Integrated Strategies to Optimize Sputum Smear Microscopy
A Prospective Observational Study

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Rationale: Smear-positive tuberculosis (TB) case detection rates are far below targets in most low-income countries. The standard approach to smear microscopy involves sputum collection over multiple days and examination of sputum smears by light microscopy (LM), an insensitive and time-consuming technique.

Objective: To determine whether two alternative approaches can increase smear-positive case detection by increasing the efficiency (single-specimen microscopy) or sensitivity (light-emitting diode [LED] fluorescence microscopy [FM]) of TB suspect evaluation.

Methods: We enrolled patients with cough of 2 weeks or more admitted to Mulago Hospital in Kampala, Uganda and collected spot and early morning sputum specimens. We compared the diagnostic accuracy of four prespecified strategies based on the number of sputum specimens collected (one specimen versus two specimens) and the type of microscopy (LM versus LED FM) using mycobacterial culture as a reference standard.

Measurements and Main Results: Two hundred thirty-three of 464 (50%) patients had culture-positive TB. There was no difference in sensitivity between single-specimen and two-specimen strategies when smears were examined with LM (55 vs. 56%; difference, −1%; 95% confidence interval [CI], −5 to −2%) or LED FM (61 vs. 64%; difference, −3%; 95% CI, −7 to +1%). LED FM was more sensitive than LM with both the single-specimen (61 vs. 55%; difference, 6%; 95% CI, 2–10%) and two-specimen strategies (64 vs. 56%; difference, 8%; 95% CI, 3–12%). Findings were similar among the HIV-infected patient subset (n = 321 patients).

Conclusions: In low-income, high TB burden settings, single-specimen microscopy and LED FM, either alone or in combination, could considerably increase identification of smear-positive TB cases.

Keywords: tuberculosis; diagnosis; smear-positive case detection

In 2008, there were more than 9 million tuberculosis (TB) cases worldwide, the highest number ever reported to the World Health Organization (WHO) (1). To address this enormous and growing burden, the WHO has adopted a new global strategy, and its first objective is to achieve universal access to high-quality diagnosis and patient-centered treatment (2). Despite recent advances in rapid diagnostics, smear microscopy remains the most widely used test in low-income countries and is likely the only means by which universal access to diagnosis and treatment can be achieved. However, sputum smear-positive case detection rates have been stagnant in many low-income countries at a level substantially below the 70% target set by the World Health Organization (1). Consequently, efforts to improve the performance of sputum smear microscopy are a high priority for global TB control (3).

The standard approach to smear microscopy involves collection of sputum specimens on at least 2 days and examination of smears using light microscopy (LM). In addition to being relatively insensitive for TB diagnosis, this approach generally requires three visits to a health care facility and does not take patient convenience or cost into account. For a variety of reasons, up to 50% of patients fail to return to provide a second specimen or receive results (4, 5). Previous studies have shown that collecting two sputum specimens 1 hour apart (i.e., same-day microscopy) instead of over 2 days can reduce patient dropout during diagnostic evaluation without sacrificing diagnostic accuracy (6–8). However, a WHO Expert Group questioned whether collecting two specimens on the same day would be feasible in busy clinics and whether TB transmission might increase if patients spend an additional hour waiting inside clinics to provide a second specimen (9).

We hypothesized that the goals of same-day microscopy—equivalent sensitivity with reduced patient drop-out compared with the conventional strategy—might be achievable by examining two smears prepared from a single sputum specimen. The single-specimen approach is simpler and sidesteps the potential concerns raised regarding operational feasibility and infection

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Microscopic examination of stained smears prepared from sputum specimens collected over 2 to 3 days is the most widely used diagnostic test for tuberculosis (TB). However, the test is relatively insensitive, and a substantial proportion of patients fail to complete the multi-day evaluation, reducing the usefulness of the test. Consequently, tuberculosis case detection rates are below target in most high-burden, low-income countries.

What This Study Adds to the Field

We found that in a setting with a high burden of TB and HIV infection, microscopic examination of two smears prepared from a single sputum specimen was as sensitive as standard two-specimen microscopy and that fluorescence microscopy using a light-emitting diode light source was more sensitive than conventional light microscopy. In combination, these strategies could both increase TB case detection rates and decrease the burden on patients and providers in low-income countries.
control. In addition, integrating light-emitting diode (LED) FM into the single-specimen strategy could further increase smear-positive case detection. Thus, our objectives were to (1) Determine whether single-specimen LM was noninferior to standard two-specimen LM, and (2) evaluate the impact of LED FM on both the single-specimen and two-specimen microscopy strategies in a population with a high prevalence of HIV and TB. Some of the results of these studies have been previously reported in the form of abstracts (10, 11).

METHODS

Study Population

We prospectively enrolled consecutive adults (age \( \geq 18 \) yr) admitted to the medical wards of Mulago Hospital (Kampala, Uganda) with cough of 2 weeks or more but less than 6 months’ duration (12). We excluded patients from the analysis if smear results were unavailable or TB culture status could not be assessed. Baseline patient evaluation included collection of spot (Day 1) and early morning (Day 2) sputum for acid-fast bacillus (AFB) smear microscopy and culture, and HIV antibody testing. Laboratory technicians provided standardized instructions on proper sputum submission (13). The study was approved by the Makerere University Faculty of Medicine Research Ethics Committee, the Mulago Hospital Institution Review Board, the Uganda National Council for Science and Technology, and the University of California San Francisco Committee on Human Research.

AFB Smear Microscopy

For both LM and LED FM, technicians prepared two direct smears from spot sputum specimens and one direct smear from early morning sputum specimens. The technicians labeled smears with random identification numbers for purposes of blinding and delivered them to the Uganda National Tuberculosis Reference Laboratory (NTRL). As previously described (12), NTRL staff performed LM (magnification \( \times 1,000 \)) on Ziehl-Neelsen-stained smears and LED FM (Lumin, LW Scientific, Lawrenceville, GA; magnification \( \times 400 \)) on auramine O-stained smears.

To test our hypotheses, we prespecified four smear microscopy strategies based on the number of sputum specimens from which smears were prepared (one specimen versus two specimens) and the type of microscopy (LM versus LED FM): (1) two-specimen LM, (2) single-specimen LM, (3) two-specimen LED FM, and (4) single-specimen LED FM (Figure 1). For each strategy, we considered patients to be smear-positive when one or more AFB were seen in at least one smear.

Mycobacterial Culture

NTRL staff cultured all specimens on Lowenstein-Jensen media as previously described (14). For one specimen per patient, NTRL staff also performed liquid culture using the BACTEC 960 MGIT system (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD). NTRL staff confirmed the identity of AFB smear-positive culture isolates using the Capilia TB (TAUNS, Numazu, Japan) speciation assay. We defined the reference standard outcome of TB as present if \( \textit{Mycobacterium tuberculosis} \) was isolated from any sputum specimen and as absent if (1) \( \textit{M. tuberculosis} \) was not isolated from any sputum specimen, and (2) at least one sputum culture was negative (i.e., not contaminated).

Statistical Analysis

We calculated the sensitivity and specificity of smear microscopy strategies in reference to mycobacterial culture results. We made prespecified comparisons of the sensitivity and specificity of different strategies using McNemar’s paired test of proportions and reported the exact binomial 95% confidence interval (CI) for sensitivity and specificity differences. We performed all analyses using Stata 10 (Stata Corporation, College Station, TX).

Our primary objective was to show that single-specimen LM was no less sensitive than standard two-specimen LM, as defined by a non-inferiority margin of 10%. We estimated that 370 tuberculosis patients would be needed to demonstrate noninferiority with 80% power and a 5% significance level using a one-sided equivalence test of correlated proportions (Power Analysis and Sample Size; NCSS, Kaysville, UT). For this calculation, we assumed that the sensitivity of two-specimen LM would be 60% using culture as the gold standard (15) and that the difference in sensitivity between single-specimen and two-specimen LM would be 0%. We expected to complete enrollment within 18 months and we planned two interim analyses at 6 and 12 months. Based on Haybittle-Peto boundary points (16), we planned to stop patient enrollment after each interim analysis if the 99.9% CI excluded more than a 10% difference in sensitivity between single-specimen and two-specimen LM.

RESULTS

We began patient enrollment in April 2009 and stopped enrollment after the second interim analysis in April 2010 because the prespecified stopping boundary was reached. During this period, 492 patients were enrolled and 28 (6%) were excluded from the analysis (Figure 2). Of the remaining 464 patients, 214 (46%) were women and 321 (69%) were HIV infected. The median age of the study population was 33 years (interquartile range, 27–40) and the median CD4 lymphocyte count among HIV-infected patients was 68 cells/\( \mu \)l (interquartile range, 20–180). Two hundred thirty-three (50%) patients had sputum specimens from which \( \textit{M. tuberculosis} \) was isolated.

Single-Specimen Versus Two-Specimen Strategies

Light microscopy. The initial smear from the Day 1 specimen detected 119 (51%) of 233 culture-positive TB cases. When the second smear examined was prepared from the same specimen, the incremental gain in sensitivity was 4%. In comparison, when the second smear examined was prepared from the Day 2 early

![Figure 1. Smear microscopy strategies. The standard approach to smear microscopy involves collection of sputum specimens over 2 days and examination of one direct smear prepared from each specimen (top panel). Single-specimen microscopy refers to a same-day microscopy strategy that involves collection of only one sputum specimen and examination of two smears prepared from this single specimen (bottom panel). With both strategies, smears can be examined either using conventional light microscopy or light-emitting diode (LED) fluorescence microscopy.](image-url)
morning specimen, the incremental sensitivity was 5%. Thus, there was no difference in sensitivity between the single-specimen and two-specimen LM strategies (55 vs. 56%; difference, −1%; 95% CI, −5 to +2%) (Table 1). There was also no significant difference in specificity between the two strategies (98 vs. 97%; difference, 1%; 95% CI, −1 to +2%). When the analysis was restricted to HIV-infected patients, the two strategies yielded similar results both for sensitivity (49 vs. 51%; difference, −2%; 95% CI, −5 to +3%) and for specificity (99 vs. 97%; difference, 2%; 95% CI, −1 to +4%).

**LED fluorescence microscopy.** The initial smear of the Day 1 specimen detected 136 (58%) of 233 TB cases. When the Day 1 specimen was nonsalivary, results were similar with LED FM. Compared with two-specimen LED FM, single-specimen LED FM was positive and LM was negative. Using culture as a reference standard, LED FM was more sensitive (64 vs. 56%; difference, 8%; 95% CI, 3–12%) and as specific (97 vs. 97%; difference, 0%; 95% CI, −3 to +2%) as LM (Table 3). When the analysis was restricted to HIV-infected patients, LED FM remained more sensitive (60 vs. 59%; difference, 9%; 95% CI, 3–16%) and as specific (97 vs. 97%; difference, 0%; 95% CI, −3 to +3%) as LM.

**Single-specimen strategy.** When both smears were prepared from the Day 1 spot specimen, LED FM and LM results were concordant in 440 (agreement, 95%; unweighted kappa, 0.87) cases. In 21 (88%) of the 24 cases with discordant results, LED FM was positive and LM was negative. Using culture as a reference standard, LED FM was more sensitive (61 vs. 55%; difference, 6%; 95% CI, 2–10%) and as specific (96 vs. 98%; difference, −2%; 95% CI, −4 to +1%) as LM (Table 4). When the analysis was restricted to HIV-infected patients, LED FM remained more sensitive (57 vs. 49%; difference, 8%; 95% CI, 2–13%) and as specific (97 vs. 99%; difference, −2%; 95% CI, −4 to +1%) as LM.

**Specimen Quality.** Of 878 specimens included in the analysis, 188 (21%) were salivary, 650 (74%) were mucopurulent, and 40 (5%) were blood-stained. The proportion of salivary specimens was greater for the Day 1 spot specimen than the Day 2 early morning specimen (24 vs. 17%; *P* = 0.009).

When the Day 1 spot specimen was salivary versus mucopurulent or blood-stained, the sensitivity of all microscopy strategies evaluated was reduced and the difference in sensitivity between single-specimen and two-specimen microscopy was increased. However, differences in sensitivity were not statistically significant between salivary and nonsalivary specimens. Compared with two-specimen LM, single-specimen LM was 5% less sensitive (36 vs. 41%; difference, −5; 95% CI, −13 to +4%) when the Day 1 specimen was salivary and 1% less sensitive (59 vs. 60%; difference, 1%; 95% CI, −5 to +3%) when the Day 1 specimen was nonsalivary. Results were similar with LED FM.

**TABLE 2. DIAGNOSTIC ACCURACY OF SINGLE SPECIMEN VERSUS TWO SPECIMEN (STANDARD) LIGHT EMITTING DIODE FLUORESCENCE MICROSCOPY**

<table>
<thead>
<tr>
<th>Overall (N = 464)</th>
<th>Single-Specimen LED FM</th>
<th>Two-Specimen LED FM</th>
<th>Difference (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Sensitivity</td>
<td>61</td>
<td>64</td>
<td>−3 (−7 to 1)</td>
</tr>
<tr>
<td>% Specificity</td>
<td>96</td>
<td>97</td>
<td>−1 (−2 to 1)</td>
</tr>
<tr>
<td>HIV-infected (N = 321)</td>
<td>% Sensitivity</td>
<td>57</td>
<td>60</td>
</tr>
<tr>
<td>% Specificity</td>
<td>97</td>
<td>97</td>
<td>0 (−1 to 1)</td>
</tr>
</tbody>
</table>

**Definition of abbreviations:** CI = confidence interval; FM = fluorescence microscopy; LED = light-emitting diode.

**TABLE 3. DIAGNOSTIC ACCURACY OF TWO SPECIMEN MICROSCOPY STRATEGIES**

<table>
<thead>
<tr>
<th>Overall (N = 464)</th>
<th>Two-Specimen LED FM</th>
<th>Two-Specimen LED FM</th>
<th>Difference (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Sensitivity</td>
<td>64</td>
<td>56</td>
<td>8 (3 to 12)</td>
</tr>
<tr>
<td>% Specificity</td>
<td>97</td>
<td>97</td>
<td>0 (−3 to 2)</td>
</tr>
<tr>
<td>HIV-infected (N = 321)</td>
<td>% Sensitivity</td>
<td>60</td>
<td>51</td>
</tr>
<tr>
<td>% Specificity</td>
<td>97</td>
<td>97</td>
<td>0 (−3 to 3)</td>
</tr>
</tbody>
</table>

For definition of abbreviations, see Table 2.
A limitation of our study is that our findings should be validated in an ambulatory population because our study included only hospitalized patients. However, several recent systematic reviews have found that although point estimates for the diagnostic accuracy of smear microscopy vary widely between studies, differences in sensitivity and specificity between microscopy strategies tend to be highly consistent across studies (9, 23). We would therefore expect that the similarities and differences in diagnostic accuracy between single-specimen and two-specimen microscopy, and between LED FM and LM, would be similar to those found in ambulatory populations.

Our results challenge the conventional dogma that sputum collection should occur over multiple days and include an early morning specimen to maximize the sensitivity of smear microscopy. A systematic review reported an average 12% absolute increase in the proportion of smear-positive patients with examination of morning versus spot specimens based on only four studies (24). However, more recent studies have shown no difference in the incremental yield of smear microscopy with spot versus morning specimens (6–8), perhaps due to differences in study populations, smear-positivity thresholds, and increased attention to sputum collection procedures. Although our results suggest that collection of an additional specimen may be warranted if the initial specimen is salivary, sputum collection on multiple days may not translate into increased smear-positive case detection after patient drop-out is considered (25).

In summary, examining two smears prepared from a single sputum specimen has the potential to improve the efficiency of evaluation for patients suspected of having pulmonary TB without sacrificing diagnostic accuracy. When combined with LED FM, a single-specimen strategy could identify infectious patients earlier and decrease the number of TB suspects in ambulatory settings who drop out before their evaluations are complete. However, additional implementation studies in routine ambulatory settings are needed to determine whether both smears can be examined and the results reported before a patient leaves the health center. Such studies should also include an assessment of whether single-specimen microscopy leads to a reduction in patient- and health system–related costs relative to collection of two sputum specimens either on the same day or over multiple days.

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**References**


### Table 4. Diagnostic Accuracy of Single-Specimen Microscopy Strategies

<table>
<thead>
<tr>
<th></th>
<th>Single-Specimen</th>
<th>Single-Specimen</th>
<th>Difference (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall (N = 464)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Sensitivity</td>
<td>61%</td>
<td>55%</td>
<td>6 (2 to 10)</td>
</tr>
<tr>
<td>% Specificity</td>
<td>96%</td>
<td>98%</td>
<td>-2 (-4 to 1)</td>
</tr>
<tr>
<td>HIV-infected (N = 321)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Sensitivity</td>
<td>57%</td>
<td>49%</td>
<td>8 (2 to 13)</td>
</tr>
<tr>
<td>% Specificity</td>
<td>97%</td>
<td>99%</td>
<td>-2 (-4 to 1)</td>
</tr>
</tbody>
</table>

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FM was 7% less sensitive (45% vs. 52%; difference, -7%; 95% CI, -19 to +5%) when the Day 1 specimen was salivary and 2% less sensitive (64% vs. 66%; difference, -2%; 95% CI, -6 to +2%) when the Day 1 specimen was nonsalivary.

**DISCUSSION**

Developing strategies to improve the efficiency and sensitivity of smear microscopy is an urgent priority for global TB control. In this study, we found that single-specimen LM was as accurate as standard two-specimen LM, but would require only one patient visit in an ambulatory setting. Sensitivity was increased both for the single-specimen and the two-specimen strategies when smears were read using LED FM. In low-income, high TB burden settings, these findings suggest that both single-specimen microscopy and LED FM, either alone or in combination, have considerable potential to increase detection of patients with smear-positive pulmonary TB.

Several recent studies have evaluated same-day smear microscopy performed using two specimens collected 1 hour apart and found the strategy to be as sensitive as smear microscopy performed using standard 2-day specimen collection (6, 7). However, to reduce the high direct and indirect patient costs and inconvenience associated with multiple health facility visits and patient failure to complete smear evaluation (17, 18), our findings suggest that collection of a single sputum specimen may be sufficient.

The idea of examining multiple smears from a single sputum specimen is an old one that has largely been forgotten. In 1949, Freiman and colleagues reported that examination of a second smear from the same specimen resulted in a 12% increase in the proportion of smear-positive specimens (19). In 1969, Rao reported increased sensitivity when multiple smears were prepared from culture-positive specimens (20). Finally, in 1993, Wilkinson and Sturm reported that performing one direct and one concentrated smear on a single specimen had increased sensitivity compared with direct or concentrated smears made from different specimens (21). Our findings are consistent with these prior observations and demonstrate for the first time the equivalence of the single-specimen and standard two-specimen microscopy strategies.

Our findings also confirm that sensitivity is increased compared with LM when sputum smears are examined using LED FM. Sensitivity was higher when LED FM was combined with either the single-specimen or two-specimen strategy. A systematic review of studies mostly from high-income and low HIV-prevalence settings reported similar findings: sensitivity was increased by 6% with LED FM compared with LM (9). LED FM also has other important advantages, including decreased time to interpret slides relative to LM and lower electric power requirements and longer lifespan relative to conventional fluorescence microscopes (22). These features make LED FM ideally suited for use in low-income countries.

**References**


