Comparison of Conventional Ziehl–Neelsen Method of Acid Fast Bacilli with Modified Bleach Method in Tuberculous Lymphadenitis

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Abstract

Introduction: Tuberculosis caused by Mycobacterium tuberculosis is a chronic infectious disease and a major health problem in developing countries, with lymphadenopathy being the most common presentation. Tuberculous lymphadenitis can be diagnosed on fine needle aspiration cytology of lymph node. Conventional Ziehl–Neelsen method for acid fast bacilli plays a key role in the diagnosis and monitoring of treatment for tuberculosis, however, with low sensitivity. Present study emphasizes the role of bleach concentration method in fine needle aspiration cytology of lymph nodes over conventional direct smear microscopy. Materials and Methods: The study included 75 patients with clinically suspected tuberculous lymphadenopathy who were referred to the Department of Pathology in a tertiary care hospital, Faridabad. Data regarding age, sex, duration and site of swelling, nature of aspirate, and cytomorphological diagnosis were documented for each patient. Results: Of the total 75 cases, 15 were positive both in conventional Ziehl–Neelsen method and bleach concentration method. By bleach concentration method, additional 34 cases showed positivity that were not revealed by conventional Ziehl–Neelsen method. Thus, a total 49 cases were positive for acid fast bacilli. Conclusion: There are problems in arriving at an absolute diagnosis in certain cases of tuberculous lymphadenitis when the aspirate shows polymorphous picture with occasional epithelioid cells and absence of typical Langhans giant cell or caseous necrosis. In the present study, acid fast bacilli positivity was established in 65.33% of the cases with the bleach method. Bleach method for detection of tubercle bacilli has a high case detection rate than that of the conventional Ziehl–Neelsen method.

Keywords: Bleach technique, conventional staining, tuberculosis

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by Mycobacterium tuberculosis and continues to be a major health problem in developing countries. India has the highest burden of TB. The World Health Organisation (WHO) statistics for 2015 give an estimated incidence of 2.2 million cases of TB for India out of a global incidence of 9.6 million.

Tuberculosis can involve any organ system in the body. While pulmonary TB is the most widespread presentation, extrapulmonary tuberculosis (EPTB: affecting outside the lung) is also an important clinical problem constituting 15–20% of all cases of TB. Lymphadenopathy is the most common presentation of EPTB. Tuberculous lymphadenitis is seen in nearly 35% of EPTB. Tuberculous lymphadenitis can be presumptively diagnosed morphologically on fine needle aspiration cytology (FNAC) of the lymph node.

Lymphadenitis is both a diagnostic and therapeutic challenge because it mimics other pathologic processes and yields inconsistent physical and laboratory findings. Diagnosis is difficult often requiring biopsy. A thorough history and physical examination, staining for acid-fast bacilli (AFB), and polymerase chain reaction (PCR) are helpful in obtaining an early diagnosis. It is also important to differentiate tuberculous from nontuberculous mycobacterial lymphadenitis because their treatment protocols vary. Treatment monitoring is more complex due to the peculiar behavior of TB lymph nodes. Situation has worsened due to a sharp increase in the incidence of new cases.

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of atypical *Mycobacteria*, poorly controlled HIV epidemic, and rise in drug-resistant TB lymphadenitis. Tuberculous adenitis is best treated as a systemic disease with antituberculosis medication. Surgical therapy along with antituberculosis medication can be beneficial in selected patients.

The clinical parameters for the diagnosis of TB in lymph nodes are neither specific nor does their absence exclude TB involvement. Conventional Ziehl–Neelsen (ZN) method for AFB plays a key role in the diagnosis and monitoring of TB treatment. Its major disadvantage is its low sensitivity, ranging from 20% to 43%.1, 4

Microscopy has many advantages when it comes to speed feasibility, and if its sensitivity could be improved, it has the potential to become an even more valuable tool for TB control programs around the world. The need for new and improved methods cannot be overemphasized. The bleach concentration method is one of the safest concentration methods for improving the sensitivity of direct microscopy for the detection of AFB. Liquefaction of specimens by sodium hypochlorite (NaOCl, bleach) and concentration of bacilli through centrifugation significantly increases the sensitivity of direct microscopy. The present study is undertaken to emphasize the role of bleach concentration method in FNAC of lymph nodes over conventional direct smear microscopy.

**Materials and Methods**

The study included 75 patients with clinically suspected tuberculous lymphadenopathy who were referred to the Department of Pathology in a tertiary care hospital, Faridabad. The cases of clinically suspected tuberculous lymphadenitis belonging to all the age groups were considered. The patients already diagnosed with TB and on antitubercular therapy initiated before FNAC lymph node were excluded from the study. Patients presenting with lymphadenopathy were subjected to a brief clinical examination. Data regarding age, sex, duration, and description of swelling-like site were documented for each patient. FNAC was performed with a 23-gauge needle and a 10-ml disposable syringe.

Tuberculous lymphadenitis was diagnosed based on the cytomorphological features of FNA smears. Cytomorphological criteria defining cytological diagnosis of reactive, suppurative, and granulomatous lymphadenitis were – reactive lymphadenitis revealed mixed population of lymphoid cells in varying proportions along with scattered histiocytes with intracytoplasmic nuclear debris (tingible body macrophages); suppurative lymphadenitis revealed lymphoid cells along with numerous intact and degenerated polymorphs against a background of necrotic debris; granulomatous lymphadenitis showed epithelioid cell granulomas with or without the presence of Langhans’ multinucleated giant cells and caseous necrosis. Cytological patterns in cases diagnosed as tuberculous lymphadenitis were (1) epithelioid granuloma with extensive necrosis, (2) epithelioid cell granulomas only, and (3) necrosis only.

**Conventional cytology technique**

Following lymph node FNA, approximately 5–6 smears were prepared on clean glass slides. One or two smears were fixed in 95% ethanol and stained with Papanicolaou stain (Pap) and hematoxylin and eosin (H and E) stains. Air-dried smears were stained with May–Grunwald–Giemsa stain (MGG) and the routine ZN method. ZN stained smears were examined for the presence of tubercle bacilli. Other smears were studied for cyt morphological evidence of TB.

**Bleach concentration technique**

The residual aspirated material in the needle was flushed out and subjected to liquefaction with 5% sodium hypochlorite (NaOCl, bleach) and stained with the routine ZN method.

**Staining procedure of routine Ziehl-Neelsen method**

Smear was prepared from the lymph node specimen on a clean glass slide and fixed by heating on a Bunsen burner flame. The heat fixed slide was placed on a staining rack or rods and the smear was flooded with working Carbol–fuchsin stain. The slide was heated gently on Bunsen burner flame until steam was noted. Boiling was avoided and heating was continued for approximately 5 minutes. It was ensured that the stain did not dry out on the slide. More stain was added if necessary. The stain was washed off the slide with water and rinsing was continued until the water became colorless. For decolorization, the slide was covered with 20% sulfuric acid for approximately 1 min. The yellow-colored complex was drained off completely. The slide was covered with methylene blue stain for 1 min for counterstaining. Wash with tap water and allow the water to drain. Air dry the slide or blot carefully. Slide was observed under a low power objective and then examined under an oil immersion objective.

**Bleach concentration technique**

Left over aspirate from the needle was rinsed with 2 ml 5% NaOCl in a clean glass test tube. After thoroughly mixing the mixture, the test tube was left for 20 min at room temperature for incubation. The specimen was centrifuged at 3000 rpm for 15 min and the supernatant was discarded. Sediment was taken on a clean glass slide and air dried, and heat fixed gently on a Bunsen burner flame. Smear was prepared and stained with the routine ZN stain. Mount in D.P.X. Slide was observed under a low power objective and then examined under an oil immersion objective.

**Results**

Tuberculous lymphadenopathy is the most common form of EPTB. The present study was undertaken to emphasize the role of bleach concentration method over the conventional direct smear microscopy for the detection of tubercle bacilli in FNA material of lymph nodes. Clinically suspected 75 cases of tuberculous lymphadenitis were evaluated.
Majority of the patients were in the age group of 16–30 years (48%) and 0–15 years (33.33%). The youngest patient was 1 year old, and the oldest patient was 75 years old [Table 1].

According to sex distribution, there were 43 males (57.33%) and 32 females (42.67%). Male:female ratio was 1.34:1. Most of the patients had lymphadenopathy of less than 3 months duration (58% cases), 3–6 months duration (20% cases), and more than 6 months duration (22% cases). Majority of the patients presented with lymphadenopathy of cervical region (53%) followed by axillary region (15%).

The aspirate was pale white and granular in 33 (44%) cases, followed by purulent aspirate in 22 (29.33%) cases. Hemorrhagic aspirate was seen in 20 (26.67%) cases. All smears were stained with H and E, Pap, and ZN stains. Majority of the cases [44 (58.67%)] were cytomorphologically diagnosed as granulomatous lymphadenopathy, followed by 22 cases (29.33%) as suppurative lymphadenopathy and 9 cases (12%) as reactive lymphadenopathy.

Among the 44 cases diagnosed as tuberculous lymphadenopathy, the cytological patterns were as follow: epithelioid granuloma with extensive necrosis (23), epithelioid granuloma only (14), and necrosis only (7).

In the present study by conventional ZN method, a total of 15 (20%) cases were AFB positive and 60 (80%) cases were AFB negative [Figure 1a].

Bleach concentration method

In the present study, newly recommended bleach concentration method was utilized for the detection of AFB in the diagnosis of tuberculous lymphadenitis. Aspirates of all 75 cases suspected of tubercular lymphadenitis were subjected to bleach concentration method and smears were stained by ZN method and thoroughly screened for AFB.

In the present study, by bleach concentration method, a total of 49 (65.33%) cases were AFB positive and 26 (34.67%) were AFB negative [Figure 1b and Tables 2, 3].

Of total 75 cases, 15 cases were positive both on conventional ZN method and bleach concentration method. By bleach concentration method, 34 additional cases showed positivity that was not revealed by the conventional ZN method. Thus, a total of 49 cases were positive for AFB by the Bleach concentration method. None of the cases diagnosed positive on routine ZN stain were missed by the bleach concentration method. AFB positivity by bleach concentration method was more in comparison with the routine ZN stain.

**Discussion**

TB can affect any organ of the body. Tuberculous lymphadenitis is the most common type of EPTB. The diagnosis of TB is easy and simple when the disease is elaborate or disseminated, however, localized involvement of extrapulmonary organ or tissue may at times pose a diagnostic problem. The clinical parameters for the diagnosis of TB in lymph nodes are neither specific nor do their absence exclude tubercular involvement. Early diagnosis of TB and initiating optimal treatment would not only enable a cure of an individual patient but will also curb the transmission of infection and disease to others in the community. Diagnostic modalities must also be tailored to the needs of the population and epidemiology of TB in that region. These include improved microscopy, usage of liquid culture for childhood and EPTB, chemical and physical detection of mycobacterial antigens in paucibacillary condition, antigen capture, antibody detection, cellular immune recognition, nucleic acid amplification, and phage assay.

**Table 1: Age distribution**

| Age distribution (in years) | Number of cases | Percentage |
|----------------------------|----------------|------------|
| 0-15                       | 25             | 33.33      |
| 16-30                      | 36             | 48         |
| 31-45                      | 7              | 9.33       |
| 46-60                      | 6              | 8          |
| 61-75                      | 1              | 1.33       |
| Total                      | 75             | 100        |

**Table 2: Correlation of cytomorphological diagnosis with bleach and conventional Ziehl-Neelsen method**

| Cytomorphological diagnosis | Bleach method | Conventional Ziehl-Neelsen method | Total | Percentage |
|-----------------------------|---------------|----------------------------------|-------|------------|
|                             | Positive for acid-fast bacilli | Negative for acid-fast bacilli | Positive for acid-fast bacilli | Negative for acid-fast bacilli |       |          |
| Reactive lymphadenitis      | 2             | 7                                | 0     | 9          | 9     | 12      |
| Suppurative lymphadenitis   | 12            | 10                               | 4     | 18         | 22    | 29.33   |
| Tuberculous Lymphadenitis   | 35            | 9                                | 11    | 33         | 44    | 58.67   |
| Total                       | 49 (65.33%)   | 26                               | 15 (20%) | 60        | 75    | 100     |

Figure 1: (a) Conventional Ziehl–Neelsen positive stain: Photomicrograph (ZN stain x100) showing acid-fast bacilli. (b) Bleach concentration method Ziehl–Neelsen positive stain: Photomicrograph (ZN stain x100) showing acid-fast bacilli.
Various techniques other than the conventional ZN staining and bleach concentration method for demonstrating AFB are Pap stain, auramine rhodamine staining (ARS), autofluorescence (AF) stain, fluorescein-diactate (FDA) vital staining, light emitting diodes (LED) microscopy, and polymerase chain reaction (PCR) technique.

Mycobacterial culture is the reference method and is time consuming. It requires specialized safety procedures in laboratories. Serological techniques lack sensitivity and specificity.[5] PCR, although rapid, is costly to be routinely used in developing countries where TB is prevalent.[5]

Pap, ARS, FDA, and LED techniques require a fluorescent microscope. Availability of fluorescent microscopes in laboratories with resource-limited settings is difficult. Advantage of fluorescent microscopy is that it can be used at lower magnifications and time required is less to examine much larger area per unit time. Pap stain avoids the use of toxic or carcinogenic substances such as phenol and rhodamine used in ARS method.

ZN method for AFB bacilli plays a key role in the diagnosis as well as in the monitoring of TB treatment.[6,7] Its major disadvantage is low sensitivity, ranging from 20% to 43%.[3,4]

Bleach concentration method is one of the safest methods for improving the sensitivity of direct microscopy for the detection of AFB. A few previous studies have shown that liquefaction of sputum by NaOCl and concentration of bacilli through centrifugation significantly increases the sensitivity of direct microscopy.[8,9] In the late 1940s, sputum liquefaction with NaOCl followed by centrifugation before acid-fast staining was implemented to improve the smear positivity for the detection of AFB. This method was slightly modified and applied in lymph node aspirates.

The present study revealed discordance between cytomorphological diagnoses and AFB positivity by bleach method in some cases. Two specimens were reactive lymphadenitis [Figure 2a and b] and 12 specimens were acute suppurative lymphadenitis; however, these were positive for AFB by the bleach method. The possible explanation for the diagnosis of reactive lymphadenitis on cytology but positive for AFB by the bleach method in cases may be due to the loss of scattered epithelioid cells among the polymorphous population of lymphoid cells. All patients responded well to antitubercular therapy. Among the 12 specimens diagnosed as suppurative lymphadenitis, these were positive for AFB by the bleach method, the likely reason could be loss of the bacilli amidst the necrotic debris.

Khubani et al. studied 55 cases of EPTB, which included 18 aspirates from body fluids, 18 from abscesses drained from various body sites, 17 from lymph nodes, and 2 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 ZN staining, and 70.90% cases positive for AFB by the bleach method.[10]

Gangane et al. studied 100 cases of TB lymphadenitis and concluded that bleach concentration method demonstrated AFB positivity in 72% of the cases. AFB positivity grade was significantly higher than with routine ZN staining making bacilli easily visible with shorter screening time. The bleach method was inexpensive, easily performed, and more sensitive and safer than the routine ZN staining.[11]

Annam et al. studied 93 cases of lymphadenopathy. Among 93 aspirates, 33.33% were positive for AFB in conventional ZN method and the smear positivity increased to 63.44% on bleach method. They concluded that the bleach method was simple and inexpensive.[12]

Chandrasekhar et al. studied 112 cases of lymphadenopathy. Among 112 aspirates, 12.5% were positive for AFB in conventional ZN method and the smear positivity increased to 60.7% on bleach method [Table 4].[13]

The present study 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. Moreover, the increased smear positivity by the bleach method is attributable to the higher density of bacilli per microscopic field obtained by the method and reduction of debris, leaving a clear field for microscopy. Thus, the preparation of samples

| Table 3: Comparison of Conventional Ziehl-Neelsen method with Bleach method for detection of acid fast bacilli |
|-------------------------------------------------|-----------------|-----------------|
| Conventional Ziehl-Neelsen method | Bleach method | Total |
| Positive | Negative | Total |
| Positive | 15 | 0 | 15 |
| Negative | 34 | 26 | 60 |
| Total | 49 | 26 | 75 |
Table 4: Comparison of acid-fast Bacilli positivity in different studies by conventional and bleach method

| Authors            | Conventional method (%) | Bleach method (%) |
|--------------------|-------------------------|-------------------|
| Khubnani et al.[10] | 21.8                    | 70.9              |
| Gangane et al.[11] | 27.0                    | 72.0              |
| Annam et al.[12]   | 33.33                   | 63.44             |
| Chandrasekhar et al.[13] | 12.5                | 60.7              |
| Present study      | 20.0                    | 65.33             |

by the bleach method reduces the time required for slide examination 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. First, because majority of patients with AFB suffer from TB and, second, because other mycobacteria are usually not present in sufficient concentration to be detected by direct microscopy. 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, however, the method is strongly recommended for all laboratories that perform direct microscopy only. In the present study, a centrifugation at 3000 rpm for 15 min yielded increased recovery of mycobacteria.

Previous studies done by various authors and the present study revealed that the bleach method for AFB detection is simple, safe, cost-effective, and has a high case detection rate. The results would be more well-organized if concentration by bleach solution, RCF, and bleach treatment is as per the time schedule and is comparable. The application of the bleach method clearly improves microscopic detection and can be a useful influence to routine cytology. This would be of advantage to the patients in receiving early and effective treatment.

**Conclusion**

There are problems in arriving at an absolute diagnosis in certain cases of tuberculous lymphadenitis when the aspirate shows polymorphous picture with occasional epithelioid cells and absence of typical Langhans giant cell or caseous necrosis, making it necessary for a definitive diagnosis. In such cases, routine ZN staining shows low sensitivity because it rarely detects AFB in aspirates. However, in the present study, we could establish AFB positivity in 65.33% of cases with the bleach method. This detection rate is far better than routine ZN staining.

It was also observed that, by routine ZN 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, in a majority of AFB positive cases, AFB was easily visible and detectable. AFB morphology was observed to be better preserved in the bleach method.

Thus, the bleach method for detection of tubercle bacilli in lymph node aspirate is more useful than the conventional ZN method. Moreover, the bleach method is safe, inexpensive, and easy to perform requiring no additional equipment.

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**Conflicts of interest**

There are no conflicts of interest.

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