*Leishmania donovani is the agent of cutaneous leishmaniasis in Sri Lanka*

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Abstract

DNA sequencing and microsatellite analyses were performed to investigate the relationship of Sri Lankan cutaneous leishmaniasis isolates to known species of *Leishmania*. This identified *L. donovani* as the agent of Sri Lankan cutaneous leishmaniasis, and showed these parasites are closely related to those causing visceral leishmaniasis in the Indian subcontinent.

Keywords: Parasitic Diseases; Protozoan Infections; Leishmaniasis, Cutaneous; Leishmaniasis, Visceral; Zoonoses; Sri Lanka

Article summary line: *Leishmania donovani* is the agent responsible for a new focus of cutaneous leishmaniasis in Sri Lanka
Infection with *Leishmania* protozoa can result in cutaneous, mucocutaneous or visceral leishmaniasis, depending on the parasite, host, and environmental factors (1). The resulting global disease burden is significant, with approximately 2 million new cases and 2.4 million disability adjusted life years per annum (2). The leishmaniases have received renewed interest because of an upsurge of cases in traditional endemic areas and the emergence of new foci of disease (3,4). One of the most dramatic examples is a new focus of cutaneous leishmaniasis (CL) in Sri Lanka (5) and since 2000-2001 over 400 cases have been reported.

Previously, a small number of isolates were characterised using multi-locus enzyme electrophoresis (MLEE) and reached the surprising conclusion that CL in Sri Lanka was caused by *Leishmania donovani* (5). However, *L. donovani* typically causes visceral leishmaniasis (VL), a potentially fatal disease and ongoing public health problem in neighbouring India, Bangladesh and Nepal, as well as in East Africa (1,2), but no cases of VL have been reported in Sri Lanka. Occasional cases of CL due to *L. donovani* have been described in other regions endemic for VL (6-9). The previous study examined a limited number of isolates identified using a single technique, MLEE (5). Whilst this is usually reliable, important exceptions were found in a recent study on *L. donovani* in East Africa (10).

Therefore, we conducted further investigations on Sri Lankan CL, increasing the number of isolates examined and using two molecular techniques.

Clinical diagnosis of suspected CL patients was parasitologically confirmed by demonstrating the presence of *Leishmania* amastigotes in skin lesions and/or promastigotes in cultures (5). Ethical approval was obtained from the Ethics Review Committee, Faculty of Medicine, University of Colombo. *Leishmania* diagnostic PCR was performed as described (11), which confirmed the identity of 15 primary isolates as members of the genus *Leishmania*. The isolates from Sri Lanka originated from Welioya (8, NE Sri Lanka), Jaffna (1, N Sri Lanka), and Galle (2, S Sri Lanka).
DNA sequencing of a single copy gene was chosen to identify *Leishmania* to the species level (10). The 6-phosphogluconate dehydrogenase (6PGDH) gene was chosen, as it shows a high degree of sequence polymorphism amongst *Leishmania* species (12), is well represented in sequence databases, and is known to differentiate the main Indian *L. donovani* zymodeme (MON-2) from those elsewhere (13). Primers for conserved regions of 6PGDH were designed using full length gene sequences of the *L. major* (MHOM/IL/1980/Friedlin) and *L. mexicana* BEL21 (MHOM/BZ/1982/BEL21) reference strains. Primers 6PGDH-F (AAT CGA GCA GCT CAA GGA AG) and 6PGDH-R (GAG CTT GGC GAG AAT CTG AC) were designed to generate a 997bp amplicon incorporating the 822 nucleotide partial 6PGDH sequence that is represented for multiple *Leishmania* species in Genbank. The partial sequences of 6PGDH genes were obtained from 11 Sri Lankan isolates from patients with CL, 2 Indian isolates from patients with VL, and 2 additional known *L. donovani* strains. These 15 new sequences and 10 publicly available sequences for species belonging to the genus *Leishmania* were used to construct a classification (Figure 1). 14 out of 17 of the *L. donovani* and *L. infantum* sequences were over 99% identical and could not be separated, the remaining three stocks were from India/Bangladesh (Ind-1, Ind-2 and BG1) and clustered together with 58% bootstrap support. Thus, *L. donovani* from Sri Lanka formed a strongly supported group with *L. donovani* and *L. infantum* from Europe and Africa. This group was quite distinct from one including *L. major* and *L. tropica*, which are the parasite species most closely related to *L. donovani* and *L. infantum* and which both cause CL in Africa and Asia. This analysis provided convincing evidence that the 11 Sri Lankan isolates examined were all *L. donovani*/*L. infantum*.

Strains of *L. donovani* from Sri Lanka were typed as zymodeme MON-37 by MLEE (5). This differs from the predominant Indian zymodeme (MON-2) in the mobility of one isoenzyme, 6PGDH itself. Therefore, the sequences were further analysed to investigate the
sequence variation underlying the isoenzyme identification. Translation of the 822 nt sequences revealed one amino acid change that was consistent with MLEE. A single nucleotide difference at position 976 was responsible for the occurrence of an uncharged asparagine (codon AAC) in MON-2, or a negatively charged aspartic acid (codon GAC) in MON-1, MON-18 and MON-37 sequences. This single change would explain the lower mobility of the MON-2 6PGDH isoenzyme, similar to the situation previously reported for glutamate oxaloacetate transaminase isoenzymes in East African L. donovani strains (10).

To analyse the relationships of the L. donovani and L. infantum strains more closely microsatellite analysis was performed (10). These data were combined with a dataset comprising 40 previously examined L. donovani/L. infantum isolates and the resulting dendrogram is shown in Figure 2. The Sri Lankan isolates clustered together and close to a group containing L. donovani isolates from India/Bangladesh/Nepal. L. infantum isolates formed a distinct cluster, as did the Sudanese L. donovani isolates and those from Kenya. This analysis reconfirms recent observations made by ourselves and others (10,14) that L. donovani isolates tend to cluster on a geographical basis, suggesting there are geographically distinct strains of this parasite. Second, although the Sri Lankan isolates form one or possibly two distinct groups, they are most closely related to L. donovani causing VL in India and distant from L. infantum parasites.

The results of this study lead to the conclusion that Sri Lankan CL is caused by Leishmania donovani. This is a significant conclusion with important implications for the epidemiology and clinical management of leishmaniasis. CL in Sri Lanka can no longer be regarded as a minor problem: an explosion of cases has occurred in the past five years, which undoubtedly under-represents the true incidence of disease, but without a single case of VL. However, the possibility of VL emerging should be considered, since subclinical infection is frequent in areas endemic for VL (15). In clinical management, CL can often be left to self-
cure, and may be preferable to active treatment, since self-cure may promote significant natural immunity to re-infection. Alternatively, anti-leishmanial drugs may be administered topically or by intralesional injection (2). However, *L. donovani* is recognised as one of the great scourges of mankind and if visceral disease does emerge as a problem in the future, more aggressive treatment of Sri Lankan CL should be considered i.e. parenteral administration of antimonials, amphotericin or oral miltefosine. Unfortunately, no drugs are currently registered for the treatment of leishmaniasis in Sri Lanka, and cryotherapy is the only available option in most health care centres. There is a need for better availability of drugs to treat Sri Lankan CL, but their introduction must be carefully monitored and critically evaluated.

This study also raises important questions about how infection with apparently identical or very similar parasites can result in radically different types of disease. Currently we can only speculate, but the answers are likely to lie with the nature of the parasites, the genetics of the human population or the contribution of sand fly vector. The data presented here demonstrate the overall close genetic similarity between all the *L. donovani* isolates examined. However, there may be some critical genetic difference between the Sri Lankan parasites that renders them less virulent than *L. donovani* from elsewhere. Clearly much work remains to be done, including investigation of possible subclinical VL infection by PCR or serology, to understand the factors behind the emergence of Sri Lankan CL due to *L. donovani*, but this must be regarded as a priority as the number of cases continues to increase.

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Dr Yamuna Siriwardana qualified from the Faculty of Medicine, University of Colombo and is currently completing postgraduate PhD training in Medical Parasitology. Her main research interest is in leishmaniasis, which she has studied in Sri Lanka and Liverpool.
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Figure Legends

Figure 1. Classification of *Leishmania* species based on the partial DNA sequence of the 6PGDH gene constructed with PHYLIP using parsimony. Numbers at branch points are bootstrap values compiled using 100 replicates. Isolates examined and the accession numbers of their 6PGDH sequences in the GenBank/EMBL/DDBJ database are as follows: 11 Sri Lankan patient isolates L59, L60, L75, L78, L80, L284, L304, L355, L330, L301, L348 (AJ888888-AJ888898); 2 Indian isolates, Ind-1, Ind-2 from splenic aspirates of VL patients in Muzafapur, Bihar (MHOM/IN/2004/Ind-1 & MHOM/IN/2004/Ind-2, AJ888900, AJ888901); 3 previously identified *L. donovani* isolates BG1 (MHOM/BD/1997/BG1, AJ888899), LEM719 (IMAR/KE/1962/LRC-L57; LEM719, AJ888902), LV9 (MHOM/ET/1967/HU3;LV9, AY168567); and *L. infantum* JPC (MCAN/ES/1998/LEM935;JPC;M5, GeneDB LinJ35.2940). In addition sequences from *L. tropica* (AY045763, AY168568), *L. major* (FV1, AF242436; 8A1, AF242436; RTC13, AY706106; JerII, AY706105; 5ASKH, AY706107), *L. mexicana* (M379, AY217723; BEL21, AY386372) and *L. amazonensis* (PH8, AY168562) isolates were analysed.

Figure 2. Classification of *L. donovani* and *L. infantum* isolates constructed using microsatellite data with parsimony in PAUP. Numbers at branch points are bootstrap values compiled using 100 replicates. Isolates formed geographically based groups (circled). Sri Lankan isolates L59, L60, L75, L78 and L80 are indicated. The tips of other branches are from a dataset of other previously analysed isolates, including all those identified as *L. donovani* or *L. infantum* and isolates from the Indian subcontinent (ref. 10).
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Figure 1

![Phylogenetic tree showing relationships between different Leishmania species and strains.]

- L. major FV1, JerII, L357
- L. major RTC13 & 8A1
- L. tropica L747 & L36
- L. major 5ASKH

- 14 L. donovani & L. infantum strains

- L. donovani BG1 & Ind-1 & Ind-2

- L. mexicana M379
- L. amazonensis PH8
- L. mexicana BEL21

5 changes