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An Enhanced Method for the Identification of *Leishmania* spp. using Real-Time PCR and Sequence Analysis of the 7SL RNA Gene Region

Lindsay G. Stevenson, **Daniel P. Fedorko**, and **Adrian M. Zelazny**

Microbiology Service, Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland

Abstract

The accurate identification of Leishmania species is important for the treatment of infected patients. Molecular methods offer an alternative to time consuming traditional laboratory techniques for species determination. We redesigned a 7SL rRNA gene based PCR and sequence assay for increased species identification. DNA extracted from 17 reference strains and 10 cultured clinical isolates was examined. Sequence comparison was used successfully to identify organisms to the complex level with intercomplex similarity ranging from 77.5% to 98.4%. Many species within each complex were discriminated accurately by this method including: *L. major*, *L. tropica*, *L. aethiopica*, *L. guyanensis*, and the previously indistinguishable *L. brasiliensis* and *L. panamensis*. The *L. donovani* complex members remain indistinguishable by this method, as are the representatives of *L. amazonensis*/*L. garnhami* and *L. mexicana*/*L. pifanoi*.

Leishmaniasis is a vector borne disease caused by protozoan parasites belonging to the genus *Leishmania*. Over 20 species of *Leishmania* are found in 88 countries worldwide; causing approximately 2 million new reported infections per year (WHO Report 2004). Due to the large number of infective species and variability in host genetics and immune status a wide spectrum of clinical presentations may occur and species determination may be complicated (Romero, Vinitius De Farias Guerra *et al*. 2001; Vega-Lopez 2003; Reithinger, Dujardin *et al*. 2007).

Research suggests that antiparasitic agents used for the treatment of Leishmaniasis demonstrates species dependent efficacy. Romero (2001) reported that meglumine antimoniate may show species dependent treatment success with *L. braziliensis* compared to *L. guyanensis* (Romero, Vinitius De Farias Guerra *et al*. 2001); and, Navin (1992) demonstrated that sodium stibogluconate produced a higher cure rate in patients with *L. braziliensis* lesions (96%) compared to those with *L. mexicana* lesions (57%) (Navin, Arana *et al*. 1992). Soto *et. al* established that miltefosine was effective against *L. panamensis* (>90% cure) but was not effective against *L. braziliensis* (Soto, Toledo *et al*. 2002). Additionally, *L. braziliensis* can be naturally azole resistant (Saenz, Paz *et al*. 1990; Navin, Arana *et al*. 1002; Rangel, Dagger *et al.* 1996). Traditional laboratory diagnostic methods for leishmaniasis include direct microscopy of skin sections and lengthy and technically demanding culture followed by analysis with multi-locus enzyme electrophoresis (MLEE).

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To provide more timely identification we previously reported on the use of the partial 7SL ribosomal RNA gene sequence, present in single copy, for complex identification (Zelazny, Fedorko *et al.* 2005). While the previous assay was useful for many species, it was unable to differentiate *L. braziliensis* from *L. panamensis*. Additionally, it was not tested in a real time format that allows for melting peak visualization rather than agarose gel electrophoresis to confirm product production. Through the use of novel primers, we extended the previously described 7SL sequenced region to include a small segment of a putative tRNA gene followed by a 98bp spacer region and a large segment of the 7SL rRNA gene. While still employing single PCR and sequencing reactions, we gain a larger sequenced area and enhanced species identification capabilities. Interestingly, the spacer region between the two genes contains conserved base differences that were especially important for distinguishing between the *L. braziliensis* and *L. guyanensis* complexes.

Strains of Leishmania used in this work were cultured and DNA was extracted as previously described (Zelazny, Fedorko *et al.* 2005). PCR was carried out with M13 tailed primers (LeishFW- 5′**GTAAAACGACGGCCAG**CATCCGTGACAGGATTCGAACC-3′) corresponding to sequence ~200bp upstream from the putative 7SL gene start sequence and (LeishRV 5′-**CAGGAAACAGCTATGAC**CGTGGGGCTCAAGTGCGGACATG-3′) corresponding to sequence at position 36 bp upstream from the end of the putative 7SL gene sequence. Real-Time PCR amplification was carried out with a reaction mixture containing 50ng of extracted DNA, 0.4μM LeishFW primer, 0.4μM LeishRV primer, 1X QuantiTect SYBR Green PCR Mastermix (Qiagen, Valencia, Ca), and 0.5 units UNG Uracil-DNA-Glycosylase (Roche Applied Science, Indianapolis, IN). The reaction was brought to 20ul total volume with PCR grade water. Real Time PCR was carried out in a Rotor-Gene RG-3000 (Corbett Research, San Francisco, CA) with a program consisting of a warming to 50°C for 2 minutes, initial denaturation at 95°C for 15 minutes, followed by 45 cycles of 95°C for 15 seconds, 65°C for 30 seconds, 72°C for 30 seconds resulting in a ~465 bp amplification product. Products used for sequencing reactions were purified using Microcon YM-100 Centrifugal Filter Devices (Millipore, Billerica, MA) following manufacture recommendations. Sequencing and analysis was carried out as previously described except using The Lasergene program suite (version 7.1.0; DNASTAR Inc. Madison, WI). Leishmania spp. sequenced regions, presented in this paper, were deposited in GenBank under accession numbers: FJ525405, FJ525406, FJ525407, FJ525408, FJ525409, FJ525410, FJ525411, FJ525412, FJ525413, FJ525414, FJ525415, FJ525416, FJ525417, FJ525418, FJ525419, FJ525420.

Seventeen Leishmania reference strains and 10 clinical isolates were examined in this study. (Table 1) These organisms represent the most commonly isolated species of Leishmania causing human disease (Jeronimo S., *et al* (2006). Nucleotide sequence information for each isolate was compared pairwise to determine percent similarity. After primer removal, a 385–387bp amplified product was seen (Figure 1). Some strains containing insertions, as compared to *L. major*, have been previously described(Zelazny, Fedorko *et al.* 2005).

The sequencing strategy used was able to clearly differentiate the tested strains to the complex level (*L. major*, *L. tropica. L. aethiopica*, *L. braziliensis*, *L. guyanensis*, *L. mexicana*, *L. donovani*) (Figure 1). Intercomplex similarity ranged from 77.5 % (*L. guyanensis* v. *L. donovani*) to 98.4 % (*L. braziliensis* v. *L. guyanensis*). In accordance with other sequence regions examined for use in identification schemes, intraspecies polymorphisms were present (Foulet, Botterel *et al.* 2007). When multiple isolates of a species were examined, intraspecies similarity ranged from 99.2 to 100%. Of four *L. aethopica* isolates tested, two isolates were conserved and two exhibited 1 or 2 bp changes. Within the four *L. braziliensis* strains examined, *L. braziliensis* MHOM/BR/84/LTB300 contained a change within the upstream putative noncoding region; and, *L. braziliensis*

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MHOM/BR/75/M2903 contained a deletion within this region. Of four *L. panamensis* isolates, two examined have a 1bp change. However, these polymorphisms did not affect the ability to identify species and all are appropriately grouped on the phylogenetic tree (Figure 2).

L. panamensis and the closely related *L. guyanensis* species isolates are distinguished by 2 conserved basepair differences and there are 5 conserved basepair changes between *L. braziliensis* and *L. panamensis* isolates allowing for a clear division in the phylogenetic tree (Figure 1, Figure 2). Additionally, while only previously separated by a single base, *L. mexicana* and *L. amazonensis* are separated by 2bp differences. Due to the small number of base changes between these species we suggest that bidirectional sequencing and a comparison of both sequences always be performed. The new assay did not allow for separation of *L. amazonensis* and *L. garnhami* or *L. mexicana* and *L. pifanoi*. As expected, the sequenced region was unable to differentiate among members of the *L. donovani* complex (*L. donovani*, *L. infantum*, *L. chagasi*). Previous work suggests that *L. infantum* and *L. chagasi* are generally considered to be genetically identical (Mauricio, Stothard *et al.* 2000) and all three species of the complex have been shown to be extremely similar (Kuhls, Mauricio *et al.* 2005;Mauricio, Yeo *et al.* 2006). Species dependent treatment schemes are not yet being employed for the *L. donovani* complex, lessening the need for species level discrimination (Chappuis, Sundar *et al.* 2007).

Here, we evaluated a Real-Time PCR assay followed by a single sequencing reaction for the identification of *Leishmania* spp. All complexes are clearly differentiated and *L. braziliensis* and *L. panamensis* are clearly separated, allowing for the option of species-specific treatment. Additionally, the primers utilized show no similarity to human DNA and no amplification was observed when the assay was preformed with DNA extracted from human material (blood, CSF, skin biopsy, joint fluid). When the PCR assay was carried out with a known positive human skin biopsy sample we were able to identify the infecting species accurately and further work will be carried out to evaluate the use of this assay for clinical specimens.

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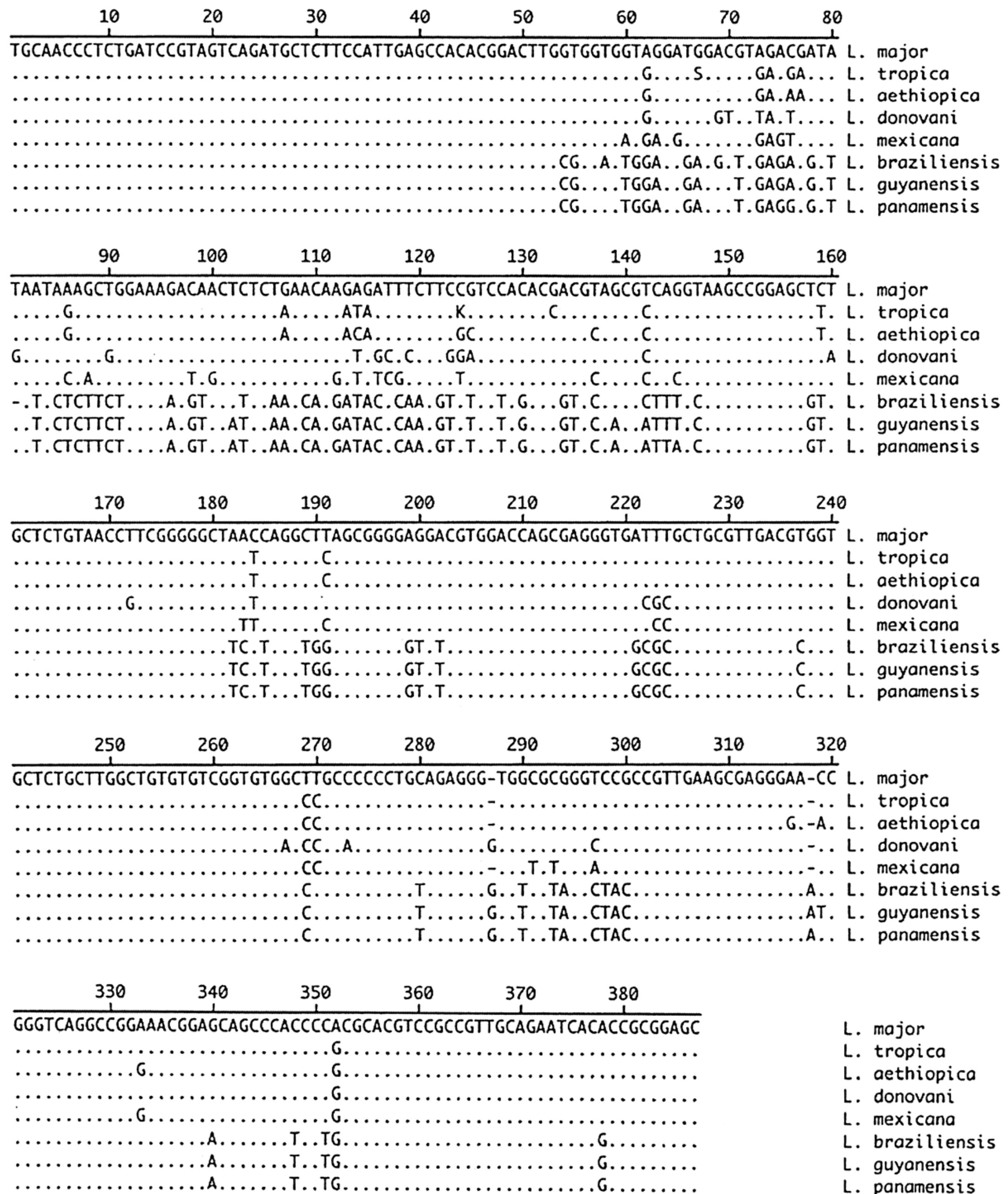

Figure 1.

Alignment of PCR amplified region from *Leishmania* spp. Representative sequence information from one isolate of each species is shown. Dots indicate identity with *Leishmania major* sequence.

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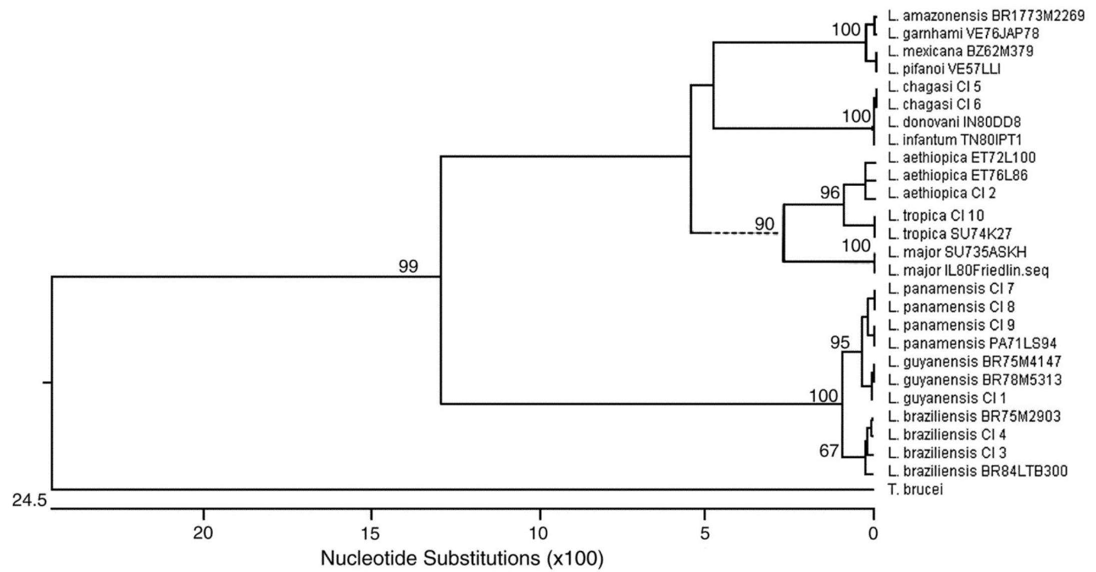

Figure 2.

Phylogenetic tree of 27 reference and clinical isolates of Leishmania spp.. constructed by the neighbor-joining method, using *Trypanosoma brucei* strain 427 homologous sequence as the outgroup. Numbers on branches represent the percentage of 1,000 bootstrap samples supporting the branch. Only values >50 are shown. CI=clinical isolate.

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