Identification of *Leishmania* spp. by Molecular Amplification and DNA Sequencing Analysis of a Fragment of rRNA Internal Transcribed Spacer 2[∇][†]

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Isoenzyme analysis of cultured parasites is the conventional approach for *Leishmania* species identification. Molecular approaches have the potential to be more sensitive and rapid. We designed PCR generic primers to amplify a segment of the rRNA internal transcribed spacer 2 (ITS2) from multiple *Leishmania* species. To validate the selected ITS2 fragment, we tested clinical specimens and compared the species results obtained by the molecular approach (PCR followed by DNA sequencing analysis) with those from the parasitologic approach (*in vitro* culture followed by isoenzyme analysis). Among the 159 patients with clinical specimens positive by both approaches, a total of eight *Leishmania* species were identified. The species results were concordant for all but two patients: for one patient, the results were *Leishmania* (*Viannia*) *guyanensis* by the molecular approach versus *L.* (*V.*) *braziliensis* by the parasitologic approach; for the other patient, the results were *L.* (*Leishmania*) *tropica* versus *L.* (*L.*) *major*, respectively. ITS2 PCR, followed by sequencing analysis, can be used to detect and discriminate among *Leishmania* species. The results confirmed our hypothesis that a region of the ITS2 gene can complement the characterization of *Leishmania* parasites at the species level. The approach we developed can be used as a diagnostic tool in reference laboratories with adequate infrastructure to perform molecular characterization of pathogens.

Leishmaniasis encompasses multiple clinical syndromes, most notably, visceral, cutaneous, and mucosal forms (9, 13, 14, 22, 27, 30). Leishmaniasis is endemic in focal areas in the tropics, subtropics, and southern Europe and is caused by species in the *Leishmania* and *Viannia* subgenera (14). Different species can be associated with diverse clinical manifestations and sequelae. Species identification can facilitate clinical management, such as decisions regarding whether/which treatment is indicated.

Isoenzyme analysis of cultured parasites is the conventional diagnostic approach for *Leishmania* species identification. Molecular approaches have the potential to be more sensitive and rapid; e.g., the results can be available within days versus weeks or months. However, *Leishmania* PCR-based assays have varied in sensitivity, specificity, and utility for genus- or complex-level versus species-level characterization (2, 3, 7, 10, 24–26, 28); some approaches have been highly sensitive for genus-level detection, e.g., PCR methods that target minicircle kinetoplast DNA (kDNA), whereas others have been tailored for particular species/settings.

We sought to develop a comprehensive molecular approach

for characterizing *Leishmania* species; we focused on the rRNA internal transcribed spacer (ITS) region, which has been sequenced by other investigators and proposed as a target for molecular typing (12, 25). Here we describe our identification and validation of a diagnostic fragment that was amplified from the ITS2 with a PCR generic primer pair that we designed. Rather than conduct a formal evaluation of the molecular approach, we focused on demonstrating proof of concept. By using ITS2 PCR, followed by DNA sequencing analysis of the amplicons, we were able to discriminate among *Leishmania* spp. to complement the diagnosis of leishmaniasis. This method has been incorporated into the Centers for Disease Control and Prevention (CDC) algorithm for the laboratory diagnosis of leishmaniasis, improving the turnaround time for diagnosis and for decisions regarding treatment of cases of infection.

MATERIALS AND METHODS

Specimen selection and handling. For the initial evaluations of the *Leishmania* ITS2—to identify what we refer to as the diagnostic fragment—we analyzed a total of 40 cultured isolates previously characterized by isoenzyme analysis and epidemiologic criteria as *Leishmania* (*Viannia*) *braziliensis*, *L.* (*V.*) *guyanensis*, *L.* (*V.*) *panamensis*, *L.* (*L.*) *mexicana*, *L.* (*L.*) *amazonensis*, *L.* (*L.*) *donovani*, *L.* (*L.*) *infantum*, *L.* (*L.*) *chagasi*, *L.* (*L.*) *aethiopica*, *L.* (*L.*) *major*, and *L.* (*L.*) *tropica* (Table 1).

To validate the diagnostic ITS2 fragment, we analyzed clinical specimens that were submitted from 2005 to 2009 to our reference laboratory at the CDC for *Leishmania* testing. Specimens were used in accordance with a CDC human subject-approved protocol; no additional specimens were obtained for the molecular analysis. Per routine, to the extent possible, the specimens were shipped by overnight mail; almost all of the specimens were from skin biopsies, and

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TABLE 1. Size and sequence variability among cloned amplicons obtained with ITS2 primers LGITSF2/LGITSR2 from 40 cultured
Leishmania reference isolates[a]

Species	Total no. of isolates (WHO; CDC)	WHO international reference code(s)[b]	Range in no. of nucleotides per amplicon[c]	No. of distinct ITS2 sequences	Microsatellite repeat(s) [starting nucleotide position(s)]
L. (V.) braziliensis	4 (1; 3)	MHOM/BR/75/M2903	383–394	8	AT (69, 118); TC (266)
L. (V.) guyanensis	4 (1; 3)	MHOM/BR/75/M4147*	386–389	4	AT (137)
L. (V.) panamensis	4 (1; 3)	MHOM/PA/71/LS94*	372–389	5	TA (68); AT (117); TC (266)
L. (L.) mexicana	4 (2; 2)	MNYC/BZ/62/M379*; MHOM/BZ/82/BEL21	436–438	5	TA (61); AT (141)
L. (L.) amazonensis	4 (2; 2)	IFLA/BR/67/PH8*; MHOM/BR/73/M2269	446–450	2	TA (63); GA (362)
L. (L.) donovani	4 (2; 2)	MHOM/IN/80/DD8*; MHOM/ET/67/HU3	416–420	4	TA (60)
L. (L.) infantum	2 (1; 1)	MHOM/TN/80/LEM235*	418–420	2	TA (60)
L. (L.) chagasi	3 (1; 2)	MHOM/BR/74/M2682*	418–420	2	TA (60)
L. (L.) aethiopica	3 (1; 2)	MHOM/ET/72/L100	424–428	4	AT (58); TC (264)
L. (L.) major	4 (3; 1)	MHOM/IL/67/JERICHO II*; MHOM/SU/73/5-ASKH; MRHO/SU/59/P-strain	430–438	3	AT (129); TG (146)
L. (L.) tropica	4 (2; 2)	MHOM/SU/74/K27*; MHOM/SU/58/L39	406–415	9	AT (69, 129); TG (147); TC (269); GA (352)

[a] The 40 cultured isolates include 17 from the WHO and 23 from the CDC. The international codes for the WHO strains are provided. For the purposes of these analyses, CDC's *L. (L.) infantum* (Old World isolate), *L. (L.) chagasi* (New World isolates), and *L. (L.) donovani* strains were defined on the basis of epidemiologic/geographic criteria.

[b] Nine WHO isolates (∗) were used to amplify the full-length ITS sequence, as were isolates from the CDC. Overall, we deposited in GenBank the complete ITS sequences for *L. (V.) braziliensis* (accession numbers FJ753374 to FJ753385), *L. (V.) guyanensis* (FJ753387 to FJ753390), *L. (V.) panamensis* (FJ948438 to FJ948446), *L. (L.) mexicana* (FJ948432 to FJ948437), *L. (L.) amazonensis* (FJ753371 to FJ753373), *L. (L.) donovani* (FJ753386, GU045589, and GU045590), *L. (L.) chagasi* (GU045591), *L. (L.) infantum* (GU045592), *L. (L.) aethiopica* (GQ920673 to GQ920677), *L. (L.) major* (FJ753391 to FJ753395), and *L. (L.) tropica* (FJ948447 to FJ948465).

[c] Ten clones per amplicon were analyzed.

specimens typically were shipped in a sterile tube containing RPMI medium that CDC provided in advance. Upon arrival at the CDC, specimens were divided into portions for the parasitologic and molecular analyses described below. When possible, stained slides (e.g., histopathology slides provided by the practitioner or impression smears/wet mounts made by the CDC) were examined by light microscopy for amastigotes; the slides were not necessarily from the same specimens that were tested by culture and PCR.

Culture and isoenzyme analysis. The culture tubes contained Novy-MacNeal-Nicolle agar with 10% defibrinated rabbit blood and an overlay of RPMI medium with 15% fetal bovine serum. The tubes were incubated at 25°C; aliquots were examined by light microscopy two to three times a week until promastigotes were noted or 4 weeks elapsed. Positive cultures were transferred from tubes to large flasks containing ~20 ml of fresh RPMI medium to facilitate obtaining large numbers of parasites for isoenzyme analysis. In brief, after the parasites again reached log phase, the liquid culture medium that was harvested from the flasks was centrifuged at $850 \times g$ for 15 min (in phosphate-buffered saline [PBS], pH 7.2), the pellet was frozen, and fresh RPMI medium was added to the flasks, which were placed back into the incubator. This process was repeated several times, using the same tube to harvest the pellet. After the final harvest, the composite pellet was treated with 50 to 100 µl of lysis buffer (0.1 M Tris, 0.1 M malic acid, 0.1 M EDTA, and 0.1 M MgCl₂); the mixture was subjected to several freeze-thaw cycles at −70°C, and the lysate was stored in liquid nitrogen pending isoenzyme analysis. Cellulose acetate electrophoresis was performed, based on protocols developed by Kreutzer et al. (15–17), targeting isoenzymes 6-phospho-D-gluconate dehydrogenase (6PGDH) and glucose phosphate isomerase (GPI).

Identification of the diagnostic ITS2 fragment. DNA was extracted from the 40 banked, frozen cultured isolates—also referred to as the reference isolates—by using a QIAamp DNA Micro Kit (Qiagen), following the manufacturer's instructions. To amplify the ITS region, we used a previously described strategy (12) and cloned the fragments as described below. To identify/select the diagnostic ITS2 fragment, we conducted multiple alignments of the full-length ITS sequence (data not shown); the sequence of the ITS2 region of each *Leishmania* species was identified by searching the GenBank database using the BLAST algorithm. On the basis of this analysis, we found a variable region within nucleotide positions 392 and 809 [using *L. (L.) donovani* GenBank entry FJ753386 as a reference], designed the generic primers LGITSF2 (GCATGCC ATATTCTCAGTGTC) and LGITSR2 (GGCCAACGCGAAGTTGAATTC)

for this region, and used this primer pair to reamplify the DNA previously extracted from the 40 reference isolates.

For PCR, the total volume per reaction mixture was 50 µl, which included 1 µl of DNA, 0.2 µM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 1.5 mM MgCl₂, and 2.5 U of AmpliTaq Gold PCR polymerase (Applied Biosystems). PCR was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosytems), using the following cycle structure: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. The amplicons were purified with a StrataPrep PCR Purification Kit (Stratagene) and cloned with a pCR2.1-TOPO vector TOP10 Kit (Invitrogen). The clones were purified by using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced by using BigDye, version 3.1, chemistry (Applied Biosystems). The sequencing reaction mixtures were purified by using Multi-Screen-HV plates (Millipore) and were analyzed on an ABI Prism 3100 sequencer, with data collection software, version 2.0, and DNA Sequencing Analysis Software, version 5.1 (Applied Biosystems). The sequences were assembled, edited, and aligned by using the DNA STAR SeqMan (DNASTAR Inc.) and the GeneStudio suite (GeneStudio Inc.) programs.

We also designed an alternative reverse generic primer named LGITS2R1 (GAATTCTCGTTTTGGTTTTTTG), at positions 773 to 794 of the *L. (L.) donovani* reference (GenBank entry FJ753386), to compensate for the CG deletion and the G/T transversion in the LGITS2R2 primer binding region of *L. (L.) amazonensis* and *L. (L.) aethiopica*, respectively. We compared the PCR results obtained with three primer combinations—LGITSF2/LGITSR2, LGITSF2/LGITSR1, and LGITSF2/LGITSR1/LGITSR2—on seven cultured isolates of *L. (L.) amazonensis* and three of *L. (L.) aethiopica*, besides at least five isolates of each of the other pertinent species (Table 1). The amplicons were resolved with 1.5% agarose gels and visualized by staining with ethidium bromide.

Validation of the diagnostic ITS2 fragment. DNA was extracted directly from the clinical specimens (i.e., from ~5 to 10 mg of tissue and ~100 to 200 µl of blood or bone marrow) by using the method described above. PCR amplifications using generic primers LGITSF2/LGITSR2 were performed in a final reaction volume of 50 µl as described above, except that the aliquot of specimen DNA was 5 (versus 1) µl, diluted at 1:1 and 1:5. Sequencing reactions were performed directly from the amplicons by using the parameters described above.

The initial molecular testing of the clinical specimens was blinded such that
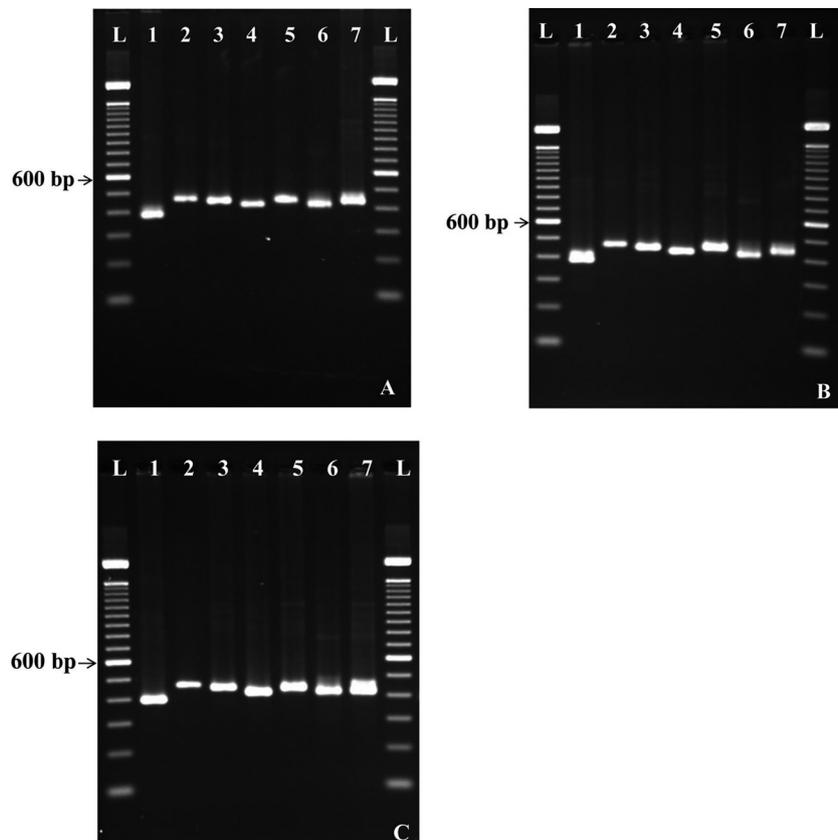
FIG. 1. (A) Agarose gel electrophoresis showing amplicons produced with generic primers LGITSF2/LGITSR1. (B). Agarose gel electrophoresis showing amplicons produced with generic primers LGITSF2/LGITSR1/LGITSR2. (C) Agarose gel electrophoresis showing amplicons produced with generic primers LGITSF2/LGITSR2. For all panels, DNA was extracted from the following cultures: lanes 1, *L.* (*V.*) *braziliensis*; lanes 2, *L.* (*L.*) *mexicana*; lanes 3, *L.* (*L.*) *amazonensis*; lanes 4, *L.* (*L.*) *donovani*; lanes 5, *L.* (*L.*) *major*; lanes 6, *L.* (*L.*) *tropica*; and lanes 7, *L.* (*L.*) *aethiopica*. Lanes L, 100-bp ladder size standards. Numbers to the left indicate the DNA fragment size in bp.

isoenzyme results were not known. However, for some specimens, if the species results differed from those obtained by the parasitologic approach (e.g., negative versus positive), the molecular testing was repeated. If residual specimen was available, DNA was reextracted; otherwise, DNA from the original extraction was reamplified. The species results obtained by isoenzyme analysis were considered the gold standard.

Quality assurance. Each DNA extraction batch included a negative control, i.e., DNA extracted from *Leishmania*-free eukaryotic cell cultures (E-6 cells in RPMI medium). For each PCR run, a tube containing water and PCR mix was used as a negative control (blank), and approximately 1 pg of the cloned full-length ITS region was used as a positive control. Separate rooms were used for DNA extraction, PCR Master Mix preparation, DNA loading onto the Master Mix, loading of the positive control, and electrophoretic analysis of amplicons.

RESULTS

Sequence divergence in the ITS2 among reference isolates. Use of the ITS2 generic primer pair LGITSF2/LGITSR2 to amplify DNA from the 40 cultured isolates produced amplicons that ranged from 372 to 450 bp in length (Table 1). The PCR results obtained by using different primer combinations—LGITSF2/LGITSR1, LGITSF2/LGITSR1/LGITSR2, or LGITSF2/LGITSR2—were comparable (Fig. 1A, B, and C), which suggests that either or both reverse primers (LGITSR1 and LGITSR2) can be used with the forward primer LGITSF2. We used LGITSF2/LGITSR2 to accomplish the validation described below.

Alignment of the ITS2 sequences showed that amplicons from the same species or even the same specimen varied in length, mainly because of AT, TC, TG, or GA microsatellite repeats (Table 1). To simplify the analysis, these discrepant repeat regions were removed, which yielded intraspecies sequences of comparable lengths. However, because of a few transitions and transversions, more than one sequence per species or isolate was found. Therefore, the few variant sequences were removed, and the resulting identical sequences were considered the consensus sequences.

In some instances, direct sequencing analysis of LGITSF2/LGITSR2 amplicons showed degraded regions, with multiple overlapping peaks after well-resolved regions, because of the regions with variant repeats. One example is the AT microsatellite starting at nucleotide positions 118 and 129 observed in the amplicons obtained from *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2903) and *L.* (*L.*) *tropica* (MHOM/SU/74/K27) WHO reference strains. Although the partial sequences sufficed for correct species identification by BLAST search, we cloned the reference strain amplicons to obtain the complete sequence of the fragment. Alignment of sequences obtained by randomly selecting one clone per species (from the identical group of sequences) showed sufficient divergence for discrimination both between and among species complexes/subgenera.

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                     1          11         21         31         51         71         81         91         101        111
L. (V.) braziliensis: GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACACACG TCTTCTCTAT .......... ....A.TATA .......... ...TATGT.G GGAGCCA.GA
L. (V.) guyanensis:   GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACACACG TCTTCTCTCT .......... ....AATATA .......... ...TATGT.G GGAGCCA.GA
L. (V.) panamensis:   GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACACACG TCTTCTCTCT .......... ....AATATA .......... ...TATGT.G GGAGCCA.GA
L. (L.) mexicana:     GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACAC..G TCTTCTCTAT ........AT ATACATTATA ...TATATAT GTGTCTGT.G GAAGCCAAGA
L. (L.) amazonensis:  GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACAC..G TCTTCTCTAT ........AT ..ACATTATA ...TATATAT GTGTCTGT.G GAAGCCAAGA
L. (L.) donovani:     GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACAC..G TCTTCTGCAC ATTATACCAT ACACAGTATA ...TATATA  TTATGTGTTG GAAGCCAAGA
L. (L.) chagasi:      GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACAC..G TCTTCTGCAC ..TATACCAT ACACAGTATA ...TATATA  TTATGTGTTG GAAGCCAAGA
L. (L.) infantum:     GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACAC..G TCTTCTGCAC ..TATACCAT ACACAGTATA ...TATATA  TTATGTGTTG GAAGCCAAGA
L. (L.) major:        GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACAC..G TCTTCTGCAT ........AT ATACAATATA ...TA.ATA. .TATGT.G   GAAGCCAAGA
L. (L.) tropica:      GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACAC..G TCTTCTGCAT ........AT ATACATTATA .....TATAA .TATGT.G   GAAGCCAAGA
L. (L.) aethiopica:   GCATGCCATA TTCTCAGTGT CGAACAAAAA ACAACAC..G TCTTCGGCAT ........AT GTATAATATA CGTTATATAA .TATGT.G   GAAGCCAAGA

  141        151        161        171        211        231        251        261        271        291        301        311
TGCGCATAT. .....ATATA TA........ .TATACGCAC CACA..CCTG TCG..AGATG AAAACTCCTC TCTGGTGGTT GCAAA..GCA CTC....... ........CT CTCTC.CTCT
TGCGCATAT. .....ATATA TA........ .TATACGCAC CACA..CCTG TCG..AGATG AAAACTCCTC TCTGGTGATT GCAAA..GCA C......... ........CT CTCTC.CTCT
TGCGCATAT. .....ATATA TA........ .TATACGCAC CACA..CCTG TCG..AGATG AAAACTCCTC TCTGGTGGTT GCAAA..GCA C......... ........CT CTCTC.CTCT
TGCACATATA TATG.ATATA TATATTGTGT .TGTGTGCAC CACA..TGTG TGTGTGGGTG AAAACTCCTC TCTGGTGCTT GCAAA..GCA CTTCCTCT.. .....TTCT  CTCTCTTTTT
TGCACATATA TATG.ATATA TA..TTGTGT .TGTGTGCAC CACA..TGTG TGTGTGGGTG AAAACTCCTC TCTGGTGCTT GCAAA..GCA CTTCCTCTCT TCCTCTTTCT CTCTCTTTTT
TGCGCATAT. ..TATATGTA TA...TATGC .TGTGTGCAC CACA....TG TGTGTGGGTG AA..CTCCTC TCTGGTGCTT GCAAA..GCA CTTTC..... ......TCT  TTTTCTCTCT
TGCGCATAT. ..TATATGTA TA...TATGC .TGTGTGCAC CACA....TG TGTGTGGGTG AA..CTCCTC TCTGGTGCTT GCAAA..GCA CTTTC..... ......TCT  TTTTCTCTCT
TGCGCATAT. ..TATATGTA TA...TATGC .TGTGTGCAC CACA....TG TGTGTGGGTG AA..CTCCTC TCTGGTGCTT GCAAA..GCA CTTTC..... ......TCT  TTTTCTCTCT
TGCCCATAT. .....ATATA TA...TATG. .TGTGTGCAC CACGCGTGTG TGTGTGCGTG AA..CTCCTC TCTGGTGCTT GCAAAAAGCA TTCTCCTTCT CC...TTTCT CTTTCTCTCT
TGCGCATAT. TATATATAT. TA...TATGT GTGTGTGCAC CGCA....TG TGTGTGGGTG AA..CTCCTC TCTGGTGCTT GCGCAAAGCA TTCTCTT.... ........TCT CTTTCTCTCT
TGCGCATATA TATGTATATA. TA...CATAT ATGTGTGCAC CGCA....TG TGTGTGGGTG AA..CTCCTC TCTGGTGCTT GCGCAACGCA TTCTCTTT.... ........TCT CTTTCTCTCT

  321        331        341        361        371        381        391        401        451        461        471
CTAT...TCT CTCTTC...T CTCA..CTTT AGAGGAGGG. ..GTTTTATA TAGAGAAGAG .......GGG TGTGG..... AAACGAGAAT TCAACTTCGC GTTGGGC (387bp)
CTAT...TCT CTCTTCTCCT CTCA..CTTT AGAGGAGAG. ..GTTTTATA TAGAGAAGAG .......AGG TGTGG..... AAACGAGAAT TCAACTTCGC GTTGGGC (387bp)
CTAT...TCT CTCTTCTCCT CTCA..CTTT AGAGGAGAG. ..GTTTTATA TAGAGAAGAG .......AGG TGTGG..... AAACGAGAAT TCAACTTCGC GTTGGGC (387bp)
CCTCCATTCT CTCCTCTCTT TTTT.CATCA AGCGAAGCGG GGG......G AGGAGGAAGG GGGGCCGAGG GGAT.....A AAACGAGAAT TCAACTTCGC GTTGGGC (437bp)
TCTCCATTCT CTCCTCTCTT TTTT.CATCA AGCGAAGAGG GGGACAAGCG AGGGAGGAGA GGGAGGGGGG GGCCGAGGGA AAACGAGAAT TCAACTTC.. GTTGGGC (450bp)
CCAT...TCT CTCCTCTCTT TTTT.CATCA AGAGAAAAA. GAG....... .AGAGGA... GGTGG..... TCG......A AAACGAGAAT TCAACTTCGC GTTGGGC (418bp)
CCAT...TCT CTCCTCTCTT TTTT.CATCA AGAGAAAAA. GAG....... .AGAGGA... GGGGGGG... TCG......A AAACGAGAAT TCAACTTCGC GTTGGGC (418bp)
CCAT...TCT CTCCTCTCTT TTTT.CATCA AGAGAAAAA. GAG....... .AGAGGA... GGGGGG...  TCG......A AAACGAGAAT TCAACTTCGC GTTGGGC (418bp)
CCAT...TCT CTCCTCTCTT TTTTTCATCA AGAGAAAAA. GAG....... .GGAGAAGAA GACACGCGGG AGTCGCGAGG AAACGAGAAT TCAACTTCGC GTTGGGC (432bp)
CCAT...TTT CTCTCCTCTT TTTT.CATCA AGAGAAAAAA GAG....... .GGAGAAAAA .......GGG GGTCG..AG. AAACGAGAAT TCAACTTCGC GTTGGGC (413bp)
CCATC.TTTT CTCTCCTCTT TTTT.CATCA AGAGAAAAAA GAG....... .GGAGAAAAA .......GGG GGTCG..AGG AAACGAGAAT TCAACGTCGC GTTGGGC (427bp)
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FIG. 2. Alignment of the variant regions of the ITS2 *Leishmania* sequences generated by PCR using generic primers LGITSF2/LGITSR2. The highlighted positions indicate the SNPs that differentiate *L. (V.) braziliensis*, *L. (V.) guyanensis*, and *L. (V.) panamensis* from each other, and the G stretch and SNP that differentiate between *L. (L.) donovani* and *L. (L.) infantum/chagasi*. The *L. (L.) donovani* sequence in the figure has a G/T transversion at nucleotide position 345 (referred to as G345T); i.e., the sequence for positions 343 to 347 is GGTGG rather than GGGGG. The variations in the LGITS2R2 primer binding regions of *L. (L.) amazonensis* and *L. (L.) aethiopica* are shown at nucleotide block 461. The original length of each sequence in the base pairs (bp) is indicated at the 3′ end of the sequence.

The *L. (L.) donovani* species complex had a stretch of Gs (a G stretch) that started at nucleotide position 343 [using *L. (L.) donovani* FJ753386 as a reference]. The reference strains of *L. (L.) infantum* and *L. (L.) chagasi* were indistinguishable; therefore, we have used the nomenclature "*L. (L.) infantum/chagasi*." However, the G stretch typically was longer for *L. (L.) infantum/chagasi* (7 or 9 Gs) than for *L. (L.) donovani* (5 Gs). Two of the four *L. (L.) donovani* strains, including the WHO strain from Asia, had a G/T transversion at position 345, referred to as G345T (Fig. 2).

Among the sequences for the three *Viannia* spp. in the analyses (Table 1), we consistently found sites for species-level identification in the clones that we sequenced from each isolate; the species were differentiated on the basis of four single nucleotide polymorphisms (SNPs), i.e., an A/C transversion at nucleotide position 59 (referred to as A59C transversion), a G220A transition, a G297A transition, and a G317A transition [positions are based on the sequences of *L. (V.) braziliensis* FJ753376, *L. (V.) guyanensis* FJ753387, and *L. (V.) panamensis* FJ948438]. *Leishmania (V.) braziliensis* was distinguished from the other *Viannia* spp. by the presence of nucleotides A, G, G, and G at positions 59, 220, 297, and 317, respectively; *L. (V.) guyanensis* and *L. (V.) panamensis* were distinguished from each other on the basis of the transition G220A. Figure 2 shows the differences that allowed species identification.

Validation of ITS2 PCR as a diagnostic tool. The ITS2 fragment was amplified directly from clinical specimens (versus cultures), the amplicons were sequenced, and the species was identified on the basis of criteria described for the reference isolates, including the SNPs and G stretch (Fig. 2). If direct sequencing analysis showed overlapping peaks because of variant microsatellites, partial sequences were used to identify the species; no cloning was done.

Species-level characterization was accomplished by this molecular approach (PCR followed by DNA sequencing analysis) for specimens from 202 patients, including 13 (6%) for whom the positive results were obtained on repeat testing (Table 2). We compared the species data with data from the parasitologic approach (*in vitro* culture followed by isoenzyme analysis). Of necessity, the comparison was limited to the 159 patients for whom species results were obtained by both approaches. For all but 2 of these 159 patients, the results were concordant. One of the two exceptions was in the *Viannia* subgenus: *L. (V.) guyanensis* was identified by the molecular approach, whereas *L. (V.) braziliensis* was identified by the parasitologic approach. DNA from this specimen was reamplified and resequenced thrice, and the same result was obtained each time. The other exception was a discordance between *L. (L.) tropica* (by PCR and sequence analysis) and *L. (L.) major* (by culture and isoenzyme analysis). DNA from these specimens was reamplified and resequenced twice, and the same results were obtained each time.

The comparative data were limited to the *L. (L.) donovani* species complex as a whole because isoenzyme analysis using 6PGDH and GPI does not distinguish *L. (L.) donovani* from *L. (L.) infantum/chagasi*. In general, the molecular approach ap-

TABLE 2. Number of patients with *Leishmania* spp. identified by ITS2 PCR followed by DNA sequencing, compared with culture followed by isoenzyme analyzes[a]

Species identified	No. of patients with positive results[b]					Comment
	Total positive by PCR (2nd attempt)	Positive by PCR only	Total positive by IE analysis	Positive by IE analysis only	Total positive by both PCR and IE analysis	
L. (V.) braziliensis	30 (5)	6	28	3	25	24 (vs 25) identified by molecular approach as *L. (V.) braziliensis*
L. (V.) guyanensis	9 (0)	4	5	1	4	5 (vs 4) identified by molecular approach as *L. (V.) guyanensis*
L. (V.) panamensis	67 (2)	10	64	7	57	
L. (L.) mexicana	33 (1)	2	34	3	31	
L. (L.) donovani complex[c]	29 (2)	15	14	0	14	
L. (L.) aethiopica	2 (1)	0	2	0	2	
L. (L.) major	23 (2)	5	22	3	19	18 (vs 19) identified by molecular approach as *L. (L.) major*
L. (L.) tropica	9 (0)	1	8	1	7	8 (vs 7) identified by molecular approach as *L. (L.) tropica*
Total no. of patients	202 (13)[d]	43[e]	177	18	159	Overall, the species results were discordant for 2 patients

[a] The generic primer pair used for ITS2 PCR was LGITSF2/LGITSR2. Overall, specimens from 220 patients were positive by one or both approaches, i.e., 202 plus 18. Among the 159 patients with specimens positive by both approaches, the results were discordant for two patients: for a patient exposed in Ecuador, the results were *L. (V.) guyanensis* by the molecular approach versus *L. (V.) braziliensis* by culture/isoenzyme analysis; for the other patient, who had traveled to Morocco, Kenya, and Spain, the discordance was between *L. (L.) tropica* and *L. (L.) major*. *Leishmania (L.) amazonensis* is not included in the Table because none of the clinical specimens were positive for this species.

[b] Positive result, species was identified. IE, isoenzyme.

[c] The sequences of the ITS2 diagnostic fragment for specimens from three patients exposed in Central America (two patients) or South America (one patient) had the GGGGG stretch; specimens from three other patients exposed in Asia, East Africa, or Central America (one each) had the G stretch with the transversion G435T (Fig. 2).

[d] The 13 patients with positive results on repeat testing are included in the total number of patients positive by PCR and not in the number of positive by IE only.

[e] Amastigotes were detected by light microscopic examination of slides for 22 of these 43 patients (for 22 of 37 patients with available slides) (see Materials and Methods).

peared to be more sensitive than the parasitologic approach for detecting parasites in this species complex; i.e., specimens were positive for 29 versus 14 patients, respectively. By using molecular criteria described for the reference isolates (Fig. 2), *L. (L.) infantum/chagasi* was the etiologic agent for all but 6 of these 29 patients, including 14 of the 15 with negative cultures. For the other six patients, the species was identified by the molecular approach as *L. (L.) donovani*; four of these six patients became infected in Central and South America, where *L. (L.) infantum/chagasi* would be the expected species (Table 2).

DISCUSSION

Various molecular approaches for diagnosing *Leishmania* infection have been described. For example, PCR methods that target minicircle kDNA can be highly sensitive for detecting *Leishmania* parasites because of the abundance of minicircles in each kinetoplast (2, 7, 10). However, high-level sequence polymorphism among minicircles is an impediment for species identification with protocols based solely on kDNA PCR. Targeting the rRNA-coding cluster poses other issues (7, 32). Highly sensitive approaches for identifying particular *Leishmania* species (or a species complex/subgenus) have been described (8, 25). However, if multiple *Leishmania* spp. need to be differentiated in a diagnostic laboratory, molecular approaches that require different PCR primers for each species have the potential for carryover contamination. Although strategies to minimize this potential have been developed (5),

contamination has been observed even when strict protocols were followed (20, 23).

Our goal was to develop a molecular approach for species-level discrimination that requires only one pair of PCR primers. We focused on the ITS region: the rRNA internal spacers are subject to less evolutionary pressure and show more sequence divergence than the coding regions and have been proposed as targets for molecular typing (1, 6, 19). We identified a region of the ITS2 adequate for our diagnostic goal and designed generic PCR primers (LGITSF2/LGITSR2) to amplify this fragment from *Leishmania* spp. associated with human infection. We found substantial differences in the ITS2 region spanned by these primers that generally allowed species identification.

During our analysis of the reference strains, we noted intraspecies variability in the AT, TC, TG, or GA microsatellite repeats, as have other investigators (12, 18, 25, 31), which initially hindered use of the ITS2 fragment for species identification. We also noted some distinct sequences because of a few transitions and transversions. In most instances, distinct sequences were found in clones obtained from the same isolate, which likely indicated the presence of multiple different ITS copies in the same genome. To exclude the possibility of erroneous incorporation of nucleotides (11, 29), we reamplified the DNA from some specimens by using *Pfu* High-Fidelity DNA polymerase (Stratagene); the results were the same as those with the routinely used *Taq* DNA polymerase (data not shown). During the development phase of the assay, we cloned reference strain amplicons to obtain the sequence of the entire

diagnostic fragment for each species, and we demonstrated that partial sequences sufficed for species identification. Therefore, in the validation phase, when we tested clinical specimens, we used direct sequencing analysis, without cloning, and in some instances relied on partial sequence data.

The distribution of species among the clinical specimens [e.g., the disproportionate number of patients infected with *L. (V.) panamensis*] reflects travel and immigration patterns among U.S. civilians evaluated for leishmaniasis; almost all of the clinical specimens we analyzed were from skin lesions. Although the PCR results obtained with different combinations of the reverse primers LGITSR1 and LGITSR2 were comparable for the isolates tested (Fig. 1A, B, and C), we had insufficient numbers of specimens positive for *L. (L.) amazonensis* and *L. (L.) aethiopica*—the species that prompted us to design the alternative reverse primer (LGITS2R1)—to compare the sensitivities of the primer pairs. We correctly identified the species as *L. (L.) amazonensis* for seven cultured isolates (including two WHO reference strains). However, this species was not identified by the parasitologic or molecular approach for any clinical specimens (Table 2). For *L. (L.) aethiopica*, the analyses included three cultured isolates (including one from WHO) and two clinical specimens (Tables 1 and 2).

The overall agreement between the molecular and parasitologic approaches was >98% when the comparison, of necessity, was limited to specimens for which a species determination was accomplished by both approaches. The two discordant results (among 159 patients) remained unresolved: for one patient, *L. (V.) guyanensis* was identified by the molecular approach versus *L. (V.) braziliensis* by the parasitologic approach (culture followed by isoenzyme analysis), and for the other patient, *L. (L.) tropica* was identified versus *L. (L.) major*, respectively. No other methods were used to evaluate the specimens; both results were epidemiologically plausible (Table 2), and the possibility of coinfection or of variant strains could not be excluded.

In general, our molecular approach differentiated among *Viannia* spp., namely, *L. (V.) braziliensis*, *L. (V.) guyanensis*, and *L. (V.) panamensis*, on the basis of four SNPs that appear to be in linkage disequilibrium with the genes that code for the isoenzymes we used to differentiate them (i.e., 6PGDH and GPI). These species, each of which likely is heterogeneous, have been associated with phenotypic (clinical) differences, which underscores the importance of species-level diagnostic techniques (4, 14, 22).

Our molecular approach did not differentiate *L. (L.) infantum* from *L. (L.) chagasi*, which many experts consider synonymous (18, 21). However, some distinguishing features in the ITS2 fragment were noted between the reference isolates of *L. (L.) donovani* versus *L. (L.) infantum/chagasi*, i.e., the length of a G stretch and, for the WHO *L. (L.) donovani* strain from Asia, a G435T transversion. Similar variation has been reported previously for some *L. (L.) donovani* strains (18). However, for several clinical specimens [four of the six classified as *L. (L.) donovani*], the approach we used appears to have resulted in misclassification of *L. (L.) infantum/chagasi* as *L. (L.) donovani*. Additional experience with more specimens from more areas of endemicity is needed to ascertain the ability of this ITS2 molecular target to reliably distinguish *L. (L.) donovani* from *L. (L.) infantum/chagasi*.

Approximately half of the patients with negative cultures but positive PCR results had slides available that were positive by light microscopic examination (Table 2), thereby confirming the diagnosis of leishmaniasis and underscoring the plausibility of PCR positivity. In some instances, negative culture (versus molecular) results might have been attributable to fastidious or nonviable parasites in the specimens we received or to contamination of the cultures, e.g., because of suboptimal handling or delayed shipment to the CDC. Lack of PCR positivity for some of the culture-positive specimens might have been attributable to heterogeneous distributions of parasites in specimens, such that the parasite/DNA concentration in the portion analyzed by PCR was below the detection limit of the assay. Preliminary data from a 40-cycle SYBR green-based real-time PCR assay under development in our laboratory suggest that positive specimens with threshold cycle (C_T) values around 35 could be negative by ITS2 PCR and/or culture (M. E. de Almeida, unpublished data).

A drawback of our approach is the need for DNA sequencing analysis, which is not yet available in most laboratories in areas of endemicity. However, the ITS2 fragment has cleavage sites for HinP1I or HhaI enzymes (GCGC) and MnlI enzyme (GAGG) that may have utility for identifying the species we evaluated except for those in the *Viannia* subgenus. A cleavage site for HphI (GGTGAN$_7$) could be used to identify *L. (V.) guyanensis* but not *L. (V.) braziliensis* or *L. (V.) panamensis*. These cleavage sites could be explored as targets for restriction fragment length polymorphism analysis to distinguish among a few species by laboratories in which DNA sequencing analysis is not yet available.

In summary, our findings confirm our hypothesis that a region of the ITS2 gene can complement the characterization of *Leishmania* parasites at the species level. The approach we developed can be used as a diagnostic tool in reference laboratories that have adequate infrastructure to perform molecular characterization of pathogens.

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