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Original Article

Ziehl Neelsen and Auramine Rhodamine staining: A comparative evaluation from North India

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Abstract

Introduction: *Tubercular lymphadenitis can be presumptively diagnosed morphologically on fine-needle aspiration cytology of lymph node. Fine-needle aspiration cytology is now widely utilized as a first line diagnostic procedure in the diagnosis of palpable masses, including peripheral lymphadenopathy. Ziehl-Neelsen (ZN) method for acid-fast bacilli plays a key role in the diagnosis and also for the monitoring of treatment in tuberculosis. Its major disadvantage is low sensitivity ranging from 20% to 43%. Present study is to compare the efficacy of ZN staining method and fluorescent method using Auramine-Rhodamine in diagnosis of Tubercular Lymph Adenitis.*

Methods: *100 samples were taken in this hospital based observational study. Patients with palpable swelling suspected to be tubercular in nature and confirmed by cytomorphology were included. Staining was done using Ziehl Neelsen and Auramine Rhodamine staining method. Comparison was made using grade for bacilli seen under the microscope.*

Results: *Maximum number of patients sample was seen in age group of 20-30 years. Female preponderance was seen. There was 59% samples where the aspirate was taken from the cervical lymph nodes. Maximum sample were seen of caseation with epitheloid cells followed by non-caseation with epitheloid cells. Positivity of aspirate was higher in staining by Auramine Rhodamine stain as compared to Ziehl Neelsen stain which was statistically significant.*

Conclusion: *The sensitivity of Auramine Rhodamine staining is higher which can be used in paucibacillary cases and it may be used in routine cases as well.*

Keywords: *Staining, Tuberculosis, Caseation, cytology.*

Introduction

Worldwide, Tuberculosis is one of the top 10 causes of death and the leading cause from a single infectious agent.[1] There are nearly 9 million new cases and 2 million deaths from tuberculosis worldwide every year. In 2017, TB caused an estimated 1.3 million deaths among HIV-negative people and there were an additional 300000 deaths from TB among HIV-positive people.[2] About 1.7 billion people, 23% of the world's population, are estimated to have a latent TB infection, and are thus at risk of developing active TB disease during their lifetime.[1]

As per the Global TB report in 2017, the estimated incidence of TB in India was approximately 28,00,000 accounting for about a quarter of the world's TB cases.[3] Diagnosis and successful treatment of people with TB avert millions of deaths each year (an estimated 54 million over the period 2000–2017), but there are still large and persistent gaps in detection and treatment.[2]

Peripheral tuberculosis lymphadenopathy has been classified into five stages. These include:

Stage 1- enlarged, firm mobile discrete nodes showing non-specific reactive hyperplasia.

Stage 2- large rubbery nodes fixed to surrounding tissue owing to periadenitis.

Stage 3- central softening due to abscess formation.

Stage 4- collar-stud abscess formation; and

Stage 5-sinus tract formation.[4]

Tubercular lymphadenitis can be presumptively diagnosed morphologically on fine-needle aspiration cytology of lymph node. Fine-needle aspiration cytology is now widely utilized as a first line diagnostic procedure in the diagnosis of palpable masses, including peripheral lymphadenopathy. Its value in the diagnosis of mycobacterial lymphadenitis in adults is well documented. Fine-needle aspiration cytology is a simple effective and safe modality for obtaining a representative sample of material from a lymph node and the diagnosis of mycobacterial lymph adenitis can be confirmed utilizing a number of different investigations, including cytomorphology, specific stains to identify the organism, culture and polymerase chain reaction. However, culture is essential for obtaining a definitive diagnosis. Unfortunately, culture is time consuming and expensive.

Ziehl-Neelsen (ZN) method for acid-fast bacilli plays a key role in the diagnosis and also for the monitoring of treatment in tuberculosis. Its major disadvantage is low sensitivity ranging from 20% to43% [3,5]. Serological techniques have the disadvantage of lack of sensitivity and specificity. Newer molecular techniques such as polymerase chain reaction, although rapid, are costly to be routinely used in developing countries where most Tuberculosis cases occur.[4] Newer investigative methods are required. Fluorescent microscopy plays an important role for detection of Mycobacteria because lower magnifications are used as well as less time is required to examine smears. Fluorescent microscopy using Auramine-Rhodamine (AR) or Papanicolaou (PAP) staining has been considered to be superior to ZN staining.[6] The method is quick and inexpensive. Purpose of present study is to compare the efficacy of ZN staining method and fluorescent method using Auramine-Rhodamine in diagnosis of Tubercular Lymph Adenitis.

Methods

Source of Sample: Fine needle Aspirates received in Department of Pathology from the patients clinically suspected as tubercular lymphadenitis.

Study Design: Hospital based observational study.

Sample Size & Sampling: 100 consecutive patients suggestive of tubercular lymphadenitis.

Study Duration: The study will begin from June-2018 till sample size is attained**.**

Inclusion Criteria

1. Patients with palpable swelling suspected to be tubercular in nature and confirmed by cytomorphology.
2. Purulent or necrotic material in the aspiration of the above mentioned swelling.

Exclusion Criteria

1. Non tubercular lymphadenitis.
2. Frankly Hemorrhagic aspirates.
3. Acellular smears. Any case diagnosed previously as tuberculosis and on anti-Koch's therapy.

All patients were clinically evaluated and an informed consent was obtained for FNAC procedure. All the limitations and complications of FNAC procedure were explained to the patient.

Cytomorphological Features: overall cell population and predominant pattern were assessed by examination under low power.

Cytomorphological categorization was done into four patterns:

1. **Caseating** tuberculosis made when necrotic debris were observed in addition to epitheloid cells, multinucleated giant cells and epitheloid granulomas.
2. **Non-caseating** tuberculosis made when no necrotic debris accompanied the other elements diagnostic of tuberculosis.
3. **Purulent with caseation** made when the smear contained necrotic debris, polymorphonuclear leukocytes and macrophages.
4. **Only caseation-** smear shows only caseating necrosis.

Staining for Ziehl Neelsen Method

1. Fix the smear of the specimen over the glass slide, either by heating or alcohol fixation.
2. Pour carbol fuschin over smear and heat gently until fumes appear. Do not overheat and allow it to stand for 5 minutes, and then wash it off with water.
3. Pour 20% sulphuric acid, wait for one minute and keep on repeating this step until the slide appears light pink in color. Wash off with water.
4. Pour methylene blue, wait for two minutes, again wash with water
5. Allow it to air dry and examine under oil immersion lens.

Staining method for Fluorescent microscopy- Auramine Rhodamine technique

1. Place the slides on a staining rack, with the smeared side facing up, the slides not touching each other
2. Flood the slides with freshly filtered Auramine-Rhodamine. Let stand for 15-20 minutes.
3. Wash well with running water, taking care to control the flow of water so as to prevent washing away the smear
4. Decolorize by covering completely with 1% acid-alcohol for 2 minutes. If not decolorized properly, acid alcohol can be used twice.
5. Wash well with running water, as before; to wash away the acid alcohol
6. Counter stain with 0.1% potassium permanganate for 30 seconds to 1 minutes.
7. Wash as before with water and slope the slides to air dry

Bacilli appear as bright yellow to orange against a dark background under fluorescent microscopy whereas bacilli appear as pink, slightly curved, beaded rods measuring 2-8 micron meter under ZN staining with background appears blue due to methylene blue. For the purpose of uniformity for examination and quantitative reporting of results, therefore the comparative grading shown in table was used in the present study. Doubtful positive were taken as scantily positive due to presence of occasional bacilli.

Table 1: Comparative grading between ZN and A-R fluorescent staining methods according to Forbes BA et al.[7]

Number of AFB seen fuchin stain (1000x magnification)	Number of AFB seen **Fluorescence stain (400x magnification)**	**Reporting**
0 AFB/300 field	0 AFB/70 field	No AFB seen
1-2 AFB/300fields	1-2 AFB/70fields	Doubtful
1-9 AFB/100fields	1-18 AFB/50fields	1+
1-9 AFB/ 10 fields	4-36 AFB/10fields	2+
1-9 AFB / fields	4-36 AFB/fields	3+
>9 AFB/field	>36 AFB/ field	4+

Results

One hundred cases clinically suspected of tuberculosis attending the various departments were studied. Fine-needle aspiration was performed on these cases which were received in Pathology department from the period of January

to September 2019. In the present study, the age of the patients ranged from one year to 70 years. The maximum incidence was in the age group of 21 to 30 years (33%). A slight female preponderance accounting for 55% (55/100) of cases was seen. Single group of lymph nodes were involved in 94 cases and multiple groups of lymph nodes were involved in 6 cases. In the former category, cervical lymph nodes were the commonest group aspirated (59%). Figure 1 shows the distribution of samples taken from various lymph node sites.

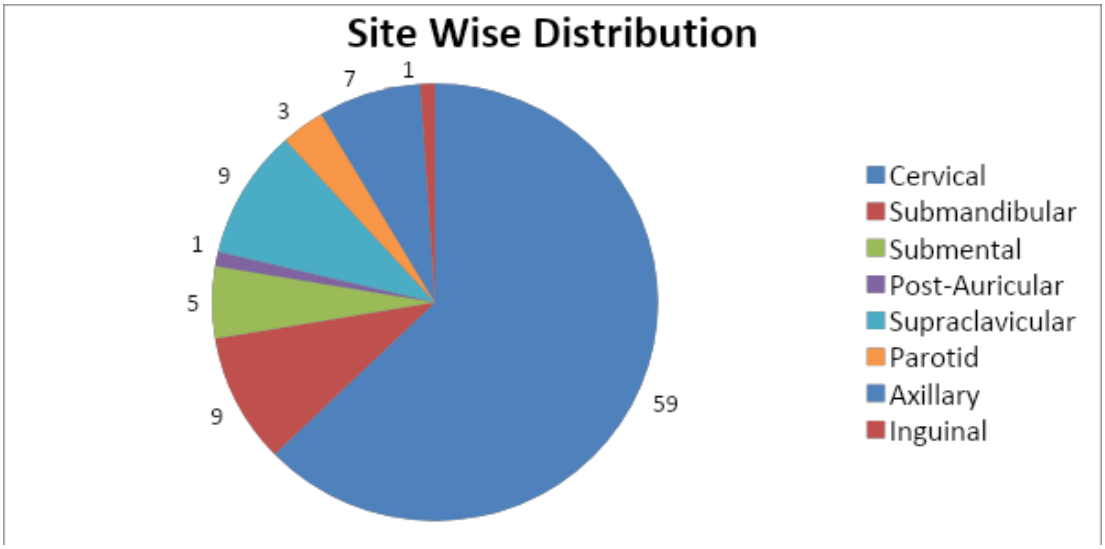

Figure 1: Site wise distribution of the lymph node lesions aspirated

On local examination of the lymph node swelling, majority of the cases were firm on palpation (71cases) and the others were soft, soft to firm and rubbery in 8 cases (8 %), 19 cases (19 %) and 2 cases (2 %) respectively. On local examination, 14 cases showed matting of lymph nodes and in 76 cases there was no matted appearance. On FNA of lymph node, the nature of the aspirate was blood-mixed in 63 cases followed by aspiration of pus and cheesy material in 32 cases and 5 cases respectively.

In the present study of the clinically suspected tuberculosis cases, the categorization of cytomorphological findings were done into four patterns with purulent with caseation (11 cases), only caseation (13 cases), caseation with epitheloid cells(52 cases) and non-caseating with epitheloid cells (24 cases). Tables 2 shows the distribution of positive and negative cases on two stains showing higher positives among AR stain.

Table 2**:** Distribution of positive and negative cases on Ziehl-Neelsen and Auramine and Rhodamine stain

	ZN-stain	**AR-stain**
Positives	36	**61**
Negatives	64	36
Total cases	**100**	**100**

Table 3: Comparison of positive and negative values on ZN and AR fluorescent stains among cases

Cytomorphological diagnosis	**Positive on ZN stain (%)**	**Positive on AR stain (%)**	**Negative on ZN stain (%)**	**Negative on AR stain(%)**
Purulent with caseation (11)	05(45%)	08(72.27)	04(36.36%)	03(27.27 %)
Only caseation (13)	06(46%)	09(69.23 %)	06(40%)	04(26.66 %)
Caseation with epitheloid cells (52)	20(38%)	34(65.38 %)	27(57.4%)	17(36.17 %)
Non caseation with epitheloid cells (24)	5(20.8%)	10(41.66 %)	21(77.7%)	19(70.%)

Cytomorphological diagnosis was differentiated on the basis of positivity among ZN and AR stains. Table 3 shows that AR stain had higher positivity as compared to ZN staining whereas table 4 shows the grading of samples between ZN and AR method of staining. There was significant difference seen between the ZN and AR staining for '+' and negatives samples. Figures 2, 3, 4, 5, 6 and 7 show the histologic visuals for various types of samples.

Table 4: Positivity and grade between ZN & AR method with p value

Gradings	Ziehl-Neelsen method	Auramine-Rhodamine method	p value*
+++	-	-	-
++	1	2	0.561
+	23	51	<0.01
Doubtful positive	12	8	0.347
Negatives	64	39	0.0004

*Chi-square test

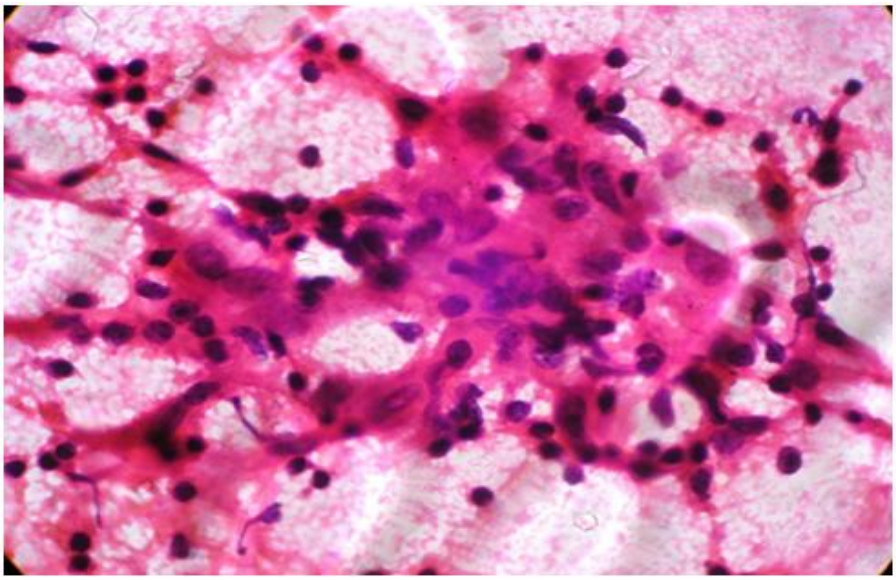

Figure 2: Caseating tuberculosis H & E X 400 (Note central granuloma with epithelioid cells & lymphocytes surrounded by necrotic material)

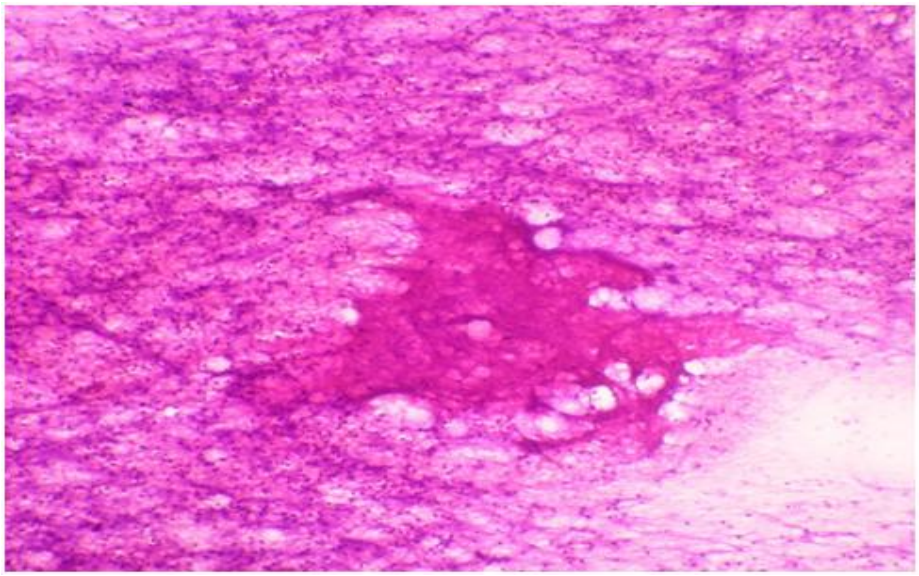

Figure 3: Caseating tuberculosis - to show evidence of caseous necrosis on cytology. H & E X 400

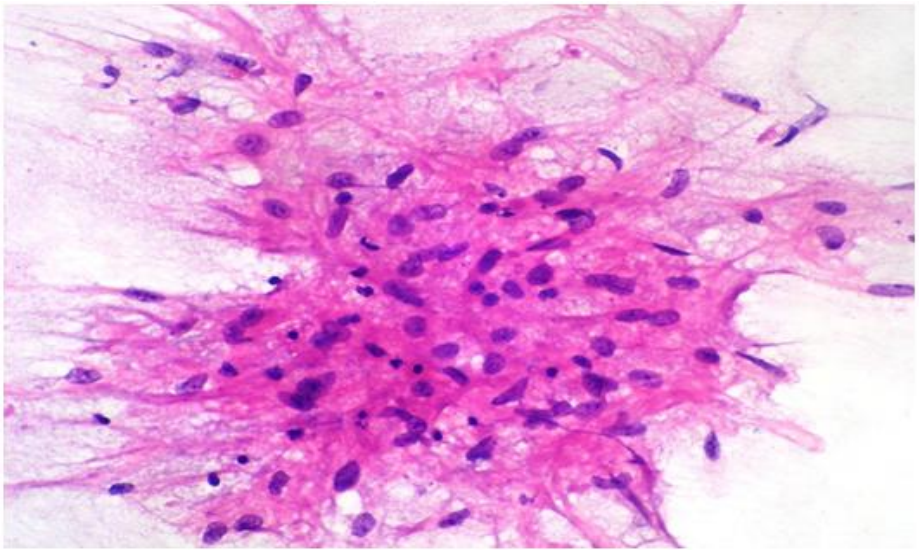

Figure 4: Non-caseating tuberculosis: showing a granuloma composed of epithelioid cells. No evidence of necrosis H & E X 400

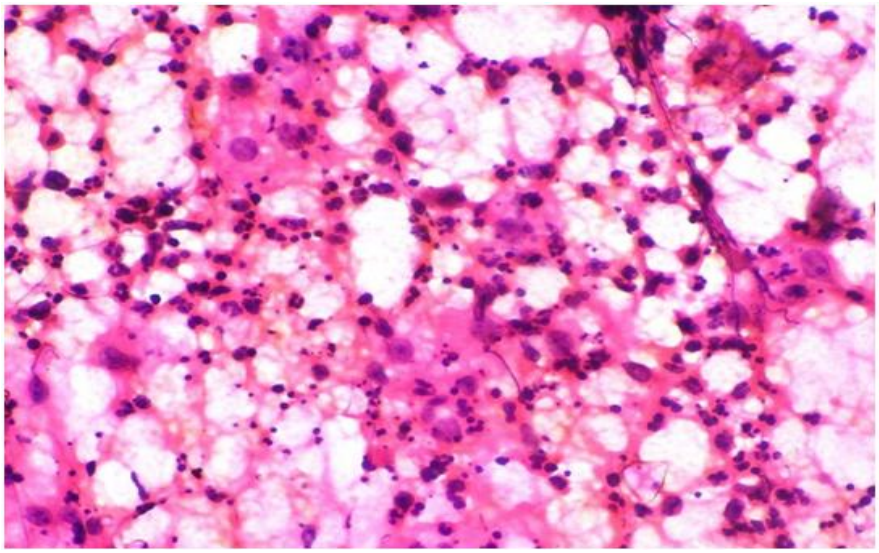

Figure 5: Acute necrotizing inflammation showing necrotic debris, polymorphonuclear leukocytes and macrophages) H & E X 400

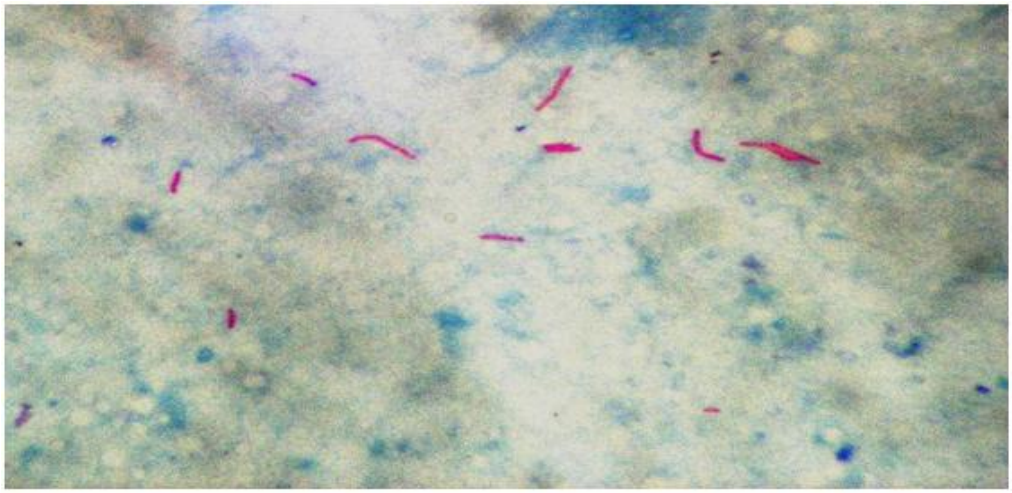

Figure 6: ZN stain Grade (2+ = 1-10AFB/ 50 oil immersion fields): Pink rod shaped acid fast tubercle bacilli. X 1000

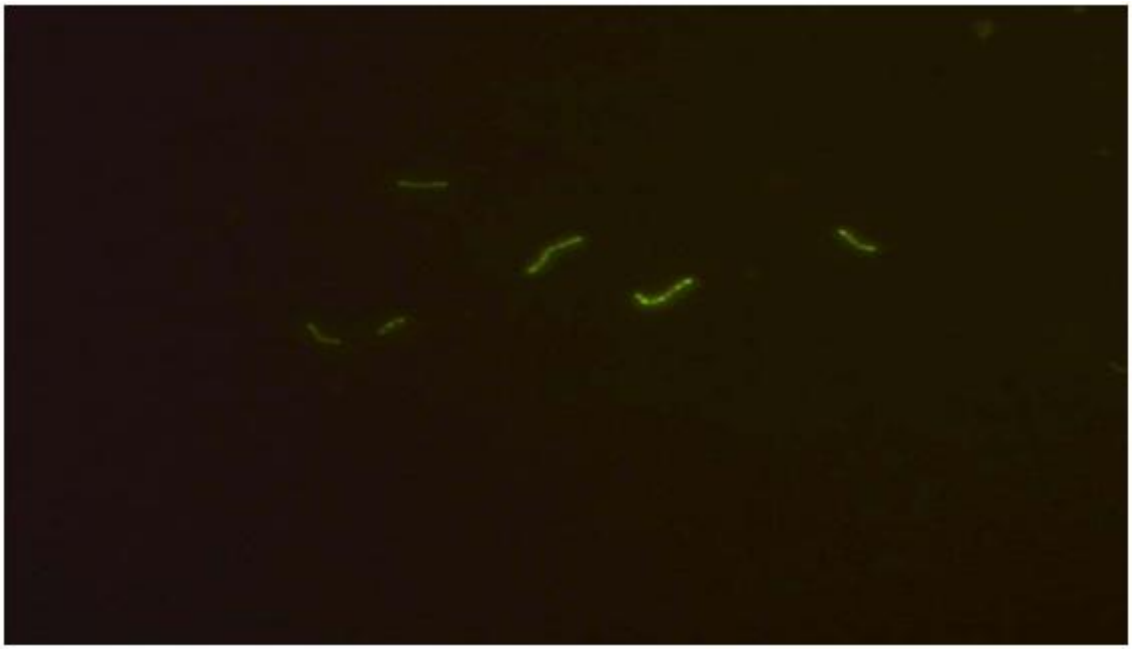

Figure 7: AR stain Grade (2+ = 11-100AFB/50fields using 20x objective): Bright yellow to orange bacilli against a dark background. X2

Discussion

In the present study, most of the patients were in the age group of 21 to 30 (33 cases) with **mean** age was **28.57** and **median** age **23.50.** In a study by Thakur et al[8], maximum cases affected were in age group of 10-30 years. In a study done by Kumar et al[9], mean age of affected adults was 40.7 years. In their study of 209 cases (of which four cases were excluded due to inadequacy), Ergete and Bekele[10] observed a similar finding with the mean and median age of their patients being 22.8 and 23 years respectively. An individual is energetically most active during this period and therefore the chances of exposure are also more. They also observed 87 males and 118 females with a male to female ratio of 0.7:1.

The cervical groups of lymph nodes aspirated were the commonest indicating that the tuberculosis in lymph nodes manifest predominantly in this region. This finding has been observed by Annam *et al*[11], wherein most of the lymph node aspirates were from cervical region constituting 72% of their total aspirations performed and by Ergete and Bekele[10] in 152 out of 209 patients.

Exposure to the tubercle bacillus can result in a spectrum of tissue changes, from caseating tuberculosis (the soft tubercle) to non-caseating tuberculous lesions (the hard tubercle).[12] If a patient's immune system is compromised, an acute necrotizing inflammation is expected. The response of tissues to the tubercle bacillus varies, depending on the tissue's immune status plus the organism's dosage and virulence. Granulomatous inflammatory tissue responses though caused by several factors, the most common in India is due to the tubercle bacillus.[12,13,14]

Shariff and Thomas[12] observed the predominant pattern to be caseating tuberculosis in 40 instances out of 70 samples. There were 11 and 17 aspirates as non-caseating tuberculosis and acute necrotizing granulomatous inflammation respectively. The present study also showed the bacilli were present more in caseating tuberculosis and acute purulent inflammation as compared to non-caseating granulomas. This observation has also supported by Shariff *et al* [42] & Ergete *et al*.[10]

Present observations are in close agreement with those of Rajwanshi[15], Raghuveer[16], Metre[17] & Bailey[18] who had also found highest rate of AFB positivity of 47.4%, 66%, 80% and 87% respectively in necrotic material with or without epithelioid cell granulomas.

The reported accuracy of the FNA cytodiagnosis of tuberculosis affecting lymph nodes varies from 87.1% [13] to 89.8% [19] to 95.4% [20] in literature. It is generally believed in literature that the Auramine-Rhodamine fluorescent technique yields more positive results than the conventional Ziehl-Neelsen method. This has been shown by the experiences of Traunt *et al*[21], Needham *et al*[22], Kuper *et al* [23] and Braunstein *et al* [24]. Needham also stresses that in his study group findings, 55% of cases which were negative on the conventional Ziehl-Neelsen method were proven to contain tubercle bacilli on the Auramine-Rhodamine fluorescent microscopy and confirmed by culture and/or animal inoculation. [22]

In present study of 100 samples, the total AFB positivity rates of 36% on ZN stain increased to 61% on fluorescent stain. This comparison of Ziehl-Neelsen and fluorescent results showed a significant p value of <0.0004 for the presence of bacilli. The possible explanation for the absence of AFB on the Ziehl-Neelsen stain in the 25 cases could be due to a decrease in the intensity of staining. It is easy to pick up bright fluorescent bacilli in dark background in fluorescent microscopy. No heating is required in Auramine – Rhodamine staining whereas in ZN staining heating is required varying from person to person (manual error). In AR method, bacilli can be picked in low power objective (20x) which was not picked up by conventional ZN method. This clearly shows that fluorescent method gives more sensitive results when compared to Ziehl-Neelsen method and successfully eliminates false-negative results in paucibacillary cases. (Table 5)

Table 5: Findings of present study and other studies conducted.

Author	ZN staining (%) positivity	Fluorescent staining(%) positivity
Krishna M et al[25]	37.5	81.82
Dagar V et al[26]	36.5	51.3
Vamsheedhar *et al* [11]	44.11	81.37
Laifangbam *et al* [27]	44.1	71.6
Present study	36	61

For a trained and experienced lab technician, each smear would take approximately a minimum of 2 minutes for 100 fields or three horizontal sweeps. In the fluorescent staining, smears are examined at much lower magnifications (typically 250x) than used for ZN-stained smears (1000x). Each field examined under fluorescence microscopy, therefore, has a larger area than that seen with bright field microscopy. Thus, a report based on a fluorochrome-stained smear examined at 250x may contain much larger numbers of bacilli than a similar report from the same specimen stained with carbol fuchsin and examined at 1000x.[28]

The bacilli were better visualized by the Auramine-Rhodamine stain in the scantily positive cases (doubtful category by Forbes grading used by Laifangbam *et al*[27]) as compared to the morphology and the Ziehl-Neelsen stain. The sensitivity of the Auramine-Rhodamine stain depends upon whether a single component has been used (Auramine or Rhodamine alone), the quality of staining reagents (freshly prepared or otherwise) as well as on time laps between the staining and the view of the results. However this study has proved that the Auramine- Rhodamine fluorescent stain provides a reliable adjuvant to the diagnosis particularly in the paucibacillary cases which are likely to be missed by the Ziehl-Neelsen method.

This fact plays an important role towards the treatment of cases particularly for those clinicians who insist on treating tuberculosis only on finding presence of acid fast bacillus positivity in the report. So, in the present study it ca be concluded that the sensitivity of AR staining method is more than conventional ZN method so fluorescent staining should be used in routine cytology.

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