Author's response to reviews

Title: Analysis of gene mutations associated with isoniazid, rifampicin and ethambutol resistance among Mycobacterium tuberculosis isolates from Ethiopia

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Version: 2 Date: 25 January 2012

Author's response to reviews: see over
Authors’ response to reviews
25th January 2012
The Editor, BMC Infectious Diseases
Re: Analysis of gene mutations associated with isoniazid, rifampicin and ethambutol resistance among M. tuberculosis isolates from Ethiopia (MS: 7162951086185106)

Dear Ms. Roselle Pangilinan,
Thank you very much for your e-mail of 16th January 2012. We are very grateful for the constructive comments forwarded by the reviewers. We have addressed the reviewers’ comments and have revised the manuscript in line with their suggestions. We have provided a point-by-point response to the reviewers’ comments.

Sincerely,
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Reviewer's report 1

Title: Analysis of gene mutations associated with isoniazid, rifampicin and ethambutol resistance among M. tuberculosis isolates from Ethiopia

Version: 1 Date: 7 December 2011

Reviewer: Pontus Jureen

The manuscript (denoted as MS from hereon) is performed on Ethiopian strains. Its essence is to evaluate the HAIN kits for detection of RMP, INH and EMB resistance by using the phenotypic BacT/ALERT system and thereafter discuss such methods suitability for in this particular tuberculosis population.

Local studies like this is important to report as the authors correctly discuss that methods that specifically detects certain mutations may show different specificity and/or sensitivity depending on the geographical setting. Thus the target population that the kit was developed for may not resemble the global situation, especially in various local tuberculosis populations.

General comments:
The MS is in a premature state and should preferably be carefully revised in regards to several aspects:

We are very thankful for the valuable comments of the reviewer. We have followed the suggestions of the reviewer to improve the quality of the manuscript.

- To the language, there are several grammatical and typographic errors as well as misspellings.

We have corrected the grammatical, typographical and spelling errors throughout the MS.

- Data should be chosen to either be presented in the running text or in the tables. As it is now the majority of the information in the results part is also given in the tables

We have revised the data presentation as per the suggestion of the reviewer.
- MS should preferably be shortened as much of the information is repeatedly given i.e. in introduction, results and discussion. This is true for both information quoted from the current literature as well as the data reported in the present MS, but not of curse the introduction for the latter.

*We have avoided the repeated information, thus the MS has shortened.*

- Throughout the MS, preferably use the HAIN kit denominations as much as possible, this instead of using the specific mutations. The HAIN testing is an indirect testing which do not give the sequence, for sequence analysis the application of DNA sequencing would be needed.

*We have tried to use the HAIN kit denominations as much as possible.*

- Genes are denoted in italic whereas the protein is denoted as norma text. This should be corrected throughout the MS.

*Corrected throughout the MS as per the reviewer’s suggestion.*

- Mutations are commonly written as Ser531Leu, not Ser531#Leu. This should be corrected throughout the MS.

*Corrected throughout the MS as per the reviewer’s suggestion.*

- Please insert numbers for each line, it is difficult to comment upon a MS without these numbers.

*We have included line numbers in the MS.*

- In terms of drug resistance; preferable use “susceptible strains” and not “sensitive strains”

*We have used “susceptible strains” instead of “sensitive strains” throughout the MS.*

Abstract:
It is not necessary to include “%” when total and fractions are given as well. Please use either or.

*We have deleted “%” as per the reviewer’s suggestion.*

**Background:**

WHO data can be updated, there are several more recent reports since 2006.

*We have updated the WHO data.*

Pg3, last sentence: “the development of “ should be deleted as these genes are indeed involved in resistance and not only involved in the development of resistance.

*The phrase “the development of” has been deleted.*

Pg3, last sentence: What is the sens. / spec. and speed based on? Any reference? Any particular method in mind? i.e. in house methods, HAIN, Cepheid?

*We have indicated a particular method, cited a reference and revised this sentence.*

Pg 4 an example of misspelling; aminoglycosides and not aminoglicosides. Please revise the MS for misspellings.

*The MS has been revised and misspellings have been corrected.*

Pg 4. DNA does not need to be explained, hardly PCR either.

*We have omitted the explanations to DNA and PCR.*

Pg 4. First paragraph last sentence. If not all, so nearly all RMP-R strains do contain mutations in rpoB.

*Corrected as per the reviewer’s suggestion.*

**Methods:**
First paragraph: How were the strains selected? Any exclusion or inclusion criteria? Were there any consideration of excluding clonal isolates / resampling of the same patients? What laboratory steps were performed before culture and HAIN?

Of 288 culture positive strains, 260 were randomly selected for this experiment, the 260 isolates included in this study were obtained from 260 patients, one strain from each patient, so, all randomly selected strains that were culture positive and identified as M. tuberculosis were included in this study. Before culture and HAIN, microscopic examination of sputum samples was performed using Ziehl-Neelsen staining technique. This paragraph has been revised.

Second paragraph: It is of less interest to quote the HAIN manual. Instead try to focus and describe what was not done according to the manual or what was chosen in the optional steps of the HIAN procedures. Was the DNA extracted from cultures or clinical samples? Which PCR program was chosen, the extended (for clinical samples) or the shorter (for cultures). Was the DNA concentration measured? What brand and model was the water bath? This could be of interest for readers as it open up opportunities for alternative apparatus than the standard Twincubator. The usage of water bath (instead of Twincubator) could also briefly be dwelled upon in the discussion part.

This paragraph has been revised as per the reviewer’s suggestion.

Third paragraph: I strongly suggest that the authors should read a book or so in basic genetics. A promotor (not promoter) region is a non coding region, thus for these regions only the nucleotides ATCG are valid. In this context the translation into amino acids T = Thr, A = Ala is completely wrong. This should be revised throughout the MS.

This paragraph has been revised and mistakes have been corrected throughout the MS. However, the spelling for the word “promoter” was correct and remains as it was instead of the reviewer’s suggestion to write as “promotor”.

Fourth paragraph: The DST testing concentrations (breakpoints) are indeed odd. WHO recommends the following concentrations: BACTEC 460 INH 0,1 mg/l, RMP 2 mg/l, EMB 2,5 mg/l and for MGIT 960 INH 0,1 mg/l, RMP 1 mg/l, EMB 5mg/l. Using other breakpoints
will likely skew the results and conclusions, I will come back to this later on. What is the rational for using these concentrations? Is there any international breakpoint recommendations for BacT/ALERT that can be referred to? For the present study this is an essential standpoint information and a reference (no 23) in the German language is not advisable as this is only comprehensible for a minority of the research society (I wonder if even all authors for this MS can read and understand reference no 23?).

As the reviewer has mentioned that BACTEC 460 and MGIT 960 have different testing concentrations for RMP and EMB, different methods can have different testing concentrations to show concordant results with those of the reference methods. We validated the BacT/Alert 3D method more than ten years ago, of course, we noticed the big difference for the INH test concentration compared to the BACTEC 460 TB method. However, it was not astonishing as the MIC of INH for the reference strain H37Rv and different wild type strains is 0,125 mg/L by BacT/Alert 3D system. The BacT/Alert method for susceptibility testing of TB strains was validated under the control of the German “Arbeitskreis Mykobakterien” (“Working group mycobacteria”), and by the support of ORGANON TEKNIKA (the former owner of the device). The method is being used by laboratories in several European countries. We have cited some publications about experiences with this method in this manuscript. The test concentrations that we have mentioned in this manuscript for BacT/Alert 3D system has also been clearly described in the book “Manual of clinical microbiology 9th ed., Vol. 1, p. 1230-1231, and p.1236 by Murray et al. 2007”. Moreover, this laboratory successfully participates in external quality assessment programmes for culture and drug susceptibility testing of mycobacteria. The first publication about BacT/Alert method was reference no 23 (ref. no 26, in the revised MS), BacT/Alert was the first method for the susceptibility testing of TB strains with a fully automated non-radioactive system. The title and the abstract of this paper including all the necessary information have also been written in English. We have replaced the German version of the title by the English version in the reference list.

Results:
The demographic paragraph and table 1; It is unclear how these contributes to the MS? These parts should either be deleted or integrated into the MS. If integrated, the demographic data should be combined and discussed with the main scope of the present study, i.e. the HAIN results. If this is not possible or there is no added value, then the demographic part should preferably be excluded.
This paragraph has been revised; the demographic data has been integrated with the HAIN results.

Second paragraph, second sentence; were there no isolates with both katG and inhA alterations?

Yes, there was no isolate with both katG and inhA alterations. We have mentioned it in the second paragraph.

Second paragraph, last sentence; a phenotypic and a genotypic method can have “concordant etc” results but not “similar” results, revise this throughout the MS.

Corrected throughout the MS

Discussion.
The first two sentences are exactly the same, word by word, as what is found in the introduction. I do believe that the authors can put more effort in writing a manuscript than this. If there are any more by me unnoted things like this, please rewrite! It will be for your own benefit as the future readers surely will interpret this as sloppiness.

We are very happy for the careful observation and kind advice of the reviewer. We have revised it and checked the MS for similar problems.

2nd paragraph; There is a meta study of the HAIN test by Bawanga F that preferably should be considered to be discussed, especially when discussing the sens. and spec. for this method.

We have considered this Meta study and discussed our findings.

pg 12 first sentence. The interpretation of the results have been extrapolated in an incorrect way. The materials in the present study are not from all Ethiopia, only the Gondor region. Also, there is no evidence that this material is representing the Gondor region. A better description of the material (in the M&M part) may convince the reader that the present material is representative for Gondor.
The interpretation of the results has been corrected.

Pg 12 end of second paragraph. Why is the presence of low level INH resistance marker (inhA) indeed low? Does the author think this is a local variation? Or do the authors think this has something to do with that they phenotypically test the strains at a 10 fold higher INH concentration than recommended for other broth based methods?

Yes, we think that the low frequency of low level INH resistance marker (inhA) is due to a local variation.

Pg 12, 3rd line from end. Write: …in the region of codon 507-533.

Corrected as per the reviewer’s suggestion.

Pg 12, last sentence, This describes the HAIN kit and thus belongs to M&M.

The sentence has deleted from the discussion and described in the M & M part.

Pg 13, ref 31 and the discussion with the comparison of the Ethiopian mutation frequencies. This reference (no 31) is unique in its way of describing mutations and their frequencies among clinical isolates. This comparison leads to skewed conclusions. The material presented in the present MS do reflect well what is found globally, although there is an exception, the high frequency (20%) of RMP-R isolates with no mutations detected by HAIN, in spite being only 3 isolates this would be more relevant to discuss.

We have added more discussion for the high frequency (20%) of RMP-R isolates with no mutations detected by HAIN. But we believe that ref. no 31(ref. no 35 in the revised MS) is not unique in its way of describing mutations. As in both cases authors used GenoType MTBDRplus to describe mutations. So this comparison is important for the discussion of our findings including the high frequency (20%) of RMP-R isolates with no mutations detected by HAIN.

Pg 13. Once again, do the authors think their results would have looked differently if a higher EMB concentration was tested for? i.e. a concentration that more resembles what is by WHO
recommended for other broth based methods. If a higher breakpoint for EMB was used, wouldn’t more strains be defined as susceptible? Would that increase sensitivity?

In the first place, our finding is supported by other previous reports that showed low sensitivity of the GenoType® MTBDRsl, like 56 % (by Huang WL et al, J Clin Microbiol. 2011 and Said HM et al Int J Tuberc Lung Dis. 2012) and 57%(by Brossier F et al, J Clin Microbiol. 2010), suggesting that there might be other mutations associated with EMB resistance. As we have mentioned earlier, we used validated EMB test concentration for the BacT/ALERT 3D system. Of course, if a higher breakpoint for EMB was used, more strains might be defined as susceptible. But it might be false susceptible result. We have revised this paragraph and added more references in the discussion part of the MS.

Level of interest: An article of limited interest

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
Reviewer's report 2

Title: Analysis of gene mutations associated with isoniazid, rifampicin and ethambutol resistance among M. tuberculosis isolates from Ethiopia

Version: 1 Date: 9 January 2012

Reviewer: Andrea von Groll

General comments:
The authors present an important study using the commercial molecular method for detection of M. tuberculosis resistant. This commercial method have been widely studied for the detection of M. tuberculosis resistant to INH and RIF since it has been recommended by WHO to be introduced in reference laboratories in several countries. As noted by the authors, each population may have a genetic variation of strains of Mycobacterium tuberculosis that could interfere with the results of this method. Thus it is important to validate with strains of the local population before its implementation in the routine.

The methodology, results and analysis of results were conducted in a scientifically appropriate and following the standards of this type of study.

The results were in line with previous studies in other populations, but it did not present any new information in relation to previous studies. Thus, to enrich the manuscript, authors are encouraged to provide more information:

We appreciate the reviewer’s valuable comments. We have provided more information.

- Major Compulsory Revisions (which the author must respond to before a decision on publication can be reached)

1. Whereas the authors performed the tests GenoType® MTBDRsl, because they only present the results for EMB and did not present the results to fluoroquinolones and aminoglycosides obtained from this test?

All isolates included in this study had no mutations conferring resistance to fluoroquinolones and aminoglycosides. This might be due to low use / access to these drugs in Northwest Ethiopia. We have included this information on the result part of the manuscript.
2. Have the authors any information on the genotypes of the MDR strains? Since all had the same mutations could have an epidemiological link.

*No, we haven’t any information on the genotypes of the MDR strains yet.*

3. Both the background and during the discussion the authors should review the sentence: “Studies have shown that mutations in the katG, inhA, kasA and ahpC genes were associated with INH resistance.”

Mutations in kasA and ahpC ... are no longer considered to be linked to INH resistance and there are other genes, such as ndh, which could be cited.

*We have revised this sentence both in the background and discussion parts of the manuscript.*

4. A important point that should be reassessed is the sentence present in the discussion: “Studies have also shown that 8% to 43% of INH resistance are defined as the low-level drug resistance mainly caused by the mutations in the promoter region of inhA gene, involving the -15, -16 and -8 locus [29]. The genoType MTBDRplus involves two wild-type probes (WT -15/-16 and WT -8) and four mutation-type probes, covering mutations of Cys15#Thr, Ala16#Gly, Thr8#Cys and Thr8#Ala, for detection of INH low-level drug resistance. In this study, we have observed that the low-level drug-resistance proportion was 6%, close to the low limit of the reported range.”

- In the paper 29 cited in this text, there is no information about the level of resistance and mutations involving the -15, -16 and -8 locus. I think that the authors should not affirm that “observed that the low-level drug-resistance proportion was 6%, close to the low limit of the reported range”, unless they have determined the minimal inhibitory concentration (MIC) to INH.

*The information about the level of resistance (8% to 43%) caused by the mutations in the promoter region of inhA gene has been stated in the paper (ref. 29, in the revised manuscript ref. 33 ). Please see the table (Mechanisms of drug resistance in M. tuberculosis) the second raw in the last column. We have revised this paragraph.*

5. To enrich the manuscript, authors are encouraged to provide more information on the non-concordant strains for INH and EMB. It would be interesting to identify the mechanism of
resistance by sequencing of the genes (whole katG, inhA promoter and gene, embCAB genes) to serve as subsides to improve molecular methods.

*It is a good idea, however, we have no the facility to do so at the moment.*

- Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

**Title:**
1. The title should not have abbreviations: M. tuberculosis should be changed to Mycobacterium tuberculosis.

*Corrected.*

**Abstract:**
Review small details like:
2. the first M. tuberculosis should also be complete: Mycobacterium tuberculosis.

*Corrected.*

3. Standardize the test name (there is a space after GenoType in the first name and not in the second name): GenoType MTBDRplus GenoTypeMTBDRsl.
The correct is GenoType® MTBDRplus and GenoType® MTBDRsl

*Corrected throughout the manuscript.*

4. Genes must be in italics: rpoB, inhA…

*Corrected throughout the manuscript.*

5. It would be interesting to insert the period of collection of isolates in the methodology.

*The period of sample collection has been stated in the methodology part.*
6. In the results: correct the position of the mutation in rpoB gene: “one at His531#Asp..”, the correct is His526#Asp
Corrected.

Text:
7. Genes must be in italics throughout the text: rpoB, inhA…
Corrected.

8. In the background, the author could add the percentage of MDR in Ethiopia.

The percentage of MDR TB in Ethiopia has been added in the background part.

9. The authors should add in the text, the country which was performed the study 31:
“In 20% of the resistant isolates, mutation was detected only at the wild type probes, which is different from the previously reported gene mutation distribution, 37% at Ser531#Leu, 3% at His526#Asp and in 60% of the isolates, mutation was detected only at the wild type probes [31]”

We have added the country where study 31(study 35, in the revised manuscript) was performed.

10. Since Patients’ history of previous treatment was associated to resistance, the authors could report in the discussion, the treatment regimen for TB and availability of these drugs in Ethiopia.

We have added information about treatment regimen for TB and availability of these drugs in Ethiopia in the background part of the manuscript.

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.

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