Identification and Differentiation of *Leishmania* Species in Clinical Samples by PCR Amplification of the Miniexon Sequence and Subsequent Restriction Fragment Length Polymorphism Analysis

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We recently developed a new PCR-restriction fragment length polymorphism (RFLP)-based assay using the miniexon sequence from the genus *Leishmania*. Here we report the application of this new genotyping method to naturally infected clinical samples for the differentiation of New and Old World *Leishmania* species. Of the newly developed assay and four currently applied diagnostic tests (i.e., in vitro cultivation, serology, and two other molecular assays using either the small subunit-internal transcribed spacer sequence or a repetitive genomic sequence), the miniexon assay showed the highest sensitivity, 89.7%, compared to 70.6, 57.1, 51.7, and 79.3%, respectively. Species differentiation was robust and reliable compared with that by two other *Leishmania* genotyping techniques. The assay provides a valuable tool for the identification of *Leishmania* directly from clinical samples and enables determination of the infecting species by a facile technique with high discrimination power. Since *Leishmania* causes a broad spectrum of diseases distinguished by different parasite and host factors, detection and characterization of the infecting species is crucial for the confirmation of a diagnosis as well as the establishment of the clinical prognosis and the initiation of an adequate therapeutic approach. The miniexon PCR-RFLP assay will facilitate such determination and might improve diagnosis and treatment of leishmaniasis.

Diagnosis of leishmaniasis can be made on the basis of clinical and epidemiological data but has to be confirmed by the demonstration of the parasite to avoid potential misdiagnosis (29). Because of differences among the *Leishmania* species in levels of virulence and in responses to the various chemotherapeutic regimens, correct identification is essential in order to determine the clinical prognosis and prescribe an appropriate species-specific therapeutic regimen (3).

At present, the classical methods used for the direct detection of the parasite include the visualization of amastigotes by microscopic examination of Giemsa-stained smears and in vitro culture of the parasite. Though microscopic examination is rapid, cheap, and easy to perform, it lacks sensitivity due to the generally low number of parasites in tissue samples (42). Culture techniques are more sensitive but require a sophisticated laboratory setup, are time-consuming, and harbor the risk of contamination (3). Indirect methods based on the analysis of specific immune responses cannot distinguish between past and current infections, and depending on the antigens used in serological assays, diagnosis can be complicated by cross-reaction of antibodies with other pathogens. Another drawback is the limited sensitivity because antibody titers may vary with the infecting species, tissue tropism, and the immunocompetence of the host (1).

Species discrimination is important not only for epidemio-

logical reasons but also for clinical reasons. *Leishmania* species were originally classified by their geographical distributions and the clinical presentations of the diseases they cause, as well as by epidemiology, sand fly vectors, or animal reservoirs (20). Since morphological discrimination of *Leishmania* species is not possible, a variety of biochemical, immunological, and molecular tools have been developed for the differentiation of the pathogenic species, such as isoenzyme, serodeme, and schizodeme analysis and hybridization techniques with species-specific DNA probes (16, 43).

With the advances in molecular techniques, a number of molecular markers and PCR protocols for the detection or identification of *Leishmania* on different taxonomical levels (genus, complex, and species) have been reported (11, 43). Target sequences for characterization include either nuclear DNA, such as the small subunit rRNA (SSU rRNA) gene (40), a repetitive genomic sequence (30), the miniexon (spliced leader) gene repeat (18), the beta-tubulin gene region (22), the gp63 gene locus (41), and internal transcribed spacer (ITS) regions (9); microsatellite DNA (36, 37); or kinetoplast DNA, such as minicircle sequences (34).

While all the above-mentioned approaches provide a multitude of valid taxonomic characters for differentiation, they are accompanied by a number of factors which limit their use in a routine diagnostic laboratory. Some of the assays are restricted to the identification of *Leishmania* at the genus level (5, 25) and do not allow species differentiation, whereas others are adapted for individual complex characterization (10, 13), which limits their applicability to restricted geographical areas. Other

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molecular approaches involve the combination of PCR amplification with DNA probe hybridization to enhance the sensitivity of the assay (33, 35), an elaborate and time-consuming procedure. The use of random amplified polymorphic DNA analysis of genomic DNA (12, 38) is limited since it requires purified parasite DNA and highly standardized PCR conditions to guarantee specificity. In addition, the results may be difficult to interpret. Assays based on the multiplex PCR technique (17) represent yet another approach but harbor the risk of reduced sensitivity caused by primer dimer formation.

The minixon gene of kinetoplastid protozoa, which is involved in the transsplicing process of nuclear mRNA, is present as 100 to 200 tandemly repeated copies per nuclear genome. Each repeat consists of three major parts: a transcribed region comprising a highly conserved 39-nucleotide exon; a moderately conserved intron, approximately 55 to 101 bp; and a nontranscribed, intergenic spacer of variable length (51 to 1,350 bp) depending on the genus and species (39). These features allow the minixon to be utilized as a genotyping marker. Based on these characteristics, we recently established a PCR assay similar to that described by Fernandes et al. (14). This PCR assay amplifies all the minixon sequences in a single reaction using universal primers, allowing preliminary discrimination between the major complexes (i.e., Old World *Leishmania*, New World *Leishmania*, and New World *Viannia* complexes) as a result of the variability in sizes of the amplification products. After restriction enzyme digestion of the PCR product, a characteristic restriction fragment length polymorphism (RFLP) pattern is produced that depends on size variations in the polymorphic spacer regions as well as mutations in the recognition sites of a chosen restriction enzyme. The minixon PCR-RFLP genotyping scheme was validated with cultured World Health Organization reference strains of *Leishmania* and cultured isolates from patients attending the polyclinic of the Swiss Tropical Institute (STI). It has proven to be a valuable tool showing high resolution, sensitivity, and an ability to discriminate between all clinically relevant *Leishmania* species (24).

The aim of this study was to optimize the minixon PCR assay for routine diagnosis of infections in naturally infected clinical samples without the need for cultivation and to compare our results with those obtained by using current methods for diagnosis of leishmaniasis. Moreover, genotyping results of the minixon RFLP system were validated by parallel analysis of the samples with two other PCR-RFLP typing schemes which are based on an uncharacterized repetitive genomic sequence of Old World *Leishmania* (30) or the ITS region of the rRNA gene (37a).

MATERIALS AND METHODS

Clinical samples. A total of 50 samples from patients presumed to have leishmaniasis were used in this study. The samples were either sent to our diagnostic unit or obtained from patients returning from areas where the disease is endemic who attended the STI polyclinic with either cutaneous signs or a tentative diagnosis of visceral leishmaniasis. Three samples were from patients with a concurrent HIV infection. The samples consisted of 25 skin biopsy specimens, 16 bone marrow biopsy specimens, 2 lesion aspirates, 4 blood samples (anticoagulated with EDTA), 2 duodenal biopsy specimens, and 1 gastric biopsy specimen. Four of the 25 skin biopsy specimens arrived as paraffin-embedded tissue samples. Samples were split on arrival; one part was used for in vitro cultivation, except in the case of paraffin-embedded tissue samples, and the other

part was processed for PCR analysis. Corresponding serum samples were available from 16 patients, and they were tested for *Leishmania*-specific antibodies by an indirect fluorescent-antibody test (IFAT). Of these, four samples were derived from patients who had traveled to the Americas, six samples were derived from patients for whom there was no information on travel history, and 19 were derived from patients with a history of travel in the Old World. In this study, a sample was considered positive when the results of at least one of the assays performed were positive. Assays were repeated independently to exclude contamination.

Specimen processing and DNA preparation. Skin, duodenal, and gastric biopsy specimens arrived in 0.9% NaCl or another physiological transport medium and were first cut into smaller pieces with a sterile scalpel in a petri dish. They were then transferred with the medium into a 2-ml tube, pelleted by centrifugation, and finally resuspended in 500 μ l of lysis buffer (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.5% sodium dodecyl sulfate).

Lesion aspirates were centrifuged at $8,000 \times g$ for 10 min and resuspended in 500 μ l of lysis buffer.

Anticoagulated bone marrow samples were centrifuged for 15 min at $800 \times g$. The buffy coat was transferred to a new tube, washed with phosphate-buffered saline (PBS), pH 7.4, and resuspended in 500 μ l of lysis buffer. Coagulated clots of bone marrow samples were treated like skin biopsy samples as described above.

EDTA-anticoagulated blood was mixed with an equal volume of PBS, pH 7.4, layered over Ficoll-Paque PLUS (density, 1.077 g/liter; Amersham Biosciences Europe GmbH, Dübendorf, Switzerland), and spun for 30 min at $400 \times g$. The interphase containing peripheral blood mononuclear cells was carefully transferred to a fresh tube, washed twice with PBS, pH 7.4, and resuspended in 500 μ l of lysis buffer.

Small sections (not more than 25 mg) of paraffin-embedded tissue samples were scratched with a sterile scalpel into a 1.5-ml tube. Paraffin was removed by being washed once with 1.2 ml of xylene and two times with 1.2 ml of ethanol. Residual ethanol was evaporated by incubating the tube for 15 min in a heating block at 37°C. The pellet was resuspended in 180 μ l of buffer ATL (QIAamp DNA Mini Kit; Qiagen AG, Basel, Switzerland), and DNA was extracted and purified by using the QIAamp DNA Mini Kit according to the QIAamp DNA Mini Tissue protocol.

Generation of the positive control template. A 498-bp fragment of the cysteine protease *cpb2* gene from *Leishmania mexicana* (accession no. AJ319727; nucleotides 1302 to 1800), designated as the positive internal control in the minixon PCR assay (6) was amplified by PCR by using primers *cpb2fw* (5'-TGA TCA CCA CGA AGG AGT GC-3') and *cpb2rev* (5'-ACG CGA GGG CAC GCG AGG-3'). Minixon primer sites were introduced by subsequent nested PCR with the composite primers *cpb2Mifw* (5'-TAT TGG TAT GCG AAA CTT CCG TGA TCA CCA CGA AGG AGT GC-3') and *cpb2Mirev* (5'-ACA GAA ACT GAT ACT TAT ATA GCG ACG CGA GGG CAC GCG AGG-3'). PCR mixtures (50 μ l) and cycling conditions for all the reactions were the same as those for the minixon PCR described below. PCR products were purified by size-selective polyethylene glycol precipitation and cloned by using the pGEM-T vector system (Promega, Madison, Wis.) according to the manufacturer's instructions. Plasmid DNA containing the positive control template was purified by using the CONCERT Rapid Plasmid Miniprep system (Invitrogen Life Technologies, Basel, Switzerland) according to the manufacturer's manual. Purified plasmid DNA was stored in Tris-EDTA, pH 8.0, containing 0.05% W-1 (Invitrogen Life Technologies).

PCR assays. For repetitive sequence PCR (*Leishmania* genotyping [LEG] PCR) (28), primers T2 (5'-CGG CTT CGC ACC ATG CGG TG-3') and B4 (5'-ACA TCC CTG CCC ACA TAT GC-3') were used to amplify a repetitive DNA sequence from Old World *Leishmania*. Two microliters of DNA solution was amplified in a 100- μ l reaction mixture containing 50 mM PCR buffer, 0.2 mM deoxynucleoside triphosphates, 1.5 mM MgCl₂, 0.5 μ M (each) primers, and 0.5 U of *Taq* DNA polymerase (Invitrogen Life Technologies). PCR amplification was carried out in a DNA Thermal Cycler 480 (Perkin Elmer, Boston, Mass.). After an initial denaturation step (5 min at 96°C), 40 cycles of 96°C for 30 s, 56°C for 45 s (annealing), and 72°C for 60 s (extension) were performed. For minixon PCR, primers Fme (5'-TAT TGG TAT GCG AAA CTT CCG-3') and Rme (5'-ACA GAA ACT GAT ACT TAT ATA GCG-3') were used to amplify the nontranscribed spacer region of the minixon sequence of *Leishmania* (24). Two microliters of DNA solution was amplified in a 50- μ l reaction mixture containing 50 mM PCR buffer, 0.2 mM deoxynucleoside triphosphates, 1.5 mM MgCl₂, 40 mM tetramethylammonium chloride, 12% dimethyl sulfoxide, 0.5 μ M (each) primers, and 0.5 U of *Taq* DNA polymerase. The positive control template was added to a final concentration of 250 fg (corresponding to approximately 70,000 plasmid copies) per reaction mix. The PCR amplification was

FIG. 1. Coamplification of *Leishmania* DNA from clinical samples and a positive control template. Black triangles denote the 540-bp band of the positive control template, and grey triangles correspond to the *Leishmania*-specific PCR signal. Lanes 1 and 2, positive samples. The PCR products of the target DNA and the positive control template are visible on the agarose gel. Lanes 3 and 4, positive samples. Target DNA outcompeted the positive control template. Lane 5, PCR inhibition is indicated because the positive control template is not visible. Lane 6, previously inhibited sample after DNA dilution. Lane 7, previously inhibited sample after subsequent purification. Lane 8, negative sample with only control template visible.

carried out in a Mastercycler gradient 5331 (Eppendorf, Hamburg, Federal Republic of Germany). After an initial denaturation (5 min at 94°C), 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s were performed.

Genotyping. Typing of the *Leishmania* species was achieved by RFLP analysis of the amplified minixon gene. Ten microliters of the minixon PCR products was digested with the restriction enzymes according to the genotyping scheme described elsewhere (24).

In order to confirm our genotyping results, DNA aliquots from all the samples found to be positive by PCR were analyzed by two other PCR-RFLP genotyping schemes based on the sequence differences in the genomic repeat of Old World *Leishmania* (30) and the ITS region of the SSU rRNA gene (37a).

Cultivation of parasites. The culture medium, a 1:1 mixture of SDM-79 (7) and SM (8) complemented with 15% heat-inactivated fetal bovine serum, was supplemented with an anticontamination cocktail consisting of penicillin G (60 µg/ml), kanamycin (100 µg/ml), chloramphenicol (10 µg/ml), and flucytosine (50 µg/ml). The medium was directly inoculated with the patient specimens and incubated at 27°C without gas phase in T-25 tissue culture flasks (Falcon, BD Biosciences, Allschwil, Switzerland). Cultures were checked for the presence of promastigote forms of *Leishmania* by examination with an inverted microscope. Microscopic examination was done every 2 days for up to 15 days.

Serology. Serum samples were tested for anti-*Leishmania* antigen antibodies by using an IFAT. Twelve-well slides were coated with imprint preparations of an *L. donovani*-infected hamster spleen and stored at -80°C. Slides were defrosted, fixed with acetone for 10 min at room temperature, and dried under ventilation. The wells on the slides were covered with 25 µl of serial twofold dilutions (1:40 to 1:1,280 in PBS, pH 7.2) of the sera and incubated in a humid chamber for 30 min at 37°C. After three washes with PBS, pH 7.2, 25 µl of fluorescein isothiocyanate-labeled goat anti-human immunoglobulin G (Biomérieux, Marcy l'Etoile, France) diluted 1:80 in Evansblue (0.1 mg/ml of PBS, pH 7.2) was applied to each well. After incubation in a humid chamber for 30 min at 37°C and three washes with PBS, pH 7.2, the slides were dried under ventilation, mounted with 50% (vol/vol) glycerol-PBS, and covered with a coverslip. Staining of the samples was assessed by examination with a fluorescence microscope and compared with that of positive and negative controls. Samples showing fluorescence with serum dilutions higher than 1:80 were regarded as positive.

RESULTS

Application of the minixon PCR assay to clinical samples.

In order to identify inhibitory effects in the PCR due to impurities in the sample and to assess tube-to-tube variations in PCR efficiencies, the test was set up with an internal positive control. The sensitivity of the assay was not compromised by

the coamplification of the positive control template (data not shown), and a concentration of 250 fg of plasmid DNA (corresponding to about 70,000 plasmid copies) per reaction mix produced a clear band in the PCR assay. From a total number of 50 clinical samples, DNA was purified and tested with the minixon PCR assay. DNA from each sample was coamplified along with the positive control template. Of the 26 DNA samples which gave a positive result, the majority outcompeted the positive control template (Fig. 1, lanes 3 and 4), whereas in the remaining samples the signals of both the *Leishmania* and the positive control were visible on the agarose gel (Fig. 1, lanes 1 and 2). The other 24 samples were negative in the assay (Fig. 1, lane 8). PCR inhibition was observed in 6 of the 50 samples (Fig. 1, lane 5). Inhibition was resolved in two samples after a 10-fold dilution, after which one sample became positive (Fig. 1, lane 6) and one was determined to be negative. The remaining four samples had to be purified again by using the QIAamp DNA Mini Kit, after which two became positive (Fig. 1, lane 7) and two were determined to be negative.

Comparison of the diagnostic assays. The minixon PCR assay was compared to the methods currently applied at the STI for the diagnosis of leishmaniasis (i.e., in vitro cultivation and serology) and a PCR assay which is based on the amplification of a repetitive genomic sequence of Old World *Leishmania* (LEG PCR assay) (28). All tissue samples, which were obtained from patients with a tentative diagnosis of leishmaniasis on clinical grounds, were tested with the LEG and the minixon PCR assays, and 38 of them were inoculated into culture. Corresponding serum samples, available from 16 patients, were analyzed for anti-*Leishmania* antigen antibodies by IFAT. Results are summarized in Table 1. For a limited comparison, only positive samples were tested by the PCR assay using the ITS region of the SSU rRNA gene (37a).

No single test could serve as a "gold standard" against which the different assays could be evaluated. Therefore, assessment of test performance (Table 2) was based on the assumptions that (i) a patient was positive for leishmaniasis when the infection was detected by at least one of the diagnostic assays and (ii) a patient was negative for leishmaniasis when results of all four assays (including serology) were negative. A *Leishmania* infection was diagnosed in 29 patients. Two infections were detected by all four methods, 12 infections were detected by

TABLE 1. Diagnostic results for clinical samples

Specimen type	Total no. of positive samples ^a	No. of samples positive by:				
		Culture	LEG PCR	Minixon PCR	Serology ^b	SSU-ITS PCR
Skin biopsy	12	6	8	12	4	6
Bone marrow	7	2	7	4	3	3
Lesion aspirate	1	0	1	1	0	1
Blood ^c	2	2	2	2	0	1
Duodenal biopsy ^d	2	1	2	2	0	1
Gastric biopsy ^d	1	1	1	1	0	1
Paraffin-embedded skin biopsy	4	0	2	4	1	1
Total	29	12	23	26	8	15

^a Leishmaniasis confirmed by at least one of the diagnostic tests.

^b For patients from whom a corresponding serum sample was available.

^c Supplemented with EDTA.

^d From patients positive for HIV.

TABLE 2. Comparison of the diagnostic assays

Assay (total) ^a	No. of positive ^b samples/no. of tested samples						Sensitivity (%)
	Skin biopsy specimens	Bone marrow samples	Blood samples	Duodenal biopsy specimens ^c	Gastric biopsy specimens	Lesion aspirates	
In vitro cultivation (38)	6/9	3/5	1/1	1/1	1/1	NA	70.59
Serology (16)	5/9	3/4	0/1	NA	NA	NA	57.14
LEG PCR (50)	10/16 ^d	7/7	2/2	2/2	1/1	1/1	79.31 (100) ^d
SSU-ITS PCR (29) ^e	7/16	3/7	1/2	2/2	1/1	1/1	51.71
Minixon PCR (50)	16/16	4/7	2/2	2/2	1/1	1/1	89.66

^a Total number of samples subjected to each assay.^b Leishmaniasis confirmed by at least one of the diagnostic tests.^c NA, not applicable.^d All negative samples were subsequently shown to be infected with New World species; therefore, sensitivity for Old World species was adjusted to 100%.^e Only samples that were previously identified as positive by other assays were tested.

three methods, and 10 infections were detected by two methods. In five patients, the presence of a *Leishmania* infection was confirmed by one method only (four cases were confirmed by the minixon PCR assay, and one was confirmed by the LEG PCR assay).

In vitro cultivation was performed for a total of 38 samples. No cultures were set up from the four paraffin-embedded specimens, and results for eight specimens are missing because either they were lost due to contamination or there was no request for this test. Twelve of 17 *Leishmania*-positive samples were detected by cultivation, of which one infection was confirmed by one other test, 13 were confirmed by two other tests, and three were confirmed by three other tests, resulting in a sensitivity of 70.6%. The remaining 21 samples with a negative culture result were also negative by both PCR assays, giving a specificity of 100%.

Sixteen serum samples were available to test by IFAT for anti-*Leishmania* antigen antibodies. This test identified eight positive samples from 14 patients whose infections were confirmed by other assays (two were confirmed by three other tests, three were confirmed by two other tests, and three were confirmed by one other test) but failed to diagnose the infection in six samples determined to be positive by other assays, giving a sensitivity of 57.1%. Two serum samples available from patients without an infection, as confirmed by all other tests, were also negative (specificity, 100%).

The LEG PCR detected *Leishmania* in 23 of the 29 positive samples, giving a sensitivity of 79.1%, but failed to diagnose the infection in six specimens that were confirmed to be positive by other tests. Parasites in all six samples for which no PCR product was obtained were subsequently identified by minixon RFLP analysis as *Leishmania* species from the New World, resulting in a sensitivity of 100% for Old World species only. Specificity of the LEG PCR assay was 100% with all samples which were also negative by all other tests, but one sample was positive only with this test and was not confirmed to be positive by any of the other tests.

The PCR-RFLP genotyping assay based on the ITS region of the SSU rRNA gene detected a *Leishmania* infection in 15 of the 29 samples and gave a sensitivity of 51.7%. Because we tested only samples previously identified as positive, we were not able to determine the specificity of the assay.

The minixon PCR detected *Leishmania* in 26 of the 29 positive samples and gave a sensitivity of 89.7% but failed to

diagnose the infection in three specimens, of which two infections were confirmed by the LEG PCR assay and positive serology and one was confirmed by the LEG PCR assay only. It is worth mentioning that these three DNA samples were all derived from bone marrow specimens and were freeze-thawed several times. All the 21 *Leishmania*-negative patients were negative by the minixon PCR assay (specificity, 100%), but it should be noted that four of the 29 positive results were not confirmed by any other test.

Genotyping of *Leishmania* spp. by RFLP analysis. All samples positive by the minixon PCR assay were subjected to RFLP analysis of the amplification product (24). No unusual or ambiguous RFLP patterns occurred, and *Leishmania* from all positive samples could be assigned to a given species according to the typing scheme. The species in 15 of the 26 positive samples was identified as *L. infantum*, that in four samples was identified as *L. major*, those in one sample each were identified as *L. tropica* and *L. mexicana*, that in three samples was identified as *L. braziliensis*, and that in two samples was identified as *L. guyanensis*.

Species in the 23 samples positive by the LEG PCR assay were typed by RFLP analysis as belonging to the Old World species *L. infantum* ($n = 14$), *L. donovani* ($n = 4$), *L. major* ($n = 4$), and *L. tropica* ($n = 1$).

DNA aliquots of the samples from the 29 *Leishmania*-positive patients were also analyzed by the PCR-RFLP genotyping assay, which is based on sequence variations in the ITS region of the SSU rRNA gene (37a). With this assay, species from positive samples were typed at the complex level. They were assigned to the *L. donovani* ($n = 7$), *L. major* ($n = 6$), *L. tropica* ($n = 1$), and *L. braziliensis* ($n = 1$) complexes.

A summary of the genotyping results is given in Table 3. There was a good correlation between the results obtained with the LEG and minixon genotyping schemes. However, the LEG PCR system cannot detect any of the New World species, and a species from four samples typed as *L. donovani* with the LEG PCR system was typed as *L. infantum* with the minixon system. There was also a good correlation between the results of the minixon and ITS genotyping schemes, except that a species from two samples typed as *L. infantum* by the minixon system was assigned to the *L. major* complex by the ITS system. These two samples were two of the four samples with a species typed as *L. donovani* by the LEG PCR system.

TABLE 3. Results from three PCR-RFLP genotyping schemes for *Leishmania* spp.

No. of patients	Species or complex identified by ^a :		
	LEG-RFLP typing	Minixxon RFLP typing	ITS-RFLP typing
1	ND	<i>L. braziliensis</i>	<i>L. braziliensis</i> complex
2	ND	<i>L. braziliensis</i>	ND
2	ND	<i>L. guyanensis</i>	ND
1	ND	<i>L. mexicana</i>	ND
1	<i>L. tropica</i>	<i>L. tropica</i>	<i>L. tropica</i> complex
4	<i>L. major</i>	<i>L. major</i>	<i>L. major</i> complex
7	<i>L. infantum</i>	<i>L. infantum</i>	<i>L. donovani</i> complex
4	<i>L. infantum</i>	<i>L. infantum</i>	ND
3	<i>L. infantum</i>	ND	ND
2 ^b	<i>L. donovani</i>	<i>L. infantum</i>	ND
2 ^b	<i>L. donovani</i>	<i>L. infantum</i>	<i>L. major</i> complex

^a ND, not determined (PCR test failed to detect the infection).

^b Discordant results.

DISCUSSION

Detection of *Leishmania* parasites in a clinical sample is necessary to confirm a suspected case of leishmaniasis. Most commonly used methods for the direct detection of the parasite (e.g., microscopic examination of Giemsa-stained smears and in vitro cultivation) lack sensitivity because of the paucity of *Leishmania* parasites in some specimens (42) or are hampered by the problem of contamination (3). Indirect methods (e.g., detection of anti-*Leishmania* antigen antibodies by serological methods) are also limited in sensitivity and are not able to discriminate between past and current infections. The advent of the PCR technology has opened new avenues in the diagnosis of leishmaniasis, and several approaches have been developed during the last two decades (11, 43). Most of these assays are based on highly repetitive genomic gene loci or extrachromosomal kinetoplast DNA sequences, but their use is confined to the detection of the parasite at genus (5, 25) or complex (10, 13) level, and therefore, their application is limited to restricted geographical areas and diagnostic settings.

Our assay was developed for use in a diagnostic setting where patients with a history of global travel are seen. It was the aim of this study to optimize and evaluate a recently developed PCR-RFLP-based genotyping assay for *Leishmania* species. The assay targets the minixxon gene of the parasite and has proven to be simple and cheap; i.e., the detection of all *Leishmania* spp. is accomplished by one PCR with all-encompassing primers. It has a sufficient sensitivity, high specificity, and the ability to differentiate between all clinically relevant *Leishmania* species by RFLP analysis of the PCR product (24).

The inclusion of a positive control template makes it possible to detect inhibitory effects in the PCR mix and also controls for tube-to-tube variation both within and between different PCR experiments. Several methods are available for the design and construction of an internal positive control, some of which were successfully used in PCR assays for *Leishmania*. These include the coamplification of the human β -globin gene in a multiplex PCR approach (27), the use of a competitive PCR assay with heterologous plasmid sequences (25), and the use of synthetic probes in the reaction (4). We used a competitive PCR assay targeting a 498-bp fragment of the *cpb2* gene of *L.*

mexicana. This fragment is slightly larger than our target sequence, has a G+C content similar to that of the minixxon sequence, and is free of any restriction sites required for subsequent RFLP analysis of the minixxon sequence. In all assays, this positive control template yielded a clearly distinguishable PCR product, and coamplification did not affect assay sensitivity or compromise amplification of the minixxon sequence and never interfered with subsequent RFLP analysis (data not shown).

Fifty patient samples were available for the validation of the assay, and samples were subjected in parallel to a second PCR assay (LEG PCR) (28). When possible, samples were cultured, and serum samples were tested for *Leishmania*-specific antibodies. As expected, PCR assays were by far more sensitive than the currently applied diagnostic methods. Successful culture is dependent on the presence of viable organisms, and growth requirements are known to differ between species (3). IFAT failed to detect six infections (those of four patients with cutaneous manifestations and those of two patients suspected to have visceral leishmaniasis) which were all confirmed by at least two other tests. This failure might reflect the generally lower sensitivity of serological assays for detection of cutaneous forms of the disease (21) or might be due to a lack of cross-reactivity with the antigens on which the assay is based (26). Furthermore, humoral responses in patients with a concurrent HIV infection or other causes of immunosuppression may be moderate or even absent (1). Indeed, all three samples obtained from HIV-positive patients were negative by the serological assay.

The LEG PCR assay was tailored for the characterization of Old World *Leishmania* and consequently failed to detect six infections by parasites subsequently typed as New World species by the minixxon assay. On the other hand, the minixxon assay failed to detect three infections by parasites typed as Old World species by the LEG assay, which might be due to lower sensitivity of the minixxon PCR. However, storage of DNA from these three patients might also have compromised the results of the minixxon PCR.

Proper sampling is crucial for the establishment of a reliable diagnostic assay for leishmaniasis, and basic questions, such as what the optimal site of sample collection is (32) and whether blood samples are sufficient for a reliable diagnosis, remain open. Hematogenous dissemination has been described for *L. donovani*, *L. infantum*, *L. tropica*, and *L. braziliensis* (J. Kubar, J. F. Quaranta, P. Marty, A. Lelievre, Y. Le Fichoux, and J. P. Aufeuve, Letter, Nat. Med. 3:368, 1997), and PCR-based assays on blood samples have proven useful for the diagnosis and monitoring of visceral leishmaniasis in adults and children in the Mediterranean region, as well as in HIV-coinfected patients (15, 19). The four blood samples in our study were processed only after the request for an additional tissue sample, and their analysis confirmed the results obtained with the other analyzed specimens.

The minixxon PCR assay was more sensitive than the conventional diagnostic methods and capable of detecting infection in a wide range of clinical samples, including paraffin-embedded tissue samples. This is of advantage since tissue biopsy specimens are often fixed and embedded in paraffin for histological examinations. Application of the PCR assay to paraffin-embedded specimens prevents repeated invasive sam-

pling if a differential diagnosis of leishmaniasis is required and might be useful for the investigation of archived samples. It is noteworthy that other PCR-based assays have reported a higher sensitivity. Yet, these assays detect the presence of *Leishmania* parasites rather than individual *Leishmania* species. Although we found the minixon assay to show a high sensitivity, other assays might be preferable for samples in which very low densities might be expected.

Prior to the development of biochemical or molecular tools for the characterization of *Leishmania*, diagnosis of leishmaniasis relied on clinical and epidemiological criteria. Over the past few years, the reports of atypical clinical manifestations have accumulated, challenging this traditional classification. This includes reports of visceral leishmaniasis caused by the dermatropic species *L. tropica* and *L. mexicana* (23) and the occurrence of cutaneous manifestations caused by *L. chagasi* and *L. infantum*, which usually cause visceral leishmaniasis (31; J. A. Rioux and G. Lanotte, Letter, Trans. R. Soc. Trop. Med. Hyg. 84:898, 1990).

Therefore, correct classification of infecting *Leishmania* species can provide important complementary information for the clinician that may result in modified clinical prognosis, as well as initiation of species-specific therapeutic approaches (3). Furthermore, identification of the infecting species is important in monitoring therapy. In areas where different species are sympatrically transmitted, discrimination between relapse of a latent infection and reinfection by another species is important in cases of reemerging leishmaniasis.

In order to appraise the validity of the minixon genotyping scheme, we typed the samples with two other PCR-RFLP-based methods, the LEG and the ITS system. Although no unusual patterns were encountered with any of the genotyping schemes and the correlation between the results of all the systems was satisfying (Table 3), some discrepancies were observed. Because the biological species concept is difficult to apply to *Leishmania* and there is as yet no agreement on the definition of the species boundaries within the genus (2), none of the genotyping systems could actually serve as a gold standard for the evaluation of the others. We observed four discrepancies, two in which data obtained with the minixon PCR suggested the presence of *L. infantum* but LEG PCR identified *L. donovani*. Although *Leishmania* has been thought to be clonal (2), hybrid forms within the *L. donovani* complex might occur. Using independent single marker genes cannot exclude this possibility. Discrepant identification of *L. infantum* and *L. major* in two cases cannot be explained but might reflect a laboratory artifact. Previously, the minixon genotyping system has been tested with both World Health Organization reference strains and culture isolates from the STI, all independently characterized by isoenzyme analysis, and the results were in good yet not perfect concordance with the species definitions (24).

In conclusion, the minixon PCR-RFLP-based genotyping assay has proven to be a reliable test for the detection of *Leishmania* in a wide range of clinical samples obtained from a travel clinic. The standardization of the test as a competitive assay including the coamplification of an internal positive control template has turned out to be a valuable tool for the assessment of PCR performance. We have demonstrated that the assay is quick and easy to perform and shows in this study

a higher sensitivity than conventional diagnostic methods. Moreover, it enables the characterization of the parasite at species level by a simple method within a reasonable time frame and provides a useful tool for the characterization of clinically relevant *Leishmania* species, which can help the clinician choose an adequate therapeutic approach.

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