Author's response to reviews

Title: Utility of the REBA MTB-Rifa(R) Assay for rapid detection of rifampicin resistant Mycobacterium tuberculosis

Authors:

Eunjin Cho (goodgene75@hanmail.net)
Isdore Chola Shamputa (shamputa@gmail.com)
Hyun-kyung Kwak (sw805@hanmail.net)
Jiim Lee (jirosenin@naver.com)
Myungsun Lee (darsunny21c@gmail.com)
Soohee Hwang (mimimaau@gmail.com)
Doosoo Jeon (sooli10kr@yahoo.co.kr)
Cheon Tae Kim (kct1082@korea.kr)
Sangnae Cho (raycho@yonsei.ac.kr)
Laura E Via (lvia@niaid.nih.gov)
Clifton E Barry III (cbarry@niaid.nih.gov)
Jong Seok Lee (cosmosljs@gmail.com)

Version: 2 Date: 3 October 2013

Author's response to reviews: see over
Reviewer: Susan S Dorman

Reviewer's report:
The authors report results of a diagnostic accuracy study that assessed the REBA MTB-Rifa assay using a large set of Mtb clinical isolates as well as microscopy smear positive sputum specimens. As applied to Mtb clinical isolates, sensitivity and specificity of the investigational assay were 98.1% and 100%, respectively. As applied to smear microscopy positive sputa, sensitivity and specificity of the REBA assay were 100% and 100%. Several novel mutations associated with phenotypic resistance to rifampin were identified and the implications were discussed. Strengths of this study include the number of specimens tested among which a high proportion were rifampin resistant, and the comprehensive assessment of the tested specimens (i.e. MICs determined, DNA sequencing performed, phenotypic DST performed).

Author response: We thank the reviewer for the positive comments.

MAJOR COMPULSORY REVISIONS
1. In the results section, please provide results for the specificity of the REBA test.

Author response: We have modified Table 1 to include both sensitivity and specificity and added appropriate language in the results section.

2. Some of the Conclusions in lines 341-344 are not clearly justified by the data and results presented. Specifically, the results do not address ‘utility’, ‘availability’ or timing of the REBA test, and do not really address testing of isolates from the Korean peninsula and surrounding regions. The sentence might be more accurately changed to ‘The findings reported herein support the accuracy of the REBA MTB-Rifa assay for the detection of RIF resistance on clinical isolates and smear positive sputum samples in South Korea.’

Author response: We agree with the reviewer’s comment and have changed that sentence accordingly.

MINOR ESSENTIAL REVISIONS
1. Inclusion of a flow diagram would be helpful to a reader in keeping track of the specimens tested and the results of investigational and conventional assays, and would be in keeping with the STARD recommendations for reporting of diagnostic accuracy studies.

Author response: We have added this as suggested as Figure 1.

2. Please clarify if tested sputa were from some of the same participants as the tested Mtb isolates – the Methods section is not clear on this point. The STARD diagram could include this information.

Author response: A figure was added as figure 1. As is clear in the flow diagram though the isolates were collected from study participants but the sputa were collected from a separate cohort that was being done as clinical
service (the anonymous results of which were exempted by the local IRB).

3. Please provide the confidence interval (e.g. 95% CI) around estimates of sensitivity and specificity. Also, recommend handling proportions consistently throughout with respect to number of places after the decimal point and with respect to inclusion of numerators and denominators.

**Author response:** The confidence intervals were added to the results section. And we have adjusted the number of significant figures to be consistent throughout manuscript.

4. In lines 264 and 266, it is unclear why the MIC values are provided as ‘less than or equal to’. The Alamar blue method should have provided an MIC value (or a narrow range of MICs). Does less than or equal to 12 mean that the MIC might be 0.25, or 1, or 2 or 12?

**Author response:** The highest drug concentration used was 64ug/ml and if isolates were still resistant in 64ug/ml, we noted it as >64ug/ml. For the lower MIC levels, we removed the ≥, > notations as these are real MICs.

**DISCRETIONARY REVISIONS**

1. Lines 272-274: would consider replacing ‘detection rate’ with ‘sensitivity’ or perhaps more correctly ‘sensitivity for detection of rifampin resistance’.

**Author response:** As suggested.

2. Discussion lines 330-338: in support of the applicability of the REBA test beyond the NMH population is the specificity of the assay as determined by your study: 277/277 (100%) as performed on clinical Mtb isolates. This might be worth mentioning.

**Author response:** As suggested.

**Reviewer:** Howard Takiff

**Reviewer's report:**

The manuscript from Cho et al describes the use of the REBA Mtb Rifa line probe assay to detect rifampicin resistance in clinical isolates and sputum samples. The work was well done and the study is interesting and perhaps relevant, especially in light of the many MDR and XDR cases in South Korea. The REBA assay seems to perform well with isolates and AFB positive sputa.

**Author response:** We thank the reviewer for those positive comments.

**Major Compulsory Revisions**

1. The only serious deficiency is that there is no real comparison with the Xpert system, which has been widely accepted for detecting Rif resistance.
The authors mention that the GeneXpert system is expensive and that the incorporation of new rpoB targets is complicated, but don’t provide adequate information to show the advantages of the REBA assay. If the authors are proposing the REBA system as a viable alternative technique, they need to give accurate estimates of the cost, labor involved and overall time to obtain a result with the REBA system, and describe how it might be employed in the daily workload in a setting with a TB and resistance burden similar to that of South Korea, and perhaps also its usage in other settings.

Author response: We agree with the reviewer but it is difficult in a country like South Korea to do this cost comparison in a meaningful way because the manufacturer of GeneXpert makes no pricing concessions and there is little consistency to date in cost of either machines or cartridges. In the precise setting where we work the GeneXpert (at $52 per cartridge) is about twice as expensive as commercial LPAs but that is likely to be very laboratory dependent, and advantages like ease of use and technical simplicity are more important in some contexts than others. Our point is that in settings with suitably trained personnel LPAs may still have some advantages, including the ability to rapidly incorporate new resistance alleles and cost. We’ve tried to capture this a little better by changing lines 109-115 in the revised version to read; ‘Despite the simplicity and utility of self-contained diagnostic tests such as the GeneXpert system, these are twice as expensive as LPAs in a country like Korea that does not qualify for preferential pricing considerations. Since a large fraction of Korean TB patients are indigent the price of such testing can be challenging. In addition, reengineering cartridges to take advantage of newly discovered resistance alleles is not trivial [14]. In settings where sufficient technical and microbiological expertise is not limiting, and self-contained molecular tests are expensive, LPAs offer an affordable, accurate and flexible alternative.’

2. In the discussion the authors try to indirectly address the XPERT’s lauded ability to detect M. tb and RIF resistance in AFB negative sputa, but if this point is included, it should be more directly addressed in a comparison of XPERT to REBA. The XPERT system has been so widely praised and advocated that it is now incumbent upon studies with other techniques to demonstrate their relevance, beyond simply a catalogue of the rpoB mutations found.

Author response: We did not assess the performance of the LPA assay in smear negative sputa, mostly because the national TB referral hospitals see very few smear negative cases so we cannot make a direct comparison. To make this clearer in the text we have added the following sentence to the conclusion of the discussion: “A limitation of this study is that the REBA MTB-Rifa® assay was not applied to sputum smear negative cases. This is primarily because our recruitment site predominantly treats confirmed TB cases in smear positive patients. The samples used in this study, therefore may not be representative of the entire South Korean TB population but are a fair representation of the difficult-to-treat TB cases being seen in the national reference hospitals.”
3. The inclusion in the title of RIF resistance correlating with MDR-TB is perhaps unnecessary, as this correlation has been well established. The inclusion of the new rpoB loci that are present in 1% of isolates from South Korea may not be justified for kits used in other parts of the world, but perhaps these regions of the gene have not been systematically examined in studies from other countries and may be more prevalent than thought.

**Author response:** We agree and have modified the title accordingly.

4. It would be worth reviewing the literature to see how many previous studies would have identified this mutation in Rif resistant isolates, but that is not essential for this publication.

**Author response:** We reviewed the literature most appropriate ones.

5. The manuscript is generally well written, although a number of minor problems with the writing are listed below, in detail, as BMC has no copy editor. The main problems are in the Discussion and Conclusions, which seems to have been less carefully edited than the other sections. It is somewhat long and rambling, with occasional run-on and confusing sentences that don’t clearly explain the points the authors wish to make.

**Author response:** We have carefully rewritten many parts of the Discussion and Conclusions to ensure it is consistent throughout.

6. The 2 novel mutations don’t seem to be included in Table 2.

**Author response:** On closer inspection we identified three novel mutations, for clarity we have bolded these in Table 2 and added a footnote to that effect.

Minor compulsory and discretionary points:

7. Background line 109: this intro is a bit too long with a repetition of things that have been stated in many articles.

**Author response:** We have shortened and better focused the introduction.

8. Line 111 “like GeneXpert COMMA

**Author response:** As suggested.

9. Line 113 - 4 The phrasing is awkward, and pricing details on XPERT might help make the argument for the REBA technique.

**Author response:** We have modified this to stress the pricing difference as suggested by both reviewers 1 and 2.

10. Line 127- 130 This sentence is long and awkward and slightly confusing.

**Author response:** See suggestion and response for reviewer 1 as well,
because this was unclear we have added a new figure and simplified the text to avoid confusion.

11. Line 172 WAS used

**Author response:** As suggested.

12. Line 174 – omit one “of”

**Author response:** As suggested.

13. Line 199 “deposited” seems inappropriate here if it means affixed to the membrane

**Author response:** We have clarified this is perpendicular to the pre-affixed oligonucleotide probes.

14. Discussion:

**Author response:** As suggested.

15. Line 273 than “in” other reports

**Author response:** As suggested.

16. Line 282 maybe eliminate “tendencies”

**Author response:** As suggested.

17. Line 264-5 was higher than in Europe, Africa and the United States, and the mutation frequency of His-526...

**Author response:** As suggested.


**Author response:** We agree, we have removed “geological” and changed this to strain genetic differences.

19. Line 292 high level phenotypic resistance (eliminate “of”)

**Author response:** As suggested.

20. Line 292 – 3 The sentence is awkward; how about something like, “frequency and distribution of mutations was similar in both sputum samples and RIF resistant isolates, however some mutations and most of the double mutations seen in the isolates were not found in the sputum samples.”

**Author response:** See response to reviewer’s comment #21
21. Line 295-97 Unclear what the authors wish to assert.

**Author response:** *We have extensively revised this paragraph to make this more clear.*

22. Line 299 – 310 This paragraph is awkward and confusing and should be rewritten.

**Author response:** *We have extensively revised this paragraph to make this more clear.*

23. Line 317-8 Please rewrite this sentence with only one “previously”, and if preserving the current form please change to ... but “it” has been noted.

**Author response:** *As suggested.*

24. Line 321 – Consider adding “sterically” preclude

**Author response:** *As suggested.*

25. Conclusions:The second sentence is long, complicated and confusing. It should be broken up and rewritten.

**Author response:** *As suggested.*

**Reviewer:** Miguel Viveiros **Reviewer's report:**

**Major Compulsory Revisions:**

Line 73-74 – Background - the sentence “XDR_TB...resistance to a fluoroquinolone and any injectable except streptomycin ...” should be revised according to the consensus definition for XDRTB from WHO, ATS, IUTALD..etc.

**Author response:** *As suggested.*

Line 74-75 – Background - the sentence “Treating patients infected with MDR-TB requires” should be revised.. MDR_TB is a clinical condition of the patient/disease and not an infectious agent.

**Author response:** *We have removed ‘infected’ and changed to ‘Treating patients with MDR-TB requires …’ as suggested.*

Line 87 - Background - the sentence “…of isolates can be correctly identified as resistant or sensitive” should be revised. Sensitive should be replaced by susceptible a more appropriate designation in laboratory medicine.
Author response: As suggested.

Line 103 - Background - The sentence “TB therapy for drug sensitive disease” should be revised. Sensitive should be replaced by susceptible a more appropriate designation in medicine and chemotherapeutics.

Author response: As suggested.


Line 108 – Background - The citation 12 and 13 are appropriate but the difficulties and less accuracy involved in the molecular detection of INH resistance are better detailed in the review work of “Alcaide F, Coll P. Advances in rapid diagnosis of tuberculosis disease and anti-tuberculous drug resistance. Enferm Infecc Microbiol Clin. 2011 Mar;29 Suppl 1:34-40.

Author response: We replaced Gegia M, et al., with Alcaide et al as suggested (ref 11 in the revised version).

Line 115 – Background - The sentence “In contrast, LPAs are extremely cost-effective and redesign to accommodate new mutations is straightforward” should be re-written since LPAs are also expensive if we consider the price of the positive and negative control in each assay plus equipment and trained personnel needed, Not as expensive as gene-expert but expensive as well and it’s not extremely cost-effective compared with Gene-Expert. Both are
cost-effective if we include the cost of management of a non-early-detected MDRTB patient but that applies to all molecular techniques for direct detection of MDRTB. The redesign of LPAs to accommodate new mutations it is not straightforward.

Author response: We agree with the reviewer but it is difficult in a country like South Korea to do this cost comparison in a meaningful way because the manufacturer of GeneXpert makes no pricing concessions and there is little consistency to date in cost of either machines or cartridges. In the precise setting where we work the GeneXpert (at $52 per cartridge) is about twice as expensive as commercial LPAs but that is likely to be very laboratory dependent, and advantages like ease of use and technical simplicity are more important in some contexts than others. Our point is that in settings with suitably trained personnel LPAs may still have some advantages, including the ability to rapidly incorporate new resistance alleles and cost. We’ve tried to capture this a little better by changing lines 109-115 in the revised version to read: ‘Despite the simplicity and utility of self-contained diagnostic tests such as the GeneXpert system, these are twice as expensive as LPAs in a country like Korea that does not qualify for preferential pricing considerations. Since a large fraction of Korean TB patients are indigent the price of such testing can be challenging. In addition, reengineering cartridges to take advantage of newly discovered resistance alleles is not trivial [14]. In settings where sufficient technical and microbiological expertise is not limiting, and self-contained molecular tests are expensive, LPAs offer an affordable, accurate and flexible alternative.’

Line 139-146 – Methods - Clinical isolates and drug susceptibility testing – the description of the DST protocol is missing as well as the standard references for this procedure.

Author response: We have added a standard reference to the methods section along with a brief description of the standard methodology – please see lines 129-135 and reference 16.

Line 190 - Methods - REBA MTB-Rifa® assay – “Specific oligonucleotides are immobilized at known locations on a membrane strip” please describe which ones are immobilized and which sequences they detect since these are the targets that allow the completion of the proposed objective of this paper “improved reverse blot assay (REBA MTB-Rifa®) for early detection of RIF resistance and assessed its ability to predict RIF resistance for MDR-TB in South Korea”

Author response: We have added full details on the probes, please see revised text lines 169-172.

Line 208 – Methods - PCR amplification and sequencing of the products – analysis of the sequenced products is missing – How it was done?
Author response: We did sequence analysis using CLC Main Workbench (CLC bio, Aarhus, Denmark) and have now added this to the methods section lines 199-200.

Line 220 - Results - Drug susceptibility patterns of MTB isolates – This paragraph and these results are better presented in a table and the results of the drug susceptibility patterns of MTB isolates of such an extensive work (492 M. tuberculosis isolates between 2005 and 2008). deserve a better presentation and analysis. The high percentage of XDR-Tb should be addressed and discussed. It’s a very important finding.

Author response: We agree that this is important but a full description of the cohort, long-term outcomes and their association with DST patterns is being prepared separately and will be submitted within the year.

Line 230 - Results - Detection of Rif resistance using the REBA MTB-Rifa® assay on DNA from cultured samples – “Alterations in the RRDR of rpoB were detected in 211 of the 492 samples analyzed. As expected, the majority of the mutations (162, 77%) detected involved three codons; 516, 531 and 533 with codon alone contributing more than half (121, 57.3%) (Table 2) of the mutations.” This sentence is very confusing for the reviewer that is used to work and analyse LPAs. Following the REBA MTB-Rifa® assay package insert and protocol the technical procedure can only validate well defined polymorphisms in codons 516, 526 and 531 plus eventually a new mutant probe (533CTG-CCG mutant probe referred in line 279) – These are the only 3-4 mutation probes that are apparently included in the improved version of the REBA MTB-Rifa® assay and the results is valid only when hybridization with the mutant probe is present simultaneously with absence of hybridization with the respective wild-type probe. If this assumption is correct it’s difficult to understand how the REBA MTB-Rifa® assay could detect all the mutations presented in table 2. The only explanation for this analysis of the results implies that the authors have merged the genomic data from the MTB-Rifa® assay with the sequencing data. This merge biases the results presented in table 1 as well as the performance and accuracy of the assays for the goals presented in the introduction of this paper. The authors are invited to analyse separately the results obtained from the application of the assay from those obtained by sequencing of rpoB gene.

Author response: We’re including the package insert for the test with this response which instructs users that failure to observe a band in a WT allele without observing a band in any mutant probe is to be interpreted as RIF-R. This is the case in most TB LPA tests we are aware of and there are numerous publications that make the same conclusion, for examples see PMIDs: 16825346, 21191055, 15728936, 20335420, 22236854. The much more widely used Haine MTBDRplusV2 uses the same algorithm (detailed in their product insert located at http://www.ipaqt.org/wp-content/uploads/2013/02/MTBDRplusV2_product-insert.pdf) - absence of a single WT band and absence of a mutation band is interpreted as RIF-R. We’re no more comfortable with that than the reviewer is which is why we confirmed these by sequencing and as it turns out the advice in the package
insert is probably correct and one can infer from absence of a WT allele (with all other controls working obviously) that there is a mutation other than that represented by the MUT probes in that particular kit.

Line 245 - Results - Detection of rifampicin resistance with the REBA MTB-Rifa® directly on smear positive sputum samples - Contrary to many published studies on direct detection of mutation for Rif resistance on smear positive samples the authors had no false negatives (amplification negative with culture positive) by lack of amplification, especially using a simple boiling protocol for extracting MTB DNA from the smear positive samples. This result is also very puzzling.

Author response: We agree it is surprising but since we only examined smear positives and our setting is in a referral hospital that sees primarily very sick patients this may explain the sensitivity of the assay. We have added a clear caveat to the end of the Discussion section to emphasize that this population may not be representative of all patients because of this.

Line 268 - Discussion – “It is worth noting that the 98.14% detection rate of Rif resistance reported in this study constituted all the M.tuberculosis isolates that harbored mutations in the RRDR of the rpoB gene, the target upon which the assay is based. Unless the new version/improved version of the REBA MTB-Rifa® assay includes all the mutations detected in this study, and this should be clearly detailed in the materials and methods section, this conclusion cannot be taken from the results presented. If the post-assay sequencing analysis of the RRDR of the rpoB gene was included, then the conclusion is obvious and reflect the molecular/mutational epidemiology data of the sample analyzed. This is exactly what the authors do from lines 282 to 297 – analysis of the mutational profile of the samples studied.

Author response: Please find the new Figure 1, in which we tried to give a clearer picture of the results in context. “It is worth noting that the 98.14% detection rate of Rif resistance reported in this study constituted all the M. tuberculosis isolates that harbored mutations in the RRDR of the rpoB gene, the target upon which the assay is based. ” was changed to “It is worth noting that the 98.1% Rif resistant isolates and 100% of smear-positive/culture positive sputum samples with Rif resistance in this study harbored mutations in the RRDR of the rpoB gene, the target upon which the assay is based. ”

Line 336 – “...source of the samples (NMH) being the biggest TB national reference hospital, and it does not negate the fact that the REBA MTB-Rifa® assay has been demonstrated to be as good as or better than other LPAs in detecting Rif resistance “. This conclusion cannot be taken form the data presented. The REBA MTB-Rifa® assay was not compared against any other LPA assay in this study.

Author response: The reviewer is correct, we have removed the sentence because no such comparison has been done in this study.
Manufacture’s instruction of REBA MTB-Rifa(R) Assay

- Please see the Table in section #6; Data interpretation, in page 3.

M&D REBA MTB-Rifa® is a rapid and simple molecular diagnostic kit that can determine rifampin susceptibility in MTB using PCR-Reverse Blot Hybridization Assay (REBA).

As more MTB that are resistance to drugs are discovered, tuberculosis is becoming threatening disease worldwide and multi-drug resistant bacteria are resistant to rifampin and isoniazid at least. Rifampin resistance of MTB caused by mutation appearance in some region of rpoB gene encoded β-subunit of RNA polymerase. This kit detects rifampin resistance of MTB by Reverse Blot Hybridization Assay (REBA) for the amplified product of region related rifampin resistant. Through PCR by using PCR premix included in Taq polymerase and biotin labeled PCR primer amplifies rpoB gene in the clinical specimen. Amplified PCR product goes through Reverse Blot Hybridization Assay (REBA) with oligo-probe of wild-type and mutant-type of rpoB bounded to REBA membrane. Finally, resistance for rifampin is determined by chromogenic reaction of hybridization of wild-type or mutant-type probe reacts with biotin labeled PCR product.
2. Product characteristics

1) Rapid and accurate detection of rifampin resistance from clinical specimens and cultured isolates.
2) Has high sensitivity and specificity.
   ⇒ 2–20 bacilli/reaction (smear 1 +; 10⁴ bacilli/ml)
3) Able to obtain identical product results as conventional culture-based DST
4) Simplified test procedure.
5) No need for expensive equipment; economical.
6) In the process of KFDA approval.

3. Basic information of REBA MTB-Rifa®

1) Rifampin resistance is a surrogate factor of multi-drug resistance.
   ⇒ Rifampin resistance is due to mutation of 69 bp hot spot area of rpoB gene, and well known to be related to it more than 98% of rifampin resistance
2) REBA MTB-Rifa® improved problems in conventional DST.
   ⇒ Because it takes few weeks to get final DST result, it has been an obstacle to rapid diagnosis and appropriate medical treatment. Therefore, rapid detection of resistant MTB strains will be able to quick diagnosis of disease and effective medication.
   ① Can be detected from cultivated or uncultivated clinical specimens
   ② Simple procedure and accurate detection using wild and mutant-type specific probe
3) Below is the information on specific probe that detects rifampin resistance and rpoB gene area targeted by REBA MTB-Rifa®.

![Diagram of rifampin resistance related region]

![Diagram of specific rifampin resistance probe region]