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Short communication

Species-specific PCR assay for *L. infantum*/*L. donovani* discrimination☆

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Abstract

Leishmania infantum and *Leishmania donovani* both pertain to the *L.* (*L.*) *donovani* complex. The status of certain strains is questioned in the literature and there are no reliable discriminative markers to identify them. Molecular tools are needed to (i) identify diagnostic markers and (ii) to allow a better understanding of phylogenetic relationships. We have developed a PCR based on *cysteine protease B* (*cpb*). This PCR discriminates between *L. infantum* and *L. donovani* with 50–100 pg of DNA. These two species are differentiated by their fragment length. Indeed, *L. donovani* strains were characterized by a 741-bp product and *L. infantum* strains by a 702-bp product, except for one strain, which revealed a heterozygous profile with the two products. This PCR does not generate amplification for other *Leishmania* or kinetoplastids and could contribute to clarify the phylogenetic status of several taxa that are also being debated, such as *L. archibaldi*.

Keywords: Cysteine protease B; *L. donovani* complex; Polymerase chain reaction

1. Introduction

Leishmania is a trypanosomatid responsible for leishmaniasis. This disease ranges in severity from a healing skin ulcer to an overwhelming visceral form. Leishmaniasis is a worldwide endemic disease, with an estimated disease burden of 2,357,000 disability-adjusted life years and 59,000 deaths per year (WHO, 2002). The most severe form (visceral leishmaniasis) is caused by the species of the *Leishmania donovani* complex. This visceral form is characterized by hepatosplenomegaly, anemia, immunosuppression, hypergammaglobulinemia and fever. The reference standard technique for *Leishmania* species typing is multilocus enzyme electrophoresis (MLEE) (Hide et al., 2001; Rioux et al., 1990). However, this method is not ideal, as it does not easily differentiate species within the *L. donovani* complex and requires a large amount of parasite. Indeed, the only isoenzymatic system (glutamate oxaloacetate transaminase, GOT E.C.2.6.1.1) that was supposed to give a discriminative pattern within the complex, has been shown not to be reliable, because some Sudanese strains identified as *Leishmania infantum* by GOT locus were shown to actually be *L. donovani* strains (Jamjoom et al., 2004). It is thus necessary to supplement this classical MLEE typing by specific PCR. The discrimination between *L. infantum* and *L. donovani* is important because its taxa

☆ *Note*: Nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers AY896776 AY896777, AY896778, AY896779, AY896780, AY896781, AY896782, AY896783, AY896784, AY896785, AY896786, AY896787, AY896788, AY896789, AY896790, AY896791.

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are morphologically indistinguishable but are associated with different epidemiology, ecology and pathology. *L. infantum* is anthropozoonotic with a dog reservoir, whereas *L. donovani* is largely anthroponotic. To develop a discriminative PCR, we focused on the cathepsin-L proteases CPB belonging to the papain-like superfamily, clan CA and family C1. They have attracted considerable attention because of their role in destruction of host protein and evasion of host immune response (Alexander et al., 1998). CPB enzymes are encoded by a tandem array located in a single locus. Within the *L. donovani* complex, Mundodi et al. (2002) have compared a *L. donovani* strain and a *L. chagasi* (syn *L. infantum*) strain and revealed at least five tandemly arranged genes. The last repeat of the *cpb* cluster was named *cpbE* for *L. infantum* and *cpbF* for *L. donovani*. We used these two *cpb* copies to design a discriminative PCR for the *L. donovani* complex. Within a sample that was representative of the genetic and clinical diversity of the *L. donovani* complex, we evaluated the use of this PCR for discrimination of the two species. Other *Leishmania* and *Trypanosoma* strains where included in this study.

2. Material and methods

2.1. Parasites

A sample of 32 strains that were representative of geographic and genetic diversity within the *L. donovani* complex was studied (Table 1). Ten parasite strains pertaining to other *Leishmania* species and to trypanosomatids were used to test PCR specificity (Table 1). All the *Leishmania* strains were typed using MLEE by the WHO reference centres of Montpellier (MON) and London (LON).

2.2. Clinical samples

Five clinical samples (spleen aspirates) were obtained in Uttar Pradesh from human patients with confirmed VL (S. Sundar, Banaras Hindu University, Varanasi, India).

2.3. Cell culture

Promastigote cultures were maintained at 26 °C by weekly sub-passages in RPMI1640 medium, buffered with 25 mM HEPES, 2 mM $NaHCO_3$ and supplemented with 10% heat-inactivated foetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were harvested by centrifugation and stored at −80 °C until DNA extraction.

2.4. Specific PCR of cpbEF copies

Genomic DNA was extracted from parasite pellets and clinical samples by classical phenol/chloroform extraction and from parasite culture (1 ml) by InstaGene™ Matrix (Bio-Rad). For the second method, after centrifugation, 200 μl of InstaGene™ matrix was added to the pellet and incubated 15 min at 56 °C, then heated 8 min at 100 °C.

The optimal conditions for *cpbEF* amplification in 30 μl were 6 pmol of each primer (forward: 5′-CGTGACGCCGGTGAAGAAT-3′; reverse: 5′-CGTGCACTCGGCCGTCTT-3′), 4.5 nmol dNTPs, 1 U Taq polymerase (Roche Diagnostics), 3 μl Buffer 10× and either 100 pg of genomic DNA or 1 μl of DNA after InstaGene™ extraction 200 μl or 1–5 μl of DNA extracted from clinical samples. Thirty cycles were necessary for amplification (denaturation 30 s at 94 °C, annealing 1 min at 62 °C and elongation 1 min at 72 °C), followed by 10 min at 72 °C. PCR amplification was also tested with 50 pg of genomic DNA. The amplification reactions were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light.

3. Results

Thirty-two DNA samples corresponding to the strains of the *L. donovani* complex were amplified using specific primers designed in this study. Optimal conditions were found using 100 pg, but 50 pg could be used by increasing the number of PCR cycles to 40. There is no amplification when less than 50 pg of DNA was used (with 30, 40 and 45 cycles) (data not shown). Two lengths of amplification products were obtained, 702 bp for *cpbE* and 741 bp for *cpbF* (Fig. 1). For the *L. donovani* and *L. archibaldi* strains, only the *cpbF* copy was obtained, whereas the *L. infantum* strains contained only *cpbE*, except one strain. The *L. infantum* strain LIPA59 (a strain from Algeria isolated from the cutaneous form of the disease) had a heterozygous pattern comprising the two *cpb* copies *E* and *F*. This heterozygous pattern has been obtained from different cultures of a given LIPA59 clone (obtained by micromanipulation) and also from different clones. Thus, this pattern was not due to a mixture of two strains or just to a contamination. Furthermore, other results based on microsatellite genotyping and sequencing of other *cp* copies (*cpa* and *cpb*) as well as *lpg2*, *amastin* and *iunh*, have confirmed the heterozygous status of this strain LIPA59 (Hide, 2004). The three Sudanese strains, originally typed by MLEE as *L. infantum*, presented a PCR product corresponding to

Table 1
Leishmania strains used in this study

Species	WHO code	Zymodeme[a]	GenBankAC no.[c]
L. donovani complex			
L. donovani	MHOM/ET/67/HU3	MON-18	*cpbF* AY896783
L. donovani	MHOM/ET/00/HUSSEN	LON-42	*cpbF* AY896785
L. donovani	MHOM/KE/67/MRC(L)3	LON-44 or 46	*cpbF* AY896786
L. donovani	IMAR/KE/62/LRC-L57	MON-37	*cpbF* AY896788
L. donovani	MHOM/SD/82/GILANI	MON-30	*cpbF* AY896784
L. donovani	MHOM/CN/00/WangJie1	MON-35	*cpbF* AY896787
L. donovani	MHOM/IN/80/DD8	MON-2	
L. donovani	MHOM/IN/61/L13	n.d.	
L. donovani	MHOM/IN/82/Patna1	LON-41	
L. donovani	MHOM/SD/68/1S	n.d.	
L. donovani	MHOM/SD/97/LEM3431	MON-30	
L. donovani	MHOM/SD/97/LEM3435	MON-30	
L. donovani	MHOM/SD/97/LEM3449	MON-18	
L. donovani	MHOM/SD/97/LEM3475	MON-18	
L. archibaldi	MHOM/SD/62/LRC-L61	MON-82	
L. archibaldi	MHOM/SD/93/GE	MON-82	
L. infantum	MHOM/FR/78/LEM75	MON-1	*cpbE* AY896781
L. infantum	MHOM/FR/82/LEM356	MON-33	*cpbE* AY896779
L. infantum	MHOM/FR/87/LEM1098	MON-1	*cpbE* AY896776
L. infantum	MHOM/FR/85/LEM716	MON-1	*cpbE* AY896777
L. infantum	MHOM/FR/85/LEM663	MON-1	*cpbE* AY896780
L. infantum	MHOM/ES/81/BCN1	MON-29	*cpbE* AY896778
L. infantum	MHOM/MA/67/ITMAP263	MON-1	*cpbE* AY896782
L. infantum	MHOM/DZ/82/LIPA59	MON-24	*cpbE* AY896790/*cpbF* AY896789
L. infantum	MHOM/GR/2003/GH14	MON-1	
L. infantum	MHOM/GR/2004/GH24	MON-1	
L. infantum	MCAN/GR/2005/GD28	MON-1	
L. infantum	MHOM/TN/80/IPT1	MON-1	
L. chagasi	MCAN/BR/84/CO910	MON-1	
L. chagasi	MHOM/BR/84/M8270	MON-1	
L. chagasi	MHOM/BR/85/M9702	MON-1	
L. chagasi	MHOM/PA/78/WR285	MON-1	*cpbE* AY896791
Reference strains[b]			
L. tropica	MHOM/SU/74/SAF-K27		
L. major	MHOM/SU/73/5ASKH		
L. mexicana	MNYC/BZ/62/M379		
L. braziliensis	MHOM/BR/75/M2904		
L. guyanensis	MHOM/BR/78M5378		
L. lainsoni	MHOM/BR/81/M6426		
L. equatoriensis	MCHO/EC/82/LSP1		
L. tarentolae	RTAR/IT/81/G8		
Trypanosoma cruzi	CL brener		
T. brucei	Jua		

[a] Zymodemes were determined with the MLEE method by the WHO reference centres of Montpellier (MON) and London (LON).
[b] Ten reference strains of other *Leishmania* species and *Trypanosoma* were used for PCR specificity test.
[c] The *cpbE* and *cpbF* sequences submitted to GenBank.

cpbF as *L. donovani*. This PCR did not generate amplification for other *Leishmania* species, representative of the genetic diversity of the genus and for *Trypanosoma cruzi* and *T. brucei*. Indeed, only some slight nonreproducible patterns sometimes appeared for *L. braziliensis*, *L. guyanensis*, *L. lainsoni*, *L. tarentolae*, *T. cruzi* and *T. brucei* when 100 ng of DNA was used for PCR instead of 100 pg. These fragment lengths varied from 200 to 450 bp (Fig. 1) and cannot be mistaken with the true *cpb* products.

The PCR products have been sequenced for 15 strains and the corresponding GenBank accession numbers are mentioned in Table 1. The difference between the two species was mainly due to a deletion of 39 nucleotides in

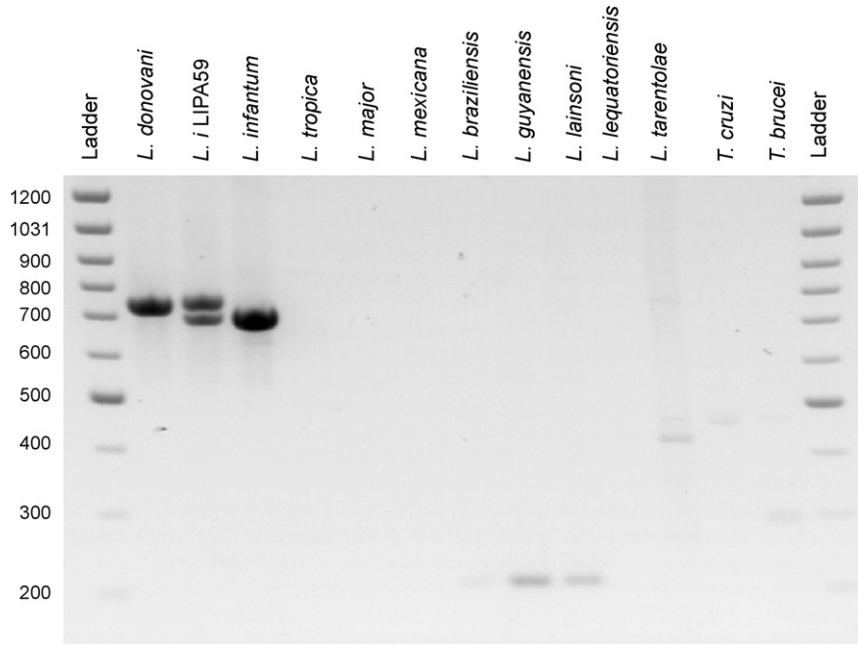

Fig. 1. Polymerase chain reaction using specific *cpbEF* primers. Amplification using 100 pg of genomic DNA was only obtained for *L. donovani* strains (*cpbF* product, 741 bp) and *L. infantum* strains (*cpbE* product, 702 bp). *L. infantum* LIPA59 revealed both *cpbE* and *cpbF* products. There is no amplification at these lengths for the other trypanosomatid species. On each side, molecular weight.

the mature domain of the *cpbE* sequences (Hide et al., in press). This deletion is responsible for the size polymorphism observed (702 bp for *cpbE* and 741 bp for *cpbF*). The amplification using DNA from Indian VL patients did not produce a PCR product and thus, this PCR was not sensitive enough for diagnosis on the clinical samples tested.

4. Discussion

This study presents a discriminative PCR of the *L. donovani* complex based on the *cpb* multigene family. It discriminates between *L. donovani* and *L. infantum* species based on a difference in product length and does not generate amplification for other trypanosomatids. The specificity of this PCR, in addition to its sensitivity (50 pg), demonstrates its usefulness in *Leishmania* typing. Furthermore, this PCR is more rapid than the classical MLEE method and does not need mass culture because DNA extraction can be performed directly on 1 ml of primary culture using InstaGene^{TM} Matrix (Bio-Rad). However, this PCR is not sensitive enough for the diagnosis on clinical samples. This negative result could be due to an inhibition by spleenic constituents but this has not been tested in this study. Nevertheless, it appears very useful for the discrimination of strains within the *L. donovani* complex (with or without mass culture) in the frame of epidemiological and taxonomic studies for example.

PCR amplification revealed the presence of a *cpbEF* heterozygous genotype for *L. infantum* LIPA59 isolated from a human cutaneous lesion. Other results based on microsatellite genotyping and sequencing of other *cp* copies (*cpa* and *cpb*) as well as *lpg2*, *amastin* and *iunh* have suggested the hybrid status of this strain LIPA59 (Hide, 2004). This strain might result from a hybridization process between the two species *L. infantum* and *L. donovani* due to a sexual recombination. Other hybrids have already been identified within the *Leishmania* genus as being between *L. braziliensis* and *L. panamensis/guyanensis* (Bañuls et al., 1997; Belli et al., 1994), *L. braziliensis* and *L. peruviana* (Dujardin et al., 1995) and *L. major* and *L. arabica* (Evans et al., 1987). However, this is the first description of a potential sexual recombination process between *L. infantum* and *L. donovani*.

The taxonomic status of strains such as *L. donovani* MON30, initially typed as *L. infantum* by MLEE, was confirmed by this analysis (Oskam et al., 1998; Quispe Tintaya et al., 2004; Zemanova et al., 2004). *L. archibaldi* and *L. donovani* revealed a common PCR pattern (*cpbF*, 741 bp) and this diagnostic PCR could contribute to clar-

ify the phylogenetic status of several taxa that continue to be debated such as *L. archibaldi* (Jamjoom et al., 2004; Mauricio et al., 2001; Oskam et al., 1998; Zemanova et al., 2004) by the comparison of their *cpbF* sequences for example.

Conflict of interest

We have no potential conflicts of interest concerning this manuscript.

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