Author’s response to reviews

Title: PLASMID-BASED HIGH-RESOLUTION MELTING ANALYSIS FOR ACCURATE DETECTION OF RPOB MUTATIONS IN MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM MOROCCAN PATIENTS

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Author’s response to reviews:

To the Editor BMC Infectious Diseases

Mr. Edward A Graviss

Rabat, July 10th, 2017

Dear Editor,

Please find enclosed the revised manuscript "Plasmid-Based High-Resolution melting analysis for rapid detection of RpoB mutations in Mycobacterium Tuberculosis isolates from Moroccan patients" to be considered for publication in "BMC Infectious Diseases", Manuscript Number: INFD-D-17-00594.
We revised the manuscript according to yours and reviewers recommendations. In particular:

1. We revised the manuscript general formatting and we added the references according to the journal format.

2. We discussed recent publications on plasmid-based HRM, which have performed direct detection on clinical samples and isolates.

3. We added more detailed description about qPCR equipment and methods in the methods section.

4. We included a section of statistical analysis in the methods section.

Looking forward to hearing from you.

Thanking you,

Yours sincerely,

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Response to Reviewers comments:

Reviewer #1:
Response: Thank you for your reviewing. We made editing and general formatting of the manuscript.

Reviewer #2:

1. The title should not include the term "rapid" as the test is being done from isolates and not directly from sputum.

Response: Thank you for this relevant comment. We modified the title accordingly (Page1:line2).

2. The manuscript should be extensively revised in terms of general formatting. The references are not added according to journal format.

Response: Thank you for your comment. Accordingly, we revised the manuscript general formatting and we added the references according to the journal format.

3. Please revise TB and MDR-TB estimates as provided in Introduction according to the latest WHO report.

Response: Thank you for your comment. We modified the TB and MDR-TB estimates according to the WHO Global Tuberculosis report 2016. (Page 5: Paragraph1).
4. Introduction should include information on other molecular tests such as the Xpert and other line probe assays as endorsed by the WHO. Xpert Ultra information can also be included.

Response: Thank you for your comment. A paragraph describing LPAs, Xpert® MTB/RIF, Xpert® MTB/RIF Ultra has been added in introduction section. (Page 6: Paragraph1).

5. What staining method was used in the qPCR? SYBR Green? More description of equipment and methods for the qPCR are needed because this is a big part of this study.

Response: Thank you for your comment. We used MeltDoctor HRM MasterMix that includes SYTO9® as a staining dye. As suggested, we added a description about the dye, which similarly to EVA®Green, has shown many advantages compared to the 2nd generation of dsDNA-specific dyes (e.g. SYBRGreen I). Moreover, we added more information about the equipment used (QuantStudio 6FLEX) which has the advantage of including an integrated HRM module that facilitates the simultaneous qPCR and HRM curves analysis, unlike older versions. (Page9: line 9 to line 19).

6. Please include a section on statistical analysis in the methods section.

Response: Thank you for your suggestion. We have included a statistical analysis section in the methods section. (Page 10, line 22 to line 25)

7. Why were only 4 mutation's chosen for the study? The mutation change should be mentioned as an amino acid change rather than a nucleotide change. Also Page 8 Lines 6, please correct S531T as S531W and H526C as H526Y.
Response: Thank you for your comment and suggestion. We have chosen 4 mutations because of their high frequency among all the mutations occurring within the RRDR based on several studies worldwide. As well, the most recent studies in Morocco, have reported these mutations as the most frequent in Mtb RIF resistant isolates from Moroccan patients.

According to your comment, we have mentioned all the mutations as an amino acid change (along the manuscript) and also corrected S531T as S531W and H526C as H526Y (Page 11: line 24).

8. The authors have used a 350 bp amplicon for sequencing, the 5 isolates which were genotypically susceptible and phenotypically resistant, did they not have the mutations mentioned in lines 8-11, page10. Some of these (codon 500, 502, 505, 538, 572, 490) will be covered by the sequencing primers used in the study. The authors should try to sequence the entire rpoB gene to look for the source for this resistance.

Response: Thank you for your relevant comment. In fact, for sequencing the rpoB fragment harboring the RRDR, we have used a pair of sequencing primers targeting a 350 bp region and thus covering the sequence from the codon 482 to codon 587. In fact, as you have noticed our primers covered the codons 490, 500, 502, 505, 538, 572 into which no mutation have been detected. Therefore, we have removed these codons from the discussion section of the manuscript (Page 14: line13 to line16).

In the context of the present study, our objective was to use sequencing only to evaluate the accuracy of our HRM assay, covering specifically the hotspot region. Otherwise, sequencing the entire rpoB gene could be a major single study to characterize RIF resistance-related mutations outside the RRDR in a statistically significant number of Mtb isolates from Moroccan patients. Hence, discovering geographically-specific rpoB mutations in further studies, would allow us to create a library of plasmid controls for multiple codons within the rpoB gene as they have proved their cost-effectiveness and accuracy associated with HRM analysis.

9. Authors should discuss recent papers on HRM which have done direct detection on clinical samples and used plasmid controls for HRM (eg. Anthwal et al 2017 Direct detection of Rifampicin and Isoniazid resistance in sputum samples from tuberculosis patients by High

Response: Thank you for your comment and suggestions. Accordingly, we have added the studies that reported the use of HRM analysis to detect RIF related mutations in isolates and directly from sputum samples (Page 15: Paragraph1).