Chapter IV
Identification of parasite species in clinical specimens
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Introduction

The infection caused by *Leishmania* spp. can lead to different clinical manifestations depending on species of infecting parasite and the genetic susceptibility of the host. The disease is normally divided into three main categories: cutaneous (CL), mucocutaneous (MCL) and visceral (VL).

Cutaneous Leishmaniasis is the most extensively studied form of the disease and is caused by several species such as *Leishmania major* and *Leishmania tropica* in the Old World and *Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania guyanensis*, *Leishmania panamensis* and *Leishmania braziliensis* in various regions of Central and South America (Goto et al., 2012).

Simple cutaneous lesions are most often self-healing but in some cases, such as those caused by *L. panamensis* and *L. braziliensis*, can progress to involve muco-cutaneous tissue. This is often a horribly disfiguring infection resulting from the chronic local destruction of tissue of the nose, mouth oro- and naso-pharynx and eyelids and can progress to affect respiratory function and hamper nutrition. MCL found in countries in South America, with the majority of disease found in Brazil, Peru and Bolivia but is also found in lesser degrees in Colombia, Ecuador, Paraguay and Venezuela (Goto et al., 2012).

Finally, visceral Leishmaniasis, the most severe Leishmaniasis form, is caused by *Leishmania donovani* (in regions of India, Pakistan, China and Africa) in the Old world and *Leishmania infantum* (in the Mediterranean region) in the New World. Visceral disease has been reported in the Middle East caused by viscerotropic strains of *L. tropica*, which has been classically thought of as an agent of CL (Weiss et al., 2009). The proliferation of parasites in macrophages in the liver, spleen and bone marrow of patients with VL gives rise to progressive hepatosplenomegaly and bone marrow suppression. Unless treated, patients develop pancytopenia and immunosuppression and are prone to super-infections with other microbes. Individuals co-infected with HIV have a particular susceptibility to developing atypical presentations, and increased severity (Okwor and Uzonna, 2013).

In a subset of patients successfully treated for VL develop Post-kala-azar dermal Leishmaniasis (PKDL), a fulminant and progressive proliferation of parasites within the skin which give rise to diffuse macular, maculo-papular or nodular lesions. It occurs mainly in India and Sudan in patients infected with *L. donovani* (Mukhopadhyay et al., 2014). In Sudan, PKDL can arise in up to 50% of patients and occurs sooner (in up to 6 months) than in
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patients in India where it has an incidence of 5–10% within 2–3 years after clearance of VL (Ganguly et al., 2010).

Generally, in the Indian subcontinent, patients presenting with clinical features suggestive of VL/PKDL have the diagnosis confirmed by the presence of parasites in Giemsa-stained smears and/or culture positivity, serological diagnosis (enzyme-linked immunosorbent assay [ELISA] or rK39 strip test), and, rarely, by molecular approaches. The causative parasites are assumed to be \textit{L. donovani}, although in recent years, studies have shown \textit{L. donovani} causing cutaneous Leishmaniasis in Sri Lanka (Siriwardana et al., 2010). In some cases, species identification becomes important for epidemiological studies and treatment. The present methods to differentiate among \textit{Leishmania} species are expensive. We used restriction fragment length polymorphism (RFLP) assay to perform the speciation.

\textbf{Materials and methods}

\textit{Leishmania} sp. typing by PCR-RFLP:

Different regions of \textit{Leishmania} genome were amplified, namely (i) ribosomal ITS1 and (ii) hsp70 following the methods described in Materials and Methods. The amplified ITS1 and hsp70 regions were digested using \textit{HaeIII} (Fermentas, Glen Burnie, MD) and analyzed the products by electrophoresis (3% agarose, 5 V/cm for 1.5 h).

DNA resequencing:

For sequencing of archived \textit{Leishmania} isolates, PCR products of the ITS1 region were purified (QIAquick gel extraction kit; Qiagen, Hilden, Germany) and then cloned into the pJET1.2 vector by blunt end ligation (CloneJET PCR cloning kit; Fermentas, Glen Burnie, MD). Recombinant plasmid DNA was used to transform \textit{Escherichia coli} DH5\textalpha; eight colonies with an ITS1 insert were selected for each sample. Plasmid DNA was purified from colonies using a Qiagen plasmid minikit (Qiagen, Hilden, Germany) and sequenced (BigDye Terminator v3.1 cycle sequencing kit; Applied Biosystems, Foster City, CA) on an automated DNA sequencer (ABI Prism 3130, Foster City, CA). DNA sequence editing and analysis were performed using Seqscape V2.5 software (Applied Biosystems, Foster City, CA, USA).

The reference sequences of the ITS1 gene from several trypanosomatid species were retrieved from GenBank and aligned with the sequence determined in this study (http://www.ncbi.nlm.nih.gov/GenBank/index.html) using ClustalW software and a phylogenetic tree constructed by the neighbor-joining method using MEGA version 5.0.
Results

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Upon routine diagnosis of patients with suspected VL or PKDL by ITS1 PCR, a different band pattern was reported that did not match the classical *L. donovani* WHO reference strain DD8 (MHOM/IN/1980/DD8, Das et al., 2011). Accordingly, we tried to identify the species using RFLP patterns of clinical specimens sourced from patients with VL or PKDL along with archived parasite isolates from a different patient population.

Different ITS1 PCR-RFLP patterns were observed among patients also in isolates

Analysis of the ITS1 PCR products of 60 patients (VL, n = 35 and PKDL, n = 25) showed two distinct trends, namely (i) a single 320-bp amplicon in 86.2% (31 VL and 25 PKDL) of samples that matched the reference strain DD8 and (ii) dual bands of 320 and 418 bp in 13.8% (4 patients with VL, Figure 1a).

![Figure 1](image-url)

Examination of the RFLP pattern of the 320-bp product revealed a pattern similar to that of DD8, having 3 fragments with sizes of 191, 75, and 54 bp, defined as “pattern A.” With regard to the 4 samples having a dual band pattern, each band was gel extracted, purified, and digested separately with *HaeIII*; the 320-bp product had an RFLP profile similar to that of DD8 (i.e., pattern A), while the larger PCR product of 418 bp remained undigested by *HaeIII* and was defined as “pattern B” (Figure 1b).

Among these 10 archived isolates, 8 were from bone marrow/splenic aspirates of patients with VL (V1 to V7), while three were from dermal biopsies of patients with PKDL (P1 to P3). The
majority of these patients (6/10) hailed from Bihar, the main zone of endemicity for VL in India that included two (V2 and P1) from areas of antimonial resistance and four (V4, V5, V6 and P2) from areas having no antimonial resistance (Perry et al., 2011). Among the remaining 4 archived isolates 3 (V1, V7 and P3) were isolated from West Bengal and 1 (V3) from Assam, whose patterns of antimonial resistance, if any, are yet to be defined.

To characterize these archived isolates, we performed ITS1 RFLP and found two variations in the PCR products (Figure 2 inset) that were verified by HaeIII digestion (Figure 2). RFLP data showed that pattern A was dominant, being present in 8/10 isolates (71.4%), and pattern B was present in 2 isolates (28.6%, Figure 2), akin to the profile obtained in clinical specimens. This lack of digestion by HaeIII has not been reported previously in Leishmaniasis and suggests unusual variations in the sequence of the ITS1 region among Leishmania strains. Although some sequence variations in the ITS1 region between strains of L. donovani have been reported, (El Tai et al., 2000), a difference of 100 bp in the PCR product has not been reported to date.
Two types of hsp70 PCR-RFLP patterns were observed among isolates

To substantiate our findings, we performed hsp70 PCR-RFLP with our archived isolates. Once again, two patterns emerged: i.e., 5 isolates matched the *L. donovani* reference strain, whereas 2 isolates (V5 and P2 which showed pattern B for ITS1 RFLP) showed another pattern (Figure 3). The latter pattern was not comparable with any other *Leishmania* species (Montalvo et al., 2010), but it was similar to that reported in 9 Indian isolates (Srivastava et al., 2010). In the clinical specimens, the hsp70 PCR did not yield any product.

**Figure 3:** RFLP analysis of the hsp70 region amplified from *Leishmania donovani* isolates. Lanes: 1, DD8; 2, V1; 3, V2; 4, V3; 5, V4; 6, V5; 7, P1; 8, P2; M, low-range DNA ladder.

Isolates showing “LdB” pattern contain several insertions from “LdA” pattern

In order to identify the organism from which this aberrant ITS1 gene was being amplified, the nucleotide sequence of the 418-bp fragment was determined, wherein we selected DD8 and P2 as representatives of patterns A and B, respectively; P2 was selected as it had undergone very few passages following transformation. We aligned two sequences denoting one sequence, “LdA,” for *L. donovani* DD8, representative of pattern A and denoting the other, “LdB,” for P2, representative of pattern B; the ClustalW alignment of the two sequences showed several mismatches and deletions (score, 73), indicating there were significant differences between them (Figure 4). To determine whether these sequence variants were due to *Taq* polymerase errors, two colonies of the variant were selected for a second round PCR using the same primers and resequenced; they were all identical to the original sequence, confirming that the observed sequence variant was not a technical error. Furthermore, two sequences were BLAST searched independently, where LdB showed a strong match with *Leptomonas seymouri* (accession no. EU623433.1).
Based on the sequence of the ITS1 region of P2 and DD8, a neighbor-joining tree was constructed that included 33 *L. donovani* Indian isolates, available at National Centre for Biological Information (http://www.ncbi.nlm.nih.gov), and an extended set of organisms (Figure 5). The tree showed that P2 was most closely related to *Leptomonas seymouri* (score, 99.0) (Figure 5) along with 21 *L. donovani* Indian isolates. The remaining 12 *L. donovani* Indian isolates were phylogenetically closely related to DD8 (Figure 5) (http://www.ncbi.nlm.nih.gov/nuccore/EU364830). Additionally, sequence analysis of these 33 isolates showed that the size of the ITS1 PCR product of the 21 isolates that matched *Leptomonas seymouri* was 418 bp, while the size of the 12 Indian archived isolates that matched with DD8 was 320 bp. Importantly, no *Hae*III restriction site was present in the 418-bp sequence, corroborating our observations (Figure 2).

**Presence of *Leptomonas seymouri* along with *Leishmania donovani* was confirmed by specific PCR**

To confirm the presence of *Leptomonas seymouri* in the clinical specimens, we designed a reverse primer from a 30-bp portion (nucleotide positions 197 to 227) unique to this organism. It was selected based on it being the inserted sequence present in the ITS1 region of
P2 following alignment of P2 and DD8 (Figure 4). The forward primer (5’CTGGATCATTTTCCGATGATACTAT3’) was designed from the common sequence (bp 1 to 25) between P2 and DD8, while the reverse primer was 5’TGCCCTCTCCTCAGCAGA3’; a partial ITS1 region was amplified at an annealing
temperature of 60°C for 30 s. Among the archived isolates, P2 and V5, which showed pattern B gave a 210-bp product, confirming these strains were *Leptomonas seymouri*. We propose that after parasite transformation, *Leptomonas seymouri* outgrew *L. donovani* as *Leptomonas* species have been reported to grow faster than *L. donovani* (Srivastava et al., 2010). In patient DNA, 4 samples appeared coinfected with *L. donovani* and *Leptomonas seymouri* as they gave the 210-bp *Leptomonas* ITS1 PCR product; additionally, 2 more samples from patients with VL showed a 210-bp product (data not shown); none of the patients with PKDL showed a 210-bp band.

**Discussions**

Species typing of the parasites causing VL/PKDL revealed 4/60 patients were concomitantly infected with *Leishmania donovani* and *Leptomonas seymouri* as also were 2 archived culture isolates among 10 studied. The occurrence of insect trypanosomatids in humans is exceptional, but reports are available that HIV-positive patients are additionally infected with nonpathogenic insect trypanosomatids (Chicharro and Alvar, 2003). In Brazil, Pacheco et al. (Pacheco et al., 1998) described a flagellate, apparently a monoxenous trypanosomatid, in a 35-year-old HIV-positive male who presented with symptoms of VL. Hybridization analyses, against a panel of many different trypanosomatids, revealed that the unknown flagellate had kinetoplastid DNA (kDNA) cross-homology only with *Leptomonas pulexsimulantis*, a parasite of a dog flea (Siriwardana et al., 2010). However, the presence of lower trypanosomatids in immunocompetent individuals is a matter of greater concern (Boisseau-Garsaud et al., 2000). Our patients had no evidence of HIV infection (testing negative for HIV), yet four of them were coinfecteed with *Leptomonas seymouri* and *L. donovani*. Additionally, *Leishmania* coinfections, including with HIV, *Plasmodium*, or *Mycobacterium tuberculosis* (Ghosh et al., 2012 and references therein) have been reported. Therefore, it may be envisaged that as VL induces a strong immunosuppression, it possibly allows nonhuman trypanosomatids to be installed in mammalian hosts.

In this study, 6.7% (4/60) patients with VL/PKDL were coinfecteed with *Leptomonas seymouri* and *L. donovani*. Interestingly, on analysis of the isolates reported in GenBank as *L. donovani*, 21/33 (i.e., 63.63%) are actually *Leptomonas seymouri*; in this study, 20% (2/10) are *Leptomonas seymouri*. Nasereddin et al. (2008) reported about 35.59% of Indian isolates obtained from patients with VL were unidentified by reverse line blot hybridization assay using *L. donovani*-specific probes, but had a ITS1 sequence similarity to *Leptomonas seymouri*. The appearance of this opportunistic infection by *Leptomonas seymouri* raises questions about the clinical relevance of this pathogen. However, to date, studies pertaining to
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the pathobiology of these opportunistic lower trypanosomatids infecting humans have been limited.

As this study had a substantial number of patients coming from zones of antimonial resistance, it raises the possibility that *Leptomonas* strains are possibly less sensitive to antimony. The *in vitro* susceptibility toward antimony of both monoxenous trypanosomatid field isolates P2 and V5 was lower than those of the other 8 strains (M. Chatterjee, personal communication), which raises the possibility of the potential contribution of *Leptomonas* to the growing incidence of unresponsiveness to antimonials reported from the Indian subcontinent; however, this must be substantiated in a larger study group to conclude whether *Leptomonas* infections influence the epidemiology, pathology, or case management of VL.