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RESEARCH NOTE

A DIAGNOSTIC ASSAY BASED ON VARIABLE INTERGENIC REGION DISTINGUISHES BETWEEN *LEISHMANIA DONOVANI* AND *LEISHMANIA INFANTUM*

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Abstract. We have developed a PCR assay that in a single reaction distinguishes between *Leishmania infantum* and *Leishmania donovani* strains on the basis of different size of the amplicon. The targeted intergenic region between putative biopterin transporter and nucleotide binding protein on chromosome 35 is highly variable, species-specific and can be amplified from clinical samples. Based on the assay, five tested *Leishmania archibaldi* and *L. infantum* strains from the Sudan and Ethiopia clearly belong to *L. donovani*, which is in accordance with a recent multifactorial analysis of these strains. The nucleotide sequence reported in this paper has been submitted to the GenBank™ with accession number EU068004.

Kinetoplastid flagellates of the genus *Leishmania* Ross, 1903 are the causative agents of leishmaniases, a group of diseases that are estimated to affect 12 million people, with 350 million people at risk and the death toll of about 57,000 worldwide annually (Desjeux 2004). The clinical symptoms range from asymptomatic or mild cutaneous leishmaniasis to severe mucocutaneous and visceral forms of the disease. In the Old World, cutaneous form is mainly caused by *Leishmania major*, *L. tropica* and *L. infantum*, whereas *L. donovani* and *L. infantum* are the main species responsible for visceral leishmaniasis. Since pathology is generally species-specific, fast and unambiguous species identification is necessary for the choice of proper therapy and prognosis of the clinical outcome.

Until the 1990s, diagnosis of these parasites was based on the microscopic detection of amastigotes in clinical samples and/or enzymatic characterisation of promastigotes obtained in culture. Only the latter method was able to distinguish among the *Leishmania* species, yet it is tedious, time-consuming and requires specialised equipment and expertise. Thus, the requirement for simple, rapid, specific and sensitive diagnostic methods for leishmaniases is obvious. In the last decade, the potential to meet these criteria was demonstrated for PCR-based methods amplifying kinetoplast DNA minicircles (Noyes et al. 1998), ribosomal intergenic regions (El Tai et al. 2001), gp63 gene (Dujardin et al. 2002), glucose-6-phosphate dehydrogenase gene (Castilho et al. 2003) and the miniexon sequence (Marfurt et al. 2003), to name the most widely used DNA targets. Amplicons usually contain single nucleotide polymorphisms or short regions characteristic for a given species or a group of strains, and provide sequence information valuable for species identification and phylogenetic analysis. However, since for almost all clinical laboratories in the endemic countries routine sequencing is not available, usually the amplicons must be digested with selected restriction enzymes to identify species-specific sites (Schönian et al. 2003, Botilde et al. 2006). In order to determine the *Leishmania* species by PCR assays yielding a single species-specific product, one has to run several reactions with different primers and a number of controls (El Tai et al. 2001, Dujardin et al. 2002, Jirků et al. 2006). Elimination of these additional steps would streamline PCR-based diagnosis, make it cheaper, less time-consuming and more user-friendly. Eventually, it would also strengthen its position among diagnostic approaches towards leishmaniases in the community of clinicians. A novel assay fulfilling such requirements for two *Leishmania* species is introduced in this work.

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In an attempt to find species-specific regions, we performed a detailed *in silico* search in the available sequence data. Such data mining allowed us to identify a 1.8 kb long intergenic region on chromosome 35 of the Friedlin strain of *L. major*, flanked by genes encoding putative biopterin transporter and nucleotide-binding proteins (L25643) (Ivens et al. 2005), in which *L. infantum* substantially differs from *L. major*. Assuming that it may be a highly variable region, the 3' end of the former gene was used to design the forward primer TGAAGCGGGACAGCTAGC (position 2003479–2003496), while the reverse primer TCTGCCGTGCTATTTAG covers the 5' part of the latter gene (position 2005292–2005308). About 10 ng of DNA from cultured promastigotes (Table 1), or an undetermined but much lower amount of DNA from amastigotes in clinical samples (Table 2), 10 pmol of each primer, reaction buffer (100 mM Tris-HCl, pH 8.8; 500 mM KCl; 1% Triton X-100; 15 mM $MgCl_2$), 0.25 mM dNTPs and 0.5 U *Taq* polymerase (Top-Bio, Prague) were processed in an Eppendorf Mastercycle through 30 cycles consisting of 60 s at 95°C, 60 s at 58°C and 90 s at 72°C. Amplicons were separated in 1% agarose gel at 80V in 0.5× TBE buffer in the presence of ethidium bromide and visualised under UV light. Selected amplicons were cloned and sequenced.

Based on the *L. major* genome sequence, the PCR product was predicted to be 1.8 kb long. Indeed, a single product of this size was obtained from all eight African strains tested, regardless of their current species affiliation (see below), and from *L. donovani* originating from India (four strains) and Saudi Arabia (two strains) and, as expected, from *L. major* (data not shown) (Table 1) (Fig. 1). However, all 21 assayed *L. infantum* strains consistently gave a strong ~1.4 kb long amplicon (Fig. 2), whereas no product was obtained with *L. tropica*, *L. aethiopica*, *L. arabica*, *L. turanica* and *L. gerbilli* (data not shown). Thus, the PCR reaction was able to distinguish between *L. infantum* and *L. donovani* by amplifying a product of species-specific size (Figs. 1, 2).

Table 1. Strains of *Leishmania* spp. used in this study in the form of DNA obtained from cultured promastigotes.

Species[a]	Lab code	WHO code	Zymodeme[c]	Origin	Type of infection or Host	Size of amplicon in bp
L. infantum	INF-01	MHOM/FR/1978/LEM75[b]	MON 1	France	Visceral	1486
	INF-02	MHOM/FR/1995/LPN114	MON 1	France	Visceral	1486
	INF-03	MHOM/1993/PM1	MON 1	Spain	Visceral	1486
	INF-04*	MHOM/FR/1997/LSL29	MON 1	France	Cutaneous	1486
	INF-05	MHOM/ES/1986/BCN16	MON 1	Spain	Cutaneous	1486
	INF-06	MHOM/PT/2000/IMT260	MON 1	Portugal	Cutaneous	1486
	INF-07	MHOM/FR/1996/LEM3249	MON 29	France	Cutaneous	1486
	INF-08*	MHOM/ES/1991/LEM2298	MON 183	Spain	Visceral	1486
	INF-14	MHOM/FR/1980/LEM189	MON 11	France	Visceral	1486
	INF-15*	MHOM/MT/1985/BUCK	MON 78	Malta	Visceral	1486
	INF-19	MHOM/ES/88/LLM175	MON 198	Spain	Visceral	1486
	INF-20	MHOM/ES/92/LLM373	MON 199	Spain	Visceral	1486
	INF-21*	MHOM/IT/94/ISS1036	MON 228	Italy	Visceral	1486
	INF-22	MHOM/IT/93/ISS800	MON 188	Italy	Visceral	1486
	INF-26*	MHOM/GR/2001/GH6	MON 98	Greece	Visceral	1486
	INF-27	MCAN/GR/2001/GD8	MON 98	Greece	Visceral	1486
	INF-28	MHOM/GR/2003/GH15	MON 98	Greece	Visceral	1486
	INF-33	MHOM/GR/2003/GH16	MON 98	Greece	Visceral	1486
	INF-34	MHOM/GR/2003/GH18	MON 98	Greece	Visceral	1486
	INF-35	MHOM/GR/2003/GH20	MON 98	Greece	Visceral	1486
	INF-36	MHOM/GR/2004/GD17	MON 98	Greece	Visceral	1486
L. archibaldi	ARC-11*	MHOM/ET/1972/GEBRE 1	MON 82	Ethiopia	Visceral	1880
	ARC-24*	MHOM/SD/97/LEM3429	MON 257	Sudan	Visceral	1880
	ARC-25	MHOM/SD/97/LEM3463	MON 258	Sudan	Visceral	1880
L. donovani	DON-09	MHOM/IN/0000/DEVI	MON 2	India	Visceral	1880
	DON-10*	MHOM/IN/1996/THAK35	MON 2	India	Visceral	1880
	DON-12	MHOM/SD/1982/GILANI	MON 30	Sudan	Visceral	1880
	DON-13*	MHOM/ET/0000/HUSSEN	LON 42	Ethiopia	Visceral	1880
	DON-16	MHOM/IN/54/SC23	MON 38	India	Visceral	1880
	DON-17*	MCAN/SD/2000/LEM3946	MON 274	Sudan	Visceral	1880
	DON-29	MHOM/IN/80/DD8	MON 2	India	Visceral	1880
	DON-31	MHOM/SA/81/KA-Jeddah	LON 42	Saudi Arabia	Visceral	1880
	DON-32*	MHOM/SA/87/VL29	LON 42	Saudi Arabia	Visceral	1880
L. infantum	INF-18*	MHOM/SD/62/3S	MON 81	Sudan	Visceral	1880
in Africa	INF-23*	MHOM/SD/97/LEM3472	MON 267	Sudan	Visceral	1880
L. tropica	TRO	MHOM/SU/74/K 27	MON 60	Former Soviet Union	?	–
L. aethiopica	AET	MHOM/ET/72/L100	MON 14	Ethiopia	*Phlebotomus arabicus*	–
L. arabica	ARA	MPSA/SA/83/JISH220	MON 99	Saudi Arabia	*Psammomys obesus*	–
L. turanica	TUR	MRHO/SU/83/MARZ-051	?	Former Soviet Union	*Rhombomys opimus*	–
L. gerbilli	GER	MRHO/CN/60/Gerbilli	?	China	*R. opimus*	–

[a]Strain identification according to isoenzyme analysis; [b]WHO reference strain; [c]zymodemes are indicated as typed by the reference laboratory, Montpellier (MON) or London (LON); *strains used for Figs. 1 and 2.

Table 2. Clinical samples of *Leishmania infantum* and *L. donovani* used in this study.

Clinical samples	Code	Zymodeme	Origin	Type of infection	Sample	Size of amplicon in bp
L. infantum	L-984	MON 1	Spain – Madrid		Human (Bone Marrow) –liver transplantant	1486
	L-1122	MON 1	Spain – Mallorca	Visceral (HIV+, Relapse)	Human (Bone Marrow)	1486
	L-1149	MON 1	Spain – Ibiza		Canine (Lymph Node)	1486
	I-288	MON 1	Portugal – Alto Douro	Visceral (HIV-)	Human (Bone Marrow)	1486
	I-337	MON 1	Portugal – Alto Douro	Cutaneous (HIV-)	Human (lesions)	1486
	I-170	MON 1	Portugal – Alto Douro		*Phlebotomus*	1486
	I-300	MON 1	Portugal – Setubal		Canine (Bone Marrow)	1486
	I-238	MON 1	Portugal – Lisboa	Visceral (HIV+)	Human (Bone Marrow)	1486
L. donovani	Don J–Q (8 samples)	?	Nepal (Siraha)	Visceral	Human (Bone Marrow)	1880

Next, using the amplification primers we sequenced three *L. infantum*, two *L. donovani* and one *L. archibaldi* amplicons (data not shown). The alignment of obtained sequences showed that, as expected, the 1.8 kb long sequence of the Sudanese strains covers the intergenic region. Unexpectedly however, in the case of *L. infantum*, the 1.4 kb long PCR product is flanked on both ends by the forward primer that anneals to the putative integral membrane protein and miss-anneals to the intergenic region (position 1469–1486). In the overlapping part, the sequence is species-specific with several

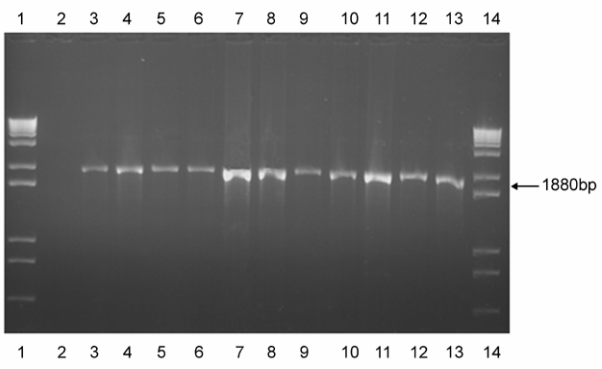

Fig. 1. Species-specificity of the PCR assay shown with a set of *Leishmania donovani*. DNA was obtained from cultured procyclics (strains DON-17, DON-13, DON-10 and DON-32) (lanes 3–6), clinical samples of *L. donovani* from Nepal (see Table 2) (lanes 7–9), cultured procyclics of Sudanese and Ethiopian "*L. archibaldi*" (strains ARC-24 and ARC-11) (lanes 10 and 11) and Sudanese "*L. infantum*" (strains INF-18 and INF-23) (lanes 12 and 13) (see text for details on species assignment). Flagellate-free human blood was used as a negative control (lane 2). 1 kb ladder DNA size marker (Gibco-BRL) (lanes 1 and 14). The size of diagnostic PCR product is indicated.

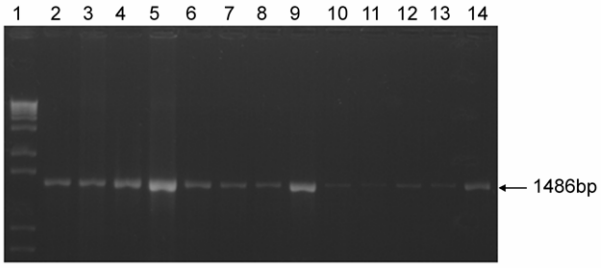

Fig. 2. Species-specificity of the PCR assay shown with a set of *Leishmania infantum*. DNA was obtained from cultured procyclics (INF-08, INF-21, INF-04, INF-35 and INF-15) (lanes 2–6), and clinical samples with previous diagnosis of *L. infantum* (984, 1122, 1141, 288, 337, 170, 300 and 238) (lanes 7–14). 1 kb ladder DNA size marker (Gibco-BRL) (lane 1). The size of diagnostic PCR product is indicated.

highly variable regions (EU068004). As predicted by the sequence analysis, the same PCR product can be obtained in *L. infantum* with the forward primer only (data not shown). Despite the fact that one primer is miss-annealing, the PCR reaction was fully reproducible with all tested *L. infantum* DNA templates.

In order to check the suitability of the above-described assay for differential diagnosis of clinical samples, bone marrows obtained by puncture from five infected humans and two dogs with clinical symptoms of visceral leishmaniasis were used (Table 2). We also tested a tissue sample of one person suffering from cutaneous leishmaniasis and DNA obtained from an infected *Phlebotomus* sp. These clinical samples were collected in 2001 and 2002 in Spain and Portugal from individuals in whom *L. infantum* was previously diagnosed using multilocus enzyme electrophoresis, with HIV co-infection detected in two cases (Campino et al. 2006). Furthermore,

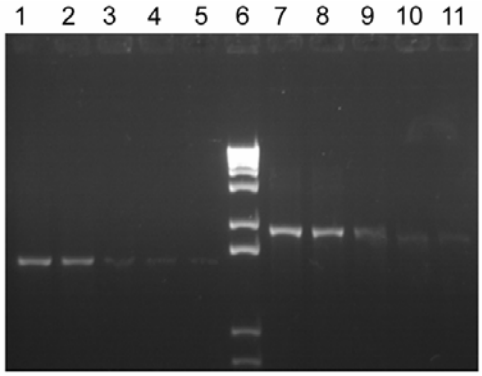

Fig. 3. Sensitivity of the assay was tested with cells of the *Leishmania infantum* and *L. donovani* strains INF-04 and DON-17, respectively. Tenfold dilutions of DNA isolated from cultured procyclics were used for PCR reactions. DNA concentrations of *L. infantum* INF-04 were 270 pg (lane 1), 135 pg (lane 2), 67.5 pg (lane 3), 45 pg (lane 4) and 27 pg (lane 5). DNA concentrations of *L. donovani* DON-17 were 9.3 ng (lane 7), 930 pg (lane 8), 186 pg (lane 9), 93 pg (lane 10) and 62 pg (lane 11). 1 kb ladder DNA size marker (Gibco-BRL) (lane 6).

bone marrow samples of eight individuals were collected by volunteers in 2002 in the rural Siraha district on the border between Nepal and India. All patients had clinical symptoms of kala-azar and were thus presumably infected by *L. donovani* (Regmi S., Southern Methodist University, Dallas, USA, pers. comm.). All human and canine samples were diagnosed by microscopy of Giemsa-stained smears (Campino L., Institute of Hygiene and Tropical Medicine, Lisbon, Portugal; Chicharro C., National Centre for Microbiology, Majadahonda, Spain; pers. comm.). DNA from drops of bone marrow tap from the filter papers or smeared material scrapped off the slides was isolated using 250 µl of lysis buffer (50 mM NaCl; 10 mM EDTA; 50 mM Tris-HCl, pH 7.4; 1% v/v Triton X-100; 200 µg/ml proteinase K) at 60°C and than extracted by phenol-chloroform extraction as described elsewhere (Maslov et al. 1996). The integrity of extracted DNA was checked by PCR with primers for the human housekeeping β-actin gene (data not shown). Next, PCR assay for the presence of *Leishmania* spp. DNA based on the internal transcribed spacer 1 (Schönian et al. 2003) was used to evaluate all clinical samples (data not shown). Several samples of randomly selected German and Czech blood donors, who have never visited countries endemic for leishmaniasis, were used as negative controls. The PCR assay was sensitive enough to detect either species in all tested human, canine and phlebotomine samples (Figs. 1, 2). When 10-fold dilutions of DNA from promastigotes of the *L. infantum* strain INF-04 and *L. donovani* strain DON-17 were used, the amplicon was obtained from less than 20 pg and 60 pg of *Leishmania* DNA, respectively (Fig. 3).

When compared with related trypanosomes, members of the genus *Leishmania* exhibit generally low genetic diversity and inter-specific differences are usually confined to single nucleotide polymorphisms, more or less evenly dispersed throughout the coding and non-coding regions (Mauricio et al. 2006, Lukeš et al. 2007). From the alignment it is apparent that the intergenic region in question belongs to highly variable regions of the *Leishmania* genome, yet the sequence remains species-specific, as exemplified by six sequences

from three species (data not shown). Multilocus enzyme electrophoresis of a number of strains from the Sudan indicated that *L. infantum* and *L. archibaldi* co-exist with *L. donovani* in this region (Pratlong et al. 2001). However, re-examination of these strains by sequencing a dozen of protein-coding genes (Mauricio et al. 2006, Zemanová et al. 2007) and other molecular markers revealed that all Sudanese strains are monophyletic and shall be considered *L. donovani* (Lukeš et al. 2007). The capacity of this assay to affiliate these strains with *L. donovani* in accordance with the large-scale multifactorial analysis, and to distinguish between the *L. donovani* and *L. infantum* strains originating from geographically distant location, shows its strength.

We have demonstrated here that the highly variable intergenic region can be used as a diagnostic tool that enables distinction between two of the medically important *Leishmania* species in a single PCR reaction. Thanks to its simplicity and reliability, it is applicable for both clinical diagnostics and epidemiological studies. Moreover, since the species-specific amplicons are derived from the same region, this assay has a potential to be optimized for an oligochromatography-based diagnostic kit.

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