Evaluation of the performance and feasibility of the fluorescein diacetate (FDA) vital staining method for follow up of Tuberculosis (TB) treatment.

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Background

TB treatment follow up

Direct Ziehl Neelsen (ZN) smear microscopy:

- Used in resource-limited settings to monitor TB treatment
- Can not distinguish live from dead bacilli
- Literature review: 10-90% of Cat1 or Cat 2 ZN smear positive TB failures are culture negative
- Patients potentially started unnecessarily on a new regimen

Mycobacterium Tuberculosis (MTB) culture:

- Gold standard method
- Not available in most resource limited settings
- Long delay for culture results

Fluorescein diacetate (FDA) staining distinguishes live and dead bacilli:

1. FDA staining of sputum smear : Intracellular uptake of FDA

FDA → Mycobacterium Tuberculosis (MTB)

2. Only in living bacilli: intracellular hydrolysis (enzymatic activity) of FDA to Fluorescein (494 / 518 nm)

3. Detection of life (fluorescent) bacilli by fluorescence microscopy

dead MTB | live MTB

Bangladesh study:
sensitivity = 99% / specificity= 82 % compared to MTB culture when used on fresh sputum specimens of TB failures

Hamid Salim A, Aung KJ, Hossain MA, Van Deun A. Early and rapid microscopy-based diagnosis of true treatment failure and MDR-TB. Int J Tuberc Lung Dis. 2006 Nov;10(11):1248-54

Objective

To evaluate the performance and feasibility of the FDA vital staining method compared to MTB culture to differentiate live from dead bacilli in ZN smear positive patients during treatment follow-up in a smear microscopy laboratory setting

Methods

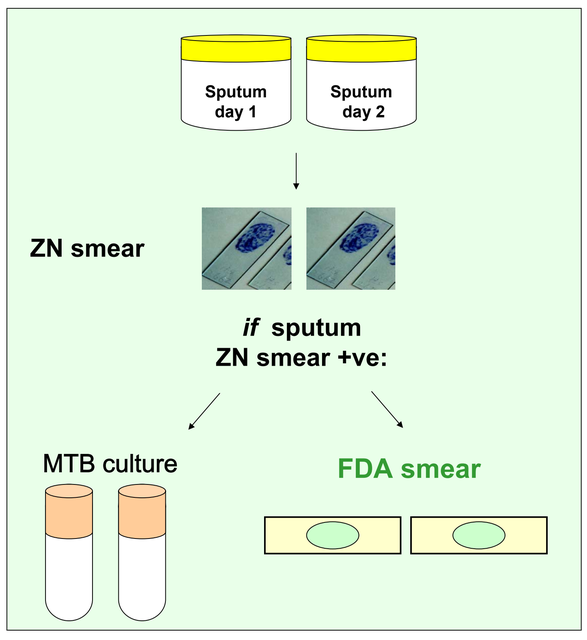
Study Design: Field evaluation of a TB diagnostic test

Study site: Outpatient chest clinic, Mae Sot, Thailand

Case inclusion criteria:

- Patient ≥ 15 years
- Under TB treatment (cat1 or cat2) since ≥ 2 months
- ≥ 1 of 2 sputum ZN smear positive (≥ 1 acid fast bacilli (AFB) /100 high power fields (HPF))

Procedure



FDA staining/reading

Staining

1. Air-dried smear from fresh sputum
2. Flood the smear with FDA (0.5 mg /ml) 30 minutes in 37 ºC incubator
3. Rinse with water
4. 1% acid alcohol 3 minutes (de-colorization)
5. Rinse with water
6. 10 minutes 5% watery phenol (safety step)
7. Rinse with water
8. Dry the smear (30 min -1 hour)
9. Read asap:

Reported result	1000x magnification	Number of fields
Negative	0 AFB / 100 HPF	100
Scanty	1-9 AFB / 100 HPF	100
1+	10-99 AFB / 100 HPF	100
2+	1-10 AFB / 1 HPF	50
3+	>10 AFB / 1 HPF	20

Microscope:
CXC21 Olympus with FRAEN After ® LED Blue
(480 nm Excitation) / Filter: 535/40 nm bandpass

Sample size and analysis:

218 ZN smear +ve cases

Sensitivity, Specificity, positive and negative predictive values (PPV, NPV):

- By specimen
- By case:
  - Positive FDA case: at least 1 out of 2 FDA positive results
  - Use of different AFB cut-off (1, 4, and 10 AFB /100 HPF) to define a positive FDA case

MTB culture, Gold standard

International Organization of Migration (IOM) Laboratory, Mae Sot ,Thailand

- Sputum decontamination (NALC-NaOH-method), spin-concentration
- 3 cultures are inoculated per specimen:
  - 1 x Liquid MGIT
  - 2 x Solid Lowenstein Jensen
- Species identification: Nitrate Reduction- and Genprobe test

Preliminary Results

28th November 2007 - September 2008 MTB culture and FDA results available for:

110 ZN smear+ve follow-up cases

- 89.3 % new cases
- 46.4% end of intensive phase
- 20.5% prolonged intensive phase
- 26.8% continuation phase
- 6.3% end of treatment
- 48% cases with 2 ZN smear +ve sputa

162 ZN smear +ve sputa

- 6.8 % sputum "saliva"

Preliminary Results (continued)

Description of samples according to the stage of TB treatment

AFB count (ZN) versus Culture positivity

	Total	Intensive phase	Prolonged intensive phase	Continuation phase	End of treatment
ZN positive sputum N (%)	162 (100)	77 (100)	35 (100)	41 (100)	9 (100)
scanty results	126 (77.8)	57 (74)	29 (82.9)	33 (80.5)	7 (77.8)
>= 1+	36 (22.2)	20 (26)	6 (17.1)	8 (19.5)	2 (22.2)
Culture positive sputum N (%)	30 (18.5)	22 (28.6)	3 (8.6)	3 (7.3)	2 (22.2)
among scanty	18 (14.3)	13 (22.8)	2 (6.9)	1 (3)	2 (28.6)
among >= +1	12 (33.3)	9 (45)	1 (16.6)	2 (25)	0

FDA performance:

Per sputum:

Cut off : 1 AFB / 100 HPF	Culture +	Culture -	Total	
FDA +	23	43	**66**	**PPV** 34.8 (23.5 – 47.6)
FDA -	7	89	**96**	**NPV** 92.7 (85.5 – 97)
Total	**30**	**132**	**162**	
% (95 % CI)	**Sensitivity 76.7** (57.7 – 90)	**Specificity 67.4** (58. 7 – 75. 3)		

Per follow up-case, using different AFB cut-off:

Cut off : 1 AFB /100 HPF	Culture +	Culture -	Total	
FDA +	19	30	**49**	**PPV** 38.8(25.2 – 53.7)
FDA -	3	58	**61**	**NPV** 95.1 (86.3 – 89.9)
Total	**22**	**88**	**110**	
	Sensitivity 86.3 (65.1 – 97.1)	**Specificity 65.9** (55 – 75.7)		

Cut off : 4 AFB /100 HPF	Culture +	Culture -	Total	
FDA +	13	14	**27**	**PPV** 48 (28.7 - 68)
FDA -	9	74	**83**	**NPV** 89 (80.4 -94.9)
Total	**22**	**88**	**110**	
	Sensitivity 59 (36.6 – 79.3)	**Specificity 84.1** (74.5 - 91)		

Cut off : >9 AFB /100 HPF	Culture +	Culture -	Total	
FDA +	8	2	**10**	**PPV** 80 (44.4 – 97.5)
FDA -	14	86	**100**	**NPV** 86 (77.6 – 92.1)
Total	**22**	**88**	**110**	
	Sensitivity 36.3 (17.2 – 59.3)	**Specificity 97.7** (92 – 99.7)		

Operational aspects of FDA vital staining method in a peripheral smear microscopy laboratory:

Feasibility

- Simple staining procedure, not time consuming (approximately 1.5 hours)
- Standard microscope equipped with LED cassette can be used for read-out
- Mean reading time for slides = 6.3 minutes (IQR: 5.4, 7.4)

Limitations:

- 20°C freezer (storage of FDA stock solution: 25 mg/ml FDA in Aceton)
- 37°C incubator (maybe required)
- fading of fluorescein signal (within hours):
  - Stained smear need to be read shortly after preparation
  - Scanty-loaded AFB-sputum may be miss-read as "fluorescein-negative"
  - Challenge for implementation of reliable quality control measures

Discussion

Low FDA performance: possible reasons

Low Sensitivity

- 78% of specimens were ZN scanty (versus 26% Bangladesh study)
  - 6/7 of false negative FDA were on scanty ZN sputum
- Study population was not limited to failure cases: Culture positivity 19 % vs 66% in the Bangladesh study
- Fading of fluorescence signal

Low Specificity

- Poor specificity on fresh specimen (67% versus 82 % specificity in the Bangladesh study)
- Scanty live bacilli may be positive in FDA but not in culture
- Preliminary observation: low Fluorescence intensity = risk of false positive ?

Considerations

Possibilities for improvement:

- Using 2 FDA smear per sputum (sensitivity)
- Considering fluorescence brightness at readout ? (specificity)
- Consider AFB cut-off for definition of FDA positive ? (specificity)
- Further investigation of FDA performance according to:
  - The ZN AFB load
  - The stage of TB treatment: ZN-failures versus others
- Further standardization/simplification of the method
- Assessment of the method on concentrated specimen
- Double staining (fluorescent markers for "live" versus "dead" in the same specimen),- feasible ?

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