Author's response to reviews

Title: Evaluation of bleach-sedimentation for sterilizing and concentrating M. tuberculosis in sputum samples

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Author's response to reviews: see over
Dear Dr Norton,

Re: Evaluation of bleach-sedimentation for sterilizing and concentrating *M. tuberculosis* in sputum samples, ref. 1884448544348339

Thank you for your consideration of our manuscript. We, the authors, appreciate the effort the reviewers have put into appraising our manuscript, and are glad they find the manuscript of interest to fellow workers in our field. We have taken on board their helpful suggestions, and in this letter hope to show that we have addressed them comprehensively with a considerably-improved manuscript as a result.

We hope that you can now consider this revised version for publication because we believe that the previous imperfections in this manuscript have been completely corrected. For your convenience, all of the reviewers’ comments, our amendments and explanations are detailed below in the order in which they appear in the manuscript, with R1, R2 and R3 denoting the reviewer (M A Hamid Salim, unnamed and Sara Irène Eyangoh respectively) who made the relevant comment. Additionally, we have also made references to recently-published literature in order to make our manuscript as up-to-date as possible.

We have also taken this opportunity to add a paragraph detailing the ethical procedures for the project that were previously only implied and are now explicitly described.

We once again confirm our contribution to this project and approve this submission with any associated revisions submitted by the corresponding author. We also confirm that this original research manuscript is not being considered by any other journal and will not be submitted elsewhere pending your decision, and that none of us are aware of any conflict of interest.

Thank you again for your consideration. Please do not hesitate to contact me if further clarification or information is needed.

Yours sincerely,

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General comments

**R1:** “I am very glad to have the opportunity to review this manuscript. The article is very well written. The background of the study, methodology and results are well presented. The discussion and conclusion are well presented.”

We thank the reviewer for these comments.

Abstract

**R2:** “Tuberculosis diagnosis by sputum smear microscopy is insensitive.”

*Insert ‘relatively’ before ‘insensitive’*

**R3:** In Abstract, background section, on the first line, authors could replace the term "insensitive" by "low sensitive" (sic).

The word ‘relatively’ has been added before ‘insensitive’ as recommended by R1.

**R2:** *The remaining 25 sputum samples were used to evaluate this technique quantitatively by making triplicate conventional direct-smears, then subjecting the remaining sputum to this bleach-sedimentation protocol, after which additional triplicate slides were made Why triplicate? Why not single? Need explanation.*

This has been clarified with the addition of the sentence:

“Slides from each sample both before and after bleach-sedimentation were prepared in triplicate to increase the precision with which the concentrations of stainable acid-fast bacilli were quantified.”

Background

**R2:** *Tuberculosis control worldwide is hampered by the low sensitivity of conventional direct-smear microscopy... Why? Need explanation.*

The reason that the low sensitivity of conventional direct-smear microscopy hinders control is now explained in this paragraph, that has been clarified as follows:

“Tuberculosis control worldwide is hampered by the low sensitivity of conventional direct-smear microscopy of sputum because this is the most widely-used laboratory test for tuberculosis in resource-poor settings but fails to diagnose patients with low concentrations of *Mycobacterium tuberculosis* in their sputum [1]. Conventional direct-smear microscopy involves smearing sputum on a microscope slide that is then stained and examined by high-power microscopy to detect the causative acid-fast bacillus, *M. tuberculosis*. Whilst inexpensive and rapid, the sensitivity per sputum sample of this technique is typically only approximately 30-70% of the sensitivity of culture [2, 3]. People with AIDS and children usually have fewer bacilli in their sputum and sensitivity is therefore lower in these patient groups [4, 5]. Consequently, reliance on direct-smear microscopy may cause the diagnosis for some tuberculosis patients to be missed or delayed, potentially increasing morbidity, mortality and transmission. Therefore, increasing the sensitivity of tuberculosis diagnostic testing is a public health priority.”

**R2:** “… the most widely-used laboratory test for tuberculosis.”

*Need reference.*

A reference has been inserted, as advised.
R2: “However, the sterilizing activity of bleach is poorly characterized for M. tuberculosis under the conditions used for bleach-sedimentation and the required concentrations and exposure times to prevent biohazard to staff are unknown [11, 12, 13, 14].”

Literature regarding Comparison of bleach sedimentation and Fluorescence Microscopy and Z-N should be mentioned.

This reference has been added (late in the discussion, last reference). We have also added to the discussion the statement that in future work, it will be desirable to include fluorescence microscopy.

Methods

R1: The sample size is very small to conclude the study results. Could you please explain the basis of calculation of sample size?

R3: The authors should give details of their sampling strategy. How do they estimate the minimal sample size (N= 72 sputum samples) to test all hypothesis?

We did not perform study size-calculations before starting the experiments for the reasons now stated in the manuscript (please see text copied below). Instead, we decided to take the alternative approach of continuing the experiment with interim analyses until the research questions were clearly answered. In fact, the first ‘interim’ analysis provided clear and statistically significant results and continuation of the experiment was not required. We do not feel that statistical power of the study is a concern because all of the analyses exceed conventional thresholds for statistical significance i.e. expansion of the study size would be redundant. We have addressed this issue by adding the following text:

“Sample size. Sample size calculations were not performed because no prior quantitative data defining the actual concentration range of acid-fast bacilli in sputum samples for the planned protocol was available. Instead, we continued the experiment until clear and statistically significant results were found comparing the concentration of acid-fast bacilli and speed of slide reading before and after bleach-sedimentation at a significance level of p=0.05. The first statistical analysis yielded statistically significant results and thus, no interim analyses were required.”

R3: What was the duration of study?

The study was carried out over 6 months. This has now been mentioned in the manuscript.

R2: “The volume and consistency (whether liquid or mucoid) was recorded.”

Quality of sputum should be used instead of these.

This sentence has been amended to read:

“The quality of each sample, as indicated by its volume and consistency (whether liquid or mucoid), was recorded.”

R2: The decontamination was then stopped by adding a 7-times excess volume of phosphate-buffered saline (PBS, pH 6.8), centrifuging at 3,000 x g for 20 minutes at room temperature and discarding the supernatant.

Why 7 times, it exceeds the volume of falcon tube.

Reference why 20 minutes.

There is no explanation about control tube. They should use control tube and inoculum size for culture not in accordance with WHO standard.

This issue has been addressed by the modification and addition of the following text:

“The decontamination was then stopped by adding a 7-times excess volume of phosphate-buffered saline (PBS, pH 6.8), centrifuging at 3,000 x g for 20 minutes at room temperature and discarding the supernatant. The addition of a 7-times excess volume of PBS and the centrifugation conditions are standard
practices for centrifuge-decontamination in several laboratories in Peru because they were found in pilot experiments to provide optimal neutralization and concentration (data not shown). The pellet was re-suspended in 34 ml PBS and then split into 17 aliquots of 2 ml each. One aliquot was used as a control to which no bleach was added and 2 ml of 3%, 6%, 10% and 15% bleach was added to quadruplet sets of the other aliquots.”

R2: “One hundred high-power fields were read per slide, and the number of acid-fast bacilli counted. If fewer than an arbitrary cut-off of 33 acid-fast bacilli were seen in 100 fields then an additional 200 fields were read to increase precision.”
Reference needed.
R2: As a quality control measure, both microscopists cross-read a random sample of approximately one in eight slides in a blinded manner to determine the degree of agreement between their readings.
Reference needed.

These issues have been clarified with the following text:

“If no more than 32 acid-fast bacilli were seen in 100 fields then an additional 200 fields were read to increase precision. This cut-off of 32 acid-fast bacilli was derived because it is the mid-point between 10 and 100 on a logarithmic scale and was selected arbitrarily to increase the precision of quantification of relatively low concentrations of acid-fast bacilli. As a quality control measure, both microscopists cross-read a random sample of slides in a blinded manner to determine the degree of agreement between their readings. For this quality control step, one in eight slides were selected for operational, work-load reasons and for these slides the re-reading by a second microscopist involved the same quantitative protocol as the first slide reading.”

R3: On page 10, "selection of bleach-sedimentation technique" authors should give more details on the five gravity bleach-sedimentation techniques to make easier understanding. Results should not be given in methods part. "These 3 techniques gave similar results" which one? These should be clearly presented to give support in their selection of the method described by Gebre-Selassie.

We have amended the relevant section by summarizing in further detail the referenced methodologies. In particular we have clarified that the latter 3 techniques gave broadly similar qualitative results with respect to smear-positivity, and the best described of these techniques was chosen to be evaluated quantitatively. We agree that in general the description of results should be avoided in the ‘Methods’ section of a manuscript and therefore tried moving the pilot finding of which technique was selected to the ‘Results’ section, as recommended by the reviewer. However, unfortunately this made the manuscript rather confusing. Clarification of this pilot finding seems to be required in the ‘Methods’ section in order for the remainder of the ‘Methods’ section to be understood. We therefore respectfully request that this pilot finding should remain in the ‘Methods’ section. The section now reads:

“In pilot experiments, five published gravity bleach-sedimentation techniques that are described in the following references [7, 8, 9, 10, 11] were compared to one another to select a technique for further assessment. For this comparison, triplicate conventional direct-smears were prepared from 16 further sputum samples. The remainder of each sample was then processed by the five gravity bleach-sedimentation techniques after which triplicate slides were produced from each bleach-processed sample. The first two techniques, bleach-sedimentation without subsequent water dilution followed by sedimentation for 3-45 minutes [7] or 12-15 hours [11], considerably reduced the number of acid-fast bacilli seen on microscopy, possibly through bleach damaging the mycobacteria (data not shown), so these techniques were not further assessed. The other published techniques that we evaluated involved adding bleach to the sputum without shaking [10], shaking at regular intervals for 15 min [9] or continuous shaking for 10 min [8] before dilution with water that was followed by sedimentation. In our pilot evaluation these three techniques gave similar qualitative results to one another in terms of the number of acid-fast bacilli seen on microscopy (data not shown). We selected the last of the three techniques, which was the method described by Gebre-Selassie [8], for further quantitative evaluation because it had the most precisely defined methodology and was reported to have produced optimal results.”
**R3: Details on ZN coloration process are not necessary. The important parameter is the fuchsin concentration, 1% or 0.3% this could explain further discordances in reading if AFB are not strong red colored.**

We have now included the concentration of carbol fuchsin used, i.e. 0.3%.

**R3: In sedimentation process specify if the sputum stand (sic) at room temperature.**

Yes, the sedimentation process was carried out at room temperature. This detail has now been included in the manuscript.

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**Results**

**R2: All M. tuberculosis in all 31 samples were killed by an exposure time of 1 minute to 15% bleach, 5 minutes to 6% bleach, or of 20 minutes to 3% bleach.**

What about control tube?

**R3: 'Bleach sterilization assessment' authors mentioned a control, but the rest of the paper however does not indicate the result of this control and its usefulness.**

**R3: Fig. 2: add the control result**

The controls yielded the expected positive result, thus justifying their use. The following sentence has been added to the ‘Bleach-sterilization assessment’ section and to the Legend for Figure 2:

“All control samples that had not been treated with bleach were culture positive.”

**R1: The study findings shows (sic) that the number of bacilli reduces significantly after bleach-sedimentation however the decrease is less in paucibacillary samples. How many samples were paucibacillary among 25 samples. Again the sample size is too small to conclude such findings.**

**R3: 2- Bleach sedimentation assessment. Authors stated that "our quantitative evaluation demonstrated that bleach sedimentation caused a significant decrease in the number of AFB visualized". Authors should use the standard scale gradation of AFB microscopy to validate this observation (exact number/ 100 field, 1+, 2+, 3+). E.g: data given in the paper could not be significantly different (sic) different 146 BAAR/ 100 fields (mean of 1.5 AFB/field) is not different from 346 AFB/ 100 fields (mean of 3.5 AFB / field),because according to the standard scheme of recording and reporting the two are graded 2+. The conventional gradation should be use instead of terms as paucibacillary or multibacillary (see WHO/IU TLAD table below).**

We agree with the reviewers and have amended the manuscript accordingly. We have added conventional sputum smear grading scores to the graph axes, to facilitate interpretation of our quantitative data by those more accustomed to sputum smear results reported as grades.

Paucibacillary samples are poorly defined and the term is variably used to refer to samples that are smear-negative but culture-positive, or samples with 1-9 AFB/100 fields, or sometimes grade + and ++ but not +++.

We have therefore avoided the term ‘paucibacillary samples’ and instead only now refer throughout the discussion of our findings to ‘relatively paucibacillary samples’ and compare these with samples ‘containing higher concentrations of stainable acid-fast bacilli’.

A strength of this research is the way the concentration of stainable acid-fast bacilli in each sample was rigorously quantified both before and after bleach-sedimentation using triplicate slides to increase precision and exact quantification of mycobacteria instead of the more conventional and much less accurate grading of slides. This reduces the clinical relevance of the research, because most labs do not assess sputum smears with such rigor. However, we did not aim to simply add to the considerable number of clinical assessments of bleach-sedimentation on slide positivity. Rather, we aimed to precisely characterize the effects of bleach-sedimentation on specific measures: the concentration of stainable acid-fast bacilli and the time required to read
a set number of microscopy fields. This approach has been informative and we have now clarified these issues in the introduction to try to better communicate these issues:

“Most microscopy studies have compared rates of microscopy positivity or the frequency of positive slides in three graded ranges (+, ++ or +++). This approach is clinically relevant but is insensitive because relatively few samples contain concentrations of mycobacteria close to the threshold between negative and positive, or close to the thresholds between microscopy grades. Thus large numbers of samples must be studied in order to detect significant differences in microscopy positivity or grading. Furthermore, any effects of an intervention on the concentration of acid-fast bacilli on the microscopy slide will be confounded by effects on the ease and speed of slide reading. Consequently, it is unclear from the published literature whether bleach-sedimentation increases or decreases the concentration of stainable acid-fast bacilli and slide reading time. To overcome these limitations, for the present research we developed a novel quantitative protocol to determine the effect of bleach sedimentation on the actual concentration of stainable acid-fast bacilli within a fixed number of microscopy fields and recorded the time it took for microscopists to examine this number of microscopy fields. By preparing triplicate slides from each sample before and after bleach sedimentation, we were able to determine whether bleach-sedimentation increased or decreased the concentration of stainable acid-fast bacilli within each sample, and independently measured effects on the speed with which slides were read. This novel approach clarified the specific effects of bleach-sedimentation and provided potential explanations for the discrepant results from previous studies.”

Discussion

R3: 1- Bleach sterilization assessment. The authors stated that "this study defined the concentrations and exposure time needed to sterilize M. tuberculosis in sputum" this is not quite exact since the authors have not assessed the quality of bleach use and do not determine the % of free chlorine content (active ingredient) of the solution; there is no possibility to know exactly what is the chlorine concentration in the different dilution 3%, 6%, 10% and 15% bleach. The term "bleach" is a generic term that encompasses numerous chemical oxidation products, and the use of term bleach without due characterization has led to confusion in the scientific literature. The methodology presents (sic) is not sufficiently informative to allow replication of the experiment; results presented here give neither answer to the concentration nor to the exposure time.

We agree this is an important and difficult issue in the entire field of bleach microbiology. In common with most other research assessing bleach utility in tuberculosis microbiology, we used commercially available fresh bleach and did not have the necessary equipment to optimally characterize this bleach but rather relied upon the manufacturer’s information. We have now included further details of the bleach used:

“Briefly, to 1 ml of sputum in a 15 ml polypropylene tube (Falcon BD, San Jose, California), an equal volume of fresh 5% bleach was added, prepared by dilution from a stock solution of 8% bleach that the manufacturer reported contained 8.09 g/100 ml free chlorine ions and had a density of 1.125 g/ml.”

We also now state in the discussion section that:

“In future research, it would be helpful to assess bleach-sedimentation effects on fluorescence microscopy and to improve quality control of the commercially available bleach, characterizing the effect of storage time on the sterilizing efficiency of bleach and actually measuring the free chlorine content of the bleach at the point of use rather than utilizing the manufacturers’ data on these concentrations.”

R3: Fig 3 is incomprehensive (sic) for the non-statistical expert. Perhaps should be removed if results are expressed using standard scale.

We regret that Figure 3 was unclear and have improved the explanation in the text, in the Figure legend and the design of the Figure itself to rectify this:

“Bleach-sedimentation assessment. Triplicate microscopy slides were prepared from each sample prior to and after bleach-sedimentation i.e. six slides were prepared from each sample. For statistical
analysis, first the geometric mean number of acid-fast bacilli seen in 100 microscopy fields was calculated for each sample from the triplicate conventional direct-smear results and also from the triplicate smears prepared from the same sample after bleach-sedimentation. The results are shown in Figure 3, in which each of the open circles represents the geometric mean number of acid-fast bacilli seen in 100 microscopy fields for triplicate, identically prepared slides. Each line joins the data derived from one sputum sample. The black diamonds represent the geometric mean of the results for all samples. These data demonstrate that bleach-sedimentation caused a significant decrease in the number of acid-fast bacilli visualized by microscopy. The geometric mean number of acid-fast bacilli per 100 microscopy fields was 166 for the bleach-sedimented smears, significantly less than 346 for the conventional direct-smears made from the same samples (Table; p=0.02)."

and

“Figure 3 - Effect of bleach-sedimentation on the concentration of acid-fast bacilli. The number of acid-fast bacilli visualized by smear microscopy is shown. Each of the open circles represents the geometric mean number of acid-fast bacilli seen in 100 microscopy fields for triplicate, identically prepared slides. Each line joins the data derived from one of the 25 sputum samples i.e. the geometric mean of triplicate conventional direct-smear microscopy slides (the left-hand end of each line) that is compared with the geometric mean of triplicate slides prepared after bleach-sedimentation (the right-hand end of each line). The filled diamonds represent the geometric mean of all 25 samples and error bars represent 95% confidence intervals. The box parallel with the y-axis indicates the sputum smear microscopy grade equivalent to the acid-fast bacilli counts per 100 microscopy fields (0 indicates none seen/100 fields; +/- indicates 1-9/100 fields; + indicates 10-99/100 fields; ++ indicates 100-999/100 fields; and +++ indicates >1,000/100 fields).”

R2: We confirmed that bleach-sedimentation resulted in more rapid slide reading compared to conventional sputum smear microscopy

Needed (sic) probable explanation about this observation.

An explanation is now offered in the following sentence:

“The decrease in slide-reading time is most likely a result of clearer microscopy fields free of cells due to the digestive properties of bleach. This may facilitate identification of acid-fast bacilli because cells may obscure acid-fast bacilli in conventional direct-smears made from untreated sputum.”

R2: Future work will focus on the optimization of bleach-sedimentation as a concentrating technique, which is a priority because we have demonstrated that this technique increases slide-reading speed and biosafety.

There is minimum risk in conventional method as per Kam et al and other international literature

There is indeed controversy concerning how safe it is to perform sputum smear microscopy under optimal and under real-world conditions. Sterilizing sputum samples suspected to contain *M. tuberculosis* can only have the potential to reduce this risk. We have therefore added a new reference (the penultimate reference in the manuscript) specifically addressing this issue and have improved the wording of this section as follows:

“The finding that bleach rapidly sterilizes sputum samples at easily achievable concentrations and exposure times demonstrates that bleach-sedimentation has the potential to improve biosafety in diagnostic laboratories.”

and

“This research validated a novel quantitative methodology, which demonstrated that gravity bleach-sedimentation decreased the numbers of acid-fast bacilli visualized in sputum smear microscopy but increased microscopy speed and potentially improved biosafety by sterilizing samples.”
Conclusions

**R2: Differences in these variables will inevitably cause heterogeneous findings from operational evaluations of bleach sedimentation in different clinical settings. Does (sic) this method suitable for NTP?**

We assume NTP to stand for ‘National Tuberculosis Programme(s)’. We have now clarified in the Background the principal objective of our research was not to determine whether or not bleach should be used programmatically, but rather to use a rigorous experimental methodology to define the actual actions of bleach-sedimentation on TB-containing sputum because of the contradictory, inconsistent results of previous studies. This issue is discussed above and is clarified in the last paragraph of the introduction. We now also clarify this issue in the last paragraph of the discussion, more explicitly stating what our study has added to the literature:

“This research validated a novel quantitative methodology, which demonstrated that gravity bleach-sedimentation decreased the numbers of acid-fast bacilli visualized in sputum smear microscopy, but increased microscopy speed and potentially improved biosafety by sterilizing samples. Previous studies have reported heterogeneous results. Some studies demonstrated that bleach sedimentation increased diagnostic sensitivity and others found a reduction in diagnostic sensitivity thus, discouraging widespread implementation. In contrast with previous studies that have focused principally on slide positivity rates, our quantitative protocol generated meaningful comparative concentration and slide-reading speed data from the analysis of multiple microscopy slides prepared from each sputum sample.

Our quantitative assessment appears to explain the contradictory results of previous studies by demonstrating that gravity bleach-sedimentation significantly reduces the concentration of acid-fast bacilli visible on microscopy, that this undesirable reduction in the concentration of acid-fast bacilli increases with the concentration of acid-fast bacilli in the sample and that bleach-sedimentation allows more of the sputum sample to be examined microscopically in the time available. Thus, the overall effect of bleach-sedimentation depends upon two main factors: the concentration of acid-fast bacilli in the sample and whether slide reading involves examining a defined number of microscopy fields or examining slides for a defined period of time. Differences in these variables will inevitably cause heterogeneous findings from operational evaluations of bleach-sedimentation in different settings. Specifically, an evaluation of gravity bleach-sedimentation in a setting with mainly relatively paucibacillary samples in which the duration of examination of each slide is fixed is more likely to demonstrate advantageous bleach-sedimentation effects than a setting in which there are few relatively paucibacillary samples and where a fixed number of microscopy fields are examined for each sample. Therefore, our quantitative findings usefully extend and clarify the results of multiple previous operational assessments of bleach-sedimentation by potentially explaining their discrepant findings.”