either isoenzymatic, kinetoplast DNA (kDNA), or nuclear DNA sequences analyses.[6–11] The methods used for each strain are indicated in Tables 1 and 2. Some strains may not have been typed, although other isolates from the same sites were confirmed.

Promastigotes of all these strains, except *Leishmania donovani* AG83, were maintained in M199-10% fetal calf serum; *L. donovani* AG83 was maintained in M199–30% fetal calf serum. Subculture was performed at ~ 96 hr, when the cells reached the stationary phase of growth.

Blood collection and human biopsy samples for PCR. Blood samples and bone marrow samples of 3 parasitologically confirmed patients with VL were obtained from the Sahibganj district of Bihar, India. The Venezuelan human cases of VL came from endemic areas in Trujillo and Falcon States, western Venezuela. As noted in Table 3, bone marrow samples were recovered from archive material collected during 1992–1998.

Extraction of DNA from promastigotes. DNA extraction from a small number of cells (10^5) was standardized as follows. The cell pellet of 10^5 *Leishmania* promastigotes was made by centrifugation at 5,000 rpm for 15 min at 4°C. The pellet was washed once in phosphate-buffered saline (PBS; pH 7.2), resuspended in 100 μL of lysis buffer (8% Chelex [Bio-Rad Laboratories, Hercules, CA], 0.33 mg/mL proteinase K), and incubated for 15 min at 65°C, then boiled for 10 min in a water bath. The sample was centrifuged at 12,000 rpm for 20 min and the supernatant collected in a fresh tube. Ten microliters of the DNA sample was then used for PCR.

Extraction of DNA from biopsy samples. Blood or bone marrow samples were kept at room temperature for 30 min. The samples were then centrifuged at 1,000 rpm for 1 min, and the supernatant was collected in a fresh tube. The supernatant was then centrifuged at 5,000 rpm for 15 min at 4°C, and the pellet was washed twice with PBS (pH 7.2). One milliliter of cold 0.5% saponin in PBS (pH 7.2) was added to the pellet. The tube was gently tapped, incubated on ice for 5 min, and then centrifuged at 4,000 rpm for 10 min at 4°C. The pellet was washed with PBS (pH 7.2) and 400 μL of NET buffer (150 mM NaCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 50 mM Tris-HCl, pH 7.5). After the wash,

TABLE 1
Old World *Leishmania* species used*

Leishmania species	Nomenclature used in text	Disease	Species/country/year	PCR telomere	Reference
L. donovani Sm	136	VL	MHOM/CN/84	+	I,k[10]
L. donovani Sc6	137	VL	MHOM/CN/86	+	I,k[10]
L. turinica KXG-2	138	CL	MRHO/CN/88	+	I,k[10]
L. infantum Xu	139	CL	MHOM/CN/94	±	I,k[10]
L. infantum Lu	140	CL	MHOM/CN/93	+	I,k[10]
L. donovani 921	141	VL	MHOM/CN/92	+	I,k[10]
L. donovani 911	142	VL	MHOM/CN/91	+	I,k[10]
L. infantum KA	143	VL	MHOM/PK/91	−	I†
L. major DE	144	CL	MHOM/PK/88	−	I†
L. tropica 1092	147	VL	MHOM/SA/91	−	I[8]
L. tropica 1095	148	VL	MHOM/SA/91	+	I[8]
L. major Si-177	149	CL	MHOM/EG/89	+	I[8]
L. tropica EP2	151	CL	MHOM/TR/93	−	S[9,10]
L. infantum EP5	152	VL	MHOM/TR/93	+	S[9,10]
L. major EP7	153	CL	MHOM/TR/94	−	S[9,10]
L. infantum EP10	154	VL	MCAN/TR/95	+	S[9,10]
L. donovani S. LAL	S. LAL	VL	MHOM/IN/94/S-Lal	+	I‡
L. donovani LSB51.1	51.1	VL	MHOM/SD/00/Khartoum	+	R
L. infantum LSB7.1	7.1	VL	MHOM/MA/67/ITMAP	+	R
L. donovani LSB51.2	51.2	VL	MHOM/SD/00/1S-2D	+	I§
L. donovani AG83	AG83	VL	MHOM/IN/83AG83	+	I‡
L. donovani UR6	UR6	VL	MHOM/IN/78/UR6	+	I,S‡
L. donovani	L0564	VL	MHOM/ET/67/HU3	+	R
L. donovani	L0571	VL	MHOM/IN/80/DD8	+	R
L. infantum	IPT1	VL	MHOM/TN/80/IPT1	+	R
L. major	Friedlin	CL	MHOM/IL/81/Friedlin	−	R
L. major	L0575	CL	MHOM/SU/59/Pstrain	−	R

* CAN = dog; CL = cutaneous leishmaniasis; CN = China; EG = Egypt; ET = Ethiopia; HOM = human; I = isoenzyme; IL = Israel; IN = India; k = kinetoplast DNA; MA = Morocco; PCR = polymerase chain reaction; PK = Pakistan; R = reference strain; RHO = *Rhombomys opinus* (great gerbil); S = DNA nuclear sequence; SA = Saudi Arabia; SD = Sudan; SU = former Soviet Union; TN = Tunisia; TR = Turkey; VL = visceral leishmaniasis.
+ in the PCR telomere assay column = detection of a visible amplification product; − = no detection of the product.
† Typed and donated by David A. Evans.
‡ Madhubala R, unpublished data.
§ LSB51.2 is a clone line of LSB51.1.

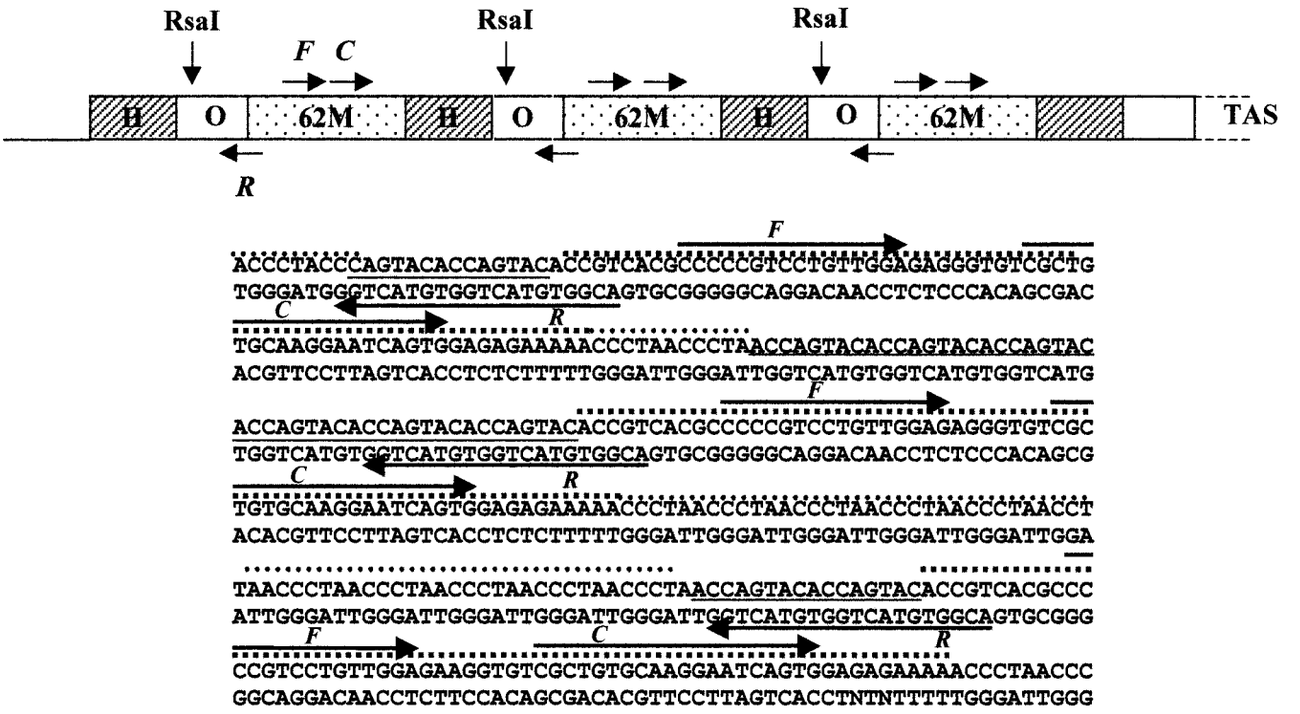

FIGURE 1. Organization of *Leishmania donovani* telomeric sequences and location of polymerase chain reaction primers. C = capture oligonucleotide; F = forward primer; R = reverse primer; TAS = telomeric-associated sequences. Hexameric sequences (H) are marked with **black dots,** 62 mer (62M) are indicated with **black squares,** and octamers are **underlined** (O). The location of *Rsa*I endonuclease cutting sites is marked by **vertical arrows.**

TABLE 2
New World *Leishmania* species and other Kinetoplastida used*

Species	Nomenclature used in text	Diseae	Specie/country/year	PCR telomere	Reference
Leishmania species					
L. chagasi	L0580	VL	MHOM/BR/74/PP75	+	R
L. chagasi	M6445	VL	MHOM/BR/81/M6445	+	k†
L. chagasi	M11862	VL	–	+	k†
L. chagasi	M2682	VL	–	–	k†
L. colombiensis	Chuao	VL	MHOM/VE/70/Chuao	–	I[7]
L. colombiensis	Talisman	VL	MCAN/VE/72/Talisman	–	I[7]
L. brazilensis	M2903	CL and MCL	MHOM/VE/75/M2903	–	R
L. guyanensis	L0566	CL and MCL	MHOM/BR/75/M4147	–	R
L. garnhami	Lby	CL	MHOM/VE/46/Lby	–	S[6]
L. mexicana	Bel 21	CL	MHOM/BZ/82/BEL21	–	R
L. amazonensis	L0570	CL	MHOM/BR/73/M2269	–	R
Other Kinetoplastida					
Trypanosoma cruzi	CL Brener	Chagas disease	–	–	–
Crithidia fasciculata	HS6	–	–	–	–

* BR = Brazil; BZ = Belize; CAN = dog; CL = cutaneous leishmaniasis; HOM = human; I = isoenzyme; MCL = mucocutaneous leishmaniasis; PCR = polymerase chain reaction; R = reference strain; S = DNA nuclear sequence; VE = Venezuela; VL = visceral leishmaniasis.
+ in the PCR telomere assay column = detection of a visible amplification product; – = no detection of the product.
† Guevara P, unpublished data.

1.25% sarcosyl and 0.25 mg ribonuclease were added. The sample was incubated 1 hr at 37°C, 0.15 mg proteinase K was added, and the sample was incubated at 50°C for 2 hr. The sample was then extracted with phenol, chloroform, and isoamyl alcohol (25:24:1) and precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. The pellet was washed with 70% ethanol, dried, and resuspended in TE (10 mM Tris, pH 8.0, 1 mM EDTA).

Recovery of DNA from bone marrow archive material. Archive material included dry bone marrow samples stained with Giemsa and stored at room temperature in cardboard holders. Stain was removed from slides containing samples

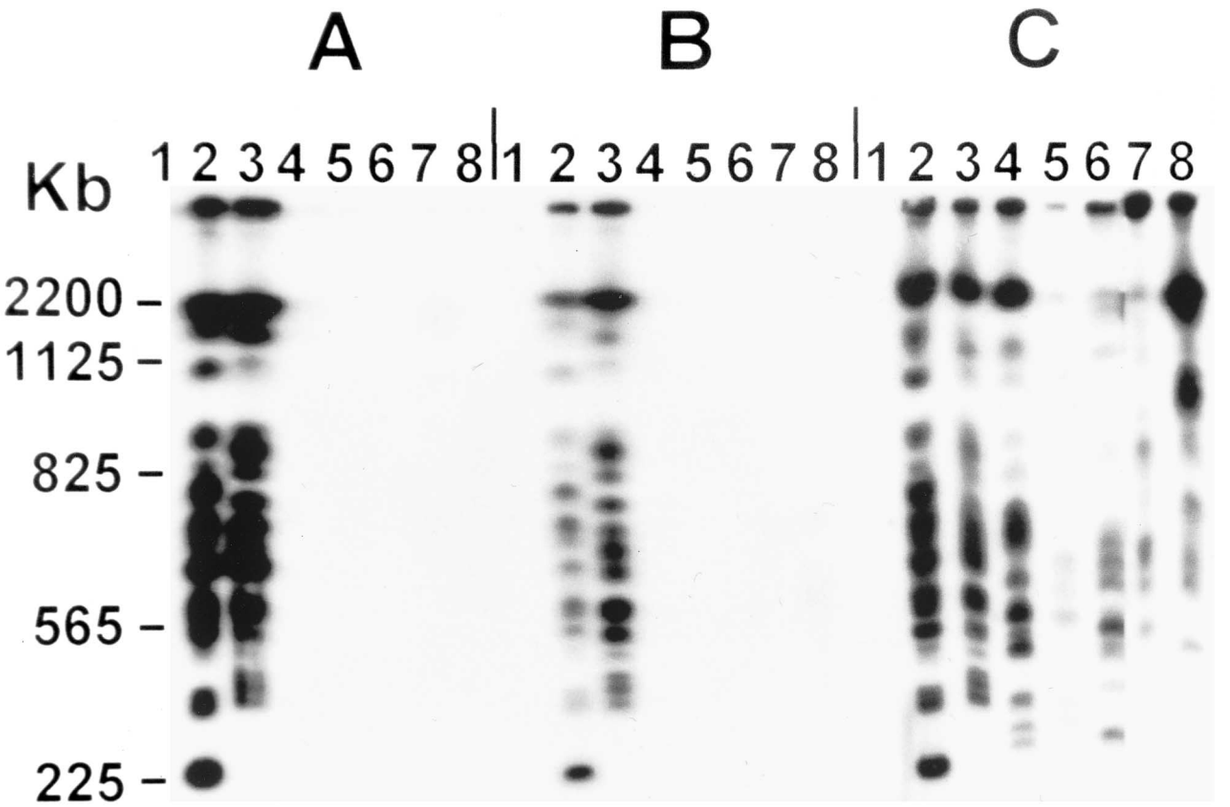

FIGURE 2. Southern blot of CHEF-separated chromosomes (chromoblots) of *Leishmania* and *Trypanosoma*. **Panel A** was hybridized with the forward primer, **Panel B** with the reverse primer, and **Panel C** with the hexameric probe $(CCCTAA)_3$. **Lane 1,** *Saccharomyces cerevisiae*. **Lane 2,** Ld 51.2. **Lane 3,** Ld 51.1. **Lane 4,** Lm Friedlin. **Lane 5,** Lb M2903. **Lane 6,** La L0570. **Lane 7,** Cf HS6. **Lane 8,** Tc CL Brener.

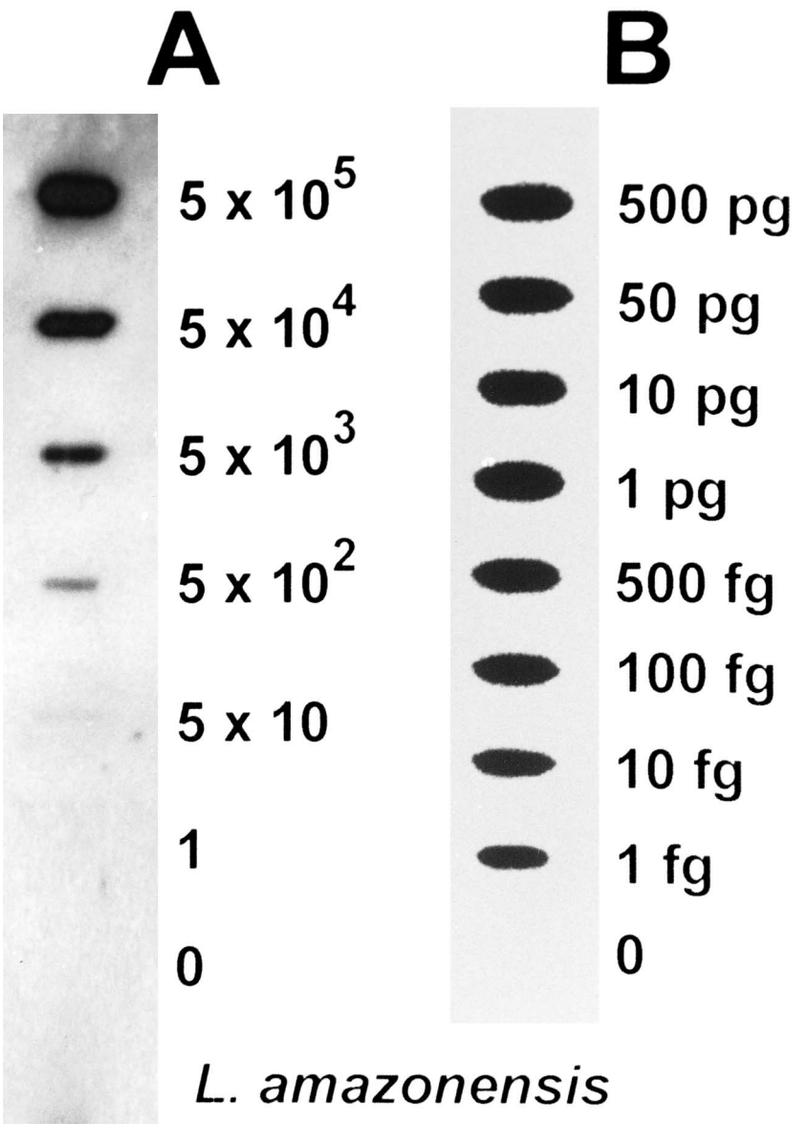

FIGURE 3. Slot blots to test sensitivity of telomeric probes and *Leishmania donovani* telomeric polymerase chain reaction (PCR) assay. **(A)** Dilutions of *L. donovani* 51.1 cells and negative controls of *Leishmania amazonensis* hybridized with digoxigenin-labeled capture oligonucleotide. **(B)** Slot blots of the PCR products performed in dilutions of pure *L. donovani* 51.1 DNA after hybridization with digoxigenin-labeled capture oligonucleotide.

with 99% acetic acid, then a wash with 1% acetic acid. Samples were scraped with a razor blade and resuspended in 100 μL of TE buffer. The suspension was incubated with 100 μg/mL proteinase K for 15 min at 60°C, then heated at 95°C for 30 min. A volume of 5 μL of each sample was used for the PCR assay.

***Leishmania donovani* telomeric PCR assay.** The overall strategy of the PCR assay used in this work is presented in Figure 1. The forward 5′-CCCCGTCCTGTTGGAG-3′ primer anchors were used on the 62-mer sequence (Figure 1B), whereas the reverse primer 5′-ACGGTGT-ACTGGTGTACTGG-3′ anchors the beginning of the 62-mer sequence and continues onto the octamer sequences. A capture oligonucleotide, 5′-CGCTGTGCAAGGAAT-CAGTG-3′, was designed for the detection of PCR products in hybridization experiments. This last step should be used in all PCR assays to double-check the assay specificity when complex samples are examined. Although we never observed any cross-reactivity in our assays, we believe this is an important practice. Oligonucleotides were purchased from Operon Technologies Inc. (Alameda, CA).

The PCR was performed with 0.8 μM of each forward and reverse primers, 0.2 mM dNTPs for 35 cycles with 1 min at 63°C, 1 min at 72°C, and 1 min at 94°C. Cycles were followed by 1 min at 63°C and 5 min at 72°C.

Agarose gel electrophoresis. The PCR products were detected with a 1% agarose gel, and genomic DNA was run on 0.7% agarose gel with a 6 V/cm gradient.

Polyacrylamide gel electrophoresis. The PCR-amplified products of *Leishmania* associated with visceral cases strains

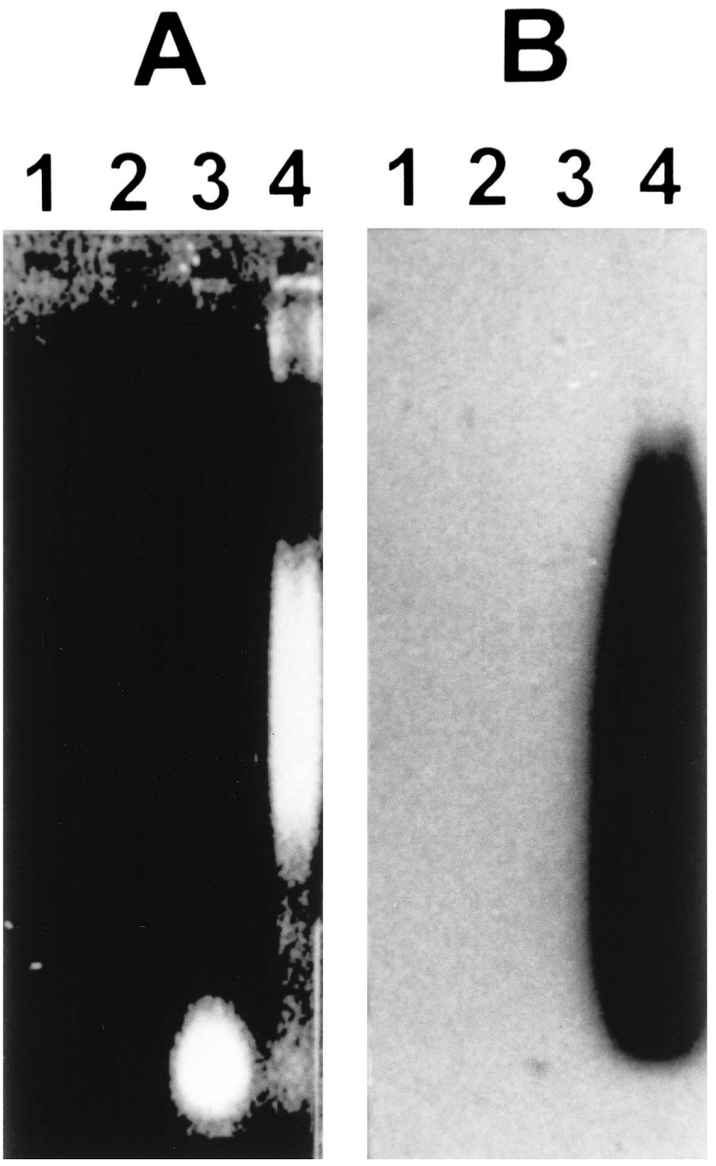

FIGURE 4. Agarose gel electrophoresis of *Leishmania donovani* telomeric polymerase chain reaction products. **(A)** Ethidium bromide-stained agarose 0.8% gel. **(B)** Southern blot of gel in A after hybridization with radioactively labeled capture probe. **Lane 1,** *Leishmania braziliensis* M2903. **Lane 2,** *Leishmania colombiensis* Chuao. **Lane 3,** *Leishmania amazonensis* L0570. **Lane 4,** *Leishmania donovani* strain 141.

were digested with restriction endonuclease *Rsa*I and resolved on 6% polyacrylamide gel electrophoresis with 0.5× tuberculin bacillary emulsion (TBE) buffer at 5 V/cm.

Pulsed field gel electrophoresis. Pulsed field gel electrophoresis of *L. donovani* strains was performed on a CHEF-DR III System apparatus (Bio-Rad Laboratories). Agarose blocks were prepared as described previously. A 1% gel was run with 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA) at 6 V/cm at 120° separation angle with 60 sec–120 sec switching time for 24 hr. The gel was then stained with ethidium bromide.

Southern, dot, and slot blots. For Southern blot tests, the gels were depurinated in 0.25 N HCl for 20 min and soaked in denaturing buffer (0.4 N NaOH, 0.6 M NaCl) for 45 min with one change of solution. The DNA was transferred to Hybond-N (Amersham Pharmacia Biotech Ltd., Paisley, UK) membrane by capillary action, and the membrane was ultraviolet cross-linked, dried, and kept for hybridization.

For dot blot, genomic DNA or PCR products were used. The DNA samples to be loaded were denatured by adding equal volumes of denaturing buffer (0.5 M NaOH, 0.25 N NaCl) and incubating at room temperature for 10 min. The denatured samples were kept on ice and diluted in 0.1× standard saline citrate (SSC) (15 mM NaCl, 1.5 mM sodium citrate), 0.125 N NaOH, such that the final volume of each sample was 200 μL. Hybond-N membrane was soaked in 0.4 M Tris-HCl (pH 7.4) for 5 min and fixed onto the dot or slot blot apparatus. Samples were loaded in the wells of

TABLE 3
Venezuelan archive cases of visceral leishmaniasis

Patient no.	Sex	Age	Symptoms at initial presentation to clinic	Bone marrow (parasites)	Treatment*	Telomeric PCR
1	F	18 months	Fever, anemia, splenomegaly	+	Glucantime	+
2	M	22 years	Fever, anemia, splenomegaly, epistaxis	+	Glucantime	+
3	M	30 years	Fever	+	Glucantime	+
4	F	24 years	Fever, hepatosplenomegaly	+	None	+
5	F	3 years	Fever, anemia, hepatosplenomegaly	−	Glucantime	+
6	F	14 months	Fever, anemia, hepatosplenomegaly	+	Glucantime	+
7	F	8 months	Fever, anemia, splenomegaly, diarrhea	+	Glucantime	+
8	F	18 months	Fever, hepatosplenomegaly	−	Glucantime	−
9	F	5 years	Fever, hepatosplenomegaly	+	Glucantime	+
10	F	5 years	Fever, hepatosplenomegaly, anorexia	+++	Glucantime	+
11	F	5 months	Fever, hepatosplenomegaly, anorexia	−	None	+
12	M	35 years	Fever, hepatosplenomegaly	++	Glucantime	+
13	Dog†	–	–	+	None	+

* Glucantime dose: 20 mg/kg for 10 days.
† Dog from the domicile of a patient with visceral leishmaniasis.
+ in the PCR assay indicates detection of a visible amplification product; − = no product detected.

dot or slot blot apparatus for 30 min, and light suction was applied until the samples drained through the wells. Membranes were washed briefly in neutralizing buffer (0.5 M NaCl, 0.5 M Tris HCl, pH 7.5), ultraviolet cross-linked and dried.

Slot blots were performed according to Howard and others.[12]

Labeling of telomeric probes. Octamer, 62-mer, and hexamer oligonucleotide probes were labeled with [γ-^{32}P] adenosine triphosphate, 3,000 Ci/mmol (Amersham Pharmacia) with T4 polynucleotide kinase.[13]

Capture oligonucleotide was labeled by synthesizing dUTP-digoxigenin tails with terminal deoxynucleotidyl transferase.[14]

Hybridizations. Hybridizations were carried out in 0.5 M Na-phosphate pH 7.2, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.1 mg/mL *Escherichia coli* tRNA at 65°C for 18 hr. After hybridization, filters were washed twice with 40 mM Na-phosphate, 0.1% SDS at room temperature for 15 min, and twice at 65°C for 15 min. Hybridization temperatures were 58°C for the hexamer probe, 54°C for the octamer probe, and 65°C for the 62-mer forward oligonucleotide

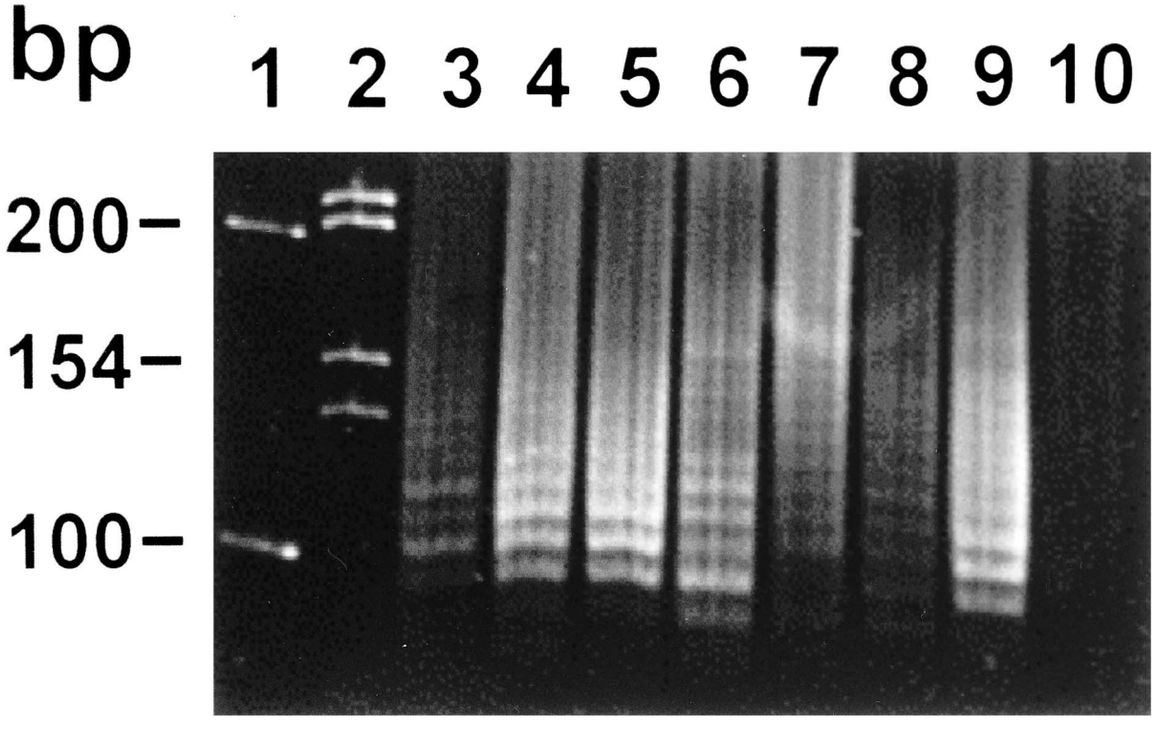

FIGURE 5. Polyacrilamide gel electrophoresis of *Leishmania donovani* telomeric polymerase chain reaction products digested with *Rsa*I endonuclease. **Lane 1,** 100-bp ladder molecular weight (MW) standard. **Lane 2,** 1-Kbp ladder MW standard. **Lane 3,** Ld 51.2. **Lane 4,** Ld 51.1. **Lane 5,** Ld LO571. **Lane 6,** Ld LO564. **Lane 7,** Li IPT1. **Lane 8,** Lch M11862. **Lane 9,** Lch L0580. **Lane 10,** Ld 51.2 total DNA.

probe. After hybridization, the last wash was at the same temperature as that used for hybridization.

For digoxigenin-labeled capture probe, hybridization was carried out in 5× SSC, 0.1% sodium-*N*-laurylsarcosinate, 0.02% SDS, and 1% blocking reagent (Boehringer Mannheim, Mannheim, Germany) at 58°C for 3 hr. After hybridization, filters were washed at room temperature in 6× SSC and 0.1% SDS for 5 min, then washed with 2× SSC and 0.1% SDS at room temperature for 15 min, and finally at 58°C for 15 min. The hybridized probe was detected by chemiluminescence with Lumi-Phos 530 according to the manufacturer's directions (Boehringer Mannheim).

RESULTS

Leishmania donovani telomeres could present the block (Beck AE, unpublished data)[15] structure shown in Figure 1A. Each block comprises a variable number of hexameric repeats, then a variable number of an octameric repeat and a single 62-mer sequence, in that order. The number of blocks per chromosome varies.[15] When chromoblots from *Leishmania* species or other Kinetoplastida were hybridized with the oligonucleotide probe derived from the 62-mer sequence (forward primer), we observed hybridization only with *L. donovani* chromosomal bands (Figure 2A, lanes 1 and 2). Similar results were obtained by a probe (reverse primer, Figure 1B) anchored on the octameric repeat that overlaps with the 62-mer sequence (Figure 2B, lanes 1 and 2). Signal intensity differences were observed between chromosomal bands of the 2 *L. donovani* strains. Figure 2C shows a positive control hybridization experiment with the telomeric probe $(CCCTAA)_3$.

To test the sensitivity of our telomeric probes, we first hybridized slot blots of *L. donovani* cell dilutions with a radiolabeled telomeric probe (see Materials and Methods). As shown in Figure 3A, we detected as few as to 50 *Leishmania* cells without PCR amplification.

When the *L. donovani* telomeric PCR was tested on purified *L. donovani* DNA and the amplification products were analyzed by slot-blot hybridizations that used the capture probe, a strong signal was observed at the maximum dilution used in this experiment (Figure 3B) equivalent to 1 fg of DNA ($\sim 5 \times 10^{-3}$ cell equivalents).

Leishmania donovani telomeric PCR products were of different sizes when resolved on agarose gels (Figure 4A and B, isolate 141). *Leishmania braziliensis, L. amazonensis,* and a local isolate later identified as *L. colombiensis* were negative (Figure 4A and B, lanes 1, 2, and 3, respectively). Separation of the PCR products by SDS-polyacrylamide gel electrophoresis produced numerous bands (Figure 5) due to variations in the numbers of hexamers and octamers per telomeric block (Figure 1) and is more evident in Figure 5 because the PCR products were digested with *Rsa*I endonuclease.

The specificity of our PCR assay was further tested by means of DNA samples from Old World *Leishmania.* The results in Table 1 and Figure 6 indicate that except for *L. infantum* KA (143), all *L. donovani* and *L. infantum* isolates were positive. A very weak signal was also observed for isolate 139, identified as *L. infantum,* but isolated from a case of cutaneous leishmaniasis. Of 5 *L. major* isolates tested, only *L. major* S1-177 (Figure 6, 149) was positive, with a ring-type hybridization devoid of signal in the center of the dot (Figure 6). Of 5 *L. tropica* isolates assayed, only isolate 148 was positive. Although identified as *L. tropica,* this isolate was obtained from a patient with VL. *Leishmania turinica* KXG-2(138) was positive with a ring-type hybridization similar to isolate *L. major* 149. The *L. donovani* telomeric PCR assay recognized all New World reference VL isolates except for 2 *L. colombiensis* isolates from Venezuela and 1 Brazilian isolate (Table 2).

Detection of patient samples. The DNA extracted from blood or bone marrow samples from 3 Indian patients with VL gave positive results when assayed on dot blots that used a telomeric probe (data not shown).

All Venezuelan archive samples with confirmed parasitological analysis (Table 3) were positive with our PCR method, and out of 2 parasitologically negative samples, one was positive and the other negative (Figure 7).

DISCUSSION

We present a novel PCR assay targeted at *L. donovani* telomeric sequences that has sensitivity and specificity for *L. donovani* and *L. infantum.* In patient samples from India, the telomeric probes detected *Leishmania* DNA even without PCR amplification, indicating a high copy number for these telomeric sequences in Indian *Leishmania* isolates.[16] This property is currently being evaluated.

Except for one *L. infantum* isolate, the assay recognized all 20 Old World VL isolates. In *L. major* isolates, 1 of 5 isolates was positive with an atypical hybridization signal. This result was unexpected because we know from our work (Chiurillo MA and others, unpublished data) and others[16,17] that the forward oligonucleotide primer of the PCR assay should not hybridize to *L. major* telomeric sequences. We also observed a single positive result out of 5 *L. tropica* isolates tested. This isolate (*L. tropica* 1095, isolate 148; Figure 6) was obtained from a patient with VL obtained during the Gulf war.[8] We are currently expanding the number of *L. major* and *L. tropica* reference isolates.

The assay in Venezuela produced the following results: all reference isolates of *L. chagasi* or *L. infantum* were positive; however, 1 Brazilian and 2 Venezuelan VL isolates were negative. The 2 negative Venezuelan VL isolates had been previously identified as *L. colombiensis,*[7] a parasite included within the *Viannia* subgenus[18] and suspected of being common in western Venezuela endemic areas (Bonfante R, unpublished data). It is interesting that in one of the endemic areas, which neighbors a *L. colombiensis*-endemic area, only *L. donovani*-related parasites were detected.

Although good PCR and other molecular assays exist for detecting *L. donovani* species,[12,19,20] the main advantages of our assay are as follows: increased specificity compared with kDNA-based assays[21] (Guevara P, unpublished data), little cross-reactivity with *L. major* or *L. tropica* strains, and for kala-azar, the possibility of using telomeric-derived probes without further amplification. This level of sensitivity is likely because of the high copy number (per diploid genome) found for related sequences in *L. donovani* isolates from India and Ethiopia.[16] The telomeres of *L. major* have sequence homology and the block structure[17] described for *L. dono-*

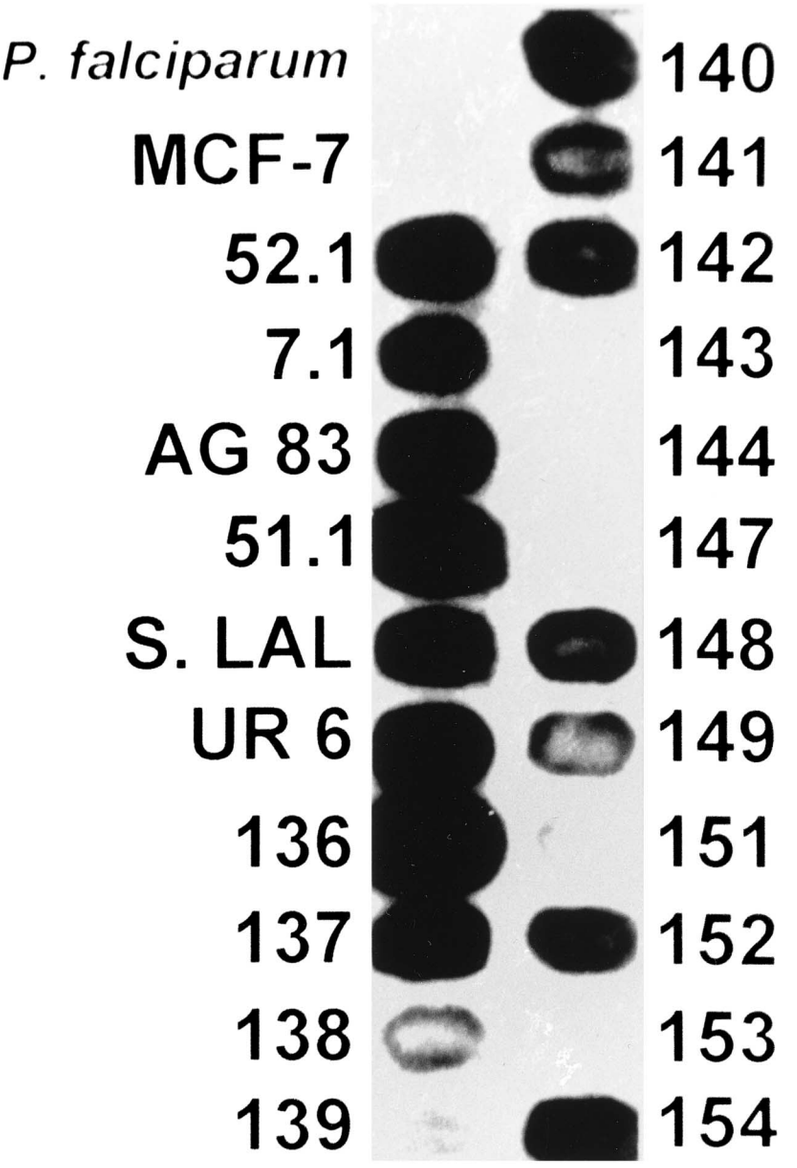

FIGURE 6. Specificity test of *Leishmania donovani* telomeric-polymerase chain reaction (PCR) in Old World *Leishmania*. Dot blots of PCR products are hybridized with radiolabeled capture oligonucleotide. The identity of isolates is indicated. *Plasmodium falciparum* and human breast cancer cell line MCF-7 DNA were used as negative controls.

vani; however, as mentioned above, the primers here hybridized to regions where *L. major* has several nucleotide changes.[15]

Finally, this PCR assay, in combination with the simple protocol for recovery of Giemsa-stained slides of bone marrow, proved useful for the retrospective study of archive material. This combination of techniques may prove to be an excellent epidemiological tool.

Acknowledgments: We thank Juanita Vitelli for technical assistance and Ian McLure for revising the English.

Financial support: This work was supported by Consejo Nacional de Investigaciones Cientificas y Tecnologicas (CONICIT) grants S1-95000524 (J. L. R.); S1-95000889 (N. A.), and 9900034 (M. A. C.); Department of Biotechnology, Government of India, New Delhi (R. M.); and (IAEA) International Atomic Energy Agency Regional Project RLA/6/026 (P. G.).

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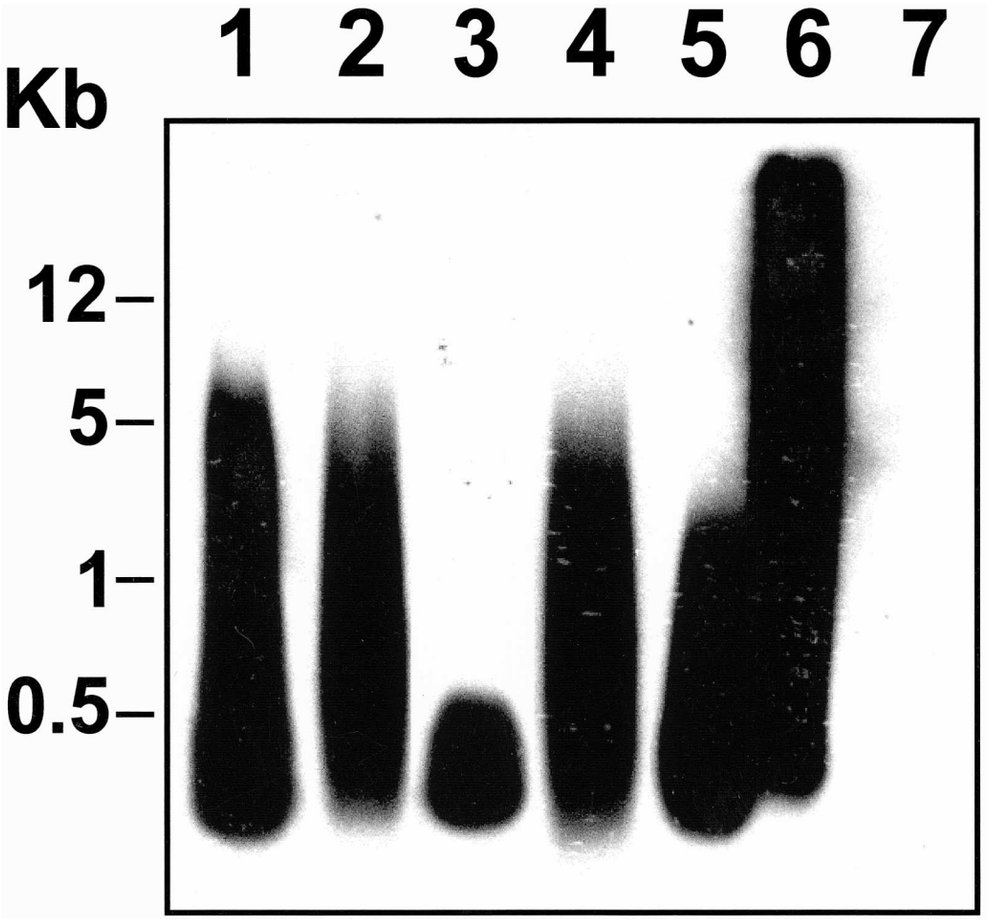

FIGURE 7. *Leishmania donovani* telomeric-polymerase chain reaction (PCR) of marrow samples from Venezuelan VL archive cases (1992–1998). The PCR products were electrophoresed on a 1% agarose gel and hybridized with a digoxigenin-labeled capture oligonucleotide (Figure 1). **Lanes 1–5,** parasitologically confirmed samples. **Lane 6,** DNA from Ld 51.2. **Lane 7,** negative control from a patient with an unrelated hematological disease.

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