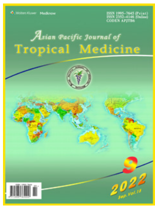

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Genetic diversity of *Leishmania donovani* isolates from cutaneous lesions of military personnel in the Mullaitivu and Kilinochchi districts of the Northern Province, Sri Lanka

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ABSTRACT

Objective: To compare the DNA sequences of *Leishmania* (*L.*) *donovani* isolated from individuals in two districts of the Northern Province with other parts of Sri Lanka and neighboring countries.

Methods: Samples were collected from military personnel at the Army Hospital, Narahenpita, Sri Lanka from November 2018 to March 2020. A portion of the samples was fixed, stained with Giemsa and observed under the light microscope. The genomic The DNA was extracted from the remaining portion of the samples using DNEasy blood tissue kit (Qiagen, Germany) and amplified using *Leishmania* genus-specific primers for molecular diagnosis initially. DNA was amplified using *L. donovani* species-specific primers by PCR and the amplified product was sequenced for comparison of nucleotide sequences.

Results: Out of 76 suspected patients, at least one biological sample of 45 (59.2%) was positive for *L. amastigotes* upon microscopy. Overall, 33 (43.4%) were positive in *Leishmania* genus-specific PCR, but only 23 (30.3%) were positive in *L. donovani* specific PCR. The dendrogram indicates that the current sequences clustered together with those from Nepal and Gampaha districts (Western Province), Sri Lanka, while the Indian and Eastern African sequences clustered separately.

Conclusions: The genetic diversity was low among the isolates, indicating a single and possibly a local point of origin. However, the similarity of Sri Lankan and Nepal strains indicate a possibility of a shared point of origin, which needs more extensive evidence to confirm.

KEYWORDS: Leishmaniasis; Sri Lanka; Phylogenetics; Kilinochchi; Mullaitivu; Northern Province

1. Introduction

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*. It is one of the major vector-borne diseases with approximately 700 000 to 1 million cases each year[1]. More

Significance

The control of leishmaniasis requires extensive knowledge of different aspects regarding parasites, vectors including molecular level changes and risk factors for transmission. There are only a few studies conducted in Sri Lanka for molecular level comparison of parasites isolated from different areas in the country. Therefore, the present study was conducted to compare the DNA sequences of parasites isolated from the northern part of Sri Lanka and to compare with parasites isolated from other foci in Sri Lanka to detect possible similarities or/and dissimilarities.

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than 98 countries and territories are endemic to at least one form of leishmaniasis, majority of which are reported from tropical countries in South-East Asia and South America[2].

The evidence for the local transmission of leishmaniasis in Sri Lanka dates back to 1992 as per published data[3]. Since then, few sporadic cases were reported from different parts of the island until a considerable increase of patients was reported after 2002, mainly as a result of the cutaneous leishmaniasis (CL) cases reported among military personnel who were serving in conflict areas in Northern Sri Lanka[4]. In 2008, leishmaniasis was listed as a notifiable disease in Sri Lanka[5]. After 2017, the number of reported cases has been exceeding 3 000 annually[6].

The major clinical form reported from Sri Lanka is CL, while visceral leishmaniasis (VL) and muco-cutaneous leishmaniasis cases have been reported less frequently[7–9]. The causative parasite strain responsible for both CL and VL in Sri Lanka has been identified as *Leishmania* (*L.*) *donovani* MON-37[10]. Among 20 species of sand flies present in the country[11], *Phlebotomus argentipes* is considered the main suspected vector[12,13]. Potential vectors are widely distributed around the island, making the vectors readily available to maintain the transmission cycle[11]. Thus, the free movement of infected individuals from conflict-free areas of the country may have resulted in the rapid transmission of leishmaniasis across the country.

Mullaitivu and Kilinochchi districts were highly affected by the civil conflict which lasted in Sri Lanka for over three decades, resulting in a significant displacement of inhabitants from the two areas. At the end of the civil conflict in 2009, inhabitants who had fled to South India started to return to their hometowns. It is assumed that these individuals and workers who returned to Sri Lanka from India have acted as a reservoir of infection[14]. Sand flies breed in moist soil rich in minerals and organic matter[15–16]. Seasonal irrigation systems, agricultural lands and animal husbandry create major breeding habitats for sand flies in Sri Lanka[17]. The development programmes, construction activities, newly established irrigation systems, agricultural lands and animal farms may have created environmental conditions suitable for sand fly breeding in these areas, further facilitating the transmission of the disease.

The control of leishmaniasis requires extensive knowledge of all the aspects regarding parasites, vectors and associated socio-demographic risk factors[5]. This special focus for leishmaniasis transmission has been characterized previously. However, molecular level comparison of the parasites isolated from this focus with other isolates in Sri Lanka is not yet been carried out to identify any potential differences. Therefore, the present study was conducted to compare the DNA sequences of parasites isolated from the northern districts with parasites isolated from other foci in Sri Lanka and other countries to detect the possible similarities or/and differences.

2. Subjects and methods

2.1. Ethics approval

The study was approved by Ethics Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka (P/94/02/2019).

2.2. Study area

Both Mullaitivu and Kilinochchi districts are located in the Northern Province (Figure 1). These areas are relatively dry with <1 250 mm rainfall annually. The average annual temperature ranges from 28 to 30 ℃, with the lowest temperature in January and highest in May[18]. The dry season extends from February to September, while the wet season is from October to January.

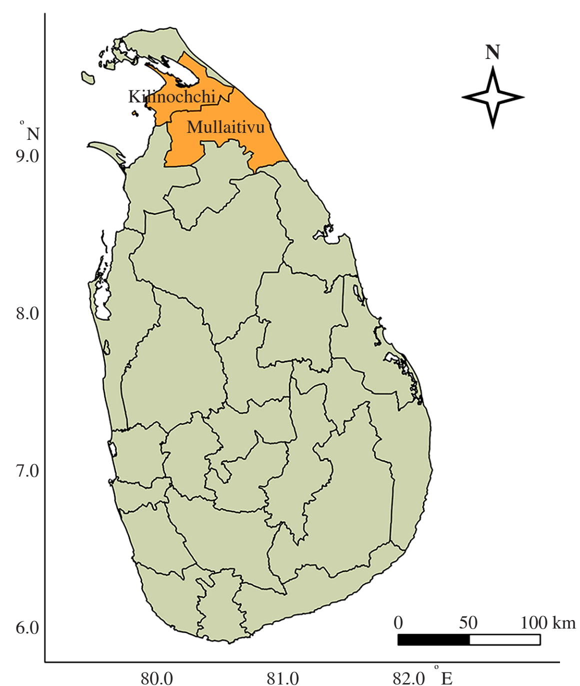

Figure 1. Geographical location of Mullaitivu and Kilinochchi districts in Sri Lanka.

2.3. Sample collection and microscopic examination

The military personnel in the camps situated in the two districts were screened and individuals with lesions suspected of causing by leishmaniasis were asked to report to the Army Hospital, Narahenpita, Sri Lanka. The study was carried out from November 2018 to March 2020 with 76 individuals being referred for confirmation of diagnosis. Each individuals' biological samples were taken using lesion aspiration, lesion scraping and tissue impression techniques following standard procedures recommended by the WHO and the Centers for Disease Control and Prevention

in the US[19]. The samples taken from each biological sampling technique were fixed on the slides with methanol, stained with Giemsa and observed by a compound light microscope under 100× magnification for the presence of amastigote forms. The microscopic observations were repeated with three observers to minimize personal errors during the diagnosis. The remaining portion of samples from the detection were transferred safely to the molecular diagnostic laboratory at the Department of Parasitology, Faculty of Medicine, University of Kelaniya, Sri Lanka for molecular analysis and stored under -20 ℃ until used for experiments.

2.4. Molecular detection of parasites

2.4.1. DNA extraction

The stored TIS samples were transferred to 1.5 mL tubes, separately. The genomic DNA was extracted using DNEasy blood tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

2.4.2. Genus specific amplification of the parasites

The genus-specific primers (F: GGTTCCTTTCCTGATTTACG; R: GGCCGGTAAAGGCCGAATAG) targeting small subunit ribosomal RNA (603 bp) of *Leishmania* spp. were used for PCR assays[20]. The amplifications were carried out in the 20 μL reaction mixture which contained 1 μL of DNA product as the template, 2 μL of 10× CoralLoad concentrate buffer (QIAGEN) with 15 mM $MgCl_2$ and loading dye, 10 μL of 2× HotStarTaq Plus Master mix (QIAGEN) with dNTP and DNA polymerase, 0.5 μL of 50 μM forward and reverse primers, and 6.6 μL of PCR water. Amplification was performed in a thermal cycler (Life ECO, Bioer) programmed for an initial denaturation step of 95 ℃ for 5 min and 40 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 50 ℃ for 30 seconds, and extension at 72 ℃ for 30 seconds. Nuclease-free, PCR grade water was used as the negative control and DNA isolated from *L. donovani* was used as the positive control.

2.4.3. Agarose gel electrophoresis

Agarose powder (Agarose S) was used for the preparation of gels with 1× TAE. The 2% agarose gels stained with ethidium bromide (0.5 μg/mL) were prepared. A volume of 4 μL of the amplified PCR products was loaded with Promega 100 bp lambda marker and gel electrophoresis was carried out at 100 V for 25 minutes to get a good separation in the amplified products. Following the gel electrophoresis, the migrated DNA was visualized and photographed under UV illumination.

2.5. DNA sequencing and confirmation of species

The samples confirmed for *Leishmania* infections were selected and they were amplified using *L. donovani* specific primers. Oligonucleotide primers F: AAATCGGCTCCGAGGCGGGAAAC; R: GGTACACTCTATCAGTAGCAC) targeting kinetoplast mini-circle sequence (792 bp) of *L. donovani* were used for PCR assays[21]. The amplifications were conducted using 20 μL of the solution containing, 1 μL of DNA product as the template, 2 μL of CoralLoad Buffer (QIAGEN) with 15 mM $MgCl_2$ and loading dye, 1.6 μL of dNTP mixture with 0.2 mM from each nucleotide, 0.06 μL of 0.3 μM forward and reverse primers, 0.41 μL of 2.5 U MightyAmp DNA Polymerase (Takara, Japan) and 15.18 μL of PCR water. Amplification was performed in a thermal cycler (Life ECO, Bioer) programmed for an initial denaturation step of 94 ℃ for 5 min and 40 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 50 ℃ for 60 seconds, and extension at 72 ℃ for 24 seconds. Nuclease-free, PCR grade water was used as the negative control and DNA isolated from *L. donovani* was used as the positive control.

The amplified products were visualized on agarose gel using the same methods described above to confirm the amplification. The PCR amplicons were purified using QIAquick PCR Purification Kit (QIAGEN) and purified products were sent to Macrogen, South Korea (Macrogen Inc., 1001, 254 Beotkkot-ro, Geumcheon-gu, Seoul, Republic of Korea) for sequencing by Sanger method. Chromatograms were analysed using BioEdit sequence alignment editor v7.0.9 software. The database search for homologous sequences was performed by submitting kinetoplast mini-circle sequences to the Basic Local Alignment Search Tool nucleotide server of the National Centre for Biotechnology Information (NCBI, USA).

2.6. Genetic diversity assessment and phylogenetic analysis

The consensus sequences were generated for each specimen by editing the obtained chromatograms using BioEdit 7.2 (Informer Technologies, Inc.). The DNA sequences were aligned with the Clustal W tool[22] of Molecular Evolutionary Genetics Analysis 10.0 (Pennsylvania State University)[23]. The sequences resulting from multiple alignment each sequence was compared with other mitochondrial genomes using the BLAST (NCBI, USA) program to confirm the species or genus level identity.

The DnaSP6 program[24] was used to obtain descriptive information on the sequences, such as the number of polymorphic sites and haplotypes per species. Intraspecies divergences of nucleotide sequence and composition were calculated using the Kimura 2-Parameter model[25]. A dendrogram was constructed by the neighbour-joining method after multiple sequence alignment[26] according to the genetic distances calculated *via* different evolutionary models. Branch support for NJ was calculated using the bootstrapping method with 1 000 replicates. The topology of the resulting phylogenetic tree was used to evaluate the species monopoly.

3. Results

3.1. Microscopical and molecular diagnosis

Samples were collected from 76 individuals. In 45 (59.2%) of the samples, *L. amastigotes* were observed by microscopy, 33 (40.8%) were detected positive by *Leishmania* genus specific PCR (Figure 2) and the subsequent *L. donovani* species specific PCR, which was done to get amplicons for sequencing, detected only 23 (30.3%) positives (Figure 3). Among them, a total of 17 isolates resulted in clear DNA sequences, which are used for sequence analysis.

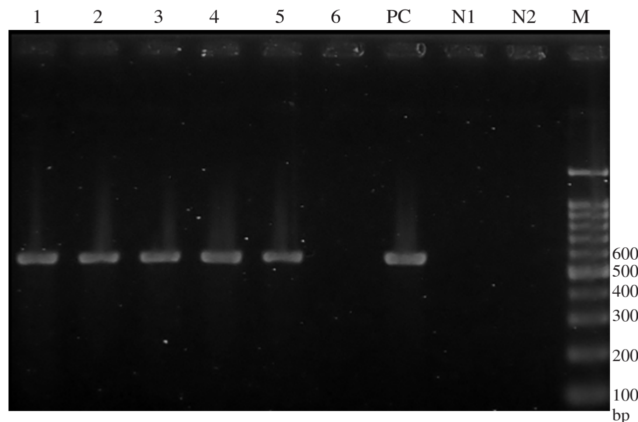

Figure 2. Results of gel electrophoresis of patient samples amplified with primers targeting small subunit ribosomal RNA of *Leishmania* spp. using *Leishmania* genus specific primers. Lane 1-6: Samples collected during the current study, Lane 7 (PC): Positive control (*Leishmania donovani* DNA), Lane 8 (N1): Negative control 1 (Previously negative patient sample), Lane 9 (N2): Negative control 2 (Nuclease free PCR water), Lane 10 (M): DNA molecular weight ladder, bp.

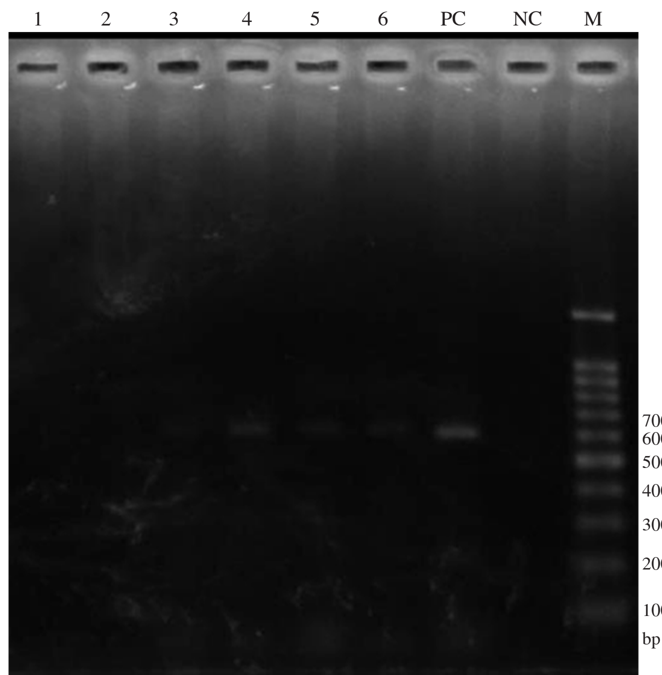

Figure 3. Results of gel electrophoresis of patient samples amplified with primers targeting kinetoplast minicircle sequences of *Leishmania donovani*. Lane 1-6: Samples collected during the current study, Lane 7 (PC): Positive control (*Leishmania donovani* DNA), Lane 8 (NC): Negative control (Nuclease free PCR water), Lane 9 (M): DNA molecular ladder, bp.

3.2. Molecular characterization

3.2.1. Nucleotide composition of L. donovani kinetoplast mini–circle DNA

The sequences obtained varied from 650 bp to 672 bp by direct sequencing. No deletions, insertions or stop codons were found, indicating that all sequences constituted functional mitochondrial products. The majority of the nucleotides consisted of adenine (A), which contributed to 35.6% of the total nucleotides, followed by cytosine (C) and thymine (T), which contributed to 33.5% and 21.2% of the total nucleotides, respectively. The guanine content was the lowest with only 9.7% relative abundance. Thus, in the sequence AT (adenine and thyamine) content is higher (mean=56.8%) relative to the GC (guanine and cytosine) content (mean=43.2%).

3.3.2. Intra–population variations

The mean genetic distance calculated with the Kimura-2-Parameter model was 0.01 for the sequences obtained during this study. The multiple alignments of nucleotides indicated the presence of only seven polymorphic sites. Three of them were singleton sites and four correspond to phylogenetically informative sites, distributed along the alignment. Only one haplotype was observed for all the sequences analyzed.

3.3.3. Phylogenetic analysis

The dendrograms generated by the neighbour-joining method (Figure 4) indicate that all the specimens collected during the current study clustered together. Interestingly, the strain isolated from Nepal and the strain L20 from Mirigama (Gampaha district, Western province) clustered with the current sequences. However, these sequences differed significantly from the strains isolated from India and Eastern Africa.

4. Discussion

This study was carried out to determine the diversity of *L. donovani* parasites causing cutaneous lesions among a cluster of military personnel deployed in the Mullaitivu and Kilinochchi districts of Sri Lanka using the kinetoplast minicircle sequence. Furthermore, the sequences were compared with those collected from other areas in Sri Lanka and its neighbouring countries. The results indicate a lower diversity of parasites among the cluster in this study. It clustered together with sequences from Nepal and the western parts of Sri Lanka (Mirigama), while Indian and Eastern African strains clustered as sister groups to this cluster. This indicates that the *Leishmania* parasites present in these areas were probably not introduced from India or Africa. It is likely to have a different route of introduction, where the source is shared with Nepal.

Although the complete suspected patients were 76, only 45

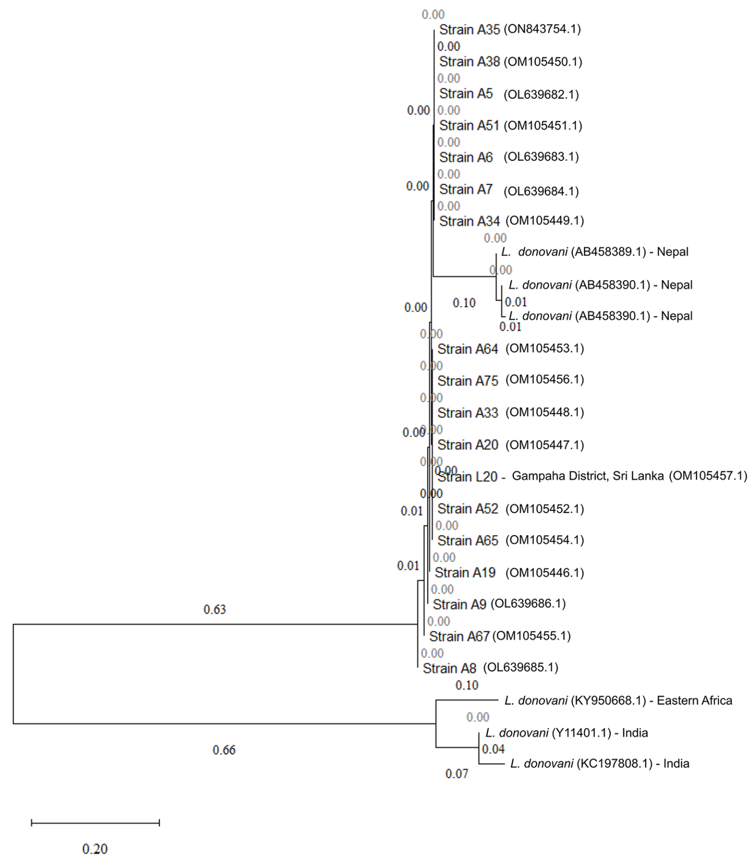

Figure 4. The dendrogram constructed for the sequences form the current study and kinetoplast minicircle sequences from other geographical areas extracted from GenBank database.

showed positive results upon microscopic examination, and only 33 patients showed positive results. Furthermore, the DNA extraction was carried out using a Spin-Column procedure to remove all the contaminants in the sample in three steps. The cycling conditions were optimized using standard optimization procedures to ensure that there are no issues with the PCR protocol. Therefore, differences among techniques could be attributed to the lower sensitivity of the genus-specific PCR at lower infection rates in some patients, rather than false positives from microscopic diagnoses. However, the PCR using *L. donovani* specific primers indicated positive results only for 23 samples. This may be a result of the lower sensitivity of these primers, which has been demonstrated in previous studies as well[27]. Therefore, more specific primers for Sri Lankan *L. donovani* strains would facilitate more extensive genetic diversity studies.

The analyzed sequences varied in length due to species differences were also observed in other studies[28]. The nucleotide composition of the kinetoplast minicircle region is slightly AT rich (the combined A and T content is higher). However, the composition is not strongly biased towards AT or GC. This is generally different from the results observed for other *Leishmania* species from South America[29]. The genetic distance calculated using the Kimura-2-Parameter model indicates that the genetic distance is very low (0.01). The lower intra-population diversity was also observed through the number of haplotypes and polymorphic sites. The geographical genetic variation of Sri Lanka *L. donovani* has been observed previously[30]. This study is also supportive of the sequences collected from Sri Lanka not being clustered apart. However, in the current study we have an additional finding by having a similarity to strains from Nepal. The lower intrapopulation genetic diversity is also evident in the neighbour-joining tree as all the specimens clustered together.

Interestingly, a single sample isolated from a patient in the Mirigama area in Sri Lanka and a sample from Nepal clustered together with the current population. This observation remains inconclusive until further studies with a much higher sample size across a wider geographical area are done. However, the Mirigama area is a newly established focus for cutaneous leishmaniasis in Western Sri Lanka[31]. The results of the current study can be used to speculate that the infection in the Mirigama area originated from the current study areas, *via* military force members. This may be used as evidence to facilitate further studies on the newly established focus in the Mirigama area of Sri Lanka. This also emphasizes the importance of active screening and treatment of military individuals before leaving deployed areas to prevent further spread of the disease into currently non-endemic areas. The results show that the

genetic diversity was very low among the isolates. The similarity between Sri Lankan and Nepal strains indicates a possibility of a shared point of origin, which needs extensive empirical evidence to confirm.

The sample size used for DNA sequencing was lower due to the funding constraints. The lower sensitivity of the sequencing primer is another caveat in this study that further reduced the sample size in sequencing analysis. However, despite the limitations, this provides valuable information to conduct more studies highlighting the importance of further studies focusing on the diversity of *L. donovani* in Sri Lanka.

Conflict of interest statement

All authors declared no financial or other relationship that might lead to any conflict of interest.

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Authors' contributions

NG and DF contributed to the study conception and design. SS and NP conducted the field surveys and sample collection. implemented the study. TW and NG contributed to the molecular assays, TW and WR contributed for descriptive DNA sequence analysis, phylogenetic analysis and interpretation. NG, DF and TW contributed in preparation of the manuscript. All authors have reviewed the manuscript and consent was given to publish.

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