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Original Research Article

Efficacy of Bleach Concentration Technique over Conventional Methods in the Detection of Mycobacterium Tuberculosis in Fine Needle Lymph Node Aspirates

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Conflict of interest: Nil

Abstract:

Background: This study was conducted to evaluate the effectiveness of the bleach concentration method over traditional techniques in the detection of acid-fast bacilli in the cytologically confirmed cases of tubercular lymphadenitis. We sought to apply the BCT (Bleach Concentration Technique) for the detection of tubercle bacilli in fine needle lymph node aspirates and compare it with the conventional Ziehl Neelsen method.

Methods: This hospital-based observational study was carried out over the course of 18 months, from December 2016 to June 2018, among 65 patients with clinically suspected tuberculous lymphadenopathy at the Department of Pathology, PES Institute of Medical Sciences and Research, Kuppam. The study was approved by the institutional ethics committee and the participants provided written informed consent.

Results: For each of the 65 cases, a cytomorphological study was performed. Overall, 23% of cases using the standard ZN approach and 46% using the bleach concentration methodology showed evidence of AFB. Conventional ZN had a 50% sensitivity and a 100% specificity. The BCT exhibited 97.9% sensitivity and 100% specificity. A statistically significant "p" value was observed in the relationship between cytomorphology and AFB positivity.

Conclusion: Compared to the traditional Ziehl-Neelsen method, the concentration of lymph node aspirates obtained using the bleach method had a much greater sensitivity for tubercle bacilli identification and a high case detection rate.

Keywords: Bleach Concentration Technique, Conventional Methods, Detection, Mycobacterium Tuberculosis, Fine Needle Lymph Node Aspirates.

Introduction

The A chronic granulomatous inflammatory condition, TB (Tuberculosis), is brought on by the MTB (Mycobacterium Tuberculosis) bacteria.[1] Gram stain frequently shows mycobacterium tuberculosis as an unstained or beaded obligate aerobe. It is a non-motile, non-spore-forming bacillus.[2] TB most frequently attacks the lungs; this is known as PTB (Pulmonary Tuberculosis). PTB patients may be contagious and exhibit fever, coughing, and an abnormal chest radiograph. While TB is primarily pulmonary in nature,[3] it can manifest as a disseminated disease or practically in any anatomical region.[4] In addition to the lungs, other organs that might harbor EPTB (Extrapulmonary Tuberculosis) include the larynx, lymph nodes, pleura, brain, kidneys, bones, and joints. In HIV-positive individuals, PTB frequently coexists with EPTB.[3] The most typical way that EPTB manifests itself is lymphadenopathy.

In approximately 35% of EPTB cases, tuberculous lymphadenitis is observed.[5] Although MTB is

present in the bodies of those who have LTBI (Latent Tuberculosis Infection), they do not have TB disease and are unable to infect others.[3] For patients presenting with superficial lymphadenopathy in developing nations with high tuberculosis prevalence, FNAC in conjunction with the ZN (Ziehl-Neelsen) stain should be the initial line of inquiry.[6] MTB must be demonstrated using microbiological, cytopathological, or histological techniques in order to provide a definitive diagnosis of tuberculosis.[7] In poor nations, direct smear microscopy is the primary method used to diagnose PTB. It can quickly, cheaply, and precisely identify the most contagious tuberculosis cases. The most reliable technique for identifying tubercle bacilli is mycobacterial culture, which has a sensitivity of 70% to 80%. This process takes a long time and calls for specific safety precautions. Therefore, the diagnosis is typically made using a combination of radiologic findings, symptoms, exposure history, and epidemiology, ideally enhanced by microscopy of smears stained with an acid-fast stain. NAAT (Nucleic Acid Amplification Tests) or microbiologic cultures should be used to confirm the existence of MTB.

There are numerous benefits to microscopy in terms of speed and practicality.[8] Granulomatous lymphadenitis in lymph node aspirates is typified by epithelioid histiocytes amidst a backdrop of lymphocytes and plasma cells. Necrosis linked to this condition can show up in the smears as a cellular granular material or it may not. ZN staining shows that acid-fast bacilli are present. The AFB is distinguished by its slender, rod-shaped, beaded look.[9] Different concentration techniques can be used to increase the sensitivity of direct microscopy in order to identify tubercle bacilli in a specimen.[10] The BCT has been previously reported for the identification of tubercle bacilli in sputum. Experiments conducted on extrapulmonary specimens have demonstrated enhanced AFB detection.[11] In the current investigation, BCT was used to demonstrate AFB (Acid Fast Bacilli) on lymph node aspirates that were cytomorphologically indicative of TB lymphadenitis.

Aims and Objectives

- To apply the bleach concentration technique in the detection of tubercle bacilli in fine needle lymph node aspirates.
- To assess the efficacy of the bleach concentration method over conventional methods in the detection of acid fast bacilli in the cytologically established cases of tubercular lymphadenitis.

Materials & Methods

This hospital-based observational study was carried out over the course of 18 months, from December 2016 to June 2018, among 65 patients with clinically suspected tuberculous lymphadenopathy at the Department of Pathology, PES Institute of Medical Sciences and Research, Kuppam. The study was approved by the institutional ethics committee and the participants provided written informed consent.

Patients presenting with clinically suspected lymphadenopathy were subjected to brief clinical examination. Data regarding age, gender, duration, description of swelling like site, number, size were documented.

FNAC was performed under strict aseptic precaution using a 22 gauge needle attached to a 5ml syringe. After aspiration of the material from the lymph node, it was expelled on to the clean glass slide and smears were made. For cytological analysis the smears were stained with hematoxylin and eosin, papanicolaou and May Grunwald Giemsa. The smears were studied for cytomorphological evidence of TB.

One smear per case was stained with Ziehl-Neelsen (ZN) stain. The slide was observed for the presence of Acid Fast Bacilli(AFB) under oil immersion objective.

The bleach method was performed with the remaining aspirated material in the needle hub or syringe, which was rinsed with 1 ml normal saline and transferred into 5 ml sterile disposable, conical screw-capped tubes. 2 ml of 6% sodium hypochlorite was added and the mixture was incubated at room temperature for 15 min by shaking at regular intervals. Centrifugation done at 3000 RPM for 15 min after adding 2 ml of distilled water to the mixture. The supernatant was discarded, and the sediment was transferred to a clean slide. The slide was air dried, heat fixed and stained by ZN method.

Control: As a control, 2 ml of distilled water was centrifuged, and the sediment was stained by ZN staining to rule out any error due to contamination. For all the cases, the ZN stained smear was examined along with the known positive control.

Inclusion Criteria: All clinically suspected cases of tubercular lymphadenitis, both genders, were referred for fine needle aspiration cytology.

Exclusion Criteria: Cases with TB of bone, uterus, fallopian tubes, abdominal TB, body fluid and metastatic lymph nodes.

Statistical Methods: SPSS version 20 was used for additional data analysis after importing the data into MS Excel 2007. The "t" test was used to analyze the numerical data. The chi-square test was used to analyze the category data. Sensitivity, specificity, and predictive values were used to express

effectiveness. A statistically significant result is defined as a "p" value less than 0.05.

Results

Table 1: Comparison between ZN and BCT

BCT	ZN		Total
	Positive	Negative	
Positive	22	24	46
Negative	1	18	19
Total	23	42	65
Correlation of significance = p<0.001			

The cytomorphological diagnosis and AFB positivity by ZN and BCT showed a statistically significant connection ($p < 0.001$). ZN staining found AFB in 23 cases, while BCT found it in 46 cases. The bleach approach missed one instance in which the traditional method tested positive for AFB.

Table 2: Comparison of Conventional ZN and BCT with Cytological Pattern

Patterns	ZN		BCT		Total
	Positive	Negative	Positive	Negative	
Pattern 1: Epithelioid granuloma with necrosis	7(38.8%)	11(61.1%)	14 (77.7%)	4 (22.22%)	18
Pattern 2: Epithelioid granuloma without necrosis	1(4.54%)	22(95.65%)	14 (60.86%)	9(39.13%)	23
Pattern 3: Necrosis without granuloma	14(58.33%)	9(39.13%)	19(82.60%)	4 (17.39%)	23
Pattern 4: Necrosis with neutrophils and macrophages	1(100%)	0	1(100%)	0	1

In cases showing epithelioid granulomas with necrosis, the ZN stinging showed AFB positivity in 38.8% of the cases, while BCT was positive in roughly 77.7% of cases.

Using the ZN approach, 4.54% of the patients in the second pattern—that is, epithelioid granulomas without necrosis in cytological smears—showed AFB positivity; however, in 60.86% of these instances, BCT found AFB.

AFB positivity by ZN technique was 58.33% in the third pattern, which is characterized by necrosis without granulomas; however, with BCT, it climbed to 82.60%.

The fourth pattern showed AFB 100% positivity by both ZN and BCT methods. The ZN technique and BCT revealed a statistically significant link between the cytomorphological patterns and AFB positivity, with a p-value of less than 0.001.

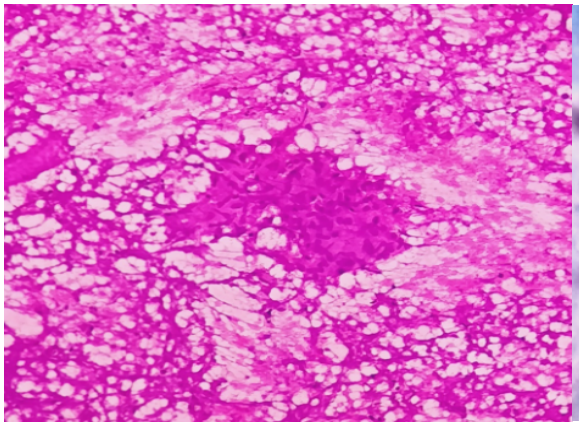

Figure 1: H&E, X200 Lymph Node Aspirate Showing Cluster of Epithelioid Cells With Background Necrosis

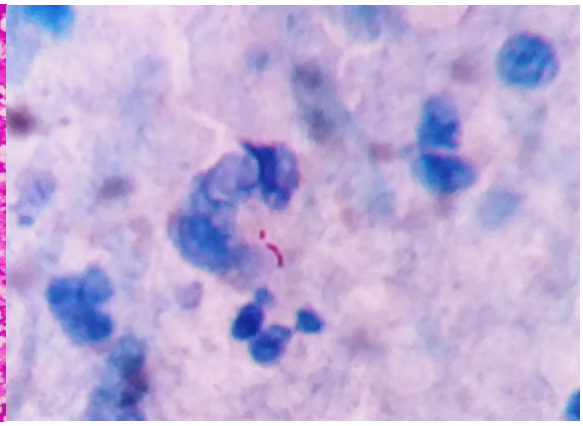

Figure 2: Conventional Zn Stain, Oil Immersion Lymph Node Aspirate Of The Same Case Showing AFB

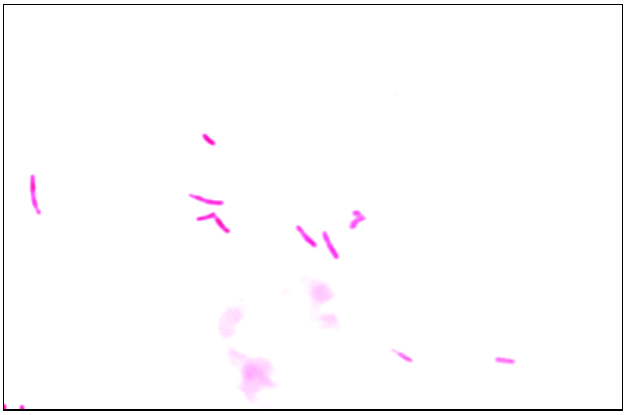

Figure 3: BCT ZN STAIN, Oil Immersion Lymph Node Aspirate of The Same Case Numerous AFB Seen Against A Clear Background

Discussion

Age Distribution: Unlike pulmonary tuberculosis, which is more prevalent in older age groups, TB lymphadenitis typically affects children and young adults, with a peak age of onset of 20 to 40 years.[12]

In a study of 75 instances, Krishna M. et al. (2017) discovered that 48% of the cases had an age range between 16 and 30 years old. In a research on 129 instances, Manika Khare et al. (2016)[13] also noted that, in 48.8% of the cases, the most common age group impacted was 16 to 30 years old. A few furher studies by Nidhi et al. (2011),[14] Chand P et al. (2014),[15] and Dagar V et al. (2016) have demonstrated that, among the population they studied, the third decade was the most frequently impacted age.

The age range of 16 to 30 years old accounted for the majority of cases in the current study (27 instances, or 41.53%), which is similar to the studies previously cited.

Gender Distribution: The gender distribution of TB lymphadenitis, which is more prevalent in women, is one of its peculiar characteristics. In addition to the bacterial factors, the patient's age, sex, diet, genetics, family history of contact, and immunological competence all have an impact on the clinical characteristics.

Due to a variety of circumstances, including pregnancy and lactation, women typically have a low metabolic rate, are malnourished, and present quiescently or with constitutional symptoms.[16] Because they are unaware of the importance of eating a nutritious diet, women from rural backgrounds do not meet their protein requirements.

In our study, the female to male ratio is 1.03:1, with no clear preponderance. Whereas various other studies done by Dandapat MC et al, Jha BC et al, Patel M et al, Khan S et al revealed a slight female preponderance. A study done by Krishna M and Gole SG showed male preponderance which is in contrast to the current study and other studies.[17-21]

The prevalence of women in many studies can be explained by the fact that women in underdeveloped nations typically have low socioeconomic and nutritional status, which might impact the immune system's ability to fight off illness.[22]

Anatomical Region: Peripheral lymphadenitis is a symptom of early post-primary tuberculosis and is thought to be part of the primary complex. The number of affected nodes varies depending on where the infection was initially focused.

In a study of 93 instances, Annam V. et al. (2009)[23] found that the cervical lymph node was impacted in the majority of cases-roughly 72% of them.

Our study revealed 76.92% of the cases showing cervical lymph node involvement which is comparable to the study done by Annam V. et al. (2009).

The most likely explanation for the cervical lymph node's involvement is that the tubercle bacilli primarily move to the lymph nodes through aerosol intake through the airway to the Waldeyers ring, where the infection is stopped at the first line of defense. After that, the cervical lymph nodes are affected.

However, other pathways of dissemination to lymph nodes, such as the tonsils and adenoids, have been hypothesized, and parenchymal lung disorders cannot fully explain the etiology of tuberculous lymphadenitis.

Nature of Aspirate: Stage 3 of the five stages of peripheral lymphadenopathy previously reported is suggestive of the establishment of an abscess that results in central softening. One of the detrimental reactions to tuberculosis is the disintegration of the tubercles' caseous centers. It is linked to hypersensitivity of the delayed kind. In more severe lesions,

liquefaction results from the breakdown of protein, lipid, and nucleic acid components by the hydrolytic enzymes of macrophages and granulocytes, which triggers an exudative response. A tuberculous lesion is confined to an immunocompetent patient until its caseous center liquefies.

In these situations, the liquefied caseum serves as a perfect medium for the AFB to proliferate, increasing AFB positivity.[24]

318 cases of clinically suspected tuberculous lymphadenitis were studied by Nidhi P et al.; 125 of these cases were found to have TB lymphadenitis. In their investigation, the most common type of aspirate, accounting for roughly 61.6% of the cases, was purulent, with cheesy material coming in second with 23.5%.

In a study of 128 cases of lymphadenitis, Dagar et al. (2016) found that 53.8% of the cases had purulent aspirates, which is the largest number of cases.

The largest number of cases in the current study-26, or 40%-showed purulent aspirate, with 12 instances (18.48%) showing cheesy white aspirate. Our work can therefore be compared to other investigations. Still, the percentages fluctuate significantly amongst researchers.

If purulent material is aspirated from a lymph node, it is necessary to differentiate between acute suppurative lymphadenitis and tuberculous lymphadenitis. When scattered epithelioid cells are absent, the cytologic images under each of these conditions may resemble one another, displaying a large number of neutrophils against a background of extensive necrotic tissue. The presence or lack of acid-fast bacilli is necessary to distinguish between these two diseases.[25]

Duration of the Swelling and the Cytological Pattern: The majority of individuals in the current study had swelling that lasted longer than three months. Necrosis without granulomas was the pattern seen in those cases, while necrosis with neutrophils and macrophages was seen in one case.

Granuloma with caseous necrosis was detected in the individuals with histories shorter than three months.

When there is sufficient immunity, the lesions calcify and/or undergo fibrosis, which helps to control the infection.

In the current study, granulomas in the smear were associated with a reduced density of bacilli in the majority of cases with swelling that lasted for less than three months.

Cytomorphological Pattern and AFB Positivity: The cytomorphological patterns in the present study were,

1. Epithelioid granuloma with necrosis with or without Langhans giant cells
2. Epithelioid granuloma without necrosis
3. Necrosis without epithelioid granuloma
4. Necrosis with macrophages and neutrophils in the background[26]

The current investigation revealed that, with equal distribution, patterns 2 and 3 were the most common.

Pattern 2, or epithelioid granuloma without necrosis, could represent an early stage of the illness. According to the literature, a compact proliferation of lymphocytes and a cluster of macrophages will occur in the presence of relatively low levels of bacillary antigens and intact immunity. Focused sampling of the node may also reveal this pattern in the FNA smears when necrosis restricts to a small area.

Similar to our study, a study by Nidhi P et al. revealed that pattern 3 was observed in the greatest number of instances, including 39.2%.

According to a study by Khan S. et al., pattern 1 was present in the majority of cases (40%) and pattern 2 in 31% of the cases. Research by Krishna M et al. and Mitra SK et al.[27] (2017) and others also revealed that pattern 1 predominated, which is at odds with the results of our investigation. Nonetheless, in all of the aforementioned investigations, pattern 3 had the highest percentage of subjects with smear positivity for AFB.

The pattern 3 cases in the current investigation had the highest percentage of AFB positive cases. In the smears with the first two patterns, a conclusive cytologic diagnosis of tuberculosis lymphadenitis might be considered; in the absence of a positive ZN stain, the third and fourth patterns might be misinterpreted as necrotizing lymphadenitis and acute suppurative lymphadenitis, respectively.[28]

In the current investigation, for instance, pattern 3 was identified in eight cases of necrotizing lymphadenitis, one case of suppurative lymphadenitis, and fourteen cases of AFB positive TB lymphadenitis. Using the ZN technique, all eight cases of necrotizing lymphadenitis tested negative for AFB. After the bleach method was applied, 5/8 instances tested positive for AFB. The bleach approach, by giving a clear background, helps in locating acid fast bacilli in such circumstances. The conventional method may have lost the bacilli among the necrotic debris, which is the likely cause of this false negative result. Our research revealed a correlation between the clinical stage and cytomorphology and the highest AFB positivity in cases with pattern 3.

Cytological Diagnosis and AFB Positivity: Cytologically, granulomas with necrosis, granulomas without necrosis, or occasionally necrosis alone, are present in TB lymphadenitis. Rather than actual

granulomas, immunocompromised persons may merely have loose collections of histiocytes.[29]

AFB positivity by ZN technique was 11% in cases of suppurative lymphadenitis, while it increased to 59.25% by BCT. AFB positive in tuberculous lymphadenitis was 71.79% by ZN, but it was 94.87% by BCT. Their research revealed a statistically significant connection between AFB positivity and cytomorphological diagnosis with a "p" value of <0.05.

Nonetheless, in 24 cases, there were differences between the bleach approach and the cytomorphological diagnosis. Of the 24 cases, 22 had positive BCT results but negative AFB results, according to the ZN technique. Out of 24 cases, two displayed cytological signs of TB lymphadenitis but were BCT-negative.

AFB positivity by ZN technique was seen in 10.9% of cases with suppurative lymphadenitis, and by BCT in 56.36% of cases. Sixty-two percent of ZN cases with TB lymphadenitis had positive AFB results. BCT raised the AFB optimism to 91.34%.

Fifty patients had differences between the BCT and the cytomorphological diagnosis. Of the fifty patients, 41 had negative ZN results but positive AFB results, according to BCT. Out of the 50 cases, 9 had cytomorphological signs of tuberculosis but tested negative for AFB by BCT.

Granulomatous lymphadenitis accounted for 50.76% of the cases in our study, which was the most common cytomorphological diagnosis. Tubercular lymphadenitis was observed in 35.38% of the cases, and smears showed positive results for AFB by ZN method. Necrotizing lymphadenitis was seen in 12.33% of the cases, and suppurative lymphadenitis was observed in 1.53% of the cases.

While regular ZN found AFB in only 29.30% of those with granulomatous lymphadenitis, routine ZN showed AFB negativity in all instances. The ZN technique consistently demonstrated AFB negative in cases of necrotizing lymphadenitis. 62.5% of the cases had BCT results indicating AFB positivity.

The number of AFB positive cases in aspirates with diagnoses of necrotizing and granulomatous lymphadenitis has dramatically increased in our study due to BCT.

Six cases in the current investigation had differences between the bleach procedure and the cytomorphological diagnosis. Five of these six instances had necrotizing lymphadenitis diagnosed because there was no AFB found using the ZN technique. By BCT, all five cases tested positive for AFB.

Treatment of the aspirate material with Sodium hypochlorite aids in creating a distinct background that makes it simple to identify the AFB.

One instance of TB lymphadenitis that was cytomorphologically confirmed-that is, with well-formed granulomas, necrosis, and positive results for AFB by the ZN method-was determined to be negative by BCT. As a result, one instance of TB lymphadenitis that tested positive by the standard ZN technique was overlooked by BCT.

Comparison of AFB Positivity by ZN and BCT: It is crucial to use specific stains for acid-fast bacilli in cases where there are neutrophilic infiltrates, necrosis, or granulomas.

In this work, we have used BCT in conjunction with the ZN technique to demonstrate AFB in smears.

Out of the 65 cases in the current investigation, 23 (35.38%) had an overall AFB positivity by conventional ZN. The BCT detected AFB in 46 instances, or 70.76%. The BCT detected AFB in 23 more cases (35.38%) than the usual technique.

ZN and BCT both detected AFB, with a statistically significant connection between cytomorphology and a "p" value of less than 0.001.

Our study is comparable to various other studies which showed improved detection of AFB by bleach concentration technique over the conventional ZN method.

Authors	AFB positivity (percentage of cases %)	
	ZN	BCT
Annam V et al[30] (2009)	33.3%	63.44%
Chandrasekhar B et al[31] (2012)	16.07%	73.2%
Patel M et al[32] (2013)	27.83%	62.6%
Dwivedi G et al[33] (2013)	35.5%	68%
Singh P et al [1](2015)	40%	63.5%
Khare M et al[34] (2016)	58.9%	89.1%
Khan S et al[35] (2018)	37%	41%
Present study	**35.3%**	**70.76%**

Research demonstrates that concentrating the aspirated material by treating it with sodium hypochlorite followed by centrifugation can greatly increase the yield of AFB.

It is thought that mycobacteria stay buoyant during centrifugation because of their lipid coat. However, following centrifugation, the bleach method has been shown to permit the deposition of bacilli at the test tube's bottom.[36] This enhanced recovery may be the result of modifications to the bacilli's surface characteristics, such as their charge and hydrophobicity, or of the specimen's denaturation, which causes flocculation and a rise in the AFB's sedimentation rate.

Clinical follow-up was performed on every instance in which BCT revealed an AFB positive result. Each of them experienced an effect from anti-tubercular treatment.

As a result, BCT increases AFB smear positivity, improving diagnostic precision. Additionally, it lessens the number of TB lymphadenitis instances that go unnoticed when AFB is overlooked using the traditional approach. This could facilitate the start of timely treatment.

Furthermore, the BCT enhances other adjuvant molecular methods, such as PCR and CB-NAAT, which may aid in the early detection of a greater number of patients.

The sodium hypochlorite used in the bleach concentration technique aids in killing the mycobacterium and hence it lowers the chance of laboratory infection.

Conclusion

Compared to the ZN method, the bleach concentration technique is more effective and superior at detecting AFB. More instances can be found when adjuvant diagnostic modalities like PCR, CB-NAAT, LPA, and LAMP are added. This could lessen the burden of disease on society and enhance both the health and economics of the country. The scientific literature on the laboratory diagnosis of tuberculosis can greatly benefit from additional research on fine needle aspirate material using molecular diagnostic techniques.

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