

Optimizing Tuberculosis Testing for Basic Laboratories

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Abstract. Optimal tuberculosis testing usually involves sputum centrifugation followed by broth culture. However, centrifuges are biohazardous and scarce in the resource-limited settings where most tuberculosis occurs. To optimize tuberculosis testing for these settings, centrifugation of 111 decontaminated sputum samples was compared with syringe-aspiration through polycarbonate membrane-filters that were then cultured in broth. To reduce the workload of repeated microscopic screening of broth cultures for tuberculosis growth, the colorimetric redox indicator 2,3-diphenyl-5-(2-thienyl) tetrazolium chloride was added to the broth, which enabled naked-eye detection of culture positivity. This combination of filtration and colorimetric growth-detection gave similar results to sputum centrifugation followed by culture microscopy regarding mean colony counts (43 versus 48; $P = 0.6$), contamination rates (0.9% versus 1.8%; $P = 0.3$), and sensitivity (94% versus 95%; $P = 0.7$), suggesting equivalency of the two methods. By obviating centrifugation and repeated microscopic screening of cultures, this approach may constitute a more appropriate technology for rapid and sensitive tuberculosis diagnosis in basic laboratories.

INTRODUCTION

Optimal tuberculosis diagnosis and drug susceptibility testing usually require culturing mycobacteria in broth.¹ However, the low sensitivity of culture without centrifuge concentration,² the cost³ and biohazard^{4,5} of centrifuges, and difficulties detecting *Mycobacterium tuberculosis* growth in broth cultures are obstacles to implementation in resource-limited settings where 95% of tuberculosis deaths occur.^{6,7} We aimed to diminish these limitations to tuberculosis diagnosis.

Centrifugation² may create biohazardous aerosols that risk laboratory tuberculosis transmission.^{4,5} Centrifugation also involves equipment purchase (approximately U.S. \$7,000 for aerosol-resistant equipment),³ annual maintenance costs, and the purchase of centrifuge tubes (approximately U.S. \$0.70 per sample).³ In contrast, filters have been used successfully to concentrate mycobacteria without major expenditure and without involving techniques likely to generate infectious aerosols.^{8–11}

Detection of *M. tuberculosis* in broth culture usually uses semi-automated radioactive- or fluorescence-based proprietary equipment,¹² but these systems may be difficult to afford and maintain in some resource-limited settings. Recently, the microscopic-observation drug-susceptibility (MODS) technique has been validated as an inexpensive broth-culture technique that sensitively diagnoses tuberculosis in 1–3 weeks.^{13–16} However, this non-proprietary technique detects tuberculosis growth in the cultures by repeated microscopic examination, which is time-consuming and relies upon the technicians' thoroughness when searching for colonies under the microscope. As a potential solution to this limitation, colorimetric redox-indicators have been used to facilitate naked-eye detection of culture positivity.¹⁷

Therefore, we conducted a proof of concept study that aimed to optimize broth culture for resource-limited settings

by replacing centrifugation with filtration and facilitating detection of positive cultures by using a colorimetric indicator of microbial growth. We evaluated the equivalency of these novel techniques compared with standard procedures.

METHODS

Clinical samples. After institutional review board approval and informed written consent were obtained, sputa were collected from outpatients with suspected or diagnosed pulmonary tuberculosis in shantytown health centers in Lima, Peru.

Sample processing and decontamination. A direct smear for Ziehl-Neelson staining was prepared from each sample. Sputa were subsequently liquefied and decontaminated by the standard NaOH-*N*-acetyl-L-cysteine method,¹ and then neutralized by dilution to a final volume of 14 mL with phosphate-buffered saline, pH 6.8. The MODS technique uses 15-mL centrifuge tubes to enable high throughput decontamination, with each tube containing only 14 mL of phosphate-buffered saline to reduce the risk of spillage.¹⁸

Post-decontamination concentration. Equal 7-mL aliquots of each 14-mL decontaminated sample were subjected to two concentration methods: centrifugation and filtration. Half (7 mL) of the decontaminated sample was centrifuged according to the standard MODS protocol,¹⁸ i.e., centrifugation at $3,000 \times g$ for 15 minutes at 17°C in aerosol-resistant centrifuge buckets. The supernatant was discarded and the pellet was re-suspended in 2 mL of culture broth, 1 mL of which was saved as a back-up (and was not used in this research) and the other 1 mL of the resuspended pellet was used in the MODS detection assay.¹⁴ In the MODS assay, this 1-mL inoculum is usually divided between several culture-wells in a 24-well tissue culture plate. However, for the present research, to facilitate comparison with the filtration technique, the entire 1 mL (i.e., one-fourth of the original sample) was inoculated into a single culture well in a six-well tissue culture plate (Falcon, Franklin Lakes, NJ).

To test for equivalence with the MODS centrifugation technique, filtration also used one-fourth of the original sample. Specifically, 3.5 mL of the decontaminated sample was aspirated

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through a filter and the remainder was saved as a back-up (and was not used in this research). A 25-mm diameter, 0.4- μ m pore size Isopore polycarbonate membrane filter⁹ was used in a reusable, sterile polypropylene filter holder (filters and holders supplied by Millipore, Billerica, MA). Because use of positive pressure to force samples through filters may risk infectious aerosol production, we instead used syringes to generate negative pressure suction to aspirate samples through filters (Figure 1A). Filters were then removed from the filter holder and sterile forceps were used to place the filter directly into wells in a sterile tissue-culture plate (Figure 1B).

Mycobacterial culture. All cultures were done in Middlebrook 7H9 broth with OADC¹⁴ that was supplemented with 50 μ g/mL of the redox indicator 2,3-diphenyl-5-(2-thienyl) tetrazolium chloride (STC) (VWR International, West Chester, PA)¹⁷ at 37°C in air. To minimize the risk of cross-contamination and occupational exposure, the culture-plates were closed with lids and permanently sealed in transparent plastic bags in which they were subjected to naked-eye and microscopic examination.^{14,15}

Culture assessment. Sealed cultures were all examined three times a week first by naked-eye for colorimetric evidence of microbial growth and then under an inverted light microscope at 40 \times magnification. The number of cording colonies characteristic of *M. tuberculosis* was counted 40 days after decontamination.² The reference standard was the total number of samples that were culture positive by either centrifugation and/or filtration.¹⁹ The sensitivities of cultures derived from samples concentrated by centrifugation and filtration were calculated as proportions of the number of samples that were culture positive in the reference standard. The rate of culture positivity was calculated as the number of positive cultures as a proportion of the total number of samples tested. Partial contamination was defined as visible contamination within a culture well that only partially obscured the culture, enabling interpretation of the presence or absence of mycobacterial

growth. Total contamination was defined as the presence of contamination that completely obscured the culture well, preventing assessment of mycobacterial growth.

Statistical analysis. Non-Gaussian data were summarized as medians with interquartile ranges (IQRs) and colony counts were compared as geometric means by using the Wilcoxon signed-rank test. Proportions were calculated with their 95% confidence intervals (CIs) and were compared by using the McNemar test. Culture time-to-positivity was analyzed by using the log-rank test. Power calculations determined that this study had 80% power at a 95% significance level to detect a 13% difference in diagnostic sensitivity between centrifugation versus filtration.

RESULTS

Study population. The 111 sputum samples were obtained from 95 patients (median age = 30 years, male:female ratio = 1.2:1) (Table 1). Sixteen patients provided two sputum samples, according to the diagnostic algorithms of their clinicians. Patients received tuberculosis treatment of a median of three days prior to sample collection, and 57% of the sputa were collected during treatment. A total of 51% of the samples were sputum smear microscopy positive. Median sputum volume was 1.5 mL (IQR = 0.5–2.0 mL). Because of the viscosity of the decontaminated sputum, only a median 0.8 mL (IQR = 0.2–1.5 mL) could be aspirated through the polycarbonate filter and the remainder of the sample was discarded. Passing all of the intended 3.5 mL through the filter was possible for only one sample. Comparison of the results of the diagnostic techniques under assessment is shown quantitatively in Figure 2 and qualitatively in Table 2. Analysis of these data is shown in Table 3.

Detection sensitivity. A total of 59% of samples were culture-positive by either centrifugation or filtration, 56% (95% CI = 47–65%) by centrifugation and 55% (95% CI = 46–64%) by filtration ($P = 0.7$, Table 3). Thus, the sensitivity to detect the 65 samples that were culture positive by either test i.e., compared with the reference-standard, was 95% (95% CI 90–100%) by centrifugation and 94% (95% CI = 88–100%) by filtration ($P = 0.7$) (Figure 2A). Excluding the cultures that were totally obscured by contamination, sample concentration by centrifugation and filtration yielded culture results with 94% agreement ($\kappa = 0.89$; 95% CI = 0.80–0.98, $P < 0.0001$). There was no significant difference in *M. tuberculosis* colony counts between cultures derived from centrifugation and

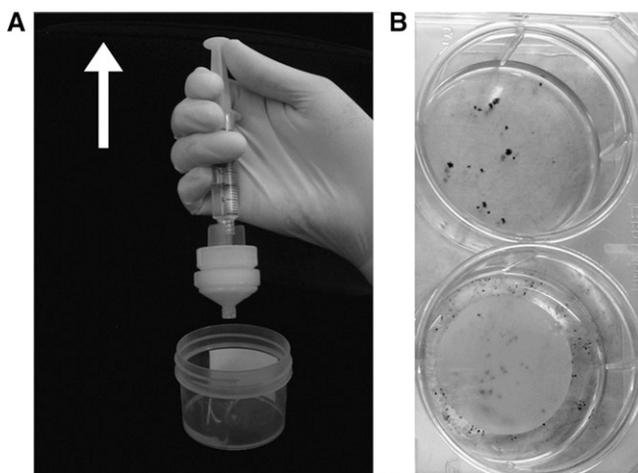


FIGURE 1. **A**, Filtration technique. Decontaminated sputum being aspirated through a filter with suction from a syringe. **B**, Culture technique. Localized (red) color change caused by the 2,3-diphenyl-5-(2-thienyl) tetrazolium chloride indicator in broth cultures indicating growth of tuberculosis colonies. The upper well contains a culture derived from the centrifuge-concentrated aliquot of a decontaminated sputum sample. The lower well contains a filter that had been used to concentrate the other aliquot of the same decontaminated sputum sample. Tuberculosis colonies are visible on and around the filter.

TABLE 1

Patient and sample characteristics*

Characteristic	Samples (n = 111)	Patients (n = 95)
Patients already taking tuberculosis treatment, % (no.)	57 (63)	55 (52)
Median treatment duration, days (IQR)	3 (0–15)	2 (0–6)
Median age, years (IQR)	–	30 (19–45)
Patient sex, % female (no.)	–	46 (44)
Sputum microscopy positive, % (no.)	51 (57)	47 (45)
Sputum culture positive by either test, % (no.)	59 (65)	53 (50)
Median sputum volume, mL (IQR)	1.5 (0.5–2.0)	–

*IQR = interquartile range.

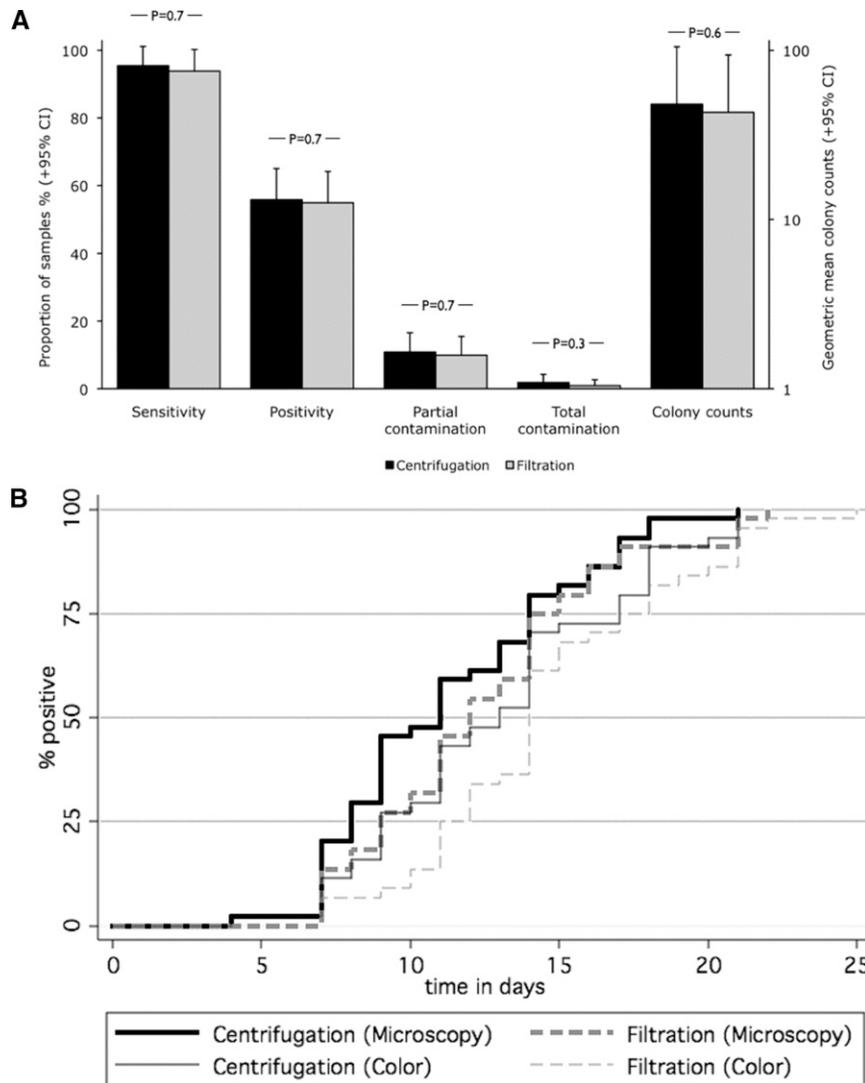


FIGURE 2. **A**, Performance of centrifugation and filtration. Black bars indicate centrifugation-concentrated aliquots and gray bars indicate filtration-concentrated aliquots. **B**, Days until tuberculosis detection for samples positive by centrifugation and filtration. Thick lines represent naked-eye colorimetric detection, solid lines represent centrifuge-concentrated aliquots, and broken lines represent filtration-concentrated aliquots. Colony count and time to positivity analyses excluded contaminated cultures.

filtration (geometric mean = 48 colonies versus 43 colonies; $P = 0.6$) (Figure 2A).

Contamination. Partial contamination that did not completely obscure tuberculosis growth had a similar frequency in aliquots concentrated by centrifugation versus filtration: 11% (95% CI = 5.0–17%) versus 10% (95% CI = 4.4–15%), respectively ($P = 0.7$). The same finding was true for contamination that totally obscured the culture well: 1.8% (95% CI = 0.0–4.3%) versus 0.90% (95% CI = 0.0–2.7%, $P = 0.3$) (Figure 2A).

Time to positivity. Culture of centrifuge-concentrated versus filtration-concentrated aliquots had similar times (Figure 2B) for microscopic-examination of cultures (median = 11 days versus 12 days; $P = 0.2$) and for naked-eye colorimetric detection of positive cultures (median = 13 days versus 14 days; $P = 0.1$). Naked-eye colorimetric detection indicated for culture positivity a median of 2 days (IQR 0–3 days) later than microscopy (Figure 2B; $P = 0.05$). Sputa collected from patients who had not yet commenced tuberculosis therapy became culture positive significantly sooner than samples

collected during treatment (e.g., for centrifuge concentration with microscopic examination the time to positive was a median of 9 days versus 12 days, respectively; $P = 0.04$). For sputum smear microscopy-negative samples, the median time to culture positivity was 14 days by all techniques (Table 3).

Colorimetric growth detection. The colorimetric indicator STC changed color in all cases of tuberculosis growth. Thus, naked-eye colorimetric screening of cultures did not show any

TABLE 2
Comparison of culture results for centrifugation-concentration and filtration-concentrated samples*

Filtration	Centrifugation		Total
	Positive	Negative	
Positive	58 (52%)	3† (3%)	61 (55%)
Negative	4 (4%)	46‡ (41%)	50 (45%)
Total	62 (56%)	49 (44%)	111 (100%)

* Microscopic and colorimetric detection of cultures yielded identical final results. Negative results include two samples with total contamination for centrifugation† and also one sample with total contamination in centrifugation and filtration.‡ Samples positive by either test are indicated in **bold** and represent the reference standard (65 culture-positive samples).

TABLE 3
Performance of culture with centrifugation and filtration*

Characteristic	Centrifugation (n = 111)	Filtration (n = 111)	P
TB detection			
Sensitivity relative to reference standard, no., % (95% CI)	62/65, 95% (90–100)	61/65, 94% (88–100)	0.7
Positivity for all samples, no., % (95% CI)	62/111, 56% (47–65)	61/111, 55% (46–64)	0.7
Specificity, % (95% CI)	100% caused by the reference-standard used (see methods section)		
Positive predictive value			
Negative predictive value	94% (87–100)	92% (84–100)	0.7
Geometric mean colony counts (95% CI)	48 (22–105)	43 (20–94)	0.6
Detection speed			
Median time to positivity by microscopy, days (IQR)	11 (8–14)	12 (9–14)	0.2
Median time to positivity by naked eye, days (IQR)	13 (9–17)	14 (11–17)	0.1
Median time to positivity by microscopy for smear-negative samples, days (IQR)	14 (13–14)	14 (13–14)	1.0
Median time to positivity by naked eye for smear-negative samples, days (IQR)	14 (14–18)	14 (14–18)	0.8
Contamination			
Total, no., % (95% CI)	2/111, 1.8 (0.0–4.3)	1/111, 0.9 (0.0–2.7)	0.3
Partial, no., % (95% CI)	12/111, 11 (5.0–17)	11/111, 10 (4.4–15)	0.7

* Colony counts were calculated after excluding samples with contamination in either test. For the calculations of sensitivity and positivity, cultures without visible *Mycobacterium tuberculosis* growth were considered to be culture negative if the culture was contaminated. CI = confidence interval; IQR = interquartile range.

false-negative results compared with subsequent microscopic examination of the same culture well. Most bacterial and some fungal contaminants also caused a color change, and naked-eye inspection of the large colonies of contaminants clearly distinguished them from mycobacteria in all but one sample that required 40× magnification examination of the sealed culture well with the inverted microscope to determine that the color change was caused by bacterial contamination, and not by the morphologically distinct cording colonies indicating *M. tuberculosis* growth.¹⁵

Workload. Considering the time required to process three sputa, concentrating by centrifugation required approximately 30 minutes (including 15 minutes of centrifugation) compared with filtration, which required less than 10 minutes. Therefore, the use of filters reduced the processing time for concentrating decontaminated sputum samples compared with centrifugation.

Scanning each culture-plate with the inverted light microscope at 40× magnification to search for cording colonies of mycobacterial growth took approximately 20 minutes. In contrast, it took less than 10 seconds for a naked-eye examination of each plate for colorimetric indication of microbial growth. When a point of color change was identified by naked-eye colorimetric inspection, subsequent microscopic examination of this specific area of color change within the sealed culture-plate for *M. tuberculosis* cording morphology to differentiate contaminants or atypical mycobacteria took approximately 10 seconds. Thus, naked-eye colorimetric detection of microbial growth considerably reduced workload compared with microscopy.

DISCUSSION

This proof-of-concept study shows that disposable filters show equivalent performance to centrifugation for sputum processing and that colorimetric naked-eye indication of microbial growth with the indicator STC increases efficiency of broth culture for tuberculosis diagnosis. Sensitivity, colony counts, time-to-positivity, and contamination rates by filtration concentration of sputum aliquots were similar to sample processing by centrifugation. Naked-eye colorimetric detection of culture positivity in comparison with microscopic inspec-

tion considerably reduced the staff time required, but delayed diagnosis slightly. Therefore, the addition of STC is a simple way to increase the efficiency of the MODS assay, at the cost of slightly delayed diagnosis.

Sputum microscopy is rapid but is relatively insensitive, especially for children and persons infected with human immunodeficiency virus (HIV) and cannot detect drug-resistant tuberculosis. Sensitive tuberculosis diagnosis and drug-susceptibility testing usually require sample centrifugation but centrifuges are expensive for laboratories in resource-limited settings and culture without sample centrifugation is generally insensitive.^{1,2} A well-maintained, refrigerated centrifuge with aerosol-resistant sealed buckets operated by experienced personnel is required to centrifuge mycobacteria-containing samples with optimal sensitivity and biosafety, but still recovers only a portion of the *M. tuberculosis* from the sample^{1,20} and leaves some of the bacteria in the supernatant. Filters have been used to concentrate mycobacteria but usually by applying suction from a vacuum pump^{8,9} and sometimes in addition to rather than in place of centrifugation.⁸ In pilot experiments, we found that 25-mm polycarbonate filters were small enough for culture of practicable size and large enough to enable a detectable volume to be filtered. In these pilot experiments, decreasing filter pore size was associated with greater *M. tuberculosis* yield but with more frequent filter blockage, which led us to use membranes with a 0.4- μ m pore size for this study, as in previous successful experiments.⁹

Although the current study did not assess cost-effectiveness, the filters cost less than U.S. \$0.50 each and the filter holders and syringes were inexpensive and reusable compared with the centrifuge that cost several thousand dollars. These observations imply that filtration may be more cost-effective than centrifugation and this issue warrants further study. Concentrating samples by filtration was also more rapid than concentration by centrifugation in our study. However, this result is dependent on the batch size and, because filtration is performed sequentially for each individual sample, centrifugation may be more rapid than filtration if ≥ 10 samples are centrifuged at once.

We have also shown that use of STC for colorimetric indication of microbial growth obviated repeated microscopic inspection of culture-negative samples, considerably decreasing

the workload of the MODS assay. *Mycobacterium tuberculosis* growth always caused color change, i.e., there were no false-negative results from naked-eye colorimetric detection of culture-positivity. The colorimetric indicator also eliminated the reliance of the MODS assay on the thoroughness of the technician when scanning culture wells for *M. tuberculosis* growth under the microscope. Most culture contamination caused large patches of color change and naked-eye examination showed that these patches were obviously not mycobacteria. However, in one culture well, a small point-of-color change required a single microscopic examination to demonstrate the absence of cording morphology, indicating that in this case the color change was caused not by *M. tuberculosis* but rather by atypical mycobacteria or contaminants.

Limitations to this study include the unblinded assessment of culture positivity because after centrifugation and filtration the aliquots were cultured in adjacent wells. Furthermore, workload data were not recorded precisely. In Peru, because only 3% of tuberculosis patients are HIV positive,² these data may not be generalizable to regions with high HIV prevalence where sputum microscopy-negative tuberculosis might be more common. This study did not test for an influence of the colorimetric indicator STC on mycobacterial growth although our unpublished data showed no effect. Although aspiration of decontaminated sputum through filters with manual suction from a sealed syringe probably avoids the aerosol production associated with centrifugation, further research is required to confirm this before the minimum necessary biosafety precautions for using this technique can be defined.

Centrifugation and filtration had similar concentrating efficiencies despite the filter becoming blocked before the entire sample was aspirated in almost all cases. Typically, less than one-fourth of the volume that was concentrated by centrifugation could be passed through the filters before they became blocked. Other liquefaction methods¹ and filters⁹ may enable more of each sample to be filtered, potentially increasing sensitivity. Future studies should also determine if concentration by filtration can be used for direct drug susceptibility testing¹⁵ and to increase the sensitivity of sputum smear microscopy.⁹ It is likely that this technique of sample concentration by filtration could be adapted to other culture techniques such as the commercial mycobacteria growth indicator tube technique, which is the subject of ongoing research.

In conclusion, disposable filters are an efficient alternative that has equivalent performance to centrifugation and may be of particular value in settings with limited financial resources. Naked-eye colorimetric indication of culture positivity with STC reduces sample reading time considerably but delays diagnosis slightly. Sensitive tuberculosis broth culture with disposable filters and STC appears to be an appropriate technology for tuberculosis culture in resource-limited settings.

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