Use of Light-Emitting Diode Fluorescence Microscopy to Detect Acid-Fast Bacilli in Sputum

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Background. Fluorescence microscopy offers well-described benefits, compared with conventional light microscopy, for the evaluation of sputum smear samples for tuberculosis. However, its use in resource-limited settings has been limited by the high cost of the excitatory light source. We evaluated the diagnostic performance of fluorescence microscopy, using novel light-emitting diode (LED) technology as an alternative to the conventional mercury vapor lamp (MVP).

Methods. Routinely collected sputum specimens from persons suspected to have tuberculosis who attended community clinics were stained with auramine O and were evaluated using 2 different excitatory light sources (MVP and LED); these specimens were then Ziehl-Neelsen stained and reexamined using light microscopy. Two microscopists independently evaluated all smears. Bacterial culture provided the gold standard.

Results. Of the 221 sputum specimens evaluated, 36 (16.3%) were positive for Mycobacterium tuberculosis by culture. Sensitivity and specificity documented for the different modalities were 84.7% and 98.9%, respectively, for the LED assessment; 73.6% and 99.8%, respectively, for the MVP assessment; and 61.1% and 98.9%, respectively, for light microscopy. $\kappa$ values for interreader variation were 0.87 for the LED assessment, 0.79 for the MVP assessment, and 0.77 for light microscopy. The mean time to read a negative smear was 1.4 min with fluorescence microscopy and 3.6 min with light microscopy, reflecting a time savings of 61% with fluorescence microscopy.

Conclusion. LED fluorescence microscopy provides a reliable alternative to conventional methods and has many favorable attributes that facilitate improved, decentralized, diagnostic services.

Sputum smear microscopy is the only diagnostic test available in most resource-limited settings for the evaluation of patients with symptoms suggestive of pulmonary tuberculosis (TB). Since the initial description of the auramine O fluorescence microscopy technique by Hagemann [1] in 1937, numerous reports have confirmed the superior performance of fluorescence microscopy, compared with Ziehl-Neelsen (ZN) staining and light microscopy [2–7]. In a systematic review of 18 studies, Steingart et al. [7] reported that fluorescence microscopy of auramine-stained smears provides similar specificity and increased sensitivity (mean improvement of 10%), compared with light microscopy of ZN-stained smears.

In addition to increased sensitivity, fluorescence microscopy also allows more-rapid screening of sputum smear specimens. From an operational perspective, this is highly advantageous, particularly when high numbers of samples are screened per day, because the majority of laboratory time is spent confirming negative smear results. According to the International Union Against Tuberculosis and Lung Disease technical guidelines for sputum microscopy, at least 5 min of screening is required to correctly identify a negative smear result when conventional light microscopy is used [8]. However, under routine field conditions, the time spent per slide is often far less than the minimum required. An operational study from Cameroon demonstrated a median sputum microscopy examination time of only 2 min.
Almost 50% of the cases detected through a thorough 10-min evaluation were missed during routine investigation [9], which demonstrates the negative impact that conventional light microscopy may have on early case detection and diagnostic delay. A comparative study reported that a mean time of 1 min to examine a sputum smear with fluorescence microscopy achieved higher sensitivity and equivalent specificity than did conventional light microscopy with an examination time of 4 min [10]. Despite the clear operational advantages of fluorescence microscopy, conventional light microscopy remains the most widely used diagnostic test in resource-limited settings.

The main reason that fluorescence microscopy is not used more widely is the limited lifespan (typically 200–300 h) and the high cost of the short-arc mercury vapor lamp (MVP), which has traditionally been used as the excitatory light source. Repeated on-and-off switching, as may occur with unreliable local power supply, shortens the lifespan even further [11]. In addition, MVPs are energy inefficient and require an extensive power supply; they may also fail catastrophically and release toxic mercury into the environment [11]. Fluorescence microscopes provided by donor agencies often fall into disuse because of high maintenance costs [12]. Light-emitting diode (LED) technology provides a cheap and reliable light source with a usable lifespan of >50,000 h; repeated on-and-off switching does not reduce its usable lifespan, and it does not pose a potential toxicity risk [11]. Initial studies indicated that LED fluorescence microscopy, with use of a royal blue LED light, offers a valid alternative to the MVP [11, 12], but data regarding its diagnostic use remain limited. Interreader variability and implementation considerations have not been evaluated under routine conditions.

Our study evaluated the diagnostic performance of auramine O fluorescence microscopy with use of an LED excitatory light source, compared with the traditional MVP. In addition, both fluorescence modalities were compared with conventional light microscopy of ZN-stained smears. Mycobacterial culture provided the gold standard.

**METHODS**

We conducted a cross-sectional, laboratory-based study. Consecutive study specimens were collected from adult patients suspected to have pulmonary TB who routinely attended 2 primary health care clinics in Cape Town, South Africa. The overall TB incidence recorded at the study setting and its immediate surroundings was 845 cases per 100,000 population in 2004 [13].

Smears were prepared from routine sputum specimens collected during a 3-month study period (April–June 2007). Standard procedures were performed for decontamination and concentration of specimens, preparation of slides, and fluorochrome staining with auramine O [14], which has an affinity for the mycolic acid contained in the cell walls of mycobacteria. With auramine O staining, mycobacteria appear as bright yellow fluorescent rods when viewed under an excitatory light source. Auramine O is excited by blue light (wavelength, 450–480 nm) and emits in the green-yellow range (wavelength, 500–600 nm). The royal blue LED light source used (350 mA, 1 W, Royal Blue Luxeon emitter; Philips Lumileds Lighting Company) has an absorption maximum of 450 nm, which matches the absorption maximum of auramine O.

Figure 1 provides an overview of the specimen collection and evaluation procedures. Research slides were prepared in 2 batches of 10–20 specimens per week. Slides were stained with auramine O and were read by 2 independent microscopists; the slides were first read with the MVP, and then read with the LED light source. An eye piece with ×10 magnification and an objective with ×40 magnification (total magnification, ×400) were used. The microscopists were blinded to all previous results. Slides were randomly reassorted after each evaluation, and separate data capture forms were used for each modality to eliminate the possibility of the first reading influencing the second. After completion of fluorescence microscopy, the same slide was ZN stained using standard methodology [8], and the stained slide was evaluated using a conventional light microscope. As recommended by the International Union Against Tuberculosis and Lung Disease technical guide, 100 fields were covered with the battlement method, with use of an eye piece...
with ×10 magnification and an oil immersion objective with ×50 magnification (total magnification, ×500) [8]. Performing a ZN stain after initial auramine O staining should not adversely affect the outcome and is accepted as standard practice (S. H. Siddiqi, personal communication). All evaluations were performed within a 2-day period.

The number of acid-fast bacilli observed was quantified according to Centers for Disease Control and Prevention guidelines (table 1). No adjustment was made for the slight difference in magnification between fluorescence (×400) and light (×500) microscopy. Specimens were routinely cultured for research purposes; because of the cost considerations, specimens for which the result of the routine diagnostic smear was found to be positive were cultured in liquid (7H9 broth) medium with use of the automated Mycobacterial Growth Indicator Tube system (BD Diagnostic Systems), and smear specimens for which the results of the routine diagnostic smear were negative were cultured on solid Löwenstein-Jensen medium.

Anonymous, unlinked data were entered into an Excel spreadsheet (Microsoft). Descriptive and comparative data analyses were performed using Stata, version 9.2 (StataCorp); 95% CIs for the χ² statistics were calculated using the bootstrap method. Sensitivity, specificity, and interreader agreement were compared among all 3 modalities, with culture as the gold standard. Ethics approval was granted by the Committee for Human Research, Stellenbosch University (protocol N07/06/136).

RESULTS

Of the 221 sputum specimens evaluated, 36 (16%) were confirmed by culture to be positive for Mycobacterium tuberculosis. Table 2 shows the yield of sputum smear–positive specimens identified with each of the diagnostic modalities evaluated. The yield achieved with fluorescence microscopy (mean yield for the LED and MVP combined) exceeded the yield achieved with light microscopy (30 [14%] of 221 specimens vs. 24 [11%] of 221 specimens; OR, 1.29; 95% CI, 0.70–2.37), but the difference was not statistically significant. The yields achieved with fluorescent microscopy using different excitatory light sources (MVP and LED) were comparable (33 [15%] of 221 specimens vs. 27 [12%] of 221 specimens; OR, 1.26; 95% CI, 0.71–2.37), although the 95% CIs of all 3 modalities overlapped. Microscopists read each slide only once; therefore, intrareader variability could not be calculated. With fluorescence microscopy, the mean time to read a negative smear result was 1.4 min; no difference was noted between the LED and the MVP. With conventional light microscopy, the mean time spent to read a negative smear result was 3.6 min, demonstrating time savings of 61% with fluorescence microscopy.

DISCUSSION

No statistically significant differences were noted, but our findings support previous studies that demonstrated the superior diagnostic performance of fluorescence microscopy, compared with conventional light microscopy [2–7]. The excellent sensitivity and specificity achieved and the short evaluation time required for fluorescence microscopy indicate the potential benefit of making the modality more widely available, especially in resource-limited settings with a high burden of TB. The time saving achieved with fluorescence microscopy did not result from a reduction in the number of fields screened (100 fields were screened with both modalities) but can be ascribed to quicker scanning of each field because of increased visibility of the mycobacteria; the decreased magnification used during fluorescence microscopy, compared with light microscopy (×400 vs. ×500), may also have contributed, particularly toward the sensitivity differences noted. Additional advantages of fluorescence microscopy include the simplicity of the fluorescence staining method, compared with ZN methods [7].

Most importantly, our study confirmed previous observations that the LED provides a reliable alternative light source for fluorescence microscopy [11, 12]; in fact, although differences were not statistically significant, the highest sensitivity and lowest interreader variability were achieved with LED fluorescence microscopy. This is an important finding, because the availability of a robust and cheap excitatory light source makes fluorescence microscopy a more feasible option in resource-limited settings. In addition, the LED light source is highly energy efficient, compared with the traditional MVP [12, 16].

Table 1. Sputum smear grading, according to the number of acid-fast bacilli (AFB) visualized, for light and fluorescence microscopy.

<table>
<thead>
<tr>
<th>No. of AFB seen</th>
<th>CDC guideline</th>
<th>Study definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>1–2 per whole smear</td>
<td>Doubtful positive</td>
<td>Scanty</td>
</tr>
<tr>
<td>Per 100 fields</td>
<td>1+</td>
<td>Positive</td>
</tr>
<tr>
<td>Per 10 fields</td>
<td>2+</td>
<td>Positive</td>
</tr>
<tr>
<td>Per single field</td>
<td>3+</td>
<td>Positive</td>
</tr>
<tr>
<td>&gt;9 per single field</td>
<td>4+</td>
<td>Positive</td>
</tr>
</tbody>
</table>

NOTE. CDC, Centers for Disease Control and Prevention. * From Kent and Kubica [15].
Table 2. Mean yield of sputum smear–positive specimens achieved with the use of 3 different microscopic modalities for 221 adults suspected to have pulmonary tuberculosis.

<table>
<thead>
<tr>
<th>Result</th>
<th>Fluorescence microscopy of auramine O–stained specimens, no. (%) of specimens</th>
<th>Light microscopy of Ziehl-Neelsen–stained specimens, no. (%) of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mercury vapor lamp</td>
<td>Light-emitting diode</td>
</tr>
<tr>
<td>Negative</td>
<td>187 (85)</td>
<td>183 (83)</td>
</tr>
<tr>
<td>Scanty</td>
<td>7 (3)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>Positive</td>
<td>27 (12)</td>
<td>33 (15)</td>
</tr>
</tbody>
</table>

NOTE. Data represent the mean yield for the 2 microscopists combined.

Table 3. Diagnostic yield and interreader variation for 2 microscopists evaluating 3 different sputum microscopic modalities for the identification of acid-fast bacilli in sputum samples from 221 adults suspected to have pulmonary tuberculosis.

<table>
<thead>
<tr>
<th>Diagnostic modality</th>
<th>No. of sputum smear specimens determined to be positive for Mycobacterium tuberculosis</th>
<th>No. of discrepant results</th>
<th>$\kappa$ value (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light microscopy</td>
<td>Microscopist 1: 23</td>
<td>Microscopist 2: 25</td>
<td>10</td>
</tr>
<tr>
<td>Fluorescence microscopy</td>
<td>Mercury vapor lamp: 25</td>
<td>Light-emitting diode: 33</td>
<td>7</td>
</tr>
</tbody>
</table>

The low energy requirements of LED fluorescence microscopy provides prospect for the development of battery–operated fluorescence microscopes that can be used in areas with unreliable or absent power supply, as is frequently the case in remote and/or resource-limited settings.

Although the idea was not formally evaluated during the study, microscopists remarked that, with the LED light source, mycobacteria remained easily visible despite the absence of a darkened environment. Microscopists are usually advised to perform fluorescence microscopy in a darkened environment, which is a major constraint in resource-limited settings where a darkened location may not be readily available. The observation that a darkened environment is not necessary requires further evaluation, but if substantiated, it would greatly enhance the practical feasibility of using LED fluorescence microscopy to provide decentralized diagnostic services. Such services have been demonstrated to improve case detection and reduce diagnostic delay, especially in rural and remote areas [17].

A previous study commented that more “scanty” smears were observed with the MVP, compared with the LED; this was potentially attributed to photobleaching of the auramine O stain after MVP exposure [11]. It is unlikely that photobleaching influenced our results; batches of auramine O–stained smears were read on the same day, and there were no differences between the numbers of scanty smears detected with the 2 modalities (7 scanty smears with the MVP and 5 scanty smears with the LED). In fact, an increased proportion of positive smear specimens were detected with the LED assessment, compared with the MVP assessment (33 [15%] specimens vs. 27 [12%] specimens), and the LED assessment was performed after MVP exposure. The improved yield was not statistically significant, but we can conclude that LED fluorescence microscopy is no less sensitive than microscopy with the traditional MVP.

Sputum culture is widely regarded to be the most sensitive test (i.e., the gold standard) for the detection of pulmonary TB, but its routine use in resource-limited settings is hampered by excessive cost, slow turnaround times, and the need for adequate laboratory infrastructure. In practice, improvements in direct sputum sample evaluation that result from improved sensitivity and/or improved access to decentralized diagnostic services remain highly relevant. There is a definite need for improved access to sputum culture to establish drug susceptibility in areas with high rates of drug-resistant TB and in HIV-infected patients who have negative sputum smear results. However, the need for rapid smear results and effective treatment of the most infectious TB cases remains paramount; in addition, fluorescence microscopy seems to be more efficient than light microscopy for detection of TB in HIV-infected patients with paucibacillary disease [2].

Our study was limited by small numbers and failed to demonstrate statistically significant differences. However, we believe that the study results are sufficient to demonstrate that LED fluorescence microscopy offers a highly feasible alternative—with at least similar diagnostic performance—to conventional...
methods. Although the inconsistent use of culture media (because of financial constraints) in our study may be criticized, this would not have introduced bias, because a single culture medium was used for each specimen. Therefore, all 3 modalities were consistently evaluated against the identical gold standard test. Performance of the ZN stain on the same slide that was initially stained with auramine O could have affected the sensitivity of the ZN stain, but this is considered to be unlikely and is accepted as standard practice. Despite this potential reservation, the decision was made to evaluate the various modalities with use of the very same slide to ensure optimal comparison, because different slides prepared from the same specimen may be highly variable.

In conclusion, our study adds to the body of evidence that demonstrates that LED fluorescence microscopy provides a valid alternative to conventional methods. Its low cost, sturdiness, energy efficiency, and perceived efficacy in the absence of a darkened environment are highly favorable attributes. In the short term, optimized microscopy offers the most realistic option for improved case finding in resource-limited settings; large-scale field trials are required to assess the advantages and feasibility of replacing conventional ZN light microscopy with LED fluorescence microscopy as a first-line diagnostic test.

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Potential conflicts of interest. All authors: no conflicts.

References