Author's response to reviews

Title: Sero-prevalence of Leishmania donovani infection in labour migrants and entomological risk factors in extra-domestic habitats of Kafta-Humera lowlands - kala-azar endemic areas in the northwest Ethiopia

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Author's response to reviews: see over
The Biomed Central Editorial Team

We would like to thank the reviewers for their excellent comments. The suggestions were highly appreciated and filled the missing parts in the MS. We tried to correct all the comments as we were advised.

Authors’ response for **Reviewer 1** (2143076614155923) (Luigi Gradoni)

**Title:** Sero-prevalence of Leishmania donovani in Labour Migrants and Entomological Risk Factors in Extra-domestic Habitats of Kafta-Humera Lowlands - Kala-azar Endemic Areas in the Northwest Ethiopia

**General**

1. Kala-azar is the name of a clinical disease resulting from the infection by Leishmania donovani. Because serological data collected were not associated with disease, the definition of “kala-azar infection” is not appropriate and should be replaced by “L.donovani infections” or “seropositivity to L.donovani” throughout the text.

   - We corrected the title by adding infection in addition to avoiding unnecessary capital letter in the title on Page 1 and Line 1-3 “**Sero-prevalence of Leishmania donovani infection in labour migrants and entomological risk factors in extra-domestic habitats of Kafta-Humera lowlands - kala-azar endemic areas in the northwest Ethiopia**”

   - “**Leishmania donovani infection**” has been used properly instead of “kala-azar” throughout the text.

2. For the non-specialist readers, introductory notes should include some concepts about the prevalence of asymptomatic L.donovani infections, the reciprocal value of serology versus leishmanin skin test to detect active/past infections, and the incubation period for full-blown disease.

   - Page 3-4 line 65-79. Agent mentioned including infection does not mean disease condition plus pros and cons of the different diagnostics.

   “In East Africa and the Indian subcontinent, VL is caused by the *L. donovani* complex, unlike Europe, North Africa and Latin America where the agent is *L. infantum* [13-14]. Ethiopia has second largest number of annual VL cases (4000–7000) in Africa, next to Sudan [15]. In endemic areas of VL, *L. donovani* infection does not necessarily mean clinical illness (2). Due to the reason not well understood, *L. donovani* infections remain asymptomatic in certain subjects and cause a lethal disease in others. The ratio of incident asymptomatic infections to incident
clinical cases in Ethiopia is 5.6:1 (4) compared to the range from 1:2.6 to 11:1 in Sudan (3) and 4:1 in Kenya (5). Leishmanin skin test (LST) and direct agglutination test (DAT) are among the immunoassays widely used in kala-azar endemic areas to determine *L. donovani* infection rates for epidemiological studies (3, 4, 5). But, kala-azar patients will not show LST positive result until 3-6 months incubation phase and less useful during VL outbreaks[16]. Furthermore, asymptomatic subjects may have to be repeatedly exposed to the parasite before they undergo LST conversion [5]. Of the several serological tests, DAT appears to be a simple and economical test with high sensitivity and specificity [17]. However, it cannot differentiate among past kala-azar, subclinical infection, and active disease [18].”

**Specific**

1. Line 31: unless otherwise proven, the VL entity of this area should be defined as “anthroponotic” and not “zoonotic”.

   Done. The word zoonotic has been deleted

2. Line 34: “which may affect”

   Done

3. Line 35: “Methods and Results” (actually, they are mostly results)
   - The abstracted structured into
     - Background:
     - Methods:
     - Results:
     - Conclusions:

4. Line 44: the figure of 116 seroreactive individuals is not shown later in the main body text.

   **Corrected now in page 10 line 210-215**

   Overall 359 blood samples were obtained from labour migrants, of these 243 (67.7%) showed no reaction for direct agglutination test while the other 116 (32.3%) were seroreactives.

5. Lines 57-62: that the cause of kala-azar in the study area is *L. donovani*, should at least be mentioned in this paragraph

   **Corrected in page 3 line 65-67**

   In East Africa and the Indian subcontinent, VL is caused by the *L. donovani* complex, unlike Europe, North Africa and Latin America where the agent is *L. infantum* [13-14].


   **Corrected as** Fuller et al., 1976 on page 4 line 85 and referenced page 17 line 354 **(Reference 20)**

7. Line 72: no data on “seroreaction” were available (one needs to calculate them)
Corrected on page 10 line 213-220

**Sero-prevalence of Leishmania donovani infection**

Overall 359 blood samples were obtained from labour migrants, of these 243 (67.7%) showed no reaction for direct agglutination test while the other 116 (32.3%) were seroreactives. In this study, titers from 1:200 to <1:800 were considered simply as reactive. Thus, the number of DAT positive study subjects (42 (11.7%)) were those with DAT results greater or equal to 1:800 titers. The 39 Individuals (10.9%) with 1:800 - 1:6400 titers were most probably has *L. donovani* infections but not kala-azar. Only 3 titers (0.8%) were found to be greater than 6400(12800) which have had high risk for development kala-azar.

8. Line 106, Population dynamics: the periodicity of collections during a year should be reported
Corrected on page 9 line 191-219 more emphasis has been given to periodicity and a figure also added.

**Population dynamics of Phlebotomus orientalis**

A total of 7443 (1748 female and 5695 male) *P. orientalis* was collected from agricultural fields (859 female & 2593 male; 3452 total) and thickets of *A. seyal* (889 female & 3102 male; 3991 total) in Adebay, Rawiyal, Baeker, Gelanzeraf and Mysegen – Mehari using 461 CDC-light trap nights in addition to *P. papatasi* (158), *P. duboscqi* (42), *P. bergeroti* (11), *P. rodhaini* (24) and *Sergentomyia* spp (91, 292). The agricultural fields and thickets of *A. seyal* have similar sand fly fauna and both habitats are characterized big cracks during November – May dry season. But, there were statistically significant differences (P=000) among the mean densities of *P. orientalis* for the two habitats in different seasons and months. The number of *P. orientalis* is slightly higher in the thickets of *A. seyal* (Table 1). Following the heaviest rain in August, the population of *P. orientalis* drops almost to zero. The number of *P. orientalis* remained low until January, the time for the begging of high temperature and cracking black soil (Figure 1). The mean density of *P. orientalis* during the November – May dry season was 11.39 ± 22.98 in agricultural fields which was lower than 25.30 ± 40.06 in thickets of *A. seyal*. March is the month with the highest overall mean number of *P. orientalis* in thickets of *Acacia seyal* (46.88 ± 71.46). Significantly, high mean number of *P. orientalis* also collected from agricultural fields in April (43.89 ±61.57). The lower mean number of *P. orientalis* in agricultural fields (0.03±0.08) and thickets of *A. seyal* (1.97±1.81) during June -August weeding season might have also been attributed to the strong wind, especially during May and June (Figure 1). Very low mean number of *P. orientalis* was obtained during September - October harvest season in agricultural fields (0.66 ± 0.65) and thickets of *Acacia seyal* (3.92 ± 7.71). Heavy rain, closing of black soil and cold weather might have played roles for reduction of *P. orientalis* during this time.
Figure 1. Population dynamics of *P. orientalis* in tickets of *Acacia seyal* (n=214) in Rawyian, Adebay, Baeker, Gelan Zeraf and Mysegen-Mehari during May, 2011 to June, 2012.

9. Line 117: jugular OR forearm veins? I guess that “jugular” is a mistake

Corrected on page 8 line 161

from the forearm veins

10. Line 123, DAT paragraph: the calculation of the cut-off dilution for *L. donovani* infection/reactivity should be reported, or relevant references provided.

Corrected on page 8 line 167-176

**Direct Agglutination Test (DAT)**

Serum samples were diluted in a dilution solution containing 0.9% NaCl solution, 0.2% (wt/vol) gelatin (Difco Laboratories, Detroit, Mich.), and 0.2 M 2-mercaptoethanol. A twofold dilution series of the sera was made, starting at a dilution of 1:100 until a maximum dilution of 1:12800. Prior to its use, aliquots of FD antigen (Royal Tropical institute *L. donovani* promastigote) (The Netherlands) were reconstituted in 5 ml of normal saline (0.9% [wt/vol] NaCl). Reconstituted antigen (50 ml) was added to each well of the microwell plate containing 50 ml of diluted serum. A 24-h incubation period at 18 to 20°C employed before the reading of the DAT. The DAT titers were grouped as negative (< 1:100), reactive (1:100 to <1: 800), most probably infected (1:800 - 1:6400) and infected and at risk of kala-azar development (> 6400 (12800).
11. Line 127: Royal Tropical Institute… of which country?

Corrected on page 8 line 171-172

…. (Royal Tropical institute L. donovani promastigote) (The Netherlands)

12. Line 177, Seroprevalence: seroprevalence results are not presented clearly. Once the cut-off for infection was defined in Methods, simply report number and percentage of individuals showing a DAT titer of: <100 (negative?); 100-799 (reactors but probably not infected?); 800-1599 (also reactors?); 1600-6400 (infected?); >6400 (12800) (infected at risk of kala-azar?)

Corrected in the method section as suggested on page 8

**Direct Agglutination Test (DAT)**

Serum samples were diluted in a dilution solution containing 0.9% NaCl solution, 0.2% (wt/vol) gelatin (Difco Laboratories, Detroit, Mich.), and 0.2 M 2-mercaptoethanol. A twofold dilution series of the sera was made, starting at a dilution of 1:100 until a maximum dilution of 1:12800. Prior to its use, aliquots of FD antigen (Royal Tropical institute L. donovani promastigote) (The Netherlands) were reconstituted in 5 ml of normal saline (0.9% [wt/vol] NaCl). Reconstituted antigen (50 ml) was added to each well of the microwell plate containing 50 ml of diluted serum. A 24-h incubation period at 18 to 20°C employed before the reading of the DAT. The DAT titers were grouped as negative (< 1:100), reactive (1:100 to <1: 800), most probably infected (1:800 - 1:6400) and infected and at risk of kala-azar development (> 6400 (12800)).

13. Line 179-180: this sentence should be moved to Methods. What is the meaning of “boarder”? Corrected:

use of the term boarder has been avoided

14. Line 182: were these high-titer positives followed-up for clinical signs of VL?

We have been communicating with them via telephone number they gave us. No risk for fewer individuals from Addis Ababa and Welita. For the individuals with DAT positive results including those with higher titers we informed them to go to the nearest treatment centers for check up in Axum Gondar or Humera when they got prolonged fever (other symptoms).

This issue mentioned in the ethics part on page 8 line 177-183.

15. Lines 234-236: this sentence is unclear, please re-phrase

- Done

**DISCRETIONARY REVISIONS**

1. Lines 36-41: the general trend from November through May, as compared with the June-July trend, should be reported first, then the March-April peaks can be mentioned. No need to write “+/- standard deviation” in the abstract.

- It has been corrected Use of standard deviation in the abstract has been avoided in the abstract

2. if labor migrants were diagnosed with VL in Humera hospitals, it means
that they have been infected early in the labor season as the disease developed before their return home. Are data available from VL diagnosed in Amhara health centers after their return?

- The incubation period of 2-6 months (Zijlstra et al., 1994) mentioned. It is possible for labour migrants to develop kala-azar while they are in Humera or Metema, where there are Humera hospital and Abdurafi Kala-azar treatment centers are found. But, most of them develop kala-azar after they returned to their home where they are treated in Gondar or Axum. As mentioned in the text the majority of migrant workers are originated from Amhara region. Gondar University Hospital Kala-azar treatment center admit a lot of kala-azar patients. To date the situation in Gondar, before X-MASS 2015 (in December, 2014), All beds in the center and the temporarily shelter 10x25m tent were occupied by the patients. In January 2015, almost all beds in the center were occupied by the Kala-azar patients.

3. Population dynamics: a graph showing the monthly density of P. orientalis could be helpful.

Study area (Page4; line 104-19) clearly described to show possible interaction between labour migrants and the vector. Complete information including Figure about the population dynamics of P. orientalis for 12 months in thickets A. seyal (the most important habitat of P. orientalis) was also included in the result section (Page8 line164-183).

“Results

Population dynamics of Phlebotomus orientalis

A total of 7,443 (1,748 female and 5,695 male) P. orientalis was collected from agricultural fields (859 female & 2,593 male; 3,452 total) and thickets of A. seyal (889 female & 3,102 male; 3,991 total) in Adebay, Rawiyal, Gelanzeraf and Mysegen – Mehari using 461 CDC-light trap nights in addition to P. papatasi (158), P. duboscqi, (42), P. bergeroti, (11), P. rodhaini (24) and Sergentomyia spp. (91, 292). The agricultural fields and thickets of A. seyal have similar sand fly fauna and both habitats are characterized by big cracks during November – May dry season. But, there were statistically significant differences (P=000) among the mean densities of P. orientalis for the two habitats in different seasons and months. The number of P. orientalis is slightly higher in the thickets of A. seyal (Table 1). Following the heaviest rain in August, the population of P. orientalis drops almost to zero. The number of P. orientalis remained low until
January, the time for the begging of high temperature and heavy cracking black soil (Figure 1).

The mean density of *P. orientalis* during the November – May dry season was 11.39 ± 22.98 in agricultural fields which was lower than 25.30 ± 40.06 in thickets of *A. seyal*. March is the month with the highest overall mean number of *P. orientalis* in thickets of *A. seyal* (46.88 ± 71.46). Significantly, high mean number of *P. orientalis* also collected from agricultural fields in April (43.89 ± 61.57). The lower mean number of *P. orientalis* in agricultural fields (0.03±0.08) and tickets of *A. seyal* (1.97±1.81) during June-August weeding season might have also been attributed to the strong wind, especially during May and June. Extremely low mean number of *P. orientalis* was obtained during September - October harvest season in agricultural fields (0.66 ± 0.65) and thickets of *A. seyal* (3.92 ± 7.71).

4. Line 170-171: please repeat here the month range for the weeding (June-July) and harvest seasons (September-October)

It has been clearly described in page 9 line 190 – 220.

The seasons and the movements of labour migrants into and out of the Metema-Humera lowlands are clearly stated (Method and result sections).

**Response to Comments to the Authors**

1. The authors try to explain ‘how and when’ the labour migrants are exposed to *L. donovani* infection. However, it does not become clear why the year when blood collection and sand fly collection were different; the blood samples were collected from the labour migrants during October and November, 2013, whereas the sand flies were collected from May 2011 to June 2012.

   - It has now corrected (justified) and included in the text on page on page 7 line 142-145.
   - Big farms in Mysegen-Mahari areas are the source of most of kala-azar patients. Just after we finished the study of population dynamics of the vector we tried to sample blood by going from one field to the other. But we could not cover Gelanzeraf and Mysegen Mahari farms due to the road. The road was out of use. We were forced to weight the next harvest season, after the road was reconstructed.

2. It does not become clear why the author assumed that the labour migrants exposed to *L. donovani* during staying in the study area? VL is reported in many areas of Ethiopia,
Meshesha Balkew, Teshome Gebre-Michael and Asrat Hailu have been working on leishmaniasis in Ethiopia for more than 30 years. Prof Asrat succeeded in establishing kala-azar centers in Gondar, Arbamich, Shiraro and Negele Borena. Meshesha Balkew, Teshome Gebre-Michael and others have been working on the vectors where cases have been reported. I, the first author of this MS, has been working on leishmaniasis for the last 10 years. Some time a situation of the same magnitude, could be expressed differently by different author. A real situation in Ethiopia has been described as follow.

CL is found in highlands while VL in lowlands. I found the vector as high as 2000 m at the periphery of Gondar. Recent work proved that all P. orientalis strains are capable of transmitting L. donovani. In Afar population, with High LST positive rate plus P. orientalis, VL cases are almost none. This is a research problem to be addressed. The border areas with Kenya (Segen Omo valleys and Somali area including Negele (oromyia))(Southern Kala-azar focus), where P. martini is the vector, everything is similar to the situation of Kala-azar in Kenya.

The distribution of kala-azar cases, sometimes, difficult to predict in the highland areas. Patients from Mehoni (Raya Azebo) and Merabete areas were treated for VL. The Addis zemen (Libo Kemkem) area is a highland area where epidemic 2005/6 erupted. But, Ashford et al., described similar situation in the neighboring village 35 years ago. Preliminary study on Libo Kemkem villages, using Leishmanin skin Skin Test (LST) proved the area to be not endemic. Probably, there is no reservoir host of VL in the area, even if the P. orientalis is plenty. The epidemic in Libo Kemkem villages was explained by anthroponothic transmission during 2005/6 season epidemic which died out soon. Now there are no cases without travel history to the Metema-Humera lowlands. Then what is emerging?

*Absence of movement of people from southern VL focus described in the text page 6 line 126 – 129 in the study area section.

“Rarely, labour migrants come from areas outside Amhara and Tigray region. No labour migrants would come from southern Ethiopia where kala-azar is endemic due to long distance
between the two areas and the presence of other better alternatives in the southern part, like gold mining and involve in tread activities in border areas with Kenya and Somalia”.

**Additional**

The important focus in the south is the Ababa Roba (Konso) focus. It is not possible even to imagine for a single person to come to Metema – Humera lowland. I know the situation in the area very well. They used to go shakiso gold mining area or to Moyale (Kenya) for thread. Almost zero chance for any body from negele Borena area to come to work in Metema – Humera lowlands. In Omo valleys, the different tribes are not known to go to even to the nearest larger towns or cities.

3. The migrants in the present study also moved from Amhara and other parts of Ethiopia. The authors should give more information on the origin of the 45 labour migrants, who were positive with DAT ($\geq 1:800$). Moreover, the length of stay of the labour migrants in the study area, whether the permanent migration or seasonal migration.

**Corrected on Page 10 line 220-223 on risk factor section**

4. The collected sand flies in the study area were identified the species morphologically and five species of Phlebotomus were observed, including *P. orientalis* and *P. rodhaini* were revealed. Although *P. orientalis* is reported as the main vector for *L. donovani* infection in Ethiopia; however, *P. rodhaini* is reported as a possible vector in transmission of *L. donovani* (Elnaiem et al., A possible role for *Phlebotomus (Anaphlebotomus) rodhaini* (Parrot, 1930) in transmission of *Leishmania donovani*, Parasites & Vector 2011,4: 238). Demonstration of *L. donovani* infection in sand flies - *P. orientalis* and *P. rodhaini*- could underline what sand fly species as the main vector for transmission of *L. donovani* in these extra-domestic habitats of Kafta-Humera Lowlands.

As the paper indicated this species is rare. Successful collection is possible via rodent bait. It can not be a vector for VL. I did human bait during my station in agriculture fields in Kafta-Humera.
I was as careful as possible to identify females outside *P. orientalis*. I couldn’t find this species. But, finding has excellent implications about reservoir hosts for future study.

4. It was confused when the labour migrant could be exposed to *P. orientalis*; during June – August (in ‘Results’) or May - June (in ‘Discussion’).

   Corrected. There would not be confusion

5. Please separate Table 1 in separate file, not embedded in the text

   - Done

6. The language of the manuscript is generally good but a carefully spelling check is urgently suggested since numerous mistakes occur.

   - Revised carefully to avoid mistakes

The minor

1. **Title and elsewhere in the text:** Prevalence is used in describing disease epidemiology; please add “infection” after ‘*L. donovani*’, otherwise use other words instead such as “Kala Azar or Visceral leishmaniasis”

   - Done

2. **Abstract:**
   1. To make it clear, please separate and heading “Results” part from “Methods”

      - Done

   2. Abstract and elsewhere in the text; for ease of reading, numbers with many digits should be divided into groups with ‘comma’

      - Done

3. Methods,
   3.1 line 37-38; Pls. replace ‘*P. orientalis/CDC*’ by “*P. orientalis/trap*”

      - Done

   3.2 line 38; Pls. add “number” after ‘The highest mean’

      - **Rephrased and made clear**

   3.3 line 36-37; what ‘(46.9) and (43.9)’ after March and April represent

      - **Corrected page 2 line 41-43.**
Materials and methods:
Subchapter ‘Study area and Sampling technique’,
1. line 87 and elsewhere in the text; replace ‘Acacia seyal’ by “A. seyal”
   - Done
2. line 91; Pls. be consistent in full name of month ‘Nov’, otherwise with all abbreviation
Subchapter ‘Study design’
   - Done
1. line 101; mistake spelling ‘adress’
   - Done
Subchapter ‘Population dynamics of P. orientalis…tickets…’, mistake spelling in
   - Done
subchapter, text in line 107; ‘p.m’, line 109; ‘Sergentomia’
   - Done
Subchapter ‘Blood sampling’
1. line 117; Pls. check ‘jugular forearm veins’
   - Done
2. line 119; Pls. replace ‘1200 cycle per second’ by “1200 cycle per minute”
   - Done
Results:
Subchapter ‘Population dynamics of P. orientalis’
1. line 150; add “.” After ‘Sergentomyia spp’
   - Done
2. Pls. be consistent in dividing numeral digits with ‘comma’ as (91,292) in line 150
   - Done
Subchapter ‘Sero-prevalence of L. donovani infection’
1. Lack of information of seroreactive results
Sero-prevalence of *Leishmania donovani* infection

Overall 359 blood samples were obtained from labour migrants, of these 243 (67.7%) showed no reaction for direct agglutination test while the other 116 (32.3%) were seroreactives. In this study, titers from 1:200 to <1:800 were considered simply as reactive and the DAT positive study subjects were 45 (12.5%), those with DAT results greater or equal to 1:800 titers (Table 2). The 42 Individuals (11.7%) with 1:800 - 1:6400 titers were most probably has *L. donovani* infections but not kala-azar. Only 3 titers (0.8%) were found to be greater than 6400(12800) which have had high risk for development kala-azar.

**Discussion:**

1. Line 210; spelling error ‘August’
   - Corrected

2. Line 210; Pls. delete – after ‘June to-.’
   - Corrected

3. Line 219 and elsewhere in the text; kala-azar is the name of disease that can be stand alone. Otherwise Pls. replace ‘kala-azar infection’ by “*L. donovani* infection”
   - Corrected

**Acknowledgement:**

Line 264; add space after ‘andScewangizaw Sime (driver)’

- Done

**References:**

Pls. check the format (position in bold, journal abbreviation without punctuation (.), word and line spacing, consistency in font, of reference.)
Table 1
1. Spelling mistake ‘tickets’
   - Done

2. For easy reading; Pls. arrange the alignment of value in each column
   - Done

Table 2
1. Colum arrangement of value alignment
   - Done

Table 3
1. Make change of font style ‘EXP’ to “Exp” in 95.0% C.I. for EXP (B)
   - Done