Title: Evaluation of PCR procedures for detecting and quantifying Leishmania donovani DNA in large numbers of dried human blood samples from a visceral leishmaniasis focus in Northern Ethiopia.

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Author's response to reviews: see over
The Editor,
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Dear Editor,

The following are our responses to the Reviewers' comments (in WORD track changes mode):

**Major revisions**

1. To consider to put the Hailu paper and the current one together in order to improve the clarity of the limitations of this research for the journal readers.

I have discussed this suggestion with my co-authors. Although we agree that the reviewer has justification for his reasoning, we will not be able to conform for a variety of reasons. Our initial decision to split the topic into two papers stemmed from the fact that our next paper, will depend heavily on the findings we present here. In addition, the Hailu paper will only be submitted in about 6 months (assuming all goes well) and in it we will not have the space to present and discuss our RT-PCR data in an appropriate fashion.

2. To review the conclusion because it is not possible with the available data to make a statement on the probability of developing symptomatic disease for individuals with a positive molecular diagnostic test. The conclusion should be restricted to the lack of reliability of the test and the choice of DNA extraction procedure. The required changes were made

3. To treat data with properly selected statistical tests to evaluate reliability. Crude data on agreement are not enough and should be adjusted by the expected agreement which would occur by chance. To clarify the findings in Table 2 [was Table 1 in the original submission] and explain samples sizes, an additional column was added giving the total number of samples in each qRT-PCR category in the initial cohort study (column# 2 in Table 2). The data itself is not amenable to statistical analysis since only one repeat test per sample was performed. We agree that if we were able to retest each sample several times, the results would have been more significant (and amenable to statistical analyses). However, we do not have sufficient material to
perform repeat DNA extractions so are unable to comply with this suggestion. We would like to reiterate our opinion that the percentage of identical results derived by repeating [albeit only once] RT-PCR analyses for a significant sample, is highly indicative of the dependability of the original results within each RT-PCR category. This level of dependability is "all" we are inferring from this assay. Such a "limited" inference does not, to our view, require any further validation. A few sentences were added to the manuscript to clarify these points (Page 12 last paragraph).

Minor essential revisions - To discuss the role of *L. major* asymptomatic infections in the region. Done. Paragraph 3 of discussion

Reviewer 2
1. I realize that the focus of the paper is on the methods but still more detail needs to be presented related to the cohort of subjects to be able to understand the context of the study. Were these subjects symptomatic or did they have signs or symptoms of VL? Were they assessed prospectively in the community or were they sampled because they sought care at a health centre. Were the lab investigators blinded to the clinical status of the subject? Done - Paragraph 2 (Samples) of the material and methods section.

2. The leading sentence in para. 3, pg 3 that indicates only 30-50% sensitivity of parasitological and serological diagnostic methods is incorrect (and inconsistent with the data discussed in the subsequent sentences). Corrected, thank you for noticing this contradiction.

3. What preventative and quality control measures were taken to ensure that positive PCR results were not due to amplification of contaminating parasite DNA?
   a. DNA extractions were performed in a room into which, live cultured *Leishmania* are never introduced
   b. The paper punches used were washed and sterilized using bleach between samples
   c. Every batch of samples extracted included a negative control (no DNA) and several positive controls with known numbers of parasites (for the standard curve).
   d. Only disposable plastic (tubes, and pipette tips) ware was used

4. Given the differences in protocol from the original study (cited as reference 12) where the JW11 and JW12 primers were used, representative melting curves should be shown to confirm that there were not non-specific products of amplification that would be quantified by the SYBR green detection method. We used the primers JW11 and JW12 to detect *Leishmania* k-DNA not for species identification. We did not use the primers JW13 and JW14 which amplify a longer segment of the minicircle DNA (REF 12). Although JW13 and JW14 can differentiate between *Leishmania* species by HRM, they are less sensitive for detection [than JW11 and JW12]
which was our primary goal. Identification of *Leishmania* species was performed based on the DNA sequencing of the ITS1 amplicon. Comments clarifying these points were added to the discussion (Pages 10-11). See also table 1 added to this revised version

5. The sequence results should be presented more thoroughly. How large a fragment was sequenced? How different were the putative *L. major* sequences from the *L. donovani* sequence (perhaps they should be shown), and how much variability is there in the ITS1 sequence within and across the *Leishmania* spp.? Which polymerase was used for the PCR amplification and was its expected level of fidelity sufficient to ensure the sequence differences were not introduced in the amplification? Was *L. major* cultured in the laboratory where the processing and PCR amplification of the dried blood samples took place (i.e. could it be a contaminant that led to erroneous amplification of *L. major* DNA in the two dried blood samples)? The ITS1 DNA sequences of the different *Leishmania* species are well characterized and available in GenBank. Many authors have submitted these sequences from different isolates all over the world. There is a significant sequence difference [exceeding 10%] between *L. major* and *L. donovani*. The ITS sequences obtained from our samples showed complete homology with either *L. donovani* (19 samples), or *L. major* (2 samples). The amplified ITS sequence was 330 bp and for such short sequence the possible introduced amplification errors caused by DNA polymerase are minimal and would not affect the fidelity of species identification. See also table 1 added to this revised version

As for the possibility of contamination: In our department many *Leishmania* species are routinely maintained. However, all the PCR work was done in a clean room where cultures are not introduced and inside a designated hood. The ITS PCRs are comprehensively controlled with 2-3 negative reactions. A statement to that effect was added to the manuscript (Page 10-11).

6. Line 4, para 3, pg 3 refers to three tests but only two are listed. corrected
7. The reference to the lower limit of detection in para. 2, pg 10 should indicate that it is per mL of blood. done
8. The last sentence in para. 2 on pg 11 raises the question of why the estimation of errors and rate of infection was not done. See response to comment 3 reviewer 1.
9. The manuscript would benefit from careful editing because there are several typographical errors and awkward sentences. done
10. The authors refer to the filter paper punches as 6 mm (presumably diameter) and r=3 mm which might be confusing. corrected and standardized
11. Are the bands at the bottom of the gels in Fig. 3 primer-dimer? Yes they are - statement added to the figure legend
Discretionary revisions:

12. The Results and Discussion sections could be combined.
We prefer to leave separate since this is essentially a technical paper and we want to ensure it remains as straightforward as possible to extract methodologies from it.

We would like to request that you adhere to the MIQE guidelines for reporting of this methodology. Done - additional details added according to these guidelines. Storage conditions (page 5), primer sequences. The number of template controls (page 6). Table 1 added with details of primers and amplicon sizes added and referred to in the text.