METHOD ARTICLE

REVISED A modified decontamination and storage method for sputum from patients with tuberculosis [version 2; peer review: 1 approved, 2 approved with reservations]

Nguyen Le Quang[1], Do Dang Anh Thu[1], Le Pham Tien Trieu[1], Nguyen Hong Hanh[1], Nguyen Huu Lan[2], Dang Thi Minh Ha[2], Guy Thwaites[1,3], Nguyen Thuy Thuong Thuong[1,3], Timothy M. Walker[1,3]

[1]Oxford University Clinical Research Unit, Centre for Tropical Medicine, Ho Chi Minh city, Vietnam
[2]Pham Ngoc Thach Hospital, Ho Chi Minh City, Ho Chi Minh, Vietnam
[3]Nuffield Department of Medicine, Centre for Tropical Medicine and Global Health, University of Oxford, Oxford, England, UK

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Abstract

Background

Collecting and storing large number of sputum samples with a view to culturing these in the future requires an efficient initial handling method. We devised a modified sputum digestion and decontamination method that maximised storage capacity and *Mycobacterium tuberculosis* (M.tb) recovery from culture while minimising laboratory workload and risk of contamination.

Methods

We collected smear microscopy positive sputum samples from patients with pulmonary tuberculosis (TB). The sputum samples were split and processed using both the standard N-Acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method and our modified method before freezing and later culturing in BD BACTEC 960 Mycobacterium Growth Indicator Tubes (MGIT) system. We assessed the Time to Positivity (TPP) and Growth Unit (GU) data.

Results

We selected 22 sputum samples to compare two digestion and decontamination methods. The samples that underwent the modified

Open Peer Review

Approval Status ? ✓ ?

	1	2	3
version 2 (revision) 20 Feb 2024		✓ view	? view
version 1 13 Apr 2023	? view	✓ view	

1. **Bhushan J. Toley**, Indian Institute of Science, Bengaluru, India
2. **Irina Kontsevaya**, Imperial College London, London, UK
3. **Virasakdi Chongsuvivatwong**, Prince of Songkla University, Kanjanavanich Rd, Kho Hong Hat Yai, Thailand

Any reports and responses or comments on the article can be found at the end of the article.

method had longer TTP ($p < 0.05$) but similar GU in comparison to standard method. Overall, 1/22 samples failed to grow in MGIT after being processed by the modified method. We then applied the modified method to 348 sputum samples with Rifampicin resistance detected by GeneXpert MTB/RIF assay, which were frozen for between 1-25 months. The overall MGIT positive, negative, and contamination rate was 90.5%, 7.8%, and 1.7%, respectively. There was no significant difference in MGIT result when samples were grouped by duration of storage or positive smear grade.

Conclusions

Our modified method yielded acceptable M.tb recovery rate and low contamination risk while allowing us to collect and store thousands of sputum samples over a long period of time for future tests.

Keywords
tuberculosis, sputum digestion, sputum decontamination, sputum frozen storage

Corresponding author: Nguyen Le Quang (quangnl@oucru.org)

Author roles: Le Quang N: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Dang Anh Thu D**: Conceptualization, Data Curation, Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing; **Pham Tien Trieu L**: Data Curation, Investigation, Methodology, Writing – Review & Editing; **Hong Hanh N**: Data Curation, Resources, Writing – Review & Editing; **Huu Lan N**: Project Administration, Supervision, Writing – Review & Editing; **Thi Minh Ha D**: Project Administration, Supervision, Writing – Review & Editing; **Thwaites G**: Project Administration, Supervision, Writing – Review & Editing; **Thuy Thuong Thuong N**: Conceptualization, Formal Analysis, Supervision, Writing – Review & Editing; **Walker TM**: Conceptualization, Formal Analysis, Funding Acquisition, Supervision, Writing – Review & Editing

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REVISED **Amendments from Version 1**

The new version addressed several key points given by the reviewers. We agreed and changed our statistical analysis approach to better represent our result. Particularly, the Figure 2 was changed to show the mean percentage of positive samples with 95% confidence interval at each months and corresponding number of samples. The figure summarised the output of a logistic regression model that uses natural cubic splines to account for the non-linear trend over time. MGIT status (pos/neg) at each month was the dependent variable, and the number of months frozen for each month was the independent co-variable. Additionally, Figure 1 was modified to give better clarity and data presentation. Secondly, we agreed with the reviewers and acknowledged our limitation of insufficient control group and reproducibility in different setting due to limited time and resources in the discussion section. Lastly, we highlighted that our study design used samples that had been frozen for longer durations that no studies had done or were comparable. Overall, we responded to the reviewers with changes in statistical analysis, data presentation, and study limitation in discussion.

Any further responses from the reviewers can be found at the end of the article

Introduction

The diagnosis of tuberculosis (TB) is not always straightforward and some clinical samples are easier to obtain than others (Harries & Kumar, 2018). Studies in patients with pulmonary TB who can produce sputum samples benefit from cheap and non-invasive sampling. However, for studies that require large numbers of sputum samples to be collected, significant sample processing and storage capacity may be needed. Furthermore, where it is only determined after sample storage which samples require further testing, an efficient initial handling process is required. Samples need to be transferred to volume efficient containers (freezer vials), maximising the chances of obtaining a *Mycobacterium tuberculosis* (*M.tb*) culture at a later date, whilst avoiding cross-contamination and minimising the risk to laboratory staff. Storage without prior decontamination has been attempted previously with some success (Shinu *et al.*, 2016; Tessema *et al.*, 2011). However, these studies did not concentrate primary sputum samples into volume efficient containers or use cryopreservative such as glycerol for long term storage of bacteria. There is thus room for logistical improvement and potentially for higher positive culture yields through the use of cryopreservatives (Shu *et al.*, 2012).

We describe a modified method for decontaminating and storing sputum samples that can be applied for studies which process large numbers of clinical samples daily and selectively culture over an extended period of time.

Methods

Ethical approval

The study was approved by the Institutional Research Board of Pham Ngoc Thach Hospital as the supervisory institution of the District Tuberculosis Units (DTUs) in Ho Chi Minh City, Vietnam (CS/PNT/20/01) and the University of Oxford (OxTREC ref 51-19), UK. IRB approval was granted on February 10th, 2020, and data collection began on March 2nd, 2020.

For initial collection of sputum samples, we specifically requested a waiver from the IRB for consent from patients we screen, and from whom we collect and store samples.

We obtained written informed consent from patients who went on to be recruited into the study. As all patients with multidrug resistance TB (MDR-TB) were recruited into the study, we obtained written consent from these patients. The other samples contributing to this manuscript were selected randomly from the archived anonymous sputum samples, which the IRB waived the need of consent for.

Study population and biological samples

This was a prospective observational cohort study in Ho Chi Minh city, Vietnam between 1st March 2020 and 31st August 2023 (42 months). All related data were collected on recruited patients within the aforementioned period, with patient outcomes and medication changes recorded for a further 12 months thereafter for patients still on treatment at 42 months. The screening population was adult patients (18 years or older) with a smear microscopy positive clinical sample or had *M. tuberculosis* detected by MTB/RIF Xpert with the expected number of about 30,000 samples. Sputum samples were collected in 50-mL Falcon tubes from all screenings from 1st March 2020. A pilot study was conducted using sputum samples collected in the first week after commencement. We adopted the modified sputum processing procedure after the results were finalized. The screening patients were recruited to MDR-TB transmission study with consent form if they had both *M. tuberculosis* detected and Rifampicin resistance detected by MTB/RIF Xpert.

Direct culture vs. culture from frozen samples

A total of 22 samples were selected randomly for a pilot experiment. No changes were made following the pilot. N-Acetyl-L-cysteine (NALC)-NaOH 2% was added to each sputum sample in a 10:1 ratio before vortexing. The liquefied samples were divided into two equal aliquots. One aliquot was processed with standard method, whereby the sample was incubated further at room temperature (RT) for up to 20 minutes, then neutralized by adding up to 40 mL phosphate buffered saline (PBS), and centrifuged at 3,220xg for a further 20 minutes. The supernatant was removed and the pellet cultured in BD BACTEC Mycobacterial Growth Indicator Tube 960 (MGIT 960, Becton Dickinson), with PANTA supplement, a cocktail of antibiotic (polymyxin-B, amphotericin-B, nalidixic acid, trimethoprim, azlocillin). The second aliquot was processed with a modified method that neutralized the sample with PBS immediately and centrifuged at 3,220xg for 20 minutes. The supernatant was removed and PBS containing 20% glycerol was added to the remaining pellet in a 1:1 volume ratio before freezing the samples at -80°C in 0.5 mL screw-capped tubes. The frozen samples were thawed after 7 days before being transferred to a fresh sterile 1.5 mL Eppendorf tube and mixed with 1 mL

NALC-NaOH 2% solution. After incubating for 20 minutes at RT, samples were centrifuged using microcentrifuges at 11,000xg for 90 seconds. The supernatant was then removed and cultured in MGIT with PANTA supplement.

Culture from samples frozen over longer time periods

Based on the pilot, we processed and froze all prospectively collected samples as described. After two years of collecting sputum samples, we needed to culture all samples for which rifampicin resistance had been detected by the GeneXpert MTB/RIF assay for the MDR-TB transmission study. These samples were all defrosted and processed according to the same method. Although we had no opportunity to compare growth to corresponding aliquots that had not been frozen in long-term storage, these samples allowed us to assess the impact of duration of freezing on mycobacterial growth.

Data collection

For each culture, MGIT culture vial was used in combination with BD BACTEC 960 system, which generated three variables of interest: "Time to positive" (TTP) in unit of days and hours (DD;HH), "Growth Unit" (GU), and MGIT result of "Positive" and "Negative". TTP unit was converted into hours while GU was analysed as generated. MGIT results were classified into "Positive" and "Negative" as generated by the system. The threshold for MGIT result of "Negative" was 42 days without any changes in GU as detected by the system; any increase in GU in less than 42 days were detected as "Positive". "Contaminated" MGIT result was added when a culture with "Positive" MGIT result showed TTP of less than three days and/or the medium showed turbidity without presence of small grains or granules. The duration of storage at -80°C for each sample was the difference in months between sample processing date and start of culture date and the Ziehl-Neelsen smear grade was recorded.

Statistical analysis

Statistical analysis was performed with RStudio (RRID: SCR_000432) version 1.2.5019 using R Project for Statistical Computing (RRID:SCR_001905) version 4.2.2. The Wilcoxon signed-rank test was performed to compare data from two processing methods. STATA (RRID:SCR_012763) version 18 was used to perform logistic regression using natural cubic splines to describe the relationship between percentage of samples that are positive in MGIT culture and the time samples were frozen for.

Results

Direct culture vs. culture from frozen samples

To test how our modified method of sputum decontamination and frozen storage affects the MGIT culture, 22 sputum samples were divided into two aliquots and processed by two methods (Figure 1). We compared the growth units from the MGIT system, an indirect measurement of microbial aerobic growth,

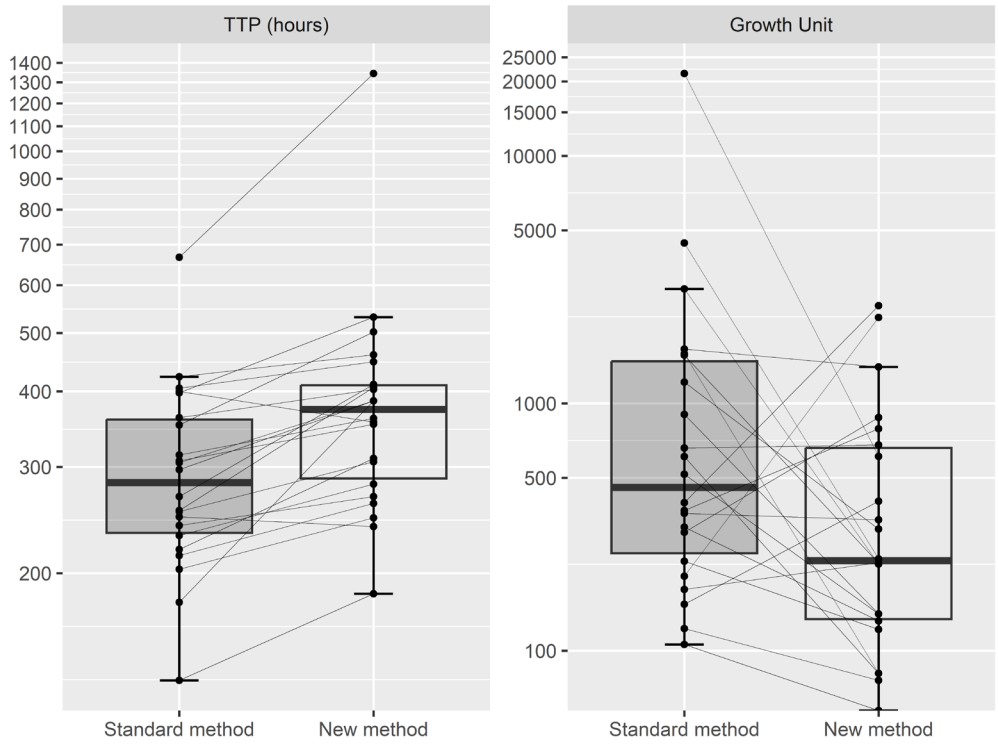

Figure 1. TTP in hours (Left) and Growth Unit (Right) reported by MGIT system between Standard method and New method (n=22). Standard method decontaminates sputum samples using NALC-NaOH 2% and cultured immediately); New method concentrates sputum samples with brief exposure to NALC-NaOH 2%, frozen in 7 days before decontamination using NALC-NaOH 2% and cultured. The vertical lines represent the tails of the boxplot. TTP, Time to positive; MGIT, Mycobacterium Growth Indicator Tubes; NALC-NaOH, N-Acetyl-L-cysteine and sodium hydroxide.

and time-to-positivity, the amount of time for a MGIT culture to reach predefined growth units. All 22 aliquots that were decontaminated and cultured immediately grew in MGIT culture, along with 21/22 to which the modified method was applied. Those aliquots that were decontaminated and cultured immediately showed a mean TTP of 303 ± 113 hours and estimated mean growth units of 1,830 ± 4,515 unit. The mean TTP and GU after the modified method was applied was 401 ± 228 hours and 536 ± 677 units, respectively. The Wilcoxon signed-rank test showed that the TTP was significantly different between the two methods in which the immediate decontamination and culture of the sputum showed faster TTP (p=0.0001), while there was no significant difference in terms of growth units ($p > 0.05$).

Culture from samples frozen over longer time periods

A total of 348 rifampicin resistant frozen sputum concentrates collected over a two year period were selected for culture (Figure 2). The mean proportion that were positive in MGIT was higher than 80% across time points. Table 1 shows samples grouped by the duration they were frozen at -80°C in months. Overall, 90.5% were positive in MGIT culture, 7.8% were negative, and 1.7% were contaminated.

The samples were further categorized by their smear microscopy grade (Table 2). The proportion positive, negative, and contaminated ranged between 86.5–96.3%, 3.7–11.7%, and 1.8–2.6%, respectively. There was no relationship between smear microscopy grade and MGIT culture result ($p > 0.05$).

Discussion

We designed a method for processing and freezing large numbers of sputum samples from patients diagnosed with TB. Large observational and randomised studies may require the storage of primary samples with the option of retrieving and culturing them at a later date. Culturing each sample upfront is prohibitively expensive whilst freezing primary samples without any decontamination step risks contamination of future cultures. Our approach uses NALC-NaOH digestion-decontamination to concentrate the mycobacteria within each sample (Ratnam & March, 1986), before and after freezing, allowing a greater number of sputum samples to be stored in a freezer in 0.5-mL cryovials, and minimising the number of contaminated cultures after defrosting.

We saw that the freezing process extends the time to culture positivity compared to culture without a prior freezing step, and also reduced the growth units reported by the BACTEC MGIT system. Such detrimental effect from freezing sputum was also observed for other microbes such as *Pseudomonas aeruginosa* (Poonja *et al.*, 2017). Although only one of our 22 samples failed to grow entirely after defrosting, freeze-thawing can lead to some samples not growing in culture (Shinu *et al.*, 2016; Shu *et al.*, 2012). Our method is not suitable for studies that assay time-to-positivity, but it may be helpful to those needing to store samples first and perform phenotypic drug susceptibility testing or molecular tests on a to-be-defined subset of samples at a later date. Our method works well when applied to 1+, 2+, and 3+ smear microscopy positive samples.

As seen elsewhere, we found that the duration of storage at -80°C did not strongly affect MGIT positivity rate (Tessema *et al.*, 2011). Our method resulted in a mean MGIT positivity rate of over 80% across all time points and above the mean obtained in a comparable study (86.0%) where sputum was frozen at -80°C for only 3 months (Shinu *et al.*, 2016). Although

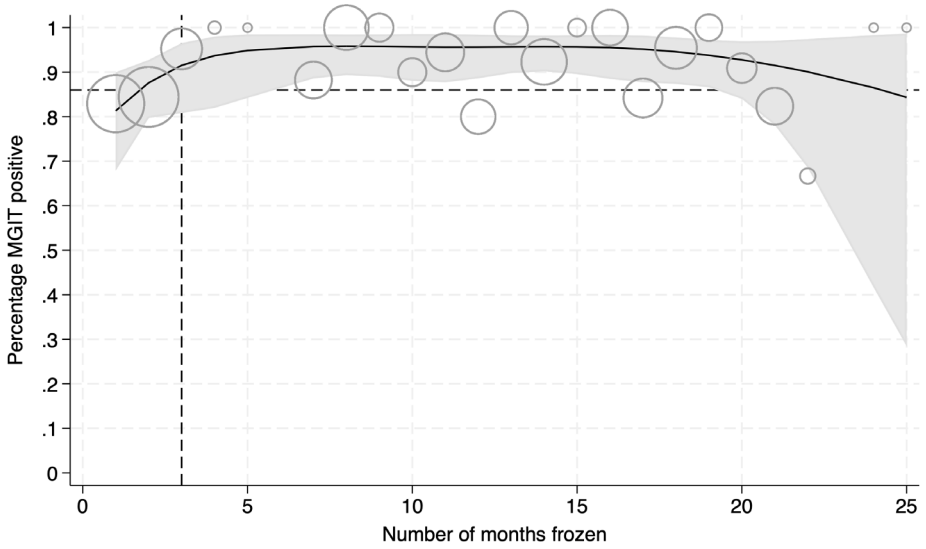

Figure 2. Percentage of sample that were MGIT positive after having been frozen. Curve defined by logistic regression using natural cubic splines. Black line shows the mean and grey shaded area the 95% confidence intervals. Circle sizes are weighted to show the relative number of samples at each time point. Dashed lines indicate the time samples were frozen for, and the percentage that were culture positive (86%), in Shinu *et al.*

Table 1. Number of MGIT samples grouped by duration of storage at -80°C (months) and the corresponding cultures result (%). MGIT, Mycobacterium Growth Indicator Tubes.

# of months stored at -80°C	Culture positive	Culture negative	Contaminated	Total
1	34 (82.9%)	7 (17.1%)	0	41
2	38 (84.4%)	7 (15.6%)	0	45
3	20 (95.2%)	1 (4.8%)	0	21
4	2 (100%)	0	0	2
5	1 (100%)	0	0	1
7	15 (88.2%)	2 (11.8%)	0	17
8	25 (100%)	0	0	25
9	10 (100%)	0	0	10
10	9 (90%)	0	1 (10%)	10
11	17 (94.4%)	1 (5.6%)	0	18
12	12 (80%)	2 (13.3%)	1 (6.7%)	15
13	14 (100%)	0	0	14
14	24 (92.3%)	1 (3.9%)	1 (3.9%)	26
15	4 (100%)	0	0	4
16	16 (100%)	0	0	16
17	16 (84.2%)	2 (10.5%)	1 (5.3%)	19
18	21 (95.5%)	1 (4.6%)	0	22
19	9 (100%)	0	0	9
20	10 (90.9%)	0	1 (9.1%)	11
21	14 (82.4%)	3 (17.7%)	0	17
22	2 (66.7%)	0	1 (33.3%)	3
24	1 (100%)	0	0	1
25	1 (100%)	0	0	1
Total	315 (90.5%)	27 (7.8%)	6 (1.7%)	348

Table 2. MGIT positive, negative, and contamination percentage classified by ZN grade (N = 348). MGIT, Mycobacterium Growth Indicator Tubes.

ZN grade	Positive	Negative	Contamination	Total
3+	79 (96.3 %)	3 (3.7 %)	0	82
2+	96 (86.5 %)	13 (11.7 %)	2 (1.8 %)	111
1+	140 (90.3 %)	11 (7.1 %)	4 (2.6 %)	155
Total	**315 (90.5 %)**	**27 (7.8 %)**	**6 (1.7 %)**	**348**

the vast majority of the sputum samples were taken before the patients started their treatment, it is possible that a very small number had already started anti-tuberculosis treatment by the time we obtained a sample. We do not have data on how many, but it is clearly the case that prior treatment risks negative sputum cultures. Our overall contamination rate was also lower than previously reported (1.7% *vs.* 5.2%) (Shinu *et al.*, 2016), suggesting that modified method of decontamination and storing sputum is unlikely to increase the risk.

We managed to recover *M. tuberculosis* from deep-frozen, processed sputum samples beyond the durations that other studies have conducted. However, there are limitations of our study. We did not include a non-frozen control group when we cultured the 348 MDR strains as these were all already frozen. We did not compare alternative methods of decontamination and storage to our own. We instead devised a method that would suit our purposes and local capacity and compared to direct-from-sample culture. This setup gave us confidence to continue with our method, which we then assessed again for the effect of time spent in the freezer on the success of future attempts at culture. We cannot therefore say that our method is better than other methods. It did however yield acceptable outcomes in terms of both culture and contamination. It is also easy and cheap to implement and has allowed us to so far collect and store over 30,000 sputum samples, some for over two years now. The method will however require validation in other laboratories in the future.

Data availability

Underlying data

Zenodo: A modified decontamination and storage method for sputum from patients with tuberculosis. https://doi.org/10.5281/zenodo.7584298.

This project contains the following underlying data:

- "22_samples_TTP_GU_method_comparison_dataset.csv" (dataset for comparing standard method and modified method using 22 sputum samples before being cultured in MGIT. MGIT is used in BD BACTEC 960 MGIT system which generates "Time to positive" hours for a culture to growth and "Growth Unit" for estimating the amount of growth.)
- "348_samples_TTP_GU_modified method_dataset.csv" (dataset for applying modified method on selected 348 sputum samples for culture in MGIT. The dataset contains "Time to positive", "Growth Unit", "ZN smear grade", and "Duration of frozen".)

Acknowledgements

We would like to thank the patients for participant in the study. We also thank all staff of the District Tuberculosis Units, Pham Ngoc Thach Hospital, and Oxford University Clinical Research Unit for their expertise and assistance that have made this study possible.

References

Harries AD, Kumar AMV: **Challenges and Progress with Diagnosing Pulmonary Tuberculosis in Low- and Middle-Income Countries.** *Diagnostics (Basel).* 2018; **8**(4): 78.
PubMed Abstract | Publisher Full Text | Free Full Text

Poonja A, Heirali A, Workentine M, *et al.*: **Effect of freezing sputum on *Pseudomonas aeruginosa* population heterogeneity.** *J Cyst Fibros.* 2017; **16**(3): 353–357.
PubMed Abstract | Publisher Full Text

Ratnam S, March SB: **Effect of relative centrifugal force and centrifugation time on sedimentation of mycobacteria in clinical specimens.** *J Clin Microbiol.* 1986; **23**(3): 582–5.
PubMed Abstract | Publisher Full Text | Free Full Text

Shinu P, Singh VA, Nair A, *et al.*: **Long-Term Storage at -80°C: Effect on Rate of Recovery of *Mycobacterium tuberculosis* From Direct Acid-Fast Bacilli Smear-Positive Sputum Samples.** *J Clin Lab Anal.* 2016; **30**(5): 567–76.
PubMed Abstract | Publisher Full Text | Free Full Text

Shu Z, Weigel KM, Soelberg SD, *et al.*: **Cryopreservation of *Mycobacterium tuberculosis* Complex Cells.** *J Clin Microbiol.* 2012; **50**(11): 3575–80.
PubMed Abstract | Publisher Full Text | Free Full Text

Tessema B, Beer J, Emmrich F, *et al.*: **Rate of Recovery of *Mycobacterium tuberculosis* from Frozen Acid-Fast-Bacillus Smear-Positive Sputum Samples Subjected to Long-Term Storage in Northwest Ethiopia.** *J Clin Microbiol.* 2011; **49**(7): 2557–61.
PubMed Abstract | Publisher Full Text | Free Full Text

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Current Peer Review Status: ? ✓ ?

Version 2

Reviewer Report 28 August 2024

https://doi.org/10.21956/wellcomeopenres.23187.r94424

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Virasakdi Chongsuvivatwong
Prince of Songkla University, Kanjanavanich Rd, Kho Hong Hat Yai, Thailand

The first part of the study having 22 specimens divided into 2 aliquots suffers from smallness of the sample size.

The second part of the study have larger sample size suffers from lacking of standard procedure control. This part showed that the results did not perfectly agree with ZN and Xpert methods. Whether the discrepancy was due to non-TB sputum or deterioration of viability of M.tb is not known.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Partly

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: TB research training.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 27 August 2024

https://doi.org/10.21956/wellcomeopenres.23187.r74935

Irina Kontsevaya
Imperial College London, London, England, UK

I have no further comments.

Is the rationale for developing the new method (or application) clearly explained?
Partly

Is the description of the method technically sound?
Partly

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Laboratory diagnostics of tuberculosis, biomarkers of tuberculosis disease and cure, treatment monitoring tools.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 21 November 2023

https://doi.org/10.21956/wellcomeopenres.20944.r69570

Irina Kontsevaya
Imperial College London, London, England, UK

The manuscript presents the results of a two-phase study of evaluation of a modified method for decontamination and storage of sputum samples collected from individuals with pulmonary tuberculosis. While in routine diagnostic it is essential to process and culture sputum samples as soon as possible for fast and precise diagnostics of tuberculosis, storage of sputum samples is often needed in longitudinal studies and randomised clinical trials where culture results might only be needed for a selected sample which cannot be determined at the time of sample collection. A method for storage of sputum in small volumes (cryovials) that does not affect the viability of Mycobacterium tuberculosis in sputum is therefore urgently needed.
The method proposed in this study is simple, it does not require additional reagents beside those used in the standard procedure, and not time-consuming. It offers a possibility to store samples in small 0.5 ml vials which is important in terms of storage space in the freezers. The study demonstrates that viability and culturability of the sputum samples after applying the modified method is not affected by freezing-thawing and also doesn't increase the contamination rate. The duration of storage also seems not to affect the MGIT positivity rate. However, it is demonstrated that storage extends the time to culture positivity and reduces growth units. Samples processed with this modified method therefore cannot be used in studies for quantitative evaluation of growth but rather in those where, for example, culture is further used for genomic studies or assessment of bacterial resistance profile.

Overall, this manuscript is of great interest for researchers conducting large studies in tuberculosis, including randomised controlled trials. However, the limitations of this study do not allow to use it as a recommendation to immediately implement the method in practice. While this can be considered a proof-of-concept, I believe further studies are needed to address the current limitations and multiple further questions that arise from this study. Below is the number of suggested further studies:

1. Direct culture vs. culture from samples frozen for a long period of time. Currently, only 22 samples were compared being cultured directly and after being frozen for 1 week. No such comparison was made for samples frozen for longer periods of time.

2. Effects of storage for longer periods, up to 5-10 years. Long-term storage is important for some types of studies, for example transmission studies on historical samples.

3. Evaluation of the method with storing samples at -20. In the study all samples were frozen at -80. In reality, many studies in tuberculosis are conducted in resource-limited settings

where sputum is collected and stored at local clinics and laboratories. In such settings, -80 freezers are often not available. Therefore, in practical terms it would be great to have a method that would allow effective storage of samples at -20 or even at +4.

4. Comparison of the proposed method with other methods of sputum storage. While it is demonstrated that this method is effective and not resource-intensive, other methods for sputum storage can also be characterised this way. To choose the best method to apply in a particular setting, comparison data are needed.

While I appreciate that these suggested studies cannot be implemented in the current manuscript, perhaps the authors could think about further studies to validate their method or motivate other groups to join the evaluation of the method to explore its feasibility.

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Laboratory diagnostics of tuberculosis, biomarkers of tuberculosis disease and cure, treatment monitoring tools.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 16 November 2023

https://doi.org/10.21956/wellcomeopenres.20944.r69006

Bhushan J. Toley
Indian Institute of Science, Bengaluru, Karnataka, India

In tuberculosis (TB) diagnostics and research, the storage and subsequent culturing of sputum samples are fundamental yet challenging tasks, especially under resource-constrained conditions or when dealing with high sample volumes. The study presented here introduces a modified method for the handling and preservation of sputum samples containing Mycobacterium tuberculosis with the aim of enhancing their viability over extended storage periods.

The standard practice of using glycerol as a cryoprotectant is maintained in this method, which is not novel in itself. However, the study proposes a second decontamination step post-thaw as a slight modification to the conventional process. This additional step is posited to potentially improve the recovery of viable bacteria after the samples have been frozen and then thawed for later use.

While the degree of innovation in the proposed method may not be substantial, it represents an incremental advance that could have practical implications for the storage duration of sputum samples. Such a modification could indeed prove beneficial in settings where the immediate processing of samples is not possible and long-term storage becomes a necessity.

This paper thus offers a potential improvement in TB sample management, albeit a modest one, which warrants attention for its possible impact on TB management protocols, especially in settings facing logistic challenges in sample processing.

I have reviewed the dataset and methodology and wish to present a series of observations that merit consideration. These insights aim to refine the research approach and enhance the validity of the findings. They are presented with the intent of constructively supporting the study's progression towards robust, reproducible results.

Control Groups and Comparative Analysis:
The study presents a novel two-step NALC-NaOH decontamination process for sputum samples. Yet, the absence of a direct comparison with a single-step decontamination control group undermines the validation of this method. To substantiate the necessity and effectiveness of the second decontamination step, it is recommended that the authors include such a control group in their analysis.
Additionally, exploring the viability of samples stored at varied temperatures—room temperature, 4°C, -20°C, as well as -80°C—could provide valuable insights into more resource-efficient storage methods. This would be particularly relevant in settings where maintaining -80°C storage is challenging.

Statistical and Long-term Storage Experiment's Shortcomings:
The statistical analysis in this study is insufficient, particularly for samples stored over 13 months, potentially skewing conclusions. Specifically, there are a larger number of samples used at lower time points and much smaller number of samples used for later time points. It is critical to adopt a more rigorous statistical model that accounts for variable sample sizes across different storage times. The claim that 90.5% of samples were viable after 25 months should be interpreted with caution. A balanced evaluation of sample viability across varied storage periods is necessary to convincingly determine the method's efficacy for long-term storage.

Reproducibility and Robustness:
Although not a pressing issue, the reproducibility of the modified method when applied in different laboratory environments or by different technicians may be addressed in future studies. Ensuring reproducibility is key to the method's robustness and broader application. Future research could enhance the study's validity by providing such reproducibility data.

Data Presentation:
The data presentation could be optimized for clarity: integrating median lines into the boxplots would provide a clear depiction of central tendencies, while simplifying TTP and GU connections with lighter lines would reduce clutter. Additionally, distinctly illustrating whiskers on boxplots would elucidate data variability and outliers, improving overall interpretability.

Literature Context:
The discussion would gain depth from a comparison of the modified method against other methods cited in current literature. This broader perspective would help situate the study within the existing research landscape.

Conclusion and final comments:
While the study introduces a promising technique potentially beneficial for tuberculosis diagnostic practices, the manuscript currently falls short in demonstrating the full validity of the proposed method due to the lack of proper control groups and robust statistical analysis. I recommend significant revisions to address these gaps before the manuscript can be considered for acceptance. The enhancements proposed here would not only strengthen the scientific rigor of the paper but also its contribution to the field.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Point of care medical daignostics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 25 Jan 2024

Quang Nguyen Le

Control Groups and Comparative Analysis: The study presents a novel two-step NALC-NaOH decontamination process for sputum samples. Yet, the absence of a direct comparison with a single-step decontamination control group undermines the validation of this method. To substantiate the necessity and effectiveness of the second decontamination step, it is recommended that the authors include such a control group in their analysis. Additionally, exploring the viability of samples stored at varied temperatures—room temperature, 4°C, -20°C, as well as -80°C—could provide valuable insights into more resource-efficient storage methods. This would be particularly relevant in settings where maintaining -80°C storage is challenging.

We thank the reviewer for the thoughtful review and detailed comments. Our study consisted of two phases: a pilot to compare one-stage and two-stage procedures, and a later stage to look at the impact on the ability to culture over longer periods of time. As part of the pilot study we therefore did have a control group. As the comparison between standard and new methods was very similar in the pilot phase, the next step was to look at samples that had been frozen for longer periods of time. We were unable to include a further control group here as the samples had already been frozen (this was retrospective). As we conducted this small study to inform how we would handle a large number of samples for an observational study that was about to start, we did not have time to collect several hundred samples prospectively and aliquot these into experimental and control groups. As the main study is coming to an end, we do not have the resource now to repeat this experiment. However, we have included a section in the discussion to acknowledge this limitation. The same goes for the idea that we repeat the experiment at different temperatures. It's a good idea, but unfortunately not possible as part of this little project.

Statistical and Long-term Storage Experiment's Shortcomings: The statistical analysis in this study is insufficient, particularly for samples stored over 13 months, potentially skewing conclusions. Specifically, there are a larger number of samples used at lower time points and much smaller number of samples used for later time points. It is critical to adopt a more rigorous statistical model that accounts for variable sample sizes across different storage times. The claim that 90.5% of samples were viable after 25 months should be interpreted with caution. A balanced evaluation of sample viability across varied storage periods is necessary to convincingly determine the method's efficacy for long-term storage.

The current text does not actually state that 90.5% were viable after 25 months. It says "Overall, 90.5% were positive in MGIT…", which is true. In the discussion we later state that the overall positivity rate was 90.5% and compare this with the positivity rate at 3 months seen by Shinu et. al.. If anything, our results would have been even better had we cultured them at 3 months and not included such a long tail. So the skew is unlikely introducing bias

when it comes to that conclusion. However, we agree that “No significant difference was seen among groups ($p > 0.05$)” is an inadequate analysis. We have therefore added a figure that shows the mean percentage of samples (plus 95% confidence interval) that were positive at each month, together with the number of samples frozen for each period of time. The figure summarises the output of a logistic regression model that uses natural cubic splines to account for the non-linear trend over time. MGIT status (pos/neg) at each month is the dependent variable, and the number of months frozen for each month is the independent co-variable. This shows that a greater percentage of our samples were positive when compared to Shinu et al.

Reproducibility and Robustness: Although not a pressing issue, the reproducibility of the modified method when applied in different laboratory environments or by different technicians may be addressed in future studies. Ensuring reproducibility is key to the method's robustness and broader application. Future research could enhance the study's validity by providing such reproducibility data.

We agree and have added a sentence to the discussion to highlight this.

Data Presentation: The data presentation could be optimized for clarity: integrating median lines into the boxplots would provide a clear depiction of central tendencies, while simplifying TTP and GU connections with lighter lines would reduce clutter. Additionally, distinctly illustrating whiskers on boxplots would elucidate data variability and outliers, improving overall interpretability.

Done.

Literature Context: The discussion would gain depth from a comparison of the modified method against other methods cited in current literature. This broader perspective would help situate the study within the existing research landscape.

We have edited the discussion a little but there aren’t additional papers that are directly comparable to our that we were able to find.

Conclusion and final comments: While the study introduces a promising technique potentially beneficial for tuberculosis diagnostic practices, the manuscript currently falls short in demonstrating the full validity of the proposed method due to the lack of proper control groups and robust statistical analysis. I recommend significant revisions to address these gaps before the manuscript can be considered for acceptance. The enhancements proposed here would not only strengthen the scientific rigor of the paper but also its contribution to the field.

Thank you. We have done our best to address your points. We hope our answers are clear and satisfactory.

Competing Interests: No competing interests were disclosed.