

Comparative Study of Auramine-O Staining and Ziehl-Neelsen for Diagnosis of Pulmonary TuberculosisNazar Abdalazeem Osman¹, Mustafa Altigani Mustafa², Mona khalafalla Hassan³, Mohamed Mohamed Tarek³¹Departments of Medical Microbiology, Faculty of Medicine, Sebha University, Sebha, Libya. ²Departments of Microbiology, Faculty of Medical Laboratory Science, Al Neelain University, Khartoum, Sudan. ³West Nile College, Department of Medical Laboratory, Khartoum, Sudan.Email: nazar585@hotmail.com

Abstract: Introduction: ZN stain is commonly used throughout the world and still remains the standard method against which new tests must be measured. FL staining is regarded as a more reliable method due to more intensive binding of mycolic acids of the bacilli to phenol auramine-O, so that the bacilli can stand out sharply against black background to allow rapid and accurate screening under low power objective. **Objective:** this study was to compare the performance of Zn stains and Auramine-O fluorescent microscopy staining techniques in detecting the presence of *Mycobacterium bacilli* in sputum. **Method:** one hundred patients suspected of having pulmonary TB, referred to Aboanga hospital were admitted to this study, sputum samples were examined by using direct and concentrated auramine O and ZN stain. **Result:** Positive concentrated auramine method result distributed as (14/3+++, 11/2++ and 30/1+), Positive concentrated ZN method distributed as (4/3+++, 10/2++ and 10/1+) while Positive direct auramine smears distribute as (4/3+++, 10/2++ and 11/1+), positive direct ZN method distributed as (2/3+++, 3/2++ and 9/1+). **Conclusion:** FM greatly improves the diagnostic value of the sputum smear especially in patients with a low density of bacilli that are likely to be missed on ZN stained smears, concentrated method on both auramine O and ZN stain more sensitive than direct method.

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1. Introduction

Tuberculosis (TB) is the major health problem in the world since 1993 when declared as a global emergency by world health organization (WHO). It is estimated that nearly one billion people will be infected with TB, 200 million develop the disease, and 35 million will die from TB during 2000- 2020⁽¹⁾. Sudan like other developing countries suffering from TB, It has high burden of tuberculosis (TB) with a prevalence of 209 cases per 100,000 of the population and 50,000 incident cases during 2009. ⁽²⁾

ZN stain is commonly used throughout the world and still remains the standard method against which new tests must be measured. A standardization of the technique was recommended by the International Union against Tuberculosis and Lung Disease (IUATLD) in 1978. ⁽³⁾

The utilization of auramine O, a fluorescent dye, instead of carbol fuchsin, was first proposed in the 1930s, ⁽⁴⁾ but found widespread application in industrialized countries only some 30 years later, after a thorough re-evaluation of the technique, using a combination of auramine O and Rhodamine. ⁽⁵⁾

Auramine fluoresces when illuminated (excited) by blue violet or ultra-violet (UV) light. It can be used to demonstrate AFB because it binds to the mycolic acid in the mycobacterium cell wall. No heating of the stain is required. After being stained with auramine,

the smear is decolorized with acid alcohol which removes the dye from the background. The smear is then washed with a weak solution of potassium permanganate to darken the background. Tubercle bacilli fluoresce white-yellow against a dark background.

In recent years, several radiometric and molecular techniques have been developed for the diagnosis of TB. Radiometric techniques reduce the turnover time for AFB ⁽⁶⁾, However, they are not suitable for Sudan because of their high cost.

Evaluation of rapid and inexpensive diagnostic methods to diagnosis pulmonary tuberculosis in Sudan has great importance, Zn stain is the most common method use to diagnosis pulmonary tuberculosis but it consider less sensitive compeer with other stain like Fluorescence stain.

The sensitivity of ZN is influenced by numerous factors, such as the prevalence and severity of the disease, the quality of specimen collection, the number of mycobacterium present in the specimen, the method of processing (direct or concentrated), the staining technique, and the quality of the examination (microscope operator expertise, time spent for smear examination). ⁽⁷⁾

The smears stained by ZN method can detect bacilli when they are at the order of 10⁵/mL of sputum, whereas fluorescent (FL) stain detects the

bacilli when they are at the order of 10^4 /mL of sputum. FL staining is regarded as a more reliable method due to more intensive binding of mycolic acids of the bacilli to phenol auramine-O. ⁽⁸⁾

The general objective of this study was to compare the performance of Zn stains and Auramine-O fluorescent microscopy staining techniques in detecting the presence of *Mycobacterium bacilli* in sputum, recommending a more sensitive method to be adopted for diagnosis of tuberculosis.

2. Materials and Methods

In this study, 100 patients suspected of having pulmonary TB, referred to Aboanga hospital, Khartoum state Sudan, between March and June 2013.

Collection of sample:

Samples were collected in clean, sterile, leak-proof, wide-mouth containers. At the time of sample collection

AFB was concerted (Homogenization of sputum), this was done by using sodium hypochlorite centrifugation technique to concentrate AFB:

- Tow ml of sputum was transferred to a screw-cap universal bottle 15–20 ml capacity.
- Equal volume of concentrated sodium hypochlorite (bleach) solution was added and mixed well.
- Leaved at room temperature for 10–15 minutes, shaking at intervals to break down the mucus in the sputum.
- Eight ml of distilled water was added, mixed well.
- Centrifuge at 3000 g for 15 minutes or at 250–1000 g for 20 minutes.
- Used a glass Pasteur pipette or plastic bulb pipette, removed and discard the supernatant fluid. Mix the sediment. When two tubes had been used, combine the two sediments. Transfer a drop of the well-mixed sediment to a clean scratch-free glass slide. Spreads the sediment to made a thin preparation and allow air-drying.
- Smear was heat-fixed and stained used Fluorescent stain and Ziehl-Neelsen technique.
- Examined it microscopically by Fluorescent and light microscope for AFB.

Preparation for ZN smears:

A small portion of the most suggestive part of the sputum sample was scooped and spread using coil type movement 2cm by 1 cm in the center of a new glass slide until a fairly even thickness was obtained. Smear was heat-fixed and covered with carol fuchsin stain, heated until vapour just begins to rise. Allow the heated stain to remain on the slide for 5 minutes. Stain was washed off with clean water and was cover the

with 3% v/v acid alcohol for 5Minutes and was washed off with clean water then s covered with malachite green stain for 1–2 minutes then washed and placed in a draining rack for the smear to air-dry, after drying, the smears were examined under the microscope using x100 oil immersion objective lens. Any definite red bacilli seen were reported as AFB positive and gave an indication of the number of bacteria present as follows:

> 10 AFB/field reported +++
 1-10 AFB/field reported ++
 10-100 AFB/100 fields..... Reported +
 1-9 AFB/100 fields..... reported the exact number

Preparation for Auramine-O smears:

The heat-fixed smears were stained with the filtered AR mixture at 37°C for 15 minutes; slide was rinsed with deiodinized water for 2 minutes. Decolonization was performed with 0.5% hydrochloric acid in 70% ethanol for 2 minutes. Slide was rinsed with deiodinized water for 2 minutes. Counterstaining was performed with 0.5% aqueous potassium permanganate for 2 minutes. Slide was rinsed with deiodinized water for 2 minutes, and air dried and examined under high power ($\times 400$) which was confirmed under oil immersion ($\times 1000$). Smears examined under fluorescent microscope. If fluorescent AFB were seen, the smear was reported as ‘AFB positive’ and gave an indication of the number of bacilli present as follows:

> 10 AFB/field reported +++
 1 -10 AFB/field reported ++
 10-100 AFB/100 fields..... Reported +
 1-9 AFB/100 fields..... reported the exact number

When no fluorescent rods were seen, the result was reported as ‘No AFB seen’.

3. Result

The proportion of positive smears detected was 55 (55%) and 24 (24%) for the concentration of auramine and ZN staining methods, respectively:

Concentrated auramine method result distributed as (14/3+++), (11/2++ and 30/1+), concentrated ZN method distributed as (4/3+++), (10/2++ and 10/1+) (Table1).

ZN method missed 30 of the 55 slides found positive by the auramine phenol method while auramine phenol detected all smear positive with ZN method.

Auramine concentrated showed positive result (3+++ in 14 slides while concentrated ZN method showed (3+++ in only 4 smears of these 14 slides, remain 10 slides showed result (2++). Result of 2++

was detected in 11 smears of concentrated auramine; concentrated ZN method detected it as (1+) in 10 smears and one negative. All (30) smears reported as (1+) by auramine showed negative result of ZN method (Table2).

Concentration auramine showed result (3+++), in 14 slides, while same samples by direct auramine showed (4/3+++), (10/2++). Result of (2++) showed in 11 samples by concentrated auramine while direct auramine detected all as (1+). 30 samples showed result of (1+) by concentrated auramine and missed all by direct auramine. (Table3).

Direct auramine method showed positive result in 25 smears while direct ZN method showed positive in 14 smears. (Table4).

Positive direct auramine smears distribute as (4/3+++), (10/2++ and 11/1+), positive direct ZN

method distributed as (2/3+++), (3/2++ and 9/1+) (Table4).

Four smears which showed positive (3+++), by auramine method, only two reeds as (3+++), by ZN method and the other two smears showed (2++). Positive (10) smears detected as (2++) by auramine, showed (1+) by ZN method except in one smear showed same result. All smears (11) positive (1+) by auramine method showed negative result by ZN method. (Table5).

Concentration ZN showed result (3+++), in 4 slides, while same samples by direct ZN showed (2/3+++), (2/2++). Result of (2++) showed in 10 samples by concentrated ZN while direct ZN detected all as (1/2++), (9/1+). 10 samples showed result of (1+) by concentrated ZN and missed all by direct ZN. (Table6).

Table 1: Comparison of performance of Concentration Auramine and Concentration ZN techniques

| Result | Concentration Techniques | |
|----------|--------------------------|-----|
| | Auramine | ZN |
| 3+++ | 14 | 4 |
| 2++ | 11 | 10 |
| 1+ | 30 | 10 |
| Negative | 45 | 76 |
| total | 100 | 100 |

Table 2: Comparison of performance in same smear between Concentration Auramine and Concentration ZN techniques

| Result | Concentration Techniques | | |
|--------|--------------------------|------------------|------------|
| | Auramine | Same smear by ZN | |
| 3+++ | 14 | 4/3+++ | 10/2++ |
| 2++ | 11 | 10/1+ | 1/negative |
| 1+ | 30 | Negative | |
| total | 55 | 24 | |

Table 3: Comparison of performance of Concentration Auramine and direct Auramine techniques

| Result | Auramine | | |
|--------|------------------------|-------------------------------|--------|
| | Concentration Auramine | Same smear by Direct Auramine | |
| 3+++ | 14 | 4/3+++ | 10/2++ |
| 2++ | 11 | 11/1+ | |
| 1+ | 30 | Negative | |
| total | 55 | 25 | |

Table 4: Comparison of performance of direct Auramine and direct ZN techniques

| Result | Direct Techniques | |
|----------|-------------------|-----|
| | Auramine | ZN |
| 3+++ | 4 | 2 |
| 2++ | 10 | 3 |
| 1+ | 11 | 9 |
| Negative | 75 | 86 |
| total | 100 | 100 |

Table 5: Comparison of performance in same smear between direct Auramine and direct ZN techniques

| Result | Direct Techniques | |
|--------|-------------------|------------------|
| | Auramine | Same smear by ZN |
| 3+++ | 4 | 2/3+++ |
| 2++ | 10 | 1/2++ |
| 1+ | 11 | Negative |
| total | 25 | 14 |

Table 6: Comparison of performance of Concentration ZN and direct ZN techniques

| Result | ZN | |
|--------|------------------|-------------------------|
| | Concentration ZN | Same smear by Direct ZN |
| 3+++ | 4 | 2/3+++ |
| 2++ | 10 | 1/2++ |
| 1+ | 10 | Negative |
| total | 24 | 14 |

4. Discussion

Microscopical diagnosis of mycobacterium tuberculosis is the highest priority in any TB control programme, it is not only use for diagnosis, and it is also part of follow up the treatment and in the prevention.

The aim of this study was to compare two conventionally used acid-fast staining methods, ZN and auramine O staining. Our results showed that the sensitivity of auramine O staining method was more than ZN method. This is in agreement with other studies. ^(8, 9, 10) Many reports showed that sensitivity of ZN ranged from 32% to 94%, and fluorescence microscopy was on average more sensitive than ZN. ⁽¹¹⁾

The use of FM significantly increases the diagnostic value of the smear, particularly where there are low-density bacilli which may escape detection on ZN-stained smears.

Our study founded that FM greatly improves the diagnostic value of the sputum smear especially in patients with a low density of bacilli that are likely to be missed on ZN stained smears, on the other hand our study also showed that, a relation between ZN missed positive smears and density of bacilli. This is in agreement with Ulukanligil and his colleague. ⁽¹²⁾ Our results also were in agreement with those of Ba and Rieder⁽¹³⁾, Guthie et al ⁽¹⁴⁾, and Pollack and Wieman ⁽¹⁵⁾, who reported that FM appears to be more likely to detect TB in smears which contain low-density bacilli.

Our result founded that, uses of concentrated method on both auramine O and ZN stain more sensitive than direct method which agree with Saroj Hooja and his colleague⁽¹⁶⁾.

In conclusion FM is more reliable than ZN; however Both ZN and Auramine-O techniques can be used in the detection of AFB in this study population. Auramine-O should remain a method of choice in this

study population whenever dealing with few samples because it showed a greater sensitivity than ZN method in the detection of AFB. Uses of concentrated method on both auramine O and ZN stain more sensitive than direct method.

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