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A Comparative Study of Fluorescence Microscopy with Ziehl Neelsen Staining for Detection of Acid Fast Bacilli in Lymph Node Aspirates

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Abstract: ***Objective: To study the cytomorphological features in fine needle aspiration smears from patients suspected of having tuberculous lymphadenitis using May Grunwald Giemsa (MGG) and Haematoxylin and Eosin stains and compare the efficacy of ZiehlNeelsen staining (ZN) and fluorescent staining for the detection of acid fast bacilli on lymph node aspirates. Materials and methods: The present study was conducted in 100 patients presenting with peripheral lymphadenopathy suspicious of tuberculous lymphadenitis. Fine needle aspirations were performed and smears from the aspirates were processed for routine cytology, the conventional ZN method, and the fluorescent method. Results: The smear positivity with ZN stain was seen in 37% of cases while the positivity increased to 73% with fluorescent method. Conclusion: Fluorescent microscopy has the advantage of speed and ease of screening, and reduces observer fatigue. The fluorescent method was found to be more advantageous than routine cytology and conventional ZN method, particularly in paucibacillary cases.***

Keywords: ZiehlNeelsen, Auramine-O, fluorescent microscopy, acid fast bacilli

1. Introduction

Tuberculosis (TB) is an ancient infection that has plagued humans since times immemorial and continues to remain a major public health problem especially in developing countries like India. It has staged resurgence in the developed world due to HIV infection and multi drug resistance acquired by the infectious agent. This has prompted World Health Organization (WHO) to declare tuberculosis as a global emergency [1]. It causes ill-health in millions of people each year and in 2015 was one of the top ten causes of death worldwide, ranking above HIV/AIDS as one of the leading causes of death from an infectious disease [2].

TB manifests clinically as pulmonary or extrapulmonary tuberculosis (EPTB), with the former being more common. The last decade has witnessed shifting trends in tubercularinfection, with EPTB emerging as an important entity. It constitutes about 15–20% of all cases of tuberculosis in immunocompetent patients and accounts for more than 50% of cases in HIV positive individuals [3,4]. The term EPTB has been used to describe isolated occurrences of tuberculosis at body sites other than the lungs [5], which includes the lymph nodes, pleura, bones, joints, brain, meninges, gastrointestinal organs, liver, genitourinary organs, peritoneum and pericardium.

Lymphadenopathy is the most common presentation of extrapulmonary tuberculosis [6,7]. The lymph nodes commonly affected include cervical, axillary, inguinal and abdominal sites with cervical lymph nodes being the most commonly affected group of nodes reported [8].

The clinical parameters for the diagnosis of TB in lymph nodes are neither specific nor do their absence exclude TB involvement [9,10]. Fine-needle aspiration cytology (FNAC) of lymph nodes in TB has varied cytomorphological features. However, the conventional ZiehlNeelsen (ZN) method for acid-fast bacilli (AFB) plays a key role in the diagnosis and also for the monitoring of treatment in TB. Its major disadvantage is low sensitivity ranging from 20% to 43% [11,12]. Mycobacterial culture is the reference method for the detection of tubercle bacilli but it is time consuming and requires specialized safety procedures in laboratories. Serological techniques have the disadvantage of lack ofsensitivity and specificity [11]. Newer molecular techniques such as polymerase chain reaction (PCR), although rapid, are costly to be routinely used in developing countries where most TB cases occur [13].

Hence, a method for the identification of AFB which is more sensitive than the ZN method is required for early detection of cases. Thus arose the need for fluorescent staining. Fluorescent staining using auramine is already in vogue for detection of AFB on sputum smears under the RNTCP. Therefore, an attempt to apply similar knowledge to lymph node aspirates was made for faster detection of EPTB.

2. Material and Methods

The present study was conducted in 100 patients presenting with peripheral lymphadenopathy suspicious of tuberculous lymphadenitis in the Department of Pathology, Government Medical College, Amritsar, after approval from the institutional thesis and ethics committee. Informed consent of the patient was taken. Relevant history of the patients was recorded as per a self- designed proforma.

FNA was performed and the material was applied to pre labelled slides and stainedwith H&E, MGG, ZiehlNeelsen and Auramine - O stains for further evaluation.

Depending on the cytomorphological features, all the cases were subdivided into six groups as follows:

Pattern I: Caseous necrosis & epithelioid cell granulomas
Pattern II: Caseous necrosis, epithelioid cell granulomas with inflammatory background (lymphocytes / acute inflammatory infiltrate)
Pattern III: Non-caseating background& epithelioid cell granulomas

Pattern IV: Caseation only
Pattern V: Purulent with caseation
Pattern VI: Purulent only

AFB on ZN stained smears were seen as pinkish, thin curvedrod-shaped bacterium coloured rods against a blue coloured background on oil immersion (1000X) and in Auramine stained smears as slender bright yellow fluorescent rod shaped bacteria against a dark background on 400X.

3. Results

Maximum cases in the present study were in the 11-20 years age group (37 %) followed by 21-30 years age group (31%). Youngest patient in our study was 1 year old while the oldest was 71 years old.Mean age at diagnosis was 24.215 years (SD 14.05) indicating a wide variation in the age of diagnosis.

Table 1: Showing Age Wise Distribution

Age group (years)	Frequency	Percent
0-10	10	10
20-Nov	37	37
21-30	31	31
31-40	10	10
41-50	6	6
51-60	3	3
61-70	2	2
71-80	1	1
Total	100	100

In our study, 44 % of patients were males and 56 % were females, showing a slight female preponderance. Male: female ratio is 1: 1.3.

Table 2: Showing Gender Wise Distribution

Gender	Frequency	Percent
Females	56	56
Males	44	44
Total	100	100

In the present study, cervical group of lymph nodes were the most common group involved accounting for 65% of cases followed by supraclavicular (13%).

Table 3: Showing Site Wise Distribution

Lymph Node	*Frequency*	*Percent*
Axillary	9	9
Cervical	65	65
Epitrochlear	1	1
Infraclavicular	1	1
Preauricular	2	2
Submandibular	7	7
Submental	2	2
Supraclavicular	13	13
Total	100	100

In our study, majority of the cases showed Pattern I constituting 49 % of cases. This was followed by Pattern V with 23% of cases and Pattern II with 14 % of cases. Other cases were constituted by Pattern III (6%), Pattern IV (6%) and Pattern VI (2%).

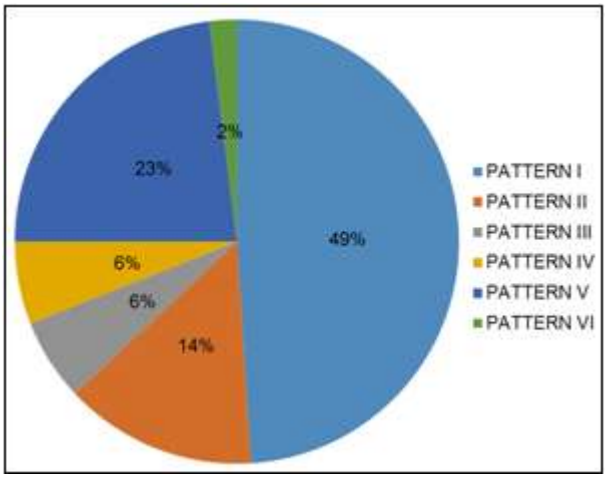

Chart 1: Showing Pattern Wise Distribution

In the present study, the smear positivity by ZN staining was 37% (37/100) cases while the positivity increased to 73% (73/100) cases with fluorescent (AO) stain.

Table 4: Corelation of Zn And Fluorescent (AO) Positivity

		FLUORESCENT (AO)		Total
		0	1	
ZN	0	25	38	63
	1	2	35	37
Total		27	73	100

0- Negative , 1- Positive

Both ZN and Fluorescent (AO) stains were positive in 35 cases and negative in 25 cases. Fluorescent staining was positive in 38cases which were negative on ZN staining. Two cases were positive on ZN, but negative on AO.

Table 5: Showing Diagnostic Test Values of ZN & AO

Diagnostic Values	ZIEHL-NEELSEN (ZN)	95% CI	FLUORESCENT (AO) STAINING	95% CI
Sensitivity	36.73%	27.22% to 47.07%	73.47%	63.59% to 81.88%
Specificity	50.00%	1.26% to 98.74%	50.00%	1.26% to 98.74%
Positive Predivtive Value (PPV)	97.3%	89.78% to 99.33%	98.63%	94.71% to 99.66%
Negative Predictive Value (NPV)	1.59%	0.40% to 6.11%	3.70%	0.92% to 13.78%
Positive Likelihood Ratio	0.73	0.18 to 3.01	1.47	0.37 to 5.91
Negative Likelihood Ratio	1.27	0.31 to 5.10	0.53	0.13 to 2.21

4. Discussion

Tuberculosis continues to be a major health problem in developing countries.FNAC is a simple, effective and safe modality for obtaining a representative sample of material from a lymph node and the diagnosis of mycobacterial adenitis can be confirmed utilising a number of different investigations, including cytomorphology, specific stains to identify the organism, culture and polymerase chain reaction.The detection of AFB is often considered as the evidence of the infected state. Thus, the laboratory plays a critical role in the diagnosis of TB [14].

The predominant cytomorphological pattern in our study was Pattern I i.e. epithelioid cell granulomas with caseous necrosis (49%) followed by Pattern V i.e. purulent aspirate with caseation (23%). This was similar to the one observed by Rajwanshi A et al [15], Chandrasekhar B et al [16]and Ergete W et al [17].

In the present study, the smear positivity by ZN staining was 37% (37/100) cases while the positivity increased to 73% (73/100) cases with fluorescent (AO) stain.

Table 6: Comparison OF ZIEHL Neelsen (ZN) and Fluorescent (AO) Positivity

Studies	Year	ZN Positivity	Fluorescent(AO) Positivity
Kumar N et al [18]	1998	33.5%	45.4%
Jain A et al [19]	2002	22%	52%
Annam V et al [20]	2009	44.11%	81.37%
Thakur B et al [21]	2013	26.67%	34.44%
Present Study	2017	37%	73%

The use of fluorescent stain greatly improves the diagnostic value of the EPTB smears with alow density of bacilli which are likely to be missed on ZN stained smears.

Table 7: Comparison of Diagnostic Tests For ZN AND Fluorescent (AO) Stains

Study	Year	Sensitivity ZN	Sensitivity AO	Specificity ZN	Specificity AO
Githui W et al [22]	1993	65%	80%	96%	97%
Hooja S et al [23]	2011	55.55%	71.85%	99.19%	99.19%
Thakur B et al [21]	2013	80.00%	88.00%	93.85%	86.15%
Abdissa K et al [24]	2015	25%	45.8%	93.8%	89.6%
Present study	2017	36.73%	73.47%	50.00%	50.00%

The fluorescent stain is regarded as a more sensitive staining method for the detection of tubercle bacilli as it can detect bacilii when they are present in theconcentration of 10^4/ ml of sputum, while ZN stain requires 10^5 bacilli/ ml to be detected and there is a stronger binding of mycolic acid of bacilli with Auramine [25]. However, no such data has been documented for lymph node aspirates.

But, there are some disadvantages of the Fluorescent method. These include fading of slides with no possibility for permanent preparations. A dark room is essential and the fluorescent microscope is costly. Trained personnel to handle the fluorescent microscope are also required. There must be a positive and negative control with every batch of staining. Sometimes, background staining can be a problem and in many instances, bright staining of artefacts may be hindrance to interpretation of results.

5. Conclusion

We conclude that the Fluorescent method is more sensitive than the ZN method as the slides can be examined under low magnification allowing for much larger areas of the smear to be screened in a short period of time. Also, it greatly improves the diagnostic value especially in paucibacillary patients that are likely to be missed on ZN stained smears. However, the fluorescent staining alone cannot be an alternative method to ZN staining. It should be used as an adjuvant along with clinical history, haematological investigations and cytological features in lymph node aspirates. Still, for the cases which are negative, but strongly suspicious for tuberculosis, additional investigations must be undertaken like CB-NAAT, Xpert MTB test etc. especially in a developingcountry like ours where TB is highly prevalent and on a rise due to the HIV epidemic.

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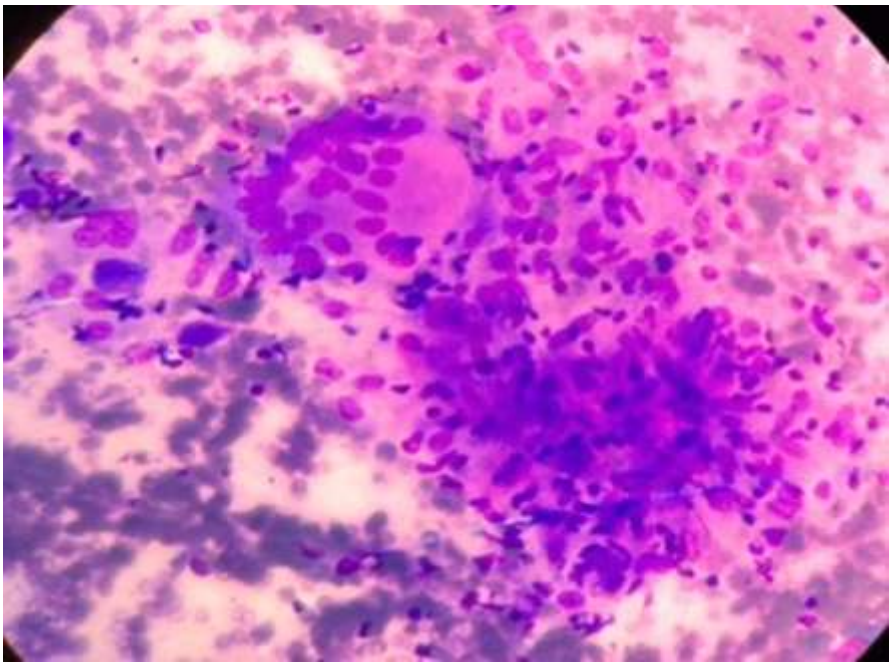

Figure 1: Photomicrograph showing epithelioid cell granuloma with giant cell (MGG, 400X)

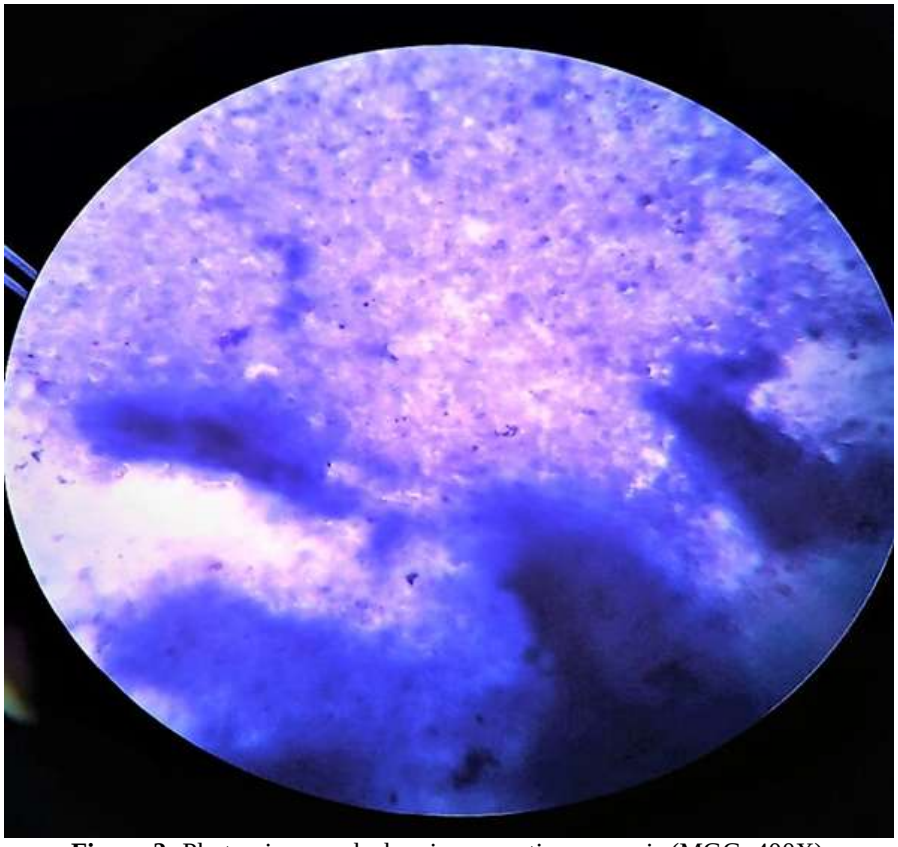

Figure 2: Photomicrograph showing caseation necrosis (MGG, 400X)

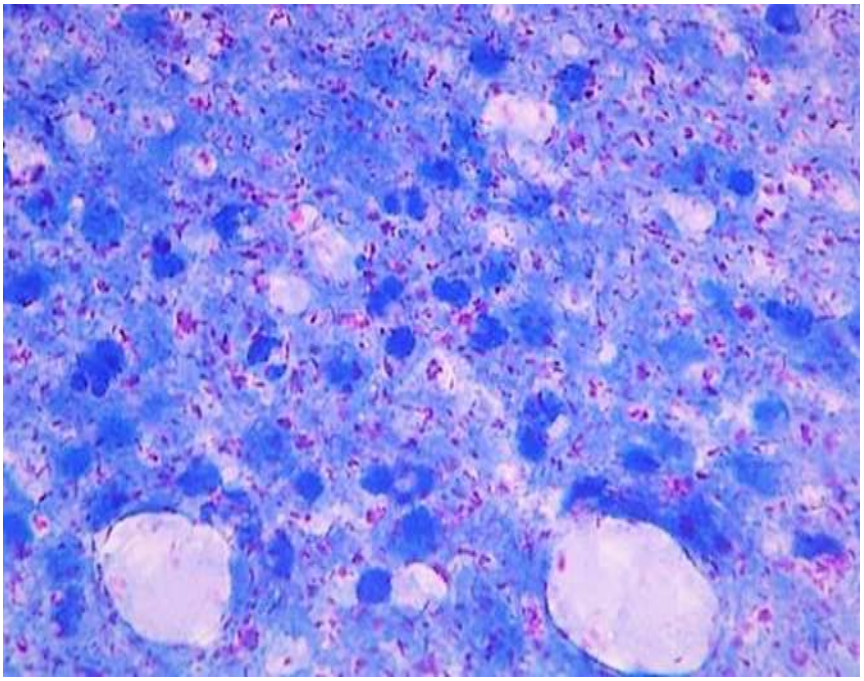

Figure 3: Photomicrograph of ZiehlNeelsen (ZN) stain showing many acid fast bacilli (1000X)

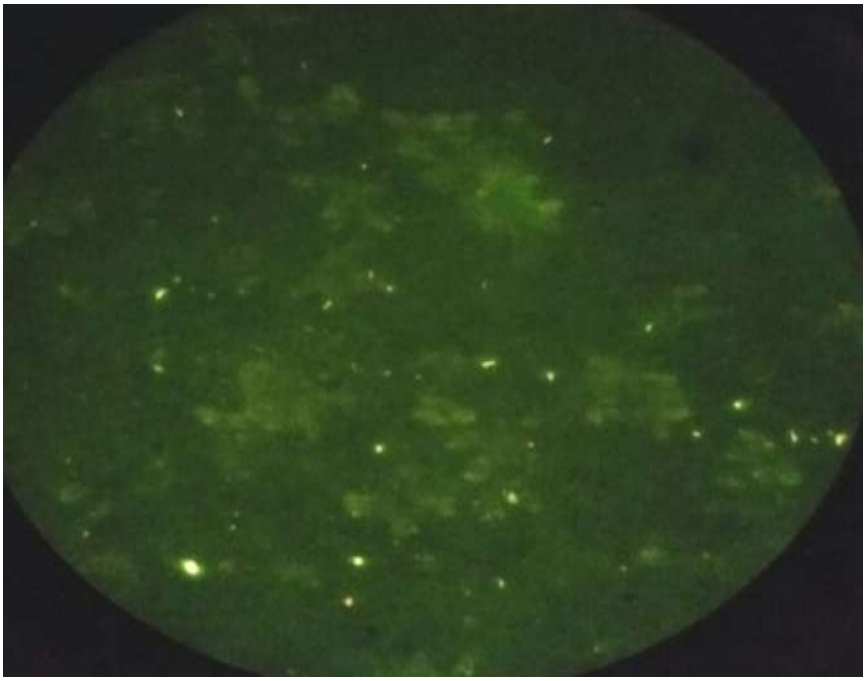

Figure 4: Photomicrograph of Fluorescent (Auramine- O) stain showing acid fast bacilli (400X)