"COMPARATIVE STUDY OF CONVENTIONAL SMEARS AND BLEACH CONCENTRATION METHOD IN DETECTION OF TUBERCLE BACILLI IN FINE NEEDLE ASPIRATION MATERIAL OF LYMPH NODES"

by

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Under the guidance of

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I hereby declare that this dissertation entitled **"COMPARATIVE STUDY OF CONVENTIONAL SMEARS AND BLEACH CONCENTRATION METHOD IN DETECTION OF TUBERCLE BACILLI IN FINE NEEDLE ASPIRATION MATERIAL OF LYMPH NODES"** is a bonafide and genuine research work carried out by me under the guidance of **Dr.B.R.YELIKAR$_{MD}$,** Professor and Head, Department of Pathology at B.L.D.E.A's Shri. B. M. Patil Medical College Hospital and Research Centre, Bijapur.

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LIST OF ABBREVIATIONS USED

1. AFB	-	Acid fast Bacilli
2. CR	-	Complement Receptors.
3. EPTB.	-	Extrapulmonary tuberculosis
4. FNA	-	Fine needle aspiration
5. FNAC	-	Fine needle aspiration cytology
6. HIV.	-	Human immuno deficiency virus
7. H & E	-	Haematoxylin and Eosin
8. LN	-	Lymphadenitis
9. MAC	-	Mycobacterium avium complex.
10. MAI	-	Mycobacterium Avium Intracellulare
11. NAOCl	-	Sodium hypochlorite
12. PAS	-	Periodic acid Schiff
13. PAP	-	Papanicolaou stain
14. PCR	-	Polymerase chain reaction
15. ROI	-	Reactive oxygen Intermediates
16. RLN	-	Reactive lymphadenitis
17. RCF	-	Relative Centrifugal force.
18. RNI	-	Reactive nitrogen Intermediates
19. SPLN	-	Suppurative lymphadenitis
20. TB.	-	Tuberculosis
21. TBLN.	-	Tubercular lymphadenitis
22. WHO	-	World Health Organization
23. Z – N	-	Ziehl – Neelsen stain

ABSTRACT

Background & Objectives: The conventional Ziehl-Neelsen staining method for acid-fast bacilli (AFB) plays a key role in the diagnosis of tuberculosis by direct microscopy. Its major disadvantage is low sensitivity. The present study was performed to emphasize the role of bleach concentration method over conventional direct smear microscopy for detection of tubercle bacilli (AFB) in fine needle aspiration material of lymph nodes.

Methods: Fine needle aspirations (FNA) were done in 109 patients with clinical suspicion of tubercular lymphadenitis. Smears from the aspirates were processed for routine cytology and the conventional Z-N method. The remaining material in the needle hub and/or the syringe was used for the bleach method. The significance of the bleach method over the conventional Z-N method and cytology was analyzed.

Results: Among the 109 aspirates 35.78% were positive for AFB on conventional Z-N method, 43.12% were indicative of TB on cytology and the smear positivity for AFB increased to 62.38% on bleach method.

Interpretation and Conclusion: The bleach method is simple, inexpensive and limits the risk of laboratory-acquired infections. The bleach method improves the microscopic detection of AFB and can be a useful contribution to routine cytology.

Keywords: Acid-fast bacilli; bleach method; fine needle aspiration; lymphnode; tuberculosis; Ziehl-Neelsen stain.

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INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by Mycobacterium Tuberculosis. It can involve any organ system in the body. While pulmonary TB is the most common presentation, extrapulmonary tuberculosis (EPTB) is also an important clinical problem.[1]

TB remains a worldwide public health problem despite the fact that the causative organism was discovered more than hundred years ago.[1] It is the second leading cause of infectious disease worldwide, leading to three million deaths annually. Approximately 8-10 million people are infected with this pathogen every year.[2,3,] To make global situation more worse TB has formed a lethal combination with HIV.[1]

India accounts for nearly one third of the global burden of TB and it is the most important public health problem facing this country. TB kills people, 14 times more than all tropical diseases combined and 21 times more than malaria. Every day more than 20,000 people become infected with tubercle bacillus, more than 5000 develop the disease and more than 1000 die from Mycobacterium tuberculosis (M.tuberculosis).[4] It has become a major barrier to socioeconomic development.[1,4]

Extra pulmonary tuberculosis is on the increase world over, which is due to HIV infection epidemic. In India, in general out patients, 10-20% of new tuberculosis cases may be extra pulmonary, while among HIV positives it could be 50%.[5]

In India and other developing countries TB lymphadenitis (TBLN) continues to be the most common form of EPTB.

Many patients present with peripheral lymphadenitis, which may remain asymptomatic for longer periods and produce disease only when the host resistance is lowered.[6] The utility of fine needle aspiration cytology (FNAC) in the diagnosis of TB lymphadenitis has been highlighted in a number of studies during the last two decades.[7-9]

In developing countries like India, the only practically available method for diagnosing extra-pulmonary tuberculosis is direct smear microscopy for tubercle bacilli of the sample from the lesion. But its sensitivity is not optimal, ranging from 9-46% when used in TB control programmes, as the minimum number of tubercle bacilli necessary to produce a positive smear results has been estimated to be minimum 5,000 to 10,000 per ml.[10]

Mycobacterial culture is the reference method for detection of tubercle bacilli, but it is time consuming and requires specialized safety procedures in laboratories and cannot be used routinely. Serologic techniques have the disadvantage of lack of sensitivity and specificity. [11] Newer molecular techniques such as PCR, although rapid, are costly to be routinely used in developing countries where most TB cases occur. [12]

Microscopy has many advantages when it comes to speed and feasibility, and if its sensitivity could be improved, it has the potential to become an even more valuable tool for TB control programmes around the world. [13]

There are various concentration methods for improving sensitivity of direct microscopy for detection of tubercle bacilli in specimen.[14] Bleach concentration method for detection of tubercle bacilli has been recently described for sputum and extra pulmonary specimens and studies have shown improved detection. [10]

Hence the present study was performed to emphasize the role of bleach concentration method over conventional direct smear microscopy for detection of tubercle bacilli in fine needle aspiration material of lymph nodes.

AIMS & OBJECTIVES OF STUDY:

1. Application of bleach concentration method in detection of tubercle bacilli in fine needle aspiration material of lymph nodes.
2. To evaluate the sensitivity of bleach concentration method over conventional direct smears in the diagnosis of tubercular lymphadenitis.

REVIEW OF LITERATURE

Tuberculosis (TB) described with different names as Consumption, Pott's disease, Phthisis, Rajyakhshma and Tapedic appears to be a disease as old as human history. Bones of prehistoric man dating back to 8000 BC have shown typical changes of TB. It has been described in India as early as 3000 BC. In Rigveda which is dated 2000 BC, it has been described as Yakshma. Sushrutha described the disease and observed it was difficult to treat. In Greek literature Hippocrates in 460 – 377 BC first described tubercle. Aristotle in 384 – 322 BC described scrofula on the skin of phthisic pigs.[15]

In the previous era, TB was aptly named as 'Captain of the ship of death'. Frascatorious (1483 – 1553) postulated that it may be transmitted in humans by airborne living particle; it was named 'contagium vivium'. The term 'tubercle' was coined by Franciseus Sylvius (1614 – 1672). He noticed tubercles in the lungs of people with 'phthisis'. Benjamin Martin (1720) suggested it may be an infectious disease. The term 'TB' was introduced by Laurent Bayle (1774 – 1816). Robert Koch on 24th March 1882 identified the specific agent Mycobacterium tuberculosis causing TB which is observed as world TB day every year by WHO.[15]

"Scrofula or the King's Evil" are the historical names of tuberculosis lymphadenopathy, involvement of superficial lymphatic especially those of the neck has plagued the mankind throughout the recorded history.[16]

The term scrofula, meaning glandular swelling (Latin) and full necked sow (French). August Hirsch stated "It denotes an inflammatory kind of tumor more particularly in the neck" first encountered in medical writings in Italy in early 16th century.[17,18]

King's Evil – It was widely believed for many centuries that the royal houses of England and France had a supernatural to gift to cure scrofula by touching the sufferers. Ceremony consisted of the king touching the afflicted subject while the court chaplain recited prayers and presented the touch piece. It was at the end of 19th century that the tuberculosis infection was identified as the cause of scrofula.[17]

Tuberculous lymphadenopathy is the commonest presentation of extra pulmonary TB in both immunosuppressed and nonimmunosuppresed population.[19,20]

In the beginning Mycobacterium bovis (M.bovis) was a common infective agent, currently most cases are caused by M.tuberculosis or atypical mycobecteria especially M.scrofulaceum and M.avium complex (MAC).[21]

The incidence of infection in nonimmunosuppresed population is due to spread of the organism from the primary focus to regional lymphnodes. This spread is self limited and contained within the regional lymphnodes.[15] Most commonly primary focus is from lung and it spreads by hematogenous or lymphatic route. When the source of infection is milk contaminated with M.bovis, the primary focus is the tonsils or the pharynx.[21]

Clinically tuberculous lymphadenopathy is most often found in the head and neck region. The posterior cervical and supraclavicular chains are the sites most commonly involved and it is less frequent in submandibular and preauricular nodes. Rare in axillary or inguinal nodes constituting less than 10% of cases.[16]

Mediastinal, peribronchial and paratracheal lymphnode TB is most often seen as a part of primary TB in children or disseminated disease in immunocomprimised host.[16] Involvement of thoracic and abdominal lymphnodes are frequently seen in HIV infected patients.[19]

The typical scenario for superficial nodal is gradual, painless enlargement over several weeks to months. Initially the overlying skin is not inflamed, but with the passage of time the epidermis becomes shiny, pink to red, faintly tender and warm to touch. Matting of nodes may occur due to periadenitis. Eventually if left untreated, the skin may be breached and a fistula formed that discharges matter that ranges in nature from serous to purulent, caseous debris.[16]

Clinical features in children can be summarized as followed: .[22]

1) **Recent infection**: Slow onset

Painless enlargement of one or a group of nodes are sometimes noticed accidentally and child appears in a state of normal health. Primary node is always largest and those draining from it are smaller. These can regress completely or can reappear after many years.

2**) Recent infection**: Acute onset

Children over 10 years of age presents with nodal swelling of rapid onset with constitutional symptoms and high fever. Nodes are easily palpable. They are highly sensitive to tuberculin test and usually proceed to early softening.

3) **Old infection**: Calcification, softening

Child can have soft painless swelling, which may not involve the skin or can have hard swelling due to calcification, which indicates infection of many years duration.[22]

Hence physical examination findings vary according to the acute or chronicity of the process.

The physical appearance of superficial tuberculous lymphadenitis has been classified into 5 stages :[3]

Stage 1: Enlarged firm, mobile and discrete nodes.

Stage 2: Large, rubbery nodes fixed to surrounding tissue due to periadenitis.

Stage 3: Central softening due to abscess formation.

Stage 4: Collar stud abscess formation.

Stage 5: Sinus tract formation.[18]

The process progress indolently and is usually not accompanied by systemic symptoms. Miliary TB should be suspected when the lymphadenopathy is generalized, localized outside the cervical chain or accompanied by systemic symptoms.[21]

In HIV infected or otherwise immuno compromised patients, the course of the desease is severe with bilateral lymphadenopathy and with systemic symptoms such as fever, night sweats and weight loss.[21]

Structure of Tubercle bacilli:

It is important to know the structure of tubercle bacilli and immunopathogensis to understand the various diagnostic modalities.

Tubercle bacilli:

Bacteria in the genus Mycobacterium are slender, aerobic rods that grow in straight or branching chains.[23] They measure 0.2 to 0.6 µ by 1 to 10 µ in size.[24] They stain weakly positive with Gram stain and they have a waxy cell wall composed of mycolic acid, which makes them acid fast.[23]

Constituents:

The cell envelope has special properties. The Mycobacterium have unique cell wall responsible for its virulence, diagnostic staining and also can induce delayed hypersensitivity.[25]

The envelope consists of two distinct parts:

- Plasma membrane
- Cell wall[24]

Cell wall:

The cell wall is composed of upper and lower segments. Beyond the membrane termed the lower segment is called cell wall core. It is the insoluble matrix of mycobacterial cell wall after removal of all soluble proteins, lipids and carbohydrates. It is composed of three covalently attached macromolecules: peptidoglycan (PG), arabinogalactan (AG) and mycolic acid – mAGP (Mycolyl Arabinogalcatan Peptidoglycan) complex. The upper segment is composed of free lipids, proteins, phosphotidylinositol mannosides (PIMs), phthiocerol containing lipids, lipomannan (LM) amd lipoarabinomannan (LAM).[26]

It can be considered that lipids, proteins, and lipoglycans are the signaling and effector molecules in the disease process; whereas the insoluble core is essential for the viability of the cell and should be addressed in the context new drug development. [26]

A. Biologic function of free Lipids :

Mycobacteria are rich in lipids and are largely bound to proteins and polysaccharides.[25] Knowledge of their roles in signaling events, pathogenesis, and the immune response is now emerging.[26]

Muramyl dipeptide (from peptiodglycan) complex with mycolic acids can cause granuloma formation whereas phospholipids induce caseous necrosis.[25]

Cord factor / TDM (Trechalse 6 6' dimycolate)

A "cord factor" has been extracted from virulent bacilli with petroleum ether. Cord formation is correlated with virulence. Virulent strains of tubercle bacilli form microscopic "serpentine cords" in which AFB are arranged in parallel chains. It induces cytokine mediated events, such as systemic toxicity, antitumor activity and release of chemotactic factors. It can cause chronic granulomas and can serve as an immunologic adjuvant.[25]

Sulfolipids

The most virulent strains were prolific in elaborating strongly acidic lipids, whereas and attenuated ones were notably deficient in these compounds. It acts as an antagonist to the fusion of the secondary lysosomes with phagosome, thus promoting intracellular survival of the pathogen.[26]

Lipoarabinomannan (LAM)

It is a lipoplysaccharide lipoglycan found in all mycobacteria. It has properties analogues to gram negative O-antigenic PAS (Lipopolysaccharide), such as nonspecific suppression of T lymphocyte activation and inhibition of antigen responsiveness of cells. LAM also inhibits interferon-gamma mediated activation of macrophages. Thus it may be

implicated in interaction of the pathogen and host cell in the down regulation of T cell responses of various types. It is also major B cell immunogen.[24]

Pthiocerol dimycocerosate (DIM /PDIM)

Major lipid of tubercle bacillus referred to as ball of wax or wax in bacillus. It is known to have wide spectrum of virulence.[26]

Analysis of lipids by gas chromatography reveals patterns that aid in classification of different species.[25]

B. Proteins:

Each type of mycobacterium contains several proteins and are associated with cell wall and are powerful immunogens. Proteins are bound to a wax fraction and on injection induce tuberculin sensitivity. They can also elicit the formation of variety of antibodies. The proteins are 23 kDa, 59 kDa etc.[25]

C. Polysaccharides:

Mycobacteria contain a variety of polysaccharides. Their role in the pathogenesis of disease is uncertain. They can induce the immediate type of hypersensitivity and can serve as antigens in reactions with sera of infected persons.[25]

Pathogenesis of TB

Route and site of infection:

M. tuberculosis is an obligatory aerobic, intracellular pathogen, which has a predilection for the lung tissue rich in O_2 supply. In most cases the tubercle bacilli enter the body via the respiratory route. The bacilli spread from the site of initial infection in the lung

through lymphatics or blood to other parts of the body. The apex of the lung and regional lymph node being favored sites. Extra pulmonary TB of the pleura, lymphatics, bone, genito-urinary system, meninges, peritoneum or skin occurs in about 15% of TB patients.[3]

Events following entry of bacilli:

- Surface binding of M.tuberculosis to macrophages.
- Phagosome – lysosome fusion.
- Mycobacterial growth inhibition / killing.
- Recruitment of accessory immune cells for local inflammatory response.
- Presentation of antigens to T cells for development of acquired immunity.[3]

Binding of M.tuberculosis to Monocytes / Macrophages

Complement receptors (CR1, CR2, CR3, & CR4), mannose receptors (MR) and other cell surface receptor molecules play an important role in binding of the organisms to the phagocytes.[3] M.tuberculosis enter macrophages by endocytosis mediated by several macrophage receptors. Mannose receptors (MR) bind lipoarbinomannan, a glycolipid in the bacterial cell and complement receptors bind opsonized mycobacteria.[23]

Phagolysosome fusion

Phagocytosed microorganisms are degraded by intra-lysosomal acidic hydrolases upon phagolysosome fusion. This highly regulated event constitutes a significant antimicrobial mechanism of phagocytes. Prevention of phagolysosome fusion is a mechanism by which M.tuberculosis survives inside macrophages.[3] Once inside the

macrophage, M.tuberculosis replicates within the phagosome by blocking fusion of phagosome and lysosome.[23]

This is an active process as live but not dead mycobacteria block phagolysosome formation, which involves inhibition of Ca^{2+} signals and blocking recruitment and assembly of the proteins which mediate phagosome lysosome fusion and mycobacterial sulphatides, derivatives of multiacylated trehlose 2-sulphate, have the ability to inhibit phagolysosome fusion.[3,23]

Thus, the earliest stage of primary TB (<3 weeks) in the non-sensitized individuals is characterized by proliferation of bacteria in the pulmonary alveolar macrophages and air spaces, with resulting bacteremia and seeding of multiple sites. Despite the bacteremia, most patients at this stage are asymptomatic or have a mild flu like illness.[23]

Macrophages by generating various antimycobacterial effector molecules like reactive oxygen intermediates (ROI)and reactive nitrogen intermediates (RNI) handle the engulfed M.tuberculosis.[3]

Evasion of host immune response by M.tuberculosis.

The immune evasion strategies are :

1) Modulation of phagosome by modulation of antigen presentation to avoid elimination by T cells.[3,27]
2) Proteins secreted by M.tuberculosis such as superoxide dismutase & catalase are anatagonistic to ROI.
3) Mycobacterial components such as sulphatides, LAM and phenolic glycolipid-I are potent oxygen radical scavengers.

4) M.tuberculosis infected macrophages appear to be diminished in their ability to present antigens to $CD4^+$ T cells, which leads to persistent infection by production of inhibitory cytokines like TGF- beta , IL-10 or IL-6.[3,27]

HIV –TB CO INFECTION

Studies have shown higher incidence of TB among HIV infected individuals. Persons with HIV infections are at increased risk of rapid progression of recently acquired infection as well as re-activation of latent infection. TB is the commonest opportunistic infection occurring among HIV positive persons. 60 to 70% of HIV positive persons will develop TB in their lifetime. Differences in HIV positive TB as opposed to HIV negative TB include a higher proportion of cases with extra pulmonary or disseminated disease.[3]

TB and HIV infections are both intracellular and known to have profound influence on the progression of each other. HIV infection brings about the reduction in CD4+ T cells, which play a main role in immunity to TB and hence formation of granuloma. TB infection also accelerates the progression of HIV disease from asymptomatic infection to AIDS to death.[3]

A potent activator of HIV replication within T cells is TNF-α, which is produced by activated macrophages within granuloma as a response to tuberculosis infection.[3]

Alternative diagnostic test, based on serology, using crude mycobacterial antigens, purified lipid and protein antigens, have been tried with varying results. Results with purified 38, 30, 16 & 27 kDa antigens to study the antibody response to different isotypers, have yielded an improved sensitivity and specificity.[3]

Since the CD4+ receptors of the T cells are bound by HIV through the gp 120 antigen, interaction of these cells with APC (antigen presenting cells) presenting antigen in the context of class II MHC (major histocompatility complex) molecules is impaired, which results in hypo responsiveness to soluble tubercle antigens.[3]

Fine Needle Aspiration Cytology

The primary purpose of FNAC of an abnormal peripheral lymph node is to decide whether or not surgical excision for histological examination is necessary.[28] The cytological examination can decide whether lymphadenopathy is due to reactive hyperplasia, infections, metastatic malignancy or lymphomas.

The first lymph node aspirations for diagnostic purpose were performed at the beginning of the century but it was not until 1921 that Guthrie described the morphological diagnostic elements for several pathological entities. In 1927, Forkner published an elaborate study in lymph node aspiration cytology and between 1930 and 1940 the works of Martin and Willis, Pavlovski, Stahel, Rohr and Weil appeared.[29,30]

Knowledge of the structural, histological features of normal lymphnode is essential in the evaluation of FNA smears from enlarged nodes, whether the pathology is reactive, infective or due to a lympho-proliferative disorder. A brief outline of the structure of a normal lymphnode is therefore included following by a more detailed description of the normal cell population.[31]

The lymphnode parenchyma is surrounded and divided by a fibrous capsule with attached septa. Anatomically it is divided into outer cortex, an inner medulla and an intervening paracortex and sinus system.[30]

The cortex contains primary and secondary follicles, the proportions varying with the state of activity of the node. Primary follicles, composed of aggregates of small resting B cells, are found in the unstimulated node. Secondary follicles develop after antigen stimulation and are composed of a narrow mantle zone of small B lymphocytes surrounding a germinal centre. Several types of cells are found in the germinal centre, the vast majority being B cells in the form of centroblasts and centrocytes. Macrophages containing phagocytosed cellular debris are also present.[30]

Mature immunoglobulin secreting B cells, familiar as plasma cells, are the principal cell type found in the medulla. The paracortex contains many small lymphoid cells which are of T-phenotype. In addition activated T-cells and immunoblasts are also present.[30]

Normal Cytology

The lymphocytes constitute 87%-99%, the plasma cells 0-5%, and remainder cells form 1%-3% of the total population in the aspirate from a normal lymphnode.[31]

1. Mature lymphocytes

The small, round lymphocyte measures 7 -10 µ in MGG (May grunwald giemsa) stained smears. It has thin rim of pale cytoplasm often visible at one edge of the cell and nuclei characterized by blocks or clumps of dense chromatin.[31]

2. Follicular center cells

Follicular cells are of following types:

(a) Small cleaved lymphocytes: These are 10-12 µ in diameter, their nuclei show deep indentation of nuclear membrane, and hence the term cleaved and is surrounded by a moderate amount of cytoplasm.[31]

(b) Small non-cleaved lymphocytes: These are larger measuring 12-20µ in diameter, they are round and have a scanty amount of cytoplasm unlike mature lymphocytes and their non-cleaved nuclei contain prominent nucleoli.[31]

(c) Large cleaved lymphocytes: These are round cells 20-40 µ in diameter and nuclei appear cleaved as a result of a deep indentation of the nuclear membrane and nucleoli are not prominent and the cells have abundant basophilic cytoplasm.[31]

(d) Large non-cleaved lymphocytes: These are 20-40µ in diameter. A moderate amount of cytoplasm surrounds a large round vesicular nucleus. The nucleus has smooth outline and contain two or three peripheral nucleoli.[31]

3. Immunoblast

These are large cells measuring 20-40µ in diameter, three times larger than small lymphocytes. The nuclei are round with irregular, finely granular chromatin and one or more eccentrically placed nucleoli, and the surrounding cytoplasm is abundant with cytoplasmic vacuolization.[31]

4. Plasma cells

In plasma cells the nuclei are eccentrically placed and possess densely packed coarse chromatin that may be arranged in a typical cart-wheel like pattern and the cytoplasm is deeply basophilic and contains a para-nuclear clear zone.[31]

5. Macrophages

These cells show wide variation in size, in the resting phase the cells measure 14-34µ in diameter. Those containing phagocytosed fragments of degenerated cells (tingible bodies) may measure upto 50µ. The nucleus measures about 13 µ in diameter and contains evenly

distributed reticulated chromatin, one to three small nucleoli may be present and at times such macrophages possess two or more nuclei.[31]

Other cells like interdigitating reticulum cells, endothelial cells can also be seen in normal lymphnode aspirate. Eosinophils, plasma cells and neutrophils may also be seen depending on the pathologic process.[31]

6. Lymphoid globules (lymphoglandular bodies)

They are the rounded cytoplasmic fragments measuring up to 8µ in diameter and scattered in the background. The fragments stain an even pale blue identical to the cytoplasm of intact cells with Giemsa stain. They are characteristic finding in smears of lymphoid tissue. They differ from necrotic debris by their regular round shape and their uniform staining.[29]

All cases of lymphadenopathy may be due to non neoplastic benign processes, primary neoplasms or metastatic diseases. FNAC may be useful in the diagnosis of such lesions. A positive FNAC diagnosis may be the first indication of a tumor and may be useful in further clinical investigation to search for occult neoplasm. As the management decision depends on the type of tumor or non tumourous process, it is important that the FNAC diagnosis for the cause of lymphnode enlargement be as accurate as possible. Study showed overall sensitivity of FNAC was 92.7% and specificity of 98.5%. FNAC is recommended as a good screening procedure in all cases of lymphadenopathy.[32]

In a retrospective analysis of 1448 lymphadenopathy cases for FNAC study emphasized its increasing popularity as the first line diagnostic procedure in the evaluation of lymphadenopathy as shown by four fold increase in number of FNAC performed in recent years.[33]

FNAC can be employed at the bedside as well as in the outpatient department without giving any prior anesthesia or any fear to the patient. Even this technique is superior to lymphnode biopsy in the sense that samples can be taken at different sites at different times without much inconvenience to the patients.[34]

In most cases, architectural distortion is minimal or absent with this procedure and hence if biopsy becomes necessary it can be done without delay.[33]

FNAC samples, not only provides an on-spot provisional diagnosis and interpretation of the adequacy of the sample. Further more, the aspirated samples can be utilized for additional studies, such as immuno marker and histochemical studies on cell blocks or cytospin preparations to enhance the diagnostic accuracy of FNAC findings.[36]

FNAC emerged as a safe and reliable diagnostic procedure in pediatric group obviating the need for excision biopsy. It has avoided unnecessary surgical trauma to the child allowing selection of the best lymphnode for excision biopsy whenever needed. No significant complications were encountered in any of the cases.[37]

FNAC of tuberculous lymphadentitis:

The cytological features of TB on FNAC might be a reflection of pathogenesis of basic lesion of the disease process viz. the granuloma.[38]

The most common cause of granulomatous lymphadenitis in developed countries is sarcoidosis but in many tropical counties and patients with immunodeficiency, most common cause is TB. Other conditions like leprosy, cat scratch disease, paracoccidiodomycosis, histoplasmosis, leishmaniasis, LGV (Lympho Granuloma Venerum), brucellosis, tularemia

and rarely foreign bodies such as talc or silica can also give rise to granulomatous lymphadenitis.[30]

Cytomorphological features of tuberculous lymphadenitis shows three major patterns:

1. Epithelioid granulomas with caseous necrosis
2. Epithelioid granulomas without caseous necrosis
3. Necrosis alone without epthelioid granulomas.[9,37]

Epithelioid granulomas are small clusters of epithelioid histiocytes which have elongated nuclei picturesquely described as banana, foot print or carrot shaped, arranged in syncytial fashion with abundant ill defined cytoplasm and single forms mixed with reactive lymphocytes with or without Langhan's giant cells.[30]

Langhan's giant cells are large multinucleated cells with their nuclei polarized in an arc at one part of the cell border or in horse shoe shaped pattern.[30]

Caseous necrosis appears granular pale stained amorphous material in the background and lacks recognizable cell remnants.[29,30] For definitive diagnosis, AFB should be identified using Z-N stain or other stains for AFB and confirmed by culture.[30]

Table No.1

Cytodiagnositc criteria for tuberculous lymphadenitiss[9]

Cytologic feature	Z-N stain	Diagnostic label.
A. Epithelioid cells ± Multinucleated giant cells ± necrosis	a) AFB positive b) AFB negative	a) Tuberculous lymphadenitis b) Granulomatous lymphadenitis likely to be of tuberculous etiology, however stain for AFB is negative.
B. Necrotic material without epithelioid cells	a) AFB positive. b) AFB negative	a) Tuberculous lymphadenitis b) Repeat FNAC advised for cytodiagnosis, Z-N staining and / or for culture of AFB.

FNAC was able to make the diagnosis of TB by demonstrating mycobacteria in a high proportion of cases. Even without mycobacteria on a Z-N stain, the cytological features of granulomatous inflammation and or caseous necrosis on FNAC are sufficient to support the initiation of treatment in endemic areas. It helps to avoid unnecessary complications due to surgical interventions and avoids the delay of chemotherapy. Hence FNAC is generally considered to be an inexpensive, reliable and sole diagnostic tool in diagnosing tuberculous lymphadenitis.[40]

The characteristic necrotic background comprising of eosinophilic granular material containing nuclear debris was described as ‘Tubercular diathesis’. Many cases lacking the typical findings and showing scattered epithelioid cells with or without granuloma or only

necrotic material with neutrophilic infiltration were diagnosed as tuberculous lymphadenitis, even though AFB were absent in these smears.[33]

In a retrospective study, criteria required to make an FNAC diagnosis of TB were reassessed with 70 cases. The sensitivity of FNA cytology with histological confirmation emphasize that all criteria for the diagnosis of TB in FNA samples must be utilized and that particular caution should be exercised in making a diagnosis of acute necrotizing TB as various lesions can show acute necrotizing granulomatous inflammation including metastatic lesions.[41]

The FNAC smears of TB were hypocellular or in some cases, normocellular but never hypercellular. FNAC on correlation with histopathology showed that its diagnostic efficacy, particularly in tubercular lymphadenitis has been reported to be as high as histopathological studies.[42]

Although culture is considered confirmatory for diagnosis, a negative culture does not exclude diagnosis of tubercle bacilli, as enlarged lymph nodes do not necessarily contain live bacilli. In situations where funds are scarce and resources limited, FNA alone is taken as diagnostic of TB.[37]

Tuberculous involvement of intercostal lymphnodes may present either cold abscesses or as granulomatous inflammation. Such abscesses are commonly assumed to be soft tissue TB. Therefore, if necrotic material is aspirated or a granulomatous infection encountered in soft tissue swellings of the chest wall, particularly in the parasternal and axillary regions, the possibility of intercostal tuberculous lymphadenitis should be considered.[43]

The diagnosis of TB is early and simple, when the disease is florid or disseminated but localized involvement of extra pulmonary organ or tissue may at times pose a diagnostic problem. FNAC offers a simple, safe, rapid and accurate diagnosis in such cases.[40]

Isolation of AFB by staining

The detection of AFB by Z-N stain technique is the most commonly used modality especially in developing countries due to the cost involved, equipment required and time involved with other modalities.[44]

The cell wall of mycobacterium, because of its lipid content has the unique capability of binding the fuchsin dye so that it is not destained by acid alcohol. This acid fast staining reaction of mycobacteria along with their characteristic size and shape, is valuable aid in the early detection of infection and the monitoring of therapy for mycobacterial diseases.[45]

Aspiration of pus from a lymph node involves a differentiation between acute suppurative and tuberculous lymphadenitis. The cytologic pictures in these two conditions are identical – numerous neutrophils lying in an abundant, necrotic background. Differentiation between these two conditions depends upon the presence or absence of AFB. While all these cases should be thoroughly screened for AFB, one clinical observation is of great help: such tuberculous lesions present as cold abscesses, while acute, suppurative lymphnodes present as hot abscesses.[44]

For bacilli to be demonstrated in smears, their number should be minimum 5,000 to 10,000 per ml of material. If the number is less than this, they may not be detected in the smears.[10]

Cytomorphologic features of tuberculous lymphadenitis in lymphnode aspirates were correlated with AFB positivity and bacillary count using 174 cases. They analyzed that in the presence of lymphocytes, epithelioid cells and langhans giant cells, AFB positivity was significantly lower while the picture was just the reverse in presence of necrosis and neutrophilic infiltration.[9]

The activated macrophages aggregate around the lesion's center to form a granuloma and effectively neutralize tubercle bacilli without causing further tissue destruction. In the central part of the lesion necrotic material assumes the aspect of caseous necrosis. When the macrophage activating response is weak, tissue destruction occurs. The lesion tends to enlarge further and the surrounding tissue is progressively damaged. At the center of the lesion, the caseous material liquefies. This liquefied caseous material contains large numbers of bacilli.[44]

It was concluded that there is an inverse relationship between the presence of granuloma and AFB positivity. In the presence of granuloma, AFB detection helps to make a definitive diagnosis of TB while in the absence of granuloma, detection of AFB is the only indicator of TB.[44]

When the smears were devoid of granulomas and show features of only acute inflammation, tuberculous etiology can be established only by doing Z-N stain routinely.[46]

Cytodiagnosis supplemented with AFB smear and culture examination helps in establishing a definitive diagnosis. A negative mycobacterial examination does not exclude the possibility of TB is evident from our cases in which smears were positive for AFB but the culture were negative and vice versa. A negative culture examination can result from prior antituberculous therapy or inadequacy of the material submitted for culture.[47]

Many patients with initial diagnosis of suppurative lymphadenitis are treated with antibiotic therapy, which is of no use. When the lympadenopathy is persistent, repeated FNAC sometimes can establish a clue to the actual pathology. This study reveals a changing face of tuberculous lymphadenitis where patients present with sole corroborative features and the cytomorphology suggests a suppurative pathology and Z-N staining is the only tool to establish the tuberculous etiology.[46]

Thus FNAC coupled with Z-N staining for AFB is very useful tool in the diagnosis of tuberculous lymphadenitis and more so in immunosuppressed individuals.[19,48]

In immunosuppressed individuals the immunologic response may be ineffective due to HIV-induced low CD4+T lymphocyte counts and reduced functional capacity as well as abnormal macrophage function. As a result, granulomas may be poorly formed or absent, with areas of necrosis containing nuclear debris and polymorphonuclear cells, often in the absence of epithelioid histiocytes and giant cells. Therefore in these patients with suppurative lymphadenitis M.tuberculosis should remain a diagnostic possibility.[19]

Mycobacterial suppurative lymphadenitis is more typically associated with MAI. The immune response in MAI infections is typically suppurative necrosis with intracellular bacterial aggregates within foamy macrophages and no granulomas.[19]

A presumptive diagnosis of mycobacterial infection can be achieved by a careful examination of Romanowsky stained smears for the presence of the unstained images of AFB. The presence of mycobacteria as negative images has been detected in Romanowsky stained smears of peripheral blood, buffy coat, bone marrow aspirate & lymphnode aspirates. These images have also described in Gram and Geimsa stained sputum samples.[49]

In Romanowsky stained smears, mycobacterium also been seen as refractile, beaded rods. The refractivity can be enhanced when the condenser is lowered and light intensified. The negative images are often more abundant in necrotic debris, thick and crushed areas that are otherwise regarded as unfit for an interpretive opinion.[49]

Closer observation of these unstained images appears as curved, forked, bundled or criss cross images within the cytoplasm of histiocytes and occasionally are seen as linear structures overlying the nucleus. These images may also be seen at an extra cellular location. Acid fast stains are necessary to confirm the presumptive diagnosis of the images and it is mandatory to stain all suspected cases for them.[49]

In contrary to other mycobacteria including tubercle bacilli, MAI has a unique staining character in addition to Z-N stain, these organisms stain positively with PAS stain. This staining characteristic is of diagnostic importance since very few bacteria of medical importance including nocardia and corynebacterium are occasionally both acid-fast and PAS positive.[49]

The detection of AFB in stained smears is the easiest and most rapid procedure which includes Ziehl – Neelsen (hot stain), Kinyoun (Cold stain) and fluorochrome stains using auramine O, with or without a second fluorochrome, rhodamine.[45]

With carbol fuchsin, the AFB stain bright red against either a blue or green background, depending on the counter stain used. Although the Z-N and kinyoun techniques are theoretically the same, it has been experience of some that the former is more sensitive in detecting lightly staining organisms, particularly some of the rapidly growing mycobacteria.[46]

The property of acid fastness is due to the thick, waxy capsule that surrounds the mycobacterial cells. For aqueous carbol fuchsin to penetrate through the wax, the capsule must be softened. This is done with heat in the Z-N procedure, much like the melting of a paraffin film in hot rays of sun. Dye that penetrates the heat softened capsule binds to the cell wall; then when the bacteria cells cool after the heat is removed, the wax again hardens protecting the bound dye from the action of the acid alcohol decolorizer (acid – fast).[45]

Obviously mycobacterial cells that are endowed with a thin waxy capsule will be more susceptible to decolorization, as may be the situation with many rapidly growing strains. In these circumstances the use of a less stringent decolorizer, such as 1% HCI (partial acid fast procedure) may disclose the innate acid fast property.[45]

In the Kinyoun or cold technique, a surface active agent is used to increase permeability of the dye through the waxy capsule however, the reformation of the waxy film may be incomplete allowing most, if all of the bound dye to be extracted by the acid alcohol decolorizer.[45]

In cold staining method using the Gabbett's modification method, reagents used were carbol fuchsin, which was same as for the standard Z-N method and Gabbetts methylene blue (methylene blue 25 gm, distilled water 1250ml, absolute alcohol 1750ml, sulphuric acid 500ml). The slide was fixed and stained with carbol fuchsin as in the standard Z-N method. It was then decolorized and counter stained at the same time with Gabbetts methylene blue solution for 20-30 sec, washed with distilled water, air dried and examined. Each side was scanned for 100 oil immersion fields. The reporting was done according to recommendations of American thorican thoracis society 1981.[50]

Fluorochrome stained bacteria are bright yellow (auramine) against a dark background, allowing the slide to be scanned under lower magnification without losing sensitivity. Modification of auramine fluorochrome stain include the addition of rhodamine, giving a golden appearance to the cells, or the use of acridine as a counter stain resulting in red to orange background.[45]

False positive reactions may be due to fluorescence of nonspecific tissue or cellular debris that can be mistaken for bacilli with 25x objective. The 40x objective should be used to confirm any suspicious form. The sharp contrast between the brightly colored mycobacteria and the dark background offers a distinct advantage in scanning the slide.[45]

Dead mycobacterial cells will also stain rhodamine and auramine, leading to a smear positive, culture negative situation about 10% of the time. This feature is also important to remember when using acid fast smears to assess treatment efficacy. The presence of AFB in fluorochrome stained smears does not necessarily indicate treatment failure – and carbol fuchsin stains should also be performed. Fluorochrome stained smears can be stained subsequently with carbol fuchsin. The opposite situation does not apply.[45]

Carbol fuchsin and auramine O dyes used in these techniques, each function by binding to mycolic acids in the mycobacterial cell wall. Smears stained with the carbol fuchsin technique must be scanned with an oil immersion objective. This limits the total area of a slide that can be scanned with a 25x objective, there by increasing the field of view and reducing the time needed to scan a given area of the slide. Fluorochrome stained smears require a strong light source either a 1200W mercury vapour or a strong blue light with a fluorescein isothiocyanate (FITC) filter.[45]

It should be emphasized that neither the auramine not the auramine rhodamine fluorochrome stain is a fluorescent antigen – antibody technique, rather each is direct physico-chemical binding of the stain to the mycolic acid rich cell wall.[46]

The fluorochrome stain offers the advantage of greater sensitivity compared with the carbol fuchsin method, since a significantly larger area of the smear can be scanned per unit of time with the auramine fluorochrome stain.[46]

Method of Reporting of AFB[9]

The grading scale (Table 2), originally recommended for grading sputum smear for AFB was adapted in studies to grade smears of lymph node aspirates stained by the Z-N method.[9]

Table 2

Grading of AFB

No. of AFB Seen	Grade	Min. No. 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
0-9 in 100 oil immersion field	Scanty	200

Despite the recent advances in mycobacteriology, early lab diagnosis of TB still relies on the examination of stained smears. It remains the cornerstone of TB diagnosis in most part of the world. There are various concentration method for improving sensitivity of direct microscopy for detection of AFB of which the bleach concentration method is the safest and easy to perform even in the laboratories where facilities for class III laboratories are unavailable.[10]

The sensitivity of specimen microscopy can be significantly augmented after liquefaction of the specimen with sodium hypochlorite (NaOCl/bleach) commonly known as household bleach, followed by centrifugation.[10]

Khubani et al. studied 55 cases of EPTB of which 17 were from lymphnode aspirates showed significant rise in the sensitivity of positivity for AFB after bleach method and they concluded that bleach method forms a cost effective, sensitive, versatile and safe procedure for demonstration of AFB and is very valuable in diagnosing cases of extra pulmonary TB and would benefit the patients to receive an early and specific treatment.[10]

Gangane N. et al. studied 100 cases of TB lymphadenitis and concluded bleach concentration method demonstrated AFB positivity in 72 % of cases. AFB positivity grade was much higher than with routine Z-N staining making bacilli easily visible with shorter screening time. The bleach method was inexpensive, easily performed and more sensitive and safer than routine Z-N staining.[51]

Annam V et al. studied 93 cases of lympadenopathy. Among 93 aspirates 33.33% were positive for AFB in conventional Z-N method and the smear positivity increased to 63.44% on bleach method. They concluded bleach method as simple and inexpensive.[52]

METHODOLOGY

Source of data:

Out-patients, in-patients and referral cases with clinical suspicion of tubercular lymphadenitis sent for FNAC to department of pathology, BLDEA'S Shri. B.M Patil Medical College, Hospital & Research centre, Bijapur.

Study period: 1st November 2007 to 30th April 2009.

Sample size:

Sample size was calculated by using the formula $n = 4pq / L^2$.

With prevalence of tuberculosis infection 30%[1] at allowable error 30% the minimum sample size was 105.

This study was carried out on 120 patients with clinically suspected tuberculous lymphadenopathy, referred to the department of cytopathology.

Of these 120 cases 11 cases were eliminated from the study because 8 aspirates were diagnosed as malignancy and 3 aspirates were inadequate. Therefore total sample size was 109 cases.

Study included cases of clinically suspected tuberculous lymphadenitis of all the age groups.

Methods of collection of data

Methods:

Patients presenting with lymphadenopathy were subjected to brief clinical examination. Data regarding age, sex, duration, description of swelling like site, number, size and association with HIV were documented for each patient.

An informed consent was taken from the patient after explaining the procedure.

After explaining the procedure to the patient, FNAC was performed under strict aseptic precautions. Aspiration was carried out with patient in lying position which exposed the target area. After stretching the overlying skin, palpable lymph node was held between two fingers of one hand and other hand used for manipulation of syringe. Disposable syringe of 10 ml with attached needle of No.22 gauge was used. Needle tip inserted into the target; suction applied by retracting the piston of syringe and the needle tip was moved in different directions within the boundaries of target to collect material. After collecting the sample, suction was released before withdrawing the needle. This allowed the collected material to stay within the needle and syringe tip. After the needle was withdrawn from the lesion, the needle was removed from the syringe and the piston was pulled back. Then, reattached the needle and expelled the material on the new clean slides and smeared gently using another clean slide. The gross appearance of aspirate was noted in each case.

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Conventional cytology technique:

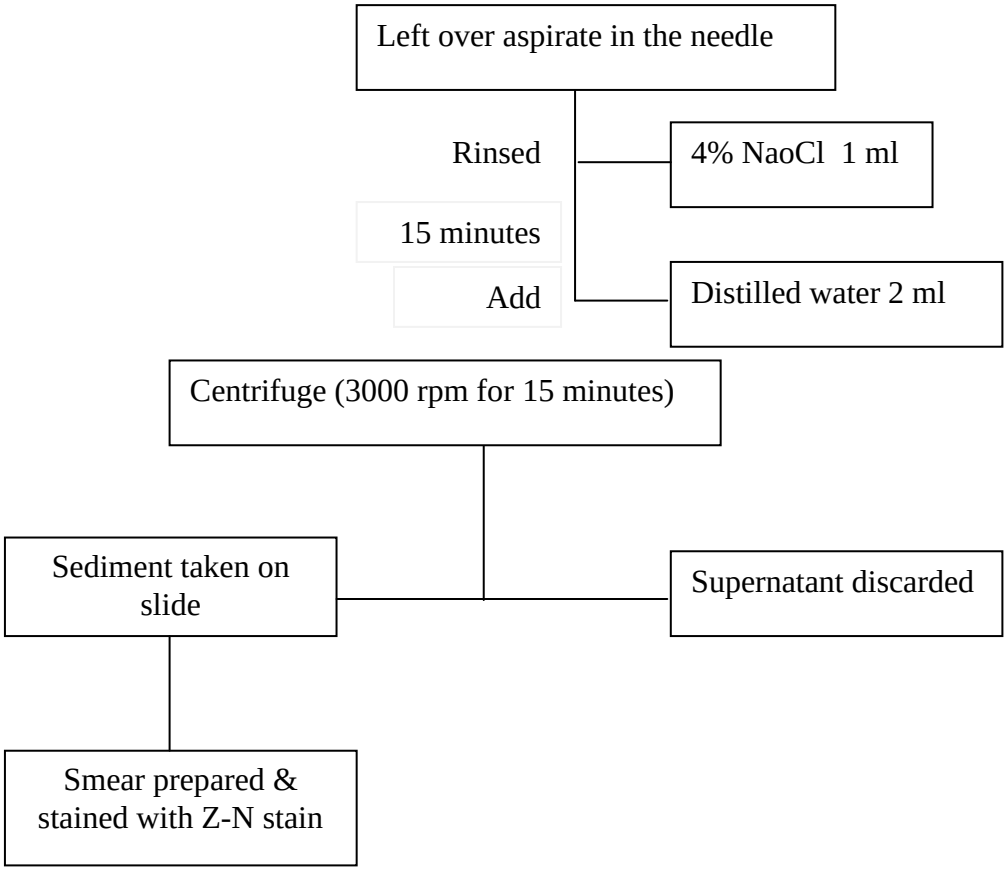
After fine needle aspiration of lymph node, sample was processed for direct microscopy by staining with H &E, Giemsa, Pap and conventional Ziehl-Neelsen stain. Ziehl-Neelsen stained smears were examined for presence of tubercle bacilli. Other smears were studied for cytomorphological evidence of tuberculosis.

Bleach concentration technique:

Left over aspirate from the needle was mixed with 1ml of 4% sodium hypochlorite. After thorough mixing the mixture was incubated for 15 min. at room temperature with frequent mixing at intervals.

An equal volume of distilled water was added and mixed thoroughly and then centrifuged at 3000 rpm for 15 min. The supernatant was discarded and smear was prepared using one drop of the sediment, air dried, heat fixed and stained by Ziehl- Neelsen staining technique. As a control 2ml of distilled water was centrifuged and the sediment was stained by Ziehl-Neelsen technique. Smears were examined under 100X for presence of tubercle bacilli.

Procedure:



Inclusion criteria:

Clinically suspected cases of tubercular lymphadenitis of all age groups.

Exclusion criteria:

Lymphadenopathy which was not suspected to be due to tuberculosis.

Statistical methods

1. Test of significance by Chi-Square (X^2) test.
2. Diagrammatic representation.
3. Sensitivity, specificity and positive predictive value.

RESULTS

Tuberculous lymphadenopathy is the most common form of extra pulmonary tuberculosis. The present study was undertaken to emphasize the role of bleach concentration method over conventional direct smear microscopy for detection of tubercle bacilli in fine needle aspiration material of lymph nodes. This study was carried out on 120 patients with clinically suspected tuberculous lympadenopathy, referred to the department of cytopathology.

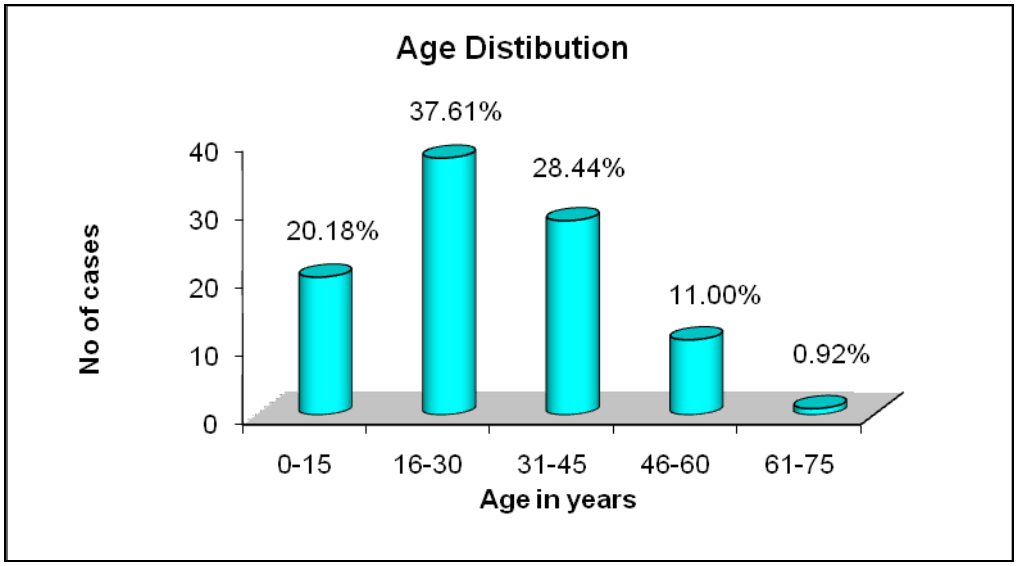
Of these 120 cases 11 cases were eliminated from the study because 8 aspirates were diagnosed as malignancy and 3 aspirates were inadequate and 109 clinically suspected tuberculous lymphadenitis cases evaluated and the results were analysed as follows :

Table No 3

Age Distribution

Age Distribution (In years)	**No. of cases**	**Percentage**
0-15	22	20.18
16-30	41	37.61
31-45	31	28.44
46-60	12	11.00
61-75	01	0.92
Total	109	100%

Graph No 1



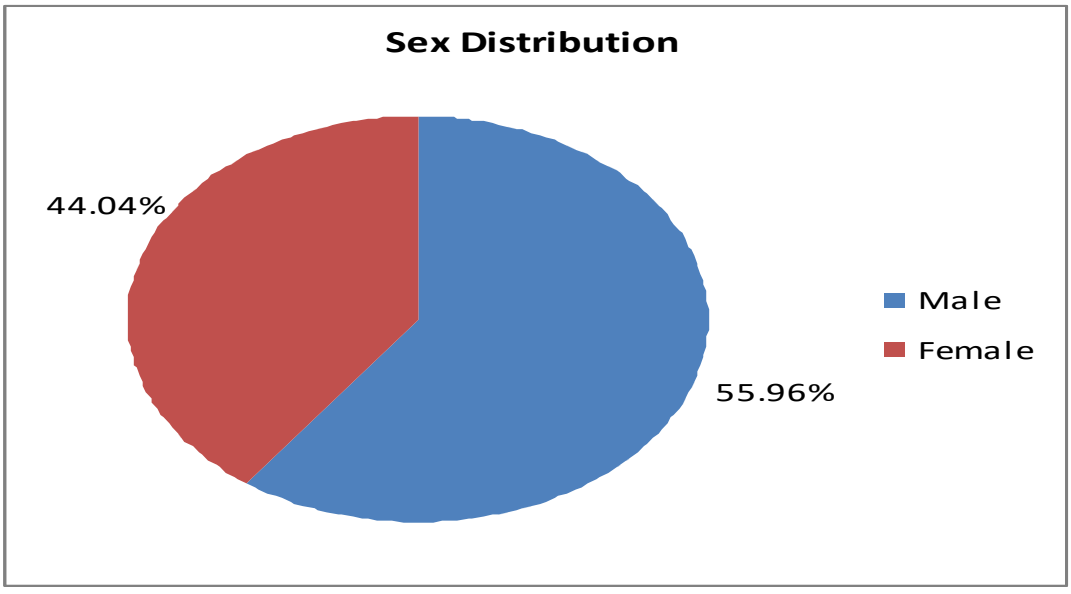
Most patients were in the age group of 16 – 30 years constituting 37.61%, followed by age group of 31-45 years constituting 28.44%.

The youngest patient was 1 year old. The oldest patient was 64 year old.

Table No 4. Sex Distribution

Sex	No of Cases	Percentage
Male	61	55.96%
Female	48	44.04%
Total	109	100%

Graph No 2



M : F ratio 1 : 0.78

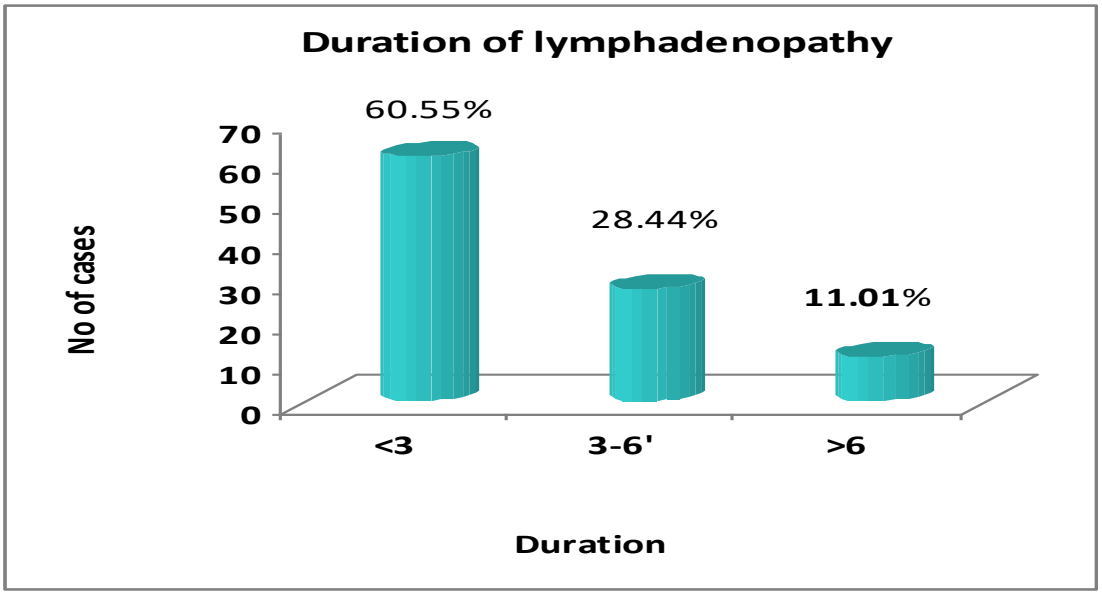
A male preponderance was seen in this study. (55.96%).

Table No 5

Duration of lymphadenopathy

Duration(In months)	No of cases	Percentage
< 3	66	60.55
3 – 6	31	28.44
> 6	12	11.01
Total cases	109	100%

Graph No 3



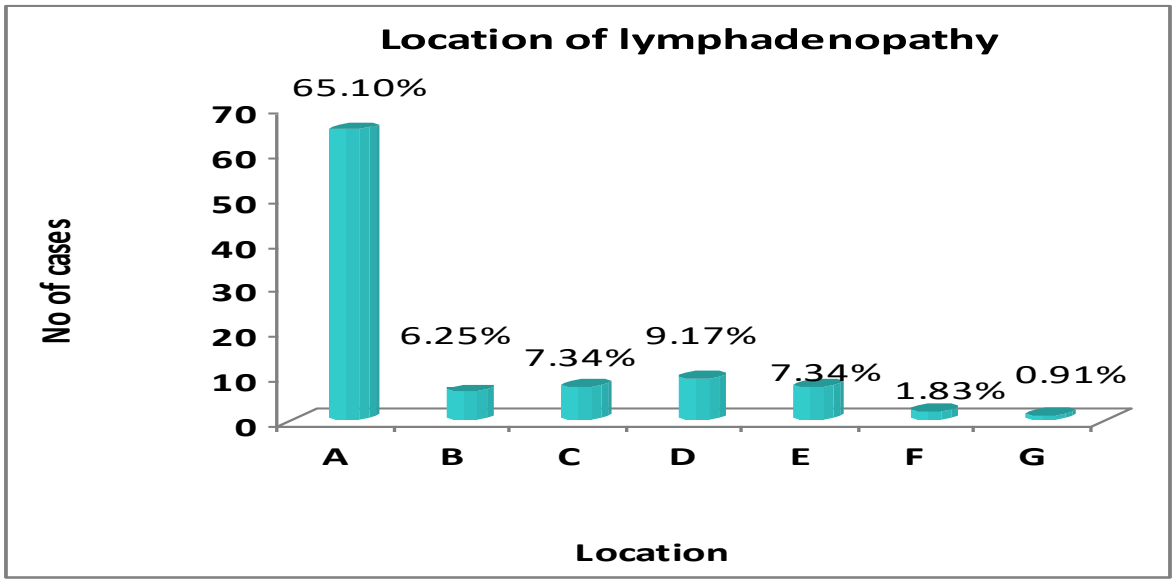
Most (66) Patients had lymphadenopathy of less than 3 months duration 60.55%, followed by 3 – 6 months duration in 31 patients (28.44%) and more than 6 months in 12 patients (11.01%).

Table No 6.

Location of Lymphadenopathy

Location	No of cases	Percentage
Cervical	**71**	**65.1%**
Supraclavicular	09	6.25%
Submandibular	08	7.34%
Axillary	10	9.17%
Inguinal	08	7.34%
Preauricular	02	1.83%
Intercostal	01	0.91%
Total cases	**109**	**100%**

Graph No 4



A) Cervical B) Supraclavicular C) Submandibular D) Axillary

E) Inguinal F) Preauricular G) Intercostal

Majority of patients presented with lymphadenopathy of cervical region,seen in 71 patients (65.10%) followed by axillary region, seen in 10 patients (9.17%).

Least number of patients seen in preauricular and intercostal regions.

Table No 07
Lymphadenopathy associated with HIV

Total cases Lymphadenopathy	Associated with HIV	Percentage
109	12	11%

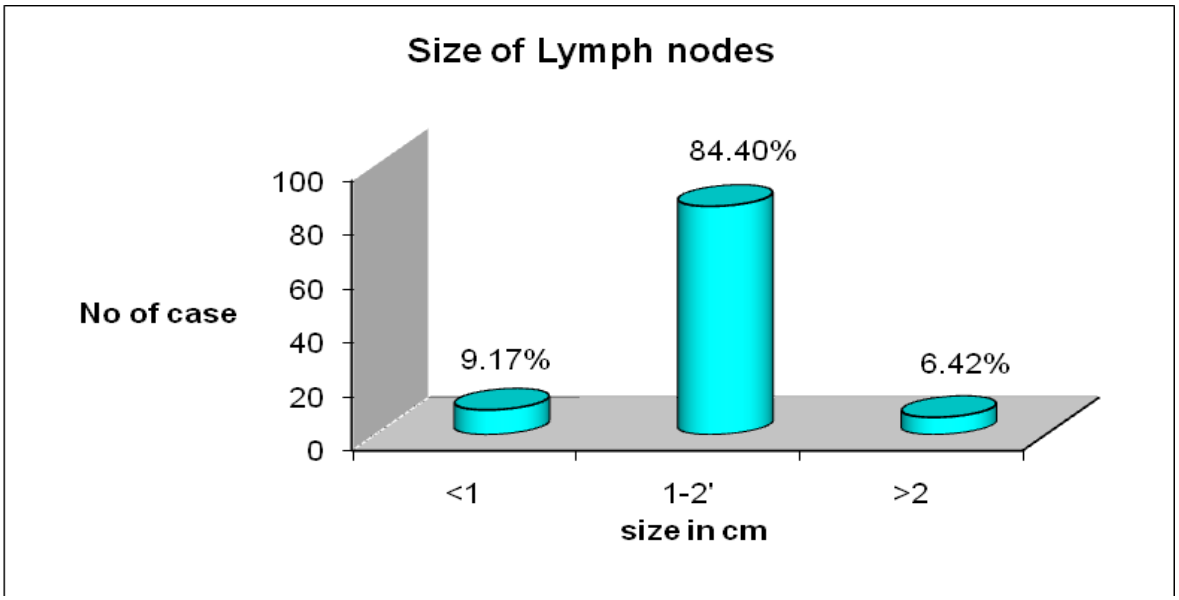
In the Present study 12 cases presented with associated HIV infection.

Table No 8

Size of lymph nodes

Size	No of cases	Percentage
< 1 cm	10	9.17%
1 – 2 cm	92	84.40%
> 2 cm	07	6.42%
	109	100%

Graph No 5



Majority of patients 84.40% presented with lymphnodes of 1 – 2 cm in size.

10 patients (9.17%) had lymphnodes of size less then 1 cm .

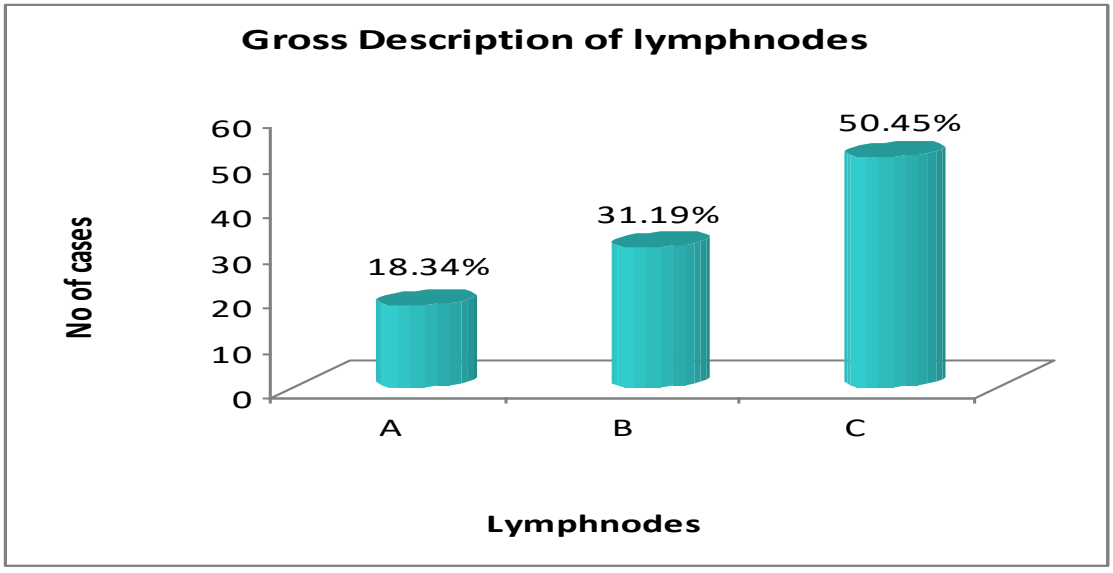
7 patients(6.42) had lymphnodes of size more than 2 cm.

Table No 9

Gross Description of lymphnodes

Lymphnodes	**No of cases**	Percentage
Single	20	18.34%
Multiple discrete	34	31.19%
Multiple matted	55	50.45%
Total cases	109	100%

Graph No 6



A) Single **B) Multiple discrete** **C) Multiple matted**

55 Patients (50.45%) had multiple matted lymphnodes, and 34 Patients (31.19%) had multiple discrete lymph nodes.

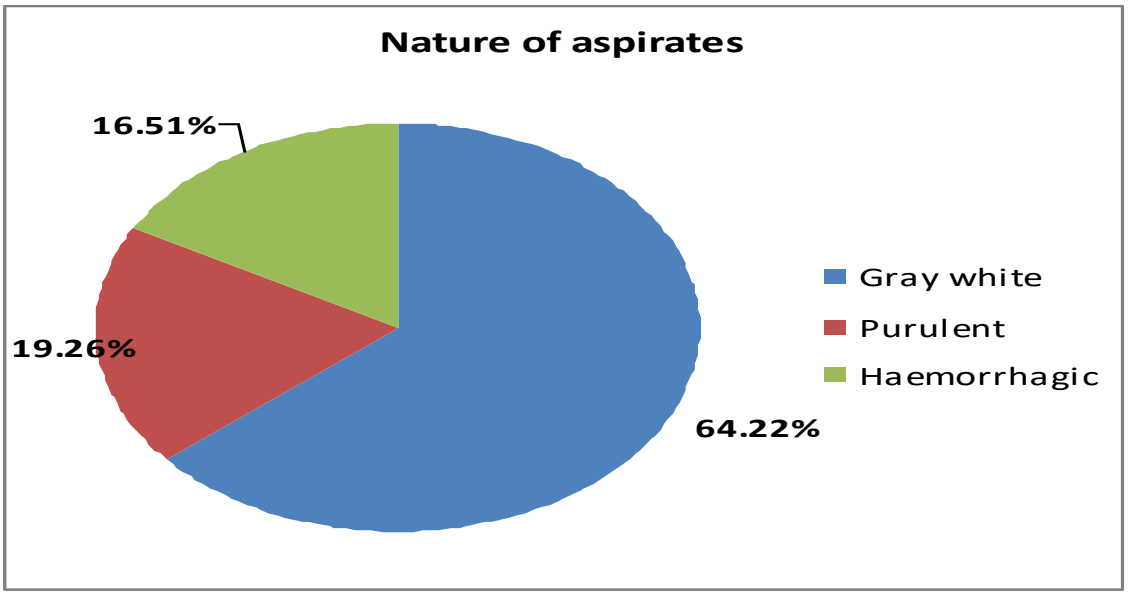
20 Patients (18.34%) had solitary lymphadenopathy.

Table No 10

Nature of aspirates

Nature	No of cases	Percentage
Grey white	70	64.22%
Purulent	21	19.26%
Haemorrhagic	18	16.51%
Total cases	109	100%

Graph No 7



Aspirate was grey white and granular in 70 cases (64.22%) constituting the major group followed by purulent aspirate in 21 cases. (19.26%).

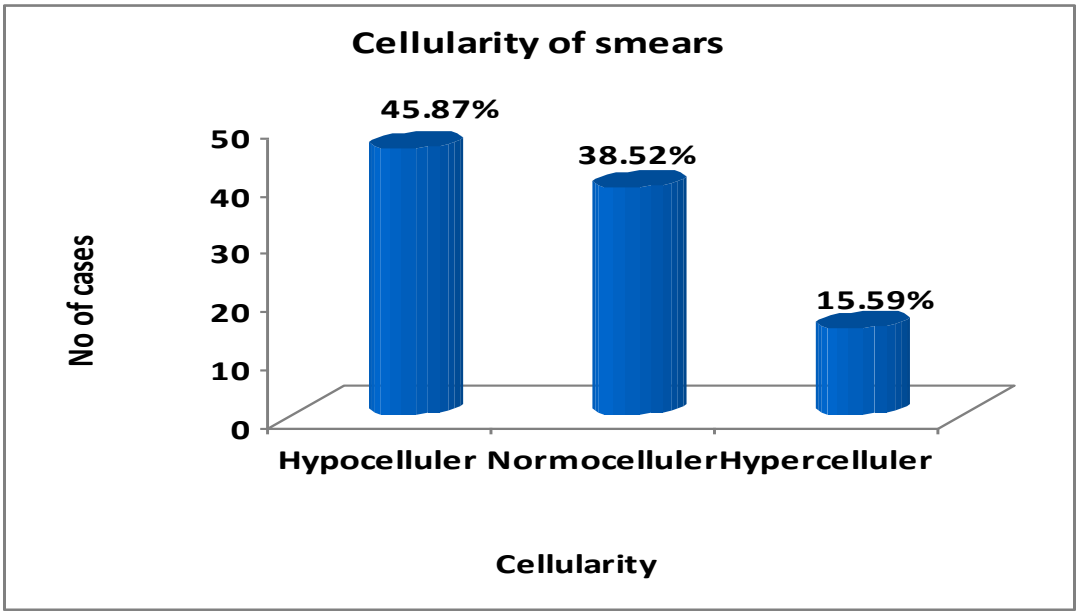
Haemorrhagic aspirate was seen in 18 patients constituting 16.51%). All smears were stained with H & E, Papaicoloau and Z – N stains and cellularity was assessed as follows.

Table No 11

Cellularity of Smears

Cellularity	No of cases	Percentage
Hypocelluler	50	45.87%
Normocelluler	42	38.52%
Hypercelluler	17	15.59%
Total cases	109	100%

Graph No 8



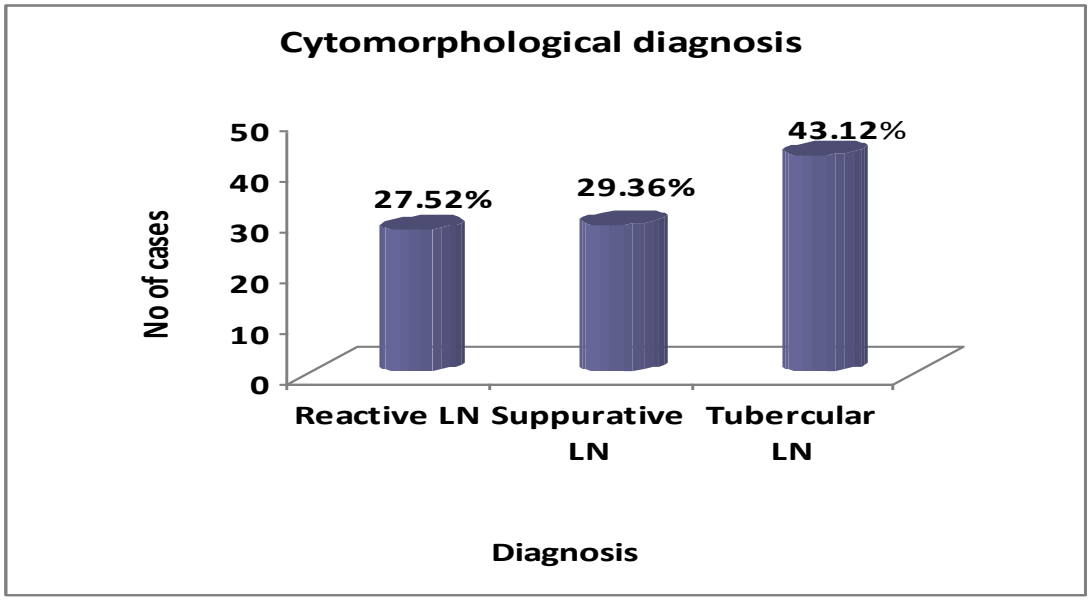
Smears were predominantly hypocellular in 50 cases (45.87%), normocellular in 42 cases and (38.53%) hypercellular in 17 cases. (15.59%).

Table No – 12

Cytomorphological diagnosis

Diagnosis	No. of cases	Percentage
Reactive LN	30	27.52%
Suppurative LN	32	29.36%
Tubercular LN	47	43.12%
Total	109	100%

Graph No 9



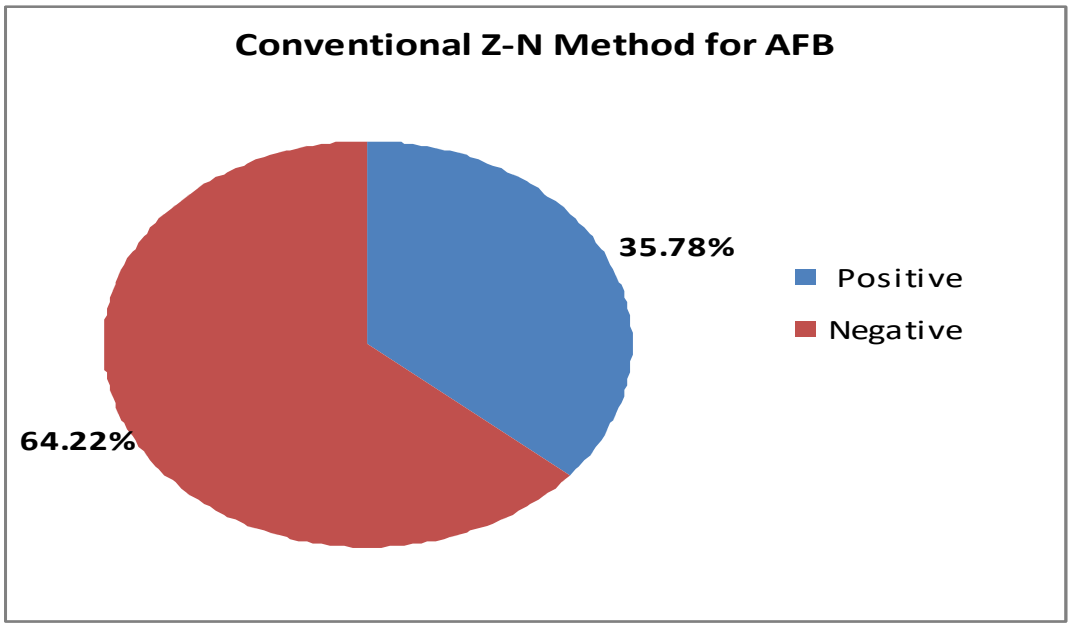
Majority of cases i.e. 47 (43.12%) were cytomorphologically diagnosed as TBLN, followed by 32 cases (29.36%) as suppurative LN and 30 cases (27.52%) as reactive LN.

Tables No – 13

Conventional Z-N method for AFB

Conventional Z-N	No. of cases	Percentage
AFB Positive	39	35.78
AFB Negative	70	64.22
Total	109	100%

Graph No 10



In the present study total 39 cases were AFB positive and 70 cases were AFB negative by conventional Z-N method.

Bleach concentration method

In the present study, newly recommended Bleach Concentration method was utilized for detection of AFB in the diagnosis of tuberculous lymphadenitis.

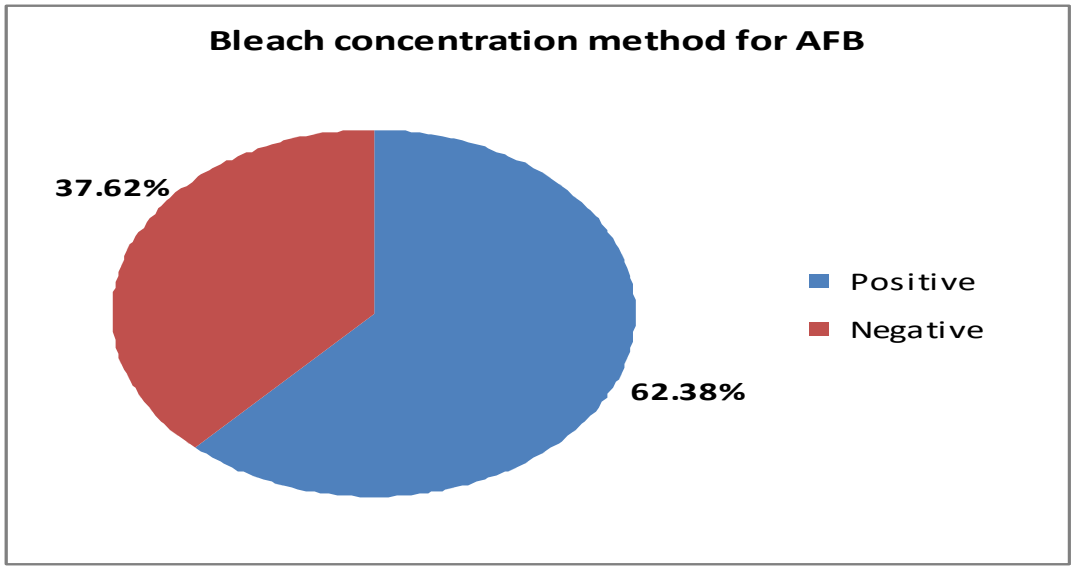
Aspirates of all 109 cases suspected of tubercular lymphadenitis were subjected for bleach concentration method and smears stained by Z-N method and searched for acid fast bacilli.

Table No -14

Bleach concentration method Z-N staining

Bleach concentration	No of cases	Percentage
AFB Positive	68	62.38
AFB Negative	41	37.62
Total	109	100%

Graph No 11



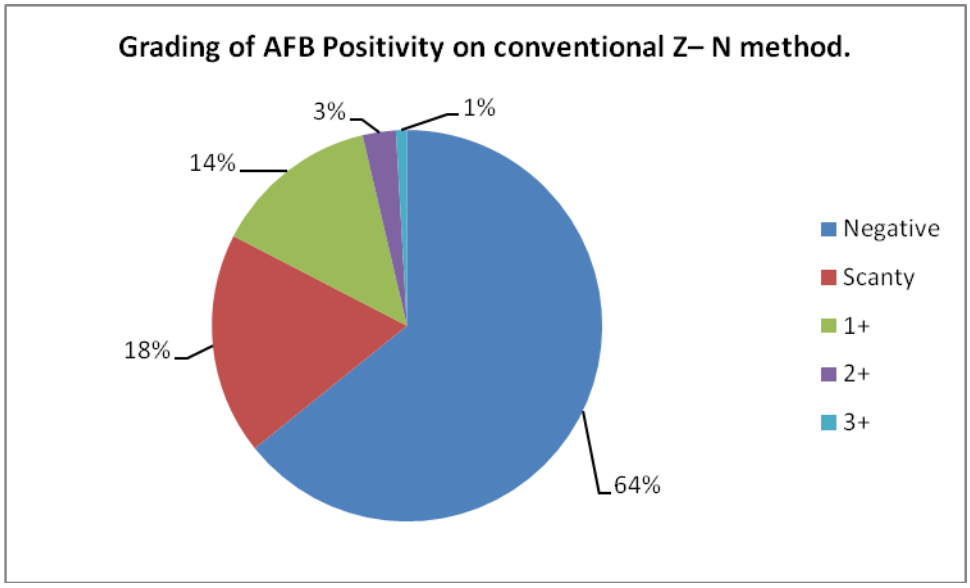
In the present study total 68 cases were AFB positive and 41 cases were AFB negative by bleach concentration method.

Table No 15

Grading of AFB Positivity on conventional Z– N method.

Grade	No of cases	Negative	Scanty	1+	2+	3+
AFB status	109	70	20	15	03	01
Percentage	100%	64.22%	18.34%	13.76%	2.75%	0.91%

Graph 12



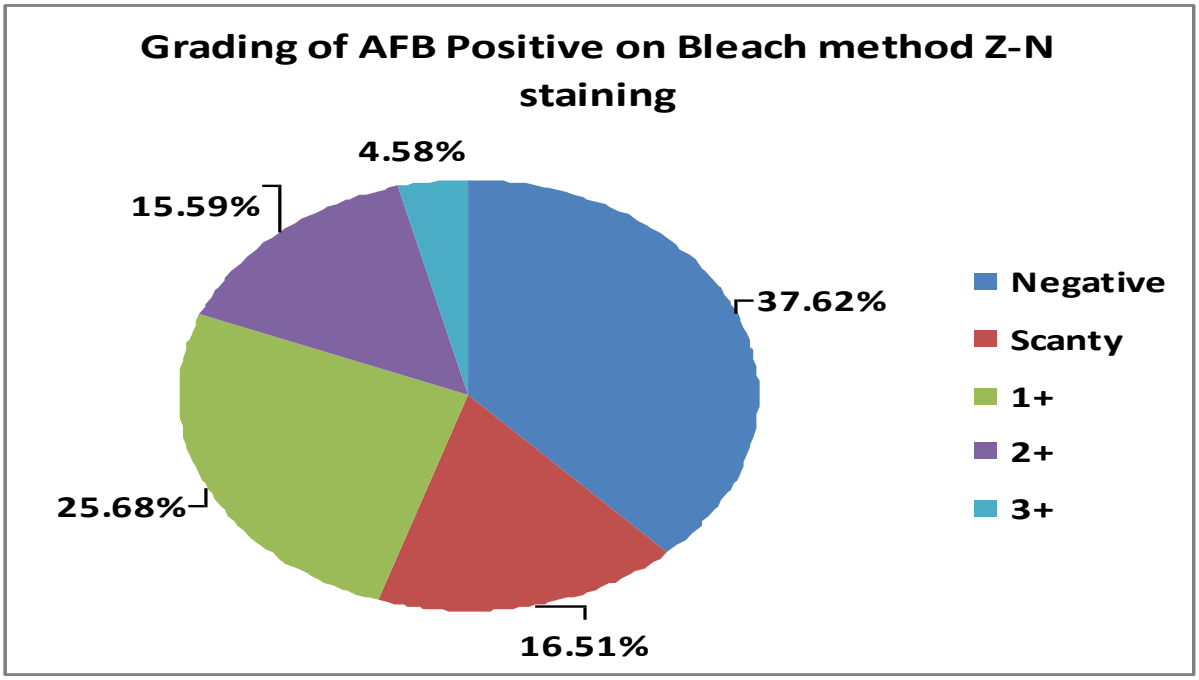
In the present study total 39 cases were AFB positive and majority of them (20) were of grade scanty , 15 cases of 1+, 3 cases of 2+ and only one case was of grade 3+.

Table No 16

Grading of AFB Positivity on Bleach Concentration method Z-N staining.

Grade	No of cases	Negative	scanty	1+	2+	3+
AFB status	109	41	18	28	17	05
Percentage	100%	37.62%	16.51%	25.68%	15.59%	4.58%

Graph - 13



In the present study total 68 cases were of AFB positive by bleach concentration method and majority (28cases) of them were of grade 1+, 18 cases of grade scanty, 17 cases of 2+ and 5 cases of grade 3+.

Of these total 109 aspirates the cytomorphological features observed were reactive lymphadenitis in 30 cases(27.52%), acute suppurative lymphadenitis in 32 cases(29.36%) & tubercular lymphadenitis in 47 cases(43.12%). There was a statistically correlation of significance(x^2 =9.18, df=3, p <0.05) between cytomorphological diagnosis, results of smears prepared by the conventional Z-N method and the bleach method

Table No 17

Correlation of Cytomorphological diagnosis with the Bleach method and the Conventional Z-N method.

Cytomorphological Diagnosis	Bleach method		Conventional Z-N method		Total
	Positive for AFB	Negative for AFB	Positive for AFB	Negative for AFB	
Reactive LN	07	23	0	30	30
Suppurative LN	18	14	04	28	32
TB LN	43	04	35	12	47
Total	68	41	39	70	109

Correlation of significance: x^2 =9.18, df=3, p <0.05

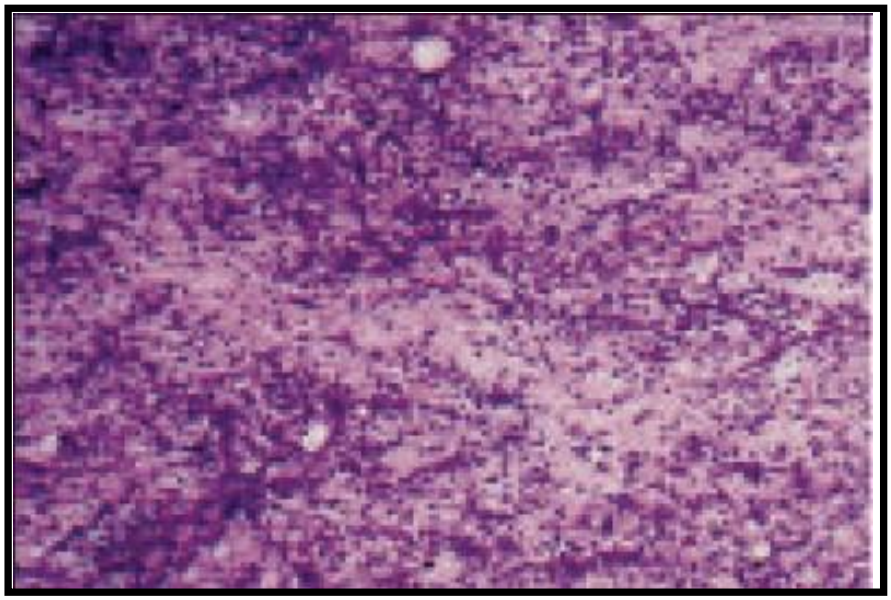


Fig. No. 1.

Microphotograph showing features of suppurative lymphadenitis (H & E X 100)

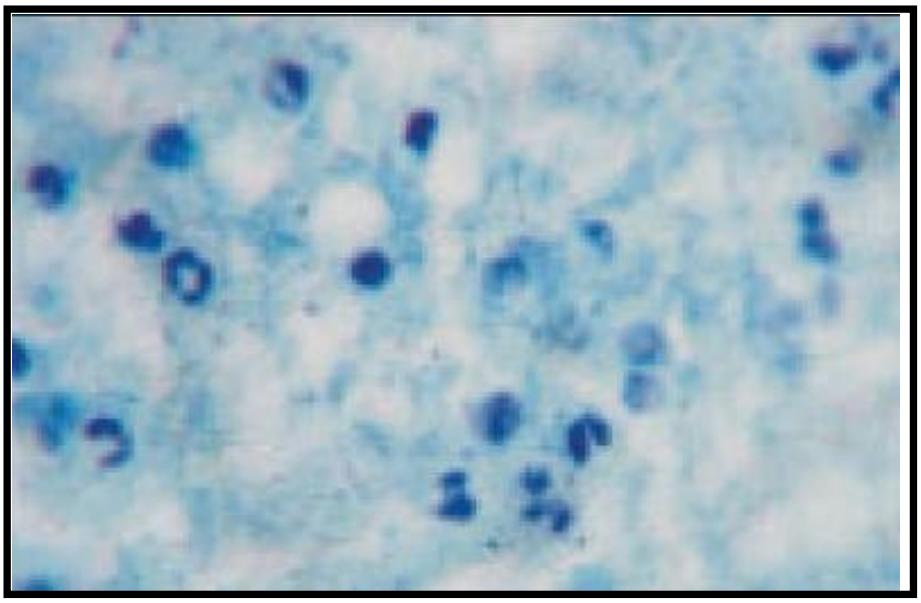


Fig No. 2

Microphotograph showing Negative for AFB by conventional method (Z-N X 1000)

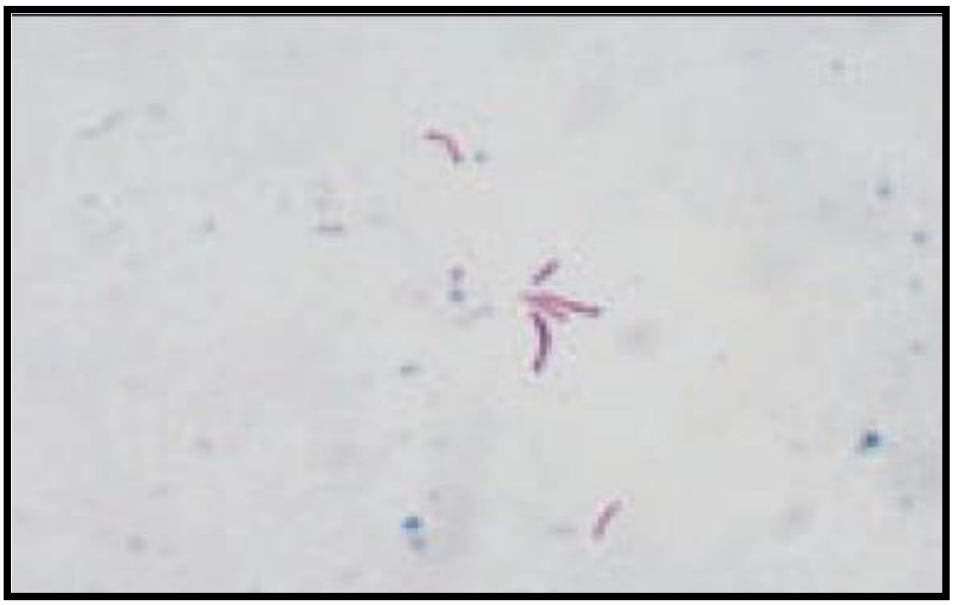


Fig. No. 3
Microphotograph showing AFB at the centre with clear background by bleach method (Z-N X 1000)

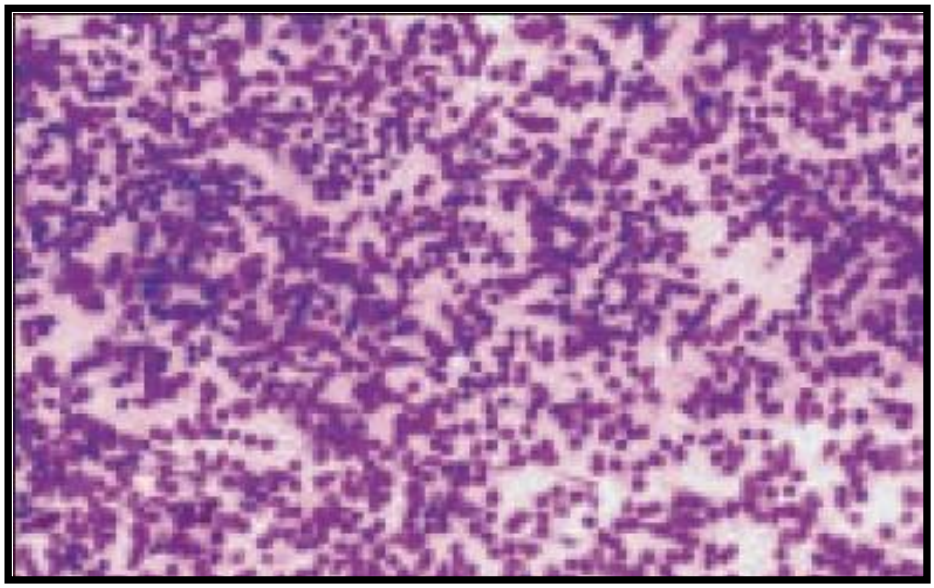


Fig No. 4

Microphotograph showing features of reactive lymphadenitis (H & E X100)

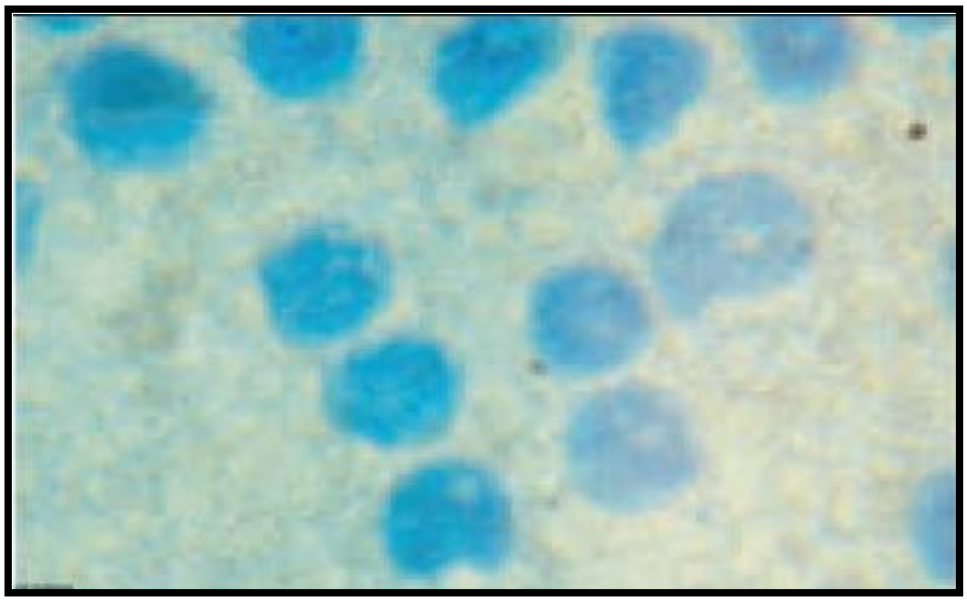


Fig No. 5

Microphotograph showing Negative for AFB by conventional method (Z-N X 1000)

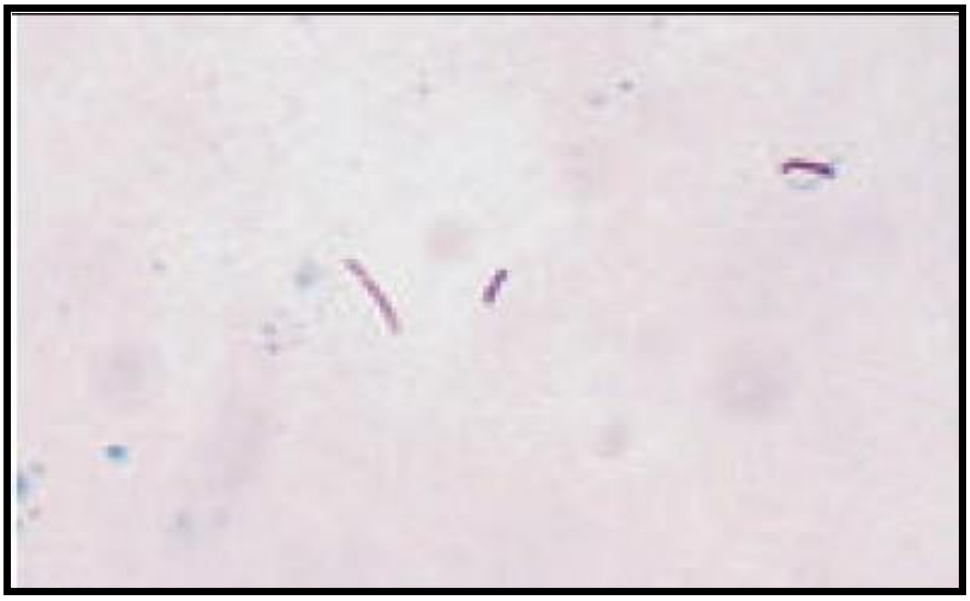


Fig. No. 6

Microphotograph showing AFB with clear background by bleach method (Z-N X 1000)

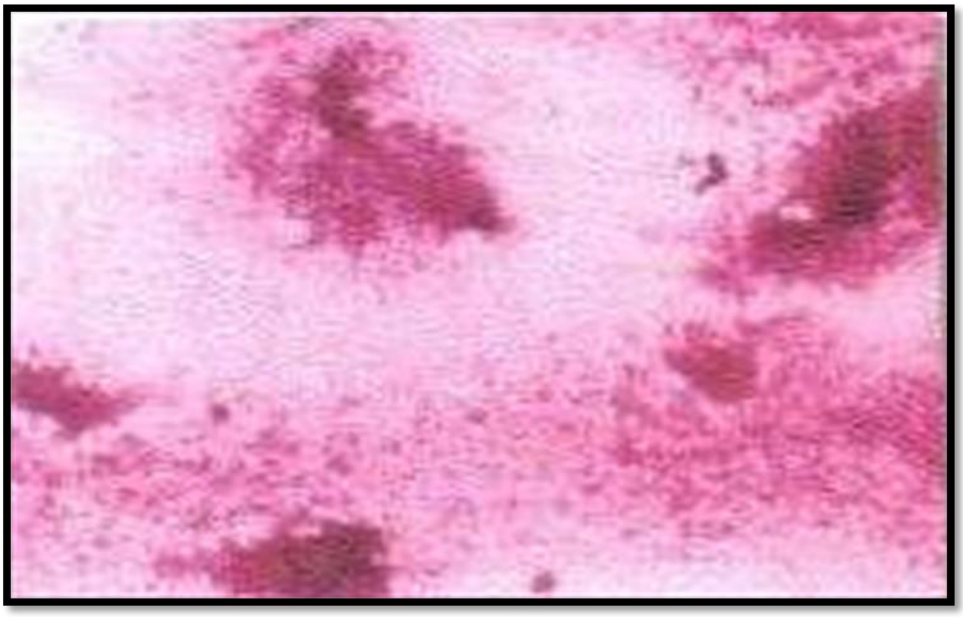


Fig No 7

Microphotograph showing caseous necrosis (Giemsa X 100)

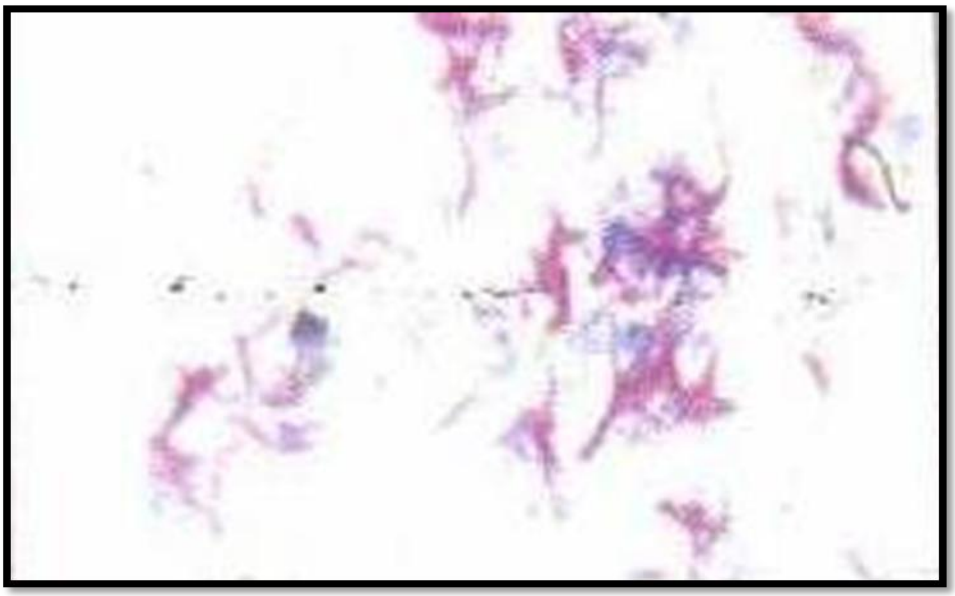


Fig No 8

Microphotograph showing AFB by bleach method (Z-N X 1000)

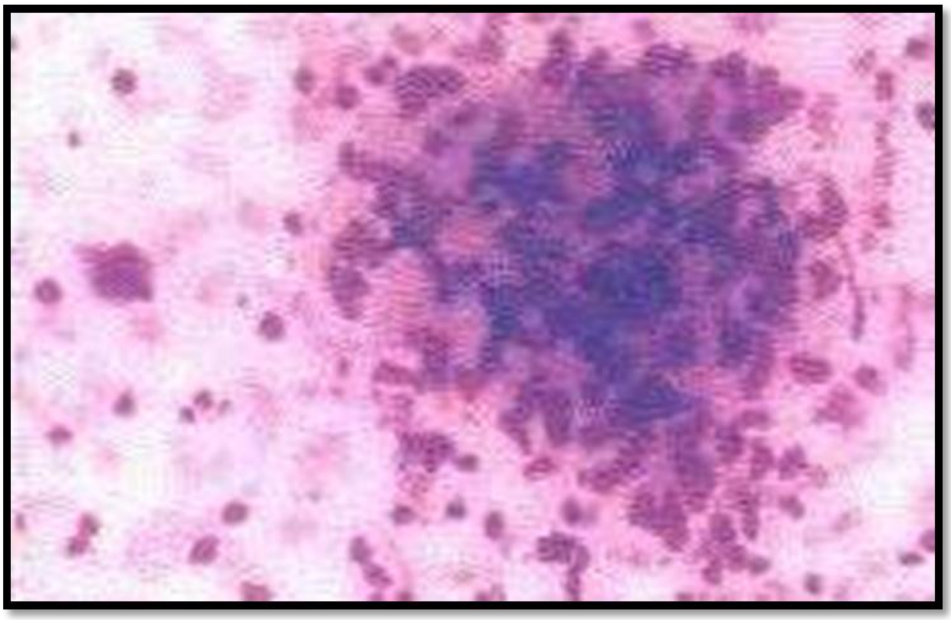


Fig No 9
Microphotograph showing granuloma (Giemsa X 400)

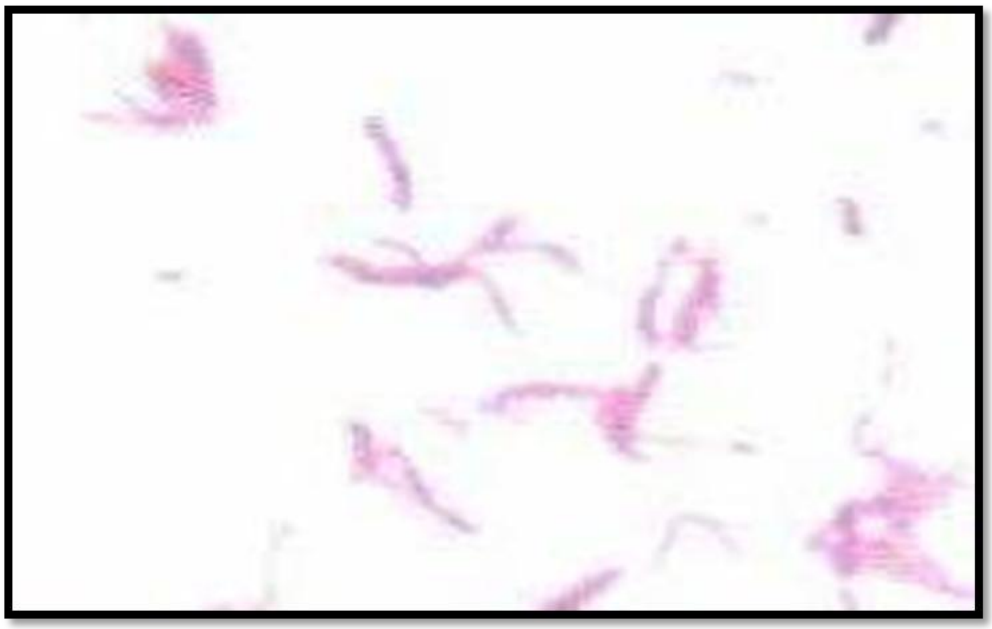


Fig No 10
Microphotograph showing AFB by bleach method (Z-N X 1000)

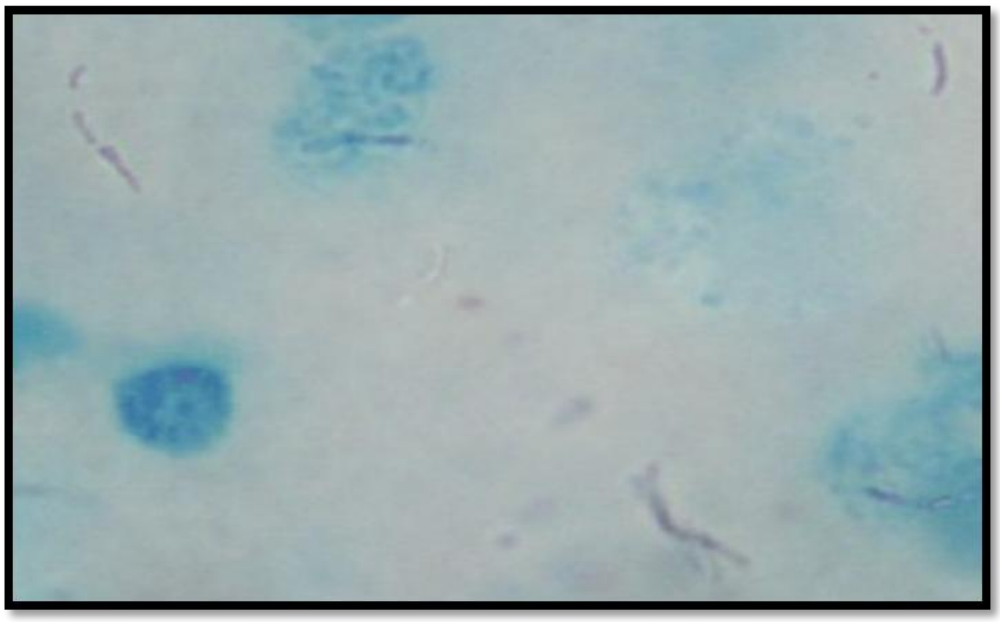


Fig No 11
Microphotograph showing atypical mycobacteria (Z-N X 1000)

Table No 18

Comparison of the Conventional Z – N method with the Bleach method for detection of AFB.

Conventional Z – N method	Bleach method		Total
	Positive	Negative	
Positive	39	00	39
Negative	29	41	70
Total	68	41	109

Correlation of significance: x^2 =36.616, df=1, p < 0.001

There was statistically significant correlation (x^2 =36.616, df=1, p < 0.001) between results of bleach method and conventional method in detection of tubercle bacilli (AFB)

Table No 19

Comparison of AFB grading by Conventional Z-N & Bleach method.

Grade	Z –N method		Bleach method	
	No of cases	%	No of cases	%
Negative	70	64.22	41	37.62
Scanty	20	18.34	18	16.51
1+	15	13.76	28	25.68
2+	03	2.75	17	15.59
3+	01	0.91	05	4.58
Total	109	100%	109	100%

In the present study more number of cases were of grade 1+, 2+ & 3+ in bleach concentration method Z-N stain compared to conventional method.

Table No 20

Evaluation of bleach method compared with conventional method.

	Bleach method	Conventional method
Sensitivity	94.44%	54.16%
Specificity	100%	100%
Positive predictive value	100%	100%

In the present study out of 72 cases finally diagnosed as TBLN, in 68 cases AFB were positive by bleach method and in 39 cases by conventional method. So thus sensitivity of bleach method was 94.44% where as the sensitivity of conventional method was 54.16%.

DISCUSSION

Tuberculosis is an ancient infection that has plagued humans since times immemorial still continues to remain a major public health problem especially in developing countries like India. The emergence of AIDS has added fuel to the existing fire of tuberculosis. The magnitude of the problem is so huge that it warrants rapid diagnosis to limit its spread.[40]

Tuberculosis is a disease that can affect any organ of the body. Tuberculous lymphadenitis is the most common type of extra pulmonary TB.[38] The diagnosis of tuberculosis is easy and simple when the disease is florid or disseminated but localized involvement of extra pulmonary organ or tissue may at times pose a diagnostic problem.[40]

The clinical parameters for the diagnosis of tuberculosis in lymphnodes are neither specific nor do their absence exclude tubercular involvement.[19] Early diagnosis of tuberculosis and initiating optimal treatment would not only enable a cure of an individual patient but will curb the transmission of infection and disease to others in the community.[53]

Diagnostic modalities must also be tailored to needs of the population and epidemiology of TB in that region. These include improved microscopy, usage of liquid culture for childhood and 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.[53]

In developing countries, microscopy of the specimen is by far the fastest, cheapest and most reliable method for the detection of AFB. In the late 1940s, sputum liquefaction with NaOCl (readily available at low cost as household bleach) and then concentration by centrifugation before acid-fast staining was implemented to improve the smear positivity for the detection of AFB. [54] This method was slightly modified and applied in cytology (i.e., only on lymph node aspirates).

The discrepancies between cytomorphological diagnosis and bleach method in the present study occurred in 29 specimens. Of the 29 specimens, 7 specimens were reactive lymphadenitis and 14 specimens were acute suppurative lymphadenitis, but these specimens were positive for AFB by bleach method. . The possible explanation for the diagnosis of reactive lymphadenitis on cytology but positive for AFB by the bleach method in both HIV positive and negative cases may be due to the loss of scattered epithelioid cells among the polymorphous population of lymphoid cells.[55] All the patients responded well for the anti-tubercular therapy. Among the 14 specimens diagnosed as suppurative lymphadenitis positive for AFB by the bleach method, the probable reason could be loss of the bacilli among the necrotic debris.

Khubnani et al.[10] studied 55 cases of extrapulmonary TB, which included 18 aspirates from body fluids, 18 from abscesses drained from various body sites, 17 from lymph nodes and two from skin scrapings. It was found that an overall 43.36% cases were suggestive of TB on cytology, 21.8% cases positive for AFB by conventional Z-N staining and 70.90% cases positive for AFB by the bleach method.

Gangane N et al [51] studied 100 cases of TB lymphadenitis and concluded bleach concentration method demonstrated AFB positivity in 72 % of cases. AFB positivity grade was much higher than with routine Z-N staining making bacilli easily visible with shorter screening time. The bleach method was inexpensive easily performed and more sensitive and safer than routine Z-N staining.

Annam V et al [52] studied 93 cases of lympadenopathy. Among 93 aspirates 33.33% were positive for AFB in conventional Z-N method and the smear positivity increased to 63.44% on bleach method. They concluded bleach method as simple and inexpensive.

Table no. 21

Comparison of AFB positivity in different studies by conventional & bleach method

Authors	Conventional Method	Bleach Method
Khubani *et al* [10]	21.8%	70.9%
Gangane N *et al* [51]	27.0%	72.0%
Annam V *et al* [52]	33.33%	63.44%
Present study	35.78%	62.38%

We have demonstrated that liquefaction of the aspirated specimen with NaOCl followed by centrifugation significantly increases the yield of AFB. This finding is of considerable interest in developing countries where smear-negative AFB has become increasingly common. The improved recovery of AFB after treatment with NaOCl might be due to changes in the surface properties of the AFB (i.e., charge and hydrophobicity) and/or denaturation of the specimen leading to flocculation and subsequent increased sedimentation

rate of the AFB.[55] Also, the increased smear positivity by the bleach method is attributable to the higher density of bacilli per microscopic field obtained by this method and reduction of debris, leaving a clear field for microscopy.[56] Thus, the preparation of samples by the bleach method reduces the time required for examination of the slides to detect AFB.

Acid-fast smear examination by the bleach method does not discriminate between tubercle bacilli and other mycobacteria. However, this is not a major problem in developing countries: Firstly, because the vast majority of patients with AFB has TB and, secondly, because other mycobacteria are usually not present in sufficient concentration to be detected by direct microscopy.[57]

Mycobacteria have a low specific gravity and may remain buoyant during centrifugation. With the occurrence of multidrug-resistant TB, the risk of laboratory infection has become a major concern. Use of the bleach method would definitely lower the risk of laboratory infection.

Because NaOCl(bleach) kills the mycobacterium, this method cannot be used on samples intended for culture, but the method is strongly recommended for all laboratories that perform direct microscopy only.

A relative centrifugal force (RCF) of 1800-2400 x g and a centrifugation time of 15-30 min have been recommended for recovering mycobacteria. One major disadvantage of the bleach method is the need for a centrifuge. In the present study, a RCF of 3000 x g applied for 15 min yielded increased recovery of mycobacteria.

In conclusion, the bleach method for AFB is simple, safe and cost-effective. The results would be more efficient if concentration by bleach solution, RCF and bleach treatment is as per the time schedule and is proportionate. The implementation of the bleach method clearly improves microscopic detection and can be a useful contribution to routine cytology. This would be of benefit to the patients to receive an early and effective treatment.

CONCLUSION

The morphologic spectrum in tuberculous lymphadenitis varies widely depending on the stage of the disease and the immunity of the host. The presence of epithelioid cells is the first step in the diagnosis of tuberculous lymphadenitis in countries such as India where TB continues to be the most common cause of lymphadenopathy compared to other non-TB causes of granuloma formation.

There are problems in arriving at a definitive diagnosis in certain cases of tuberculous lymphadenitis when the aspirate shows polymorphous picture with occasional epithelioid cells and absence of typical Langerhans giant cell or caseous necrosis, making it necessary to resort to excisional biopsy for a definitive diagnosis. In such cases, routine Z-N staining is having low sensitivity because it rarely detects AFB in aspirates. However, in the present study, we were able to establish AFB positivity in 62.38% of cases with the bleach method. This detection rate is much better than routine Z-N staining.

It was also observed that by routine Z-N staining, most of the aspirates had scant AFB positivity and searching for them was a tedious, time-consuming exercise compared to the bleach method. By the bleach method, the majority (58.82%) of positive cases showed grades of AFB positivity that were above “scanty,” making them easily visible and detectable. AFB morphology was observed to be better preserved in the bleach method. Similarly, the bacilli were seen in clumps in a thin background, making the screening process easier, faster and less strenuous on the eye.

Moreover, there are many other advantages of this technique over routine Z-N staining. Sodium hypochlorite (NaOCl) can effectively kill mycobacteria. This makes the specimen safe to handle, but of course unsuitable for mycobacterial culture. The bacilli can

be easily detected against the clear background. However, as the smears are thin and not easily visible to the naked eye, extra care is required in labeling and staining the correct side of the slide.

Thus the bleach method for detection of tubercle bacilli in lymph node aspirate is more sensitive than the conventional Z-N method. Moreover, the bleach method is safe, inexpensive and easy to perform and requires no additional equipment.

SUMMARY

The present study was undertaken to emphasize the role of bleach concentration method over conventional direct smear microscopy for detection of tubercle bacilli in fine needle aspiration material of lymph node in a study period of November 2007 to April 2009.

1) This study was carried out on 120 patients with clinically suspected tuberculous lympadenopathy, referred to the department of cytopathology.
2) Of these 120 cases 11 cases were eliminated from the study because 8 aspirates were diagnosed as malignancy and 3 aspirates were inadequate and 109 clinically suspected tuberculous lymphadenitis cases evaluated and the results were analyzed.
3) Predominant age group involved was 16-30 years (37.61%) followed by 31-45 years (28.44%).
4) Male preponderance was seen in the study (Male 55.96% & Female 44.04%)
5) Most of patients presented with lymphadenopathy of less than 3 months duration seen in 60.55% of cases.
6) Lymphadenopathy predominantly involved cervical region seen in 65.10% cases followed by axillary region in 9.17% cases.
7) 91.83% of cases had lymphadenopathy of more than 1 cm in size. In that majority presented with multiple nodes (81.66%). Matting of lymphnodes was seen in 50.45% of cases.
8) Nature of aspirate was grey white in 64.22% of cases, purulent in 19.26% cases and hemorrhagic in 16.51% of cases.
9) Smears were predominantly hypocellular in 45.87% cases followed by normocellular in 38.52% cases and hypercellular in 15.59% cases.

10) Out of 109 cases, 47 (43.12%) were cytomorphologically diagnosed as TBLN, followed by 32 cases (29.36%) as suppurative LN and 30 cases (27.52%) as reactive LN.

11) Overall AFB positivity is seen in 39 cases (35.78%) by conventional smear Z-N method.

12) Out of 39 positive cases 20 cases were scanty, 15 were 1+, 3 cases were 2+ and only one case was of 3+ grade by conventional method.

13) Overall AFB positivity is seen in 68 cases (62.38%) by bleach concentration method Z-N smear.

14) Out of 68 positive cases 18 were scanty, 28 were 1+, 17 cases were 2+ and 5 cases were of 3 + grade by bleach method.

15) Sensitivity of bleach method was 94.44% and sensitivity of conventional method was 54.16%.

16) Thus the bleach method for detection of tubercle bacilli in lymph node aspirate is more sensitive than the conventional Z-N method. Moreover, the bleach method is safe, inexpensive and easy to perform and requires no additional equipment.

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PROFORMA

B.L.D.E.ASSOCIATION'S

SHRI B.M.PATIL MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTRE, BIJAPUR – 586103.

DEPARTMENT OF PATHOLOGY

"COMPARATIVE STUDY OF COVENTIONAL SMEARS AND BLEACH CONCENTRATION METHOD IN DETECTION OF TUBERCLE BACILLI IN FINE NEEDLE ASPIRATION MATERIAL OF LYMPH NODES"

PROFORMA

Name	:	IP No/PO.No	:
Age and Sex	:	Date	:
Occupation	:	Ref by	:
Address	:		

Chief complaints :

History of present illness :

Treatment history :

HIV Status :

Physical examination :

General physical examination :

Systemic examination :

Local examination :

Clinical diagnosis **:**

MACROSCOPY **:**

Site :

Number :

Size :

Consistency :

Matted/Discrete :

Nature of aspirate :

MICROSCOPY :

Cytomorphological diagnosis :

Conventional smear Z – N Stain:

Bleach concentration method :

Smear Z – N stain :

IMPRESSION :

CONSENT FORM

BLDEA'S SHRI. B.M. PATIL MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTRE, BIJAPUR-586103

RESEARCH INFORMED CONSENT FORM

TITLE OF THE PROJECT	:	"COMPARATIVE STUDY OF CONVENTIONAL SMEARS AND BLEACH CONCENTRATION METHOD IN DETECTION OF TUBERCLE BACILLI IN FINE NEEDLE ASPIRATION MATERIAL OF LYMPH NODES"
P.G. GUIDE	:	DR.B.R.YELIKAR$_{MD}$ PROFESSOR & HOD, DEPARTMENT OF PATHOLOGY
PRINCIPAL INVESTIGATOR	:	DR.RAGHUNATH RAO PG STUDENT, DEPARTMENT OF PATHOLOGY.

PURPOSE OF RESEARCH:

I have been informed that the present study is to compare conventional smear and bleach concentration method in detection of tubercle bacilli in fine needle aspiration material of lymph nodes.

PROCEDURE:

I understand that I undergo detailed history and clinical examination after which lymph nodes will be aspirated and subjected to cytological examination.

RISK AND DISCOMFORTS:

I understand that there is no risk involved and I may experience mild to moderate discomfort during the fine needle aspiration procedure.

BENEFITS:

I understand that my participation in the study will have no direct benefit to me other than the potential benefit of the treatment.

CONFIDENTIALITY:

I understand that the medical information produced by the study will become a part of hospital record and will be subjected to confidentiality and privacy regulations of the hospital. If the data is used for publications the identity of patient will not be revealed.

REQUST FOR MORE INFORMATION:

I understand that I may ask more information about the study at any time.

REFUSAL FOR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and that I may refuse to participate or may withdraw from the study at any time.

INJURY STATEMENT:

I understand that in the unlikely event of injury to me during the study I will get medical treatment but no further compensations.

I have read and fully understood this consent form. Therefore I agree to participate in the present study.

_____ _____

Participant / Guardian Date:

_____ _____

Signature of Witness Date:

I have explained the patient the purpose of the study, the procedure required and possible risk and benefit to the best of my ability in their own language.

_____	_____
Investigator / P.G.	Date:
_____	_____
Witness to Signature	Date

KEY TO MASTER CHART

1. TBLN.	-	Tubercular lymphadenitis
2. HIV.	-	Human immuno deficiency virus
3. CER	-	Cervical
4. S/C	-	Supra clavicular
5. S/M	-	Submandibular
6. AXL	-	Axillary
7. ING	-	Inguinal
8. P/A	-	Preauricular
9. I/C	-	Intercostal
10. SPLN	-	Suppurative lymphadenitis
11. RLN	-	Reactive lymphadenitis
12. M	-	Multiple
13. S	-	Single
14. D	-	Discrete
15. MT	-	Matted
16. NT	-	Not tested
17. NEG	-	Negative
18. POS	-	Positive