# Comparison of Fluorescent and Ziehl-Neelsen Staining in Fine Needle Aspirates of Tuberculous Lymphadenitis

**Akash Bhat, [1] M Jayaprakash [2]**

1. Intern, Father Muller Medical College, Father Muller Road, Kankanady, Mangalore, Karnataka 575002
2. Professor, Dept of Pathology, Father Muller Medical College, Father Muller Road, Kankanady, Mangalore, Karnataka

## Abstract

*Background: Tuberculosis remains a global health problem with an enormous burden of disease. Tuberculous lymphadenitis being the most common extra pulmonary manifestation 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. Aims: The aims of our study was to compare fluorescence staining with Auramine-O with the conventional Ziehl-Neelsen (ZN) stain for detecting tubercle bacilli in tuberculous lymph nodes by Fine Needle Aspiration Cytology (FNAC) and study the efficacy and advantages of using the Auramine-O stain on lymph node aspirates under fluorescent microscopy. Methods: We performed a prospective study of sixty-six patients clinically suspected to have tuberculous lymphadenitis from April 2016 to October 2016 at a tertiary care teaching hospital. Clinical and demographic data were collected. FNAC samples were obtained and stained with Papanicolaou and May Grunwald Giemsa stain to assess cytomorphological features. Staining of samples with ZN stain and AO stain was done to identify tubercle bacilli and compare the same. Results: Positive staining was noted in 2.7 % cases with AO stain and 16.7 % with ZN stain. 10.6% cases showed lymphocytosis. Overall sensitivity, specificity and PPV of IF in comparison to ZN stain was 45.45%, 81.82%, 33.33% respectively. Overall sensitivity, specificity and PPV of ZN in comparison to IF staining were 33.33%, 88.8%, and 45.45%, respectively. Conclusions: IF staining is a more effective, easy and rapid alternative to ZN staining is detecting TB bacilli through FNAC, especially in paucibacillary cases.*

*Keywords: Tuberculosis, tubercular lymphadenitis, fine needle aspiration cytology, Ziehl-Neelson stain, Auramine-o Stain.*

**Address for correspondence:** Dr. Akash Bhat. Intern, Father Muller Medical College, Father Muller Road, Kankanady, Mangalore, Karnataka 575002. Email: akashbhat.m@gmail.com

## Introduction

Tuberculosis (TB) is said to be one of the most infectious diseases in the world, caused by a bacillus, Mycobacterium tuberculosis, which is known for its property of being acid fast. The year 2014 witnessed an estimated 9.6 million new TB cases. Deaths caused by TB are unacceptably high. However, with timely diagnosis and proper treatment, almost all patients with TB can be cured.[1]

Tuberculosis primarily and typically affects the lungs and causes pulmonary tuberculosis. Extra-pulmonary TB affects commonly the lymph nodes but other structures like, intestine, skin, bones, joints, meninges etc., can also be affected. Lymph nodes in tubercular lymphadenitis have been classically described as multiple, matted and hard with draining sinuses. Cervical nodes are the most commonly affected nodes in tuberculous lymphadenitis, accounting for approximately 63% of cases.

***The stages of tubercular lymphadenitis are as follows:***
1. Lymphadenitis
2. Periadenitis
3. Cold abscess
4. 'Collar stud' abscess
5. Sinus

Typically, most of the cases of tubercular lymphadenitis are seen in the stages 1 and 2. The involved lymph nodes may be discrete and firm[2].

Fine-needle aspiration cytology is now widely utilized, and is a first line diagnostic procedure in the diagnosis of many palpable masses, including enlarged peripheral lymph nodes. Even though laboratory parameters in conjunction with clinical features can suggest the diagnosis, these features are non-specific and can be seen in other conditions as well[2]. Fine needle aspiration cytology (FNAC) can suggest a diagnosis of tuberculosis in the presence of granulomas, Langhan's giant cells and caseous necrosis. However, confirmatory evidence is provided by the detection of tubercle bacilli, as granulomatous lymphadenitis can be caused by a variety of conditions.

Ziehl-Neelsen stain is commonly used to detect the acid-fast tubercle bacilli. It is one of the most commonly used methods around the world, especially in developing countries, due to its simplicity and cost effectiveness. It is a Carbol-fuchsin based staining technique, using the three component hot process and is used as a standard staining technique for the detection of Mycobacterium tuberculosis bacilli. Though excellent results are yielded by the technique, it is a method that requires a considerable amount of skill and judgement. Modification of the traditional ZN stain such as the three component Kinyoun stain (ZN-cold method) has been developed to cut short the 'Processing Time' and to increase the Laboratory productivity. In the year 1917 Kaiserling was the first person to observe the spontaneous fluorescence of a suspension of human tubercle bacilli. Kuper and May[3] added rhodamine to auramine fluorescence staining for acid-fast organisms in tissue sections and found that it gave better contrast and appreciation of bacilli. WHO manual of Laboratory services in Tuberculosis Control gives a method for quantification of acid-fast bacilli observed by Fluorescent Microscopy so as to obtain a grading comparable to that obtained under a magnification of 1000x after staining with carbolfuschin. This comparative grading method has been adopted by the RNTCP.[4] Fluorescence staining has better sensitivity and has the advantage of speed and ease of screening and less observer fatigue. The fluorescent method is more advantageous than the conventional ZN method, particularly in pauci-bacillary cases. This study was done to compare the efficacy of fluorescence microscopy by Auramine-O staining with that of conventional Ziehl-Neelsen staining in detection of M. tuberculosis in lymph node aspirates is suspected cases of tuberculosis.

# Materials and Methods

It was a prospective study conducted at Department of pathology, Father Muller medical college from April to October 2016 to compare fluorescence staining with Auramine-O with the conventional ZN stain for detecting tubercle bacilli in lymph node FNAC and study the efficacy and advantages of using the Auramine-O stain on lymph node aspirates under fluorescent microscopy.

### Inclusion Criteria
All cervical lymph node aspirate samples from cases suspected to have TB in the study period.

### Exclusion Criteria
1. FNAC samples that were not suspected TB cases and not submitted for AR stain.
2. Cases where in enough material was unavailable for both ZN and fluorescence stain were excluded from the study

Demographic data and clinical details of the patient, diagnosis were noted. FNAC was done from suspected cases of tuberculosis by a standard protocol and under aseptic precautions using 23g needle. The aspirated material was spread over glass slides after proper labelling of the slides. Minimum of two passes were done and four slides were prepared (2 wet & 2 air dried). The unstained slides were kept aside. Wet smears were stained with Papanicolaou (PAP) staining and the air dried smears were stained with May Grunwald Giemsa (MGG) staining and observed under light microscope to note cytological morphological features. Ziehl-

Neelson (ZN) stain and Auramine-O (AO)stain were done to detect presence of AFB. ZN stained smears were examined under oil immersion of a light microscope, Olympus CX21 with 1000x magnification. The AO stained smears were examined using blue light under Leica DM1000 (LED) microscope with Leica microsystems' filter cubes and Osram HBO short arc mercury lamp after blinding. Slides were screened using 10x objective. Bacilli were confirmed by the 40x objective. Presence or absence of AFB was noted in both the tests. Statistical Analysis: The obtained data was tabulated and analyzed for age and sex ratios, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) by Chi square test.

# Results

66FNAC samples were studied. Four slides were prepared from all the samples and each was stained with PAP, MGG, ZN and AO stain respectively. PAP and MGG smears were done to study the cyto-morphological features. ZN stained smears were studied under light microscope and AR stained smears were studied under fluorescent microscope. In ZN stained smears the TB bacilli were seen as red rods under light microscope and in AO stained preparation the bacilli appeared yellow-green rods when viewed under fluorescent microscope, as seen in Figs 1 and 2.

Of the 66 cases, there was slight female preponderance as shown in Figure 3.

The age of the patients ranged from 7-years to 72-years. Most of the patients belonged to the age group of 21-40years, as seen in Figure 4. This was also observed in male and female subgroups.

As seen in table 1, various presenting symptoms such as fever, cachexia, cough, night sweats, loss of appetite and fatigue were noted. Fatigue and fever were the most common presenting symptoms.

22.7% cases stained positive with immunofluorescence staining, 16.7% with ZN staining and 10.6% cases showed lymphocytosis, as seen in table 2.

Table 3 shows that 5 cases were positive by both ZN and immunofluorescence methods. Ten cases were positive on immunofluorescence, but negative on ZN staining, while 6 cases were

positive on ZN staining, but negative on immunofluorescence.

Sensitivity, specificity, PPV, NPV and overall accuracy of both the stains has been shown in table 4. Immunofluorescence was more sensitive, while ZN staining was found to be more specific. Likewise, the positive predictive value for ZN staining was higher, while immunofluorescence had a higher negative predictive value.

**Table 1**: +ve & -ve cases with presenting symptoms

|  | -ve | | +ve | | Total | |
| --- | --- | --- | --- | --- | --- | --- |
|  | Count | % | Count | % | Count | % |
| Fever | 44 | 66.7 | 22 | 33.3 | 66 | 100 |
| Cachexia | 54 | 81.8 | 12 | 18.2 | 66 | 100 |
| Cough | 57 | 86.4 | 9 | 13.6 | 66 | 100 |
| Night Sweats | 62 | 93.9 | 4 | 6.1 | 66 | 100 |
| Loss of Appetite | 51 | 77.3 | 15 | 22.7 | 66 | 100 |
| Fatigue | 28 | 42.4 | 38 | 57.6 | 66 | 100 |

**Table 2**: +ve &-ve cases of IF staining, ZN staining.

|  | -ve | | +ve | | Total | |
| --- | --- | --- | --- | --- | --- | --- |
|  | Count | % | Count | % | Count | % |
| Immunofluorescence Staining | 51 | 77.3 | 15 | 22.7 | 66 | 100 |
| ZeihlNeelsen Staining | 55 | 83.3 | 11 | 16.7 | 66 | 100 |
| Lymphocytosis | 59 | 89.4 | 7 | 10.6 | 66 | 100 |

**Table 3**: Comparison of of+ve & -ve cases

|  |  | Ziehl Neelsen Staining | | Total |
| --- | --- | --- | --- | --- |
|  |  | +ve | -ve |  |
| IF Staining | +ve | 5 33.3% 45.5% | 10 66.7% 18.2% | 15 100.0% 22.7% |
|  | -ve | 6 11.8% 54.5% | 45 88.2% 81.8% | 51 100% 77.3% |
| Total |  | 11 16.7% 100% | 55 83.3% 100.0% | 66 100.0% 100.0% |

**Table 4**: Sensitivity, specificity, PPV, NPV

|  | IF v/s ZN | ZN v/s IF |
| --- | --- | --- |
| Sensitivity | 45.45 | 33.33 |
| Specificity | 81.82 | 88.8 |
| PPV | 33.33 | 45.45 |
| NPV | 88.24 | 81.82. |

**Table 5**: Comparison of smear positivity of both ZN staining and IF staining of our results with various other studies.
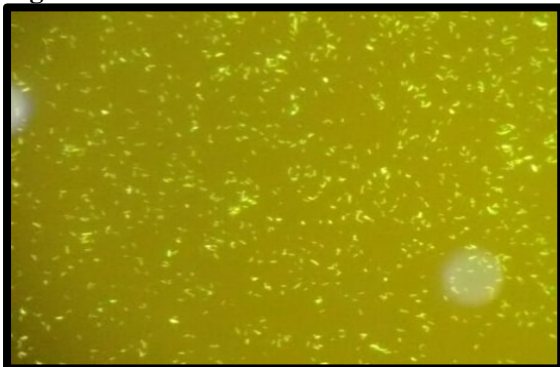
|  | Smear positivity ZN | Smear positivity IF |
| --- | --- | --- |
| Present study | 16.7% (11/66) | 22.7% (15/66) |
| Annam et al;[6] | 44.11% (45/102) | 81.37% (83/102) |
| Jain et al;[10] | 22% | 52% |
| Desai K et al;[11] | 21.42% | 14.28% |
| Prasanthi et al;[12] | 45% | 29% |

## Discussion
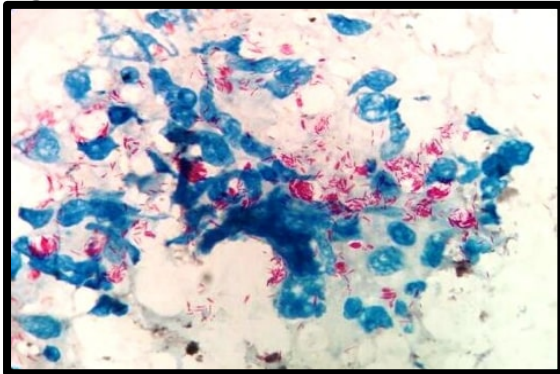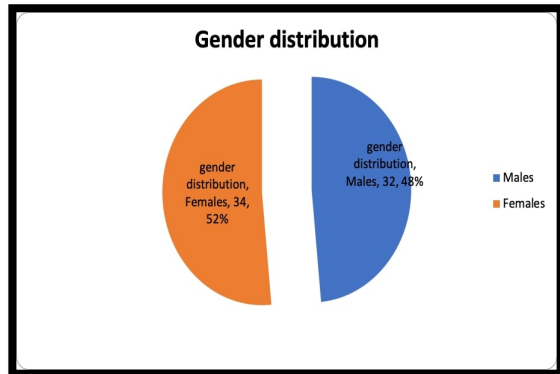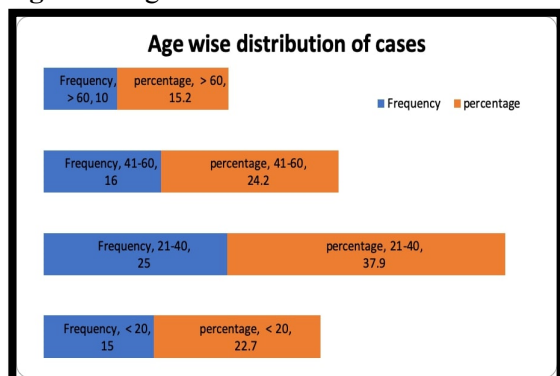
The diagnosis of tuberculosis is based on poorly validated symptom-based algorithms, very often resulting in a non-definitive diagnosis. Despite the introduction of various diagnostic procedures, microscopic examination of mycobacterium tuberculosis still continues to be an essential, cost effective 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.[5] 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 AR, is highly recommended because of its increased sensitivity and ease of interpretation when compared to that of the ZN method.[6] In our study it was found that when compared to male patients female patients were suspected more for the diagnosis of TB as shown in Figure 1. It was also found that frequency of suspected TB patients were more between the ages of 21- 40 as shown in Figure 2. Similar findings were reported in a nationwide study conducted by the ICMR in 1969 has reported increased incidence of Tuberculosis in 25-34 year age group.[7] In another study by Krishna et al; also found higher incidence of suspected cases in a similar age group (20-40 years) and most cases (60.23%) were females[8].

It was established that for IF, the sensitivity was in the range of 44 to 45%, specificity in the range of 81 to 82%, negative predictive value was in the range of 88 to 89%, and the positive predictive value was found to be in the range of 32 to 33% as shown in table 4. In a study by Akther et al;[9] they found smear positivity by ZN stain to be 26.2 % and 64.2% by fluorescence stain. Annam et al; found 44.11% of cases positive for acid fast bacilli (AFB) on the conventional ZN method, while the smear positivity increased by 81.37% on the modified fluorescent method.[6] Jain et al; found 22% positivity by ZN stain and 56% positivity by fluorescent method.[10] Another study of the 88 lymph node aspirates by Krishna et al; , the smear positivity for AFB on the ZN method was

**Figure 1**: Ziehl- Neelsen Stain



**Figure 2**: Auramine-O Stain



**Figure 3**: Sex ratio –frequency & percentage



**Figure 4**: Age wise distribution of cases.

37.5% (33/88) while the positivity increased to 81.82% (72/88) on the AR fluorescent method.[8] As shown in Table 5 itis evident that smear positivity by IF was more when compared to the smear positivity by ZN staining.

## Conclusion

This study was aimed to know the efficacy of IF staining as an easy and alternative method to detect TB bacilli when compared to ZN staining. We concluded that AO stained smears are more effective in detecting TB cases when compared to that of ZN stained smears. Fluorescence staining has better sensitivity and has the advantage of speed and ease of screening and less observer fatigue when compared with ZN staining particularly in paucibacillary cases.

## Acknowledgment

***Conflict of Interest:*** *None declared*
***Source of Support:*** *Nil*
***Ethical Permission:*** *Obtained*

## References

1. Global Tuberculosis Report 2015. WHO. 2015;https://apps.who.int/iris/handle/10665/191102 (Accessed 14 April. 2016)
2. Thakkar K, Ghaisas S, Singh M. Lymphadenopathy: Differentiation between Tuberculosis and Other Non-Tuberculosis Causes like Follicular Lymphoma. Frontiers in Public Health. 2016;4.
3. Kuper S, May J. Detection of acid-fast organisms in tissue sections by fluorescence microscopy. J Pathol 1960; 79:59–68.
4. RNTCP Manual for Sputum Smear Fluorescence Microscopy. New Delhi. Central TB Division Directorate General of Health Services.
5. Bhumbla U, Gyaneshwari. A comparative study of Ziehl-Neelsen staining and auramine staining in sputum sample for the diagnosis of pulmonary tuberculosis. IJBR. 2014; 5:383-5.
6. Annam V, Kulkarni M, Puranik R. Comparison of the modified fluorescent method and conventional Ziehl-Neelsen method in the detection of acid fast bacilli in lymph node aspirates. Cytojournal.2009; 6:13.
7. Silver H, Sonnenwirth A, Alex N. Modifications in the fluorescence microscopy technique as applied to identification of acid-fast bacilli in tissue and bacteriological material. J Clin Pathol 1966 Nov;19(6):583-8.
8. Krishna M, Kumar A. Tuberculous mycobacteria bacilli fluorescence and compare with Ziehl- Neelsen stain in fine-needle aspiration cytology of tubercular lymphnode. International Journal of Otorhinolaryngology and Head and Neck Surgery. 2016;2(2):66.
9. Akther S, Fernandes H, Goyal G. Comparison of auramine-O stain with Zeihl-Neelson stain in suspected cases of tubercular lymphadenitis. IJRTSAT. July 2015:15(3):524-5.
10. Jain A, Bhargava A, Agarwal Sk. A Comparative Study of Two Commonly Used Staining Techniques for Acid Fast Bacilli In Clinical Specimens. Ind J Tub 2002;49:161-2.
11. Desai K, Malek S, Mehtaliya C. Comparative study of ZN staining v/s fluorochrome stain from pulmonary and extrapulmonary tuberculosis. Gujarat Medical Journal 2009; 64(2): 32-34.
12. Prasanthi A. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. Indian Journal of Medical Microbiology. 2005;23(3):179.