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Detection and Identification of *Leishmania* Species from Clinical Specimens by Using a Real-Time PCR Assay and Sequencing of the Cytochrome *b* Gene[▽]

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Visceral and cutaneous leishmaniases are heterogenous entities. The *Leishmania* species that a given patient harbors usually cannot be determined clinically, and this identification is essential to prescribe the best species-specific therapeutic regimen. Our diagnosis procedure includes a real-time PCR assay targeted at the 18S rRNA gene, which detects all *Leishmania* species but which is not specific for a given *Leishmania* species. We developed a species identification based on sequencing of the cytochrome *b* (cyt *b*) gene directly from the DNA extracted from the clinical specimen. The sequences were analyzed using the Sequence Analysis/Seqscape v2.1 software (Applied Biosystems). This software is designed to automatically identify the closest sequences from a reference library after analysis of all known or unknown polymorphic positions. The library was built with the *Leishmania* cyt *b* gene sequences available in GenBank. Fifty-three consecutive real-time PCR-positive specimens were studied for species identification. The cyt *b* gene was amplified in the 53 specimens. Sequencing resulted in the identification of six different species with ≥99% identity with the reference sequences over 872 nucleotides. The identification was obtained in two working days and was in accordance with the multilocus enzyme electrophoresis identification when available. Real-time PCR followed by sequencing of the cyt *b* gene confirmed the diagnosis of leishmaniasis and rapidly determined the infecting species directly from the clinical specimen without the need for the isolation of parasites. This technique has the potential to significantly accelerate species-adapted therapeutic decisions regarding treatment of leishmaniasis.

The leishmaniases are a group of parasitic diseases of major and growing public health importance (12, 22). Leishmaniasis is endemic in many countries that are destinations for millions of travelers or migrant workers from Northern countries, including patients with immunodepression, each year (9). About 21 *Leishmania* species have been reported to cause human infection (12). Some species causing cutaneous leishmaniasis, mainly *Leishmania braziliensis* and to a lesser extent *L. panamensis* and *L. guyanensis*, are associated with the risk of delayed mucosal leishmaniasis, and the response to antileishmanial agents is influenced by the species (16, 23). Although the clinical presentation of cutaneous leishmaniasis is influenced by the infecting species (13, 22), on an individual basis, the clinical presentation is not specific enough to allow a reliable species determination (1, 7, 13, 32). Identification of the species can also help predict the risk of dissemination in immunocompromised patients (10, 11). Thus, species identification is important to determine the clinical prognosis and to select the most appropriate therapeutic regimen to be administered to each individual.

The reference positive diagnosis methods for leishmaniasis, i.e., direct smear examination and culture, have important limitations (8). Direct examination requires some expertise and lacks sensitivity. Culture is labor-intensive, and the result is not known for weeks. The "gold standard" for *Leishmania* species identification is multilocus enzyme electrophoresis (MLEE) (27). MLEE requires culture and isolation of the parasites, a process sometimes jeopardized by bacterial contamination. Moreover, the result of MLEE is available several weeks or months after the diagnosis. Thus, while MLEE is essential for epidemiological studies, this technique is not rapid enough to guide first-line therapeutic decisions. So in most instances, clinicians determine the first course of therapy based on epidemiological data only.

By contrast, PCR assays provide results in one or two working days. We routinely perform a conventional PCR test that is more sensitive than in vitro culture for the diagnosis of *Leishmania* infection (6). We report here the upgrading of our conventional PCR to a real-time PCR format that improves the reliability of the positive diagnosis and obtains quantitative results to assess treatment efficacy (4). We have therefore developed a diagnostic strategy including a positive diagnostic step followed by identification using sequencing. The first step is a real-time PCR assay targeted at the 18S rRNA gene region. The second step of species identification uses the cytochrome *b* (cyt *b*) gene sequences known to be polymorphic among *Leishmania* species (15).

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TABLE 1. Primers and probes used in the present study[a]

Primer (sequence)	Modification
Positive diagnosis using quantitative real-time PCR	
LEI-18S1 (5′-ACTCACGGCCTCTAGGAATGA-3′)	None
LEI-18S2 (5′-TCGATCTCCACACTTTGGTTCT-3′)	None
LEI-18S3 (5′-AGAATTTCACCTCTGACGCCCCAGT-3′)	5′LCRed640 3′Ph
LEI-18S4 (5′-GCTGTAGTTCGTCTTGGTGCGGTCT-3′)	3′ FITC
Species identification based on the cyt *b* gene	
LEI-CYTB9 (5′-TTATGGTGTAGGTTTTAGTYTAGGTT-3′)	None
LEI-CYTB10 (5′-CCATCCGAACTCATAAAATAATGT-3′)	None
LEI-CYTB11 (5′-TTTGTTATTGAATWTGAGGWAGTGA-3′)	None
LEI-CYTB12 (5′-TGCTAAAAAACCACTCATAAATATACT-3′)	None

[a] Y, C or T; W, A or T. FITC, fluorescein isothiocyanate.

MATERIALS AND METHODS

Clinical specimens and DNA extraction. DNA was extracted from biological samples (blood, bone marrow aspirate, or skin biopsy or aspirate) using the High Pure PCR Template Preparation kit (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. Following the centrifugation and washing steps, total DNA was eluted from the spin columns with 50 μl of elution buffer, 5 μl of which was used for the PCR and 45 μl of which was stored at −30°C.

Real-time PCR assay. Two primers and a pair of hybridization probes were selected to amplify a conserved 152-bp region using real-time PCR (Table 1). The amplification was carried out using a LightCycler instrument (Roche Molecular Biochemicals, Meylan, France), and a single fluorescence reading for each sample was taken at the annealing step. PCR was set up in a final volume of 20 μl with the Faststart DNA Master Hybridization Probes kit (Roche Diagnostics, Meylan, France), 4 mM $MgCl_2$, each primer and probe (Proligo, Paris, France) at concentrations of 0.5 μM and 0.25 μM, respectively, and 0.25 μl of uracil-DNA-glycosylase (Biolabs, Courtaboeuf, France). The reaction mixture was initially incubated for 1 min at 50°C, followed by a 8-min step at 95°C. Amplification was performed for 50 cycles of denaturation (95°C for 10 s; ramp rate, 20°C/s), annealing (60°C for 10 s; ramp rate, 20°C/s), and extension (72°C for 15 s; ramp rate, 20°C/s). PCR-positive specimens were cultured on NNN medium (8) and then, whenever positive in culture, sent to the reference center (Montpellier, France) for MLEE typing.

Species identification by nucleotide sequencing of the cyt *b* gene. Identification was performed with the real-time PCR-positive samples obtained from 2003 to 2005. Two sets of primers were designed (Table 1) to obtain two overlapping PCR fragments to sequence 872 bp of the cyt *b* gene with both strands. Amplification was carried out in a 50-μl reaction mixture containing 2.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2 mM each dATP, dTTP, dGTP, and dCTP; 0.2 μM each primer; and 1.25 U of AmpliTaq Gold polymerase (Applied Biosystems, Courtaboeuf, France). After an initial denaturation step at 95°C for 10 min, samples were amplified for 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Specific amplification products were purified using the HighPure PCR Product Purification kit (Roche Diagnostics, Meylan, France) and were then sequenced using the DiDeoxy Terminator cycle sequencing kit v1.1 protocol (Applied Biosystems, Courtaboeuf, France). The reaction products were run on a ABI PRISM 310 genetic analyzer (Applied Biosystems) and analyzed with Sequence Analysis/Seqscape v2.1 software (Applied Biosystems, Courtaboeuf, France). This software is designed to automatically identify genotypes from a reference library after analysis of all known or unknown polymorphic positions.

A local reference library was created using the 13 reference strains whose sequences are available in GenBank (Table 2). Between nucleotides 83 and 954, 186 positions are variable. This local library allows an easy and rapid comparison of sequences of clinical specimens with the reference sequences. The software underlines the differences observed between the reference and the clinical sequences. This allows the visual checking and manual correction if necessary, avoiding the cumbersome reading of the chromatograph. The identification of a submitted clinical sequence is given with a percentage of homology. In addition to the clinical specimens, we also sequenced 9 reference strains maintained in our laboratory for several years, 12 WHO reference strains, and the MON-45 reference strain obtained from the Centre National de Référence of Montpellier, France, and cultivated only once in our laboratory to obtain DNA.

TABLE 2. Reference strains used in the present study

Leishmania species	Reference strain	GenBank access no. or source
L. donovani donovani	2525M-C2-2M	AB095957
L. donovani infantum	MHOM/TN/80/IPT1	AB095958
L. donovani chagasi	MHOM/BR/74/PP75	AB095959
L. tropica	MHOM/SU/58/Strain OD	AB095960
L. major	MHOM/SU/73/5ASKH	AB095961
L. aethiopica	MHOM/ET/72/L100	AB095962
L. mexicana mexicana	MHYC/BZ/62/M379	AB095963
L. amazonensis	MHOM/BR/73/M2269	AB095964
L. garnhami	MHOM/VE/76/JAP78	AB095965
L. braziliensis	MHOM/BR/75/M2904	AB095966
L. braziliensis	MHOM/EC/88/INH-03	AB095967
L. panamensis	MHOM/BR/71/LS94	AB095968
L. guyanensis	MHOM/BR/75/M4147	AB095969
L. aethiopica	MHOM/ET/72/L100	Local laboratory
L. tropica	MHOM/SU/74/K27	Local laboratory
L. mexicana	MHOM/MX/93/CRE47	Local laboratory
L. major	MHOM/SU/73/5ASKH	Local laboratory
L. guyanensis	MHOM/BR/75/M4147	Local laboratory
L. infantum	MCAN/GR/94/CRE69	Local laboratory
L. donovani	MHOM/IN/80/DD8	Local laboratory
L. braziliensis	MHOM/BR/84/LTB300	Local laboratory
L. amazonensis	LMA MPRO/BR/72/M1841	Local laboratory
L. infantum	MHOM/TN/80/IPT1	WHO strain
L. donovani	MHOM/IN/80/DD8	WHO strain
L. chagasi	MHOM/BR/74/PP75	WHO strain
L. major	MHOM/SU/73/5ASKH	WHO strain
L. aethiopica	MHOM/ET/72/L100	WHO strain
L. braziliensis	MHOM/BR/84/LTB300	WHO strain
L. panamensis	MHOM/PA/71/LS94	WHO strain
L. amazonensis	MHOM/BR/73/M2269	WHO strain
L. garnhami	MHOM/VE/76/JAP78	WHO strain
L. tropica	MHOM/SU/74/K27	WHO strain
L. guyanensis	MHOM/GF/79/LEM85	MON-45 reference strain
L. mexicana	MHOM/BZ/82/BEL21	WHO strain
L. pifanoi	MHOM/VE/57/LL1	WHO strain

Nucleotide sequence accession numbers. The cyt *b* gene sequences described in this paper have been deposited in GenBank under accession numbers EF579895 to EF579916.

RESULTS

The cyt *b* gene sequences found in GenBank (Table 2) were used to build a local library, and the percentage of identity was in keeping with data reported previously (15). The 53 consecutive real-time PCR-positive specimens, the 9 reference strains cultured in our laboratory, and the 13 WHO reference strains were all positive for the amplification of the cyt *b* gene. *L. amazonensis* (one reference strain, one WHO strain, and one clinical specimen), *L. guyanensis* (one reference strain, one WHO strain, and four clinical specimens), *L. mexicana* (one reference strain and one WHO strain), and *L. tropica* (one reference strain, one WHO strain, and two clinical specimens) sequences were all identical to the GenBank reference sequences. This result was obtained even though the GenBank and the local or WHO reference strains could have different names (Table 2). For instance, the name of the WHO *L. panamensis* strain is MHOM/PA/71/LS94 and not MHOM/BR/71/LS94 as referenced in GenBank.

Table 3 shows the differences between the local and WHO reference strains, the clinical specimens, and the GenBank data. The local *L. aethiopica* reference strain had 11 nucleotides that were different from the GenBank sequence, although they harbored the same name. For *L. major*, the 25 clinical specimens had the same sequence, with only two nucleotides that were different from the reference sequence.

TABLE 3. Sequence variations observed between the GenBank reference strains, the local reference strains, the WHO reference strains, and the 53 clinical specimens tested[d]

Species	Nucleotide at position:												
	104	209	221	248	389	393	434	584	651	779	843	932	938
L. infantum MHOM/TN/80/IPT1[a]	T	T	A	C	A	C	A	G	A	C	T	G	T
L. infantum MCAN/GR/94/CRE69[b]	T	T	A	C	A	C	T	A	C	C	T	G	T
L. infantum MHOM/TN/80/IPT1[c]	T	T	A	C	A	C	T	A	C	C	T	G	T
L. donovani 2525M-C2-2M[a]	C	T	A	C	A	C	A	G	A	C	T	A	C
L. donovani MHOM/IN/80/DD8[b]	T	C	T	T	G	T	T	A	C	C	C	G	C
L. donovani MHOM/IN/80/DD8[c]	T	C	T	T	G	T	T	A	C	C	C	G	C
L. chagasi MHOM/BR/74/PP75[a]	T	C	T	T	G	T	T	A	C	C	C	G	C
L. chagasi MHOM/TN/80/IPT1[c]	T	T	A	C	A	C	T	A	C	C	T	G	T
13 clinical specimens	T	T	A	C	A	C	T	A	C	C	T	G	T
2 clinical specimens	T	T	A	C	A	C	T	A	C	T	T	G	T
Position	490	624											
L. major MHOM/SU/73/5-ASKH[a]	C	T											
L. major MHOM/SU/73/5-ASKH[b]	C	T											
L. major MHOM/SU/73/5-ASKH[c]	C	T											
25 clinical specimens	T	C											
Position	111	121	139	150	483	490	493	585	667	837	880		
L. aethiopica MHOM/ET/72/L100[a]	T	T	A	C	T	T	T	T	G	T	T		
L. aethiopica MHOM/ET/72/L100[c]	T	T	A	C	C	C	T	T	G	T	T		
L. aethiopica MHOM/ET/72/L100[b]	C	C	G	T	C	C	C	A	T	C	C		
Position	195	262	807										
L. braziliensis MHOM/BR/75/M2904[a]	T	A	T										
L. braziliensis MHOM/EC/88/INH-03[a]	G	T	T										
L. braziliensis MHOM/BR/84/LTB300[b]	A	T	C										
L. braziliensis MHOM/BR/84/LTB300[c]	T	T	T										
5 clinical specimens	G	T	T										
Position	355												
L. panamensis MHOM/BR/71/LS94[a]	G												
L. panamensis MHOM/PA/71/LS94[c]	G												
1 clinical specimen	T												

[a] GenBank reference strain.
[b] Local reference strain.
[c] WHO reference strain.
[d] Nucleotide location refers to the *L. infantum* MHOM/TN/80/IPT1 sequence (GenBank accession number AB095958).

For *L. braziliensis*, the five clinical isolates had the same sequence as one GenBank sequence. The other GenBank sequence and the other two reference strains had one or two nucleotides that were different. For *L. panamensis*, the clinical isolate had one nucleotide difference with the reference strain. For *L. infantum*, 13 clinical specimens had the same sequence as one of our local reference strains, identical to the sequences of the *L. infantum* and *L. chagasi* WHO strains. Only 2 clinical samples had one base difference from the other 13 clinical specimens. The local and WHO reference strains of *L. donovani* had sequences that were identical to the sequence of *L. chagasi* reported in GenBank. Despite these minor differences, each clinical specimen was unambiguously assigned to a species with ≥99.8% similarity with at least one reference sequence. All of these changes were synonymous for the *L. donovani* group and for *L. major*. For the other species, most of the changes were synonymous: 8 of 11 and 2 of 3 for *L. aethiopica* and *L. braziliensis*, respectively. For *L. panamensis*, the change led to a Gly-Val substitution.

Thus, among the 53 clinical specimens, six different species were identified (Table 4). Culture failed in 9 of 44 specimens (20%) and could not be performed with 9 other specimens, mainly because of the paucity of the available material. Among the 18 culture-negative specimens, the cyt *b* gene sequencing identified 7 *L. major*, 7 *L. infantum*, 2 *L. braziliensis*, 1 *L. guyanensis*, and 1 *L. tropica* isolates. For the 35 culture-positive samples, the MLEE typing data were in accordance with the identification based upon cyt *b* gene sequencing. No specific MLEE type could be associated with a given sequence for a given species (Table 4).

Among the six species, five were known to be only dermotropic, and none of these five was identified in blood or bone marrow specimens. For these dermotropic species, the identification was in accordance with the geographical origin of the patients. The only visceral species of our series, *L. infantum*, was isolated from blood and marrow specimens but also from three skin biopsies, all from North African patients (Table 4). One of these three patients was human immunodeficiency virus positive. An *L. infantum* isolate was secondarily recovered from a blood culture for this patient. The other two patients had unknown human immunodeficiency virus status, and they were cured with intralesional injections of meglumine antimoniate.

TABLE 4. Origin, cyt *b* identification based on sequencing, and MLEE typing of the 53 real-time PCR-positive clinical specimens

Clinical specimens	Geographical origin	cyt b identification	MLEE typing result[a]
Nasal biopsy	Amazonia	*L. braziliensis*	MON-43
Skin biopsy	Amazonia	*L. braziliensis*	ND
Skin biopsy	Bolivia	*L. braziliensis*	MON-43
Skin biopsy	French Guyana	*L. braziliensis*	ND
Skin biopsy	Peru	*L. braziliensis*	ND
Skin biopsy	Amazonia	*L. guyanensis*	ND
Skin aspirate	French Guyana	*L. guyanensis*	MON-45
Skin aspirate	French Guyana	*L. guyanensis*	MON-131
Skin aspirate	French Guyana	*L. guyanensis*	MON-45
Blood	Algeria	*L. infantum*	MON-24
Skin aspirate	Algeria	*L. infantum*	MON-24
Skin biopsy	Algeria	*L. infantum*	ND
Node biopsy	Croatia	*L. infantum*	MON-1
Blood	France	*L. infantum*	ND
Blood	France	*L. infantum*	MON-1
Blood	France	*L. infantum*	MON-24
Blood	France	*L. infantum*	MON-1
Blood	France	*L. infantum*	ND
Blood	France	*L. infantum*	ND
Blood	France	*L. infantum*	ND
Blood	France	*L. infantum*	ND
Bone marrow aspirate	France	*L. infantum*	MON-24
Skin biopsy	France	*L. infantum*	ND
Blood	Portugal	*L. infantum*	MON-1
Blood	Spain	*L. infantum*	MON-1
Node biopsy	Algeria	*L. major*	ND
Skin aspirate	Algeria	*L. major*	MON-25
Skin aspirate	Algeria	*L. major*	MON-25
Skin aspirate	Algeria	*L. major*	MON-25
Skin aspirate	Algeria	*L. major*	MON-25
Skin biopsy	Algeria	*L. major*	MON-25
Skin biopsy	Algeria	*L. major*	MON-25
Skin biopsy	Algeria	*L. major*	MON-25
Skin biopsy	Algeria	*L. major*	MON-25
Skin biopsy	Algeria	*L. major*	ND
Skin biopsy	Algeria	*L. major*	ND
Skin biopsy	Algeria	*L. major*	ND
Skin aspirate	Burkina Faso	*L. major*	MON-74
Skin aspirate	Burkina Faso	*L. major*	MON-26
Skin biopsy	Mali	*L. major*	MON-74
Skin biopsy	Mali	*L. major*	ND
Skin biopsy	Mauritania	*L. major*	MON-74
Skin aspirate	Morocco	*L. major*	MON-25
Skin biopsy	Morocco	*L. major*	MON-25
Skin aspirate	Senegal	*L. major*	ND
Skin biopsy	Senegal	*L. major*	MON-74
Skin aspirate	Tunisia	*L. major*	MON-25
Skin aspirate	Tunisia	*L. major*	MON-25
Skin aspirate	Tunisia	*L. major*	MON-25
Skin biopsy	Tunisia	*L. major*	ND
Skin aspirate	Costa Rica	*L. panamensis*	MON-100
Skin biopsy	Afghanistan	*L. tropica*	ND
Skin aspirate	Syria	*L. tropica*	MON-76

[a] ND, not done.

DISCUSSION

Conventional PCR assays developed for the diagnosis of leishmaniasis are more sensitive than direct examination and in vitro culture (6, 14). However, conventional PCR assays harbor the risk of contamination and, therefore, the risk of false-positive results. A real-time PCR assay markedly reduces the risk of contamination with previously amplified products, as the reaction tubes remain closed during the detection process (3). Moreover, the use of enzymatic prevention with uracil-DNA-glycosylase practically excludes the risk of false-positive results due to carryover. The emergence of real-time PCR assays has resulted in the progressive withdrawal of conventional PCR assays for diagnosis in clinical laboratories (25). Several DNA targets for the diagnosis of leishmaniasis using real-time PCR assays have been reported. They include small subunit rRNA genes (2, 35), kinetoplast DNA (19, 24), and 18S rRNA gene sequences (29). All are repeated genes, a feature that should increase the sensitivity of the positive diagnosis. We focused on the 18S rRNA gene. Compared to a previous report (29), we modified the primers to shorten the amplicon (152 versus 478 bp), and we targeted probes to constant regions for a more reproducible quantification.

Species identification was based upon sequencing of the cyt *b* gene. This identification is particularly useful for travelers with cutaneous leishmaniasis, as the *Leishmania* isolate that they harbor potentially belongs to numerous species. As opposed to Africa, where *L. major*, *L. tropica*, and *L. infantum* rarely circulate in the same area, in South America and in the Middle East, areas where dermotropic *Leishmania* species can be found consistently overlap. For example, a patient returning from a single Peruvian area may be infected with *L. braziliensis*, *L. panamensis*, *L. guyanensis*, or *L. amazonensis*. *L. braziliensis* infection is best treated with 10 to 20 injections of pentavalent antimony (16), whereas *L. panamensis* and *L. guyanensis* infection very often heals after a limited number of injections (31) or even a single injection (28) of pentamidine or with orally administered miltefosine (30). In our cohort, we isolated and identified six different *Leishmania* species, another illustration of this heterogeneity in travelers.

Our work on the cyt *b* gene underlines some difficulties in using the GenBank data and reference strains. In our laboratory, our so-called reference strains presented sequences different from those in the GenBank database. Some of them could have undergone shifts after several subcultures or misidentification of tubes after several handlings. More importantly, differences were observed between the GenBank data and the WHO strain sequences freshly obtained from the reference center of Montpellier, France. This could have also been due to repetitive cultures in different laboratories. This hypothesis is supported by the fact that most of the changes observed were synonymous. Another possibility could be that stocks and strains are not clonal. However, we did not observe ambiguities in reading the sequence chromatograms, which should have been the case if several different *Leishmania* clones had been mixed in relatively equal quantities. Thus, this latter hypothesis seems to us to be unlikely. Whatever the explanation for the differences between the GenBank data and the WHO reference strains, it should be wise to regularly check the stocks and strains used in the laboratory to detect any genetic drift over time.

In order to identify the *Leishmania* complex or species to which an isolate belongs, several strategies have been reported. Restriction fragment length polymorphism after PCR has been described previously (21, 33), but this technique does not provide data that are easily computerizable and exchangeable between laboratories. Some identification techniques rely on

the melting curves obtained after amplification using FastStart DNA Master SYBR green (24, 29). Because different species have the same melting point (29), these melting curves are not reliable enough to support an identification process ultimately leading to a therapeutic decision (29). Another interesting strategy is multiplex PCR, with each amplification reaction including a specific primer/probe combination (34). The targeted gene for this *Leishmania* complex identification is the glucose phosphate isomerase (GPI) gene. However, only a single GPI locus could be detected in the *L. mexicana* genome (26). As a consequence, we selected a multicopy gene to aim at optimizing the identification directly from the clinical specimens, thus bypassing the cumbersome culture step. We chose the cyt *b* gene, which is located in the maxicircle component of the kinetoplast for which there are about 50 copies. The cyt *b* gene has the possibility to individualize the 13 human-pathogenic species representing the most common causative agents of leishmaniasis in the New World and the Old World (15). There is enough nucleotide sequence variation among *Leishmania* species genomes for identification purposes: 245 nucleotide positions were polymorphic, and 190 positions were parsimony informative (15). cyt *b* sequencing has recently been used with success for species identification in Argentina (18) and in Pakistan (17). In our series, sequencing of the cyt *b* gene provided unambiguous results for the 53 clinical diagnoses.

The cyt *b* gene could have been used for both positive diagnosis and species identification. However, handling of amplified DNA for diagnosis and identification increased the risk of carryover and, therefore, the risk of false-positive results. Moreover, the polymorphism observed within the DNA sequence of the cyt *b* gene precluded the selection of one probe common to all the species. This would have led to a set of several primers/probes, as was done for the GPI gene (34). The systematic use of several PCR sets including hydrolyzed or hybridization probes on a routine basis is more expensive than sequencing. Besides, sequencing of the cyt *b* gene allows identification to the species level and not only to the complex level (34). Nevertheless, the polymorphism observed is too limited within a species to be used as an infraspecific typing method. Other methods, such as the analysis of microsatellite markers (5), are more appropriate for this specific issue.

The proposed strategy, which includes a positive diagnosis using real-time PCR and sequencing of a given gene, is expensive with regard to the currently used equipment and biochemicals necessary for the reactions. Our double-step strategy could not be justified in other settings where the species is almost always the same and where other diagnostic means might be as efficient. In India, for instance, the detection of circulating antigens should be more cost-effective for the diagnosis of *L. donovani* infections (20). However, real-time PCR and sequencing are now rapidly performable at a reasonable cost in hospitals sharing the costly equipment necessary for molecular diagnostics. The positive result allows the exclusion of differential diagnoses and species identification to start specific therapy even in the case of a culture-negative specimen. The advantages of our identification process in terms of better and earlier adaptation of therapy counterbalance the cost of the technique. In many instances, proper species identification markedly reduces the risk of severe adverse events as well as the duration of hospitalization, not to mention the reduction of the number of injections, an important improvement, especially for children.

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