Author's response to reviews

Title: Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis

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Author's response to reviews: see over
Dear BioMed Central Editorial Team,

Thank you and the four reviewers for your thorough consideration of our manuscript. We have carefully addressed the issues raised by the reviewers and have modified the manuscript accordingly. Below, please find our responses to the questions raised, followed by a point-by-point listing of changes to the manuscript which are indicated using italic font.

**Reviewer 1: Dr. Robert Colebunders**

1. The typing error in the discussion has been corrected.
   
   **Discussion:** Unfortunately, we are unable to provide information what exactly are the differences between the two versions of the test.

2. The majority of HIV-infected patients had not started antiretroviral therapy at enrolment.

3. The reviewer asks about a hypothesis why the DLR was high in patients with fever. We assume that fever is a kind of unspecific marker of TB and that it therefore increases the positive DLR. However, theoretically it is also possible that fever somehow changes the excretion of LAM so that the test gets more specific without a total loss of sensitivity.
Reviewer 2: Dr. Ben Marais

1. The reviewer advised to shorten the explanations on diagnostics likelihood. Please see the shortened description below.

Materials and Methods: *Diagnostic test performance (Sensitivity, specificity, predictive values and likelihood ratios)* was calculated only in the groups with defined TB status (A&B as positives, C as negatives) using the “diagt” Stata component. The diagnostic likelihood ratio (DLR) [26] compares the probability of obtaining a correct test result with that of containing an incorrect test result for positive and negative test results respectively. Good tests should have a positive DLR that is well above unity and a negative DLR that is close to zero. DLR calculation uses the following formulas: positive DLR = sensitivity / (1-specificity); negative DLR = (1-sensitivity) / specificity.

2. We agree that well-defined symptoms are important in order to standardise diagnostic evaluations. A table of definitions is found in the Addendum.

3. The reviewer asked for the proportion of HIV-infected patients who have sputum-smear positive TB. We included further statistics in regard to the TB and HIV prevalence.

Results: The TB prevalence in HIV positive patients with defined TB status (groups A, B and C) was 57.5 %, and 26.7 % in HIV negative patients of the same groups. 18 % of all HIV-infected patients (n = 291) had sputum smear-positive TB.

4. We performed uni- and multivariate regression analysis to answer the question if the difference between the diagnostic performance in men and women can be explained by the fact that women are more often HIV infected. In this case HIV infection would be a confounding factor. The analysis in regard to sensitivity showed that both factors have an independent influence (sensitivity, multivariate analysis: HIV status, p = 0.040, RR = 2.62; sex, p = 0.118 [non significance most likely due to small sample size n=69], RR = 0.69).

Results: When combined in a multivariate Poisson regression model with LAM sensitivity as the outcome (true positives coded as “0”, false negatives coded as “1”), the influence of HIV status and sex was less prominent than in separate univariate models. However, HIV status still had a significant association with LAM sensitivity (P = 0.040), and the association of sex with LAM sensitivity was still evident, although not significant anymore (P = 0.118).

5. The language error has been corrected.

Results: The overall sensitivity of the LAM-ELISA in patients with culture confirmed pulmonary M. tuberculosis infection (groups A and B) was 50.7 %

6. In accordance to the reviewer’s comment, the additional explanation for the interpretation of the positive DLR has been deleted.
Results: This is also reflected by a strong increase in the positive DLR (from 4.16 to 19.6) that is not totally offset by the increase in the negative DLR (0.56 to 0.77).

7. To avoid misunderstanding, figure 1 has been changed. In the new version, the outside values are also shown. We have chosen box plot as a common way to visualise numerical data.

8. We think further investigations are needed to answer questions on the role of glomerular integrity and LAM excretion since no scientific data have so far published on this issue.

9. Following the advice of the reviewer, we introduce the terms “organism load” instead of “burden of diseases.

Discussion: Detection of M. tuberculosis antigens especially in body fluids other than sputum has the following theoretical advantages: a) opportunity to quantify the organism load, b) possibility of high specificity, c) independence of a functioning immune response, d) applicability also in extrapulmonary TB [29].

10. We agree that it was rather unlikely that the combination of the diagnostic test performance with clinical parameters would have added diagnostic advantages. However, we wanted to provide a most comprehensive analysis including important clinical data.

11. We have extended the discussion on positive LAM-ELISA results in patients with non-tuberculous mycobacterial infections.

Discussion: In-vitro analysis has shown that the LAM-ELISA can detect both M. tuberculosis and non-tuberculous mycobacterial species, but the latter only at significantly higher concentrations [19]. At first sight it seems that the LAM-ELISA in our study determines mycobacterial infections other than tuberculosis in almost 9 % of the patients. However, the fact that a similar percentage of controls are also LAM-ELISA positive suggests that the positive results in patients with solely NTM infection are not really attributable to cross-reactivity of the test.

12. We are thankful for the important comment on the effect of more precise reference diagnostics compared to previous studies. If all NTMs in this study were classified in a study without mycobacterial differentiation as TB culture positive samples, the sensitivity would be lower (34.2 %) and the specificity would be unchanged under the assumption that the LAM results remain the same in both situations.

Discussion: Unlike most of the previous LAM-ELISA studies, our study employed molecular biological differentiation between M. tuberculosis and non-tuberculous mycobacteria (NTM) which should actually improve the apparent sensitivity of the test by improving the specificity of the gold-standard. However, despite the use of more precise reference diagnostic, we observed a lower sensitivity instead.

As the reviewer stated, the LAM ELISA theoretically has the potential to diagnose extrapulmonary TB. However, in this study we did not evaluate the assay in this sub-group.
13. We now use in all parts of the manuscript a common definition of fever and the usual notation (≥37.5°C). The definition was chosen under consideration of a variety of definition in the scientific literature (Sund-Levander M, Forsberg C, Wahren LK. Normal oral, rectal, tympanic and axillary body temperature in adult men and women: a systematic literature review. Scand J Caring Sci. 2002 Jun;16(2):122-8).

14. The heading of column 2 in Table 1 has been changed following the reviewer’s advice.

Table 1: Symptoms during 3 months prior to enrolment

15. The Swahili terms for cough (=kikohozi) and chest pain (=maumivu ya kifua [generalized] or kichomi [sharp]) are different. Usually, patients can clearly differentiate between those terms.

Reviewer 3: Dr. Gerd Michel

1. We have added the information that the test is now produced by Inverness Medical Innovations, Inc.

   Introduction: The test is now distributed as Clearview® TB ELISA by Inverness Medical Innovations, Inc., Waltham, USA.

2. Unfortunately, the manufacturer does not provide information about the potential technical factors which might have led to the different performance of the test versions. We regret that details such as the type of used antibodies for antigen capture are not available.

3. Following the advice of the reviewer we have replaced the misleading word ‘unprocessed’.

   Discussion: In the present study, a direct antigen-capture ELISA for mycobacterial lipoarabinomannan in boiled and centrifuged urine is comprehensively evaluated for its diagnostic value.

4. We agree that in this stage high expectation should not be raised. Therefore, we have changed the statement that the test might be helpful as a supplemental tool in HIV-associate TB

   Abstract: The question whether the assay is suitable as a supplemental device in the diagnosis of HIV-associated TB, requires further investigations.

   Discussion: In our opinion, further investigations are needed to elucidate if the LAM-ELISA, in this stage of development, is valuable as a supplemental tool for the diagnosis of HIV-associated TB.

5. We have also added a clear statement that the manufacturer does not disclose important technical details.
Discussion: The manufacturer’s information on reagents and test configuration only provides a broad picture on the principle of the test, but lacks important specifications such as the type of antibodies used for antigen capture.

Reviewer 4: Dr. Stephen Lawn

1. We appreciate the author’s suggestion on how to improve logical structure of our manuscript. We have divided the Results section by inserting sub-headings.
   Sub-headings of the Results section: Characteristics of the study population; Diagnostic performance of the LAM-ELISA; Association between performance of the LAM-ELISA and patient characteristics; Optical density values; Urinalysis results and LAM-ELISA performance.
   We used the new structure throughout the manuscript.

2. We also include recent results presented at the 16th Conference on Retroviruses and Opportunistic Infections 2009.
   Discussion: Data regarding the LAM ELISA presented at the 16th Conference on Retroviruses and Opportunistic Infections 2009 by Mutetwa et al. [31] support our findings. This group reports an overall sensitivity of 44 % and an overall specificity of 89 %.

3. The title has been changed as suggested by the reviewer.
   Title: Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis

4. Following the advice of the reviewer we included the following characteristics in table 1.
   Table 1: Sex, mean age, mean weight, and mean body temperature.
   BMI data can unfortunately not be reported because the height was not measured.

5. Only two patients were pregnant in the observation period. One patient was assigned to group I and therefore her data were not included into the analysis. The second woman was in group C and did not have proteinuria.

6. We kindly ask the reviewer to accept the existing classification. In our opinion it follows a logical concept, since B and B NTM can be clearly differentiated in the entire manuscript.

7. We followed the reviewer’s suggestion and now present numerators and denominators in table 3.
   Results: The proportion of LAM positive patients in relation to their classification group and HIV status is shown in Table 3.

8. The box plot shows the upper and lower quartiles, p75 and p25, and thus the interquartile range iqr = p75 - p25 (box). The adjacent values are the highest value not greater than p75 + 3/2 iqr and the lowest value not less than p25 - 3/2 iqr. The adjacent values are marked graphically on a box plot by the ends of the whiskers drawn out from each central box. All this is in accordance with the usual conventions regarding box plots. The
following link provides further information http://analysis.thomson-pharma.com/SpotfireWeb/(S(1rkxyrmaigeujl55fsflqvtx55))/Help/dxpwebclient/stat_adjacent_values_and_outliers.htm. We are convinced that after adding the outside values to the graph (suggestion of Dr B. Marais) it becomes obvious that the data of all groups are represented accurately.

9. We performed uni- and multivariate regression analysis to answer the question if the difference between the diagnostic performance in men and women can be explained by the fact that women are more often HIV infected. In this case HIV infection would be a confounding factor. The analysis in regard to sensitivity showed that both factors have an independent influence (sensitivity, multivariate analysis: HIV status, p = 0.040, RR = 2.62; sex, p = 0.118 [non significance most likely due to small sample size n=69], RR = 0.69).

Results: When combined in a multivariate Poisson regression model with LAM sensitivity as the outcome (true positives coded as “0”, false negatives coded as “1”), the influence of HIV status and sex was less prominent than in separate univariate models. However, HIV status still had a significant association with LAM sensitivity (P = 0.040), and the association of sex with LAM sensitivity was still evident, although not significant anymore (P = 0.118).

10. In order to render more precisely the differences to the publication of Boehme et al., we have also displayed the proportions in regard to sex and HIV status as recommended by the reviewer.

Discussion: This study by Boehme et al. recruited fewer female (151 f / 140 m vs. 95 f / 136 m; P = 0.014) and more HIV-infected (172 HIV+ve / 119 HIV–ve vs. 147 HIV+ve / 66 HIV–ve; P = 0.023) participants than the present trial.

11. We have extended the discussion on positive LAM-ELISA results in patients with non-tuberculous mycobacterial infections.

Discussion: In-vitro analysis has shown that the LAM-ELISA can detect both M. tuberculosis and non-tuberculous mycobacterial species, however latter only at significantly higher concentrations [19]. At first sight it seems that the LAM-ELISA in our study determines mycobacterial infections other than tuberculosis in almost 9% of the patients. However, the fact that a similar percentage of controls are also LAM-ELISA positive suggests that the positive results in patients with solely NTM infection are not attributable to real cross-reactivity of the test.

12. Following the advice of the reviewer we have also included a discussion on the association between LAM-ELISA result and CD4 counts with emphasis on patients who are considered to be TB negative according to the gold standard.

Discussion: Because the sensitivity of sputum microscopy and culture techniques in HIV infected persons with advanced immunodeficiency is low, it is be theoretically possible that false negative gold standard assessments could have led to a misclassification of some LAM-ELISA results and
thus caused the low specificity of the assay. However, the influence of CD4 counts on LAM-ELISA results adjusted for TB status, HIV status, sex and age in a multivariate model was small and far from significant. Furthermore, the group of HIV+ve, LAM+ve TB-ve persons had relatively high CD4 counts when comparing them to HIV+ve LAM+ve TB+ve. Consequently, this is an unlikely explanation for the low specificity of the test.

13. We added the following to give more information on QA/QC according to WHO guidelines at our laboratories, which are also serving clinical TB trial.

Materials and Methods: The microbiology and the molecular biology laboratory of the Mbeya Medical Research Programme were operating according to standardised protocols and to quality control and assurance procedures.

14. The time period of the recruitment is described as follows.

Materials and Methods: 300 adults with symptoms of pulmonary tuberculosis who had been referred from health facilities of Mbeya urban and rural districts were recruited at the TB clinic of the Mbeya Medical Research Programme between July and September 2007.

15. Two urine samples were obtained at the same day of recruitment (day 1). Two samples were chosen, because the excretion of LAM antigen in urine might possibly be depending on variations during the day.

Methods: Each of the two urine samples that were both collected on day 1 of recruitment was divided into two aliquots for duplicate analysis.

16. The agreement between the LAM-ELISA results of sample 1 and 2 of each patient was 89%.

17. Results: In 89% of all patients both urine samples were either LAM positive or negative, only in 11% a disagreement of the two LAM –ELISA results was found.

18. The majority of HIV-infected patients had not started antiretroviral therapy at enrolment.

19. We added a sentence on the advanced stage of TB disease in our study population.

Discussion: The real life situation is also obvious in the fact that most of the patients were suffering from several TB associated symptoms for more than 3 months prior to enrolment which indicates an advanced state of diseases.

20. The legend of Figure 2 has been changed following the advice of the reviewer.

Figure 2: Maximum optical density of the two samples by participant classification (median [line], interquartile range [box], upper and lower adjacent values [whiskers], and outside values [dots]); N for each category is shown in Table 3.
To the Editor: Sabina Alam

1. We have added details on obtaining informed consent from the patients. Materials and Methods: The purpose and the procedures of the study were explained thoroughly to the attending TB suspects. Only persons who gave voluntarily written informed consent in the presence of a witness were enrolled in the study.

2. We have structured the abstract according to the provided guidelines.

We hope that the revised version of the manuscript meets all requirements for publication.

I remain respectfully,
Yours,

Dr. Klaus Reither