A COMPARATIVE STUDY OF AURAMINE STAINING USING LED FLUORESCENT MICROSCOPY WITH ZIEHL-NEELSEN STAINING IN THE DIAGNOSIS OF PULMONARY TUBERCULOSIS

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ABSTRACT: Background: Tuberculosis remains world's leading cause of death from a single infectious agent. Pulmonary tuberculosis is the most common presentation of tuberculosis (TB). For developing countries with a large number of cases and financial constraints, evaluation of rapid and inexpensive diagnostic methods has a great importance. **OBJECTIVE:** To study the efficacy of Fluorescent Light Emitting Diode (LED) microscopy in the diagnosis of pulmonary TB in comparison to Ziehl-Neelsen (ZN) staining of sputum samples from patients suspected of pulmonary Tuberculosis of Dr.B.R.Ambedkar Medical College, Bangalore. **MATERIALS AND METHODS:** 634 sputum samples collected from suspected cases of pulmonary Tuberculosis were processed and subjected to ZN and Auramine-O (AO) staining for detection of TB. Positive smears were graded according to standard WHO criteria. **RESULTS:** Out of 634 sputum smears, 10.57% and 16.56% were positive by ZN and AO respectively. AO was found to be superior to ZN in detecting TB cases and also AO staining was able to detect more paucibacillary cases than ZN.

KEY WORDS: Ziehl-Neelsen staining, Tuberculosis, Auramine-O, LED microscopy

INTRODUCTION: Tuberculosis continues to be the world's most important infectious cause of morbidity and mortality among adults. Nearly 9 million people develop TB disease each year, and estimated 1.6 million die from the disease [1].

Microscope remains the most appropriate method till date, but it suffers from low sensitivity for paucibacillary samples, found more frequently among immune compromised patients co- infected with HIV [2]. This seriously jeopardizes the quality of Ziehl Neelsen (ZN) microscopy, further reducing its yield [3].

Direct microscopy for Acid Fast Bacilli (AFB) is a widely used method for the diagnosis and confirmation of pulmonary Tuberculosis. First of all, ZN stain has low sensitivity relative to fluorescent stain [4] and culture [5], secondly it takes more time to scan at least 300 field. Thirdly, it needs experienced microbiologists. Fourthly, it often misses the paucibacillary TB cases especially

when the patient is co-infected with HIV. Technical error is also more common in cases of ZN staining.[6]

The advantages of fluorescence staining procedure are that it is simpler and can be examined at a lower magnification than ZN (40x Vs 100x). It has been estimated that Fluorescent microscope (FM) may take upto 75% less time than ZN [4]. This advantage would be a tremendous benefit for overburdened laboratory system in many low resource settings.

Recently it has been demonstrated that low cost LEDs could be a viable alternative to Mercury vapour lamps used in FM. LEDs can produce very narrow spectrum of light and are able to excite auramine stain without the production of UV light. LEDs have an expected bulb life up to 50000h (compared with 200h for a conventional mercury bulb), produce minimal heat and contain no hazardous materials and hence the power consumption is much lower. Finally it has been reported the image quality remains good outside of a dark room [7].

In view of this, the need for quicker and more sensitive methods than those currently used for diagnosis of TB has become imperative. We, therefore, performed a study comparing the reliability of LED and ZN stained sputum smears in the diagnosis of pulmonary tuberculosis.

MATERIALS AND METHODS: This comparative study was conducted in the department of microbiology Dr. B.R.AMC, Bangalore from September 2012-to January -2013. A total of 634 sputum samples were collected from patients suspected of pulmonary TB.

2 sputum samples were collected on 2 consecutive days from each patient (One spot and one early morning sample) in clean, sterile, heat proof, wide mouth containers. The processing of samples was carried out in a bio-safety cabinet. Each sample was then subjected to ZN staining and Fluorescent Auramine -O (AO) staining.

ZN stained smears were examined using oil immersion objective (x1000) and AO stained smears using 40x objective (x400) under fluorescence microscope [8]. The results were graded in a defined manner as per the guidelines of International Union against Tuberculosis.

Table 1:Grading chart (who, Iuatld, 2007)

Result (WHO scale) 1000 x field=HPF	Bright fields x 1000 magnification: 1length = 2cm=100HPF
Negative	Zero AFB / 100 HPF
Scanty	1-9 AFB / 100 HPF
1+	10-99 AFB / 100HPF
2+	1-10 AFB /1HPF (on average 50 HPF)
3+	>10AFB /1 HPF (on average 20 HPF)

HPF= high power field, AFB =Acid fast bacilli

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Table 2: Grading chart (who, Iuatld, 2007) for led fluorescent microscopy:

Result (WHO scale)	LED fluorescent microscopy (400 x :1 length =40 fields =200HPF)	Minimum number of fields to be examined
1000 x field=HPF		
Negative	Zero AFB / 1 length	40
Scanty	1-19 AFB / 1 length	40
1+	20-199 AFB / 1 length	40
2+	5-50 AFB / 1 field on average	20
3+	>50 AFB / 1 field on average	8

For the present study 2+ and 3+ were classified as multibacillary and 1+ and scanty as paucibacillary [9].

RESULTS: Out of 634 sputum samples collected, 67 and 105 sputum samples were found to be positive for AFB by ZN and AO staining respectively. The ZN smear positivity rate and the AO smear positivity rate in this study was 10.57% and 16.56% respectively [Table 1 and Chart 1].

Table 3: Comparison of ZN and Auramine staining reports:

Staining method used	No. of Positive smears	No. of Negative smears
ZN stain	67 (10.57%)	567 (89.43%)
Auramine stain	105 (16.56%)	529 (83.44%)

Table 4: Distribution of positive slides by grading and technique used:

Grading	ZN Staining	Auramine Staining
Scanty	1(1.49%)	18(17.14%)
1+	2(2.99%)	8(7.62%)
2+	28(41.8%)	35(33.33%)
3+	36(53.73%)	44 (41.90%)
Total	67	105

Table 5: Distribution of paucibacillary and multibacillary cases detected by ZN staining and LED fluorescent microscopy.

Staining method	No. of paucibacillary cases	No. of multibacillary cases	Total no. of positive cases detected
ZN staining	3(4.55%)	64(95.52%)	67
Auramine staining	26(24.76%)	79(75.24%)	105

Table 6: Comparison of LED fluorescent microscopy (Auramine staining) with ZN staining method:

Type of staining method used:	Fluorescent microscopy (Auramine stain) smear positive	Fluorescent microscopy (Auramine stain) smear negative	Total
ZN smear positive	66(10.41%)	1(0.16%)	67
ZN smear negative	39(6.15%)	528(83.28%)	567
Total	105	529	

DISCUSSION: Current recommendations for the control of Tuberculosis emphasize early case detection so as to allow treatment of patients and thereby limit the transmission of bacilli.

ZN stain can detect bacilli when they are in the order of 10^5 /ml of the sputum, whereas a more sensitive AO stain can detect in the order of 10^4 /ml of sputum. In the present study out of the 634 samples examined, 67(10.57%) and 105(16.56%) TB cases were detected by ZN and AO staining methods respectively. Similar results have also been reported by studies done by Suria kumar *et al*, 2012[10]. Whereas higher smear positivity rates were shown by K Prasanthi *et al*, 2005(50% by ZN, 69% by AO) [9] and Ulukanligil *et al* (67.6% by ZN, 85.2% by AO)[11].

The results of this study suggest that there is no need for culture as the gold standard, given the high consistency between results obtained with the two techniques. The high consistency of the two methods resolves a pre-eminent question of quality assurance, using a system of internal quality control without recourse to culture or external proficiency testing. Nevertheless, recourse to culture could be valuable, particularly for microscopically low scanty results, both for confirmation of microscopic findings and for internal quality control[12]

It was observed that a total no. of 39 sputum smears which were negative by ZN method were positive by AO staining method and that AO staining with LED Microscopy was more efficient over ZN stain in determining paucibacillary cases have also been proved in this study. AO staining could detect 26 paucibacillary cases, whereas ZN staining detected only 3 of them. This was in concordance with studies done by Laifangbam *et al* 2009[13]. Failure to detect and hence to treat paucibacillary cases can be effectively prevented by the use of fluorescent LED microscopy. The false positivity with fluorescent staining was very low (0.16%) in our study which was in accordance with study done by Khatun *et al* (2011).[14]

In this study AO staining was found to be 6.15% more effective than ZN staining. This shows that fluorochrome staining of sputum smears in comparison to that of ZN staining is a better method of microscopy. FM LED therefore appears to be a more sensitive technique than ZN due to its ability to detect low bacilli load especially in HIV positive subjects.

CONCLUSION: Hence our study concludes that Fluorochrome staining with LED is more efficient over ZN stain in detecting Tuberculosis bacilli in sputum, especially the paucibacillary cases and also FM LED has been found to be less time consuming as compared to ZN method (1000x) in the diagnosis of TB. FM with LED is easier to use, quicker and cheaper especially in centers where large numbers of sputum specimens are processed.

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Figure 1: Photomicrograph of Ziehl-Neelsen stain sputum smear showing TB Bacilli (1000x) as bright pink to red, beaded or barred forms, while the tissues cells and other organism are stained blue. : (a) AFB++ and (b) AFB +++

Figure 2: Photomicrograph of Auramine-O stain sputum smear by LED microscopy (400x). The bacilli are seen as yellow luminous organism in a dark field (a) AFB++ and (b) AFB+++

Chart 1: Bar diagram shows the positive smear results of ZN staining and by Auramine O staining by LED fluorescent microscopy.

Chart 2:

Chart 3: