Leishmania Mini-exon Genes for Molecular Epidemiology of Leishmaniasis in China and Ecuador

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The mini-exon gene is unique and is tandemly repeated in the *Leishmania* genome. The transcribed region is highly conserved, but the non-transcribed spacer region is distinct in length and in sequence among different *Leishmania* species. The usefulness of PCR amplification of the *Leishmania* mini-exon gene was examined for molecular epidemiology of visceral and cutaneous leishmaniasis. We previously described a PCR method for amplification of the mini-exon gene and obtained positive amplification in bone marrow aspirates of patients with visceral leishmaniasis in China. In this study, we have cloned and sequenced two PCR products from the patients. The sequences of two products revealed 100% identity and showed more similarity to the mini-exon gene of *L. donovani* Indian strain than those of *L. donovani* complex in Africa and South America. We also applied this PCR method to the diagnosis of cutaneous leishmaniasis. We obtained positive PCR amplification in skin biopsy materials taken from patients with cutaneous leishmaniasis in Ecuador. Since this PCR amplification is simple and requires only a pair of primers to detect all *Leishmania* species distributed in Ecuador, the method may be a useful tool for the detection of parasites, not only from patients, but also from sandflies and reservoir animals in this area of endemicity.

Keywords : Visceral leishmaniasis, Cutaneous leishmaniasis, PCR, Mini-exon gene, Epidemiology
Note: Nucleotide sequence reported in this paper is available in the DDBJ data base under the accession number AB017336.

INTRODUCTION

Leishmania causes a wide spectrum of diseases known as cutaneous, mucocutaneous and visceral leishmaniasis (kala-azar). For the diagnosis of leishmaniasis, a number of different probes derived from nuclear DNA and kinetoplast DNA have been used for identification of *Leishmania* species by various methods, including PCR and Southern hybridization [1-3, 6, 7, 13-16]. Among DNA probes, the mini-exon gene is highly specific and sensitive because the gene is present as tandemly repeated copies in the *Leishmania* genome but is absent from mammalian hosts and sandfly vectors. Furthermore, the non-transcribed intergenic spacer region is distinct in length and in sequence among different *Leishmania* species [3,6], although the transcribed region is highly conserved. Thus, comparison of the mini-exon gene sequence is used for understanding the evolutionary and geographical relationships among *Leishmania* species [3].

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In our previous paper, we reported a nested PCR method based on amplification of the *Leishmania* mini-exon gene for the diagnosis of visceral leishmaniasis [10]. We demonstrated that the nested PCR procedure is sufficiently sensitive to detect a single *Leishmania* cell. The parasite DNA was detected not only in bone marrow aspirates but also in buffy coat cells of patients with active kala-azar in the Xinjiang-Uigur Autonomous Region in China, where kala-azar cases have been recorded annually [4].

In this study, we extended our previous work [10] by performing DNA sequence analysis of PCR products amplified from these Chinese patients with kala-azar. We confirmed that the PCR products were derived from the *Leishmania* mini-exon gene. The sequences of the products showed more similarity to the mini-exon gene of Indian *L. donovani* reference strain than those of *L. donovani* complex reported from Africa and South America. In addition, we also verified the usefulness of PCR amplification of the mini-exon gene for the diagnosis of cutaneous leishmaniasis. We applied this method to skin biopsy materials collected from patients with cutaneous leishmaniasis in Ecuador, where we have been conducting a consequential survey on leishmaniasis since the 1980s [5].

MATERIALS AND METHODS

Parasites

We used 15 WHO reference *Leishmania* strains, including *L. aethiopica* (MHOM/ET/72/L100), *L. amazonensis* (MHOM/BR/73/M2269), *L. braziliensis* (MHOM/BR/84/LTB300), *L. chagasi* (MHOM/BR/74/PP75), *L. donovani* (MHOM/IN/80/DD8), *L. garnhami* (MHOM/VE/76/JAP78), *L. gerbilli* (MRHO/CN/60/GERBILLI), *L. guyanensis* (MHOM/BR75/M4147), *L. infantum* (MHOM/TN/80/IPT1), *L. major* (MRHO/SU/59/P), *L. mexicana* (MHOM/BZ/82/BEL21), *L. panamensis* (MHOM/PA/71/LS94), *L. pifanoi* (MHOM/VE/57/LL1), *L. tropica* (MHOM/SU/60/OD), and *L. tropica* (MHOM/SU/58/StrainOD). These reference strains were kindly provided by D. A. Evans (London School of Hygiene and Tropical Medicine, UK), and G. Grimaldi Jr. (Institute of Oswardo Cruz, Brazil). We also used other isolates, such as *L. donovani* (MHOM/SU/00/2S-25M-C2) [8], *L. major* (MHOM/ML/90/Tokai-L1) [17], *L. mexicana* (IAYA/EC/92/ALI-3) [9], and *L. panamensis* (MHOM/EC/87/G-05) [9]. Promastigotes were grown in USAMRU medium (Difco blood agar medium) [18] or Schneider's Drosophila medium at 25℃. The parasites collected were stored in absolute ethanol at -20℃ or cryopreserved in liquid nitrogen before use.

Clinical samples

Bone marrow aspirates were taken from the sphenoid bone of kala-azar patients in Kashi in the Xinjiang-Uigur Autonomous Region of Northwestern China [10]. Bone marrow smears were stained with Giemsa for microscopic examinations. The aspirates were inoculated in NNN medium, and the remaining samples were stored in absolute ethanol for subsequent DNA isolation.

Skin biopsy samples were taken from the edge of ulcer or nodule lesions of patients with cutaneous leishmaniasis in Ecuador by a biopsy punch and were stored in absolute ethanol. Parasite isolation was made by performing syringe aspiration of tissue materials from the margins of active skin lesions of patients; the aspirates were inoculated into USAMRU medium and cultured at 25℃. Smear preparations were also made from the aspirates for microscopic examination. Parasite isolation from sandflies was also performed by the inoculation of gut materials containing parasites into the nose or the foot pads of hamsters. Biopsy materials were taken from lesions of the infected animals and passed into the cultures.

Bone marrow cells of uninfected Japanese children were supplied by the Jikei Hospital in Tokyo. Skin biopsy samples were collected from healthy Japanese and Ecuadorian adult volunteers. Normal mouse skin samples were also collected. DNA was similarly extracted from these negative control tissues. Informed consent was obtained from all patients or their guardians and all volunteers involved in this study.

DNA isolation and PCR assay

DNA was extracted from parasites, bone marrow aspirates, skin biopsy samples and negative control tissues by a standard phenol chloroform method or by using a DNA

extraction kit (Blood & Cell culture DNA Kit, QIAGEN Inc., Chatsworth, CA). PCR amplification was carried out in a reaction mixture of 20 μl containing 10 mM Tris-HCl (pH 9.3), 50 mM KCl, 1.5 mM $MgCl_2$, the four deoxynucleoside triphosphates at a concentration of 0.2 mM each, 10% dimethylsulfoxide, each primer at a concentration of 0.5 μM, 10-100 ng of *Leishmania* or tissue DNA, and 0.05 units/μl Taq DNA polymerase (Amersham Pharmacia Biotech Ltd., Uppasale, Sweden). We used a pair of primers, S-1629 (5' gggaattCAATAT/AAGTACAGAAACTG) and S-1630 (5' - gggaagcTTCTGTACTT/ATATTGGTA) [3,15]. Lowercase letters in the primers indicate noncomplementary bases. Each reaction mixture was overlaid with 50 μl of paraffin oil. The PCR was run in a thermocycler (Perkin-Elmer Corp., Norwalk, CT) as follows. The samples were first denatured at 95℃ for 5 min and were then subjected to 35 cycles of denaturation at 95℃ for 1 min, annealing at 50℃ for 30 s, and extension at 72℃ for 1 min. The final extension was at 72℃ for 10 min. The PCR products in 10-μl aliquots were electrophoresed in 2% agarose in 1x TAE (40 mM Tris-acetate and 1 mM EDTA) at 100 V. The gels were stained with 0.5 μg of ethidium bromide per ml and photographed.

Cloning and sequencing of PCR products

The PCR products were size-excised from agarose gels and cloned into the vector pCR2.1 (TA-cloning Kit, Invitrogen Corp., Carlsbad, CA). Nucleotide sequencing of the clones was performed by the dideoxy termination method (PE Applied Biosystems, Warrington, UK). DNA sequences were analyzed by DNASIS-Mac v3.4.

RESULTS

Amplification of the mini-exon gene repeat in different *Leishmania* species

We determined the size of the mini-exon gene in different *Leishmania* species. As reported in previous papers [3,15], the DNA banding pattern of amplified mini-exon gene repeats was *Leishmania* complex-specific (Fig. 1). The DNA ladder corresponded to multimeric forms of the mini-exon gene repeat of each *Leishmania* species. The viscerotropic species of the *L. donovani* complex, such as *L. donovani*, *L. infantum* and *L. chagasi* gave rise to amplified products of approximately 450 bp (Fig. 1, Ld complex). The *L. tropica* complex, including *L. tropica*, *L. major*, and *L. aethiopica* also yielded products of around 450 bp (Fig. 1, Lt complex). Parasites classified into the *L. mexicana* complex, such as *L. amazonensis*, *L. mexicana*, *L.*

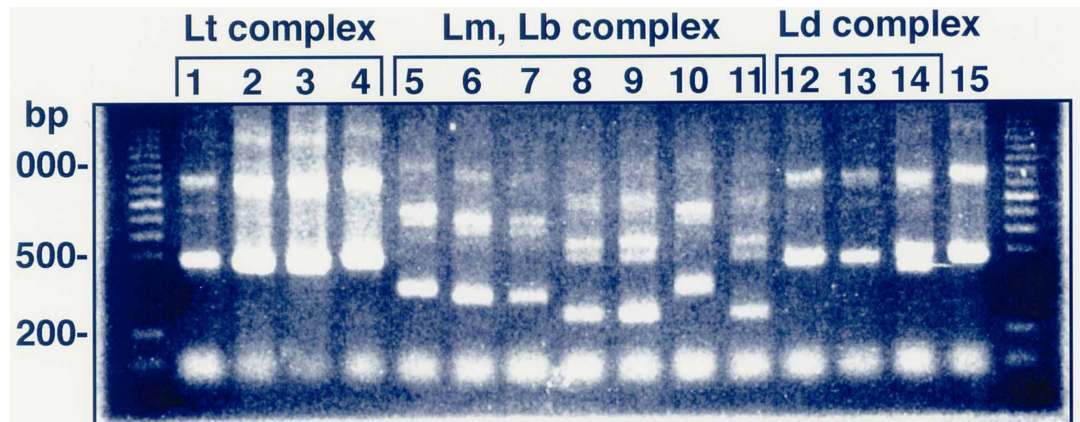

Fig. 1 PCR amplification of the mini-exon genes for different *Leishmania* species. Lane 1, *L. major* (P); lane 2, *L. aethiopica* (L100); lane 3, *L. tropica* (OD); lane 4, *L. major* (Tokai-L1); lane 5, *L. pifanoi* (LL1); lane 6, *L. garnhami* (JAP78); lane 7, *L. amazonensis* (M2269); lane 8, *L. guyanensis* (M4147); lane 9, *L. braziliensis* (LTB300); lane 10, *L. mexicana* (BEL21); lane 11, *L. panamensis* (LS94); lane 12, *L. chagasi* (PP75); lane 13, *L. infantum* (IPT1); lane 14, *L. donovani* (DD8); lane 15, *L. gerbilli* (GERBILLI). Parasites in lanes 1-4, lanes 5-7 and 10, lanes 8-9 and 11, lanes 12-14 belong to the *L. tropica* (Lt), *L. mexicana* (Lm), *L. braziliensis* (Lb) and *L. donovani* (Ld) complexes, respectively.

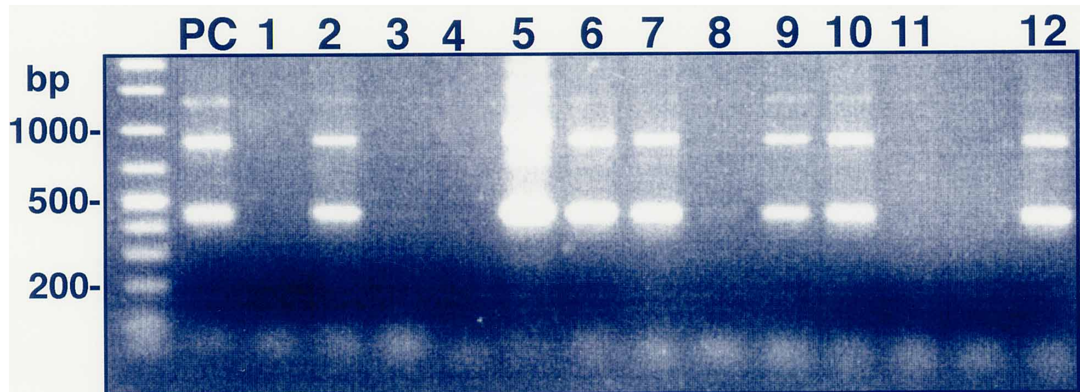

Fig. 2 PCR detection of the *Leishmania* mini-exon gene for DNA samples from bone marrow aspirates of kala-azar patients in China. The lane number corresponds to the patient number. PC, positive control using *L. donovani* DNA.

pifanoi and *L. garnhami*, yielded amplification products of 320-350 bp (Fig. 1, Lm complex). Finally, approximately 250-bp amplification products were generated from dermotropic *Leishmania* species belonging to the *L. braziliensis* complex, e.g., *L. braziliensis*, *L. guyanensis* and *L. panamensis* (Fig. 1, Lb complex).

PCR detection of the *Leishmania* mini-exon gene for DNA samples from bone marrow aspirates of patients with kala-azar in China

Amplification of the mini-exon gene was observed in nine of 12 bone marrow DNA samples from kala-azar patients in China (Fig. 2, lanes 1-12). The PCR products were characterized by the presence of a ladder of 3 bands, as described previously [10]. Although only faint bands were detected from two DNA samples (Fig. 2, lanes 3 and 8), PCR positivity was confirmed for these samples in different experiments. The DNA ladder seen was in multiplicity of ~450 bp, which was consistent with that observed in the *L. donovani* (2S-25M-C2) positive control (Fig. 2, PC). Bone marrow samples from these nine patients were also positive for parasites in smear preparations and cultures [10].

Comparison of DNA sequences of the *Leishmania* mini-exon gene amplified from kala-azar patients with those of *L. donovani* complex

The 450-bp PCR products from two Chinese kala-azar patients (patient ID numbers, KS-6 and KS-16) (Fig. 2, lanes 5 and 12) were cloned and sequenced. Both PCR products contained the expected leishmanial exon, intron and non-transcribed spacer regions. The DNA sequence of the two PCR products was identical and was referred to as KS-6/16. Alignment of the mini-exon gene intron and spacer sequence from these patients and viscerotropic *Leishmania* species is shown in Fig. 3. The sequence of the mini-exon gene from Chinese kala-azar patients (KS-6/16) was 96.9%, 94.6%, 93.7%, 92.1% and 88.4% identical to *L. donovani* (India, DD8), *L. donovani* (Sudan, 1S), *L. donovani* (Kenya, MRC-74), *L. infantum* (Tunisia, IPT1) and *L. chagasi* (Brazil, BA3), respectively.

Amplification of the mini-exon gene for the diagnosis of cutaneous leishmaniasis in Ecuador

The mini-exon gene repeats were generated from skin biopsy materials of patients with cutaneous leishmaniasis in Ecuador. A typical result is shown in Fig. 4. A DNA ladder was generated from the DNA sample collected from the skin lesion of a patient (patient ID number 4 at El Calmen). The ladder corresponded to multimeric forms of ~250-bp mini-exon gene repeat in *L. braziliensis* complex. The DNA banding pattern was indistinguishable from that observed in the DNA sample of *L. panamensis* (G-05), which was isolated from another patient in a different endemic area in Ecuador (Fig. 4, lanes 4 and 5); this biopsy material was also positive for parasites in smears and cultures.

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KS-6/12      GTATGCGAAA CTTCCGGAAG CTGTCTTCCG GCAACATTTT GGAAGCGCGC
Ld (DD8)     GTATGCGAAA CTTCCGGAAC CTGTCTTCCG GCAACATTTT GGAAGCGCGC
Ld (1S)      GTATGCGAAA CTTCCGGAAC CCGTCTTCCG GCAACATTTT GGAAGCGCGC
Ld (MRC-74)  GTATGCGAAA CTTCCGGAAC CTGTCTTCCG GCAACGTTTT GGAAGCGCGC
Li (IPT1)    GTATGCGAAA CTTCCGGAAC CTGTCTTCCG GCAACATTTT GGAGGCGCGC
Lc (BA3)     GTATGCGAAA CTTCCGGAAG CTGTCTTCCG GCAACATTTT GGAAGCGCGC 50

KS-6/12      AGGCG--CTC TTTTTTTTTG TGTGCGTGTG TGTGGCGGCG GGCCCCCCTG
Ld (DD8)     AG-CG---TC CTTTTTTTTG TGTGCGTGTG TGTGGCGGCG GGCCCCCCTG
Ld (1S)      AGGCG--CTC TTTTTTTTTG TGTGCGTGTG TGTG-TGGCG G-------CG
Ld (MRC-74)  AGGAG--CTC -TTTTTCTTG TGTGCGTGTG TGTGGCGGCG GGCCCCCCTG
Li (IPT1)    GGGCGTCCTT TTTTTTTTTG TGTGCGTGTG TGTGGCGGCG GGCCCCCCTG
Lc (BA3)     AG-CGCC--- --TTTTTTTG TGTGCGTGTG TGTGGCGGCG GGCCCCC-TG 100

KS-6/12      CGGTCCCGCG CGGTCGCCGG CGGCTTCCGT AGCGGTGGCC CCGCCGCGCC
Ld (DD8)     CGGTCCCGCG CGGTCGCCGG CGGCTTCCGT AGCGGTGGCC CCGCCGCGCC
Ld (1S)      -GGTCCCGCG CGGGCGCCGG CGGCTTCCGT AGCGGTGGCC CCGCCGCCCC
Ld (MRC-74)  CGGTCCCGCG CGGTCGCCGG CGGCTTCCGT AGCGGTGGCC CCGCCGCGCC
Li (IPT1)    CGGTCCCGCG CGGTCGCCGG CGGCTTCCGT AGCGGTGGCC CCGCCGCGCC
Lc (BA3)     CGGTCCCGCG CGGTCGCCGG CG-CTTCCGT AGCGGTGGCC CCGC-GCGCC 150

KS-6/12      GAGGGGGCCG CGACGCCG-C GGGGCAGGCG TCCCCCAAGG -CGCCGCGTG
Ld (DD8)     -AGGGGGCCG CGACGCCG-C GGG-CAGGCG TCCCCCAAGG -CGCCGCGTG
Ld (1S)      GAGGGGGC-G CGACGCCG-C GGGGCAGGCG TCCCCCCAGG -CGCCGCGTG
Ld (MRC-74)  GAGGGGGC-G CGACGCCGGC GGGGCAGGCG TCCCC--AGG -CGCCGCGTG
Li (IPT1)    GAGGGGGCCG CGACGC---- GGGGCAGGCG TCCCCCAANG GCGCCGCGTG
Lc (BA3)     GAGGGGGCCG CGACGCCGGC GGG-CAGGC- TCCCC--AAG GCGCCGCGTG 200

KS-6/12      ACTGGCGCAC CGGGCACGGG GCCTGCGT-G CGCGCAGCCG TTTCCGTCTC
Ld (DD8)     ACTGGCGCAC CGGGCACGGG -CCTGCGT-G CGCGCGGCCG TTTCCGTCTC
Ld (1S)      ACTGGCGCGC CGGGCACGGG GCGTGCGT-G CGCGCGGCCG TTTCCGTCTC
Ld (MRC-74)  ACTGGCGCAC CGG-CACGGG -CCTGCGT-G CGCGCAGCCG TTTCCGTCTC
Li (IPT1)    ACTGNNAC-C GGGTCACGGG GCCTGCGTAG CGCGCAGTTG TTTCCGTCTC
Lc (BA3)     ACTGGCG-AC CGGGCG-GGG GCCTGCGATG CCGGCAGCCG TTTCCGTCTG 250

KS-6/12      CGCGGGGCGC CGCCCGCCGC GGTGTGTGCC AGGGCGCGGC GCCC-CGCAC
Ld (DD8)     CG---GGCGC -GCCCGCCGC GGTGTGTGCC AGGGCGCGGC GCCC-CGCAC
Ld (1S)      CGCGGGGCGC CGCCCGCCGC GGTGTGTGCC AGGCCGCGGC GCCC-CGCAC
Ld (MRC-74)  CGCGGG-CGC CGCCCGCCGC GGTGTGTGCC AGGGCGCGGC ----------
Li (IPT1)    CGCGGG-CGC -GCCCGCCGC GGTGTGTGCC AGG--GCCGC GCCC-CGCAC
Lc (BA3)     GGCCCGGC-- CGCCCGCCGC GG-GTGTGCC AGGGCCGGCC GGCCGCCCAC 300

KS-6/12      CGCCCGGCG- CGAGGCG--A GCCCGGTGCG CGGCCATGGT GGTGACGCGC
Ld (DD8)     CGCCCG-CG- CGAGGCG--A GCCCGGTGCG CGGCCATGGT GGTGACGCGC
Ld (1S)      CGCCCGGCG- CGAGGCG--A GCCCGGTGCG CGGCCATGGT GGTGACGCGC
Ld (MRC-74)  ---------- ---------- ---------- ----CATGGT GGTGACGCGC
Li (IPT1)    CGCCCGGCGT CGAGGCGTCA GCCCGGTGCG CGGCCATGGT GGTGACACGC
Lc (BA3)     CGCCCGG-GC CGAG-CG--A GCCCGGTGCG CGGCCATGGT GGTGACGCGC 350

KS-6/12      GGGCCCGTGC GCGGAGAACA TCCGCCCCGC GAATGCGGGC TGTGGGTGTG
Ld (DD8)     GGGCCCGTGC GCGGAGAACA TCCGCCC-GC GAATGCGGGC TGTGGGTGTG
Ld (1S)      GGGCCCGTGC GCGGAGAACG TCCGCCCCCC GAATGCGGGC TGTGGGTGTG
Ld (MRC-74)  GGGCCCGTGC GCGGAGAACC GCCGCCCCGC GAATGCGTGC TGTGGGTGTG
Li (IPT1)    GGGCCCGTGC GCGGAGAACC TTCCGCCCGC GAATGCGGGC TGTGGGTGTG
Lc (BA3)     GGGCCCGTGC -CGGAGAAC- CTCGCGC--C GAATGCGCGC TGTGGGTGTG 400

KS-6/12      ACGGCTTTC
Ld (DD8)     ACGGCTTGC
Ld (1S)      ACGGCTTGC
Ld (MRC-74)  ACGGCTTGC
Li (IPT1)    ACGGCTTTC
Lc (BA3)     ACGGCTTTC
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Fig. 3 Alignment of intron and non-transcribed spacer sequences of the mini-exon gene for *Leishmania donovani* complex. The sequences are derived from PCR products of Chinese kala-azar patients (KS-6/12, DDBJ accession number AB017336), *L. donovani* (India, DD8, GenBank accession number X69443), *L. donovani* (Sudan, 1S, X62143), *L. donovani* (Kenya, MRC-74, X69456), *L. infantum* (Tunisia, IPT1, X69445), and *L. chagasi* (Brazil, BA3, X69446). The alignment was made with DNASIS-Mac v3.4. Identical nucleotides among at least three sequences at the same position are highlighted.

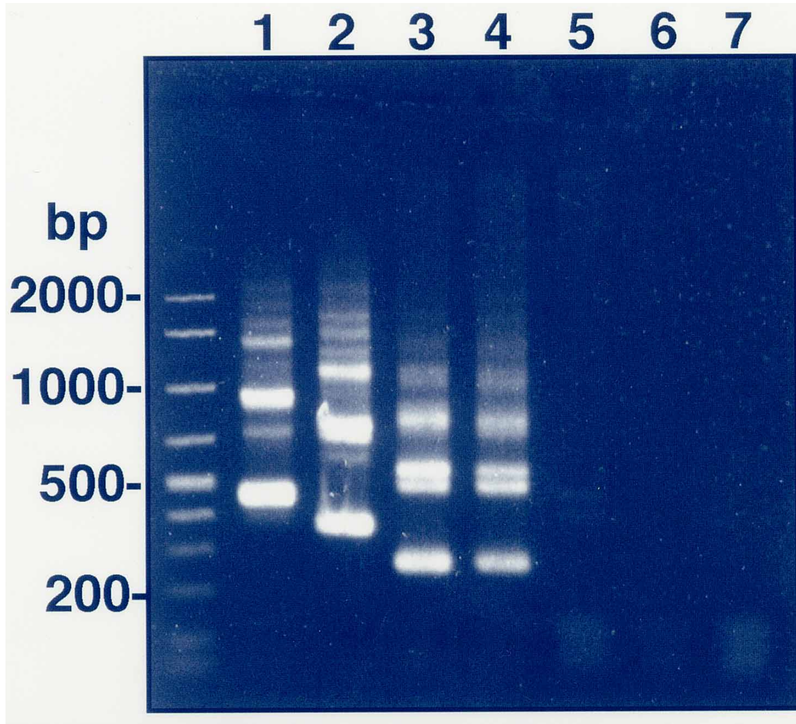

Fig. 4 PCR detection of the *Leishmania* mini-exon gene for the diagnosis of cutaneous leishmaniasis in Ecuador. Lane 1, *L. tropica* (StrainOD); lane 2, *L. mexicana* (ALI-3); lane 3, *L. panamensis* (G-05); lane 4, patient skin; lane 5, uninfected human skin; lane 6, uninfected mouse skin; lane 7, distilled water.

DISCUSSION

In this study, we determined the DNA sequence of PCR products amplified from two patients with kala-azar in Kashi in the Xinjiang-Uigur Autonomous Region of Northwestern China. The sequence data revealed that both PCR products were derived from the *Leishmania* mini-exon gene and showed more similarity to the *L. donovani* gene in India than those of other *L. donovani* complex in Africa and South America (Fig. 3). *Leishmania* isolates collected in China were initially characterized by their isoenzyme profiles [19]. Additional Chinese isolates were recently analyzed by restriction fragment length polymorphism of kDNA and nuclear DNA. The results indicated that Chinese *Leishmania* isolates from patients with kala-azar were genetically heterogeneous and appeared to be different from viscerotropic strains in other countries [11]. Since parasite isolation from kala-azar patients cannot be easily done, PCR amplification of the mini-exon gene followed by sequence analysis may be useful for understanding evolutionary and geographical relationships among *Leishmania* species that cause kala-azar in China and the surrounding countries.

PCR amplification of the mini-exon gene is useful for rapid identification of parasites because it requires only one pair of primers for detection of all *Leishmania* species [3]. We have applied this PCR method for the diagnosis of cutaneous leishmaniasis in Ecuador. Our studies revealed that the *Leishmania* mini-exon gene can be amplified from alcohol-fixed skin biopsy materials collected from patients with cutaneous leishmaniasis (Fig. 4). In Ecuador, at least six human pathogenic *Leishmania* species are distributed [5]. In the lowland Pacific Coastal region, *L. panamensis* is a dominant species, and small numbers of *L. amazonensis*, *L. mexicana* and *L. major*-like parasites were detect-

ed. The main distribution of *L. braziliensis* is limited to the Amazon Basin. In contrast, in the highland Andean Mountain regions, *L. mexicana* is dominantly distributed, and a *L. major*-like species has also been detected. Since the *L. braziliensis* and *L. mexicana* complex are easily distinguishable by the size of their mini-exon gene repeats, PCR amplification of the mini-exon gene may be a useful and simple method for the detection of *L. panamensis* and *L. mexicana* not only from patients, but also from sandfly vectors and reservoir animals for further epidemiological studies of leishmaniasis in Ecuador. Further species identification can be performed by polymorphism-specific arbitrary primed PCR with species specific primers under stricter conditions [12].

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