TITLE: Field evaluation of a simple fluorescence method for detection of viable *Mycobacterium tuberculosis* in sputum during treatment follow up.

RUNNING TITLE: FDA smear microscopy
AUTHORS and AFFILIATIONS

Birgit Schramm1# (Birgit.Schramm@epicentre.msf.org), Cathy Hewison2
(Cathy.Hewison@paris.msf.org), Laurence Bonte3 (Laurence.Bonte@paris.msf.org), Warren
Jones3*(wjones@iom.int), Olivier Camélique4 (ocamelique@yahoo.fr), Ronnatrai Ruangweerayut5
(ronnatrai@yahoo.com), Witaya Swaddiwudhipong5 (swaddi@hotmail.com), Maryline Bonnet6
(Maryline.BONNET@geneva.msf.org).

1Epicentre, Paris, France; 2Médecins Sans Frontières (MSF), Paris, France; 3 International Organization
for Migration (IOM) Bangkok, Thailand; 4MSF, Bangkok, Thailand; 5Mae Sot General Hospital, Ministry
of Public Health, Tak, Thailand; 6 Epicentre, Geneva, Switzerland.

KEYWORDS: Mycobacterium tuberculosis, smear microscopy, treatment monitoring, fluorescein
diacetate viable staining

# Corresponding author: Email: Birgit.Schramm@epicentre.msf.org, Phone: +33(0)1402155457,
FAX: +33(0)14021 5500

* Present address: International Organization for Migration, Regional Mission for East and Central Africa, Nairobi
Kenya
ABSTRACT

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%CI: 70.3-92.6]) and specificity low (66.1% [59.5-72.2]). The good negative predictive value (94.8% [90.1-97.8]) and negative likelihood ratio (0.2) suggest using it to rule-out treatment failure in settings without access to culture.
Monitoring the response to Tuberculosis (TB) treatment is essential to detect failure or drug resistance early (13). The only monitoring tool available in resource-limited settings is sputum smear microscopy using Ziehl-Neelsen or Auramine staining (12,13). Smear-positivity at month 3 or later should be investigated with Mycobacterium Tuberculosis (MTB) culture and drug-susceptibility testing (DST), while smear-positivity at month 5 or later defines treatment failure (13). Importantly, smear microscopy can not 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 (1,5,8,10,11,13). These patients are at risk of receiving an unnecessary prolonged- or new treatment regimen in settings with limited or no access to MTB culture. Culture is the only test that can identify viable bacilli, but 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), in combination with smear microscopy (2,3).

We assessed the performance of the FDA vital staining method compared to MTB 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 month 2, 3 (if positive at 2), 5 and 6 for treatment of new TB cases, and at month 3, 4 (if positive at 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 (12). Only Zn-positive specimens were included in the study and subjected to FDA smear microscopy within a median of 2 days upon specimen collection following 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’ BRL014 Oxoid Ltd, with 0.05% Tween80). After air-drying, FDA smears were examined by fluorescence microscopy at 1000x magnification using an Olympus CX21 microscope equipped with a FluorLedBlue® (480nm) LED cassette (Fraen Corporation Srl, Italy) and a 535/40 nm band-pass filter. An FDA+ smear was defined by at least 1 fluorescent bacilli/100 High Power Fields (12). The remaining specimen was sent for MTB culture at the International Organization for Migration (IOM) laboratory in Mae Sot. Specimen decontamination used N-Acetyl-L-Cysteine-Sodium-Hydroxide, with 2% NaOH final
volume concentration for 15 minutes. One liquid Bactec MGIT 960 and 2 solid egg-based Lowenstein Jensen (LJ) cultures were inoculated per specimen. Positive cultures were tested by Zn smear and identification of MTB species versus Non-Tuberculous mycobacteria (NTM) was by 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, “MTB positive” if ≥ 1 of 3 cultures were positive, and “MTB-negative” otherwise.

Unreadable FDA smears, 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 Zn scanty positive and 69.1% were derived from end of intensive phase or end of prolonged intensive phase of treatment (“delayed treatment responder specimens”) (figure 1). Among the total 288 specimens 50 (17.4%) were MTB culture positive, 13 (4.5%) were NTM positive, 224 (77.8%) culture-negative and 1 (0.3%) contaminated. In total, 125 (43.4%) of specimens were FDA smear positive, 100 (80%) of these were scanty. The sensitivity of FDA smear was 83.7% and 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%), p=0.002 (Wald test). The negative predictive value (NPV) was 95% 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 of high AFB-load (80% ≥ 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 MTB, especially
from paucibacillary specimens have been killed during decontamination (7). Furthermore, “viability” of bacilli as defined by FDA positivity (fluorochrome activation by enzymatic activity) may not always correspond to bacilli “viability” as defined by culture (ability to multiply) (4,6). This may be more pronounced among specimens derived from early treatment phases. It is of note that the specific LED system used (480nm cassette) and smear reading at 1000x magnification did not confer a very strong fluorescent signal. A potent fluorescence signal for FDA smears can be achieved using the standard LED set-up for auramine (450nm cassette and 510nm long pass filter) combined with reading at 200x magnification (Armand 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 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 2nd line regimen. In programmes with limited access to MTB culture, the FDA method may help identifying cases (FDA positive) requiring culture assessment. Such application needs further evaluation. Additional evaluation is also needed in populations with higher proportions of confirmed failure.
ACKNOWLEDGEMENT

This study was supported by Médecins Sans Frontières, Paris, France.

We thank the entire Médecins sans frontières team in Mae Sot and Bangkok, specifically Jadee Tawiwongg kamton, Kraywa Seangraoengsong, Paw Lweh Hay Sonklinprai, Mako Rakarsrom and Muewapho Kulapkeeree for FDA smear microscopy and patient data collection, and Chongon Tantavanich, Sein Sein Thi, Andres Romero, YinWin Khin and Marianne Gale for support during study implementation, and Sarala Nicholas for support during data analysis. We thank Armand Van Deun, Md. A. Hamid Salim and Md. Anwar Hossain for sharing their expertise on the FDA method and for technical support. We thank Warithorn Madilokkowit and Kittisak Amornpaisarnloet for MTB culture and technical support and Anne-Laure Page for comments on the manuscript.
REFERENCES


Included and analyzed
N=288 Zn+ specimens
98.6% category 1 treatment
1.4% category 2 treatment

End of intensive phase
N=131 specimens
(45.5%)
123 (93.6%) mucopurulent
8 (6.1%) salivary
95 (72.5%) Zn scanty+
36 (27.5%) Zn ≥ 1+
40 (30.5%) MTB+
88 (67.2%) no growth
3 (2.3%) NTM+
0 contamination
65 (95.6%) mucopurulent
3 (4.4%) salivary
51 (75.0%) Zn scanty+
17 (25.0%) Zn ≥ 1+
5 (7.3%) MTB+
61 (89.7%) no growth
2 (2.9%) NTM+
0 contamination
69 (94.5%) mucopurulent
4 (5.5%) salivary
63 (86.3%) Zn scanty+
10 (13.7%) Zn ≥ 1+
3 (4.1%) MTB+
61 (83.6%) no growth
8 (10.9%) NTM+
1 (1.4%) contamination
13 (81.2%) mucopurulent
3 (18.7%) salivary
14 (87.5%) Zn scanty+
2 (12.5%) Zn ≥ 1+
2 (12.5%) MTB+
14 (87.5%) no growth
0 NTM+
0 contamination

Prolonged intensive phase
N=68 specimens
(23.6%)
Continuation phase
N=73 specimens
(25.3%)
End of treatment
N=16 specimens
(5.6%)

Zn+ = Ziehl Neelsen-positive sputum smear, MTB+ = *Mycobacterium tuberculosis*-positive culture
NTM+ = *Non-tuberculous mycobacteria*-positive culture

Depicted are the number of included Zn+ follow up cases and their respective Zn+ sputum specimens with specimen characteristics
TABLE 1  FDA smear performance, total and by treatment phase.

<table>
<thead>
<tr>
<th>Definition</th>
<th>Total</th>
<th>Delayed responder</th>
<th>Suspect failure</th>
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<tbody>
<tr>
<td></td>
<td>specimens</td>
<td>specimens</td>
<td>specimens</td>
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<tr>
<td></td>
<td>N=273*</td>
<td>N=194</td>
<td>N=79</td>
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<tr>
<td>FDA+ specimen</td>
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</tr>
<tr>
<td>C+</td>
<td>41</td>
<td>37</td>
<td>4</td>
</tr>
<tr>
<td>C-</td>
<td>76</td>
<td>59</td>
<td>17</td>
</tr>
<tr>
<td>FDA - specimen</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C+</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>C-</td>
<td>148</td>
<td>90</td>
<td>58</td>
</tr>
<tr>
<td>SE, % [95%CI]</td>
<td>83.7 [70.3-92.6]</td>
<td>82.2 [67.9-91.9]</td>
<td>100 [39.7-100]**</td>
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<tr>
<td>SP</td>
<td>66.1 [59.5-72.2]</td>
<td>60.4 [52.1-68.3]</td>
<td>77.3 [66.2-86.2]</td>
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<tr>
<td>PPV</td>
<td>35.0 [26.4-44.4]</td>
<td>38.5 [28.8-49.0]</td>
<td>19.9 [0.5-41.9]</td>
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<tr>
<td>NPV</td>
<td>94.8 [90.1-97.8]</td>
<td>91.8 [84.5-96.4]</td>
<td>100 [93.8-100]**</td>
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<td>LR+</td>
<td>2.5 [1.9-3.0]</td>
<td>2.1 [1.6-2.6]</td>
<td>4.4 [2.9-6.7]</td>
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<tr>
<td>LR-</td>
<td>0.2 [0.1-0.5]</td>
<td>0.3 [0.1-0.5]</td>
<td>0.0</td>
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</table>

C+ = MTB positive, C- = MTB negative, SE = sensitivity, SP= specificity,
LR= positive and negative likelihood ratio

* N=15 specimens excluded: N=1 FDA smear not readable, N=1 culture contaminated, N=13 NTM positive
** one-sided 97.5 % confidence interval