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Development of species-specific PCR and PCR-restriction fragment length polymorphism assays for *L. infantum*/*L. donovani* discrimination

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ABSTRACT

Discrimination of *Leishmania infantum* and *L. donovani*, the members of the *L.* (*L.*) *donovani* complex, is important for diagnosis and epidemiological studies of visceral leishmaniasis (VL). We have developed two molecular tools including a restriction fragment length polymorphisms of amplified DNA (PCR-RFLP) and a PCR that are capable to discriminate *L. donovani* from *L. infantum*. Typing of the complex was performed by a simple PCR of *cysteine protease B* (*cpb*) gene followed by digestion with *Dra*III. The enzyme cuts the 741-bp amplicon of *L. donovani* into 400 and 341 bp fragments whereas the 702 bp of *L. infantum* remains intact. The designed PCR species-specific primer pair is specific for *L. donovani* and is capable of amplifying a 317 bp of 3' end of *cpb* gene of *L. donovani* whereas it does not generate an amplicon for *L. infantum*. The species-specific primers and the restriction enzyme were designed based on a 39 bp insertion/deletion (indel) in the middle of the *cpb* gene. Both assays could differentiate correctly the two species and are reliable and high-throughput alternatives for molecular diagnosis and epidemiological studies of VL in various foci.

1. Introduction

Leishmania is a genus of protozoan flagellates that cause a broad spectrum of diseases, ranging from self-limiting localized cutaneous lesions to visceral leishmaniasis (VL) with fatal spontaneous evolution (WHO, 2002). Natural transmission of *Leishmania* is carried out by a certain species of sandfly of the genus *Phlebotomus* in the Old World or *Lutzomyia* in the New World. The visceral form, also known as black sickness or kala-azar in Asia, is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia and is complicated by serious infections. It is the most severe form of the disease and, left untreated, is usually fatal. Although confirmed cases of VL have been reported from 66 countries, 90% of the world's VL burden occurs on the Indian subcontinent, Brazil, Nepal, and in Sudan (http://www.who.int/leishmaniasis). VL is most commonly caused by species pertaining to the *Leishmania donovani* complex: *L.* (*L.*) *donovani* in the Old World and *L.* (*L.*) *infantum* or *L.* (*L.*) *chagasi* in the Old World and the New World, respectively, but on occasion, other *Leishmania* spp. such as *L.* (*L.*) *amazonensis* in Latin America (Almeida et al., 1996) and *L.* (*L.*) *tropica* in the Middle East (Hyams et al., 1995; Parvizi et al., 2008; Alborzi et al., 2008) or Africa (Mebrahtu et al., 1989) have been isolated from patients with visceral manifestations. Dermotropism of *L. donovani* complex species has also been observed in human infections caused by *L.* (*L.*) *infantum* in the Mediterranean basin and North Africa, *L.* (*L.*) *donovani* in Sudan, and *L.* (*L.*) *chagasi* in the New World (Bem-Ismael et al., 1992; Frank et al., 1993; Noyes et al., 1997; Belli et al., 1999). The clinical features of VL caused by different species are different, and each parasite has a unique epidemiological pattern.

Currently, multilocus enzyme electrophoresis (MLEE) is the generally accepted "gold standard" for the identification and classification of isolates of *Leishmania* (Hide et al., 2001; Rioux et al., 1990). By this method, strains are divided into groups with identical enzyme patterns, called "zymodemes". The main criticism of this approach is that genotypes are assayed indirectly, with the consequence that nucleotide substitutions may not be observed in synonymous sites or in nonsynonymous sites, if it is assumed that subsequent changes in the amino acid composition do not lead to different electrophoretic mobilities. In contrast, post-translational modifications may change the electrophoretic mobilities, despite identical genotypes. Furthermore, the method is quite slow, laborious, and costly because

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it requires culturing and obtaining the profile of 10–20 different enzymes. In case of the *L. donovani* complex, this method is not ideal, as it does not easily differentiate species within the complex and requires a large number of parasites. 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 *L. 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 are morphologically indistinguishable but are associated with a different epidemiology, ecology and pathology. *L. infantum* is anthropozoonotic with a dog reservoir, whereas *L. donovani* is largely anthroponotic.

Recently, Hide and Bañuls (2006) developed a discriminative PCR, by focusing on the cathepsin-1 proteases CPB belonging to the papain-like superfamily, clan CA and family C1. These genes have attracted considerable attention because of their role in destruction of the host protein and evasion of the host immune response (Alexander et al., 1998; Hide and Bañuls 2008). 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*. They used these two *cpb* copies to design a discriminative PCR for the *L. donovani* complex. These two species were 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. This PCR does not generate amplification for other *Leishmania* or kinetoplastids. However, the close length of the two PCR products (only 39 bp differences) may cause a problem in diagnosis of the species (1) when using normal agarose gel electrophoresis (Fig. 1), (2) where both species are not available for comparison, and (3) there are heterozygous or hybrid specimens. In this study, the sequence of these two *cpb* copies were analyzed to explore the potential of these targets to design higher resolution PCR-based methods such as species-specific PCR and PCR restriction fragment length polymorphism (PCR-RFLP) to discriminate *L. donovani* from *L. infantum*.

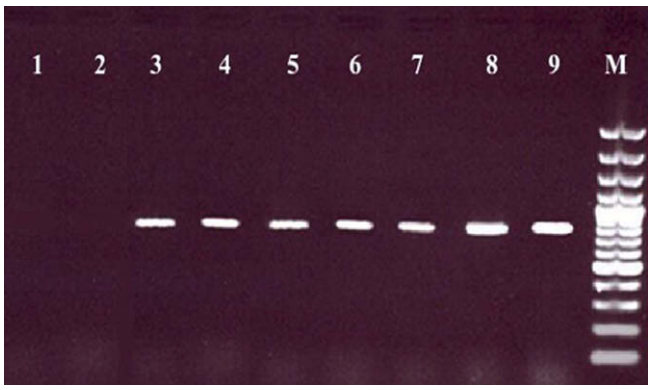

Fig. 1. Species-specific PCR of the *L. donovani* complex *cpbEF* gene. The PCR products (702 bp for *L. infantum* and 741 bp for *L. donovani*) are electrophoresed in 1% agarose gel. Lanes: 1, negative control *L. tropica* (MHOM/IR/03/Mash-878); 2, negative control *L. major* (MHOM/IR/75/ER); 3, *L. donovani*: *cpbF* (IPER/IR/2007/HS10); 4, *L. donovani*: *cpbF* (IPER/IR/2007/AS110); 5, *L. donovani*: *cpbF* (MHOM/ET/00/HUSSEN); 6, *L. donovani*: *cpbF* (IPER/IR/2007/AK118); 7, *L. donovani*: *cpbF* (IPER/IR/2007/HZ6); 8, *L. infantum*: *cpbE* (IPER/IR/2007/AS327); 9, *L.infantum*: *cpbE* (MCAN/IR/97/LON 49); M, 100-bp plus ladder (Fermentas).

2. Material and methods

2.1. Parasite culture and DNA preparation

The sources and geographical origins of the strains included in this study are listed in Table 1. Six *L. donovani* strains and one *L. infantum* strain were found in sand flies captured in Ardabil Province, the most important VL focus in north western Iran. Parasite promastigotes were maintained in NNN medium and cultured in RPMI 1640 medium supplemented with 15% fetal calf serum. After the promastigotes were harvested, they were washed in buffered saline (Desjeux and Alvar, 2003). DNA was extracted by the classical phenol–chloroform method, suspended in TE (Tris–EDTA) buffer, and stored at 4 °C. Some DNA of the *L. donovani* complex strains was received from the reference center Montpellier, France. The strains were representatives of the complex including *L. d. donovani*, *L. d. infantum,* and a hybrid of both species *L. d. donovani*/*infantum*.

2.2. PCR amplification of cpbEF

PCRs were performed in a volume of 30 μl containing 6 pmol of each primer (forward: 5′-CGTGACGCCGGTGAAGAAT-3′; reverse: 5′-CGTGCACTCGGCCGTCTT-3′), 4.5 nmol dNTPs, 1 U *Taq* DNA polymerase, 3 μl buffer 10× and 1–5 μl of DNA extracted from samples. After an initial denaturation step of 5 min at 95 °C, 30 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. The amplification reactions were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light. The rest of PCR products were used for further PCR-RFLP to discriminate *L. donovani* from the *L. infantum*.

2.3. Restriction of PCR fragments

To select appropriate restriction enzyme/s for discrimination of *L. infantum* from *L. donovani* all of the published sequences of *cpbEF* gene of the *L. donovani* complex were screened. A proper restriction site for *Dra*III enzyme was found on the 39 bp indel of the *cpbEF* gene. The *cpbEF* PCR products of *L. donovani* complex were subjected to digestion with the enzyme in a total volume of 20 μl, with approximately 0 ± 5 μg of DNA (10–15 μl PCR products) and 5 U of restriction enzyme in the recommended buffer, overnight at the recommended temperature. Restriction fragments were separated at 120 V for 1 h in 2% agarose, and etidium bromide staining.

2.4. Primer design and species-specific PCR

Sequences of the *cpbEF* gene of *L. donovani* complex were obtained from gene banks and were aligned with the help of the program Clustal X (Thompson et al., 1997). This allowed us to identify a *L. donovani* specific sequence element for the design of an appropriate primer. The sequence was found on the 39 bp variable site of the *cpbEF* gene that showed nucleotide identity only with *L. donovani* species and sequence discrepancy with the rest of 702/741 bp PCR products of the *L. donovani* complex (Fig. 2). The following forward primer 5′-ACCAGCTGCGCTGGCGATGCACTGA-3′ (DonF) was designed and used with the reverse primer mentioned above (Hide and Bañuls 2006) to test its specificity for *L. donovani* strains. The specificity of the primer was tested against strains of the *L. donovani* complex through the thermal program used for amplification of the *cpbEF* gene. Amplification reactions were performed in 25 μl con-

Table 1
Leishmania strains used in this study.

International code	Species	Origin	Host	Accession No.
MHOM/FR/87/LEM1098	*L. infantum*	France	Human	*cpbE* AY896776
IPER/IR/2007/AS327	*L. infantum*	Iran	Sand fly	*cpbE* EU637907
MCAN/IR/97/LON 49	*L. infantum*	Iran	Dog	*cpbE* EF653268
MHOM/ET/00/HUSSEN	*L. donovani*	Ethiopia	Human	*cpbF* AY896785
IPER/IR/2007/HS10	*L. donovani*	Iran	Sand fly	*cpbF* EU637913
IPER/IR/2007/AS110	*L. donovani*	Iran	Sand fly	*cpbF* EU637912
IPER/IR/2007/HZ6	*L. donovani*	Iran	Sand fly	*cpbF* EU637911
IPER/IR/2007/AS259	*L. donovani*	Iran	Sand fly	*cpbF* EU637910
IPER/IR/2007/AK11	*L. donovani*	Iran	Sand fly	*cpbF* EU637909
IPER/IR/2007/AC1	*L. donovani*	Iran	Sand fly	*cpbF* EU637908
MHOM/DZ/82/LIPA59	*L. donovani/infantum*	Algeria	Human	*cpbE* AY896790/ *cpbF* AY896789
MHOM/IR/75/ER	*L. major*	Iran	Human	EU482830
MHOM/IR/03/Mash-878	*L. tropica*	Iran	Human	EU727198

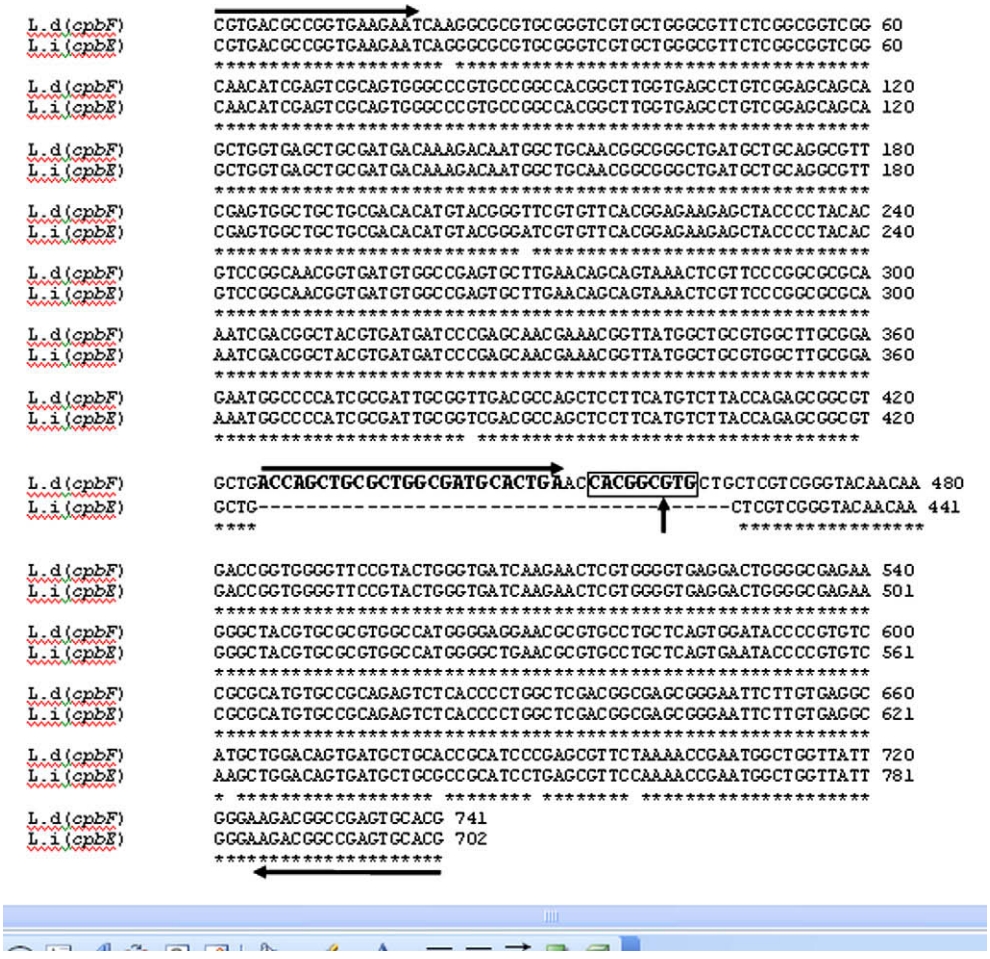

Fig. 2. *cpbEF* gene sequence of the *L. donovani* complex representatives (L.d: *L.donovani*, AY8967785; L.i:*L. infantum*, AY896776). The horizontal arrows indicate the positions of the forward and reverse primers used in the PCRs. The species specific primer (DonF, 425-449 bp) for *L. donovani* is bold. The restriction site (452-460) of *DraIII (AdeI)* enzyme is bold and boxed. The vertical arrow shows digestion point of the enzyme. Gaps/strikes, and * show polymorphisms (substitutions or indels) and identical sites respectively.

taining the following: 1 μl of the DNA, 20 pmol of each primer, 0.2 mmol/l dNTPs, 10 mmol/l Tris–HCl, 50 mmol/l KCl, 0.1% Triton-X 100, 1.5 mmol/l $MgCl_2$, and 1 U *Taq* polymerase. The products were analyzed by electrophoresis in 1.5%, w/v, agarose gels, and product size was estimated with comparison to a DNA ladder (100-bp DNA ladder, Fermentas). We have designed a few primers on the *cpbEF* gene to use them as species-specific primer for *L. infantum* but unfortunately no appropriate sequences were found.

3. Results

3.1. cpbEF and species-specific PCR

The PCR of *CPBEF* was first used to confirm the taxonomic status of the specimens if they belong to the *L. donovani* complex. For strains belonging to the *L. donovani* complex, the PCR assay against *cpbEF* gene using universal primers of Hide and Bañuls (2006) resulted in product sizes of 702 bp for *L. infantum* and 741 bp for *L. donovani*

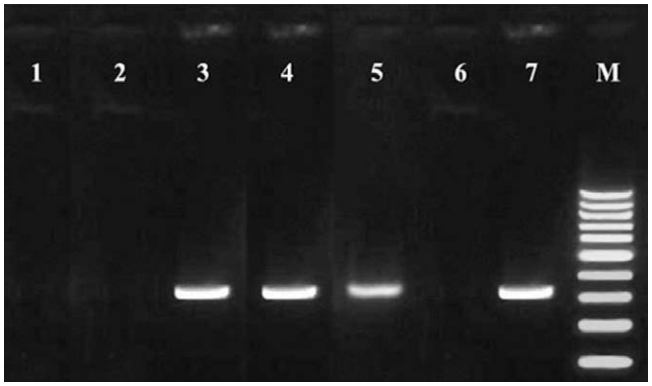

Fig. 3. Species-specific PCR (317 bp) of *L. donovani cpbF* gene. Lanes: 1, *L. tropica* (MHOM/IR/03/Mash-878; 2, *L. major* (MHOM/IR/75/ER); 3, *L. donovani*: *cpbF* (IPER/IR/2007/HS10); 4, *L. donovani*: *cpbF* (IPER/IR/2007/AS110); 5, *L. donovani*: *cpbF* (MHOM/ET/00/HUSSEN); 6, *L. infantum*: *cpbE* (IPER/IR/2007/AS327); 7, *L. donovani/infantum*: *cpbF/cpbE* (MHOM/DZ/82/LIPA59); M, 100-bp ladder(Fermentas).

srains (Fig. 1). However, the strains of *L. donovani* could not be typed easily from *L. infantum* strains according to their amplicon sizes (Fig. 1). This PCR does not generate amplifications for *L. major* and *L. tropica*. The species-specific primer of *L. donovani* (DonF) based on the PCR of the *cpbEF* gene was validated for all of the *L. donovani* strains (Table 1). Also, the specificity of the designed PCR primer, for the *L. donovani* strains, was confirmed by testing different strains of *L. infantum*, *L. major*, and *L. tropica* species, which produced a negative PCR result (Fig. 3). For all of the strains belonging to *L. donovani*, the PCR assay resulted in product size of 317 bp (Fig. 3). In addition, the hybrid strain of *L. donovani/infantum* from Algeria yielded 317 bp amplicon as well.

3.2. PCR-RFLP of cpbEF gene

Sequence analysis of the *cpbEF* gene revealed an appropriate and specific restriction site "*Dra*III (*Ade*I)" for *L. donovani* strains. The restriction site of the enzyme (CACNNNGTG) was found within the 39 bp variable site (39 bp insertion in *L. donovani*) of the *cpbEF* gene (Fig. 2). Based on this restriction site, a PCR-RFLP assay was designed to easily distinguish *L. infantum* from *L. donovani* species. Restriction digestion of the *cpbEF* PCR product with the enzyme

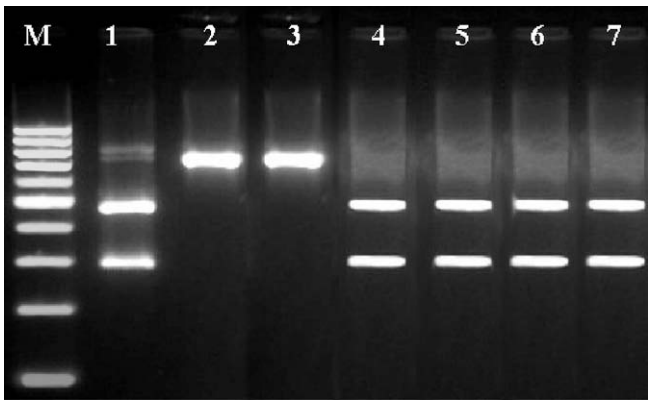

Fig. 4. *cpbEF* gene PCR-RFLP analysis of selected strains of *L. donovani* complex with the use of the restriction enzyme *DraIII*. Lanes: M, 100-bp ladder (Fermentas); 1, *L.donovani/infantum*: *cpbF/cpbE* (MHOM/DZ/82/LIPA59); 2, *L. infantum*: *cpbE* (MHOM/FR/87/LEM1098); 3, L. infantum: *cpbE* (IPER/IR/2007/AS327); 4, *L. donovani*: *cpbF* (MHOM/ET/00/HUSSEN); 5, *L. donovani*: *cpbF* (IPER/IR/2007/HS10); 6, *L. donovani*: *cpbF* (IPER/IR/2007/AS110); 7, *L. donovani*: *cpbF* (IPER/IR/2007/HZ6).

*Dra*III gave two distinct patterns that were associated with the presence of the restriction site or 39 bp insertion in *L. donovani* strains (Fig. 4). *L. donovani* (Fig. 4, lanes 4–7) showed a profile of 284 and 457 bp bands, whereas *L. infantum* (Fig. 4, lanes 2 and 3) remained intact and showed the 702 bp original PCR products. The PCR product of *L. donovani* portion (the 741 bp) of the *L. donovani/infantum* hybrid were digested into 284 and 457 bp fragments, whereas *L. infantum* portion of the hybrid with 702 bp remained intact (Fig. 4, lane 1). All strains of *L. donovani* from Iran, Ethiopia, and Algeria showed identical RFLP profile.

4. Discussion

Here we describe the development of two reproducible discriminating PCR-based molecular typing methods, based on the amplification of the *cpb* gene, which were able to characterize and distinguish strains of *L. donovani* from *L. infantum*. The species-specific PCR is easier and faster than the PCR-RFLP method, however, we could find only a primer for *L. donovani* and none for *L. infantum*. Therefore, one might question that lack of amplicon for *L. infantum* is due to technical problem rather than specificity of the primer. On the other hand, PCR-RFLP requires an extra post-amplification step, but since the profile of both species is present, it generates more simple interpretable results.

Both methods are based on the different sizes and sequence polymorphism (39 bp indel) of the obtained *cpbEF* amplicons. These indel sites have been already observed in all strains of the *L. donovani* complex (Hide and Bañuls 2006). Generally, *cpbE* produce 702 bp and *cpbF* 741 bp amplicons. They showed that all of the *L. donovani* strains got only the *cpbF* copy, whereas the *L. infantum* strains contained only *cpbE*. Therefore, both assays presented here could discriminate between *L. donovani* and *L. infantum* species. Interestingly, the PCR-RFLP also could determine hybrid status of the strains. As it shows in Fig. 4, the RFLP profile of the hybrid strain of *L. infantum/donovani* comprises both the *cpbE* and *cpbF* gene. The methods we used in this study worked very well for a few strains originating from a very wide geographical range including Asia (Iran), Europe (France), and Africa (Algeria and Ethiopia). However, since a set of interesting polymorphisms in the *cpb* gene such as copy sequence or expression according to the parasite's life stage has been reported in the *L. donovani* complex (Hide and Bañuls, 2008), further experiments with a wide range of strains is needed to confirm the utility of the assays for discrimination of the complex.

During the last decade, several molecular markers with different resolution levels such as PCR-RFLP (Mauricio et al., 2004; Quispe Tintaya et al., 2004), RAPD-PCR (Hide et al., 2001; Zemanova et al., 2004), and multilocus microsatellite typing (MLMT) (Bulle et al., 2002; Ochsenreither et al., 2006) have been developed for strain typing as well as for population genetic studies of the parasite. Most of these assays target kinetoplast DNA or noncoding regions, such as intergenic sequences of repeated genes and microsatellite sequences. Recently, Haralambous et al. (2008) based on the amplicon size of *K26* gene, developed a PCR method capable of species/strain discrimination of *L. donovani* complex strains. Although the method was very good and in many cases could easily distinguish *L. donovani* from *L. infantum*, the method was more practical to distinguish geographical strains than species since there were some identical or very close PCR products for both species of *L. donovani* and *L. infantum*. Another phylogenetic study by Lukes et al (2007) using a multifactorial genetic analysis including DNA sequences of protein-coding and noncoding regions, as well as microsatellites, RFLPs, and RAPD markers of 25 geographically representative strains of *L. donovani* complex could not resolve between all *L. infantum* and *L. donovani* strains. They

showed that the genotypes are strongly correlated with geographical (continental) origin, but not with current taxonomy or clinical outcome. For example, all East African strains constituted a common clade, independently of whether they have been designated in the past as *L. infantum*, *L. donovani* or *L. archibaldi*.

In conclusion, the developed molecular typing methods not only have sufficient discriminatory power but also have other advantages. They are very simple and cost-effective methods because typing of a parasite belonging to the *L. donovani* complex can be accomplished by simple PCR when species-specific primers are used, or species identification is achieved by restriction-fragment length polymorphism (PCR-RFLP). Furthermore, they are specific, rapid, and highly reproducible. PCR or PCR-RFLP could be carried out directly on cultures and other biological forms such sand flies. Also they can be performed directly on clinical samples and tissue of reservoirs, avoiding parasite isolation and culturing. However, in this study we have not tested the methods for clinical samples and their sensitivity for detection of parasites in clinical samples should be tested.

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