

## The Yield of Sputum Smear Direct Microscopy Using Ziehl-Neelsen Stain in Comparison with Lowenstein Jensen Culture on the Diagnosis of Pulmonary Tuberculosis in Tripoli-Libya

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### Abstract

**Background:** In the developing countries, Tuberculosis is one of leading causes of morbidity and mortality. The detection and identification of *Mycobacterium tuberculosis* is considered a great challenge for clinical microbiologists. Albeit Ziehl-Neelsen (ZN) smear is being a rapid diagnostic tool for TB, the culture on Lowenstein Jensen (LJ) medium is considered the golden diagnostic tool for TB in the developing countries.

**Objectives:** To evaluate the validity and reliability of ZN smear of acid-fast bacilli compared to the culture on LJ medium.

**Design and Settings:** This study is the first in Libya which was conducted from January 2016 to December 2016 in the Administration of TB & Leprosy Control, National Centre for Disease Control, Libya.

**Patients and methods:** The retrospective analytic comparative cross-sectional study included patients visiting TB OPD clinic at NCDC, including transferred patients from other healthcare facilities and hospitals. A total of 2783 specimen of suspected pulmonary TB cases were processed for both direct smear and culture. The direct smears were stained with ZN method (1% Carbol-fuchsin, 3% Hydrochloric acid Ethanol and 0.1% methylene blue). Specimens were observed under 100-x oil immersion lens, whilst cultures were inoculated on LJ medium after digestion and decontamination of the clinical specimens.

**Results:** Of total 2783 study subjects, 203 (7.29%) were smear positive while 327 (11.74%) were culture positive. Out of 203 smear positive pulmonary cases, 154 (47.094%) were found to be positive on LJ culture. A total of 2407 pulmonary cases were negative on the smear and LJ culture for *Mycobacterium tuberculosis*.

**Conclusion:** This preliminary study indicated the low sensitivity of sputum smear direct microscopy for early diagnosis of TB, while culture on LJ remains the golden standard modality.

**Keywords:** *Mycobacterium tuberculosis*; AFB; ZN Stain; Pulmonary TB; LJ Medium

**Abbreviations** TB: Tuberculosis; ZN: Zeihl-Nelson Stain; LJ: Lowenstein Jensen; NCDC: National Center for Disease Control; WHO: World Health Organization; AFB: Acid Fast Bacillus; SN: Sensitivity; SP: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value; MTB: *Mycobacterium tuberculosis*; PTB: Pulmonary Tuberculosis; OPD: Out Patient Department

### Introduction

Tuberculosis (TB) is a major air-borne infectious bacterial disease. Despite the downward trend of TB, it continues to be a challenging public health problem for its emergent morbidity and mortality worldwide, especially in developing countries [1,2]. In 2013, 9 million people with TB infection estimated by the World Health Organization (WHO), and 1.7 million died from the disease [3]. Over the past decade, TB is on rise in Libya as a major public health problem. The

prevalence of TB was assessed by many surveys in Libya. In 2010, a retrospective study was conducted in Northwestern Libya, revealed an estimated incidence of pulmonary TB cases among total population (Libyans & non-Libyans) calculated as 0.09 cases/100,000 and estimated prevalence of total pulmonary TB cases (Libyans & non-Libyans) calculated as 14 case/100,000 [4].

The common symptoms of the PTB are Cough (especially if lasting for 3 weeks or longer) with or without sputum production, coughing up blood (hemoptysis), night sweats, moderate fever, anorexia, unexplained weight loss, chest pain, loss of appetite and fatigue [5]. However, the principle diagnosis of TB depends on the complexion of history, physical and radiographic evidence and by the quantitative presence of AFB in a direct smears and cultures [6]. Where, the submission of sputum at least three specimens, preferably collected on three consecutive days, to the laboratory for acid-fast bacilli (AFB) smear and culture, which is recommended by most of the standard guidelines for the clinical microbiology laboratories.

Although there are studies implying that two specimens may be as sensitive as three specimens [7-10]. Newly, the WHO also recommended decreasing the number of specimens to be examined for screening of TB cases from three to two, in laboratories where a well-functioning external quality assurance (EQA) system exists [8]. In highly epidemic countries, this would extremely reduce the number of specimens needed to be collected and processed from each patient in ruling out TB, which results in trimming laboratories workload. Sputum smear direct microscopy has a significant value either clinically or epidemiologically in the detection of AFB and remains the most widely used diagnostic test for PTB in most developing countries, despite the convenience of new radiometric and molecular diagnostic techniques in the developed world [11-13].

While culture on LJ medium is considered time consuming, necessitates special procedures and requires proficient technicians, it is cheaper than the molecular techniques. Therefore, remains the gold standard for the diagnosis of TB in developing countries [14].

This study has been conducted to analyze and evaluate the validity and reliability of sputum smear direct microscopy and its contribution in the ultimate diagnosis of PTB.

## Materials and Methods

**Setting:** This is analytic comparative cross-sectional study was carried out in the Administration of TB & Leprosy Control, National Centre for Disease Control, Libya.

**Study period:** January 2016 to December 2016.

### Inclusion criteria:

- Patients of all age group and both genders
- Newly clinical suspected cases of Pulmonary TB, patients underwent ZN stain, and confirmed with LJ culture.

### Exclusion criteria:

- Patients who had received anti-tuberculosis treatment in the previous months
- Samples suggestive of saliva
- Contaminated samples
- Samples rapidly diagnosed by ZN, but confirmed with PCR instead of LJ
- Any other sample collected instead of sputum (i.e. body fluid samples).

**Samples:** A total of 3253 samples from different patients of both gender and all age groups were collected, of which 470 samples were excluded according to the exclusion criteria. Therefore, 2783 sputum samples processed for microscopy by Ziehl-Neelsen stain and cultured on Lowenstein-Jensen medium.

**Laboratory methods:** The study subjects were patients visiting TB-OPDs and referred patients from healthcare facilities of Tripoli, Libya. Clinical suspects of PTB, presented with fever, night sweats, fatigue, anorexia and weight loss, were requested to provide three early-morning sputum samples over 2 days, one in the spot and two in the following day, since *Mycobacteria* were in the highest concentration. Afterward, all sputum specimens were labeled and transported instantly to the reference laboratory where processed within 48 hours by laboratory technicians trained in mycobacteriology, maintaining standard laboratory guidelines for TB.

Collected sputum specimens were decontaminated and digested with 4% N-Acetyl-L-Cysteine-Sodium Hydroxide (NaOH); phosphate buffer (Ph 6.8) was added up to 50-ml; samples were concentrated via centrifuged at 3000 × g at 45°C temperature for 15 minutes; Supernatant was discarded and sediment was re-suspended in (1-2 ml) of phosphate buffer (Ph 6.8); suspension mixed and inoculated onto the slants of LJ medium [11,15]. Once specimen processed and culture inoculated, smear was prepared using ZN stain method [1% Carbol-fuchsin, 3% Hydrochloric acid Ethanol and 0.1% methylene blue] for sputum smear direct microscopy [16].

The reading of sputum smear direct microscopy was based upon the quantitative presence of AFB in sputum samples under light microscopy in which the standard method of reporting was adopted according to WHO manual sputum grading (Table 1). In assessing the sensitivity and specificity of the smear procedures, culture was considered as the reference gold standard. Cultures were evaluated twice in the first week then weekly up to 40 days with visual inspection and scored positive, contaminated or negative if no growth occurred after 8 weeks of incubation. Extensive quality control has been implanted in the NCDC TB-Laboratory necessitating the reduction of False Positive results via rechecking the random positive and negative smears by an experienced microbiological technician for cross-contamination.

No. of AFB	No. of oil immersion field	Results
No AFB	100	Negative
1-9	100	Scanty
10-99	100	Positive (+)
1-10	One	Positive (++)
>10	One	Positive (+++)

**Table 1:** The standard method of reporting according to WHO manual sputum grading [17,18].

## Statistical analysis

Analyses date was performed using SPSS 24.0. Which sensitivity, specificity, PPV & NPV were calculated in reference to the outcomes defined above.

## Results

### Data presentation and analysis

A total of 3253 suspects were enrolled in the study from January 2016 until September 2016. Of these, 0.18% (n=6 of 3253) were unable to submit sputum early-morning specimen; 1.56% (n=51 of 3253) did not have culture performed; 2.12% (n=69 of 3253) cases were contamination; 8.91% (n=290 of 3253) were extra pulmonary, 1.66% (n=54 of 3253) were diagnosed via DNA probe. In 85% (n=2783 of 3253) suspects the data set was complete and included results for ZN and culture.

However, gender distribution for specimens was (n=1923) for males and (n=860) for females. Although for PTB was 244 males and 83 females respectively. The mean age for PTB was 37.3815.01 years, with male to female ratio 2.94:1 respectively. While in males, the highest incidence was 42.6% (n=104 of 244) in the age group (26-40), instead

of females the highest incidence was 34.9% (n=29 of 83) in the age group (0-25).

#### Sensitivity and specificity of direct sputum smear microscopy

Of the 2783 pulmonary specimens, AFB was detected from one or more sputum specimens by smear microscopy in 7.29% (n=203 of 2783), while 11.74% (n=327 of 2783) were culture positive. Out of 203 smears positive pulmonary cases, 75.86% (n=154 of 203) were found to be positive on LJ culture. A total of 6.21% (n=173 of 2580) pulmonary cases were negative on the smear but were found to be positive on LJ culture for *Mycobacterium tuberculosis* as shown below in Table 2.

ZN smear sensitivity of the pulmonary specimens is considerably low, but high positive predictive value (PPV) and negative predictive value (NPV) as shown in (Tables 2 and 3).

Results	Culture positive	Culture negative	Total
Smear positive	154	49	203
Smear negative	173	2407	2580
Total	327	2456	2783

**Table 2:** Comparison of AFB smear with culture results in 2383 pulmonary cases.

As culture considered as the gold standard, therefore the sensitivity, specificity, PPV, and NPV for smear is 47.094%, 98.004%, 75.862% and 93.294% respectively as shown on (Table 3).

Sensitivity	Specificity	PPV	NPV
47.094%	98.004%	75.862%	93.294%

**Table 3:** Sensitivity, specificity, PPV and NPV of AFB smear microscopy for pulmonary specimens.

## Discussion

TB is one of the leading causes of morbidity and mortality all over the world, killing about 2 million people each year. It is estimated that about one-third of the world population are infected with TB and most cases are in the underdeveloped countries [19].

TB control strategies necessitate developing strategies for accurate and rapid detection of TB patients as advocated by WHO, helping in isolation for such cases to provide preventive therapy to contacts [20]. The diagnostic modalities in resource-poor settings should have certain standardized features of being sensitive, specific, reliable, cost effective, safe, simple, and easily applicable. Sputum smear direct microscopy is considered the most rapid, effective and presumptive test in establishing the diagnosis of PTB.

Although considerable efforts have been made to improve the sensitivity of sputum smear microscopy in the diagnosis of PTB, it still lacks sensitivity [21]. Sputum smear microscopy depends mainly upon the nature of the specimen, quality, quantity, bacterial content and the extent of viable organisms especially in pediatric age group, so culturing of MTB has become tremendously important in the last few decades [22].

According to our results, the highest incidence for males was in the age group (26-40 years), but for females it was in the age group (0-25 years). Our results were against those obtained by Shargie and

Lindtjorn [23], who found that the highest number of tuberculosis cases was in the age group between 15 and 24 years for males and females. Obtaining expectorated sputum from children for detection of AFB is difficult and its examination is of low yield [24,25] hence the low results for patients who are less than 15 years in male and females, (n=2 of 216) and (n=4 out of 76) respectively. Sputum induction has higher yield than expectorated sputum in children, and the use of sputum induction for obtaining TB diagnostic specimens in children is increasing. Sputum induction is performed via administration of aerosolized heated saline combined with salbuterol (or similar drug to minimize wheezing), followed by suctioning to capture the expectorated sputum [25].

As per our results the contamination rate was 2.12%. Contamination rate of 2 to 5% of sputum specimens is acceptable as a general rule for LJ medium. If fewer than 2% of specimens are contaminated the process may be killing many of the mycobacteria as well as contaminants (the treating time should be decreasing). If more than 5% of the cultures are contaminated, the decontamination process is inadequate (the treating time should be increasing) [26,27].

Consistent with our study's results, sputum microscopy was found to have 47.09% sensitivity (SN), 98.00% Specificity (SP), PPV 75.86% & NPV 93.29% (Table 3). Compared with the findings depicted in Arslan et al (2014) in Pakistan, our results substantially agree with the nearly the same SP and NPV but contradicts with the SN and PPV findings, 97%, 93%, 71% and 98% respectively [28]. Therefore, our findings have important implications for public health problem. First, 7.18% (n=173 of 2407) have PTB and are not detected when sputum-smear microscopy alone is used. Second, supporting WHO recommendations that AFB direct microscopy examination is solely insufficient for the case detection of PTB in high-burden-settings due to its low sensitivity at operational level and culturing remains the golden diagnostic modality in the diagnosis of PTB in the developing countries [11-14].

In conclusion, admitting sputum smear direct microscopy is rapid, cheap and specific test for early diagnosis of TB, its sensitivity is considered low compared with culture on LJ medium which is still thought to be the golden diagnostic test in the diagnosis of PTB. Nevertheless, further studies are required in different study settings that should be receiving support from the national TB control program.

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## Conflict of Interest Statement

None

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