Tropical Medicine and Health Vol. 36 No. 4, 2008, pp. 171-179

Original article

Morphological and molecular studies on Sri Lankan *Leishmania*

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Received 9 September, 2008 Accepted 24 October, 2008 Published online 25 November, 2008

Abstract:
Cutaneous leishmaniasis (CL) is an emerging disease in Sri Lanka, more than 400 cases having been reported since 2001. However, the morphology and taxonomic status of the Sri Lankan strain of *Leishmania* is not known yet. Therefore, it is important to study the morphology and to analyze the phylogenetic position to predict the risk and expansion of the disease and thereby to develop an effective control programme. Morphology of the amastigote of the Sri Lankan isolate was checked by light microscopy and electron microscopic observation. Presence of amastigotes within macrophages was confirmed in skin biopsy samples. The promastigote had the characteristic appearance of a kinetoplastid cell in cultures. The kinetoplast minicircle DNA has been used for diagnosis of *Leishmania* for a long time and also for phylogenetic studies on trypanosomatid flagellates. The kinetoplast minicircle was amplified using PCR and subsequently sequenced from samples obtained from Sri Lankan patients with cutaneous lesions. Mitochondrial cytochrome b gene has been recently shown to be useful for identification and phylogenetic analysis of the genus *Leishmania*. The nucleotide sequence of the cytochrome b gene of Sri Lankan *Leishmania* was determined using the semi-nested PCR and 620 bp of this gene obtained. Phylogenetic analysis using these sequences unambiguously indicated that Sri Lankan isolate of *Leishmania* belongs to *L. donovani* complex. However, the Sri Lankan isolate forms a distinct lineage within the complex and probably represents a new branch.
Keywords: Sri Lankan *Leishmania*, cytochrome b gene, phylogenetic analysis, minicircle, kinetoplast DNA
The sequences of kinetoplast minicircle DNA and cytochrome b gene of the Sri Lankan isolate of *Leishmania* used in this study are available in the EMBL, GenBank and DDJB data bases under the accession numbers: DQ205333, DQ205334, DQ276851, DQ276852, DQ276853, DQ276854, DQ276855, DQ276856, DQ276857.

INTRODUCTION

Leishmaniasis has a worldwide distribution, being endemic in at least 88 countries. The World Health Organization (WHO) considers it to be one of the most important parasitic diseases, with approximately 350 million people at risk. The clinical outcome is variable depending upon numerous factors [1]. Twenty of 30 species in the genus *Leishmania* cause disease in humans. The leishmaniases have attracted renewed interest because of the re-emergence of cases in traditional leishmaniasis endemic areas and the emergence of new foci of disease [2, 3].

Cutaneous leishmaniasis (CL) or oriental sore, is reported to be caused primarily by *L. tropica, L. major, L. aethiopica, L. mexicana, L. amazonensis, L. panamensis, L. guyanenis, L. peruviana* and *L. braziliensis* but may be caused by any of the *Leishmania* species which infect humans. Localized cutaneous leishmaniasis (LCL) is a vector-borne disease commonly caused in the old world by *L. tropica* or *L. major* but reports are now appearing of isolated cases due to *L. donovani* from Kenya [4], Yemen [5] and India [6]. However, *L. donovani* typically causes visceral leishmaniasis (VL) which is a fatal disease and a major public health problem in India, Bangladesh, Nepal as well as in East Africa [7, 8].

CL has recently emerged as an important public health problem in Sri Lanka [9]. For decades, it was considered an exotic disease, but the first locally acquired case was reported in 1992 and it was confirmed as *Leishmania* by light microscopic examination [10]. In 2003, isoenzyme analysis

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of 5 isolates from 4 CL patients in Sri Lanka showed that they were all *Leishmania donovani* zymodeme MON-37, the parasite that also causes VL in East Africa [9]. More than 400 cases of cutaneous leishmaniasis have been reported from the Northern, North Central, Central, North Western, Eastern and Southern Provinces in Sri Lanka since 2001 [11,12]. The skin lesions of patients were detected in exposed areas like the face, arms, and legs, and were 5-20 mm in diameter. Some lesions lasted from 4 to 10 months as blisters without any change, but some changed into ulcerations and some lesions lasted for more than 2 years. DNA studies have demonstrated that the causative organism of this cutaneous leishmaniasis is *L. donovani* [11, 12]. Thus, *L. donovani* from Sri Lanka was found to be grouped with *L. donovani* and *L. infantum* from Europe and Africa which is quite distinct from the group that includes *L. major* and *L. tropica* [11].

Although the Sri Lankan *Leishmania* has been identified as *L. donovani* it presents as the cutaneous form at the present time. Keeping this in view, the aim of the present research was to study the morphology of the Sri Lankan isolate, since it has not yet been clarified and also to carry out further molecular analyses to promote phylogenetic study and diagnosis.

MATERIALS AND METHODS

Sample collection

Aspirates from skin lesions (4-10 months duration) of cutaneous leishmaniasis (CL) patients in Sri Lanka (from Welioya and Anuradhapura areas) were collected in rabbit-blood agar medium between August 2004 and August 2005. These two areas belong to the northeast and north central provinces respectively and are located in the dry zone of Sri Lanka, receiving <900 mm rainfall per year. Samples were named according to the International Code for labeling *Leishmania* isolates recommended at the 1980 UNDP/ WHO meeting in Washington D.C. (Table 1). The diagnosis of cutaneous leishmaniasis was made mainly on the basis of clinical findings and confirmed by examination of Giemsa-stained tissue smears. Skin biopsy specimens were obtained (with the consent of the patient) under aseptic conditions. All patients included in this study had never been abroad. Collected samples were cultured and maintained in the Department of Environmental Health Science, Kochi Medical School, Japan. This study was reviewed and approved by the Health Ministry of Sri Lanka and the Institutional Ethical Committee of Kochi Medical School.

Culture method of *Leishmania*

Sri Lankan *Leishmania* samples collected from patients were transferred into fresh rabbit blood agar medium and incubated for one week at 25℃ in a rotating incubator. Cells were washed in a solution containing a high concentration of streptomycin and penicillin (streptomycin 100ug/ ml and penicillin 100iu /ml) and transferred into fresh rabbit blood agar medium once weekly for three weeks and incubated under the same conditions. After three weeks, cells were pelleted by centrifugation (3500rpm, 3-5min.) and transferred into 5-10 ml Schneider's Drosophila medium with 15-20% (vol/vol) fetal bovine serum (Cansera International INC, Canada) and incubated at 25℃ for two weeks.

Cells of other reference *Leishmania* species such as *Leishmania amazonensis* (MHOM/BR/73/M2269), *Leishmania donovani* (MHOM/IN/80/DD8) and *Leishmania major* (MHOM/SU/73/5ASKH) were pre-cultured in rabbit blood agar medium for one week at 25℃ and transferred into Schneider's Drosophila medium with 5-10% (vol/vol) fetal bovine serum and incubated at 25℃ for two weeks for the comparative studies.

Electron microscopy

The electron microscope analysis of the skin biopsy samples obtained from the lesions of the infected patients was performed as described by Watanabe et al. [13]. Ultra-thin sections were examined with a Hitachi H-7100 electron microscope (Hitachi, Tokyo, Japan; accelerating potential, 75kV). Electronmicrographs were taken of clearly visible

Table 1: Details of the samples used for the present study

International Code	Patient Code	Place	Parasite Isolation	Lesions
MHOM/LK/2004/SLT	SLT1	Anuradhapura	Positive	right arm, neck
MHOM/LK/2005/SLR	SLR1	Welioya	Positive	ear, left arm
MHOM/LK/2005/SLR	SLR2	Welioya	Positive	left wrist
MHOM/LK/2005/SLR	SLR5	Welioya	Positive	right arm
MHOM/LK/2005/SLR	SLR6	Welioya	Positive	left arm, forehead
MHOM/LK/2005/SLR	SLR7	Welioya	Positive	right arm, ear
MHOM/LK/2005/SLR	SLR8	Welioya	Positive	right wrist
MHOM/LK/2005/SLR	SLR9	Welioya	Positive	neck

Note: Anuradhapura and Welioya belong to the north central and north east provinces of Sri Lanka located in the dry zone. Samples were collected from August 2004-2005.

cells.

DNA extraction

Parasite cells were harvested by centrifugation for 15 min at 350g after two weeks in the logarithmic phase of growth. Genomic DNA was extracted from the promastigote pellet ($1x10^9$ cell count) using the Easy-DNA™ Kit (Invitrogen Corporation, Carlsbad, California) for genomic DNA isolation in accordance with the manufacturer's instructions.

PCR amplification of cytochrome b gene

For PCR amplification of the cytochrome b (*Cyt* b) gene of Sri Lankan *Leishmania* parasites, oligonucleotide primers were designed on the basis of consensus sequences of *Cyt* b and other genes present in the maxicircle of a number of *Leishmania* spp. Lcyt F1, Lcyt F2 and Lcyt R1 specific primers were used for PCR amplification to obtain a band of about 600bp. Primer sequences are as follows. LcytF1: 5'-TGT ACR ATG ATG TCG TAT TG-3', LcytF2: 5'-CGA TGA TGT CGT ATT GAG GTC-3', LcytR1: 5'-GCT AAA AAA CCA CTC ATA AAT ATA C-3'. The first PCR reaction was done using the primers Lcyt F1 and Lcyt R1. Then a nested PCR was carried out using the outer forward, inner forward and the reverse primers (Lcyt F1, Lcyt F2 and Lcyt R1). PCR reactions were done in total volume of 25µl. Each reaction mixture contained DNA template, 10 pmol of each primer, 2.5mM of each dNTP, 1unit of *Z-Taq*™ polymerase, 10x *Z-Taq*™ buffer (TaKaRa, Japan). The PCR conditions were as follows: initial denaturation at 94 ℃ for 3min, followed by 31 cycles of 94 ℃ for 30 sec, annealing at 50 ℃ for 30 sec and extension at 72 ℃ for 1 min and final extension at 72 ℃ for 5min. PCR was carried out in a My Cycler ™ Thermal cycler (BioRad, USA). Semi-nested PCR was also carried out under the same conditions. PCR product was purified by QIA quick PCR purification column (QIAGEN, GmbH, Hilden, Germany). Purified product was ligated into PGEM® T-vector system (Promega, USA).

PCR amplification of kinetoplast minicircle DNA

Primer sequences used to amplify the minicircle were (KP1) 5'-GGG GTT GGT GTA AAA TAG G-3' and (KP2) 5'-TGA ACG GGG TTT CTG TAT-3', which were also used to diagnose Indian isolate of *L. donovani* [14]. The reaction was carried out in a total volume of 25µl. Each reaction contained the following: 100ng of DNA template 10 pmol of each primer, 2.5mM of each dNTP, 1unit of *Z-Taq*™ polymerase and 10x *Z-Taq*™ buffer (TaKaRa, Japan). The PCR conditions were as follows: initial denaturation at 94 ℃ for 3 min, followed by 31 cycles of 94 ℃ for 30 sec, annealing at 50 ℃ for 30 sec and extension at 72 ℃ for 1 min and final extension at 72 ℃ for 5 minutes. The minicircle PCR products were separated by electrophoresis on agarose gels, purified using the Gene Clean 11 Kit (Q.BIO gene, North America) and ligated to pGEM® -T Vector system (Promega Corporation, Madison, USA).

Subcloning and DNA sequencing

Purified PCR products ligated into pGEM® T-vector system were transformed into *Escherichia coli* JM109 cells. Plasmid DNA from positive clones was extracted using an alkaline SDS method and three clones per each isolate were sequenced on both strands using vector-specific primers. DNA sequencing was carried out in an ABIPRISM 310 automated sequencer (Perkin-Elmer Corporation, USA).

Sequence alignments and phylogenetic analysis

Sequence similarity searching was performed using the NCBI BLAST programme (http://www.ncbi.nlm.gov/blast/). Multiple sequence alignments were done using the programs CLUSTAL W and GENETYXMAX (ver. 6.0). Phylogenetic analysis was performed using distance and parsimony methods in MEGA (ver.3.1). For distance analyses, the Kimura 2-parameter model was used to construct the distance matrix and the trees were inferred from this using the neighbor- joining (NJ) and maximum parsimony (MP) approaches. Heuristic method in MP approach was used to obtain the MP trees. Minimum Evolution method was used for the phylogenetic analysis of amino acid sequences. Bootstrap resampling (1000 replicates) was performed for each method to assess tree topology. Numbers of transitions, transversions and amino acid differences among *Leishmania* species were also calculated using the same programme.

RESULTS

Cutaneous lesions of the patients observed in this study were present on the face, neck and arms. Four patients had lesions on the arms and face or neck region while the other four had lesions only in one region (Table 1). The location of the lesions suggests that uncovered areas of the body are more prone to clinical manifestations of CL infection. Lesions were 4-6mm in length, dry and scaly and appeared papulonodular. No hepatomegaly or splenomegaly was detected in these patients.

It was found that the culturing of Sri Lankan *Leishmania* was very difficult with slow growth rate and low multiplication compared to other reference *Leishmania* species. We could subculture and maintain the Sri Lankan *Leishmania* only in Schneider's Drosophila medium.

Light microscopic observation of the Giemsa-stained

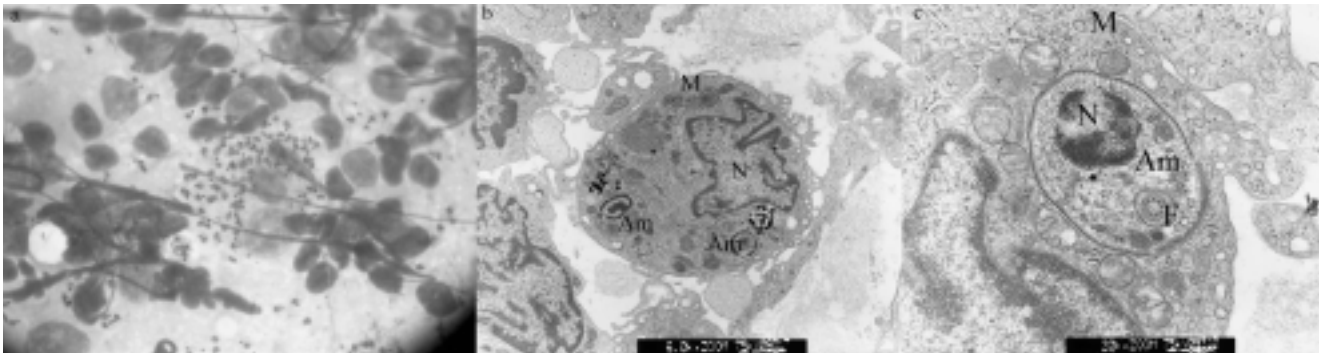

Fig. 1. (a) Presence of *Leishmania* amastigotes within the macrophage of a skin biopsy sample from a cutaneous leishmaniasis patient in Sri Lanka (Giemsa stained x1000) (b). Electronmicrograph of a skin biopsy sample from a cutaneous leishmaniasis patient in Sri Lanka (x6000). **Am,** amastigotes; **M,** macrophage; **N,** nucleus (c). Electronmicrograph of a cross section of amastigote present inside a macrophage (x20,000). **M,** macrophage; **Am,** amastigote; **N,** nucleus of the amastigote; **F,** flagellum (9+2) arrangement of microtubules.

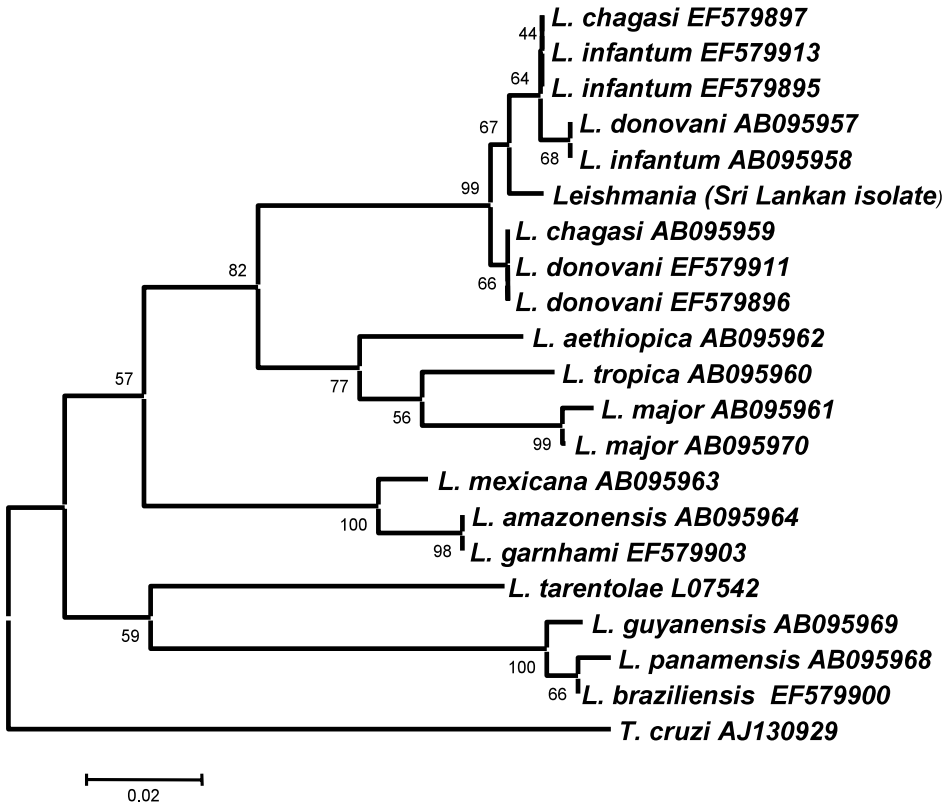

Fig. 2. (A) Phylogenetic tree rooted by outgroup (*Trypanosoma cruzi*), inferred from partial cytochrome b nucleotide sequence of Sri Lankan *Leishmania* using the Kimura 2-parameter model of sequence evolution and the neighbor joining method (NJ) of tree construction (1000 bootstrap) in MEGA program. Scale bar indicates the proportion of sites changing along each branch. Accession number of Sri Lankan *Leishmania* (DQ205333).

skin biopsy samples of patients revealed the presence of *Leishmania* cells inside the macrophages (Fig.1a). Electronmicrographs of skin biopsy samples of the patients revealed that the presence of large amastigotes inside macrophages of length of 2.1μm and width of 1.48μm (Fig.1b), resembling those of other *Leishmania* species [15]. A kinetoplast and the 9+2 arrangement of the microtubules were visible. Promastigotes of Sri Lankan *Leishmania* cells were oval or

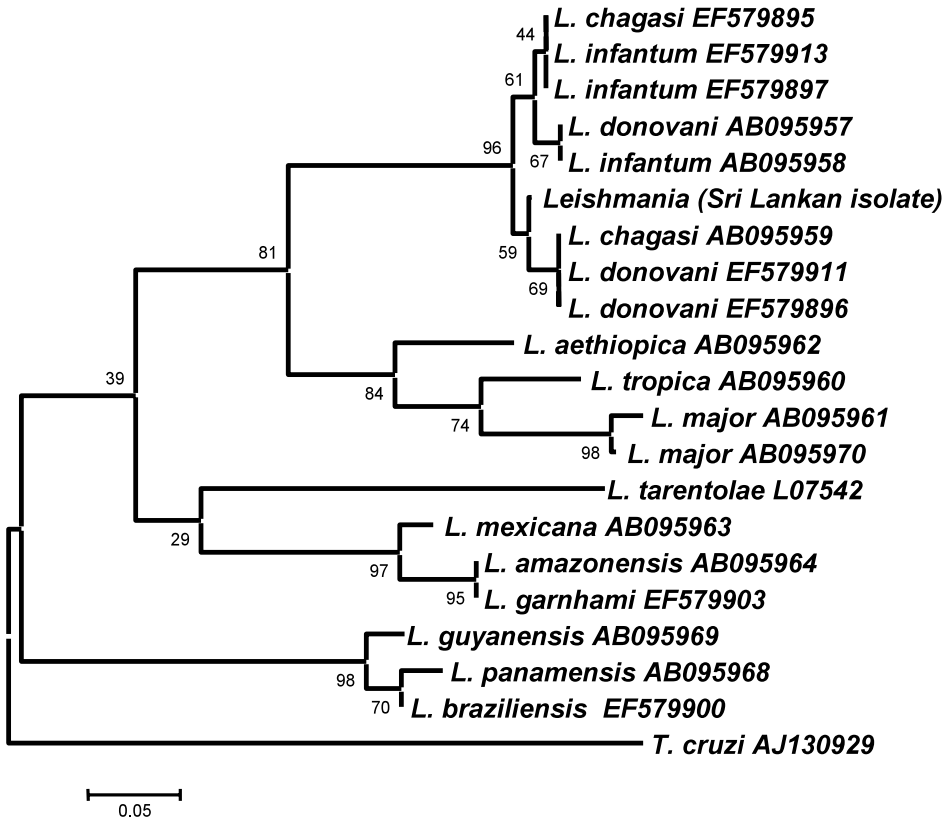

Fig. 2. (B) Phylogenetic relationships of Sri Lankan *Leishmania* with members of the genus *Leishmania* inferred from the amino acid sequence of a portion of the cytochrome b gene using Kimura 2-parameter model and the Minimum Evolution (ME) method of tree construction, MEGA version 3.1 program (1000 bootstrap).

pyriform in shape in cultures.

Cytochrome b PCR products obtained from eight Sri Lankan *Leishmania* samples analysed were about 600bp and found to be A/T rich. Usage of A is 26.9% and the T is 50%. The sensitivity of this PCR reaction was very high with all samples producing positive results. Nucleotide sequences obtained from the cytochrome b PCR products of Sri Lankan *Leishmania* samples were identical. The Sri Lankan sequence aligned with those from members of the *L. donovani* complex without any gaps in the sequence alignment. At the nucleotide level, two nucleotide substitutions (all transitions) were observed between *L. chagasi* (MHOM /BR/74/PP75)(AB095959) and Sri Lankan *Leishmania* isolate. About three base substitutions were observed relative to *L. donovani* (AB095957) and *L. infantum* (AB095958). Furthermore, many transitions and transversions (more than 10) were detected between the Sri Lankan *Leishmania* isolate and other *Leishmania* species that cause cutaneous leishmaniasis such as *L. tropica*, *L. major* etc. Multiple alignment of the inferred amino acid sequences from members of the *L. donovani* complex revealed only one amino acid difference between the Sri Lankan *Leishmania* isolate and *L. chagasi* (MHOM/BR/74/PP75)(AB095959). Two amino acid differences were present between *L. infantum* (MHOM/TN/80/IPTI) (AB095958) and *L. donovani* (2525 M-C2-2M) (AB095957). There were many amino acid differences (13-20) between the Sri Lankan sequence and other species causing cutaneous leishmaniasis.

Phylogenetic analyses using nucleotide or inferred amino acid sequences of cytochrome b gene clearly demonstrate that the Sri Lankan *Leishmania* isolate falls within the *L. donovani* complex (Fig. 2) but is not identical in sequence to any other member of the complex. However, pairwise differences are small between members of this complex. The neighbor-joining (NJ) tree inferred from the nucleotide sequence of cytochrome b revealed that there are two distinct branches within the *L. donovani* complex (Fig. 2A). One branch consists of *L. chagasi* (MHOM/BR/74/PP 75) (EF579897), *L. infantum* (MCAN/GR/94/CRE69) (EF 579913), *L. infantum* (MHOM/TN/80/IPT1) (EF579895) *L. donovani* (2525M-C2-2M) (AB095957), *L. infantum* (MHOM/TN/80/IPT1) (AB095958), and the Sri Lankan *Leishmania* strain. Within this group Sri Lankan isolate forms a separate lineage. Whereas, *L. chagasi* (MHOM/BR/

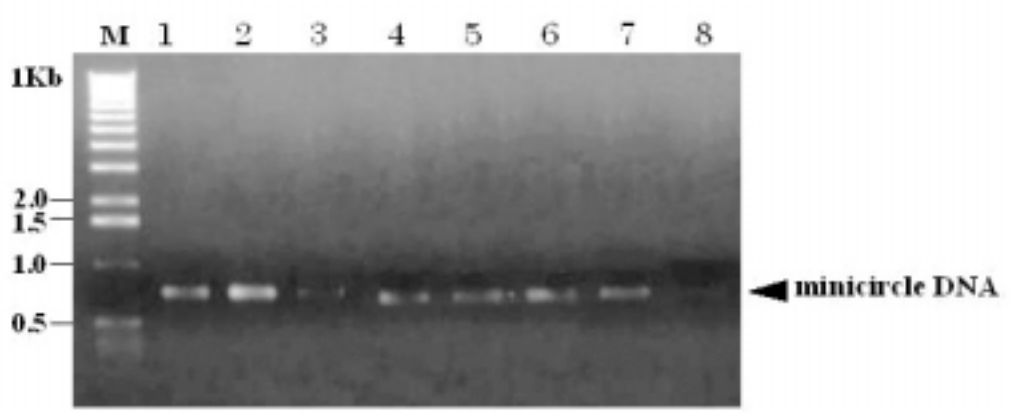

Fig. 3. (A) Size of the PCR products of kinetoplast minicircle DNA obtained from Sri Lankan *Leishmania* samples. Lane M, The DNA size marker , 1kb DNA ladder; lane 1, SL/T/1; lane 2, SL/R/1; lane 3, SL/R/2; lane 4, SL/R/5; lane 5, SL/R/6; lane 6, SL/R/7; lane 7, SL/R/8; lane 8, SL/R/9.

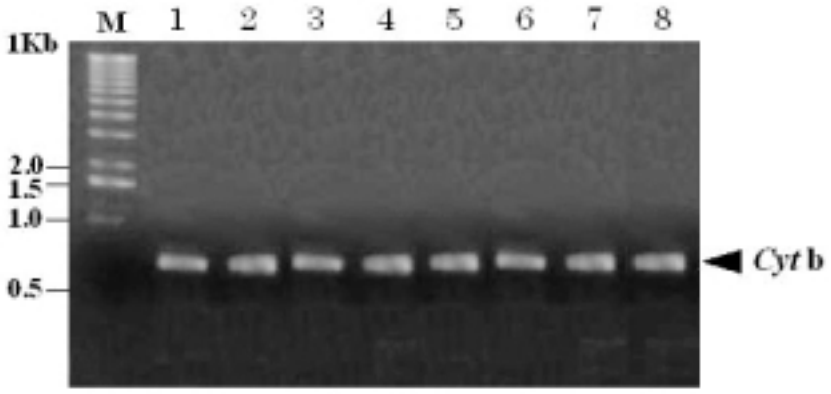

Fig. 3. (B) Size of the PCR products of Sri Lankan *Leishmania* cytochrome b (*Cyt* b) gene (600bp). Lane M, The DNA size marker, 1kb DNA ladder; lane 1, SL/T/1; lane 2, SL/R/1, lane 3, SL/R/2; lane 4, SL/R/5; lane 5, SL/R/6; lane 6, SL/R/7; lane 7, SL/R/8; lane 8, SL/R/9.

74/PP75) (AB095959), *L. donovani* (MHOM/IN/80/DD8) (EF579911) and *L. donovani* (MHOM/IN/80/DD8) (EF 579896) form the second group. *Leishmania aethiopica*, *L. tropica* and *L. major* that are known to be the causative organisms of CL in the world form a separate group in the tree. Minimum Evolution tree obtained using the amino acid sequence of *Cyt* b gene also revealed a similar topology (Fig. 2B), and Sri Lankan *Leishmania* isolate forms a separate branch within the *L. donovani* complex. In this tree Sri Lankan isolate branches out with a group consisting of *L. chagasi* (MHOM/BR/74/PP75) (AB095959) and two other Indian strains, *L. donovani* (MHOM/IN/80/DD8) (EF 579911) and *L. donovani* (MHOM/IN/80/DD8)(EF579896).

A distinct DNA band of approximately 700 base pairs corresponding to kinetoplast minicircle DNA was detected from Sri Lankan *Leishmania* positive samples using the primers designed by Singh et al. [14] (Fig. 3). This PCR reaction showed high specificity and sensitivity for the samples producing a very clear band at the first PCR reaction even in the presence of low DNA quantities. Therefore, this could be a highly reliable PCR for purposes of diagnosis. Nucleotide sequence analysis of this DNA fragment revealed the presence of the universal minicircle conserved sequence block (CSB) and a variable region. Eight kinetoplast minicircle sequences were analyzed and showed the highest similarity (in BLAST searches) to *L. donovani*. Multiple sequence alignment of the kinetoplast minicircle sequence of Sri Lankan *Leishmania* with closely related *L. donovani* strains revealed the presence of base substitutions and gaps in the variable region. Therefore, pair-wise comparisons of the 109 nucleotides in the minicircle conserved sequence block (CSB) of Sri Lankan *Leishmania* isolate was carried out with closely related species of *Leishmania* to confirm the highest percentage of similarity. This analysis revealed 83% similarity to the *L. infantum* (MHOM/ES/81/LEM307) (AF188701) and *L. chagasi* (MHOM/BR/74/

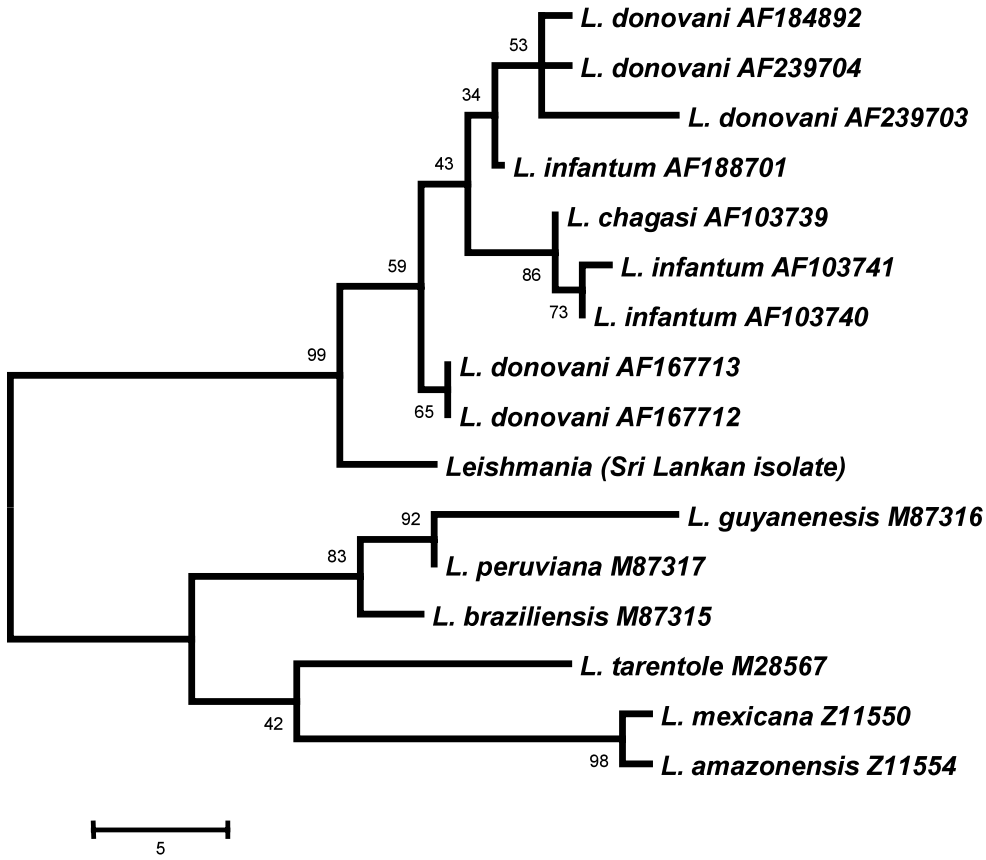

Fig. 4. Phylogenetic relationship of Sri Lankan *Leishmania* with the members of genus *Leishmania* inferred from the conserved region of the kinetoplast minicircle using the Maximum Parsimony (MP) method, MEGA version 3.1 program (1000 bootstrap). Scale bar indicates the proportion of sites changing along each branch.

PP75) (AF103739). There was 81% similarity to the *L. donovani* (MHOM/KE/79/GG96) (AF239704) and the difference was high with *L. panamensis* (loc173)(AF118474) and *L. guyanensis* (AB095969) species that cause cutaneous leishmaniasis in the world. A phylogenetic tree (MP approach) was constructed using the minicircle conserved sequence block to identify the position of the Sri Lankan strain within the *L. donovani* complex. It produced the same topology obtained for *Cyt* b gene (Fig. 4). The Sri Lankan *Leishmania* was again placed within the *L. donovani* group but it forms a separate branch. Within the *L. donovani* complex, *L. donovani* Kenya strains (MHOM/KE /73/MRC74) (AF239703), (MHOM/KE/75MUTINGA)(AF 184892) and (MHOM/KE/79/GG996)(AF239704) group together while *L. infantum*, *L. chagasi* strains form another group. Sri Lankan *Leishmania* branched with two Indian strains, *L. donovani* (MHOM/IN/82/NANDI) (AF167713) and *L. donovani* (MHOM/IN/80/DD8) (AF167712). *L. amazonensis*, *L. mexicana*, *L. guyanensis*, *L. peruviana* and *L. brazilensis* form a separate group in this tree. The same topology was obtained for the NJ tree as well (figure not given).

All sequences obtained from Sri Lankan *Leishmania* isolates are deposited in the GenBank under the Accession numbers **DQ 205333,DQ 205334,DQ 276851 up to DQ 276857.**

DISCUSSION

Location of the lesions suggests that uncovered areas of the body are more prone to clinical manifestations of CL infection. This was reflected by the occupational and clothing habits of the patients observed in this study. Microscopic findings of smear samples and electron microscopic observation of skin biopsy samples revealed the presence of amastigotes within macrophages. It revealed that the Sri Lankan *Leishmania* amastigote has the characteristic appearance of a kinetoplastid cell. The Sri Lankan *Leishmania* could be subcultured and maintained only in Schneider‘s Drosophila medium supplemented with 20% fetal bovine serum, while other reference Leishmania strains could easily be cultured in Schneider’s Drosophila medium supple-

mented with 10% fetal bovine serum under the same conditions. This may be due to the difference in nutrient requirement of the Sri Lankan *Leishmania* compared to other *Leishmania* strains.

The previous isoenzyme analysis of Sri Lankan *Leishmania* revealed that all isolates studied (collected in the same localities as our samples) were *Leishmania donovani* zymodeme MON-37 and differed from the Indian zymodeme (MON-2) in the mobility of one isoenzyme, 6-phosphogluconate dehydrogenase (6PGDH) [9]. Although isoenzyme analysis has been used as the gold standard for identification of *Leishmania*, it has drawbacks such as difficulties encountered in comparing the raw data from different laboratories and in analysis of new strains in the context of existing data [16]. In the present study, Sri Lankan *Leishmania Cyt* b gene and kinetoplast minicircle DNA sequences were analyzed to investigate the sequence variation to evaluate the results of the previous isoenzyme identification. The kinetoplast minicircle sequence alignment with the sequences of most related *L. donovani* strains revealed 83% similarity to the *L. infantum* (MHOM/ES/81/LEM 307)(AF188701) and *L. chagasi* (MHOM/BR/74/PP75) (AF103739) with many gaps and base substitutions. There was 81% similarity with the *L. donovani* (MHOM/KE/79/GG96) (AF239704). Multiple alignment of the inferred amino acid sequence of *Cyt* b gene revealed one amino acid difference between the Sri Lankan *Leishmania* isolate and *L. chagasi* (MHOM/BR/74/PP75) and two amino acid differences between *L. infantum* (MHOM/TN/80/IPTI) and *L. donovani* (2525M-C2-2M). Base substitutions were also identified at the nucleotide level of *Cyt* b gene. These data suggest that Sri Lankan *Leishmania* has nucleotide and amino acid differences in its genes compared to the members of the *L. donovani* complex. Minicircle DNAs have been used as a target gene for the identification and phylogenetic analysis of *Leishmania* species by molecular methods for many years, although they have variations in their sequences [17, 18]. More recently, the sequences of *Cyt* b genes from various *Leishmania* species were assessed and shown to be a very good standard for determination of the species [19]. In this study, sequences from the *Cyt* b gene and kinetoplast minicircle conserved sequence block of Sri Lankan *Leishmania* was used for the phylogenetic analysis. It revealed that the Sri Lankan *Leishmania* strain clustered together with the members of the *L. donovani* complex. The tree topology was highly supported by the bootstrap value in the NJ method. The same topology was obtained for the MP trees as well. But the Sri Lankan *Leishmania* isolate forms a separate branch in these trees. The results of the phylogenetic analysis revealed that the Sri Lankan *Leishmania* branched with the *L. donovani* Indian strains, (MHOM/IN/82/NANDI) (AF167713) and (MHOM/IN/80/DD8) (AF 167712)) within the *L. donovani* complex. This analysis reconfirms recent observations [20, 21] that *L. donovani* isolates tend to cluster on a geographic basis and that the strains are geographically distinct. Siriwardana et al. [11] also demonstrated that the Sri Lanka isolates clustered together and close to a group containing *L. donovani* isolates from India, Bangladesh and Nepal based on microsatellite studies. But it is premature to comment further on the relationship of Sri Lankan *Leishmania* with members of the *L. donovani* complex at this stage since further molecular research such as genotyping should be carried out to confirm the relationship.

Currently, Sri Lankan leishmaniasis presents in the cutaneous form, but results of the nucleotide sequence analysis revealed that the Sri Lankan *Leishmania* isolate belongs to the *L. donovani* complex, members of which typically cause VL. Therefore, it is possible that the visceral form may appear in Sri Lanka in the future. The dermotropic zymodemes have the ability to transform into visceral forms and similar phenomena have been reported from Mediterranean countries [22, 23, 24]. Sub-clinical infection is frequent in VL-endemic areas [25]. Therefore, it is essential to carry out constant epidemiological studies as well as pathogenic studies to confirm the disease pattern to plan an efficient and effective control program.

The origin of the Sri Lankan leishmaniasis is still unclear and suggests several possibilities. Emergence of leishmaniasis in a particular area requires specific sand fly vectors, other mammalian hosts plus environmental features such as appropriate climatic and vegetation zones. The patients observed in this study were from the dry zone of Sri Lanka which has abundant sand flies. The presence of a large number of insect vectors may be one reason for the occurrence of the disease in this area.

We suggest, therefore that it is very important to carry out further molecular research as well as epidemiological and pathogenic studies to plan an efficient and effective control program for the control of leishmaniasis in Sri Lanka.

In conclusion, the morphological analysis revealed that the Sri Lankan *Leishmania* has an appearance characteristic of *L. donovani* cells. Phylogenetic analysis revealed that the Sri Lankan isolate clusters with members of the *L. donovani* complex but that forms a distinct separate branch.

ACKNOWLEDGEMENTS

We would like to thank Prof. David Blair, James Cook University, Australia for the invaluable review of this manuscript. We also extend our thanks to all the staff members in

our laboratory, Department of Environmental Health Sciences in Kochi Medical School for their encouragement and help.

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