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Original Research Article

Role of autofluorescence technique in detection of mycobacterial bacilli on fine needle aspiration cytology in tubercular lymphadenitis in comparison to conventional methods

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ABSTRACT

Background: Lymphadenopathy is the most common presentation of extrapulmonary tuberculosis. Fine needle aspiration cytology (FNAC) has emerged an important role in the evaluation of peripheral adenopathy as a possible non-invasive procedure. Conventional Ziehl-Neelsen (ZN) method for acid fast bacilli (AFB) plays an important role in the diagnosis and the monitoring of treatment in tuberculosis. Fluorescence microscopy using auramine-rhodamine (AR) or Papanicolaou (PAP) staining has been considered superior to ZN staining.
Methods: The study was conducted in tertiary care centre for a period of 2 years. A total number of 75 patients were included in the study. Four smears made from each aspirate: three air dried smears were stained with Giemsa, ZN, and AR stains and one was wet fixed for PAP stain for autofluorescence. Aspirate sent for culture over Lowenstein-Jensen medium was taken as a reference method.
Results: Seventy five aspirates reported as tuberculous lymphadenitis on cytomorphology were stained with various methods. Autofluorescence was of more diagnostic utility for detection of AFB when cytologically only necrosis (64.71%) or granulomatous lymphadenitis (42.11%) was seen. With necrotising granulomatous lymphadenitis reported cases, Auramine Rhodamine was positive in maximum cases (66.7%) and was significant statistically. Overall taking culture as gold standard ZN stain was most specific (100%) and Auramine Rhodamine was most sensitive (82%).
Conclusions: There is a definite advantage of autofluorescence in detecting mycobacteria over Ziehl-Neelsen and auramine rhodamine stain as it is more sensitive as well as an inexpensive technique. Autofluorescence can be a useful addition to routine cytology for early diagnosis and effective treatment.

Keywords: Tuberculosis, Autofluorescence, FNAC, Lymph node

INTRODUCTION

Tuberculosis (TB) is a major health problem in developing countries and lymphadenopathy is the most common presentation of extra pulmonary tuberculosis.[1,2] The exact cause of these enlarged lymph nodes is often difficult to establish by history, physical examination and radiographic studies alone. Fine needle aspiration cytology (FNAC) has an important role in the evaluation of peripheral adenopathy as a possible non-invasive alternative to excision biopsy.[3]

The cytological criteria for the diagnosis of tubercular lymphadenitis have been clearly defined as epithelioid

cell granulomas with or without multinucleated giant cells and caseation necrosis.[4] Though culture is essential for a definitive diagnosis but it normally take weeks for identification and sensitivity is also relatively low in paucibacillary conditions.[5,6] Conventional Ziehl-Neelsen (ZN) method used for detection of acid fast bacilli (AFB) plays an important role in the diagnosis and the monitoring of treatment in tuberculosis.[7] Its major disadvantages are low sensitivity, time consuming and oil immersion use.

Fluorescent microscopy plays an important role for detection of Mycobacterium because it is less time consuming and lower magnifications can be used to examine smears. Fluorescence microscopy using Auramine-Rhodamine (AR) or Papanicolaou (PAP) staining has been considered superior to ZN staining.[8,9] The reason being that this method is quick and inexpensive in early diagnosis of mycobacterial infection in the cytological specimens, but it shows proclivity towards observer bias and problems associated with artefacts.

This study was aimed to find out cost effectiveness, rapidity, sensitivity and specific technique that can be used routinely in developing countries for early diagnosis and effective treatment of tuberculous lymphadenitis. In this study, the correlation of the cytomorphological features with various techniques in FNA smears from patients who were suspected of having tuberculous lymphadenitis was demonstrated. Several staining techniques including fluorescent microscopy with Auramine Rhodamine staining (ARS), autofluorescence (AF) and conventional ZN method to detect Mycobacterium and to compare it with culture of lymph node aspirates in cytology was done.

METHODS

The study was conducted in tertiary care centre of Haryana – PGIMS, Rohtak for a period of 2 years (2014-2016). The sample size was calculated using the patient load data in the institute and thus 75 patients were included in the study.

Relevant information regarding patient were recorded and symptoms like fever, chills, night sweats and weight loss were noted. Personal and past history regarding any cancer, immunocompromised status, tuberculosis or contact of tuberculosis was also included. Physical examination of nodes including size, site and consistency (hard, rubbery, soft), tenderness, discrete, fixed or matted was done. Patients with any malignant condition or inadequate sample were excluded from the study.

Four smears were made from each aspirate: three air dried smears were stained with Giemsa, ZN, and AR stains and one wet fixed with PAP stain for autofluorescence. Aspirate sent for culture over Lowenstein-Jensen medium was taken as a reference method. The cytomorphological evaluation was made on the established criteria- amorphous granular necrotic debris, epithelioid histiocytes and multinucleated/ Langhans giant cells in a background of reactive lymphocytes and plasma cells. PAP and AR stained slides were examined under fluorescent microscope using the blue excitation filter (450–480 nm). Mycobacterium appeared as greenish yellow, slender, and slightly curved rod-shaped (400X). ZN stained smears were examined for AFB under oil immersion (1000X) using light microscopy which appeared as pinkish, thin curved rod-shaped bacterium measuring 0.5 to 3 micrometer and sometimes beaded. Culture yield was of brown granular colonies of mycobacteria.

RESULTS

The cases belonged to the age group ranging from 1.5–70 years. Maximum number of patients were in 10-30 years range (Table 1). Female preponderance was noted accounting for 57.3% (M: F-1:1.34) of cases. Most of these patients (69.3%) presented without constitutional symptoms and about 70% of them had history of tuberculosis contact. (Table 2) Patients presented to OPD with complains of lymphadenopathy with cervical (60%) being the most common site among them. These lymph nodes were mostly discrete with rubbery in consistency.

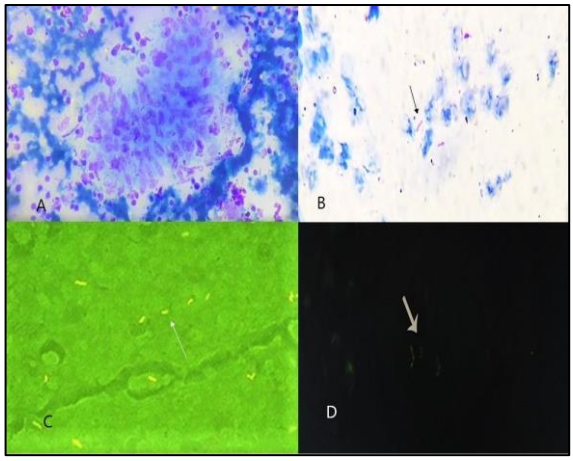

Figure 1: (A) Leishman-stained smear showing epithelioid cell granuloma (400X). (B) Ziehl Neelsen staining for AFB is positive showing rod like beaded bacilli. (C) Auramine Rhodamine staining showing yellow bacilli. (D) Autofluorescence in mycobacteria demonstrating as rod shaped beaded bacilli.

Among other investigations done in such cases, ESR was found to be high (>20 mm/hr) in more than 50% patients, sputum for AFB positivity seen in 48% patients and 54.7% were Mantoux positive.

FNAC yielded pus (39) in maximum number of cases followed by blood mixed aspirate (33). Microscopically, three patterns – necrotizing granulomatous lymphadenitis (52%), granulomatous lymphadenitis (25.3%) and

necrosis only (22.7%) were seen. Out of a total of 75 cases, 52% (39 cases) had pus aspirate, 44% (33 cases) had blood mixed aspirate and only 4% (3 cases) had cheesy aspirate.

Table 1: Distribution of case according to age.

Age in years	No. of patients	Percentage
<10	05	07
10-30	37	49
30-50	24	32
>50	09	12

Table 2: Distribution of cases according to constitutional symptoms.

Constitutional symptoms	No. of patients	Percentage
Nil	52	69.3
Present	23	30.7
Total	75	100

Among 39 patients in which FNAC yielded pus, ZN stain was positive in 58.97%, Auramine Rhodamine in 64.1% and autofluorescence in 66.67% cases. "P" value was found to be significant ($p<0.05$) for Auramine Rhodamine and autofluorecence stains. Taking culture as gold standard specificity was highest with ZN stain (100%) and sensitivity (83%) were more compared to other two stains. (Figure)

Similarly, in 33 cases with blood mixed aspirate, 45.45% were positive on ZN stain, 51.5% in Auramine Rhodamine and 48.48% in autofluorescence. None of these values were significant except for culture method. Thus, with culture as gold standard, ZN was 100% specific and Auramine Rhodamine was both specific and sensitive.

With cheesy aspirate into consideration, each method could detect just one case out of 3, thus sensitivity and specificity was same for all i.e. 100%.

Autofluorescence was of more diagnostic utility for detection of AFB when cytologically only necrosis (64.71%) or granulomatous lymphadenitis (42.11%) was seen. With necrotising granulomatous lymphadenitis reported cases, Auramine Rhodamine was positive in maximum cases (66.7%) and was significant statistically. In all such cases ZN stain was most specific and Auramine Rhodamine along with autofluorescence was most sensitive.

Considering Mantoux test results, almost all methods were in concordance but autofluorescence was the most useful in detecting AFB. ZN stain was found to be most specific and Auramine Rhodamine most sensitive.

So overall taking culture as gold standard ZN stain was most specific (100%) and Auramine Rhodamine was most sensitive (82%). (Table 3)

Table 3: Comparative analysis of various parameters used in the study.

N=75												
Cytomorphological findings		**ZN staining**		**Auramine Rhodamine**		**Auto Fluoresence**		**Culture**		**Montoux test**	
Necrotizing granulomatous lymphadenitis	Positive	23	37.33%	25	34.67%	25	33.33%	30	40%	22	29.33%
	Negative	16	14.67%	13	17.33%	14	18.67%	09	12%	17	22.67%
P value (chi-square)		0.193 (1.70)		0.016 (5.86)		0.041 (4.19)		0.000 (15.3)		0.352 (0.866)	
Granulomatous lymphadenitis	Positive	07	9.33%	07	9.33%	08	10.67%	09	12%	08	10.67%
	Negative	12	16%	12	16%	11	14.67%	10	13.33%	11	14.67%
P value (chi-square)		0.220 (1.51)		0.220 (1.51)		0.461 (0.542)		0.806 (0.603E-01)		0.461 (0.542)	
Necrotizing lymphadenitis	Positive	09	12%	10	13.33%	11	14.67%	10	13.33%	11	14.67%
	Negative	08	10.67%	07	9.33%	06	8%	07	9.33%	06	08%
P value (chi-square)		0.798 (0.663E-01)		0.440 (0.597)		0.198 (1.66)		0.440 (0.597)		0.198 (1.66)	

DISCUSSION

Tuberculosis is one of the major air borne infectious bacterial disease and it remains a worldwide health problem.10 TB lymphadenopathy accounts for approximately 30-40% of TB cases and also as one of the most frequent causes of lymphadenopathy.[3,4] A careful history and physical examination is often suggestive of pulmonary TB. Acid-fast bacilli (AFB) stain and cultures are the most specific components of diagnosis, but the chances of a positive smear depends heavily on the extent of bacterial load in pulmonary involvement and is not

very sensitive. Therefore, culture examination is desirable for establishing the diagnosis before initiating the treatment.[11,12] FNAC is considered to have an important role in the evaluation of peripheral adenopathy as a non-invasive simple and economic alternative to excision biopsy. FNA averts the physical and psychological trauma that is frequently encountered with open surgical biopsy. In addition, it is convenient for patient and physician and can be performed on outpatients. It is relatively painless and provides good correlation between cytomorphological and histopathological features.[22]

Extra pulmonary tuberculosis is an obscure process and more difficult to diagnose compared to pulmonary TB. History of exposure from pulmonary TB is highly suggestive of lymphatic TB in a given clinical setting. Diagnosis of tuberculous lymphadenitis is established by histopathological examination along with demonstration of acid-fast bacilli (AFB) and culture from lymph node aspirate.

The diagnostic modalities can be divided into: primary diagnostic studies and ancillary diagnostic studies.[13] On FNA caseating granulomas in smear with AFB positivity is considerably sensitive and specific for the diagnosis of TB. Overall, the diagnostic accuracy of lymph node FNAC ranges from 71.3% to 97%.[28,32] Other features like nonspecific noncaseating granulomas with lymphoid aggregates or Langerhans giant cells in areas of extensive caseous necrosis may support a diagnosis of probable tuberculosis.[13]

M. tuberculosis can be detected in excisional biopsies by using culture technique in 70–90% of cases. Excisional biopsy is preferable as incisional biopsy may be associated with sinus tract formation. Identifying Mycobacteria by culture still represents the cornerstone on which the definitive diagnosis is based. Although culture can be performed with aspirated specimen, the positive rates are at times significantly lower in aspirated specimen as compared to biopsy specimen (17% versus 80%).[14] Culture is considered the diagnostic gold standard. However, current methods require 3 to 6 weeks for cultivation and identification of species. Hence, a few modern rapid methods have been developed. These include microcolony detection on solid media, septic check AFB method, microscopic observation of broth culture, the BACTEC 460 radiometric system, BACTEC MGIT 960 system, MB/BacT system, and ESP II culture system.[15] Of these, currently MGIT culture is the gold standard.

Molecular diagnosis or nucleic acid amplification (NAA) are nowadays used to detect mycobacterial DNA instead of detection of Mycobacteria. These method have higher sensitivity and are much rapid and are also capable of identifying the species and drug resistance earlier compared to conventional methods.15 Amplified molecular tests for detecting M. tuberculosis are PCR-based technique which is fast and useful for the demonstration of mycobacterial DNA fragments in patients with clinically suspected mycobacterial lymphadenitis. A systematic review of NAA using PCR technique in tuberculous lymphadenitis revealed to be highly variable and with inconsistent results (sensitivity, 2–100%; specificity, 28–100%), with more favourable performance from commercial assays and with sample sizes of more than 0.20 uL.16 Based on these inconsistent results, these PCR-based tests are not recommended and TB Gene Xpert is preferred. The RNTCP guidelines also recommend TB Gene Xpert to be used on every FNAC or biopsy sample of lymph node for its high sensitivity and specificity in diagnosing TB and rifampicin resistance.

The tuberculin skin test (TST) is positive in the majority (74–100 percent) of patients with tuberculous lymphadenitis (in the absence of HIV infection), but not sufficient to establish the diagnosis. A negative test does not exclude the diagnosis, especially in immunosuppressed individuals.[17]

Interferon-gamma release assays have high sensitivity and specificity rates in diagnosis of TB lymphadenitis, but positive test results are not sufficient to establish a diagnosis. The Indian government banned serological antibody tests in 2012, and both Standards for TB Care in India (STCI) and International Standards for TB Care (ISTC) discourage the use of IGRAs for the diagnosis of active TB.[18]

Sputum smear microscopy is the one that has very close relation with infectiousness, patient who are sputum smear positive and culture positive are for more likely to be infectious than culture positive but smear negative.[19]

Chest radiography findings consistent with active pulmonary TB should prompt sputum cultures; if positive, evaluation for miliary TB should be pursued.[20]

Gross and microscopic findings of tuberculous lesions in fine-needle aspirates are well documented in literature. The gross appearance of the aspirates can be blood-mixed, cheesy, and purulent. FNA cytological diagnosis of lesions likely to be of tuberculous etiology or due to nontuberculous mycobacteria (NTM) requires demonstration of epithelioid granuloma with or without necrotic material.[27] Based on the morphological findings, Bailey et al found distinguished 2 groups: cases with distinct epithelioid granuloma and those with no granulomas but large amounts of necrotic debris with variable numbers of polymorphonuclear cells, histiocytes, and lymphocytes.[28]

Several conditions such as mycosis, bacterial, and viral adenitis may mimic similar morphology on cytology as Mycobacterium tubercular adenitis. In such cases, laboratory investigations may be essential to establish the cause, because treatment and prognosis may differ. Demonstration of Mycobacterium tuberculosis bacilli on fine needle aspirate is necessary for an early and accurate

treatment. Specific treatment may decrease morbidity and prolong life expectancy.[23]

Auramine-Rhodamine requires less time, low power examination and is superior to ZN staining as auramine combine more readily with mycolic acid than the conventional carbol-fuchsin acid-fast stain, but it involves the use of toxic and carcinogenic substances. AF on PAP stained smears is a safe, inexpensive and exposure-free diagnostic procedure along with more positive results when compared to AR and ZN stain. Single PAP stained smear can be used for both routine light microscopy for cytological diagnosis and fluorescent microscopy showing bacilli. Mycobacteria on fluorescence is seen as brilliant yellowish green bacilli, thin, and slightly curved with polar enhancement and sometimes even beaded appearance.[23]

Previous studies for detection of AFB from various clinical specimens, comprising sputum, CSF, fine needle aspirate, pus, and miscellaneous body fluids which were examined by ZN and AR staining techniques, showed that AR was 86.6% sensitive as compared to 67.3% sensitivity by ZN stain with more marked difference in extra pulmonary samples.8 In other studies on FNA smears of lymph nodes, autofluorescence was found to be more sensitive than ZN staining, but as compared to cytodiagnosis, it was less sensitive.[9,24,25] The present study was conducted to find out which method is more effective in detecting Mycobacterium and to compare AR and AF with conventional ZN method on lymph node aspirates in cytology. Thus, highest sensitivity was observed with Auramine Rhodamine followed by autofluorescence and least with ZN staining. ZN stain was found to be more specific than AF.

Our study showed only marginal difference in sensitivity between these three methods. Though fluorescent techniques (auramine rhodamine and autofluorescence) showed better outcomes but as they yielded more false positive incidence, the specificity decreased. The reasons for false positive may be due to sampling problem and artefacts. Some organisms such as spore-forming like Bacillus subtilis, nonspore forming bacteria like Staphylococcus aureus, Nocardia, budding yeast exhibit spontaneous emission spectra following excitation at specific wavelengths may pose problem in the detection of Mycobacteria.[26] Also air-drying artefacts in Papanicolaou stained smears may cause problems in identifying Mycobacteria by fluorescent microscopy. There is also increased frequency of false positivity on AF due to subjective biased errors and interobserver variability.

Their number can be decreased by familiarizing with the method of procedure and developing strict diagnostic criteria to avoid over diagnosing fluorescent particles as microorganisms. Sensitivity of Auramine is more due to its property to detect organisms in specimens with low density of mycobacteria.

Sensitivity of Auramine Rhodamine was found to be highest in all the cases irrespective of aspirate and similarly specificity was highest with ZN stain. The reason for this observation could be due to presence of more number of acid fast bacilli in necrotic aspirate and these fluorescent dyes can detect viable and non viable bacilli which may be missing on ZN staining.

Highest AFB positivity using ZN stain and auramine rhodamine stain were seen in cases showing both granuloma and necrosis microscopically was 58.97% and 66.67% respectively. Autofluorescence on Papanicolaou stained aspirates showed highest AFB positivity in 64.71% cases that showed only necrosis microscopically and minimum with granuloma alone. This character may be attributed to the presence of non viable bacilli due to partially treated status and bacteriostatic substances which were missed on culture.

In developing countries like India, with limited resources, the diagnosis is still based on poorly validated symptoms based algorithm, often not resulting in a definitive diagnosis. Thus, this study was conducted to evaluate the various diagnostic techniques for detection of mycobacterium tuberculosis.

Diagnosis is mainly based on clinical features, cytomorphological patterns, histopathology, and identification of acid fast bacilli and the isolation of mycobacterium tuberculosis from the clinical specimen. Cytomorhology though simple but not specific as other opportunistic infections may cause similar morphology. Therefore, culture of aspirate is also done for confirmation. Cultivation of bacilli is the most sensitive method and is considered gold standard but it takes several weeks for result to appear. It is also influenced by inadequate sampling, variable bacterial load and commencement of therapy prior to diagnostic procedure. In our study its sensitivity was observed to be 68% and ranges from 8% to 75%.[24,34]

ZN staining though simple but needs technical proficiency and experience in identification AFB. Sensitivity of this stain ranges from 0% to 77.8% with our study showing 76%.[32,33]

To combat these problems fluorescent microscopy came to rescue as turnaround time for diagnosis reduces. Sensitivity in our study was found to be 82% and range from 32% to 88%.[35,23] Autofluorescence method in various studies demonstrated sensitivity 65% to 96%.[9,23] Sensitivity was about 78% in our study.

The recent development of light emitting diode provides a cheap and reliable light source.

Thus, it was observed in our study that although fluorescent stains were found to be more sensitive but when specificity was considered, conventional ZN staining was found to be most specific.

Even when all the methods of detection were combined, it was observed that 19 cases were still undetected which were suspected of tuberculosis on cytology. Thus, no single method is sufficient enough for definite diagnosis and there is need to improve these techniques for better detection and early diagnosis of tuberculosis.

Limitations

There is chance of technical as well as interpretative error in the FNA cytologic diagnosis of TB. The sample may not be representative or adequate. ZN stain to demonstrate bacilli in the smears, their number should be 10,000-100,000/ml of material therefore, chances of false negative increases. Fluorescent dye needs requirement of unstable and expensive fluorescence reagents, hindering the use of fluorescent microscopy in developing countries. Also its carcinogenic effects have caused problems in health workers.

CONCLUSION

In developing countries were TB is widely prevalent, delay in treatment is unacceptable, as it results in more spread of disease in the community. Lymph Node TB is the most common form of extra pulmonary TB which is different from pulmonary TB in terms of diagnosis and management. The most usual sign and symptom is the appearance of a chronic, painless mass in the neck, which is persistent and usually grows with time. Because of no other remarkable symptom, its diagnosis and distinction needs a high index of suspicion, and application of a variety of diagnostic modalities. This study explores the utility of autofluorescence as an adjunct to routine cytology as it is simple, rapid and cost-effective screening technique especially in developing countries where tuberculosis is widely prevalent and shows continued presence of infection in the community. However, limited and cautious use of AF is necessary because of its increased false positivity rate and subjective-biased errors. AF should be used in addition to other ancillary techniques.

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