Author's response to reviews

Title: Investigation of the high rates of extrapulmonary tuberculosis in Ethiopia reveals no single driving factor and minimal evidence for zoonotic transmission of Mycobacterium bovis infection

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Version: 2
Date: 10 January 2015

Author's response to reviews: see over
Reviewer No 1 report and responses:

Title: The Enigma of Extrapulmonary Tuberculosis in Ethiopia: no single driving Factor but possible Roles for Zoonotic Transmission of Mycobacterium tuberculosis and a Complex Interplay of Host and Pathogen Genotypes

Version: 1 Date: 17 September 2014

Reviewer: Leen Rigouts

Reviewer's report:
Major Compulsory Revisions
None

Authors’ response: Thanks.

Minor Compulsory Revisions
1. Lines 183-184: Why were patients with disseminated disease excluded? What was their number?
   Authors’ response: The patients with disseminated TB were excluded because our focus was on exploring the epidemiology of peripheral tuberculous lymphadenitis (TBLN) rather than all forms of TB that involved lymph nodes. TB lymphadenitis is a well-recognized distinct form of clinical manifestation separate from disseminated TB. Although it is very difficult to totally exclude any pathologic involvement (parenchymal lesion) of the lungs in TB lymphadenitis, TBLN is clinically restricted to the lymph nodes as opposed to manifestations in both the lung and the lymph nodes. Dissemination to other non-contiguous sites such as the central nervous system is again different from restriction of clinical manifestation to lymph nodes.
   Only patients diagnosed with pulmonary TB or TBLN were recruited, therefore the number of excluded patients is not known.

2. Lines 195-202: This information is not formulated in a clear, understandable way for an outsider.
   Authors’ response: Text changed to clarify.
   
   Was: Sputum and FNA samples were stored at 4°C (or on ice) at the field sites and during transportation to the TB laboratory of the Armauer Hansen Research Institute (AHRI) in Addis Ababa where they were further processed within five days after sampling. However, due to long distances between many collection sites and AHRI, samples were commonly stored at the field site at -20°C (to maintain sample quality) until transport to AHRI. In most cases, samples were processed at AHRI within five days of receipt but where this was not possible they were stored at -20°C until processed.
   
   Changed to: Sputum and FNA samples were stored at 4°C at the field sites and during transportation to the TB laboratory of the Armauer Hansen Research Institute (AHRI) in Addis Ababa where they were further processed within five days after sampling. For remote collection sites (NFJ), samples were commonly stored at the field site at -20°C until transport to AHRI.
3. Lines 264-265: Was association with lineage excluded?
Authors’ response: The possible effect of lineage on recovery of organism from sputum upon culture has not been investigated. It is difficult to associate relative effect of lineage on yield of the AFB positive cultures when the organism fails to grow (unless direct lineage typing with molecular techniques is applied on the sputum, a technique we did not have). The negative effect of delayed sputum transport on mycobacterial yield is well known, a fact which prompted us to propose a likely factor that could at least partly explain the difference seen in Table 1.

4. Line 271: Somewhat confusing what ‘AFB positivity’ refers to; culture or smear. Usually it is restricted to smear results.
Authors’ response: Corrected by adding “culture” to sentence: “with an average yield between the collection sites of 79% and 38% culture positivity, respectively (Table 1).”

5. Lines 196-300: not clear how the (lack of) association was determined if pulmonary TB patients did not have HIV testing done.
Authors’ response: The lack of association between TBLN and HIV is discussed in the discussion. Therefore we have deleted “, but there was no association between HIV positivity and TBLN diagnosis” on line 299 here as it does not belong to the result section. For additional comments, please see page 7 and limitations of study.

6. Line 491: What about the impact of population density in urban settings as a factor of higher transmission in an urban setting?
Authors’ response: We did not investigate this factor specifically. Up to date – and with the given data stated in the manuscript – we can only observe that more cases are reported in urban areas. We think it has rather to do with access (thus the ‘likely’ in the phrase) than the transmission force for TBLN and TB in general. Our intent of mentioning this is really to open the scope of possible research that should more closely capture this aspect (i.e. access to hospital/health centre-based studies [and such studies rather reflect access] – vs. population-based studies that better capture incidences – compared between rural and urban settings).

It is correct that urban settings favour higher transmission of TB than rural areas. Nevertheless, Ethiopia is less urbanized (<15%) but has a higher TBLN rate than many other countries around the world. We emphasized access to health facilities as a major factor here considering its significance in the context of Ethiopia where the TB burden is high.

Level of interest: An article of importance in its field

Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Reviewer No 2 report and responses:

Title: The Enigma of Extrapulmonary Tuberculosis in Ethiopia: no single driving factor but possible Roles for Zoonotic Transmission of Mycobacterium tuberculosis and a Complex Interplay of Host and Pathogen Genotypes

Version: 1  Date: 14 November 2014

Reviewer: Wael elamin

Reviewer's report:
Summary:
The authors attempt to clarify factors that influence the rate of extrapulmonary TB in Ethiopia, a populous country with a high burden of TBLN. In previous work using molecular typing, the authors have shown a minimal role for M. bovis (Firdessa et al., 2013), despite it being endemic in Ethiopian Cattle. The authors also collected epidemiological, demographic and clinical data, from the previously mentioned study which is presented in this paper. Their findings have excluded zoonotic transmission of M. bovis, HIV co-infection and lineage diversity as major factors in the increased incidence of TBLN in Ethiopia. These findings are in themselves useful to set a direction for further investigations and studies.

A) Major comments:
1. The article title suggests a complex interplay of host and pathogen genotypes; however, the manuscript provides no evidence to substantiate the statement. The authors have previously characterised the TB isolates for large sequence polymorphisms and spoligotyping, and SNP analysis (Firdessa et al., 2013), however the samples were stratified according to the geographical regions; without further human population or genetic analysis. The conclusion of the abstract (line 116-117) stating “genetic features of the pathogen and/or the host population”; should also be revised or further evidence to support the statement provided in the manuscript.

Authors’ response: This study has taken into account several relevant factors that could explain the high rate of EPTB in Ethiopia. The result of our study indicates that none of these factors alone explains this fact, but here we suggest that interplay between several of these factors may have an impact on these high rates. We have rephrased the conclusion of the abstract to the following “The study suggests a complex role for multiple interacting factors in the epidemiology of extrapulmonary TB in Ethiopia, including factors that can only be derived from population-based studies, which may prove to be significant for TB control in Ethiopia”.

B) Minor comments:
1. Line 128: “is the most frequent form”: please provide a reference.
2. Line 133-135: “Zoonotic transmission can occur through the aerosol route during close contact with animals leading to pulmonary disease”: Please provide a
reference.
Authors' response: References provided.

2. Line 136: “M. bovis is primarily transmitted through consumption of contaminated milk and is therefore often associated with TBLN”: Please consider removing the word therefore.
Authors' response: The word “therefore” has been removed.

3. Line 148: it would seem likely: Consider using the word plausible rather than likely.
Authors' response: The word “likely” has been replaced by “plausible”.

4. Line 258: (from where patients were recruited in this study) – I am not sure if the statement is necessary.
Authors' response: As the ethnic groups of other suburban areas of Addis Ababa may not be “Oromo” we like to keep this sentence for clarification.

5. Lines 289-292: Was there any correlation with the volume of aspirate?
Authors' response: Unfortunately, the true volume of FNA sampled from each patient was not recorded and therefore correlations with other parameters were not possible to perform.

6, Lines 298-299: I could not comprehend the statement “Five HIV positive patients were confirmed with TBLN by culture”.
Authors' response: We have tried to make this sentence more clear by changing to: “Five of these 14 HIV positive patients were confirmed with TBLN by positive mycobacterial culture”

7. Lines 333-338: Please rewrite to make more comprehensible.
Authors' response: A minor modification was made adding “M. tuberculosis”:
“Analysis of demographic factors among pulmonary TB patients showed a borderline statistical difference between TB contact and M. tuberculosis lineage causing the disease; patients infected with Lineage 4 reported “contact with other TB patient” more often than patients infected with Lineage 3 did (OR=1.6, CI95% 1.0-2.7; p=0.064). The analogous comparison in TBLN patients showed no significant difference.”

8. Lines 374-375: Please provide a reference for the sentence - In comparison; prevalence rates of 1-10% for bovine TB in Ethiopian zebu cattle grazing in pasture are relatively low.
Authors' response: Two references added.

9. Line 382: The authors state despite the fact these countries are endemic for bovine TB, however the reference 26 (Diguimbaye-Djaibé et al., 2006) is specific for Chad, with no reference for the other mentioned countries.
Authors' response: References added.
10. Line 481: Please provide a reference for Sudan and Somalia rates.  
Authors’ response: References added on HIV-TB co-infection rates.

C) Discretionary revision
Discussion: Lines 488-495 – urbanisation is a recognised risk factor for TB infection and is not specific to Ethiopia; please refer to (Hargreaves et al., 2011) to support your argument.

References
Authors’ response: we found this helpful and added suggested reference.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interests
Reviewer No 3 report No 1 report and responses:

Title: The Enigma of Extrapulmonary Tuberculosis in Ethiopia: no single driving Factor but possible Roles for Zoonotic Transmission of Mycobacterium tuberculosis and a Complex Interplay of Host and Pathogen Genotypes

Version: 1     Date: 21 November 2014

Reviewer: Eric Walter Pefura-Yone

Reviewer’s report:
This study attempts to identify factors that may explain the high incidence of extra-pulmonary tuberculosis in Ethiopia. This is an interesting topic but the approach used in the paper does not allow easily understand of the results. Several major revisions seem necessary.

Major Compulsory Revisions
Title
1. The title does not reflect the content of the manuscript.
Authors’ response: We acknowledge the opinion of the reviewer and have reconsidered the title and suggest the following: “Investigation of the basis for high rates of extrapulmonary Tuberculosis in Ethiopia reveals no single driving factor and emphasizes the surprising paucity of evidence for zoonotic transmission of Mycobacterium bovis infection”

Abstract
The result and conclusions section of abstract should be rewritten according to the below comments.
Authors’ response: We have addressed the comments below as advised.

Background
1. The introduction is too long and does not pose the question straightforward.
Authors’ response: We have shortened the introduction to some extent and have spelled out the reason for this study earlier in the introduction. We have added the sentence: “What are the risk factors that can explain this high rate of extrapulmonary TB in Ethiopia?” to the background section.

2. There is no need to state the signs of TBLN. The sentence containing this statement is not well written
Authors’ response: Sentence has been modified to “Although pulmonary TB is the most common manifestation, an estimated one million people (~15%) develop extrapulmonary TB, of which TB lymphadenitis in the cervical lymph nodes (TBLN) is the most frequent form.”

3. I don’t understand the relationship between consumption of raw milk (with M. bovis contamination) and cervical TBLN.
Authors’ response: We are not sure what aspect the author is referring to or actually means. Raw milk consumption is a common event in Ethiopia and we thought infected cattle could pass *M. bovis* on to humans through raw milk. As the author would agree, *M. bovis* is presumed to enter the draining lymph nodes of the buccal mucosa upon ingestion of contaminated raw milk and cause lesion. This has been generally accepted as an important pathogenetic mechanism for scrofula in children when *M. bovis* was prevalent in Europe (although draining from the lung could provide another route for cervical lymphadenitis). Recent reports seem to confirm relationship of oral ingestion of mycobacteria in milk and cervical lymphadenitis. Doran P, Carson J, Costello E, More S. An outbreak of tuberculosis affecting cattle and people on an Irish dairy farm, following the consumption of raw milk. Ir Vet J. 2009 Jun 1;62(6):390-7. doi: 10.1186/2046-0481-62-6-390.)

4. Please, it is not possible to conclude that there is no relationship between extra-pulmonary TB and *M. bovis* infection based on the fact that only 1% of TB patients had *M. bovis* infection (line 150-line 154).

Authors’ response: Agreed. The message we are trying to pass is that *M. bovis* is not a major reason for the high rate of extrapulmonary TB in Ethiopia, “the high incidence rate of extrapulmonary TB reported in Ethiopia is likely due to other factors”.

5. Why metadata and not data?

Authors’ response: We have changed from “metadata” to “data”

Methods
There are major methodological problems in the manuscript.

1. The one major problem is that there is no information about HIV status of patient with pulmonary TB. Extra-pulmonary TB is more frequent in patient with HIV infection.

Authors’ response: We have repeatedly observed that the proportion of co-infection with HIV is similar for pulmonary and extrapulmonary TB patients in Ethiopia

References:


Data were not collected on HIV status of the pulmonary TB patients enrolled in this study. We have indicated this as a limitation. Nevertheless, regarding TBLN, the proportion of HIV positive TBLN patients among those tested was low (3.6%) and did not suggest that HIV was a cause for the high TBLN rate in Ethiopia (as noted earlier).

2. The diagnosis of TBLN should not only be based on fine needle aspiration but
also on lymph node biopsy.

Authors' response: We have evaluated the performance of fine needle aspirate (FNA) against excision biopsy in the Ethiopian setting and found it to be comparable. Several investigators have recommended FNA as a better option in clinical diagnosis of TB lymphadenitis. [Reference - Chand P, Dogra R, Chauhan N, Gupta R, Khare P. Cytopathological Pattern of Tubercular Lymphadenopathy on FNAC: Analysis of 550 Consecutive Cases. J Clin Diagn Res. 2014 Sep;8(9):FC16-9. doi: 10.7860/JCDR/2014/9956.4910. Epub 2014 Sep 20.]

Some experts do not recommend excision biopsy for the diagnosis of tuberculous lymphadenitis anymore (eg Ammari FF et al. Tuberculosis of the lymph glands of the neck: a limited role for surgery. Otolaryngology - Head and Neck Surgery 2003; 4:576-580). This has prompted many ethics review committee members to question excision biopsy as first line measure for the confirmation of cervical tuberculous lymphadenitis in Ethiopia (Personal experience of the PI, AA). Excision biopsy is invasive, requires surgical facilities and is not logistically manageable in such a large study in Ethiopia. It is recommended where malignancy is suspected (hard fixed lymph nodes – please see algorithm in Supplement figure S1A)

3. The paragraph concerning sites of study could be shortened because you have already published some results related to the current study.
Authors' response: The paragraph has been rephrased as advised.

4. You stated that: “you included volunteer patients”. The risk of selection bias is high. Why you didn’t use a random sampling method?
Authors' response: By “volunteer” we meant informed consent, as an essential requirement to conduct investigation. We recruited patients presenting themselves to health facilities consecutively during the study period.

5. Please, you should summarize the key clinical data collected in the test.
Authors' response: We have now mentioned the cardinal symptoms and their duration: fever, night sweat, weight loss, poor appetite, weakness, cough, and blood in sputum as well as information on nodes such as number, size, and consistency in brackets. The list is also shown in supplementary Tables S2, S3

6. Once again, the collection of specimens section is too long.
Authors' response: It has now been shortened as suggested.

7. What were the conditions to include or exclude an independent variable in the logistic regression models?
Authors' response: We present univariate analyses (crude odds ratio) and adjusted odds ratio (with adjustment to the expected most important confounders sex, age and site) for the clinical data and culture outcomes (supplementary data). We expect interactions and co-linearity between different signs and thus do not further include / exclude other variables than these. However, we have recalculated Table 2 showing explanatory variables for presenting with TBLN vs. PTB to show the derived ORs with the full model (including all variables).
8. Please, specify the significance threshold.
Authors’ response: We have added in the methodology ‘The statistical significance threshold was set at 0.05, p ≤ 0.05.’

9. How did you create the binary variable for lineages?
Authors’ response: We created binary outcome variables for Lineages 3, 4 and 7 (the respective lineage vs. all other lineages) to test associations with explanatory variables such as pulmonary TB and TBLN. The section on statistical analysis was adopted according to the points mentioned here and above.

10. Please, why did you restrict multivariable adjustment only for sex and site?
You should also include all variables that appear to be significant in univariable analysis in multivariable regression models. This is very important to adjust all significant variables (for example raw milk consumption and contact with animals) before drawing a conclusion about independent associated factors. It is possible that patients who are often in contact with the animals are also those who also consume more often raw milk.
Authors’ response: We agree – and we have added in Table 2 the ORs obtained with all variables included in the full model. We do not do any further backward selection. Note that having adjusted to all explanatory variables only changed the result in the sense that regular and direct contact with live animals was significantly associated with TBLN in both the uni- and multivariate analysis. Sentences referring to consumption of raw milk and living with animals were rephrased to no longer mention their significant association.

Results
1. Please, sample collection should be replaced by sample collected.
Authors’ response: Headline has been changed to “Study population and Sample collected”

2. I think that the results section should start by study population and sample collected. You should give the descriptive statistics of the participants and information on sample collected in this paragraph.
Authors’ response: We have made some modifications in this paragraph and moved the following sentence to next section:
“, and isolated acid-fast bacilli from 1212 of these patients (Table 1).“

3. There is no need to explain the reason why you chose different health centers to conduct this study. This is a results section and not the discussion section. Please, you should consider remove the explanation in the result section.
Authors’ response: Agreed that results section should avoid discussion. Nevertheless, here, we are showing the characteristics of the study participants with
regard to ethnicity. A sentence has been removed “In addition to their geographic
diversity, the different sites allowed sampling of multiple ethnic groups”.

4. Parts of the results section are about the relation between clinical features and
positivity of culture. This will be removed because it is not in line with the
objectives of the study. The aim of the study is to compare patients with
pulmonary TB to those with TBLN in order to fine risk factors of TBLN.
Authors’ response: The sentences have been modified to exclude any discussion in
results. “Not surprisingly” has been deleted.

5. Please, in the subheading “demographics”, please, just give the fact.
Explanation and interpretation should be stated in discussion section.
Authors’ response: We have deleted the following paragraph:
“These 954 patients had previously been included in the molecular epidemiology
analysis [10], and were known to have been infected with a strain from the M.
tuberculosis complex. As reported in previous studies [15, 16] and references
therein, TBLN was significantly associated with the youngest age group (≤20 years)
and with more females than males.”

The text “From our initial hypothesis that the high prevalence of extrapulmonary TB
may reflect zoonotic transmission, we anticipated that TBLN patients might have
close contact with farm animals and/or consume raw milk. This was in fact the
case.” has been moved to discussion section under subheading “zoonotic TB- M.
tuberculosis”

6. In the result section, there is no need to cite previous published data.
Authors’ response: With the deletion of the previous paragraph, we have taken 3
references away as well, in accordance with what the reviewer’s comment advised.

7. Please, concerning lineage analysis, you should compare PTB to TBLN and
not describe the characteristics of patient with a specific lineage.
Authors’ response: The paragraphs do indeed compare PTB to TBLN regarding
lineages.

8. Is there any relationship between TB site and lineage?
Authors’ response: If by TB site, the reviewer is referring to body site (lymph node
versus lung): we discussed that “Across Ethiopia as a whole, we did not observe any
association between M. tuberculosis lineage and disease presentation (Figure 2).
Increased representation of Lineage 4 in contrast to Lineage 3 in TBLN (data not
shown) was however observed in the north of the country but it only reached
statistical significance in Gondar (Table 3)”.

Discussion
1. Please, you should summarize the main results of the study early in discussion
section.
Authors’ response: We appreciate that the conventional form of presentation is to
summarize the main findings early in the discussion. In this particular case we
thought that providing the background to why we find the reasons for the high rate of extrapulmonary TB in Ethiopia enigmatic is a better approach to start with. The discussion is given in subsections and we have summarized the main findings at the end. We hope the reviewer will find this an acceptable option.

2. The discussion is too long and not reflected the results of the study. Many paragraphs are hypotheses.
Authors’ response: The fact that no single causal factor was evident from the results required that we look at multiple possibilities. Nevertheless, we have tried to be brief in all sections.

3. The limitations of the study are not acknowledged. For example: selection bias, lack of HIV test in pulmonary TB patients.
Authors’ response: We have made some modifications acknowledging the limitations: Line 511-518 discuss limitations.

Conclusions
The conclusions are not supported by the results.
Authors’ response: We believe that the conclusions are presented in a way that reflects the results. It now reads: “The study suggests a complex role for multiple interacting factors in the epidemiology of extrapulmonary TB in Ethiopia, including factors that can only be derived from population-based studies, which may prove to be significant for TB control in Ethiopia.”

Minor Essential Revisions

Figures and Tables
1. Please, all the abbreviations in tables should be explained (TB, AFB for example).
Authors’ response: Abbreviations given (see Table 2)

2. p-values indication should not be placed in the title of table 2.
Authors’ response: This has been corrected for and they have been placed as footnotes instead.

3. What is rel% (Table 2).
Authors’ response: Rel% = PTB versus TBLN, showing the relative proportions of PTB and TBLN per category

4. In figure 2, is it percentage?
Authors’ response: Yes, this has now also been added to the Figure legend.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable
**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**
I declare that I have no competing interests