Improved tuberculosis smear detection in resource-limited settings: Combined bleach concentration and LED fluorescence microscopy

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A B S T R A C T

Improved diagnostics for tuberculosis is a high priority in resource-limited settings (RLS). Sputum concentration and fluorescence microscopy (FM) are standard techniques in developed countries where appropriate biosafety precautions are possible. Recently, inexpensive fluorescent lenses using LED light sources have made auramine-based FM more feasible in RLS. Sterilization of sputum with bleach protects lab personnel and, combined with concentration, increases the sensitivity of microscopic detection. We compared the effect of both bleach concentration and FM with LED based lenses to culture for the detection of tuberculosis in military medical hospitals in Addis Ababa, Ethiopia. Three sputum specimens were obtained from 409 patients (1227 total). Standard Ziehl-Neelsen (ZN) or auramine staining were compared with direct or bleach-concentrated specimens. The prevalence by culture was 26%. Sensitivity of microscopic diagnosis was increased both by bleach concentration (14%) and auramine staining (5%). The overall yield of smear positivity varied from 21% for direct ZN to 27% for auramine after concentration (P<0.00001, Cochran test for matched proportions). Twenty-nine HIV+ patients were diagnosed with TB, but ten (34%), would have been missed with direct ZN staining. Bleach concentration and auramine staining with new LED fluorescent systems are cost-effective and safe methods to increase the diagnostic yield of smears, including in HIV-infected patients.

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1. Introduction

Tuberculosis is a major health problem worldwide, particularly in resource-limited areas where the majority of the 9.3 million new cases of TB in 2008 were diagnosed. 1 Sub-Saharan countries with high prevalences of HIV have been particularly hard hit. Thirteen of the 15 countries with the highest incidence of TB also have the highest HIV prevalence rates, 2 resulting in a co-infection rate of more than 30%. 3 HIV-infected patients are 20 times as likely to develop active tuberculosis in endemic areas, 3 yet the diagnosis is particularly challenging because they present with atypical chest x-rays and fewer acid fast bacilli in their sputum. Despite the development of rapid, molecular techniques to diagnose tuberculosis and faster liquid culture methods, direct sputum smears stained by the Ziehl-Neelsen (ZN)
method remain the mainstay of diagnosis in most resource-limited settings (RLS). Estimated sensitivity is only 70% overall and less in HIV-infected patients. The WHO has made the development of improved diagnosis of TB a major focus with a goal of a universal 70% detection rate by 2015.

Several studies have found enhanced sensitivity after concentration of mycobacteria by centrifugation following decontamination with bleach. Concentration in bleach not only sterilizes the sputum, decreasing the risk to laboratory personnel, but also enhances smear positivity by ZN smears by an average of 6–20%. Detection of smear positive cases may also be improved approximately 10% with the use of fluorescence microscopy. The development of new, relatively inexpensive LED powered fluorescent lenses has now made this technique possible in all countries. To our knowledge, this prospective study is the first to compare the added sensitivity of both bleach concentration and LED fluorescence microscopy to direct ZN staining using cultures as the gold standard.

2. Experimental materials and methods

2.1. Study design

From November 2007 until June 2009, a cross sectional study was performed in which three sputum samples were obtained for microscopic diagnosis of TB from patients at either Armed Forces General Teaching Hospital, Bella Defense Referral Hospital or Federal Police Hospital. Following informed consent, patients were instructed to collect three sputum specimens by the standard protocol in Ethiopia: the first at clinic, followed by a morning sputum with a third specimen in clinic the next day. All patients received voluntary counseling and were offered a rapid test for HIV, if they were willing. The HIV results were only provided with the coded specimen number. The sample size was based on the hypothesis that auramine fluorescence will be more sensitive than direct ZN staining by approximately 15% (80% vs. 65%) based on previous studies in Kenya. With 100 positive cultures as the gold standard, this difference can be detected with a power of 80% at a 5% significance level, based on McNemar Test Power Analysis. The study protocol was approved by Institutional Review Boards at Armed Forces General Teaching Hospital and the University of California, San Diego. Follow-up clinical information was not available on the patients.

2.2. Acid fast stains and microscopy

Direct smears were made on each of the 1227 coded specimens and stained with the standard ZN protocol and auramine staining. For auramine-O staining, slides were heat-fixed using a flame. The slides were then flooded with auramine-O stain for 15 min at room temp. After rinsing with tap water, slides were decolorized for 2 min with acid alcohol (0.5% HCl-ethanol), rinsed, and counterstained 2 min with 0.5% aqueous potassium permanganate. Following rinsing, the slides were air dried and scanned for a minimum of 3 min using 10X ocular and Lumin 40X objective lens (400X total magnification) with LED lamp light source (Lumin Lens, W.R. Sanborn Co., Solana Beach, CA, USA) on a standard microscope (Figure 1). The remaining samples were split for bleach concentration or pooled for concentration and culture on LJ slants.

For bleach concentration, at least 1 mL of specimen was inoculated for 15 min at room temperature with an equal volume of commercial household bleach (3.5–5% NaOCl). The sample was transferred to a 15 mL capped conical centrifuge tube and distilled water added to 15 mL total volume. After centrifugation at 3000 g for 15 min, 1–2 drops of the sediment were added to duplicate slides and processed for ZN and auramine staining. The activity of the bleach was tested daily with commercial pool chloride testing kits to maintain free chlorine between 1 and 10 parts per million. Smears were graded using the WHO/IUATLD scale.

Any smear with ≥ 1 AFB/100 high-powered fields was considered positive. Quality control of the smears was performed by having the auramine and ZN smears read by different technologists without knowledge of the results, and blindly re-reading all of the positive and 10% of the negative slides using a standard Olympus fluorescence microscope at the Ethiopian Health and Nutrition Research Institute (EHNRI).

2.3. Mycobacterial cultures

To ensure that the bleach treatment successfully sterilized all mycobacteria in sputum samples, a pilot study was performed at UCSD. Cultures of Mycobacterium tuberculosis, M. avium complex, and M. kansasii were suspended at final concentrations of 5 × 10⁸ organisms/mL in Middlebrook broth and 0.5 mL (5 × 10⁷) and 1.0 mL (1 × 10⁸) were added to sputum samples. An equal volume of 5% commercial bleach was added to each sputum, and the specimen was mixed and incubated for 15 min at room temp. An aliquot of each specimen (0.5 mL) was inoculated onto Middlebrook and LJ slants and incubated for eight weeks. An aliquot of each of the three sputum specimens from each patient was combined, sent to EHNRI, concentrated by the standard N-acetylcyesteine method, and plated on LJ slants. Growth of M. tuberculosis was confirmed by standard biochemical tests.

2.4. Data management and statistical analysis

Smear and culture results were entered in log books by the coded patient number. The final results were entered on a spreadsheet using Microsoft Excel® software (Microsoft Corp., Redmond, WA, USA) and imported into Stata 11.0.
(Stata Corporation, College Station, TX, USA). Smears were considered positive if any one of the three specimens was positive. To be considered negative, all smears had to be negative. The sensitivity, specificity, positive and negative predictive values were compared to culture results. Intra and inter-reader comparisons were calculated by the $\kappa$ coefficient.

3. Results

Patients ranged in age from 4 to 89 years old. Gender information was available for 366 patients: 19.3% were female and 80.7% male. Each patient gave informed consent, was assigned a code number, and was offered HIV testing. Duplicate smears were made from each specimen, aliquots were combined from three specimens for culture, and additional duplicate slides were made following bleach decontamination and concentration.

Results of the direct ZN smear only were made available to the physicians. The concentrated and auramine-stained slides were read blindly by different laboratory personnel. The cultures were sent to the central lab at EHNRI and were concentrated and cultured on LJ slants. Almost 26% of patients had at least one positive culture, 26.9% were smear positive by any method, and four cultures were positive when smears were negative (Table 1). Eight smear positive cultures did not grow, of which four were contaminated and could not be evaluated. At least six (of 12) smears were positive from the other four patients, so the cultures were likely false negative. False negative cultures could have resulted from patients already on therapy, delays in planting or loss of viability during sample processing.

Quality control of the auramine method was performed by blindly re-reading all of the positive and 10% of the negative slides using a standard Olympus fluorescence microscope at EHNRI with a kappa coefficient of 1.0 intra-reader and 0.99 inter-reader correlations. All smears were confirmed at secondary reading except for five auramine concentrated stained slides, which were negative at EHNRI. All five specimens grew in culture, so they likely represent false negative results, which may be due to loss of material from the concentrated slides. The high intra-reader coefficient of variability of 1.0 likely may reflect the difficulty of having a truly blinded study with a small number of trained personnel. However, intra-reader reliability was still high (0.99), when the previous results were unknown to the readers.

The performance of each method of diagnosis, direct Ziehl-Neelsen (D/ZN), concentrated Ziehl-Neelsen (C/ZN), direct auramine (D/Au), and concentrated auramine (C/Au) were compared to culture results. Sensitivity was highest (94%) with specimens that were both concentrated with bleach and stained with auramine (Table 2). Sensitivity was enhanced substantially by bleach concentration (increased from 75% to 89% or 14% overall) and less so by auramine staining (increased from 89 to 94% or 5% overall). The specificity for all methods was equivalent at 98--99%. The positive predictive value was highest with the direct ZN at 98%, and the negative predictive value was greatest (98%) for bleach concentration plus auramine. The overall yield of smear positivity varied by method from 21% for direct ZN to 27% for concentrated auramine ($P < 0.00001$, Cochran test for matched proportions).

The detection of TB is particularly important in HIV+ patients who may have fewer bacilli in their sputum. HIV antibody results were only available on 144 patients (35.2%), however, 29 of the 106 patients diagnosed with TB in this study (27.6%) were known to be HIV+. Although the numbers are small, 10 of the 29 HIV+ patients (34%) would have been missed by smears without bleach concentration and/or auramine fluorescence (Table 3).

4. Discussion

The goal of this study was to improve TB diagnostics in research-limited settings, especially in HIV+ patients with a low mycobacterial load. Ethiopia was an appropriate test site as it ranks seventh among the 22 highest burden countries for TB. In addition, diagnosis by microscopy has been underused or ineffective based on a recent study that found that only 34% of the new pulmonary cases of TB were diagnosed with a positive smear. We sought to bridge the gap in procedures that could readily be instituted in RLS.

### Table 1

<table>
<thead>
<tr>
<th>Smear method</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>LJ culture positive</td>
<td>102</td>
<td>25.9</td>
</tr>
<tr>
<td>LJ culture negative</td>
<td>4</td>
<td>0.7</td>
</tr>
<tr>
<td>LJ culture contaminated</td>
<td>4</td>
<td>3.4</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
<td>409</td>
</tr>
</tbody>
</table>

a Three specimens were collected from each patient. Smears were read individually, but the three specimens were pooled before they were inoculated onto LJ slants. Smear results are included as positive if $>1$ mycobacterium was detected per 100 high power fields by any method.

### Table 2

<table>
<thead>
<tr>
<th>Smear method</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/ZN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/ZN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D/Au</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/Au</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3

Positive diagnostic smear method in 29 HIV+ patients.

<table>
<thead>
<tr>
<th>Smear method</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>All methods positive (D/ZN, C/ZN, D/Au, C/Au)</td>
<td>19</td>
<td>66</td>
</tr>
<tr>
<td>Only concentrated smears positive (C/ZN, C/Au)</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>Only concentrated auramine positive (C/Au)</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

D/ZN: Direct Ziehl-Neelsen; C/ZN: Concentrated Ziehl-Neelsen; D/Au: Direct auramine; C/Au: Concentrated auramine.
We tested bleach concentration because a number of studies have shown that it increases sensitivity by approximately 20%, and it sterilizes the sputum, decreasing the infection risk to laboratory personnel. We first confirmed that standard household bleach killed all mycobacteria in sputum inoculated with 10⁶ organisms in 15 min. To assure potency of the bleach, we monitored the free chloride concentration daily with widely available and inexpensive swimming pool testing strips. We found that bleach concentration was readily adopted by laboratory personnel and increased sensitivity by 14%. The concentration step allows rapid reporting of results, but requires a tabletop centrifuge and distilled water, which smaller laboratories may lack. Overnight sedimentation in bleach has also been shown to enhance sensitivity by approximately 5% and may be more suitable for these settings. A more recent review of studies of bleach centrifugation suggests that the median improvement over direct staining might only be 6% with a small decrease in sensitivity of 3%. Auramine fluorescence staining after concentration has been the decades-old standard method for TB microscopy in developed countries, but has required BSL3 (biosafety level 3) facilities for concentration and expensive, high-maintenance fluorescent microscopes. Recently, a number of light-weight, inexpensive lenses (<US$1200) have been developed, which use LED light sources, which can be attached to any microscope, are operational in ambient light, and have expected lifetimes of >10 years. The increase in sensitivity from auramine staining in this study (5%) is less than the average from Steingart’s review (10%) of studies using standard mercury vapor short arc lamps. A recent review comparing two different LED lens systems to standard FM found that the LED lens had a false negative rate of 6–14%, which our study of a larger number of smears with cultures as the gold standard did not confirm. Our false negative smear rate was <1% whether compared to standard FM or culture. In addition to increased sensitivity, auramine staining results in significant time savings, with higher sensitivity at one minute of screening than standard microscopy at four minutes with equivalent specificity.

Pulmonary TB diagnosis in RLS requires validation of new methods in HIV+ patients who often present diagnostic challenges. The prevalence of HIV in the overall Ethiopian military was 7.2% in 2003, several years before this study. We found that 34% of HIV+ patients with TB would have been missed by standard ZN staining. Thus bleach concentration and auramine staining should improve diagnosis in HIV+ patients as well.

We used mycobacterial cultures on solid agar as the gold standard for diagnosis of active pulmonary tuberculosis. Liquid culture systems, including MODS (Microscopic Observation of Broth-Dilution Susceptibility Assay) and the MGIT system (BD Diagnostics, Sparks, MD, USA), require BSL3 standards per WHO recommendations, which are only now being built in regional laboratories in Ethiopia. Four smear negative patients were culture positive, reflecting the higher sensitivity of culture methods when specimens contain as few as 10–100 viable organisms. Four patients who were smear positive, but culture negative, had all their smears confirmed on review, and thus may have had false negative cultures. We did not have access to charts to determine if these patients were already on treatment, and loss of viability during processing and planting could not be ruled out.

Other studies have shown that the incremental benefit of a third smear is low, <5%. Indeed, in our study, no patient was diagnosed based on the third smear alone. Studies in Ethiopia and Malawi have shown that the requirement for three smears may delay treatment from 2–4 weeks, resulting in the loss of a significant number of patients to follow up care.

Our prospective, blinded study has demonstrated that bleach concentration and auramine staining and detection using a small, fluorescent LED based lens system improve sensitivity by 19% without any additional infrastructure, a significant difference in resource-limited areas. These techniques should permit Ethiopia and other RLS to detect 70% of TB cases by smear microscopy, a goal the WHO has set for the year 2015.

Authors’ contributions: SLR, GM, EG, EL, CM and JAM designed the study protocol; GM, MJ, EG, MG, and EL performed the training, testing, and data acquisition; CM and JAM did the statistical analysis; and SLR drafted the manuscript. All authors read and approved the initial and revised manuscripts. SLR and JAM are guarantors of the paper.

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Conflicts of interest: None declared.

Ethical approval: This study was approved by the IRBs of University of California, San Diego and Armed Forces General Teaching Hospital, Addis Ababa, Ethiopia.

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