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Field Evaluation of a Simple Fluorescence Method for Detection of Viable *Mycobacterium tuberculosis* in Sputum Specimens during Treatment Follow-Up

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Simple tuberculosis (TB) treatment monitoring tools are needed. We assessed the performance of fluorescein-diacetate (FDA) smear microscopy for detection of viable *Mycobacterium tuberculosis* in sputum specimens ($n = 288$) of TB cases under treatment compared to culture (17.4% culture positivity). FDA sensitivity was moderate (83.7% [95% confidence interval {CI}, 70.3 to 92.6]), and specificity was low (66.1% [59.5 to 72.2]). The good negative predictive value (94.8% [90.1 to 97.8]) and negative likelihood ratio (0.2) suggest using this method to rule out treatment failure in settings without access to culture.

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Monitoring the response to tuberculosis (TB) treatment is essential to detect failure or drug resistance early (12). The only monitoring tool available in resource-limited settings is sputum smear microscopy using Ziehl-Neelsen (ZN) or auramine staining (11, 12). Smear positivity at month 3 or later should be investigated with *Mycobacterium tuberculosis* culture and drug susceptibility testing (DST), while smear positivity at month 5 or later defines treatment failure (12). Importantly, smear microscopy cannot distinguish viable from dead bacilli. A significant proportion of patients on treatment may, however, continue to cough up dead bacilli from necrotic lung cavities, thus remaining "smear positive" although responding to therapy (1, 5, 8, 9, 10, 12). These patients are at risk of receiving an unnecessary prolonged or new treatment regimen in settings with limited or no access to *M. tuberculosis* culture. Culture is the only test that can identify viable bacilli, but it requires several weeks to report results and needs a high level of expertise and laboratory infrastructure. Recent studies proposed a simple and instant method for TB treatment monitoring, based on a common fluorescent viability marker, fluorescein-diacetate (FDA) (4), in combination with smear microscopy (2, 3).

We assessed the performance of the FDA vital staining method compared to *M. tuberculosis* culture in a peripheral smear microscopy laboratory in Mae Sot, Thailand. Sputum specimens were collected during routine TB treatment monitoring from pulmonary TB cases at months 2, 3 (if positive at month 2), 5, and 6 for treatment of new TB cases and at months 3, 4 (if positive at month 3), 5, and 8 for treatment of previously treated TB cases. Two consecutive specimens per time point were processed by the standard direct Ziehl-Neelsen (ZN) smear microscopy (11). Only ZN-positive specimens were included in the study and were subjected to FDA smear microscopy within a median of 2 days upon specimen collection according to published FDA procedures (2). FDA stock solution (FDA F1303 [Molecular Probes, Inc.], 25 mg/ml in acetone, stored at −20°C) was used to prepare fresh staining solution (0.5 mg/ml) in phosphate-buffered saline (pH 7.3, Dulbecco A BR0014 [Oxoid Ltd.], with 0.05% Tween 80). After air drying, FDA smears were examined by fluorescence microscopy at ×1,000 magnification using an Olympus CX21 microscope equipped with a FluoLedBlue (480-nm) light-emitting diode (LED) cassette (Fraen Corporation Srl, Italy) and a 535/40-nm band-pass filter. An FDA-positive (FDA^+) smear was defined by at least 1 fluorescent bacillus/100 high-power fields (11). The remaining specimen was sent for *M. tuberculosis* culture at the International Organization for Migration (IOM) laboratory in Mae Sot. Specimen decontamination used *N*-acetyl-L-cysteine-sodium hydroxide, with a 2% NaOH final volume concentration for 15 min. One liquid Bactec MGIT 960 and two solid egg-based Lowenstein-Jensen cultures were inoculated per specimen. Positive cultures were tested by ZN smear, and identification of *M. tuberculosis* species versus nontuberculous mycobacteria (NTM) was performed by the rapid nucleic acid hybridization method (Gen-Probe Accuprobe *Mycobacterium tuberculosis* complex culture identification test; Biogenentech). A specimen was defined as "contaminated" if all 3 culture media were contaminated, "*M. tuberculosis* positive" if ≥1 of 3 cultures was positive, and "*M. tuberculosis* negative" otherwise. Unreadable FDA smears and culture-contaminated or NTM-positive specimens were excluded from the performance analysis. The study was approved by the Comité de Protection des Personnes, Saint Germain en Laye, France, and the Ethical Review Committee of the Ministry of Public Health, Thailand.

Two hundred eighty-eight ZN^+ specimens from 215 treatment follow-up cases were included between December 2007 and March 2009. Of them, 77.4% were scanty ZN positive (i.e., with 1 to 9 bacilli/100 high power fields [HPF]) and 69.1% were derived from the end of the intensive phase or the end of the prolonged intensive phase of treatment ("delayed treatment responder spec-

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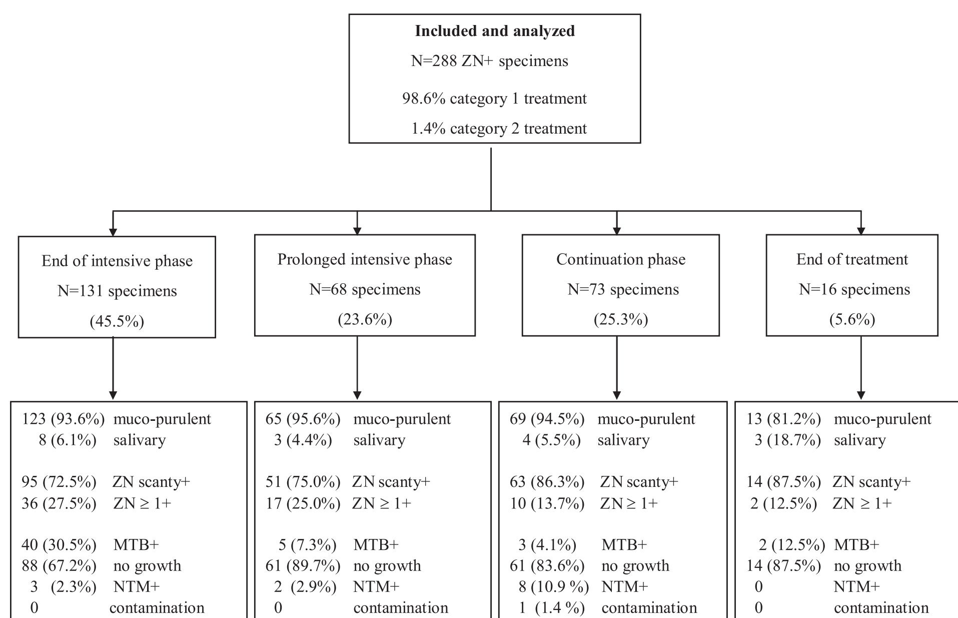

FIG 1 Numbers of included ZN$^+$ sputum specimens with specimen characteristics. ZN+, Ziehl-Neelsen stain-positive sputum smear; MTB+, *Mycobacterium tuberculosis*-positive culture; NTM+, nontuberculous mycobacterium-positive culture; ZN scanty+, 1 to 9 bacilli/100 HPF; ZN ≥ 1+, ≥10 bacilli/100 HPF.

imens") (Fig. 1). Among the total 288 specimens, 50 (17.4%) were *M. tuberculosis* culture positive, 13 (4.5%) were NTM positive, 224 (77.8%) were culture negative, and 1 (0.3%) was contaminated. In total, 125 (43.4%) of specimens were FDA smear positive, and 100 (80%) of these were scanty positive. The sensitivity of FDA smear was 83.7%, and its specificity was 66.1% (Table 1). False-positive FDA results were significantly more frequent among scanty FDA-positive results (67/93, 72%) than among highly positive FDA results (≥1+) (9/24, 37.5%), with a *P* value of 0.002 (Wald test). The negative predictive value (NPV) was 94.8%, and the negative likelihood ratio (LR−) was 0.2 (Table 1). A subset performance analysis by treatment phase indicated a significantly higher specificity among specimens from suspected treatment failures than among specimens from delayed treatment responders ($P = 0.011$, Wald test) (Table 1).

The overall performance of FDA was lower than previously reported (2). This may be explained by notable differences in the study populations. Hamid et al. included exclusively specimens from suspected failure cases, which were largely culture positive (61.4%) and mainly with high acid-fast bacillus (AFB) loads (80% with scores of ≥1+). The present study population was characterized by mostly scanty ZN-positive specimens from delayed treatment responders with a low culture positivity rate. Indeed, the FDA accuracy seemed lower in paucibacillary specimens. Furthermore, FDA specificity may have been underestimated due to false-culture-negative results. With a very low culture contamination rate, it is likely that some *M. tuberculosis* organisms, especially from paucibacillary specimens, have been killed during decontamination (7). Furthermore, "viability" of bacilli as defined by FDA positivity (fluorochrome activation by enzymatic activity)

TABLE 1 FDA smear performance, total and by treatment phase[a]

Specimen type (*n*)	No. with FDA result: Positive	Negative	% SE (95% CI)	% SP (95% CI)	% PPV (95% CI)	% NPV (95% CI)	LR$^+$ (95% CI)	LR$^-$ (95% CI)
Total (273[b])			83.7 (70.3–92.6)	66.1 (59.5–72.2)	35.0 (26.4–44.4)	94.8 (90.1–97.8)	2.5 (1.9–3.0)	0.2 (0.1–0.5)
C$^+$	41	8						
C$^-$	76	148						
Delayed responder (194)			82.2 (67.9–91.9)	60.4 (52.1–68.3)	38.5 (28.8–49.0)	91.8 (84.5–96.4)	2.1 (1.6–2.6)	0.3 (0.1–0.5)
C$^+$	37	8						
C$^-$	59	90						
Suspected failure (79)			100 (39.7–100)[c]	77.3 (66.2–86.2)	19.9 (0.5–41.9)	100 (93.8–100)[c]	4.4 (2.9–6.7)	0.0
C$^+$	4	0						
C$^-$	17	58						

[a] Abbreviations: C, culture; C$^+$, *M. tuberculosis* positive; C$^-$, *M. tuberculosis* negative; SE, sensitivity; SP, specificity; PPV, positive predictive value; NPV, negative predictive value; LR$^+$, positive likelihood ratio; LR$^-$, negative likelihood ratio.
[b] Fifteen specimens were excluded: the FDA smear was not readable for 1 sample, 1 had culture result "contaminated," and 13 samples were NTM positive.
[c] One-sided 97.5% confidence interval.

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may not always correspond to bacillus "viability" as defined by culture (ability to multiply) (6). This may be more pronounced among specimens derived from early treatment phases. It is of note that the specific LED system used (480-nm cassette) and smear reading at ×1,000 magnification did not confer a very strong fluorescent signal. A potent fluorescence signal for FDA smears can be achieved using the standard LED setup for auramine (450-nm cassette and 510-nm-long pass filter) combined with reading at ×200 magnification (A. Van Deun, personal communication).

In this second study assessing the FDA method, the performance was not accurate enough to propose the FDA smear method as a stand-alone tool for TB treatment monitoring. However, in this study population with few confirmed failures, the good NPV and LR− may suggest using the method to rule out treatment failure and avoid an unnecessary second-line regimen. In programs with limited access to *M. tuberculosis* culture, the FDA method may help in identifying cases (FDA positive) requiring culture assessment. Such application needs further evaluation. Additional evaluation is also needed in populations with higher proportions of confirmed failures.

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