Author's response to reviews

Title: A first insight into the genotypic diversity of Mycobacterium tuberculosis from Rwanda

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Author's response to reviews: see over
RESPONSE TO REVIEWERS’ COMMENTS

Reviewer's report 1
Title: A first insight into the genotypic diversity of Mycobacterium tuberculosis from Rwanda
Version: 2 Date: 26 July 2012
Reviewer: Christopher McEvoy
Reviewer's report:
Reviewer response: Gafirita et al, A first insight into the genotypic diversity of Mycobacterium tuberculosis from Rwanda.

General Comments.
The authors produced a valuable first report on M. Tuberculosis strain diversity in Rwanda, a country for which such studies have been neglected. In addition, drug resistance profiles of M. tb isolates and patient HIV status are available and the impact of the report is thus strengthened. The techniques used to determine strain, HIV status and drug resistance are well established and the manuscript is simple and straightforward. Apart from the minor points detailed below the paper is suitable for publication.

Specific Comments
1. Pg 3, 2nd paragraph. Minor Essential Revision.
“Currently, the only data......drug resistance studies,..... Please provide references for these drug resistance studies.

Response: Yes, this has now been included as reference 14 of the revised manuscript.

2. Pg 3, 3rd paragraph. Minor Essential Revision.
The first sentence suggests that all of the TB patients were from Kigali whereas in the “Study setting” section on pg 4 it states that they were from “several health units in Rwanda”. Please clarify this. If possible mention whether patients were from a localised region of Rwanda (eg Kigali) or whether they were located country-wide.

Response: This has been corrected. The National Reference Laboratory (NRL) is located in Kigali. According to the National TB algorithm, NRL receives samples from all parts of the country mainly: new cases with contact of known MDR patients; cases that had been on treatment for three months and remained sputum smear positive; and retreatment cases. Therefore this study consists of all country-wide cases that were found at NRL between March and Sept, 2009.

3. Pg 10, 2nd paragraph. Discretionary Revision
The largest cluster and 31.8% of the total isolates belong to SIT52. Is this SIT known to be present in neighbouring countries? Has the author’s previous work in Uganda identified this strain? What can be inferred regarding migration, geography etc regarding this strain?

Response: Yes, this isolate was found to be 7.6% (26/344) of isolates in a study in Central Uganda (ref 18) and 4.8% (6/125) of isolates from South Western Uganda which borders Rwanda (ref 27), and none was seen in a collection of 130 isolates from Northern Tanzania (ref 28). But generally, this genotype together with the related SIT 135 and SIT 148 are known to be
the commonest strain types causing TB in east central Africa (ref 4) as indicated in the discussion on page 10 of the revised manuscript.

The authors point out that high clustering rates (as seen in this study) are associated with high transmission frequencies which, in turn, are associated with HIV positive sero-status. However, the proportion of HIV positive sero-status in the unique (non-clustered) strains is 19/36 (53%), by my quick count, compared to only 45.7% overall. Please comment.

Response: We regret this error. Spoligotyping cannot tell strain transmission at a local level, but can only give a picture of global strain traffic in relation with the global database. This has now been corrected.

5. Pg 11. Discretionary Revision
A major finding of this study was the extremely high proportion of MDR cases and in particular the rapid rise in MDR since previous studies (refs 32 & 33). I feel that this should be emphasised more and comment made on possible reasons for the higher number of retreatment cases seen in this study which might be the cause of the higher MDR rate. Comment could also be made on the study of Umubyeyi et al (Int J Infect Dis, 2008) who found low rates of resistance to second line anti-TB drugs. It would be interesting to know whether this resistance rate has also increased.

Response: The Umubyeyi et al study of 2007 comprised of new cases and very few retreatment patients unlike this study (as clarified above and in the introduction of the revised manuscript) which had more of retreatment cases hence MDR suspects. This has been clarified in the introduction. Regretably, the current study did not look at second line drugs so we have no data to compare with.

All changes made are in red text in the revised manuscript.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests
Reviewer's report 2
Title: A first insight into the genotypic diversity of Mycobacterium tuberculosis from Rwanda
Version: 2 Date: 26 July 2012
Reviewer: Matthew Bates
Reviewer's report:
- Major Compulsory Revisions

1. Abstract, results:
If the speciation and typing is the main objective and purpose of the paper, then I think these findings should come at the end of this section.

Response: We agree with the reviewer. The first sentence of the conclusion: “Mycobacterium tuberculosis is the most prevalent species of Mycobacterium tuberculosis complex in Rwanda, and SIT 52 (T2) the predominant strain” tells us about the species (M. tuberculosis) and main genotype (SIT 52) seen in the study.

Was the MDR data generated by the study or simply taken from the National Reference Laboratory records? I suspect the latter as no mention of culture DST in methods. If so source of DST data needs to be stated. Also... the high levels of MDR are quite striking... especially the 30.4% in new treatment cases. The study design needs to explain the rationale for what cultures were analysed. Were they chosen at random, if so how? If they were not randomly chosen then by what criteria were they selected?

Response: The MDR data were taken from National Reference Laboratory Records. According to the National TB algorithm, NRL receives samples from all parts of the country mainly: new cases with contact of known MDR patients; cases that had been on treatment for three months and remained sputum smear positive; and retreatment cases. Therefore this study consists of all country-wide cases that were found at NRL between March and Sept, 2009. This has now been explained in the study design section on page 5 of the revised manuscript.

In the discussion you state “This, to the best of our knowledge, is the first report describing the species and strain diversity of M. tuberculosis complex isolates from TB patients in Rwanda” This suggests you 151 isolates are randomly representative of the population of strains in Rwanda. Based on the MDR rates I suspect this is not true. The study design needs to be more explicit in how isolates were selected and the discussion needs to acknowledge this limitation.

Response: We agree with the reviewer, that this was not a cross sectional study and may thus not be truly representative. But since the recruiting clinics were country wide as explained in the study design, the findings give good reference picture for future country-wide studies. We have stated this limitation in the discussion section on page 11 of the revised manuscript.

2. Introduction:
You state “Currently, the only data available on MTC in Rwanda focuses on drug resistance studies, and less is known about the prevalent species and strains, and how these relate with host demographic characteristics as well as drug resistance of the strains.”41% of your samples are
MDR, which shows a massive bias towards drug resistance. What is the current data you refer to? Not referenced.

Response: We regret this error. Three references have now been inserted (14, 15 and 16), and these refer to the drug resistance studies in Rwanda.

If there are any studies with species or typing data from Rwanda then these need to be presented in the introduction and your data needs to be compared with them in the discussion. If data is unpublished then suggest discussion with Rwanda NTP to include it in this paper, or at least refer to their internal reports and documentation.

Response: We agree with the reviewer. The previous molecular study (Umubyeyi, A.N., et al., Molecular investigation of recurrent tuberculosis in patients from Rwanda. Int J Tuberc Lung Dis, 2007. 11(8): p. 860-7) focused on transmission of MDR strains by MIRU-VNTR but did not mention species and spoligotypes of the starains. This has now been discussed on page 11 of the revised manuscript.

3. Materials and Methods, Ethical Considerations: Was this a prospective or a retrospective study? No mention of recruiting and consenting patients in abstract. Maybe consent and ethics approval was through a linked prospective study? What were the inclusion and exclusion criteria of the consenting study and what were the sites that the patients were recruited from? 4.

Materials and methods, study setting: If it was a prospective study then how many sites were there, how many patients were recruited from each site and what was the rationale for choice of sites and sample number from each site – presume inclusion criteria is ‘suspicion of TB’ – this must be defined.

Response: We regret the oversight of this very important aspect of the write up. A new section “ethical issues” has been added in the materials and methods section of the revised manuscript. This study was approved by both the Institutional Research and Ethics Committee of Kigali Health Institute (where JG was faculty), and Rwanda National Ethics Committee. Samples were shipped to Uganda for molecular analysis (where JG was an MSc student of molecular Biology) with proper materials transfer agreements between the College of Health Sciences of Makerere University and the National Reference Laboratory in Rwanda. Each participant in the study filled a consent form retrospectively during treatment follow-ups, provided by the health facility staff, after thorough explanations about the purpose of the study. Inclusion criteria were all culture positive for TB patients aged 18 years and above. Samples were brought to the NRL from all parts of the country as explained at the beginning of the “study design” section.

5. Results
HIV sero-status: What about it? Suggest throughout resulting summarizing key finding in section headings. Is there a link between HIV status and spoligotype or not? I think you are trying to answer this question but your answer is not clear?

Response: There was no association between HIV sero-status and any particular spoligotypes. We have stated this in the last sentence of this section.
6. Conclusion: If no significant difference by HIV status is one of your key findings then you need to show these stats on table 1 and bring this finding to the fore in the discussion.

Response: From our previous experience with two studies in Uganda (references 18 and 27) and literature from a study in northern Tanzania (reference 29), we did not expect to find any association between spoligotypes and HIV status, so this was not a key finding in this study.

7. What are the limitations of your study and what is the next question arising from your findings? What is the next study you want to do?

Response: We acknowledge that our sample collection may not reflect a national picture; hence a future national survey could genotype all isolates so as to give a clear situation of strain types, as well as transmission pattern in this locale.

**- Minor Essential Revisions**

8. Abstract, Background: “of” instead of “f”. Response: This has been corrected.

9. Abstract, Setting: put “National Reference Laboratory” here and remove from design to reduce repetition. Response: This has been corrected

10. Abstract, Objective: Remove “technique so as”. Response: This has been done.

11. Abstract, Design: Where these 151 cultures from sputum? Please specify. Response: Yes, the cultures were from sputum samples, and it has been indicated.

12. Abstract, Results: Give figures/percentages for the “predominance” of T2 and SIT 52. Response: Figures indicated. T2 = 58.3% (88/151), SIT 52 = 31.8% (48/151).

13. Materials and Methods, Study Setting: Remove “26,338km2” and replace with population density, if indeed the purpose is to illustrate that Rwanda has quite a high population density compared to other countries in the region... which is of relevance to TB.

Response: This has been done as advised.

14. Materials and Methods, DNA extraction: Your statement “Only thermolysates with enough harvest were subjected to DNA extraction using standard protocols [18], while the rest were used directly for PCR in subsequent analyses” does not explain the rationale for this approach. Also method of DNA extraction should be briefly stated although reference is supplied... sonication? QIAGEN extraction kit?

Response: This section has been re-written for clarity as seen on page 6 of the revised manuscript
15. Results, demographics: Give IQR for age... as more descriptive than range and helps one get an ideal of the age distribution. Was the difference in median ages in men and women statistically significant? Mann Whitney. Un less you analyse your speciation and typing results by age then this break down is probably not required. What about TB treatment history and HIV status? Just describe what you have shown in table 1... which could do with some percentages as well as raw data to make it easier to interpret.

Response: The IQR has been given in the revised manuscript. Indeed, we are not speciating and genotyping by age, hence obviating the necessity for these analyses. Percentages now included in the table.

16. Results, Table 1: Susceptible to what? Maybe just put “Non-MDR”. What does “not interpretable” mean? Give percentages out of each row. Put non-MDR (including the three mono-resistant isolates) and MDR side by side (so 70 vs 64). The mono-resistance probably doesn’t need to be tabulated –just stated in body text. Get rid of “not interpretable” and just explain in footer and in body text why 17 isolates were excluded.

Response: We agree with the reviewer. The table has been modified as advised, and we have now explained in the footer that “susceptible” means sensitive to both isoniazid and rifampicin.

17. Whenever you give a percentage give the fraction from which it is derived and vice versa eg. Results, spoligotyping, “revealed that 35/48 strains in SIT52 were retreatment cases while 11 of the 12 cases in SIT125 were retreatment” – also... is the second “retreatment” supposed to read “new treatment”? Response: This has now been corrected.

18. Discussion, paragraph one: Is there previous data from Rwanda or not? It’s eluded to elsewhere as you state previous studies have focussed on MDR... which I feel is also the focus of your study. In this paragraph state what your key findings are and then discuss them in more detail in the paragraphs that follow. Discuss speciation results first, then typing... so same order as in results. The second paragraph of the discussion contains just one reference. This section is supposed to compare your findings with that found elsewhere... select 3 or 4 key papers which give a solid picture of the spoligotypes found in other setting and compare your findings with them.

Response: We agree with the reviewer. This paragraph has now been well discussed, relating it to three previous studies from Uganda and one from Northern Tanzania. The corrections are on page 10 of the revised manuscript. The speciation now appears before strain diversity.

19. Discussion, third paragraph: You state “The 17 clusters identified in this study comprised 76.2% (115/151) of the sample, signalling a high transmission rate within the population” – why does this signal a high transmission rate? In the following sentence add an ‘s’ to ‘rate’. Still on transmission rates: you suggest the link between HIV and TB transmission is traced back to one study in Kenya? This statement should simply refer to a review or a couple of seminal studies in which this was shown. In your results section on the 17 clusters, you make no mention of HIV status, yet you are discussing it? Response: This was an error, since this technique cannot tell transmission at a local level but only gives traffic of strains at a regional/global level. This sentence has now been deleted.
- Discretionary Revisions
20. Abstract, Background: – “spread”... consider re-phrasing using “transmission” Response: this has been changed.

21. Abstract, Design: “speciation and strain typing” instead of “identification and typing” Response: This has been changed.

22. Abstract, Results: I don’t think MDR definition needs to be in abstract – suggest put in methods. Response: The authors prefer to define this early in the write up.

23. Abstract results: suggest condensing “Among the 151 isolates, 64 (42.4%) were multidrug resistant (MDR, resistant to both isoniazid and rifampicin) while two were resistant to rifampicin and one was resistant to isoniazid. Additionally, 94 of the 151 isolates were retreatment cases, of which 48 (51.1%) were MDR cases. Of the 46 newly presenting cases, 14 (30.4%) were MDR” to “Among the 151 isolates, 64 (42.4%) were multidrug resistant (MDR) with 3 cases on mono-resistance. Of 94 of the retreatment cases, 48 (51.1%) were MDR and of 46 newly presenting cases 14 (30.4%) were MDR”. Response: We thank the reviewer; this has been changed as assisted.

24. Introduction: The first sentence of the introduction is clumsy and needs to be rephrased. MTC is very nicely defined in the abstract. This detailed definition could be removed from abstract (where the detail is possibly not necessary, as MTC is a well known concept, and used to start introduction. Response: We agree with the reviewer, the clumsy statement has been refined and redundant information removed.

25. Methods, Ethical considerations: The statement “Clinical isolates and patient data were treated anonyously. Laboratory codes were used for all strains and patient data throughout the study, with no possibility to identify the patients except investigators only” could be removed. This is all standard procedure. Response: Thank you, this has been removed.

26. Not clear why laboratory analysis had to be done in Uganda? Always good to use local facilities and build capacity locally where possible. Response: JG was a Master’s student at Makerere University in Uganda and was required to do his research from this institution. Collaboration has been established to have similar studies done at his home institution so as to transfer the technology.

27. Materials and methods, study setting: move “Sample processing, confirmatory microscopy as well as culture and susceptibility testing were performed here. Colonies were harvested in 400μl of sterile Tris-EDTA (TE) buffer, heat inactivated at 80oC for two hours and then shipped to the Department of Medical Microbiology at the College of Health Sciences, Makerere University, for identification and typing” to sample processing section. Sample transport and decontamination can all be in one section. Response: We agree, and this has been moved.
28. Materials and methods: suggest combine study setting and study design sections as these are intrinsically linked and would reduce word count and repetition. Then have a separate section on ‘patient recruitment’, with all the HIV VCT info.

Response: The two sections have been done. However, we did not perform recruitment and HIV VCT as these were done (and patients treated) and the national program.

29. Materials and Methods, RD analyses and spoligotyping: this section is not clearly explanatory to a non-TB diagnostics specialist.

Response: We have provided ground breaking references which discuss the techniques in detail and we wish to reduce on word count here.

30. Results, demogrpahics: statements like “The demographic data of the patients were such that...” are wordy and unnecessary. Just say “59.6% (90/151) of isolates were from male patients”. You then don’t need to state the opposite figure for female patients as this is self explanatory.

Response: This has been corrected.

31. Results, Figure 1: Not sure what HIV status adds to this figure? What do you want to show? State what you want to show with respect to HIV and spoligotypes in body text.

Response: We wished to give the reader the opportunity of comparing, side by side, drug resistance in strains isolated from the two sero groups for strains that did not cluster. The clustered strains, on the other hand, were well discussed in the body text in the 3rd paragraph on page 8.

32. Results, spoligotyping: “Analysis of drug resistance in the major clusters revealed that SIT 52 (T2) with 48 strains had 34/65 (52.3%) of the total isoniazid resistant strains in the sample, 35/66 (53%) of the rifampicin resistant strains and 34/64 (51.3%) of the MDR isolates while SIT 125 (T2) with 12 strains had all eight isoniazid resistant strains being rifampicin resistant, hence MDR”. – this is a long sentence and is hard to follow. Suggest fragment?

Response: Yes, the sentences has been fragmented into three as now seen on page 8.

33. Discussion: no need to state that Uganda neighbours Rwanda. Response: Deleted

34. Discussion: discuss speciation first, then typing. Response: This has been done.

35. Discussion: MDR section: again you need to explain your recruitment better for the reader to understand your findings with respect to MDR-TB in Rwanda as a whole.

Response: This has been done.

All changes made are in red text in the revised manuscript

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

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests
Reviewer's report
Title: A first insight into the genotypic diversity of Mycobacterium tuberculosis from Rwanda
Version: 2 Date: 6 May 2012
Reviewer: Prasit Palittapongarnpim
Reviewer's report: Minor Essential Revisions
Specific comments

Abstract
Page 2 Line 2: “f” should be “of”. Response: Thank you, this has been corrected.

Page 2 Line 23: “different” should be “difference”. Please correct the P value in the parenthesis. Response: Thank you, these have been corrected.

Materials and Methods
Page 4, Line 25
Study Design: The fact that not all samples were genotyped indicate a possible bias for sample collection. More description of the selection criterion, “the highest ZN score”, is required.

Response: Thank you. The study design has been modified and sections have been merged to address the concerns of all the reviewers as indicated on

Page 4, Culture and drug susceptibility testing
Explicit description on how the bacterial isolates were identified as MTC is needed.

Response: Positive cultures were subjected to Ziehl-Neelsen (ZN) staining for confirmation of mycobacterial growth, and isolates were later confirmed as MTC at the molecular level by a PCR typing panel as described in the last section on page 5 of the revised manuscript. This has been clarified in the second paragraph of the revised manuscript on page 5.

Page 4 analysis and spoligotyping.
The target of analysis is the 16S rRNA gene not the 16S rRNA itself. Since the gene is present in every bacterium and very conserved, more details, such as primer sequences or exact positions of primers, need to be given so that the readers can evaluate the specificity of the primers.

Response: Thank you, this has now been corrected. Additionally, in the interest of word count, we have included a statement and three references (4, 23 and 24) where primer sequences and amplification conditions can be found.

Results
Page 8 Line12: This sentence seems self-conflicting. A Euro-American lineage should not be M. africanum.

Response: We agree. We have now included the word “erroneously” to show that it was an error in that study (ref 26), but a later study (ref 4) found that it was actually M. tuberculosis.
Page 8 Line 18 and 20: should be “isoniazid”. **Response:** Thank you, it has been corrected.

Page 8 Last paragraph: This paragraph should include a description on whether there are any statistical significant associations between any genotypes and drug resistance patterns.

**Response:** There were no significant statistical associations between genotypes and drug resistance. This statement has been included in the second sentence of page 9 of the revised manuscript.

Page 9 Last paragraph: There should also be a description on whether there are any statistical significant associations between any genotypes and HIV-serostatus of the patients.

**Response:** There were no statistically significant associations between any genotypes and HIV-sero status of the patients. This statement exists as the last sentence of the last paragraph on page 9 of the revised manuscript.

**10 Discussion**

Page 10 Paragraph 2 The authors reason that high genetic diversity of isolates in Rwanda was due to high level of transborder influx of strains due to human migration. However, at the same time, they seem to cite data indicating that the predominant strain in Uganda does not appear frequently in Rwanda. This discrepancy need to be explained. It may also be useful to indicate how many strains identified in Uganda were also found in Rwanda.

**Response:** We understand the concern of the reviewer. However, Uganda is not the only country neighboring Rwanda. There is DR Congo, Tanzania and Burundi, and most of these areas are not well studied in terms of TB and strain diversity. For strain types in Uganda, studies in central and South Western indicate that SITs 52, 125, and 135 are shared are common in both countries.

Page 10 Paragraph 3 There are a few precautions that need to be taken to conclude the high transmission rate in Rwanda. First, spoligotyping is known to have relatively low discriminating power compared to other popular genotyping methods. Isolates in the same spoligo cluster are usually differentiable by IS6110 RFLP or VNTR typing or SNP typing. Second, the period of sample collections was short, just for 7 months. It is unlikely that two patients with the same genotypes in this study are the results of direct transmission events during the study period. All these facts should be discussed.

**Response:** We agree with the reviewer, spoligotyping is mainly used to track regional or global strain traffic but not local transmission. The statement has been deleted from the body of the manuscript.

Page 11 Line 11-13: This sentence cannot be accurate. The gene coding for 16S-rRNA cannot be deleted as M. tuberculosis is known to contain a single copy of the gene. Its deletion would render the bacterium completely unviable. The absence of amplification product of 16S rRNA gene must be explained by another reason. In reverse, the ability to amplify the 16S rRNA gene may not be considered as evidence in species identification of M. tuberculosis because the gene region is very conserved. Some sets of primers can actually amplify a vast range of bacterial species. The information regarding the primer sequences should be provided in the manuscript.
Response: We agree with the reviewer, However, there seems to be a mis-interpretation of the sentence in question:

“Deletion analysis using 16S-rRNA, RD9 and TbD1 loci showed that all the strains investigated were characterized by presence of both 16S-rRNA and RD9 loci, and deletion in the TbD1 regions, a pattern confirming that they all were M. tuberculosis strict sense.”

This sentence does not indicate at all that the 16S-rRNA gene is deleted; in fact we wrote that “all the strains investigated were characterized by presence of both 16S-rRNA and RD9 loci”.

But to avoid this confusion, we have changed the words ‘deletion analysis’ in the manuscript to “regions of difference (RD) analysis”. Once again, in the interest of word count, we do not see the justification to show primer sequences that are already published, but have instead referred the reader to references 4, 26 and 27 where these sequences and amplification conditions were stated and evaluated.

All changes made are in red text in the revised manuscript

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: 'I declare that I have no competing interests'