



## MEDICO RESEARCH CHRONICLES

ISSN NO. 2394-3971

DOI No. 10.26838/MEDRECH.2021.8.5.559

Contents available at [www.medrech.com](http://www.medrech.com)

### APPLICATION OF FLUORESCENT MICROSCOPY FOR IMPROVED DETECTION OF ACID FAST BACILLI IN SPUTUM SMEAR PREPARATION

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#### ARTICLE INFO

##### Article History

**Received:** September 2021

**Accepted:** November 2021

**Keywords:** Tuberculosis; Ziehl-Neelsen; Auramine – O Staining; Fluorescence; Microscopy; Sputum smear.

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#### ABSTRACT

**Background:** Tuberculosis remains a global health problem with an enormous burden of disease. Ziehl-Neelsen stain is the most commonly used method worldwide to detect tuberculous lymph node aspirates, however, Auramine – O Staining is being preferred owing to its high sensitivity, rapidity and ease of screening. The aim of present study was to compare the efficacy of fluorescent microscopy using Auramine O stain with conventional microscopy using Ziehl- Neelsen stain as a screening method to detect clinically suspected cases of pulmonary tuberculosis. **Method:** A total of 100 clinically suspected cases of pulmonary tuberculosis referred to RNTCP laboratory from chest and TB OPD Medicine and Surgery and all other inpatient and out-patient departments were studied with detail clinical history and sputum examination using Ziehl–Neelsen and Auramine O staining. **Results:** Among 100 cases, 28% cases were detected sputum smear positive by Ziehl-Neelsen staining and 30% cases were detected by Auramine O stain using fluorescence microscope. Maximum sputum smears positive (36.6%) cases were in the age group of 31-40 years with male predominance (75%). Auramine O stain showed higher grading in 4 cases in which one case was scanty on Ziehl-Neelsen smear showed positive 1+ by Auramine O and other 2 cases which were negative by Ziehl-Neelsen stain were showed 1+ result on Auramine O stain. One case which showed 2+ grading on Ziehl-Neelsen stain showed 3+ grading with Auramine O stain. Fluorescence microscopy using Auramine O showed better grading than Ziehl-Neelsen stain. **Conclusion:** Fluorescence microscopy is more superior in detecting sputum smear positive cases over Ziehl-Neelsen stain.

#### ORIGINAL RESEARCH ARTICLE

2021, [www.medrech.com](http://www.medrech.com)

## INTRODUCTION

Tuberculosis (TB) still remains a global health problem affecting one third of world population. Early diagnosis of TB is very important for therapeutic reasons and to control the spread of infection. For developing countries with a large number of cases and financial constraints, evaluation of rapid and inexpensive diagnostic methods has a great importance [1]. The sputum microscopy is the main case finding tool in tuberculosis control programmes. In India under Revised National Tuberculosis Control programme (RNTCP), Ziehl Neelsen method is the recommended procedure for staining tubercle bacilli. However, for a better implementation of control programme, there is a need for a better case finding tool. Therefore, fluorescence microscopy is a rapid, useful and reliable tool for the detection of acid fast bacilli (AFB) [2, 3].

However, fluorescence microscopy offers well described benefits compared with conventional light microscopy for the evaluation of sputum smear samples for tuberculosis. Fluorescence microscopy of Auramine-O stained smears provides similar specificity and increased sensitivity compared with light microscopy of Ziehl-Neelsen stained smears. Fluorescence microscopy also allows more rapid screening of sputum smear specimens particularly when high numbers of samples are screened per day because the majority of laboratory time is spent confirming negative smear results [4].

Auramine O staining based on direct fluorescence is superior alternative to Ziehl-Neelsen stain due to its high sensitivity. Fluorescent microscopes with light emitting diodes are being evaluated by World Health Organization for increase in sensitivity and specificity, ease of use and for less time being required to scan the slides in a low power objective. It is estimated in a meta-analysis that fluorescent microscopy is having approximately 10% greater sensitivity in

diagnosis of pulmonary tuberculosis [5]. Hence the present study was undertaken to evaluate and compare the diagnostic performance of fluorescence microscopy using Auramine O-stain with conventional microscopy using Ziehl- Neelsen stain for improved detection of AFB in sputum for diagnosis of suspected cases of pulmonary tuberculosis.

## MATERIALS AND METHODS

This observational study was carried out in the Department of Pathology at Tertiary Care Centre during the period from December 2010 to April 2013. A total of 100 clinically suspected cases of pulmonary tuberculosis referred to RNTCP laboratory from chest and TB OPD, Medicine, Surgery and all other inpatient and out-patient departments were included in the study. Patients on AKT and those completed treatments within three months were excluded.

To obtain good sputum sample patients were instructed properly and sufficient amount of thick muco-purulent sputum coughed out deeply from lungs was collected. Total 200 sputum specimens including one spot and one early morning were collected. Total 400 sputum smears were made from 200 sputum specimens. From each specimen two smears were prepared and stained one by Ziehl-Neelsen stain and one by Auramine O stain. Preparation of Sputum Smears was done as per current guidelines of the Revised National Tuberculosis Control Programme [6]. The sputum smears prepared were stained one by Ziehl-Neelsen method [7] and examined under oil immersion by using light microscope and the other slide was stained by Auramine -O stain [8, 9] and examined first using low power objective (10X and 20X) followed by high power objective (40X) using NIKON ECLIPSE 80 I fluorescence microscope. Positive smears were graded into four grades of positivity using the 20x objective along with 10x eyepiece. Smears were examined carefully by scanning at least 100 fields before

reporting a smear as negative. In smears stained by Ziehl-Neelsen method. AFB were seen as pink slightly curved rods 2-8 microm in diameter. In smears stained by Auramine O in a positive smear AFB were seen as slender bright yellow fluorescent rods standing out clearly against a dark background. The numbers of AFB seen in the smears were recorded according to the recommendations of

National Tuberculosis Control Programme. Interpretation of smears were done by grading them as shown in table 1. The smears were graded by comparative grading system suggested by Manual for sputum fluorescence microscopy by central TB division Directorate General of Health Services Ministry of Health and Family Welfare Govt. of India.

**Table 1:** Comparative Grading [9]

RNTCP ZN staining grading (using 100x oil immersion objective and 10x eye piece)	Auramine O fluorescent staining grading (using 20 or 25x objective and 10x eye piece)	Reporting/ Grading
10 AFB/field after examination of 20 fields	100 AFB/field after examination of 20 field	Positive 3+
1-10 AFB/field after examination of 50 fields	11-100 AFB/field after examination of 50 field	Positive 2+
10-99 AFB/100 field	1-10 AFB / field after examination of 100 fields	Positive 1+
1-9 AFB/100 field	1-3 AFB/100 fields	Doubtful positive/repeat
No AFB per100 fields	No AFB per 100 fields	Negative

[AFB-acid fast bacilli].

## OBSERVATIONS AND RESULTS

A total of 100 cases were studied during a study period, out of them maximum i.e. 73(73%) were male and 27(27.0%) female with male to female ratio of 2.7:1. Among 100 cases, 28% cases were sputum smear positive by Ziehl-Neelsen method using conventional

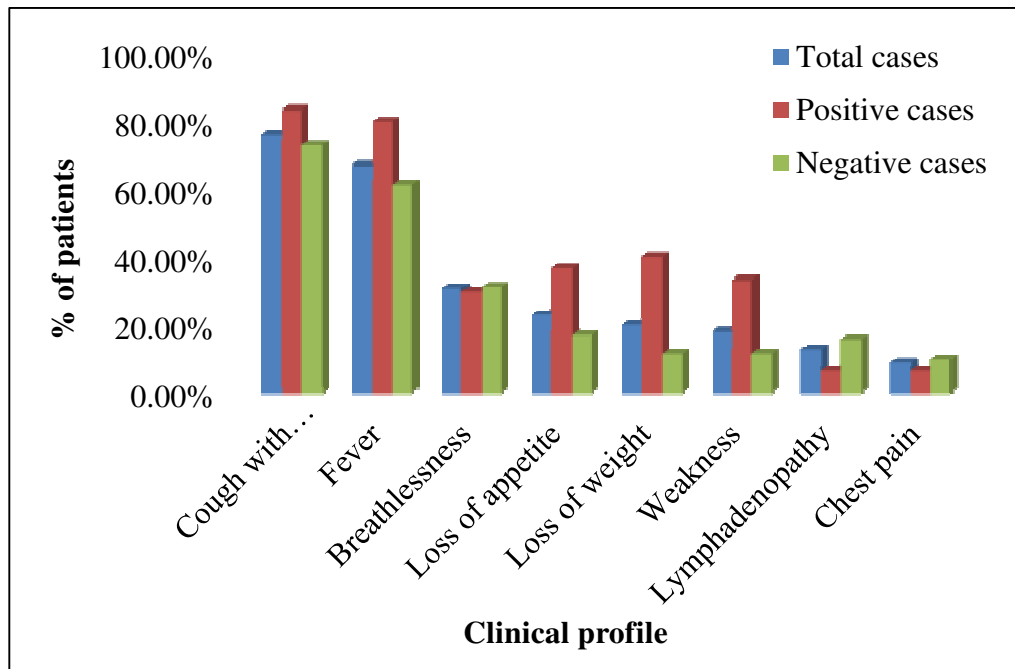
microscopy and 30% cases were positive by Auramine O stain using fluorescence microscopy. Maximum sputum smears positive (36.6%) cases were in the age group of 31 to 40 years followed by 41 to 50 years (26%) with male predominance (75%) as shown in table 2.

**Table 2:** Demographic profile of positive/negative cases

Demographic data		Total cases (n=100)	Positive Cases (n=30)	Negative Cases (n=70)
Age Group (Years)	< 10	01 (01.0%)	Nil	01 (01.4%)
	11-20	07 (07.0%)	01 (03.4%)	06 (08.6%)
	21-30	18 (18.0%)	05 (16.7%)	13 (18.6%)
	31-40	33 (33.0%)	11 (36.7%)	22 (31.4%)
	41-50	17 (17.0%)	08 (26.0%)	09 (12.9%)
	51-60	10 (10.0%)	01 (03.4%)	09 (12.9%)
	61-70	11 (11.0%)	03 (10.0%)	08 (11.4%)
	>71	03 (03.0%)	01 (03.4%)	02 (02.8%)
Gender	Male	73 (73%)	25 (75%)	48 (68.57%)
	Female	27 (27%)	05 (25%)	22 (31.42%)
	Ratio (M:F)	2.7:1	5:1	2.1:1

Among 100 clinically suspected cases of pulmonary tuberculosis as well as among 30 sputum smear positive cases, cough with

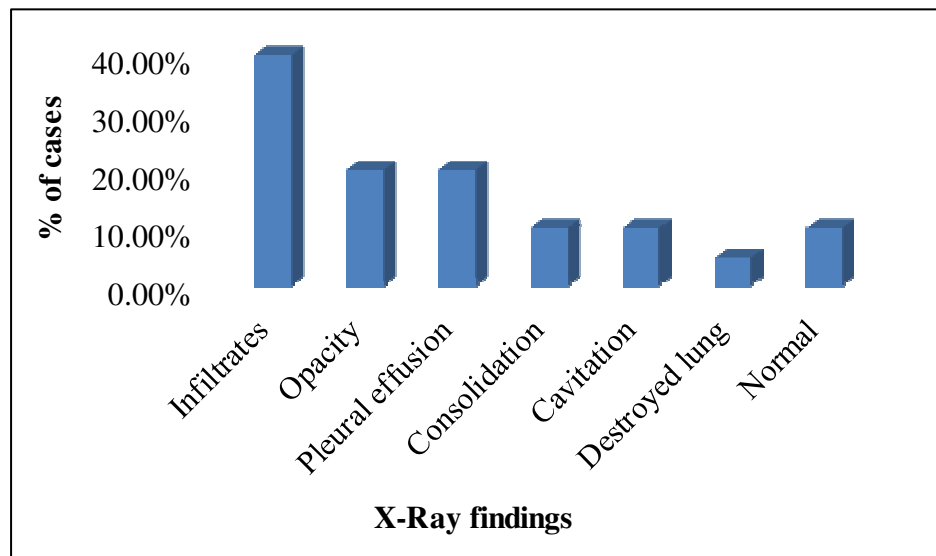
expectoration with fever was the commonest clinical feature observed as depicted in figure 1.



**Figure 1:** Distribution of cases as per clinical features

Among 30 smear-positive cases, an x-ray was available in 20 cases. The commonest x-ray finding was infiltration which was noted in 40% cases followed by opacity and pleural

effusion in 20% cases consolidation and cavitation in 10% cases as shown in figure 2. Fibrosis and military shadow was not observed.



**Figure 2:** Showing X-ray findings in sputum smear positive cases (n=20)

Haematological parameters in positive cases showed mean hemoglobin 9.64 gm% with maximum cases (76.7%) in the range between 8.1 to 12 gm%. The mean total leukocyte count was 9556/cumm. Maximum cases (56.7%) were in the range of 7100 to

11000/cumm. The mean erythrocyte sedimentation rate was 38.7 mm at the end of the first hour. 46.6% of cases between the range of 31 to 40 mm and 13.3% showed ESR > 50 mm at the end of the first hour by Wintrobe's method, (Table 3).

**Table 3:** Haematological parameters in sputum smear positive cases (n=30)

Parameters	Range	No. of cases	Percentage
Hb (Mean 9.64 gm%)	6.1-8	06	20.0
	8.1-10	12	40.0
	10.1-12	11	36.6
	>12	01	03.3
ESR (Mean 38.7 mm at the end of 1 <sup>st</sup> hr.)	<10	Nil	Nil
	10--20	01	03.3
	21-30	05	16.6
	31-40	14	46.6
	41-50	06	20.0
	>50	04	13.3
TLC (Mean 9556 /cmm)	3-7	07	23.3
	7.1-11	17	56.7
	11.1-14	01	03.3
	>14	05	16.7

Out of 100 cases graded on Ziehl-Neelsen staining showed 3+ grading in 10 cases, 2+ grading in 6 cases, 1+ grading in 11, cases and only one case was graded scanty. 72% cases were sputum smear negative. Out

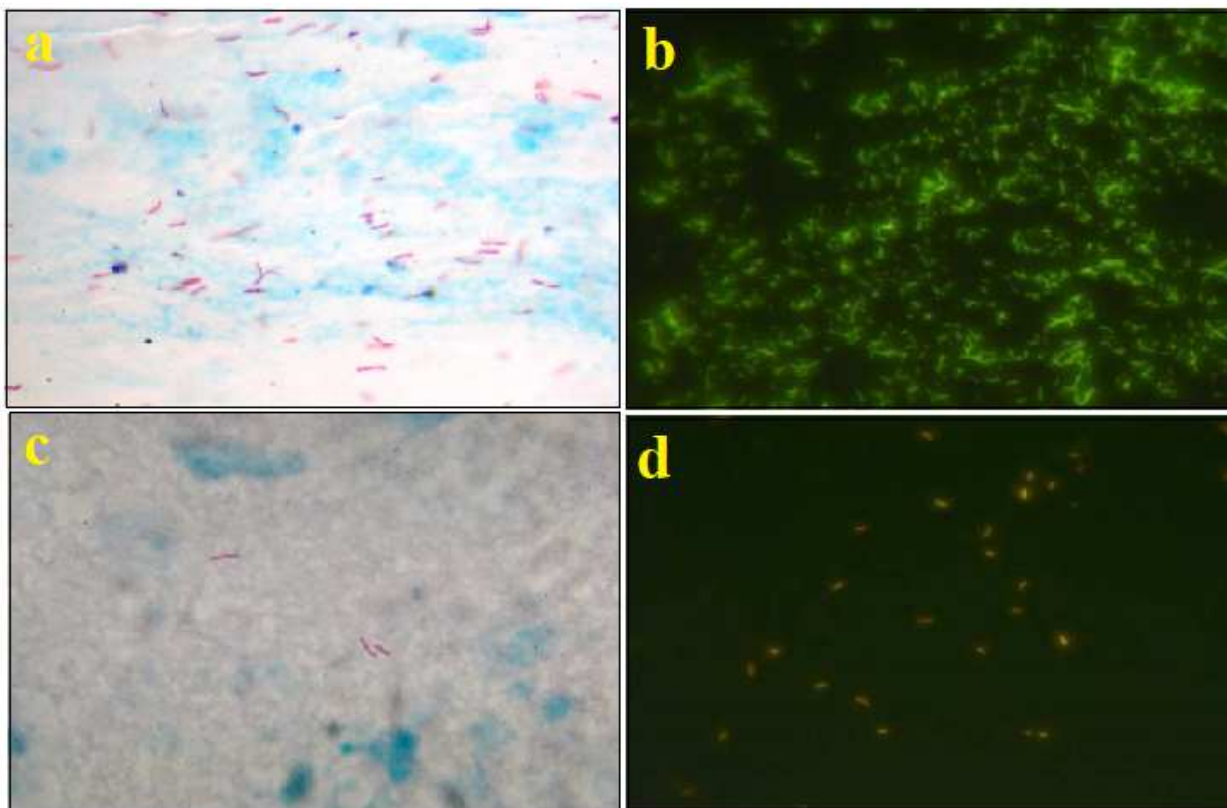
of 100 cases graded on Auramine O staining showed 3+ grading in 11 cases, 2+ grading in 5 cases and 1+ grading in 14 cases. 70% cases were sputum smear negative, (Table 4).

**Table 4:** Grading on sputum smear examination

Stain	Grading					Total
	Negative	Positive				
		3+	2+	1+	Scanty	
Zn (%)	72	10	06	11	01	100
AO (%)	70	11	05	14	Nil	100

Statistical analysis cannot be applied to the data in the present study as the difference in positivity (2%) and difference in grading

was too small as compared to sample size in other studies.



**Figure 3:** a) ZN Stain showing afb positive (3+); b) Auramine O stain showing AFB positive (3+); c) ZN showing AFB positive (scanty); d) Auramine O showing AFB positive (1+)

## DISCUSSION

Tuberculosis is a disease of the respiratory system, caused by *Mycobacterium tuberculosis*. It has a great impact on morbidity and mortality in the developing world. The diagnosis of tuberculosis is based on poorly validated symptom-based algorithms, very often resulting in a non-definitive diagnosis. Despite the introduction of various diagnostic procedures, microscopic examination of *mycobacterium tuberculosis* still continues to be an essential, cost effective diagnostic modality which helps in establishing the diagnosis and identifying tuberculosis infected patients. It also helps in grading of the disease depending upon the bacillary load [10]. Since the early 1940's, there has been a lot of comparison of the

fluorescent method with the conventional ZN method, this was implemented to improve the smear positivity for the detection of AFB. The use of a fluorochrome acid-fast stain, such as AO, is highly recommended because of its increased sensitivity and ease of interpretation when compared to that of the ZN method [11]. In the present study males were preponderance over female with male to female ratio 2.7:1. Previous studies also showed male preponderance with male to female ratio ranges from 1.85:1 to 2.7:1 [5, 12, and 13]. Out of 30 sputum smear positive cases maximum cases were found in the age group of 31 to 40 years (36.6%) followed by 41-50 years (26.7%) which is correlated with the findings showed by Chakraborty [14] and Rao S [15]. Present study also showed cough with

expectoration and fever the most common presenting feature which in according with previous studies [16, 17].

Out of 30 sputum smear positive cases, X-rays were available in 20 cases. The common X-ray finding was infiltration which was observed in 8 cases (40%) followed by opacity and pleural effusion in 4 cases (20%) each. Consolidation and cavitation were observed in 2 case (10%) each. However, fibrosis and military shadow were not found in current study. In 2 cases (10%) x-ray findings were within normal limit. These findings are in accordance with previous studies [16-18]. The mean haemoglobin level was 9.64 gm% in sputum smear positive cases, with range from 6.1 to 12 gm%. Maximum cases were observed in the range of haemoglobin level from 8.1 to 12 (76.7%). Mean total leucocyte count (TLC) was 9556 /cumm with maximum cases in the range from 7100 to 11000 /cumm (56.7%). Seven cases (23.3%) were in the range 3000 to 7000 /cumm 5 cases (16.7%) TLC count was more than 14000 /cumm. Mean erythrocyte sedimentation rate (ESR) in was 38.7 mm at the end of first hour. Maximum (46.6%) were in the range of 31 to 40 mm. 6 cases (20%) showed ESR in the range of 41 to 50 mm, 5 cases (16.6%) were between 21 to 30 mm and 4 cases (13.3%) showed ESR more than 50 mm at the end of first hour. These findings are correlated with the study conducted by Aziz et al [16] and Kaur et al [17]. In the current study, anaemia was present in most of the patients with haemoglobin level range between 6.1 to 11 gm%. Also raised erythrocyte sedimentation rate was 80% showed ESR in the range 30 to 60 mm at the end of first hour 20% of patients showed ESR in the range of 10 to 30 mm.

Out of total 100 suspected cases of pulmonary tuberculosis 28 cases (28%) were positive by Ziehl-Neelsen stain and 30 (30%) were positive by AO stain. Two more cases were detected by AO stain. The difference of 2 cases (2%) positivity by fluorescence

microscopy can be comparable with the findings of Kumar et al [19]. The current study showed difference in grading in four cases (2 cases negative on ZN but grade 1+ on Auramine O. One cases were scanty on ZN but 1+ with Auramine O. One case 2+ on ZN showed 3+ on Auramine O) with higher grades of positivity showed by Auramine O stain using fluorescent microscopy.

**Case No. 39:** Male 68 years presented with low grade fever, chest pain and loss of appetite. He was a known case of hypertension. His x-ray findings showed bilateral extensive flappy opacities. Clinical features and x-ray findings were suggestive of tuberculosis however Ziehl-Neelsen stain was negative. The case was considered as sputum smear negative pulmonary tuberculosis. Auramine O stain showed grade 1 positivity.

**Case No. 75:** Male 36 years presented with low grade fever and generalised weakness. He was a known case of HIV. His x-ray findings showed infiltrates in both the lungs. Ziehl-Neelsen stain was negative. Auramine O stain showed grade 1 positivity. The case was considered as sputum smear negative pulmonary tuberculosis. Auramine O stain showed grade 1 positivity.

Though the sample size was less in present study, there is a definite increase in the number of positive cases with the use of fluorescence microscopy using Auramine O. Our findings support previous studies that demonstrated the superior diagnostic performance of fluorescence microscopy compared with conventional microscopy. From public health point of view, a single active case missed is a public health threat. Early diagnosis of tuberculosis and initiating optimal treatment would not only enable cure of an individual patient but will also curb the transmission of infection and disease to others in the community. Of the several distinct components of tuberculosis control programme case finding remains the cornerstone for effective control of disease.

**CONCLUSION**

Fluorescence microscopy is more superior in detecting sputum smear positive cases over Ziehl-Neelsen stain. Fluorescence microscopy should be adopted in tertiary secondary or even comprehensive health centres for detection of suspected cases of tuberculosis for better management of the disease. This would go a long way in reducing the burden and consequences of the disease.

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