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Original Research Article

# Comparision of pap stained smears by using auto fluorescence method with ziehl-neelsen method for detection of acid fast bacilli (AFB)

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ABSTRACT

**Introduction:** The Ziehl-Neelsen staining method though it plays an important role in detecting acid fast bacilli (AFB) by direct microscopy its low sensitivity makes to look for the other alternatives.
**Aims & Objectives**: This study was undertaken to evaluate the utility of auto fluorescence method on Papanicolaou stain (Pap) in comparison tothe traditional Ziehl-Neelsen stain (ZN) method in detection of AFB from lymph node aspirates.
**Materials and Methods:** Fine needle aspirations (FNAs) were done in 153 patients with clinical suspicion of tubercular lymphadenitis. Smears from aspirate were processed for routine cytology for Hematoxylin & Eosin (H&E), Giemsa, Pap, Ziehl-Neelsen (ZN) and Auramine (AR) staining. Pap stained smears were examined for AFB for their autofluorescence under fluorescent microscope using the blue excitation filter. ZN stained smears are examined for AFB under oil immersion of compound microscope. The efficacy of the autofluorescence method by Pap in detection of acid fast bacilli (AFB) over the conventional Ziehl-Neelsen stain was analyzed by taking AR as a standard control for AFB.
**Results:** Tuberculous lymphadenitis was diagnosed in 36 cases out of 153 clinicall suspected cases by cytology, culture and microscopy**.** The sensitivity of ZN was 57.14% and specificity was 99.15% while the sensitivity of AF was 96.5% and the specificity was 91.8%.
**Conclusion**: It was a novel method of detection of AFB, cheaper, easily available and less time consuming than other methods. AR also had high sensitivity rates as AF.



## 1. Introduction

Tuberculosis is a major health problem affecting mankind in developing countries and is Mycobacterium tuberculosis.[1] It is an airborne disease and most commonly affects lungs. It has currently affected over one third of the world's population and more than 1.5 million deaths every year. Extra pulmonary involvement is seen in 15 to 20% of cases. The most common presentation of extra pulmonary tuberculosis is lymphadenopathy.[1–4] The specific cause of peripheral lymphadenopathy is often challenging to obtain with history, physical examination, and radiographic studies alone. Fine needle aspiration cytology (FNAC) has got a key role in evaluation of these cases as a possible alternative to excisional biopsy.[1]

The demonstration of Mycobacterium tuberculosis in FNAC smear is most commonly done using Ziehl-Neelsen (ZN) staining, but it has low sensitivity and the screening is cumbersome. On the other hand Fluorescent microscopy with fluorochrome dyes has high sensitivity and specificity but is more expensive. Culture is considered as gold standard in diagnosing tuberculosis but it is time consuming, on an average it may take 3 to 6 weeks to get the report.[3]

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Hence as per the need, the present study is undertaken to correlate autofluorescence method on Papanicolaou stain with conventional Ziehl-Neelsen stain method in detection of AFB in lymph node aspirates as it is simple, rapid, cost effective & most frequently utilized stain for cytological specimens.

## 2. Materials and Methods

Patients of both OPD and IPD, referred to the department of Pathology in Maheswara medical college, Patancheruvu, for fine needle aspiration (FNAC) of lymph node from suspected cases of tuberculosis were included in the study. The study was conducted between November 2019 to April 2021. The study was initiated after obtaining Institute's ethics committee approval.

### *2.1. Inclusion criteria*

1. All the patients clinically diagnosed of TB lymphadenitis referred for FNAC were included in the study.

### *2.2. Exclusion criteria*

1. The patients already diagnosed with TB.
2. Patients already on antitubercular therapy.
3. Patients having small non palpable lymph nodes, skin diseases were excluded from the study.

A prospective study of 153 samples of suspected cases of tuberculosis satisfying the inclusion and exclusion criteria belonging to all age groups referred to the Pathology department were included in the study

A detailed history of patients was elicited and complete general physical examination and systemic review of the patients was undertaken. Informed consent was taken from the patients for the FNAC procedure. A 23-gauge needle and a 10-ml disposable syringe were used.

Tuberculous lymphadenitis was diagnosed based on the cytomorphological features of FNA smears. Cytomorphological criteria.

Reactive lymphadenitis - mixed population of lymphoid cells with scattered histiocytes with intracytoplasmic nuclear debris.

Suppurative lymphadenitis - lymphoid cells with numerous intact and degenerated polymorphs against a necrotic debris background.

Granulomatous lymphadenitis - epithelioid cell granulomas with or without the presence of Langhans' multinucleated giant cells and caseous necrosis. [Figures 1, 2 and 3]

Cytological patterns in cases diagnosed as tuberculous lymphadenitis were (1) epithelioid granuloma with extensive necrosis, (2) epithelioid cell granulomas only, and (3) necrosis only.[5,6]

Following the procedure, 5–6 smears were prepared on clean glass slides. These Smears were subjected to H&E, Giemsa, Pap, Ziehl-Neelsen (ZN) and Auramine stains. ZN stained smears were examined for AFB under oil immersion of compound microscope and Auramine stained smears were scanned under fluorescence microscope. Culture was taken as a gold standard method. Contaminated cultures were excluded from the study.

### *2.3. Statistical analysis*

The data was analysed using Microsoft Xcel. Sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) were obtained for each method, using culture as the reference. Sensitivity, specificity, PPV, and NPV were calculated according to the following formulae: Sensitivity = (a/[a+c])×100; specificity = (d/[b+d])×100; PPV = (a/[a+b])×100; and NPV = (d/[c+d])×100; where a is the number of true positives, b is the number of false positives, c is the number of false negatives, and d is the number of true negative samples.[7]

## 3. Results

153 Patients clinically suspected of having tuberculosis with lymphadenopathy were included in the study. Predominant age group involved in this study was 21-30 years (23.5%) followed by 11-20 years (20.9%). Both sexes were equally affected. Females were 78 & males 75. About 60.5% of the cases presented with lymphadenopathy of less than 3 months duration. Lymphadenopathy predominantly involved cervical region in 109 (71%) cases followed by Sub-mandibular in 31 (20%) cases, supra-clavicular in 8(5%) and axillary in 5 (2%) cases. Nature of the aspirate was hemorrhagic in 118 (77.77%) cases. Out of 153 cases 54 (35.29%) were diagnosed as TB lymphadenitis based on cytomorphological features. Among these 54 cases culture positivity was observed in 36 cases (66.66%). So further analysis was made for these 36. Most common cytomorphological pattern encountered in this study was necrotizing lymphadenitis 19 (67.8%). Overall AFB positivity was seen in 16 cases (8.7%) by ZN method. Based on number of bacilli per high power field grading was done on for both ZN stained and Pap stained smear (AF). This grading was done depending on number of bacilli per high power field (Table 1).

### *3.1. Z-N staining results*

AFB was seen as bright red rods against a blue background. They were straight to slightly curved, beaded rods, measuring approximately 0.5 cm × 3 cm, occurring singly, in pairs, and as small groups. Out of the 36 TB lymhadenitis cases AFB positivity was seen in 16 (44.4%)

### 3.2. Pap staining results

Fluorescent bacilli appeared as slender, often beaded, yellow green, straight or slightly curved rods against a dark background. (Figure 5). Out of 36, 28 (77.7%) were positive by this method.

AFB positive and AR negative was seen in 2 cases and AR positive and AFB Negative in 12 cases.

Highest positive cases of AFB was seen in necrotizing lymphadenitis (Table 2). The sensitivity of AF method was 96.5% where as ZN was only 57.14% (Table 3). Thus the AF method for detection of AFB in lymph node aspirate was more sensitive than conventional ZN method. Moreover, the method was safe, inexpensive and easy to perform and requires no additional staining.

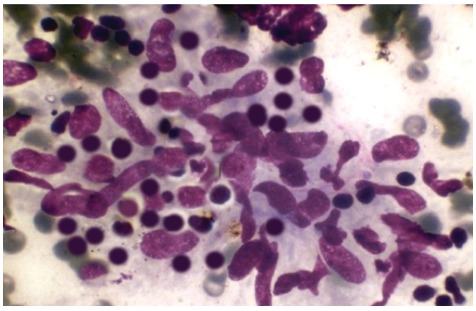

**Figure 1:** Microphotograph showing features of Granulomatous lymphadenitis (PAP, 400X)

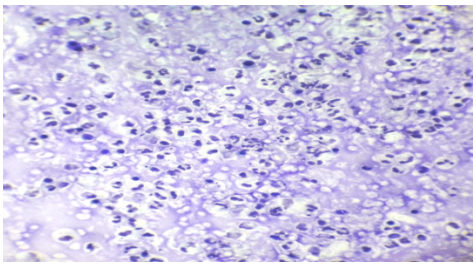

**Figure 2:** Microphotograph showing features of Necrotizing lymphadenitis (PAP 100X)

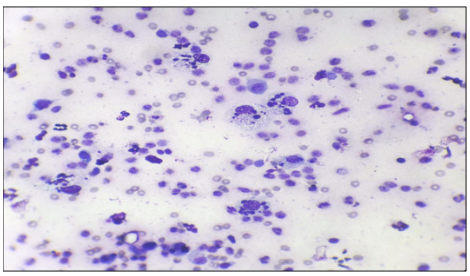

**Figure 3:** Microphotograph showing features of acute suppurative lymphadenitis (PAP, 100X)

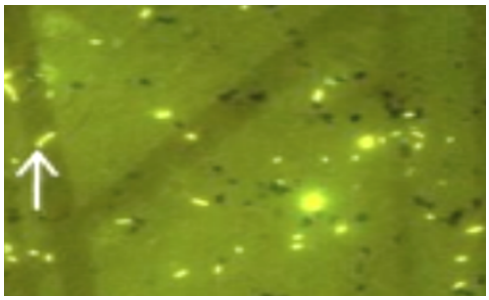

**Figure 4:** Microphotograph showing AFB under fluorescence microscope by AR method (400X)

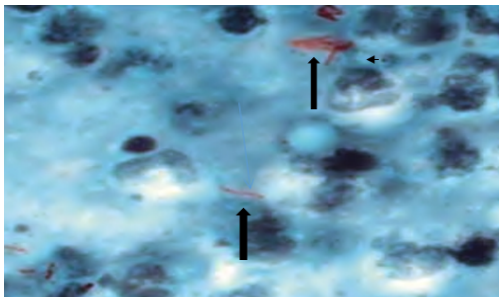

**Figure 5:** Microphotograph showing AFB by conventional method (ZN Stain, 1000x)

**Table 1:** Showing grading of acid fast bacilli based on number of AFB /100 HPF.

| No. of AFB seen | Grade | Minimum number of fields to be examined |
|---|---|---|
| >10 per oil immersion field | 3+ | 20 |
| 1-10 per oil immersion field | 2+ | 50 |
| 10-99 in 100 oil immersion field | 1+ | 100 |
| 1-9 in 100 oil immersion field | Scanty | 200 |

**Table 2:** Cytomorphological patterns in various TB Lymph node aspirates

| Cytomorphological pattern | ZN positive | AF positive |
|---|---|---|
| Necrotizing Lymphadenitis | 10 (62.5%) | 19(67.8%) |
| Granulomatous Lymphadenitis | 05(31.25%) | 06(21.42%) |
| Acute suppurative | 01(6.25%) | 03(10.71%) |

**Table 3:** Taking culture as a reference method, analysis of statistical values of ZN and AF.

| | ZN | AF |
|---|---|---|
| **Sensitivity** | 57.14% | 96.50% |
| **Specificity** | 99.15% | 91.79% |
| **PPV** | 93.75% | 57.00% |
| **NPV** | 91.02% | 99.71% |
| **Accuracy** | 85.62% | 69.28% |

*3.2.1. Fluorescent stain*

Sensitivity = true positive=28/28+1(negative on culture)x100=96.5%

Specificity=true negative=125/137x100=91%

Positive predictive value=16/28x100=57%

Negative predictive value=125/125x 100=100%

*3.2.2. ZN stain*

Sensitivity=True positive-16/28x100=57.14%

Specificity=True negative=125/125x100=100%

Positive predictive value=16/16x100=100%

Negative predictive value=125/137x100=91.2%

**Table 4:** Comparison of the results of this study with previous studies 2, 5, 14

| Autofluorescence study | Parul joshi et al [8] | Wright et al. [2] | Wright et al. [5] |
|---|---|---|---|
| Sensitivity 96.5% | 95% | 65.9% | 67% |
| Specificity 91.8% | 81.81% | 73% | 97% |
| Negative predictive value 99.7% | 94.7% | 61.7% | 66% |
| Positive predictive value 57% | 82.6% | 76.5% | 97% |

## 4. Discussion

TB has troubled mankind since ages and continues to persist as a major health problem especially in developing countries like India.

TB lymphadenitis is the most common type of manifestation of extra pulmonary TB. Diagnosis of TB is easy when the disease is extravagant but often difficult if there was a involvement of extra pulmonary organs where establishing the diagnosis is really challenging.

Diagnostic modalities must be tailored to population and epidemiology of TB in that region, which includes microscopy, culture, new modalities like AF in diagnosis of extrapulmonary TB, chemical and physical detection of mycobacterial antigens in paucibacillary condition, antigen capture, antibody detection, cellular immune recognition, nucleic acid amplification and phage assay.

In developing countries, the diagnosis of TB is made on symptoms based algorithms. The diagnosis of TB by cytomorphology is not a new entity. Before, the diagnosis was made based on the patterns Granulomatous, Caseous necrosis, Langhan's type of giant cells. But several viral infections, bacterial infections will also present with the similar cytomorphological patterns. So laboratory test plays an important role to establish the cause of such lymphadenopathy.

Autofluorescence is simple, sensitive, and inexpensive, but is not widely used. It allows the detection of tubercle bacilli without the need of hazardous and costly fluorochrome dyes.[9] It requires a fluorescent microscope, which are now available in most routine laboratories. However, in the past, it has been reported not to be highly specific, as the specificity is dependent on the experience of the operator, and as in ZN staining, it cannot differentiate between the various Mycobacterium.

UV-induced fluorescence as a diagnostic modality for detection of pathogens was first described by Graham (1983), and Mann (1983).[10,11] Ghali et al. (1984), first demonstrated autofluorescence of Pneumocystis cariniiin Pap stained smears. Their study showed eosin to be responsible for the autofluorescence. The main components of Pap stain being OG-6, EA-65 or EA-50. (cytoplasmic stains).[12] Kupper et al. in 1995, demonstrated the autoflourescence of MTB which can be easily differentiated from other mycobacteria and non-mycobacterial species.[13]

The ZN stain was the most common diagnostic modality used to detect AFB in lymphadenitis because of its less cost and easy availability. The low sensitivity is the major disadvantage of this method. Other molecular techniques such as Polymerase chain reaction, are not used routinely in developing countries because of the cost, infrastructure and skilled personnel. Though the sensitivity of Fluorescent microscopy using dyes like Auramine is high but these are carcinogenic and toxic. But AR is superior than ZN stain in detection of AFB because on ZN Stained smears it is very hard to identify AFB on low bacterial load smears and it takes lot of time to search bacilli on high power field (100x). So in search of new modalities Autofluorescence for detection of AFB was explored. This technique was simple, fast and cost effective especially in developing countries like India. The AFB typically fluoresce as green, slender, rod-shaped bacilli.[14] Other bacteria, such as Nocardia, can also exhibit this feature.

Many similar studies were done on Lymph node aspirate to know the accuracy of ZN over AF. Most of them showed similar statistical values of ZN and AF as in the present study and it was noted that the AF stain had high sensitivity 96.5%, and high NPV 99.7%. (Table 4)

The most common pattern encountered in the present study was Granulomatous lymphadenitis while Caseating necrosis was the most common pattern observed in study conducted by Brijesh et al. It may be due to regional differences. In comparison with other studies on FNA smears of lymph node aspirates, AF was more sensitive than ZN in terms of staining, but in comparison with cytodiagnosis, it was less sensitive.[8]

Many studies which were conducted for detection of AFB from various specimens, like sputum, CSF, FNA, pus, and other body fluids which were examined by ZN and AR stains also, showed similar results that AR was more sensitive as compared to ZN stain.

In the present study we had made a comparison between AF and AR stains. It was found that AF was more superior than AR. Similar results were found in the study conducted

by Brijesh et al.[1] But 11 cases of AF positive were found AR negative. However, there are very little availability of resources in literature on Comparison between AF and AR stains.

Organisms such as B.Subtilis, S.aureus, Nocardia may produce flourescene. Even air drying artifacts or stain precipitates on PAP stained smears may mimic Mycobacteria.[15,16] There is increased rate of false positivity in AF due to intraobserver variability.

## 5. Conclusion

In conclusion, this study was done to evaluate the utility of fluorescence microscopy by using normal PAP stained smears via AF method which showed high sensitivity rates. It was a novel method of detection of AFB, which is cheaper, easily available and less time consuming than the other methods. AR also had high sensitivity rates as AF but the Auramine dye which was used in this method has carcinogenic properties.

## 6. Source of Funding

None.

## 7. Conflict of Interest

None.

## 8. Acknowledgement

None.

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