Application of polymerase chain reaction with specific and arbitrary primers to identification and differentiation of *Leishmania* parasites

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**Abstract:** Two oligonucleotide primers Lsmc1 and Lsmv1 derived from the conserved and the variable region of a major class kinetoplast DNA (kDNA) minicircle (pLURkE3) of *Leishmania* strain UR6 were used for the polymerase chain reaction (PCR) in order to amplify a 461-bp fragment from the kDNAs of different *Leishmania* species. These primers amplify the specific fragment from the kDNAs of cutaneous species only. The cutaneous species can further be distinguished by randomly amplified polymorphic DNA (RAPD) analysis of the kDNAs of these organisms using arbitrarily chosen oligonucleotides. The arbitrary primers also generate polymorphic DNA fingerprints at the genomic level with different *L. donovani* isolates. The results indicate that the PCR and arbitrarily primed PCR (AP-PCR) may be extremely useful approaches for identifying and distinguishing *Leishmania* parasites.

**Key words:** *Leishmania* species; Polymerase chain reaction; Polymorphism; Strain differentiation

**Introduction**

The protozoan parasites of the genus *Leishmania*, the causative agent for leishmaniasis, present a complex set of clinical features ranging from a self-healing cutaneous lesion through the metastasizing muco-cutaneous form to the often fatal visceralizing form [1]. The severity of the clinical manifestations is largely determined by the particular infecting *Leishmania* species and in some cases the subspecies, and the treatment varies accordingly [2].

*Leishmania* are members of the order Kinetoplastidae. They possess a massive, compact network structure of DNA, the kDNA within the single mitochondrion of the organism. This kDNA is comprised of two components, maxicircles and minicircles which are topologically interlocked within the kDNA network [3].

The remarkable kDNA network has recently emerged as an important tool in the classification and identification of the organism [4]. The discovery of minicircle sequences unique to species [5,6] and their use as species-specific probes has been found to be a promising solution to the problem of characterization of unknown isolates [5].

The polymerase chain reaction (PCR) offers an alternative approach to parasite detection and
characterization [7]. Using defined oligonucleotide primers for PCR, specific amplification of parasite DNA can be achieved [7] and, using arbitrarily chosen primers for PCR, polymorphism in the amplified DNA can be generated. This AP-PCR can distinguish between strains of almost any organism [8].

In this study, we have conducted PCR with primers derived from the major class minicircle of a *Leishmania* species (*Leishmania* strain UR6) which identifies cutaneous *Leishmania* species and AP-PCR which generates polymorphic DNA patterns in the kDNA of these cutaneous *Leishmania* species. The same arbitrary primers were also used with PCR to study polymorphism in the genomic DNA of different *L. donovani* isolates.

**Materials and Methods**

**Parasites and culture conditions**

*Leishmania* strain UR6 (MHOM/IN/1978/UR6) was originally isolated from the bone marrow of a patient admitted in the Calcutta School of Tropical Medicine. Since then, promastigotes have been maintained in Ray's modified medium [9] with subculture at 72-h intervals. This strain has an isozyme pattern similar to *L. tropica* as tested in the laboratories of Professor J.A. Rioux, University of Paris, France and Dr. R. Killick-Kendrick of the Imperial College of London. *L. tropica major* LRC408 is also routinely maintained in the laboratory in Ray's modified medium [9]. Other *Leishmania* strains used were *L. donovani* MHOM/IN/89/GE-1, MHOM/IN/89/GE-2, MHOM/IN/89/GE-4, MHOM/IN/GE-6 and MIIOM/IN/83/AG-83, which are maintained in mice [7].

**DNA extraction and quantification**

kDNA was isolated as described [10] and total nucleic acid was isolated from the strains by phenol extraction and ethanol precipitation [11]. The DNA was dissolved in TE buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA) and the final concentration was estimated by agarose gel electrophoresis and ethidium bromide staining.

**Development of primers**

Full-length minicircles from *Leishmania* strain UR6 promastigotes were linearized with *EcoRI* and cloned into the *EcoRI* site of the plasmid pGEM4Z [11]. A recombinant plasmid, pLURkE3, representing the major class minicircle population, was sequenced [11]. The minicircle sequences were analyzed using the software program DNASIS which uses a modification of the Needleman-Wunsch algorithm [12], and appropriate primers for PCR were synthesized.

The M13/pUC Forward sequencing primer, API (5'-CGCCAGGTTTTCCAGTCAC-GAC-3') and Reverse sequencing primer, AP2 (5'-TCACACAGGAAACAGCTATGAC-3') designed for other purposes and chosen arbitrarily for these experiments, were obtained from Promega, USA.

**PCR amplification with target primers**

Primers Lsmc1 (5'-CGTTCTGCGAAAAATCG-GG-3') and Lsmv1 (5'-TATTGCTTTATTCC-TAT-3') were synthesized in automatic DNA synthesizer (Generator, Du Pont). PCR reaction mixtures (20 μl) contained 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 0.5 mM MgCl2, 0.01% gelatin (w/v), 200 μM each of dATP, dCTP, dTTP and dGTP, 0.1 μM of each primer, 10 ng kDNA or 25 ng total nucleic acid and 1.2 units of *Taq* DNA polymerase (Perkin Elmer, Cetus). Each reaction was overlaid with mineral oil and amplification was performed in a Thermal cycler (Techne, USA) programmed for 30 cycles of 1 min at 94°C, 2 min at 50°C and 2 min at 72°C. At the end of the PCR cycles all tubes were incubated for 10 min at 72°C to allow the amplification process to go to completion. Products were analyzed by electrophoresis in 1.2% agarose gel in TAE buffer (0.04 M Tris acetate, 0.002 M EDTA; pH 8.0). The gels were stained with ethidium bromide (5 μg ml⁻¹) destained in water and photographed under UV illumination.
PCR amplification with arbitrary primers

A typical 20-μl reaction contained 0.1 μM of each arbitrary primers, 25 ng of total nucleic acid and all other components as described above. The reaction buffer was adjusted to 2.5 mM with respect to MgCl₂. A high Mg^2+ concentration was selected to enhance the stability of primer-template interactions. An annealing temperature of 40°C and 40 amplification cycles were adopted, followed by 10 min extension at 72°C. Products were analyzed following electrophoresis as above.

Results and Discussion

Development of primers

The full-length nucleotide sequence of the kDNA minicircle pLURkE3 from Leishmania strain UR6 was compared with those known for other species of Leishmania [13,14]. The primers chosen for amplification were derived from the conserved and the variable region of pLURkE3 as illustrated in Fig. 1. Since the primers are 461 bp apart, the resulting product of amplification reactions using these oligonucleotides and the kDNAs from UR6-related strains would be expected to be 461-bp fragments.

PCR amplification of Leishmania DNA with kDNA specific primers

PCR amplification of kDNA and total cellular DNA using the above specific primers was carried out on 22 different Leishmania strains. Figure 2 shows that amplification of 10 ng purified kDNA samples or 25 ng of total nucleic acid from different species of Leishmania produces 461-bp fragments from Leishmania strain UR6, L. tropica major (LRC 408), L. tropica (L. 571) and L. major (WR 309), the Leishmania species causing cutaneous leishmaniasis. These primers are, however, unable to amplify the DNA fragments from species causing visceral and mucotutaneous leishmaniasis.

AP-PCR on cutaneous Leishmania species

The kDNAs of cutaneous Leishmania species tested exhibited the same PCR pattern when specific primers were used. Polymorphisms in DNA fingerprints generated by AP-PCR can distinguish between slightly divergent strains of any organism. To determine whether the polymorphism obtained by AP-PCR would allow discrimination between these Leishmania species, M13/pUC forward (AP1) and reverse sequencing primers (AP2) were used as arbitrary primers for random amplification of kDNAs from the cutaneous Leishmania species. The annealing temperature was kept at 40°C. At this low temperature, the primers can be expected to anneal to many sequences with a variety of mismatches. Sequences between these positions will be PCR amplifiable. The polymorphic band pattern thus obtained clearly differentiated the species (Fig. 3). Despite the small number of amplified fragments, the polymorphism generated was informative enough to discriminate the kDNAs of the cutaneous species which gave identical PCR patterns when specific primers were used.

Polymorphisms in L. donovani isolates by AP-PCR

To determine whether fingerprints obtained by AP-PCR allow discrimination between strains of the same species, the AP-PCR products from five isolates of L. donovani were electrophoresed on a single gel (Fig. 4). The same AP1 and AP2 primers were used as arbitrary primers. Identical band patterns were obtained in two L. donovani isolates (GE1 and GE2), while the other three isolates (GE4, GE6 and AG83) showed distinct banding patterns indicative of genetic polymorphism. This result probably reflects sequence differences between the genomes of L. donovani isolates. This AP-PCR appears to be a highly promising tool which allows the differentiation of even closely related strains of the same species and in this respect it is similar to isozyme studies [15].

To summarize, our data indicate that PCR and AP-PCR can be successfully applied to differenti-
Fig. 1. Schematic diagram of a *Leishmania* strain UR6 major class kDNA minicircle showing the position of the primers used. The primers sequences (Lsmc1 and Lsmv1) are shown below the diagram, with arrows indicating the direction of DNA synthesis for each primer. Numbers within brackets denote the position of nucleotide sequences. E and P represent EcoRI and PstI sites.

Fig. 2. PCR amplification using the primers Lsmc1 and Lsmv1 of kDNA minicircles from various *Leishmania* species. 10 ng of kDNA was used in each PCR in (A), (B) and (C) and 25 ng of total nucleic acid was used in each PCR in (D). (A) Lanes: 1, marker, ∅ × 174 DNA digested with *Hae*III; 2, no DNA; 3, pLURKE3; 4, *Leishmania* strain UR6; 5, *L. tropica* major (L.RC 408); 6, *L. mexicana* (L. 577); 7, *L. chagasi* (L. 684); 8, *L. major* (WR 309); 9, *L. guyanensis* (L. 565). (B) Lanes: 1, no DNA; 2, marker as in (A); 3, *L. amazonensis* (Josefa strain); 4, *L. amazonensis* (PH 815); 5, *L. braziliensis* (M2903); 6, *L. chagasi* (PP75); 7, *L. guyanensis* (M4147); 8, *L. panamanensis* (LS94); 9, *Leishmania* UR6. (C) Lanes: 1, marker as in (A); 2, no DNA; 3, *L. tropica* (L. 571); 4, *L. donovani* (Peking); 5, *L. infantum* (IPTI); 6, *Leishmania* UR6. (D) Lanes: 1, no DNA; 2, marker, pBR322 digested with *Hpa*II; 3, *L. donovani* (GE1); 4, *L. donovani* (GE2); 5, *L. donovani* (GE4); 6, *L. donovani* (GE8); 7, *L. donovani* (DD8); 8, *L. infantum* (Visceral isolate from a dog in Spain); 9, *L. donovani* (AG83); 10, *Leishmania* UR6.
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