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Development of a direct species-specific PCR assay for differential diagnosis of *Leishmania tropica*

Milan Jirků[a,b,1], Eva Zemanová[a,b,1], Amer Al-Jawabreh[c,d], Gabriele Schönian[c], Julius Lukeš[a,b,*]

[a]*Institute of Parasitology, Czech Academy of Sciences, 37005 České Budějovice, Czech Republic*
[b]*Faculty of Biology, University of South Bohemia, České Budějovice, Czech Republic*
[c]*Institute of Microbiology and Hygiene, Charité, Humboldt University, Berlin, Germany*
[d]*Islah Medical Laboratory, Islah Charitable Social Society, Jericho*

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Abstract

We have developed a polymerase chain reaction assay for differential diagnosis of *Leishmania tropica*, based on simple amplification of the target region. The assay detects less than 5 protozoan cells, was tested on human samples and an experimentally infected animal, and is appropriate for clinical laboratories in countries where leishmaniasis is endemic.

Keywords: Kinetoplastids; *Leishmania tropica*; PCR assay; Differential diagnosis

1. Introduction

Kinetoplastid flagellates of the genus *Leishmania* are causative agents of leishmaniasis, 1 of the 6 most deadly human parasitic diseases. The pathology of leishmaniasis varies from painless cutaneous lesions to a visceral disease with clinically rich symptoms and a very serious prognosis. In humans and other vertebrates, *Leishmania* flagellates occur as tiny round amastigote stages that can be found in, or cultured from, skin ulcers, bone marrow, aspirate fluid, and/or biopsies from visceral organs. Due to their low abundance, diagnosis based on finding *Leishmania* cells is very difficult, whereas histopathologic and immunologic diagnostic tools fail to discriminate among species and have suboptimal sensitivity. Yet, establishing the species responsible for infection is critical for proper treatment and control measures because the clinical prognosis, treatment protocols, and control schemes differ for individual *Leishmania* species.

Until quite recently, cultivation of flagellates obtained from clinical material was necessary to obtain sufficient amount of cells required for multilocus isoenzyme electrophoresis, a laborious and rather sophisticated method that enables unambiguous species determination (Pratlong et al., 2001). Unfortunately, its routine use is confined to a handful of specialized laboratories, whereas determination in the field and standard clinical laboratories is usually based on geographic origin of the strain and clinical manifestation of the disease.

Molecular techniques such as polymerase chain reaction (PCR) bear promise to improve this situation. PCR assays for species-specific diagnosis of leishmaniasis in clinical material target small subunit rRNA gene (van Eys et al., 1992), miniexon locus (Ramos et al., 1996), kinetoplast DNA minicircles (Noyes et al., 1998; Salotra et al., 2001), telomeric repeats (Chiurillo et al., 2001), internal transcribed spacer (Schönian et al., 2003), gp63 (Dujardin et al., 2002), glucose-6-phosphate dehydrogenase (Castilho et al., 2003), and nuclear repetitive sequence (Gangneux et al., 2003). Amplicons obtained by some of the abovementioned PCR assays contain single nucleotide differences or short regions characteristic for a given species or group of strains and provide sequence information valuable for phylogenetic analyses of the genus *Leishmania.* Because in most clinical laboratories, routine sequencing is impossible, the amplified

* Corresponding author. Institute of Parasitology, Czech Academy of Sciences, 37005 České Budějovice, Czech Republic. Tel.: +42-0-38-7775416; fax: +42-0-38-5310388.
E-mail address: jula@paru.cas.cz (J. Lukeš).
[1] These authors contributed equally to this work.

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fragments must be digested with selected restriction enzymes. This is not a trivial procedure because it requires expensive and unusual restriction enzymes, sufficient amount of PCR product, and additional technical controls. Designing of a test based on direct PCR amplification of a species-specific fragment would therefore represent an important step toward fast, affordable, and reliable diagnostics of leishmaniasis. Herein, we present a *Leishmania tropica*-specific PCR test that distinguishes this species from the other causative agents of the New and Old World leishmaniases.

2. Materials and methods

2.1. Parasites

Nine World Health Organization (WHO) reference strains and 1 new strain of *L. tropica* originating from geographically distinct locations were used in this study. Total DNA isolated from the following kinetoplastids was used as control: *Leishmania aethiopica*, *Leishmania amazonensis*, *Leishmania arabica*, *Leishmania archibaldi*, *Leishmania braziliensis*, *Leishmania donovani* (3 strains), *Leishmania gerbilli*, *L. guyanensis*, *L. infantum* (2 strains), *Leishmania major* (6 strains), *Leishmania mexicana*, *L. turanica*, *Trypanosoma brucei* 29-13 (all Trypanosomatina), *T. brucei* 29-13, and *Trypanoplasma borreli* Tp-Tt (Bodonina) (Table 1). Total DNA was isolated from 10^5 to 10^7 flagellates using the DNA isolation kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France).

2.2. PCR conditions

L. tropica species-specific primers B6-F (GCTCTGCCCACGCACACACAG) and B6-R (CGGTGCCTGCCAAGTA) were used in 25 μL of PCR reaction mixtures containing 10 pmol of each primer, reaction buffer (100 mmol/L Tris–HCl, pH 8.8; 500 mmol/L KCl, 1% Triton X-100; 15 mmol/L $MgCl_2$), 0.25 mmol/L of dNTPs, 1 U of Taq polymerase (Top-Bio, Prague, Czech Republic), and varying amount of DNA (see below). The reaction mixtures were amplified in Eppendorf Mastercycler at 94 °C for 5 min followed by 30 cycles, each consisting of 30 s at 94 °C, 30 s at 68 °C, and 30 s at 72 °C, and a final extension of 10 min at 72 °C. PCR products were detected by electrophoresis in 1% agarose at 80 V in the presence of ethidium bromide.

2.3. PCR sensitivity

The sensitivity of the standard PCR procedure was determined using the *L. tropica* SU23 (Table 1). Cultured

Table 1
Strains of *Leishmania* and *Trypanosoma* spp. used in this study

Species	WHO strain code	Country of origin	Hosts	LT-1 PCR
L tropica Vedha	MHOM/TR/99/Vedha EP41	Turkey	Human	+
L tropica SU23	MHOM/TR/1998/SU23	Turkey	Human	+
L tropica 75	MHOM/PS/02/34JnF4	Palestine	Human	+
L tropica 68	MHOM/PS/02/ISL676	Palestine	Human	+
L tropica 63	MHOM/PS/01/LRC-L838	Palestine	Human	+
L tropica 60	MHOM/PS/01/ISL592	Palestine	Human	+
L tropica 57	MHOM/PS/01/ISL593	Palestine	Human	+
L tropica L810	IARA/IL/00/Amnunfly1	Israel	Human	+
L tropica OD	MHOM/SU/58/OD	Former Soviet Union	Human	+
L tropica K27	MHOM/SU/74/K27	Former Soviet Union	Human	+
L. aethiopica	MHOM/ET/72/L100	Ethiopia	*Phlebotomus arabicus*	–
L. amazonensis	MHOM/BZ/82/M2269	Belize	Human	–
L. arabica	MPSA/SA/83/JISH220	Saudi Arabia	*Psammomys obesus*	–
L. archibaldi	MHOM/ET/72/GEBRE1	Ethiopia	Human	–
Leishmania brasiliensis	MHOM/BR/75/M2903	Brazil	Human	–
L. donovani	MHOM/IN/80/DD8	India	Human	–
L. donovani	MHOM/ET/67/HU3 (LV9)	Ethiopia	Human	–
L. donovani	MHOM/IN/96/THAK35	India	Human	–
L. gerbilli	MRHO/CN/60/Gerbilli	China	*Rhombomys opinus*	–
L. guyanensis	MHOM/BR/75/M4147	Brazil	Human	–
L. infantum	MHOM/CN/80/STRAIN A	China	Human	–
L. infantum	MHOM/FR/95/LPN114	France	Human	–
L. major	MHOM/SU/73/5-ASKH	Turkmenistan	Human	–
L. major	MHOM/PS/00/ISL506	Palestine	Human	–
L. major	MHOM/DZ/98/LPS13	Algeria	Human	–
L. major	MHOM/SN/96/DPPE23	Senegal	Human	–
L. major	MHOM/IL/80/Friedlin	Israel	Human	–
L. major	MTAT/KE/8?/NLB089A	Kenya	Human	–
L. mexicana	MHOM/BZ/73/BEL21	Belize	Human	–
L. turanica	MRHO/SU/83/MARZ-051	Former Soviet Union	*R. opinus*	–
T. brucei	29–13	Laboratory strain		–
T. borreli	Tp-Tt	Czech Republic		–

promastigotes of this strain were counted using a cell counter (Beckman Z2) and resuspended in 500 μL of whole human blood. Twenty microliters of proteinase K was added to 100 μL of anticoagulated blood; the volume was adjusted to 220 μL with phosphate-buffered solution, and 200 μL of the AL buffer (Qiagen) was added. The mixture was incubated for 10 min at 70 °C, and the DNA was extracted using the DNeasy Tissue Kit (Qiagen). DNA extracted from the equivalent of 10, 10^2, 10^3, and 10^4 cells in human blood was resuspended in 50 μL of distilled water. The isolation was performed twice in parallel. For PCR amplification, 2.5 μL of the sample DNA was used. The volume of extracted DNA was equivalent to the number of promastigotes using the following formula: [number of cells used for the extraction/dilution volume at the end of the DNA extraction], e.g., the DNA extraction from 400 promastigotes diluted 1:50 means that 1 μL contains the DNA of 8 promastigotes. The PCR protocol was tested to the amount of DNA theoretically equivalent to 0.5 promastigote cell.

2.4. Clinical material

To check for the validity of PCR as a differential diagnostic test, we evaluated sensitivity and specificity using 18 randomly selected clinical samples of different types taken from Palestinian patients who were referred to the Islah Medical Laboratory in Jericho for diagnosis between the years 2000 and 2004. With 56% and 44% of the samples obtained from children and adults, respectively, 44% patients had a single lesion, 28% had 2 lesions, and the same percentage had multiple lesions. The distribution of lesions was as follows: 72% in the head and neck, and 17% and 6% in the upper and lower extremities, respectively. Twenty-eight percent of the lesions were ≤1 month old, 56% were 1–2 months old, and 17% were 3 months old and older. The clinical samples were diagnosed by microscopy of Giemsa-stained smears and confirmed and genotyped by internal transcribed spacer 1 (ITS1)-PCR (Schönian et al., 2003).

Three types of positive samples were used, of which 8 were tissue and blood blotted on 3-mm filter papers, 8 were unstained tissue slides, and 2 were 5 × 5-mm batches obtained from Giemsa-stained smears. Giemsa-stained slides prepared from 10 blood samples of randomly selected healthy native German blood donors from the Charité Hospital in Berlin who have not visited a tropical country were used as a negative control. Again, the negative control samples were checked using microscopy and ITS1-PCR. The integrity of extracted DNA was checked by amplifying the human housekeeping β-actin gene. In the case of filter paper samples, 2 punched discs were incubated in 250 μL of lysis buffer (50 mmol/L NaCl; 50 mmol/L Tris, pH 7.4; 10 mmol/L EDTA, pH 8.0; 1% vol/vol Triton X-100; 100 μg of proteinase K per milliliter) at 60 °C overnight. DNA was extracted from the lysates by phenol–chloroform extraction as described elsewhere (Meredith et al., 1993). As for the smears, the material was scrapped off the slides and treated as described above.

2.5. Experimental infection

Male golden hamster (*Mesocricetus auratus*) was infected by a subcutaneous injection of 10^6 stationary phase promastigotes of *L. tropica* SU23 (Table 1) into the rear right leg. Five months after the injection, the hamster was anesthetized and tissue scrapings were obtained from the central and peripheral regions of the wet ulcer that developed in the site of injection. DNA from this material was isolated as described above. DNA was also isolated from the left ear of the hamster, as well as from the tail and ear of a control laboratory mouse.

2.6. Nucleotide sequence accession number

Nucleotide sequence data reported in this article have been submitted to the GenBank database with the accession number AY919872.

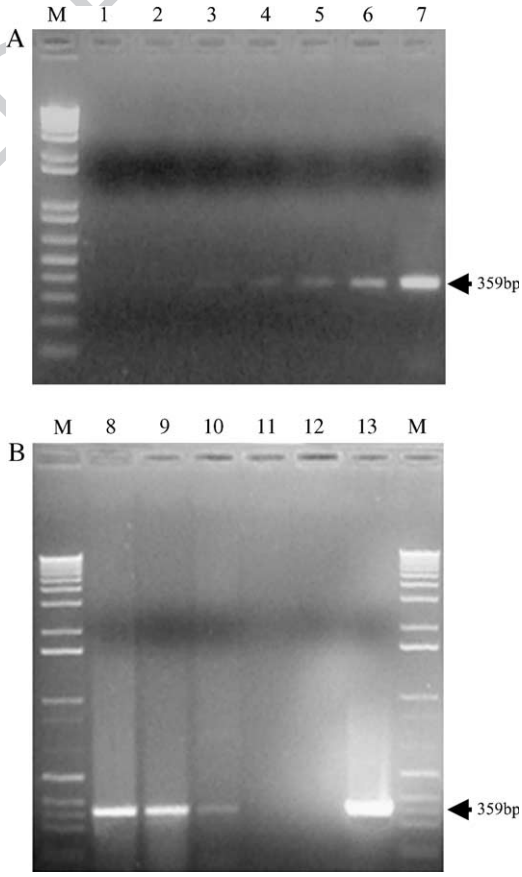

Fig. 1. Sensitivity of the LT-1 assay tested with cells of the *L. tropica* strain Vedha mixed with human blood (A) and with the *L. tropica*-positive and negative animals (B). (A) Tenfold dilutions of promastigotes were used for PCR reactions as follows: the equivalent of 0.5 cell (lane 2), 5 cells (lane 3), 50 cells (lane 4), 500 cells (lane 5), and 5000 cells (lane 6). Flagellate-free human blood was used as a negative control (lane 1). DNA isolated from 10^5 cultured promastigotes served as a positive control (lanes 7 and 13). (B) DNA isolated from peripheral (lane 8) and central part of the lesion (lane 9), ear (lane 10) of an infected golden hamster, and ear (lane 11) and tail (lane 12) of a noninfected mouse was used. Q4

3. Results and discussion

One amplicon specific for the *L. tropica* strain Vedha, amplified in the frame of an extensive RAPD analysis (Zemanová et al., 2004), was cloned and sequenced. The 359-bp long fragment, labeled LT-1, has 87% similarity with an intergenic region derived from the *L. major* Friedlin chromosome 31. Primers B6-F and B6-R derived from the 5′ and 3′ ends of the amplicon were tested with 9 other *L. tropica* strains originating from geographically distinct locations that were previously unambiguously shown to belong to *L. tropica* (Schönian et al., 2001, 2003). At the annealing temperature of 68 °C, the primer pair produced a single and abundant approximately 360-bp fragment from all *L. tropica* strains, whereas the PCR was negative with DNAs of other New and Old World *Leishmania* species (*L. aethiopica*, *L. amazonensis*, *L. arabica*, *L. archibaldi*, *L. braziliensis*, *L. donovani*, *L. gerbilli*, *L. guyanensis*, *L. infantum*, *L. major*, *L. mexicana*, and *L. turanica*), and *T. brucei* and *T. borreli*. To exclude the presence of PCR inhibitors, using primers and conditions described elsewhere (Schönian et al., 2003), we amplified the ITS1 region from all the DNA samples tested (data not shown).

The LT-1 PCR assay was sensitive enough to detect an equivalent of less than 5 parasite cells per reaction in human blood spiked with 10-fold dilution of the *L. tropica* promastigotes (Fig. 1A). In another sensitivity test, DNA isolated from various tissues of an experimentally infected golden hamster was used (Fig. 1B). Although the parasite was shown to be very rare in some parts of hamsters infected in the same way (Svobodová and Votýpka, 2003), all DNA samples were positive. No amplification occurred from DNA isolated from ear and tail of a *Leishmania*-free control mouse (Fig. 1B).

In a double blind experiment, the LT-1 assay was evaluated for its sensitivity and specificity using 18 samples of 3 types (see Materials and methods) obtained from skin lesions of patients examined in Jericho, Palestinian Authority. Previously, these samples were evaluated by the ITS1-PCR assay (Schönian et al., 2003). Both healthy individuals and patients infected with *L. major*, regardless of the sampling method, were negative, giving a specificity of 100%. On the other hand, all *L. tropica* patients were unambiguously positive, giving a sensitivity of 100%.

L. tropica is a perplexing parasite (Jacobson, 2003) with a wide geographic distribution, ranging from the Greek Islands to India on the east–west axis and from Turkmenistan to Namibia from north to south. Because it is so widespread, *L. tropica* overlaps with other causative agents of leishmaniasis, especially *L. aethiopica*, *L. donovani*, *L. infantum*, and *L. major* (Ashford, 2000). Besides the fact that *L. tropica* seems to be a species with exceptionally high intraspecific diversity (Schönian et al., 2001; Schnur et al., 2004), it causes variable clinical manifestations predominately associated with dry and wet lesions (Jacobson, 2003; Jaffe et al., 2004). Moreover, it has also been associated with visceral infections of US military personnel (Magill et al., 1993) and patients in Kenya (Mebra et al., 1989). The LT-1 assay is suitable for clinical studies because it performed equally well with a spectrum of samples and is as sensitive as other PCR-based assays used to detect *Leishmania* in clinical material (Marfurt et al., 2003a; Noyes et al., 1998; Schönian et al., 2003). However, its major advantage rests in its simplicity because all other PCR-based tests for the species diagnosis of *Leishmania* require a 2-step protocol. After the ITS1 region, small subunit rRNA or spliced leader RNA genes are amplified; only a specific pattern produced by restriction enzyme digestion enables to distinguish the species from which the amplicon is derived (Marfurt et al., 2003a, 2003b; Schönian et al., 2003). The LT-1 assay has also a potential for epidemiologic studies when it comes to the identification of infected sand flies and putative animal hosts. *L. tropica* infections seem to be transmitted anthroponotically in urban areas in Asia and rather zoonotically in rural places in the Middle East and Africa, but the animal reservoirs have yet to be identified. We hope that the identification of at least one species-specific region or a sequence motif, such as the one presented here for *L. tropica*, can be done for every pathogenic *Leishmania* species. Such data may eventually lead to a multiplex PCR assay that would allow the identification of a species within a few hours using simple PCR apparatus and agarose gel equipment. Search for such regions in other *Leishmania* species is under way.

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