Author’s response to reviews

Title: Evaluation of Mycobacterium tuberculosis viability in OMNIgene-SPUTUM reagent upon multi-day transport at ambient temperature

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Author’s response to reviews:

Dear Reviewers,

Please find enclosed our revised version of the manuscript “Evaluation of Mycobacterium tuberculosis viability in OMNIgene-SPUTUM reagent upon multi-day transport at ambient temperature” by Tagliani et al and the point by point reply to your comments.

We indicated the lines of the manuscript that were revised according to the comments made (reference is made to the manuscript version containing the track changes).

Reviewer reports:

Norbert Heinrich (Reviewer 1): Review INFD-D-17-00730
General: this paper describes a validation study for OMNIGENE sputum, a stabilizing reagent for preserving samples for later culture. The main comparison seems to be between culture and further testing at the site of collection in Albania, and at the site of receipt of OMNIGENE - preserved samples in Milan, Italy, with some delay of testing in Milan. The paper is well written and merits publication after some changes.

The fact that results for the two methods are generated in two differing laboratories is the main weakness of this study, since TB decontamination and culture are procedures that are not very well standardisable and results differ between labs. This is appropriately mentioned in the limitations section. Another weakness is that the number of positive cultures is not extremely high.

Nevertheless the presented data are of great interest; since there is an urgent need for published information on this reagent - which could solve pressing problems in TB sample shipment and remote analysis. The problem of contamination when sending samples for remote analysis in many high burden settings makes quality diagnostics unavailable for a large part of persons in need of testing.

The main statement of this paper is correct in emphasizing that an approach of splitting samples and sending them at room temperature to a distant (reference) lab in OM-S is a viable option, based on the data presented. What this data cannot support are claims of sample processing method’s equivalence due to the mentioned limitation.

The manuscript has been modified accordingly (line 30; line 125-127 and line 135).

In general, analysis at the remote lab would probably benefit of a combination of culture and molecular analysis, since there is some loss of viability observed at remote analysis.

In detail:

Abstract: it is appropriate to add that such transport media will make available quality diagnostics to a large population in need.
Introduction

Line 44: sentence not complete

Text modified accordingly (lines 45-47)

Methods:

Line 77: how was the sample size calculated - or was this a convenience study?

According to our sample size calculation, a minimal sample size of 310 sputum samples was required to achieve a minimum power of 80% to detect a change in the percentage value of sensitivity from 0.70 to 0.90, with 95% confidence interval and an estimated prevalence of smear positive samples of 10% (AFB smear positive prevalence in Albania is between 10 and 15%).

Line 79: how was the sample divided - was there any homogenization done prior to this step? Was sample split into portions of equal volume?

Each sample was manually split into two portions of approximately equal volume using a sterile swab stick and randomly assigned to one treatment method. No homogenization step was performed prior to this procedure.

Line 102: student’s t test requires a normal distribution of the sample, which in my experience in MGIT TTP is not the case.
We thank the reviewer for pointing this out. The distribution is indeed not normal, therefore we re-analyzed the data using a Mann-Whitney U test. Text in the method section was modified accordingly (line 107).

Results:

Line 109: this reads like the entire sample was either decontaminated or treated with OM-S; but in methods there is mention of a splitting of sample. Please clarify.

Text modified accordingly (line 114).

Line 113: if sample are mixed with equal portions of OM-S, and sample volumes differ, how can 1/3 rd of the sediment always be 0.5 ml? Did you resuspend it after spinning with 1,5ml? When comparing TTP, it is important to state fractions of total sample inoculated.

The reviewer is correct, regardless of the decontamination method, sediments were re-suspended in 1,5 ml and 0,5 ml inoculated into each MGIT tube (line 85; line 92). The same procedure was used for both treatment groups to allow for comparison.

Line 117/118: please clarify which samples were analysed where.

Samples in OM-S were centrifuged, inoculated into MGIT tubes and analyzed at the TB Supranational Reference laboratory (SRL) in Milan, Italy (lines 91-93). Samples decontaminated by NALC/NaOH standard method were processed at the TB National Reference Laboratory (NRL) in Tirana, Albania (lines 83-89). Both laboratories collected the variables analyzed in the study, while SRL Milan performed the overall data analysis.

Line 119: since samples with OM-S and NALC treatment are not processed in the same lab, one cannot say that OM-S "reduced" contamination - baseline contamination rates in the two labs might be fundamentally different.
We addressed this comment modifying the text accordingly (lines 125-127).

Line 137: what does "rescued" mean - positive or negative result? Negative here might be false negative. Propose to avoid this word; since this sounds overly supportive for OM-S.

Text has been modified accordingly (line 146).

Line 183: please explain the disadvantages of CPC.

Text has been modified accordingly (line 200)

Discussion: Line 190: how are the 17.7% loss of viability calculated? Overall there where 45 culture pos samples in Nalc/NaOH, of which 9 where negative in OM-S, making 20%?

The original calculation did not take into account one sample for which MTBDRplus testing was also negative since we could not exclude the possibility of it being a “true negative”. However, we have now revised the manuscript taking a more conservative approach and considered in the calculation all 9 culture negative samples out of 45 (representing 20%) (line 206).

Figure 1: change title to "comparison of time to culture positivity between OM-S preservation and Nalc-OH decontamination, dependent on time in OM-S.

Title has been changed following reviewer’s suggestion.

Further, there are four symbols in OM-S groups with a TTP above 41 days; while the methods section states that culture in MGIT was performed for up to 41 days - and I assume all samples without a positive signal up to that time were declared negative. Either update methods or clarify on these.
We thank the reviewer for pointing this out. We used an extended protocol incubating samples up to 56 days rather than 41. The method section has been updated to include this important information (line 93-94).

Max Salfinger (Reviewer 2):

The authors study a very important aspect of the laboratory diagnosis of tuberculosis, the pre-analytical phase.

Title: Is the title indeed appropriate? multi-day transport at ambient temperature?

The title is appropriate since the average transport time was 8 days in the absence of refrigeration.

Line 77-time period/season of the study?

Sample collection: from mid-March to mid-April (2016), with the last samples collected being inoculated at the end of April.

Line 78-first assessed by smear microscopy - direct or concentrated?

Sample were first assessed by direct smear microscopy.

Line 81-processed for MGIT, reference 5 recommends MGIT and LJ - was a LJ inoculated as well?

Our study protocol included only liquid culture by MGIT.

Line 83-MGIT with PANTA?
Yes, all samples, regardless of the processing method (i.e. OM-S treated and NALC/NaOH decontaminated) were inoculated into MGIT tubes containing PANTA. PANTA was reconstituted in 15 ml of growth supplement and 0.8 ml of PANTA/growth supplement mixture was added to each MGIT tube.

Line 84-what is the CPC concentration in the OM-S reagent?

To our knowledge, OM-S does not contain CPC.

Line 87-shipped in ambient temperature… how long were the samples in transit? To which temperature exposed during transit? Overnight courier or regular mail?

Samples were sent by DHL Express service from Tirana to Milan. Given the nature of the samples, upon arrival to the airport in Milan, specimens were subjected to phytosanitary inspection by the Ministry of Health and sent to Rome. After that, they were sent back to the laboratory in Milan. Therefore, samples were in transit for an average of 8 days at ambient temperature (not refrigerated).

Line 88-how were the samples stored and at which temperature (samples for Italy)?

Samples were sent to Italy in batches on a weekly-bi-weekly basis. They were stored at room temperature in the laboratory (i.e. on the lab bench) until shipping. As described above the shipment occurred at ambient temperature (not refrigerated).

Line 90-are there any studies published on the effect of CPC on MGIT?


Line 93-instead of ‘DE’ use Germany.
According to Table 1, there are only 37 TB positive cultures and not 42 - please clarify. As indicated in table 1, the total number of OM-S treated culture positive samples were 36 (also positive in the NALC/NaOH treatment group), plus 1 (negative in the NALC/NaOH treatment group), plus 5 (contaminated in in the NALC/NaOH treatment group), for a total of 42 positive culture samples.

Contamination rate is 13%; however, it should be <10% according to reference #5 - any explanations? This is concerning since these samples were collected in-house.

We agree with the reviewer that ideally the contamination rate for MGIT culture should be <10%, however, this is not often the case even in National TB Reference laboratories (NRL). Reasons are different. As NRL, the laboratory in Tirana receives samples from the entire country with delays and/or sub-optimal conservation of the samples during the shipping. In addition, the NRL often receives samples from patients affected by different pulmonary infections such as chronic obstructive pulmonary disease or cystic fibrosis and in those cases a higher contamination rate is observed.

An additional factor that may contribute to a higher contamination rate is the lack of maintenance of the biosafety cabinets (BSC) used for sample processing (i.e. HEPA filters have not been changed in years at the NRL).

Among 48 AFB smear positive samples, 33 resp. 35 were culture positive - does this mean 15 resp. 13 samples were AFB positive and culture negative or AFB positive and culture positive for nontuberculous mycobacteria (NTM)? Please provide more details.

In the NALC/NaOH group 7 out of 48 (14.6%) samples were AFB positive /culture contaminated and 6 out of 48 (12.5%) were AFB positive /culture negative. These 6 samples were from presumptive TB cases that were started on anti-TB treatment based on smear microscopy results during the sample enrollment period, thus possibly explaining the culture negativity. In the OM-S group, among the 15 AFB positive / culture negative samples, 6 were positive in the NALC/NaOH group thus indicating a loss of viability upon OM-S treatment, 3 were contaminated in the NALC/NaOH group and 6 were negative also in the NALC/NaOH group for the same reason mentioned above (i.e. patient started on anti-TB treatment).
Line 123-9 resp. 10 were AFB negative and culture positive for TB. With OM-S, there were a total of 42 TB culture positive and with conventional a total of 45. The proportion of AFB smear positive is 33/42 (79%) for OM-S and 35/45 (78%) for conventional. While the culture positivity of 13% resp. 14% is reasonable for samples from TB suspects, the AFB smear positivity of 79% resp. 78% is high. Any explanations?

We agree with the reviewer that the proportion of culture positive/AFB smear positive samples is indeed high. This is most likely due to delays in TB diagnosis in Albania, with individuals with signs and symptoms of TB presenting late to the diagnostic site.

Line155-Replace 'DE' with Germany

Text modified accordingly (line 170)

Line 156-Out of 51 specimens tested… according to Line 94, only the samples from TB confirmed cases were processed for LPA [there are only 48 TB positive cultures], please clarify.

We agree with the reviewer on the lack of clarity and have modified the text accordingly (line 172). LPA was performed on all samples that had a culture positive results regardless of the decontamination method used (i.e. 42 culture positive samples in the OM-S treatment group plus the 9 culture negative in the OM-S group but culture positive in the NALC/NaOH group).

Line 171-As far as this reviewer understands, the authors compared only samples at most 1 day at ambient temperature (in-house) versus several weeks at ambient (25C) temperature. The real challenge is the field experience with uncontrolled temperature exposure during shipping; however, this aspect has not been studied.

We agree with the reviewer that this is indeed an important aspect that should be closely monitored especially in tropical and sub-tropical regions. We assumed that in this specific study setting the temperature exposure was less a concern given the temperate climate of both Albania and Italy and an average temperature of 15-20°C during the study period (mid-March - end of April 2016).
Line 190-17.7%, please provide the numerator and denominator.

The manuscript has been revised considering all 9 OM-S culture negative samples out of 45 culture positive in the NALC/NaOH group (20%) (line 206). As explained above, we originally excluded one sample that was both culture and MTBDRplus negative (i.e. 8 out of 45; 17.7%) since we could not exclude it was a true negative sample.

Discussion: Include Tazir M et al Tubercle 1979 60(1):31-36

Reference Included (ref 11) (line 276)

References #6 and #8 are identical

References were revised.

Reference #11 not referenced in text.

Text was modified accordingly, now reference 12 (line 200).