Author's response to reviews

Title: Evaluation of Microscopic Observation Drug Susceptibility assay for diagnosis of multidrug-resistant tuberculosis in Viet Nam

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Author's response to reviews: see over
Reviewer 1: Ruth Mc Nerney

We would like to thank you for your helpful comments. We would like to apologise for the omission of page numbers, line numbers and tables. Tables were uploaded to the website but were omitted from the final pdf. We have now revised the manuscript to include page and line numbers and the missing tables.

A. Major compulsory revisions

1. The conclusion in the abstract and the text should be modified to better reflect the results which suggest low predictive values for the method under study (MODS).

   We have added comment on the low predictive values of MODS in diagnosis of MDR-TB in the abstract (lines 46 - 52), result (lines 309 - 312) and discussion (lines 410 - 413) as requested. We agree that this is important information and improves the manuscript.

   **Results**

   2. The actual number of each MOTTS species should be recorded.

   The actual number of each MOTT species has been added to the result section, as requested (lines 273 - 274).

   3. MODS data on MOTTS was excluded from the analysis. It is important that this data is included as it is a potential source of false positive results.

   The isolates identified as MOTT by biochemical tests were included in the analysis of sensitivity and specificity for detection of TB. DST-LJ was not performed for isolates identified as MOTT according to routine practice in the laboratory and so DST-LJ results was not available for inclusion in the analysis. However, none of these isolates represent false positive MDR diagnoses by MODS. Since submitting the manuscript for publication, we have performed LiPA MYCOBACTERIA line probe assay on these isolates which identified 4 as actually *M.tuberculosis* and 3 as MOTT species. The data in
the manuscript has been modified accordingly. Data on MOTT had been added in the results section, as requested (lines 274 - 282).

**Discussion**

4. The authors should discuss the clinical significance of low levels of resistance to INH.

We have expanded discussion of the clinical significance of low levels of resistance to INH in the discussion (lines 436 - 439), as requested. The clinical significance of low-level resistance to INH as defined by laboratory breakpoints has not been established. We agree with the reviewer this would be valuable.

5. The authors have not discussed other rapid culture systems such as thin layer agar which is a serious omission.

Information on Nitrate Reductase Assay and Thin Layer Agar Assay has been added to the introduction to describe the evidence from systematic reviews and the WHO policy statement on the use of noncommercial culture and DST methods (lines 83 - 95).

**B. Minor revisions**

6. For the results in the abstract CI could be provided rather than the numbers found/tested.

The numbers found/tested have been replaced with confidence intervals in the abstract, as requested (lines 43 - 48).

**Introduction**

7. The first sentence should be rephrased. The worldwide occurrence of MDR-TB has been documented, rather than there has been worldwide documentation.

We agree this is an important distinction and have rephrased the sentence, as requested (line 58)
8. The comments on the GeneXpert test should be expanded. The test is called Xpert MTB/RIF. It has been evaluated in many settings, and in some has been shown to give false positive RIF results.

We have replaced GeneXpert by XpertMTB/RIF (line 102).

Discussion on false positive RIF resistance by XpertMTB/R has been added in the introduction (lines 107 - 109).

9. Viet Nam (with a space) is used in the text but not in the title.

A space was added to the word VIETNAM in the title, as requested (line 4).
Reviewer 2: Lucia Barrera

A. Major compulsory revisions

Abstract

1. Results related to cross contamination and risk of misdiagnosis of MDR TB due to the presence of non tuberculous mycobacteria should be mentioned.

We have modified the abstract to clarify this issue “Cording in MODS was unable to correctly identify 3 MOTT samples by MODS resulting in 3 false positive TB diagnoses. None of these samples was diagnosed as MDR-TB by MODS”, as suggested by the reviewer (lines 40 – 42). See also response (3) to reviewer one.

Methods

2. Inclusion criteria might be incompletely described. Detection of TB by conventional culture is too high (52.6%) and does not seem to correspond to suspects newly presenting to a hospital. Was this population screened by other method?

The high TB detection rate is due to the study being conducted at the tertiary referral hospital for TB in Ho Chi Minh City which is a high burden setting. The conventional culture detection rate in the study (52.6%) reflects the normal situation of the TB suspect population presenting to this hospital. Many patients will have been assessed by clinical evaluation and chest X-ray at the primary hospital before referral. This population therefore represents the diagnostic population presenting to the TB reference hospital rather than a general hospital. All patients presenting for TB diagnosis at PNT Out Patient Department and fulfilling stated inclusion criteria were included during the time of the study.

3. Volume of bacterial suspension inoculated in the plates is not specified.
The volume of bacterial suspension inoculated in the plates as a positive control has been added to the methods section, as requested (line 166); 0.5ml suspension was used for preparation and inoculation.

**Results**

4. **Tables are missing.**

We apologise for the omission of all three tables (table 1, 2 and 3) from the pdf file. The tables were uploaded in the website submission but were not included in the final pdf. The tables are now included in the article file.

5. **Specific mutations detected by MAS-PCR are not presented.**

We have added the mutations which can be detected by MAS-PCR in the methods section to clarify (lines 207 – 209). The specific mutations identified by MAS-PCR in the discrepant analysis are presented in table 2 and table 3, which was not in the reviewers’ pdf.

6. **Spoligotyping results are not presented.**

The spoligotyping data was used to identify possible incidents of cross contamination. Genotyping of the isolates was not the primary aim of this study. We therefore have not included spoligotyping data in the interests of brevity. We are happy to include this data in the supplementary table if it is considered of interest.

7. **The title “Resolve discrepancy in resistant isolates…” is not correct.**

The title “Resolve discrepancy in resistant isolates…” has been replaced by “Resolution of discrepant results” (line 319).

8. **Sixth paragraph under this title. Reference 18 is not correct. Interpretation of INH false susceptibility may be eliminated, it is presented in the discussion.**

We would like to thank the reviewer for this point, the incorrect reference has been changed to (Minion et al., 2010 – Reference 9) (line 353). The rationale for mutation
detection is described in the results section. The interpretation is presented in the
discussion.

9. Time to detection: It might be necessary to revise statistical calculation for the
difference between the time to detect grow by MGIT and MODs. Data and curves
(figure 3 ) do not seem to be statistically different.

We have re-checked our calculations and are happy to state that (as reported) the time
to detect MODS is indeed highly significantly shorter than the time to detect MGIT
(p<0.0001 according to the Wilcoxon signed rank test).

Significance of the results can be explained by 3 factors:

- The large sample size (327 samples)

- A clear and clinically relevant difference in median times to positive of 2 days (as
reported)

- As both tests were done on the same samples, a paired test (i.e. the Wilcoxon signed
rank test) was used which increases power.

Of note, the pairing of samples was not explicit in the Figure 3 or in the main text and to
clarify this, we have added information regarding the proportion of patients for whom
MODS was quicker than MGIT to the manuscript (lines 375 – 377).

Discussion

10. Paragraph 10: Risk of misdiagnosis of TB and, eventually, of drug-resistant TB
using MODS method should be commented. It was not possible to differentiate non
tuberculous mycobacteria by cording observation. This may pose a serious
drawback in settings with high prevalence of environmental mycobacteria. Besides,
because of this limitation the method is not advantageous in relation to biosafety
requirements as it is necessary to open the microtrite plates to identify the isolates.
In Viet Nam, more than 90% TB suspects with a mycobacterial infection are infected with *M. tuberculosis*, TB suspects are currently diagnosed by smear microscopy, as in most high burden settings. A smear positive patient will receive TB treatment according to WHO guidelines without isolate identification. If MODS is used in place of or in addition to smear microscopy the detection rate may be higher than smear and therefore case detection rates will increase. We agree with the reviewer that the ability to differentiate MOTT would be helpful, however we do not consider it an essential priority given the limited resources and low prevalence of MOTT among TB suspects in this setting. Application of MODS in low TB burden settings where MOTT represents a significant proportion of cases is unlikely to be optimal due to availability of greater resources and the labour-intensiveness of MODS plate reading. The applicability of MODS in high-TB burden, high HIV-prevalence settings remains to be determined.

B. Minor Essential revisions

**Methods**

11. Avoid repetition of information (e.g. reference methods are presented under titles Definition and Statistics).

   We have merged and revised the paragraphs ‘definition’ and ‘statistics’ under a single heading to avoid repetition *(lines 225 – 247)*.

**Results**

12. Text under the title MAS-PCR corresponds to Methods or, eventually, to discussion.

   We agree with the reviewer. We have moved the ‘MAS-PCR’ paragraph referred to by the reviewer from the results section to the methods section *(line 212 – 215)*.

13. Below the title “Resolve discrepancy…,” in paragraph 1: Four (not three) samples were discrepant for RIF resistance.
Overall, there were 4 samples discrepant for RIF resistance. Of these, three samples were discrepant for RIF resistance only and one sample was discrepant for both RIF and INH. We have revised the sentence to clarify this point (lines 322 – 324).

**Discussion**

14. It is worth considering the direct DST-LJ should have produced earlier results than indirect DST-LJ.

We agree that direct DST-LJ would have produced earlier results than indirect DST-LJ. However, results using direct DST-LJ are inconsistent and not reliable due to bacterial clumping and low bacterial load in sputum or body fluid samples. Therefore, indirect DST-LJ has been recommended as the gold standard for detection of drug-resistant isolates by WHO and it is the generally accepted reference standard for DST of *M. tuberculosis*. Furthermore, indirect DST is the currently applied technique at this hospital and in most reference laboratories in high burden settings and therefore we considered it the appropriate comparison to current practice for turnaround time.

**Acknowledgements**

15. Revise first sentence.

We have revised the acknowledgements as requested (lines 482 - 484).

**References**

16. It should be presented in accordance to instructions for authors.

The reference format has been corrected.

C. Discretionary revisions

17. Nitratase and colorimetric methods are not mentioned in the introduction where the authors bring up the tests endorsed by WHO for rapid detection of MDR TB.
Information on Nitrate Reductase Assay and colorimetric methods has been added to the introduction (lines 83 - 95).

18. Sample collection section may be eliminated from Methods. It is no relevant.

The sample collection section has been removed from the methods, as requested.
Reviewer 3: Howard Takiff

A. Major compulsory revisions

1. The data is probably sound, but the three tables described in the text were not included in the material to review, so it is difficult to assess completely the adequacy of the data in supporting the conclusions. The introduction and discussion are a bit long.

We apologise for the omission of all three tables (table 1, 2 and 3) from the pdf file. Tables were uploaded in the website submission but were not included in the final pdf. The tables are now included in the article file.

2. The intro reviews other techniques, which is appropriate but could be more concise.

There have been several policy statements issued by WHO in recent years on novel TB diagnostics, we therefore aim to put MODS in context with recent development in the field. Reviewers 1 and 2 have requested the addition of information on Thin Layer Agar (TLA), Nitrate Reductase Assay (NRA) and colorimetric methods in the introduction section (lines 83 – 95).

3. The discussion also needs to be shortened, focused and tightened

The findings of the study are surprising because the sensitivity of MODS for the detection of both INH and RIF resistance is lower than reported in other settings. We therefore discussed the potential reasons for this and believe that it is appropriate. The question of whether 0.4µg/ml or 0.1µg/ml is the optimal breakpoint for INH resistance in MODS is controversial and we have included our opinion that the use of 0.4µg/ml results in low sensitivity for the detection of INH resistance which is supported by our data and is also the conclusion of a recent meta-analysis.

4. The authors should state why MGIT wasn’t also used for detection of resistance.

MGIT-DST is too expensive for use in this setting (lines 80 – 82). As stated above in response to reviewer 2, DST-LJ is the currently applied technique at this hospital and in most
reference laboratories in high burden settings and therefore we considered it the appropriate comparison to current practice. Financial limitations for the study also precluded the use of MGIT-DST as the reference standard.

5. The results of sequencing should be shown in detail. Perhaps this data is in the missing tables.

The relevant results of sequencing are presented in table 2.

6. Besides promoter mutations, mutations in the inhA coding region also confer resistance.

We agree with the reviewer that other resistance mechanisms besides inhA promoter -15C-T and katG 315 mutation are likely to be responsible for the INH resistance in some discrepant isolates. Mutations in the inhA coding region are rare and they also confer a low MIC to INH so may represent a likely culprit. Mutations in the inhA coding region have not been identified to date in this setting (unpublished data). We therefore did not investigate the inhA coding region as the reason for discrepant INH results. Financial constraints prevented further investigation of these factors, but we agree with the reviewer that data which explains the reasons for discrepancies between different DST methods for M.tuberculosis would be valuable.

7. Without the tables it is hard to follow how the discrepant results were resolved.

Please see our response to point 1.

8. More data on the cross contamination would be helpful…if two isolates had the same spoligotype, but it’s one of the most common ST’s in the population, it might not be cross contamination.

Spoligotyping was used to evaluate possible cross contamination. As mentioned by the reviewer if two STs were identical we were not able to exclude cross contamination. We have added a sentence to clarify this for the reader (lines 245 - 247). However, the maximum possible cross contamination rate in this study was 1.4% which is very low for a liquid culture technique and confirms the findings of previous studies which show that cross
contamination rates are low for the MODS technique. We therefore did not consider the spoligotyping data to be essential to the manuscript and have not included it in the interests of brevity. We are happy to include the spoligotyping data as a supplementary table if the editor considers it of interest.

9. If the gold standard is LJ, how was the performance of LJ evaluated? Compared to MGIT?

MGIT and LJ were the gold standard reference tests. Therefore they were not methods under evaluation.

10. H37Rv is used as a control but not mentioned in the Methods section

H37Rv was used as a control and is mentioned in the methods section: “Daily, one susceptible clinical isolate (H37Rv), one INH-resistant clinical isolate and one RIF-resistant clinical isolate were used as the controls. A McFarland 0.5 (approximately 10^4 CFU/ml) suspension of each isolate was made and diluted. 100-fold (10^2 CFU/ml). A 0.5ml volume of the final suspension was used as the inoculum.” (lines 163 - 167).

11. The section “DST results by MODS” and the following section should be combined. The way the results are separated makes it more confusing, and not only in this section.

We have edited and merged these paragraphs as suggested (lines 298 – 301).

12. The Results section could be written more concisely to make it more easily understood. The way it is written makes the reader have to work to figure out what the authors wish to say, especially in the section on discrepant results. Having the tables should make this easier, but even with them, the writing in the results needs to be improved and tightened.

The omitted tables are included in the resubmission. We have revised the results section.

13. Were discrepant results repeated on LJ?
The discrepant results were not repeated on LJ. Resolution of discrepant results was attempted using a third method (MAS-PCR) in order to detect possible mutations which would confirm resistance in the isolates.

14. How is it that some isolates weren’t available to test again? What about the control wells without antibiotics?

The control wells were sampled and subcultured on LJ. However, in some cases, the subcultures did not grow. This is probably due to the very low volume of sparse culture in the MODS wells (1ml).

15. In comparing time to results, do the authors really intend to use the median time (half of the tests were completed in a shorter time, half longer), or is it the mean (average) time they wish to indicate?

We would like to clarify that we indeed wish to summarize times by their median and interquartile range. We believe that this gives a more appropriate summary of the time to positive (which has a rather skewed distribution) than the mean.

16. In the last paragraph of the Results, “The final fungal contamination rate of MGIT and LJ” should be “rates”. There are many examples of errors like this.

We have corrected this error, as requested. (lines 398).

17. In the last sentence before the discussion, what is the meaning of “nonsense mutation on ropB gene by sequencing and resistant by DST-LJ”? A nonsense mutation generally means a stop for translation (UGA, UAG or UAA), but that couldn’t be possible because rpoB is essential. Do the authors mean a mutation that doesn’t alter the amino acid encoded? The sentence is incomprehensible.

We have corrected this error to ‘synonymous mutation’ (line 335).

18. The principal problem is that the manuscript is very sloppily assembled. There are all manner of typing mistakes, awkward prose, bad grammar, verb agreement
mistakes, plurals that should be singular and visa versa, and other outright errors (ropB should be rpoB). Also the three tables were not included.

We apologise for these errors and we have corrected the manuscript (lines 327, 334, 340, 442).

19. For example, the first sentence of the Results section “Detection of drug-resistance”... “Although there were 373 samples positive by either MGIT or LJ, 9 samples were identified as Myco other than TB (M.fortuitum or M. Chelonae) which were identified by standard biochemical tests and therefore DST-LJH was not done for these samples.” This sentence has so many errors it’s hard to know where to begin: “there were 373 samples..., 9 sample were identified as ....which were identified” Not all MOTT are M. fortuitum or M. chelonae. If they were identified as these, that should be stated. Why include the abbreviation MOTT if it is the only time it is used, except in Figure 1, where it is defined again in the legend? If biochemical tests were performed, mention of these belong in the Materials and Methods section.

We have revised this section of the results, as requested (lines 272 – 285).

The biochemical identification test are mentioned in the methods section “All cultures positive by MGIT or LJ were subcultured on LJ (Becton Dickinson) for indirect DST, standard biochemical identification (Niacin and Nitrate) and archiving“ (lines 188 - 189).

20. Perhaps some of the awkward syntax could be explained by English not being the author’s mother tongue, but that doesn’t explain or justify the sloppy preparation of the ms. Some of the co-authors have names that suggest they might be native English speakers who should be able to resolve some of the awkwardness of language.

We have corrected the manuscript, as requested.

21. For example, in Methods, “Exclusion criteria....or a prior DOSE of TB therapy.” Does that mean prior treatment, or having taken even a single dose of TB therapy?
A prior dose of TB therapy means a patient was excluded from the study if they had taken a single dose of TB therapy, as interpreted by the reviewer (line 133).