Author’s response to reviews

Title: Genetic diversity of Leishmania donovani that causes cutaneous leishmaniasis in Sri Lanka: a cross sectional study with regional comparisons

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Author’s response to reviews:

Reviewer 01:

Ibrahim Hassan Babikir, PhD (Reviewer 1): Title: Genetic diversity of Leishmania donovani that causes cutaneous leishmaniasis in Sri Lanka

Authors: Udeshika Lakmini Kariyawasam, BSc; Angamuthu Selvapandiyan, PhD; Keshav Rai, MSc; Tasaduq Hussain Wani, MSc; Kavita Ahuja, MSc; Mizra Adil Beg; Hasitha Upendra
Dear authors,

First of all, it was a pleasure for me to read your article. It is a very interesting report. After reading the manuscript the following raised points represent a suggestion to add to the manuscript:

**Background**

Q1. This section looks good and well written; however, there are too much cited references. It would be enough when needed to cite 2-3 references for your documentation.

R1. Authors agree with the reviewer and the number of references has been reduced as suggested.

Q2. - Materials and Methods (Page 4, line 89) please, correct to "Methods" or "Patients and Methods". As you deal with patients (human) in your study not only materials.

R2. The subheading ‘Materials and Methods’ was used as per journal guidelines to authors hence, it has been retained.

Q3. What was the type of the study?

R3. The study was done as a case only cross-sectional study. The relevant information is now given to page 04, line 92.

Q4. I think it will be more informative when you start this section with some information regarding your patients, study settings and population. Sample size and sample technique.

R4. The relevant details are now given on Page 04 lines 92-101.
Q5. Methods (Page 4, line 105) what type of in-vitro culture has been used, incubation and interpretation of results.

R5. The relevant information is now given on page 05, lines 112-117 as given below.

Diagnosis was confirmed in all study subjects by the demonstration of Leishmania parasites under oil immersion (X1000) microscopy of Giemsa-stained smears of lesion aspirates or slit-skin scrapings of CL patients and bone marrow aspirates of VL patients, either directly or following in-vitro culture. Parasites were grown in M199 medium (Gibco, Invitrogen,USA) supplemented with 15% heat inactivated FBS (Gibco, Invitrogen,USA) with Penicillin-Streptomycin (Sigma-Aldrich, USA) and the cultures were maintained at 26oC.

Q6. Some parts of your results look like discussion, please state only your results and move these comments to the discussion section. e.g.:

- line 190 (indicating the highly diverse nature of the Indian isolates)
- line 193 (that can be considered as an indication for …)
- line 199 (suggestive of …)
- line 209 (which might demonstrate the effects of …)

R6. Corrections have been made accordingly and relevant statements have been moved to page 10, lines 254-263 in the discussion section as given below.

However, the highest haplotype diversity and nucleotide diversity were observed for the Indian parasite sequences indicating the highly diverse nature of the Indian isolates when compared to the Sri Lankan and Nepalese counterparts.

Indeed, a recent population expansion or an existence of an excess number of alleles often observed in genetic hitch-hiking was evident from Fu’s F value test for Sri Lankan sequences, where a negative Tajima’s D test implies purifying selection. In contrast, positive F value observed in both Nepal and Indian sequences are an indication of deficient alleles, suggestive of a recent population bottleneck, and a positive Tajima’s D points towards a balancing selection.

Q7. The association between L. donovani in Sri Lanka and Nepal in the genetic diversity, population structure, and phylogenetic relationships is not fully understood.
High levels of haplotype and nucleotide diversity were observed in Sri Lankan, Nepalese and Indian sequences. However, the highest haplotype diversity and nucleotide diversity were observed for the Indian parasite sequences indicating the highly diverse nature of the Indian isolates when compared to the Sri Lankan and Nepalese counterparts.

Indeed, a recent population expansion or an existence of an excess number of alleles often observed in genetic hitch-hiking was evident from Fu’s Fs test for Sri Lankan sequences, where a negative Tajima’s D test implies purifying selection. In contrast, positive Fs value observed in both Nepal and Indian sequences are an indication of deficient alleles, suggestive of a recent population bottleneck, and a positive Tajima’s D points towards a balancing selection.

This study confirms that, sequences of the Sri Lankan, Indian (VL and PKDL) and Nepalese (VL and PKDL) parasites, clustered within the L. donovani complex, while CL causing parasites obtained from skin lesions of Indian patients, formed a sister cluster with L. tropica. None of the Sri Lankan CL and VL parasites were found to be associated with L. infantum, a closely related subspecies within the L. donovani complex [5, 59].

Q8. The genetic differentiation between CL-DR and CL-S to antimonial drug resistance is not clear.

R8. There were three distinct sub-clusters seen within the Sri Lankan group, comprising sequences from i) visceral leishmaniasis (VL) patients, ii) cutaneous leishmaniasis patients (CL-S) and iii) CL cases that showed poor response to the standard drug SSG (CL-DR). However, no specific clustering was observed in relation to their geographical origins within Sri Lanka. The phylogenetic differentiation within CL-S, CL-DR and VL may be due to the genetic mutations/variations associated with these three clinical groups (viz. VL, uncomplicated CL and CL with poor response to antimonial drugs, which is currently used as the first line of treatment in Sri Lanka) [64]. These details have been presented more clearly now on page 12 lines 284-291.

Q9. The role of genetic diversity of L. donovani in Sri Lanka to their antimonial sensitivity is not clear.

R9. Our findings point towards genetic mutations/variations associated with the three clinical groups; viz. VL, uncomplicated CL and CL with poor response to antimonial drugs, which is currently used as the first line of treatment in Sri Lanka. It is possible that multiple genetic strains of L. donovani exist within Sri Lanka, as reported from other geographic regions in the world. Since Sri Lanka is an island close to India, the parasites may have also been in circulation
within the country long enough to adopt such independent genetic characteristics. Sri Lankan Leishmania parasites may have co-evolved with the ecological settings along with its vector and possible reservoir hosts. However, this remains as a mere speculation with precise origin of the Sri Lankan L. donovani parasites being debatable and not addressed.

Q10. (Page 11, line 271) please correct, (There were 3), any number less than 10 please write in alphabet. (There were three) (line 275) the 3 clinical groups (the three clinical groups).

R10. The relevant corrections have been introduced to Page 12 lines 284 and 289.

Q11. Please try to well describe the above points in the discussion section before add them to the conclusion section.

R11. Conclusion section has been changed accordingly on page 13 lines 320-328.

This study reveals for the first time, the extent of genetic diversity of L. donovani in Sri Lanka and a clear clustering nature of local parasites according to their antimonial sensitivity and tissue localization. Furthermore, the genetic differentiation between CL-DR and CL-S suggests a likely genetic basis for poor responsiveness to antimonial drugs and possible drug resistance. Overall, parasite genetic variations associated with specific functional characteristics are likely to influence the disease phenotype, which is of clinical relevance and significance. Studies are underway to use whole genome sequence information with finer mapping, to investigate the parasite genetic variations associated with distinct clinical manifestations.

Reviewer 02:

Mohammed Abdelgadir Abdelmahmoud, MSc (Reviewer 2): Dear Authors,

The manuscript submitted above covers very important problem, like the etiology of leishmaniasis in tropical and sub-tropical countries like Sri Lanka, India as well as Nepal, which have high burden and clinically poor prognosis in such regions. Although, there were genetic variations and modification to the causative agents recently, so I recommend the suggested changes below;

Q1- Suggested changes of the manuscript title is needed to;

- Genetic Diversity and Geographical Distribution of L.donovani in Srilanka, Comparing to other Strains from India and Nepal: a cross sectional study.
R1. The manuscript of the title has been changed to Genetic diversity of Leishmania donovani that causes cutaneous leishmaniasis in Sri Lanka: a cross sectional study with regional comparisons

Q2- Would you kindly please, State the study design clearly in manuscript title and in material and methods section, eg; This was cross sectional study? and when the study was conducted or carried out? and how long so far?

R2. This has been now introduced to page 04 lines 92-97 as shown below.

A case only cross-sectional study was carried out to represent all the administrative provinces of the country over one year period (Fig. 1). Study participants were selected from above administrative provinces of the country who were laboratory-confirmed as either CL (n=34) or VL (n=4). Patients with other skin diseases and who were negative for the laboratory diagnosis were excluded. Within the CL group, there were six patients (n=6), who had a history of poor response to the routine local treatment of intra-lesional sodium stibogluconate (IL-SSG).

Q3- How did you choose the study participants? and how did select or control them? Is there any exclusion criteria? Like Muco-subcutaneous Leishmaniasis? How did you justify them? would you Give eligibility criteria, to avoid selection bias.

R3. Study participants who were laboratory-confirmed as either CL (n=34) or VL (n=4) were selected from above administrative provinces of the country. This is now mentioned in page 04 lines 93-95.

Q4- Is there any variables rather than study regions? Like the study participants? The clinically significance? Age, Sex? Type and Genus of vectors? And were they affect the outcome of the study objective?

Since this is an observational genetic study a minimum number of 30 samples were selected from each geographic area. However, the age, sex and type and genus of the vector were not considered.

Q5- Some of figures and tables were repeated, so delete one.

R5. Since figures and tables demonstrate different indices and different types of analysis, they have been retained.
Q6- About statistical analysis, how did you analyze data? what was version did you choose?

R6. This information is mentioned on pages 06 lines 142-155 and 07, lines 157-169 and 171-182 under genetic analysis section.

Q7- About Ethical consideration, would you kindly please include ethical approval letter number?

R7. The relevant information is now given on page 14 line 334 under Ethical consideration section.