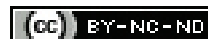


# Utility of Conventional ZN Staining and Modified Bleach Techniques in Detection of Acid Fast Bacilli from Aspirates of Suspected Cases of Tuberculous Lymphadenitis

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## ABSTRACT

**Introduction:** Tuberculosis (TB) is a significant public health problem in many developing countries, including India. Diagnosing tuberculous lymphadenitis, in particular, poses challenges as it requires the demonstration of Acid-Fast Bacilli (AFB) using the conventional Ziehl-Neelsen (ZN) technique. The Modified Bleach Technique, which involves liquefying the aspirated specimen with sodium hypochlorite followed by centrifugation, has been described to enhance the yield of AFB, improving sensitivity and diagnostic accuracy.

**Aim:** To highlight the role of the bleach concentration method compared to conventional ZN staining in detecting AFB in Fine-Needle Aspiration (FNA) material from clinically suspected cases of tuberculous lymphadenitis.

**Materials and Methods:** A prospective cross-sectional study was conducted at the Department of Pathology, MVJ Medical College and Research Hospital, Bengaluru, Karnataka, India from November 2020 to December 2022. Fine needle aspiration material from 100 cases of clinically suspected tuberculous lymphadenitis was included. In each case, part of the aspirate was processed for ZN staining, while the remaining material was used for modified bleach staining and Cartridge Based Nucleic Acid Amplification Test (CBNAAT). Patient data, including age, sex, duration and site of swelling, nature of aspirate, and cytomorphological diagnosis, were documented. The data was entered into a Microsoft excel spreadsheet and analysed using

the Chi-square test, with a p-value <0.05 considered statistically significant.

**Results:** The age range of the study participants was 1-80 years. Out of 100 cases, 13 were excluded due to inadequate material. The most common lymph node site was cervical 69 (79.3%), followed by axillary 8 (9.1%) and supraclavicular 6 (6.89%). Among the 87 cases, reactive lymphadenitis was diagnosed in 36 (41.3%), suppurative lymphadenitis in 12 (13.8%), necrotising granulomatous lymphadenitis in 12 (13.8%), granulomatous lymphadenitis in 15 (17.3%), and necrotising lymphadenitis in 12 (13.8%). Out of the 100 cases included in the study, 32 cases were confirmed as tubercular lymphadenitis by CBNAAT. Among these 32 cases, conventional ZN stain was positive for AFB in only 3 cases (9%), whereas the Modified bleach stain was positive in 16 cases (50%). The results were compared with CBNAAT findings, and the modified bleach technique showed sensitivity (59.4%), specificity (100%), Positive Predictive Value (PPV) (100%), Negative Predictive Value (NPV) (80%), and Accuracy (85%).

**Conclusion:** The Modified bleach method of ZN staining is a simple, safe, inexpensive, and easy-to-perform technique. With the bleach method, AFB was easily visible and detectable in the majority of AFB-positive cases. Additionally, the bleach method preserved the morphology of AFB better. It improves the microscopic detection of AFB, leading to a definitive diagnosis of TB on cytology.

**Keywords:** Cartridge-based nucleic acid amplification test, Conventional ziehl-neelsen staining, Fine needle aspiration, Modified bleach concentration method, Sodium hypochlorite, Tuberculosis

## INTRODUCTION

The TB is a common public health problem and the second leading cause of infectious disease worldwide, with an estimated 1.3 million deaths globally. India has the highest TB burden, accounting for one-fourth of the global incidence [1]. Tuberculous lymphadenitis is the most prevalent form of extrapulmonary TB, with cervical lymph nodes being the most commonly affected [2]. Various diagnostic tests are available for detecting TB, like conventional Ziehl-Neelsen staining, fluorescent staining, mycobacterial culture, molecular methods like Polymerase Chain Reaction (PCR), and CBNAAT [3]. The gold standard for TB diagnosis is culture on LJ medium, but it is time-consuming and requires specialised safety procedures in laboratories.

The FNAC followed by conventional ZN staining plays a crucial role in diagnosing tubercular lymphadenitis. However, the sensitivity of conventional ZN staining in detecting AFB is relatively low, ranging from 9% to 46% [4]. This sensitivity can be significantly improved by

liquefying the sample using chemical reagents and then concentrating it through centrifugation or sedimentation before acid-fast staining [3]. Previous studies have demonstrated that liquefaction of sputum using Sodium Hypochlorite (NaOCl, bleach) and concentration of bacilli through centrifugation significantly enhance the sensitivity of direct microscopy [5,6]. Therefore, the present preliminary study aimed to explore the use of the bleach (NaOCl) method in Fine Needle Aspiration Cytology (FNAC) of lymph nodes, building upon previous literature.

**Modified bleach staining method:** The FNAC aspirate was mixed with 2 mL of 4% sodium hypochlorite. The mixture was incubated for 15 minutes, followed by the addition of an equal volume of distilled water. After centrifugation at 3000 Rotation per Minute (RPM) for 15 minutes, the supernatant was discarded, and one drop of sediment was stained with ZN staining. The Modified Bleach concentration method for detecting AFB utilises a 2-5% concentration of NaOCl, which digests the necrotic material and inactivates the mycobacteria without altering their structure. This

allows for staining and observation of the mycobacteria, even when they are killed. This technique is simple, requires no expertise, and is cost-effective [7]. The present study aimed to compare the diagnostic accuracy ZN staining technique with the Bleach concentration method in relation to CBNAAT, which serves as the gold standard for diagnosing Tubercular lymphadenitis and to evaluate the role of the Modified bleach technique of ZN staining in diagnosing TB lymphadenitis.

## MATERIALS AND METHODS

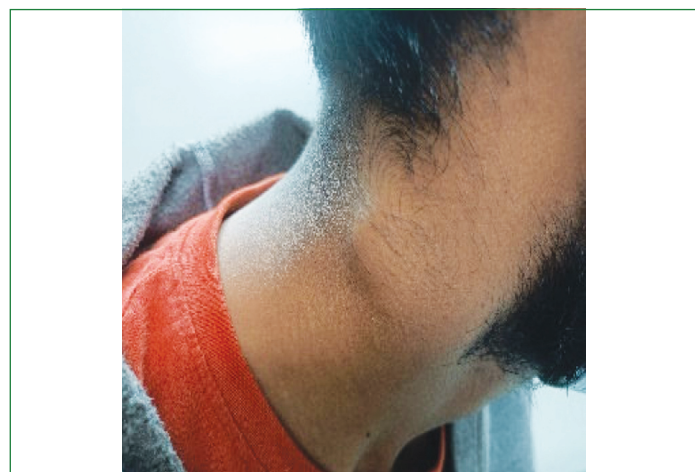
A prospective cross-sectional study was conducted at Department of Pathology, MVJ Medical College and Research Hospital, Bengaluru, Karnataka, India, including 100 cases of clinically suspected tubercular lymphadenitis that presented to the Medicine/Surgery/Pulmonology OPD over a period of two years, from November 2020 to December 2022. The study received approval from the Institutional Thesis and Ethics Committee (No. 452), and informed consent was obtained from each patient. FNAC was performed using a 22-gauge needle in the Department of Pathology, following aseptic precautions. Cases where the material was inadequate for modified bleach staining and CBNAAT were excluded from the study.

**Inclusion criteria:** Patients with palpable lymphadenopathy and clinical suspicion of TB lymphadenitis were included in the study, irrespective of age, sex, and site of lymph nodes. Detailed clinical history was obtained from each patient, including information on fever, cough, weight loss, past history of TB (pulmonary or extrapulmonary), past or recent history of Anti-Koch's Treatment (AKT), and history of contact with tubercular patients.

**Exclusion criteria:** Cases where the FNAC material was inadequate for cytology processing, modified bleach technique, fluorescent staining, and CBNAAT were excluded from the study.

### Study Procedure

For each case, part of the aspirate was used for routine cytology processing and staining. This included two air-dried smears, one for giemsa stain (to assess the cytomorphology of lymph nodes) and another for conventional ZN staining. The remaining aspirated material was liquefied with a 5% sodium hypochlorite (NaOCl) solution in a test tube at room temperature for 30 minutes, followed by centrifugation at 3000 rpm for 15 minutes. The supernatant was discarded, and smears were made from the sediment on clean glass slides, which were then stained by ZN staining. The slides were mounted and screened. As a control, 2 mL of distilled water was centrifuged, and the sediment was stained by ZN staining to rule out any errors due to contamination during testing of each specimen. Additionally, part of the aspirate was sent for CBNAAT. The bacilli were graded according to the Revised National Tuberculosis Control Programme (RNTCP) guidelines [Table/Fig-1] [8,9].



[Table/Fig-1]: Gross examination of lymph nodes- Enlarged cervical lymph node.

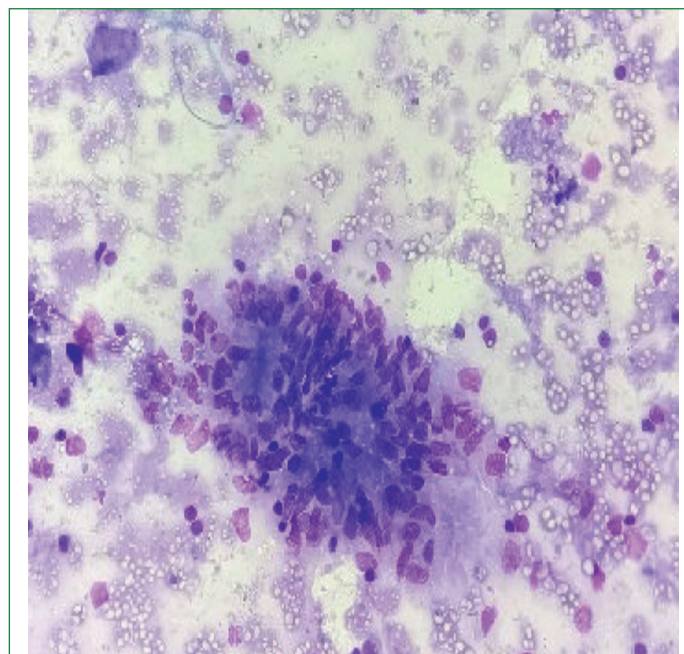
## STATISTICAL ANALYSIS

The data was analysed using Statistical Package for Social Sciences (SPSS) version 17.0. These results were compared with the CBNAAT findings, and sensitivity, specificity, PPV, NPV, and diagnostic accuracy were calculated accordingly. The p-value was calculated using the Chi-square test, and a p-value <0.05 was considered statistically significant.

## RESULTS

Out of the 100 cases included in the study, 13 cases were eliminated due to inadequate FNAC material. The most common age group affected was 15-29 years, with 27 out of 87 cases (31%). The youngest patient was a five-year-old, and the oldest patient was a 74-year-old. There was a slight male preponderance, with a male-to-female ratio of 1.12:0.8. Fever, cough, weight loss, and loss of appetite were the presenting symptoms.

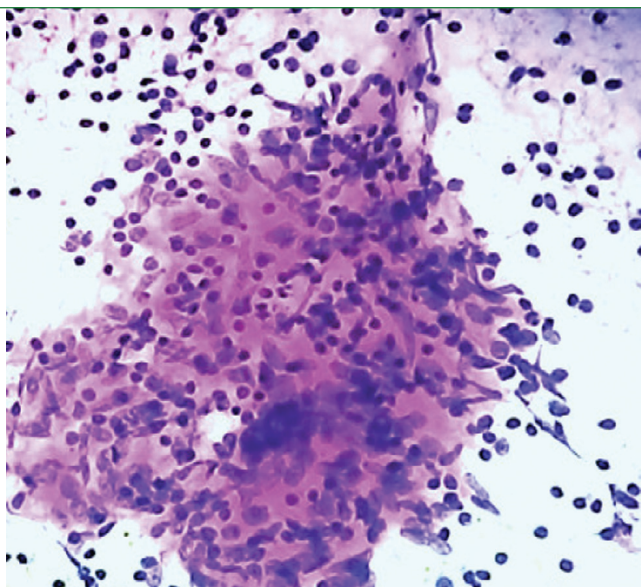
The cervical group of lymph nodes [Table/Fig-1] was the most commonly affected, with 69 out of 87 cases (79.31%). The cytomorphological diagnosis of reactive lymphadenitis was made in 36 cases (41.3%), followed by 15 cases (17.3%) of granulomatous lymphadenitis [Table/Fig-2], 12 cases (13.8%) of suppurative lymphadenitis, 12 cases (13.8%) of necrotising granulomatous lymphadenitis [Table/Fig-3], and 12 cases (13.8%) reported as necrotising lymphadenitis [Table/Fig-4]. Among the 87 cases of lymph node enlargement, 32 cases (36.7%) were confirmed to be of tubercular aetiology by CBNAAT. Out of these cases, AFB were detected only in 3 cases (9.3%) by conventional ZN staining, whereas 16 cases (50%) showed AFB positivity by the modified bleach technique of ZN staining. Maximum AFB positivity was seen in necrotic lymph nodes (66%) by conventional staining and (50%) by the modified bleach technique. None of the cases diagnosed as reactive lymphadenitis morphologically were positive for AFB on smears or CBNAAT positive for tubercular bacilli [Table/Fig-5-10].



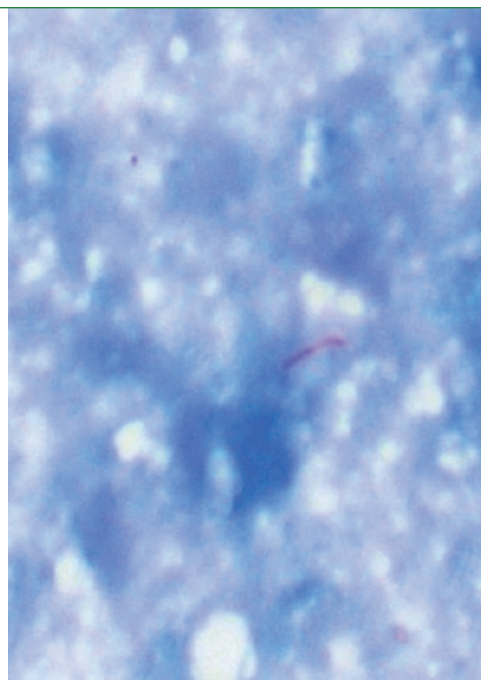
[Table/Fig-2]: Granuloma, aggregate of epithelioid histiocytes (Giemsa stain 40X).

On comparing the modified bleach technique with conventional ZN staining, the former showed a significantly higher rate (50%) of AFB detection on cytology smears. In the present study, the authors evaluated two staining methods for AFB, namely the conventional ZN method and the modified bleach method, with CBNAAT as the gold standard test. The conventional ZN stain method showed a sensitivity of 9.37%, specificity of 100%, PPV (100%), NPV of (65%), and diagnostic accuracy of 66%. These values increased when using the modified bleach method, with sensitivity of 59.4%, specificity of 100%, PPV of 100%, NPV of 80%, and diagnostic accuracy of 85%.

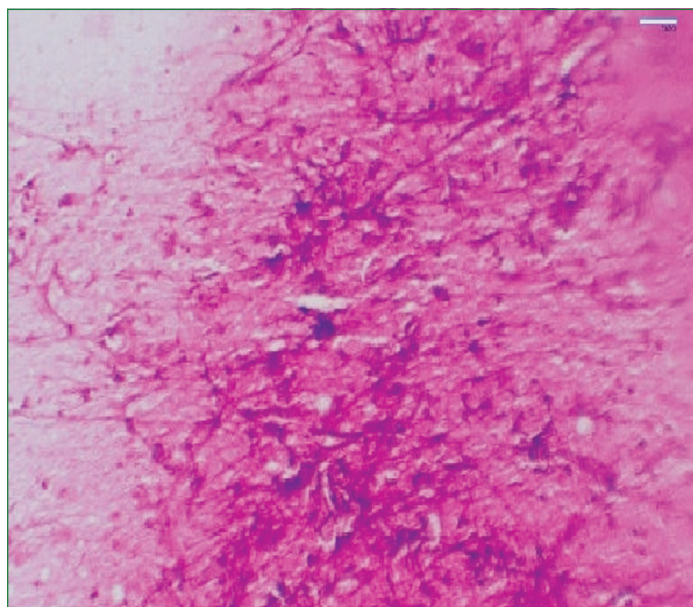




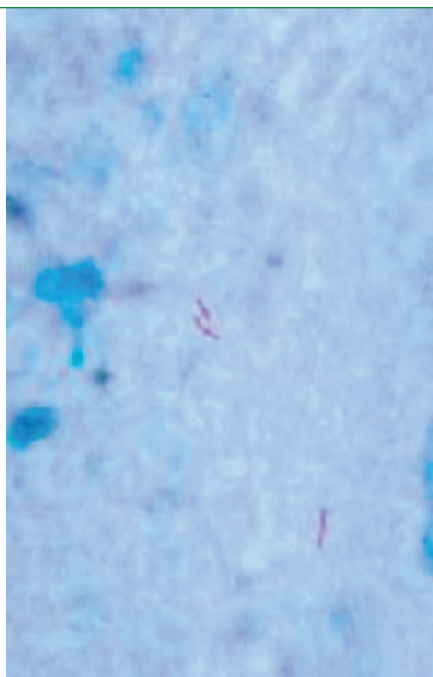
**[Table/Fig-3]:** Necrotising granulomatous lymphadenitis, cluster of epithelioid histiocytes in a necrotic background (H&E, X40).



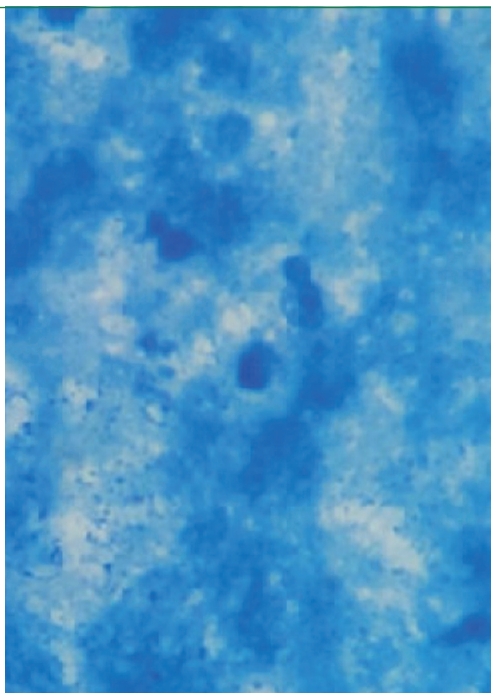
**[Table/Fig-6]:** Scanty AFB (X1000).



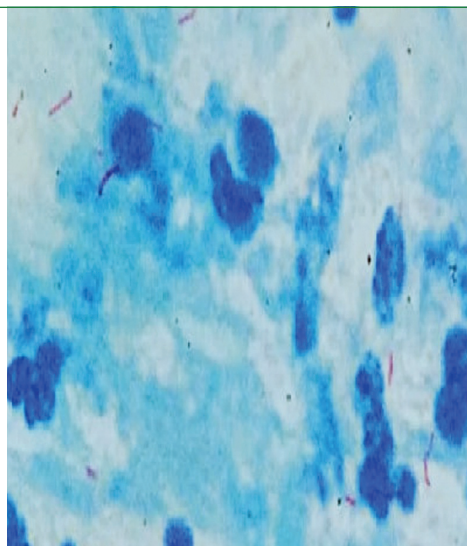
**[Table/Fig-4]:** Necrotising lymphadenitis (H&E, x40).



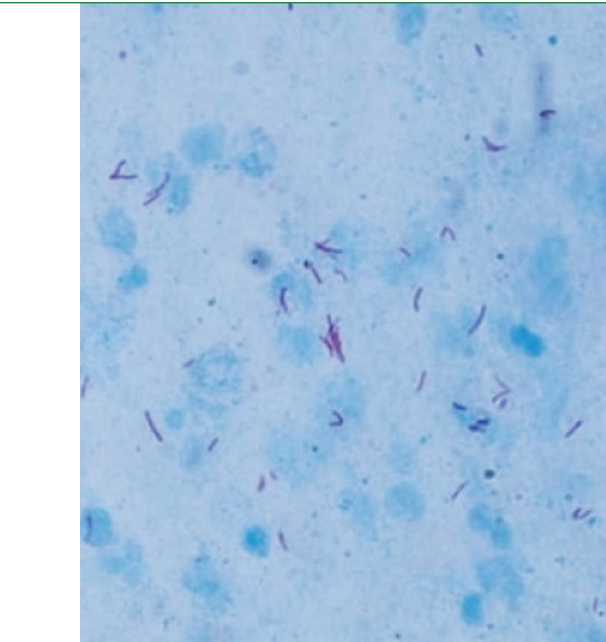
**[Table/Fig-7]:** Grade 1+ for AFB (X1000).



**[Table/Fig-5]:** Conventional ZN stain, negative for AFB (X1000).



**[Table/Fig-8]:** Modified bleach technique of ZN staining- Grade 1+ for AFB (X1000).



[Table/Fig-9]: Grade 2+ for AFB (X1000).

No. of AFB seen	Grade	Min. No. of fields to be examined
>10 bacilli/oil immersion field	3+	20
1-10 bacilli/oil immersion field	2+	50
10-99 bacilli/100 oil immersion field	1+	100
1-9 bacilli/100 oil immersion field	Scanty	100

[Table/Fig-10]: Grading of Ziehl-Neelsen staining for AFB by modified bleach method [8,9].

The results showed increased sensitivity in detecting AFB by the modified bleach method, with greater diagnostic accuracy compared to the conventional ZN stain method, which had lower sensitivity and diagnostic accuracy [Table/Fig-11-13].

Diagnosis	No. of cases	CBNAAT confirmed cases of TB	ZN staining by conventional method	ZN staining by modified bleach method
Reactive LN	36	0	0	0
Suppurative lymphadenitis	12	6	0	2
Necrotising granulomatous lymphadenitis	12	10	1	4
Granulomatous lymphadenitis	15	5	0	2
Necrotising lymphadenitis	12	11	2	8
Total	87	32	03	16

[Table/Fig-11]: Comparison of AFB positivity by conventional ZN staining, modified bleach technique and CBNAAT.

CBNAAT			
		Positive	Negative
Conventional ZN method	Positive	3	0
	Negative	29	55
CBNAAT			
		Positive	Negative
Modified bleach method	Positive	16	0
	Negative	16	55

[Table/Fig-12]: Comparison of conventional ZN method with that of modified bleach ZN method.

## DISCUSSION

Tuberculosis (TB) is a worldwide health problem that causes significant morbidity and mortality [10]. Tubercular lymphadenitis is the most

Statistical analysis	Conventional ZN method	Modified bleach method
Sensitivity	9.37%	50%
Specificity	100%	100%
Positive Predictive Value (PPV)	100%	100%
Negative Predictive Value (NPV)	65%	77%
Diagnostic accuracy	66%	81%

[Table/Fig-13]: Evaluation of conventional ZN method and modified bleach method with CBNAAT (Gold standard test).

common form of extrapulmonary TB [11]. The gold standard for diagnosing TB is culture on L.J medium, which is time-consuming [9,10]. Newer methods like Polymerase Chain Reaction (PCR) yield faster results but are expensive and not easily accessible. Therefore, the usage of ZN staining and microscopic detection of AFB is widely used for diagnosing TB lymphadenitis, as this technique gives relatively quicker and more reliable results. However, conventional ZN staining has low sensitivity and specificity. Hence, the ZN technique for detecting AFB has been improved by the bleach concentration method to increase the sensitivity and diagnostic accuracy of ZN staining for AFB detection on cytology [12-14].

In the present study, the greatest number of cases presenting with lymphadenopathy were between the ages of 15 and 29 years (27 cases, 31%), which is comparable to studies conducted by Thakur B et al., (60%) and Rasool G et al., (50%) [15,16]. There was a male predominance, and the male-to-female ratio was 1.12:0.8, consistent with studies conducted by Bhakti K and Kangle R (1.14:1) and Subhan Ali R et al., (1.26:1) [17,18]. Cervical lymph nodes were the most commonly involved group of lymph nodes, with 69 cases (80%). This finding resembled other studies carried out by Khare M and Kaushal M, (71.3%) and Sharma KC and Yadav A, (88.2%) [19,20].

Predominant patterns reported on cytology were reactive lymphadenitis in 36 cases (41%), followed by granulomatous lymphadenitis (17%), suppurative lymphadenitis, necrotising lymphadenitis, and necrotising granulomatous lymphadenitis (14% each). These findings were consistent with a study conducted by Bhakti K and Kangle R, which reported granulomatous lymphadenitis (20%), reactive lymphadenitis (52.3%), and suppurative lymphadenitis (9%) [17]. Khan S et al., reported granulomatous lymphadenitis (31%), necrotising granulomatous lymphadenitis (40%), and necrotic lymph nodes (29%), while Hemalatha A et al., reported granulomatous lymphadenitis (25%), necrotising granulomatous lymphadenitis (25%), necrotic lymph nodes (17%), and reactive lymphadenitis (49%) [21,22].

The maximum AFB positivity in our study was observed in necrotic lymph nodes. Out of the three cases showing AFB positivity on conventional ZN staining, two were in necrotic nodes. Similarly, eight out of sixteen cases where AFB was detected using the modified bleach technique were in necrotising lymphadenitis. According to some authors, higher detection rates of AFB in necrotic lymph nodes might be due to the concentration of the bacilli in the necrotic portions of the tubercle [21,22]. Vimal S et al., have stated that AFB positivity shows an inverse relationship with the presence of epithelioid cell granulomas and is directly proportional to the presence of necrotic material [23].

Purushothaman G et al., Bhakti K and Kangle R, and Gangane N and Singh R, have shown maximum AFB positivity in suppurative lymphadenitis on cytology using the modified bleach method [3,17,24]. This is attributed to the digestion of neutrophils and cellular debris by the modified bleach technique, resulting in a clearer background where AFB can be readily identified [3,17,24]. In the present study as well, two cases of suppurative lymphadenitis that were negative for AFB on conventional ZN staining showed AFB positivity on the modified bleach method of ZN staining.



In the modified bleach technique, the use of NaOCl causes liquefaction of the aspirated specimen, and further centrifugation of this specimen significantly increases the rate of detecting AFB in ZN-stained smears. NaOCl treatment brings about changes in the charge and hydrophobicity of AFB and also denatures the specimen, leading to flocculation and increased sedimentation of AFB [12,25]. The significant increase in the rate of AFB detection by this technique may be due to the higher density of bacilli obtained per microscopic field using this method and the reduction of debris, leaving a relatively clean background in smears [12].

In a study conducted by Gangane N and Singh R, on 100 cases of tubercular lymphadenitis, it was found that the modified bleach concentration technique detected AFB in 78% of cases, whereas the conventional ZN staining only showed AFB positivity in 27% of cases [24]. Additionally, in 58% of cases, the modified bleach method exhibited a higher grade of AFB positivity compared to the routine method [24]. In the present study as well, one case with scanty bacilli on conventional ZN staining was upgraded to Grade-I, and two cases that showed Grade-I positivity on ZN staining displayed a higher concentration of bacilli on the modified bleach method, resulting in an upgrade to Grade-II. The morphology of the bacilli appeared to be better preserved with the modified bleach technique; they were thicker and longer than the routine ZN smears. This could possibly be attributed to the swelling of the bacilli in the salt solution. Therefore, the effective use of the modified bleach technique increased the detection rate of AFB and reduced the screening time [12,21,24].

In another similar study conducted by Khare M and Kaushal M, AFB positivity increased from 58.9% to 89.1% when implementing the modified bleach concentration method of ZN staining [19]. The mean screening time of ZN-stained smears for AFB detection decreased from 23.36 minutes to 8.9 minutes when using the bleach concentration method, as the AFB positivity grade was much higher than with routine ZN staining.

In the present study, the authors observed increased sensitivity in detecting AFB using the modified bleach method, which resulted in greater diagnostic accuracy compared to the conventional ZN stain method, which exhibited lower sensitivity and diagnostic accuracy. These findings were consistent with studies conducted by Aalmeen G et al., (92.05%), Dhote AR et al., (80.27%), and Rioki JN et al., (85%), which demonstrated higher diagnostic accuracy for the modified bleach technique compared to the conventional ZN staining technique [26-28].

### Limitation(s)

In certain cases of tuberculous lymphadenitis, arriving at an absolute diagnosis can be challenging when the aspirate displays a polymorphous picture with occasional epithelioid cells, the absence of typical Langhans giant cell or caseous necrosis, and a more necrotic background. This situation necessitates a definitive diagnosis. In such cases, routine ZN staining exhibits low sensitivity as it rarely detects AFB (acid-fast bacilli) in aspirates. However, the modified bleach method has shown higher AFB positivity. This detection rate is significantly better than routine ZN staining. With routine ZN staining, most of the aspirates exhibited scant AFB positivity, and searching for them was a tedious and time-consuming exercise compared to the bleach method.

### CONCLUSION(S)

Early and definitive diagnosis of TB is essential for efficient treatment. The material obtained from FNA can be subjected to various tests to confirm TB, including conventional ZN staining, modified Bleach technique of ZN staining, fluorescent staining, CBNAAT, and culture. The modified bleach technique is a simple, safe, and cost-effective

method. It increases the concentration of bacilli by centrifugation and clears the necrotic background, resulting in better visualisation of AFB. A clearer background aids in detecting bacilli, especially in cases where cytomorphological diagnosis indicates suppurative lymphadenitis and antibiotic therapy has been ineffective. This method has an obvious advantage in timely detection and early treatment. Therefore, the present study validates the utility of the concentration of AFB by the modified bleach method for detecting AFB in lymph node aspirates. The sensitivity (50%) and diagnostic accuracy (81%) of the modified bleach method are far better than those of the conventional ZN method.

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