Co-infection of Leptomonas seymouri with Leishmania donovani in Indian Leishmaniasis

Running Title: Co-infection of Leptomonas seymouri with Leishmania donovani

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Abstract

Leishmania donovani is considered as the causative organism of visceral leishmaniasis (VL) and Post Kala-azar dermal leishmaniasis (PKDL). Testing of 4/29 DNA from VL and PKDL patients as also 2/7 field isolates showed an aberrant ITS1-RFLP pattern which upon sequencing strongly matched with Leptomonas seymouri thus confirming its presence in Indian Leishmaniasis.
Visceral leishmaniasis (VL) is a vector-borne disease caused by replication of parasites of the *Leishmania donovani* complex (*L. donovani* and *L. infantum*) within the macrophage-phagocytic system. In the Indian subcontinent and parts of Africa, its transmission is anthroponotic (2) with Post kala-azar dermal leishmaniasis (PKDL) being a sequel of VL and is characterized by a macular, maculo-papular or nodular rash (11). Generally, in the Indian subcontinent, patients presenting with clinical features suggestive of VL/PKDL are confirmed by presence of parasites in Giemsa stained smears and/or culture positivity, serological diagnosis (ELISA/rK39 strip test) and rarely by molecular approaches. The causative parasites are assumed to be *L. donovani* although in recent years, studies have shown *L. donovani* causing cutaneous leishmaniasis in Sri Lanka (18). Additionally, a lower trypanosomatid, *Leptomonas seymouri* has been detected in isolates from patients with VL, but not in clinical specimens (19).

Molecular diagnosis of leishmaniasis is often by polymerase chain reaction (PCR) typically targets the Internal transcribed spacer 1 (ITS1), separating the genes coding for ssu rRNA and 5.8S rRNA (7). Additionally, isolates have been characterized by restriction fragment length polymorphism (RFLP) analysis of ITS1 region (8) or gene fragment encoding 70 kDa heat shock protein (hsp70, 13), the latter being among the first kinetoplastid genes to be cloned and characterized owing to their conserved nature (10). Upon routine diagnosis of patients suspected with VL/PKDL by ITS1 PCR, a different band pattern was reported that did not match with the classical *L. donovani* WHO reference strain DD8 (MHOM/IN/1980/DD8, 7). Accordingly, this study was undertaken to study the RFLP patterns of clinical specimens sourced from patients with VL or PKDL along with archived parasite isolates from a different patient population.

The study population included 29 patients from January 1st, 2010 – January 31st, 2012 who were admitted to the School of Tropical Medicine, Kolkata with clinical features of
VL (n = 23) or PKDL (n = 6). Clinical materials included peripheral blood from patients with VL or lesional skin biopsy from patients with PKDL after obtaining informed consent. The diagnosis of VL/PKDL was confirmed by rK39 strip test (20), ELISA for anti-leishmanial antibodies and PCR of the ITS1 region of Leishmania sp (7). The study received approval from the Institutional Ethical Committee of the School of Tropical Medicine, Kolkata, and Institute of Postgraduate Medical Education and Research, Kolkata.

In addition, our study included archived Leishmania isolates (n = 7, V1-V5 and P1, P2), obtained from patients with VL (n = 5) or PKDL (n = 2); all except V5 presented at the School of Tropical Medicine between 2006-2011. In patients with VL, parasites were isolated from spleen/bone marrow aspirates (7) while for PKDL, a 3 mm punch biopsy from a nodule was collected in Medium 199 (M199) supplemented with 20% heat inactivated fetal calf serum (FCS), penicillin G (50 IU/ml) and streptomycin (50 µg/ml). The material was passed through a 230 µm sterile iron mesh and finally resuspended in 1.5 ml of the same medium and after incubation at 24°C, culture growth was evident after 5-10 days. After transformation from amastigotes to promastigotes, they were gradually adapted into M199 supplemented with 10% FCS, penicillin G (50 IU/ml), and streptomycin (50 µg/ml) and subcultured every 2-3 days, inoculum being 1×10^6/ml. When parasites reached the range of 10^7, we cryopreserved (approximately 1 × 10^7 parasites per cryo vial) in freezing medium (M199 containing 30 % FCS and 7.5 % DMSO).

All the archived strains were typed by ELISA using species specific L. donovani monoclonal antibody (5) and PCR-RFLP (8). For PCR, DNA following isolation from peripheral blood, skin biopsy and isolates (QIAamp DNA mini kit, Qiagen, Hilden, Germany) was eluted in 200 µl elution buffer. Different parts of Leishmania were amplified namely (i) ribosomal ITS1 (8) and (ii) hsp70 (13). Amplification reactions were performed in 25 µl (JumpStart™ REDTaq® ReadyMix™ Reaction Mix, Sigma Aldrich Chemicals, St Louis,
MO, USA) in a Master cycler (Eppendorf, Hamburg, Germany). The amplified ITS1 and hsp70 regions were digested using HaeIII (Fermentas, Glen Burnie, MD, USA); briefly, reactions were carried out using 1U of HaeIII, 1X buffer, 5 µl of the amplicon (approximately 100 µg of DNA) and incubated at 37°C for 3 h (for ITS1) or overnight (for hsp70). The digested product was analyzed by electrophoresis (3% agarose, 5 V/cm for 1.5 h) along with a 100 bp DNA Ladder or GeneRuler™ Low Range DNA ladder (Fermentas, Glen Burnie, MD, USA) and visualized in G-BOX gel doc (Syngene, Cambridge, UK) using Gene Tools software (version 4.01.04).

For sequencing of archived Leishmania isolates, PCR products of 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, USA). Recombinant plasmid DNA was used to transform Escherichia coli DH5α; eight colonies with an ITS1 insert were selected for each sample. Plasmid DNA was purified from colonies using a Qiagen Plasmid Mini kit (Qiagen, Hilden, Germany) and sequenced (BigDye Terminator v3.1 cycle sequencing kit, Applied Biosystems, Foster City, CA, USA) on an automated DNA sequencer (ABI Prism 3130, Foster City, CA, USA). DNA sequence editing and analysis was performed using Seqscape V2.5 software (Applied Biosystems, Foster City, CA, USA).

The reference sequences of 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, Accession no. JN848802) using ClustalW software and a phylogenetic tree constructed by Neighbor-Joining method using MEGA version 5.0 (21).

Blood was sourced from patients with VL (n = 23) and lesional skin biopsies from patients with PKDL (n = 6, Table 1); 58.62% of patients hailed from Bihar (17/29) and
amongst them, 11 (64.70%) were from zones with antimonial resistance (17). Of the remaining twelve patients, eleven were from West Bengal and one from Chhattisgarh, whose areas of antimonial resistance, if any, have not been defined.

Analysis of the ITS1 PCR products of these 29 patients showed two distinct trends namely (i) a single 320 bp amplicon in 86.2% (19 VL and 6 PKDL) of samples, that matched with the reference strain DD8 and (ii) dual bands of 320 and 418 bp in 13.8% (4 patients with VL). Examination of the RFLP pattern of the 320 bp product revealed a pattern similar to DD8 having 3 fragments of MW 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 a RFLP profile similar to DD8 i.e. pattern ‘A’, while the larger PCR product of 418 bp remained undigested by HaeIII and was defined as pattern ‘B’.

Amongst the seven archived isolates studied, five were obtained from bone marrow/splenic aspirates of patients with VL (V1-V5) while two were from dermal tissue of patients with PKDL (P1 and P2). Majority of these patients (5/7, except V1 and V3) hailed from Bihar, the main endemic zone for VL in India; among them, two (V2 and P1) were from areas of antimonial resistance and three (V4, V5 and P2) were from an area having no-antimonial resistance (17). The remaining two archived isolates (V1 and V3) were isolated from a patient of West Bengal and Assam respectively whose patterns of antimonial resistance, if any, are yet to be defined.

All archived strains showed strong binding with D2, a L. donovani species specific monoclonal antibody (12) and absorbances obtained were comparable with that obtained with DD8 (MHOM/IN/1980/DD8), the L. donovani reference strain; accordingly, they were typed as L. donovani. To further characterize these archived isolates, we performed ITS1-RFLP and found two variations in the PCR products (Figure 1, inset) that was verified by HaeIII.
digestion. RFLP data showed that pattern ‘A’ was dominant, being present in 5/7 isolates (71.4%) and pattern ‘B’ in 2 isolates (28.6%, Figure 1) akin to the profile obtained in clinical specimens. This non-digestion by HaeIII has previously not been reported in leishmaniasis and suggests unusual variations in the sequence of ITS1 region among *Leishmania* strains. Although some sequence variations in the ITS1 region between strains of *L. donovani* have been reported, (8, 16), a difference of 100 bp in the PCR product has to date not been reported.

To substantiate our findings, we performed hsp70 PCR-RFLP in 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 (Data not shown). The latter pattern was not comparable with any other *Leishmania* sp. (13), but was similar to that reported in 9 Indian isolates (19). In the clinical specimens, the hsp70 PCR did not yield any product (data not shown).

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 pattern ‘A’ and ‘B’ respectively; P2 was selected as it had undergone very few passages following transformation. We aligned two sequences denoting ‘LdA’ to *L. donovani* DD8, representative of pattern ‘A’ and LdB to P2, representative of pattern ‘B’; the ClustalW alignment of the two sequences showed several mismatches and deletions (score 73) indicating that there were significant differences between them (Figure 2A). 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*
Based on the sequence of the ITS1 region of P2 and DD8, a Neighbor-Joining tree was constructed including 33 *L. donovani* Indian isolates available at National Centre for Biological Information, USA (http://www.ncbi.nlm.nih.gov) and an extended set of organisms (Figure 2B). The tree showed that P2 was most closely related to *Leptomonas seymouri* (Score 99.0, Figure 2B) along with 21 *L. donovani* Indian isolates. The remaining 12 *L. donovani* Indian isolates were phylogenetically closely related to DD8 (Figure 2B, http://www.ncbi.nlm.nih.gov/nuccore/EU364830). Additionally, sequence analysis of these 33 isolates showed that MW of the ITS1 PCR product of the 21 isolates which matched with *Leptomonas seymouri* was 418 bp, while 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 with our observations (Figure 1).

To confirm the presence of *Leptomonas seymouri* in the clinical specimens, we designed a reverse primer from a 30 bp portion (197-227 bp nucleotide position) 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 2A). The forward primer (5’ CTGGATCATTTTCCGATGATACTAT 3’) was designed from the common sequence (1-25 bp) between P2 and DD8 while the reverse primer was 5’ TGCCCTCTCTCACACAGCA 3’; a partial ITS1 region was amplified, annealing temperature being 60°C for 30 sec. 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* sp. have been reported to grow faster than *L. donovani* (19). In patient DNA, 4 samples appeared co-infected 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.
Based on this analysis, we conclude that clinical specimens (4/29) isolated from patients with VL/PKDL were concomitantly infected with *Leptomonas seymouri* as also were two archived culture isolates amongst seven studied; importantly, they phylogenetically clustered more closely to the monoxenous parasite, *Leptomonas seymouri*. The occurrence of insect trypanosomatids in humans is exceptional, but reports are available that HIV positive patients are additionally infected with non-pathogenic insect trypanosomatids (6). In Brazil, Pacheco *et al*. 1998 (15) 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 kDNA cross-homology only with *Leptomonas pulexsimulantis*, a parasite of a dog flea (18). However, the presence of lower trypanosomatids in immunocompetent individuals is a matter of greater concern (4). Our patients had no evidence of HIV infection (they tested negative for HIV), yet four of them were co-infected with *Leptomonas seymouri* and *L. donovani*. Additionally, *Leishmania* co-infection with other organisms include HIV (3), *Plasmodium vivax* (1), *Mycobacterium tuberculosis* (9) have been reported. Therefore, it may be envisaged that as VL induces a strong immunosuppression, it possibly allows non-human trypanosomatids to be installed in mammalian hosts.

In this study, 13.8% (4/29) patients with VL/PKDL were co-infected 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, 28.57% (2/7) are *Leptomonas seymouri*. Nasereddin *et al.*, 2008 (14) 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 with *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 the pathobiology of these opportunistic lower trypanosomatids infecting humans is limited.

As this study had a substantial number of patients coming from zones of antimonials resistance, it raises the possibility that *Leptomonas* strains are possibly less sensitive to antimony. The *in-vitro* susceptibility towards antimony of both monoxenous trypanosomatid field isolates P2 and V5 was lower than the other 5 strains (Chatterjee M, 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 influences the epidemiology, pathology or case management of VL. Taken together, this study emphasizes the importance of estimating the extent of opportunistic pathogens in leishmaniasis.

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References:


Table 1: Clinical features of Study Population

<table>
<thead>
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<th>Features</th>
<th>Patients with VL (n = 23)</th>
<th>Patients with PKDL (n = 6)</th>
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<tr>
<td>Age (years), Mean ± SD</td>
<td>30.7 ± 19.2</td>
<td>31.6 ± 16.6</td>
</tr>
<tr>
<td>Median (range)</td>
<td>25.5 (1-70)</td>
<td>25 (13-57)</td>
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<tr>
<td>Sex ratio, Male:Female</td>
<td>16:7</td>
<td>5:1</td>
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<tr>
<td>History of VL (%)</td>
<td>NA</td>
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<td>Interval between cure of VL and onset, years (Mean ± SD)</td>
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<td>Median (Range)</td>
<td>NA</td>
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<td>Spleen size (cm), Mean ± SD</td>
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<td>Liver size (cm), Mean ± SD</td>
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<td>Range</td>
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NA = Not applicable
Figure legends

Figure 1: RFLP analysis of ITS1 region amplified from *Leishmania donovani* isolates

Lane 1 = DD8; Lane 2 = V1; Lane 3 = V2; Lane 4 = V3; Lane 5 = V4; Lane 6 = V5; Lane 7 = P1; Lane 8 = P2; Lane M = Low range DNA ladder. *Inset:* PCR assay of ITS1 region from *Leishmania donovani* isolates. Lane M = 100 bp ladder; Lane 1 = PCR control (water); Lane 2 = DD8; Lane 3 = V1; Lane 4 = V2; Lane 5 = V3; Lane 6 = V4; Lane 7 = V5; Lane 8 = P1; Lane 9 = P2.

Figure 2: Determination of the presence of *Leptomonas* sp.


B: Neighbor-Joining tree based on p-distances of the ITS1 sequences of *Leishmania donovani* and P2; outgroup: *Leptomonas* sp. The analysis is based on an alignment of 1234 nucleotides. Distances are measured along the horizontal branches, according to the scale shown. Bootstrap values above 70% are indicated at the internodes. * indicates sequence of *L. donovani* isolates of India collected from GenBank and • indicates the sequence of P2.