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Comparative Evaluation Between Modified Bleach Method and Conventional Z-N Staining Method in Improved Detection of Acid–Fast Bacilli in Tubercular Lymphadenitis

Md. Shajjad Hossain[1], Saequa Habib[2], Saumitra Chakravarty[3], Md. Shahrior Nahid[4], Mohosina Sultana[5]

[1]Lecturer, Department of Pathology, Dhaka Dental College, Dhaka, Bangladesh; [2]Professor, Department of Pathology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh; [3]Assistant professor, Department of Pathology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh; [4]Medical Officer, Department of Pathology, National Institute of Laboratory Medicine and Referral Centre, Dhaka, Bangladesh; [5]Lecturer, Department of Pharmacology, Dhaka Dental College, Dhaka, Bangladesh

Abstract

Background: This cross-sectional study was carried out first in Bangladesh to compare a newer 'modified bleach method' and traditional Z-N staining method for improved structural and morphological detection of Acid-Fast Bacilli (AFB) in Tubarcular lymphadenitis. **Objectives:** This study was aimed to compare the effectiveness of modified bleach concentration method with the conventional Z-N method to detect Acid fast bacilli (AFB) for the diagnosis of Tuberculosis in lymph node cytology. **Methodology:** This was a cross-sectional study conducted from January 2019 to January 2022 in the Department of Pathology at Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh. This study executed both conventional Z-N method and modified bleach concentration method in each sample for total 98 tubercular lymphadenitis cases. Then the data were compared with each other and with GeneXpert which was the reference control for this study. **Results:** This study revealed that the Bleach method detected more positive AFB than the Z-N method. Among the 98 cases, a total 63 cases were AFB positive (64.29%) and 35 cases (35.71%) were AFB negative by bleach concentration method. On the other hand, 51 (52.04%) cases were AFB positive, and 47 cases (47.96%) were AFB negative by Z-N staining method. Not only that but among the 47 negative cases of the conventional Z-N method, 13 cases were found to be positive in modified Bleach method. **Conclusion:** In conclusion, the application of the bleach method clearly improves microscopic detection and can be a useful adjunct to routine cytology.

Keywords: Tuberculosis; modified bleach method; traditional Z-N method; lymphadenitis

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Introduction

Tuberculosis has been researched with medical knowledge by memory since 9000 eras[1]. Recently, Bangladesh ranks sixth among the top twenty-two high-burden countries (HBC) of tuberculosis by the World Health Organization (WHO). And 51 per 100000 of the population died in one year with the complications of Tuberculosis (TB)[2].

Tuberculous lymphadenitis (TBL) is a common form of extra-pulmonary tuberculosis (EPTB), and the leading cause of lymphnode swelling in venues with a high frequency of tuberculosis[3]. In its early stage, tubercular lymphadenitis still remained a diagnostic challenge for clinicians. Because many patients present with peripheral lymphadenitis, which can go unnoticed for longer durations and only spread illness when the host's immune system is impaired[4]. Since then, fine-needle aspiration cytology (FNAC) has constantly been practiced as a simple, safe, reliable, and economical method of establishing the diagnosis of lesions and masses in various sites and organs and is the most accessible and convenient diagnostic aid[5].

Direct smear microscopy for acid-fast bacilli from the lesion sample is the only viable bacteriologic approach for diagnosing extrapulmonary TB in most

Correspondence: Dr. Md. Shajjad Hossain, Lecturer, Department of Pathology, Dhaka Dental College, Mirpur-14, Dhaka, Bangladesh; Email: shetukmc42@gmail.com; Cell no.: +8801756866753; +8801767696985; ORCID: https://orcid.org/0009-0003-0622-1728

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low-income countries[6]. FNAC alone offers poor specificity because the morphologic spectrum of tuberculous lymphadenitis varies greatly depending on the stage of the disease and the host's immunity[7]. Though cytomorphologically, the presence of epithelioid cell granulomas, caseous necrosis, and Langhans giant cells are characteristics in the diagnosis. However, similar features can be seen in lesions other than TB, such as fungal infections, other inflammatory causes, and sarcoidosis. Regrettably, the technique's sensitivity is not optimal, ranging from 9.0% to 46.0% when used in control programs[8]. The need for new, improved, low-cost techniques cannot be neglected[9].

Here is the hope that many researchers have indicated that if the sample is liquefied with one or more chemical reagents and then concentrated by centrifugation or sedimentation before acid-fast staining, the performance of smear microscopy can be greatly improved. As a result, many researchers have experimented with various ways such as the modified bleach concentration method on FNA material from lymph nodes, abscesses, skin scrapings, and bodily fluids[10]. This method clears necrotic and cellular debris, resulting in a clear field with easy vision, thereby increasing AFB microscopical detection[11].

Thus, many studies demonstrated an increase in the smear positivity for AFB by the modified bleach method in comparison with the conventional ZN method. Also, this approach is safe, affordable, requires no skill, and is simple to use[13]. Now- a - days in low & middle-income countries, the modified bleach method is the most widely studied and practiced method due to its statistically significant improvement in sensitivity over the Z-N method[14].

Another method, Polymerase Chain Reaction (PCR) has been accepted as the reference method (gold standard) while compared to the bleach method with the Z-N method[15]. Though culture of mycobacteria is considered to be the cornerstone for TB diagnosis, PCR remains an even more sensitive technique as it detects even nonviable mycobacteria[16]. This study was aimed to compare the effectiveness of modified bleach concentration method with the conventional Z-N method to detect Acid fast bacilli (AFB) for the diagnosis of tuberculosis in lymph node cytology.

Methodology

Study Settings and Population: This cross-sectional study was undertaken in the Department of Pathology at Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh from January 2019 to January 2022 to emphasize the role of the bleach concentration method over conventional direct smear microscopy for the detection of tubercle bacilli in fine needle aspirate material of lymph nodes. All extrapulmonary lymphadenitis patients (both male and female) who came to Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh during that particular period were selected for this study. However, the patient who had been diagnosed as malignant or who had received Anti TB drugs were excluded from this study. The patients presenting with clinically suspected tubercular lymphadenopathy were included in this study. After taking consent from patients, lymph node aspirates were taken with proper precautions and measures. This study was performed by both conventional Z-N and modified bleach concentration bleach method for each sample.

Study Procedure: For the modified bleach concentration method, aspirated materials were transferred to a conical screw-capped tube. Here 2ml of 5.0% NaOCl solution was added. The mixture remained incubated for 30 minutes at room temperature and shaking was done at regular intervals. The conical tube containing the mixture was concentrated by centrifugation after the addition of 2 ml of distilled water. After centrifugation, the supernatant was carefully discarded and the sediment was transferred with a sterile pipette on to clean the sterile glass slide. Two smears were made from the sediment and the smear area was marked by a marking pencil. Subsequently, the slide was air dried, heat-fixed, and stained with the Z-N method. To rule out any error due to contamination while testing each specimen, 2ml distilled water was centrifuged as a control and the sediment was stained with Z-N staining.

For the Z-N method, Smear was prepared from the lymph node aspirates. The heat-fixed slide was placed on a staining rack or rods and the smear was flooded with a working Carbol–fuchsin stain. Then the stain was washed off the slide with water and rinsing continued until the water became colorless. For decolorization, the slide was covered with 20% sulfuric acid or 3% v/v acid alcohol for approximately 1 min but not more than 3 minutes. Dipping in the acid solution and subsequent water wash was continued until decolorization was completed. The slide was covered with methylene blue stain for 1 min for counterstaining. Clearing with Xylene and subsequent air drying or blotting of slides was done carefully. The

slide was observed under a low-power objective and then was examined under an oil immersion objective.

After the execution of the conventional Z-N method and modified bleach concentration method for each sample then the data were compared with each other and with GeneXpert (which was the reference control for this study). This cross-sectional study was undertaken to emphasize the role of the bleach concentration method over conventional direct smear microscopy for the detection of tubercle bacilli in fine needle aspirate material of lymph nodes. A total of 107 Patients presenting with clinically suspected tubercular lymphadenopathy were included in this study. After obtaining consent from patients, lymph node aspirates were taken with proper precautions and measures. This study was performed by both conventional Z-N and modified bleach concentration bleach method for each sample.

Statistical Analysis: A structured questionnaire form was used to collect information about each subject's medical history, aspiration details, demographic characteristics, and investigation results. All data regarding patients was recorded methodically in a data sheet. The statistical analysis was carried out using the Statistical Package for Social Sciences version 23. Statistical significance was considered at 95% confidence level. To test any association Mcnemar's Chi-square Test Inter- Class Correlation test (ICC test) were used. In all cases significance level was considered at P value < 0.05.

Ethical Clearance: All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional research ethics. Formal ethics approval was granted by the local ethics committee (Ref: IRB/NINS/…..). Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Results

This study found that the AFB positivity on Z-N staining and modified Bleach method in contrast with the correlation among 6 cytomorphological patterns. In every pattern no. of AFB positive cases were more in the modified bleach method than Z-N staining method (Table 1).

This study revealed that, Bleach method detected more positive AFB than Z-N method. Table 2 shows that among the 98 cases, total 63 cases were AFB positive (64.29%) and 35 cases (35.71%) were AFB negative by bleach concentration method. On the other hand, 51 (52.04%) cases were AFB positive, and 47 cases (47.96%) were AFB negative by Z-N staining method (Table 2).

Table 2: Direct Comparison of the Conventional Z-N Method with the Modified Bleach Method in the Detection of Acid-Fast Bacilli

AFB Staining	Modified Bleach Method	Z-N staining Method	P value
Positive	63 (64.29%)	51(52.04%)	≤ 0.05
Negative	35(35.71%)	47 (47.96%)	
Total	**98 (100%)**	**98 (100%)**	

This study also compared the detection rate of AFB between the conventional Z-N method and modified Bleach method. Among the detected 51 positive cases of conventional Z-N method, all of them were found to be positive in the modified Bleach method. Not only that but also among the 47 negative cases of the

Table 1: Cytomorphological pattern and AFB positivity on routine Z-N staining and Modified Bleach Method

	Routine Z-N staining		Modified bleach method		Total
	AFB negative	AFB positive	AFB negative	AFB positive	
Pattern 1	43 (43.87%)	**31(72.0%)**	12(27.9%)	21(48.83%)	22(51.16%)
Pattern 2	7(7.1%)	**6(85.7%)**	1(14.28%)	5(71.4%)	2(28.5%)
Pattern 3	19(19.3%)	**9(47.3%)**	10(52.6%)	7(36.8%)	12(63.2%)
Pattern 4	7(7.14%)	**2(28.6%)**	5(71.4%)	1(14.3%)	6(85.7%)
Pattern 5	3(3.06%)	**1(33.3%)**	2(66.7%)	0(0.0%)	3(100%)
Pattern 6	19(19.38%)	**9(47.4%)**	10(52.63%)	2(10.52%)	17(89.47%)

Cytomorphological Pattern19: The pattern 1 represents epithelioid granulomas with Langerhans giant cells and caseous necrosis. Pattern 2 represents numerous clusters of epithelioid cells in a reactive background. Pattern 3 represents mostly caseous necrotic material with few epithelioid cells. Pattern 4 represents caseous necrotic material with few lymphocytes and histiocytes; no epithelioid cells. Pattern 5 represents only caseous necrosis without any other cell types. Pattern 6 represents tuberculous abscess showing predominantly neutrophils along with degenerating epithelioid cells and necrotic material.

Table 3: Comparison of the Detection of AFB by Modified Bleach Method over Conventional Z-N Method

Z-N Method	Bacterial Meningitis		Total	P value
	Positive	Negative		
Positive	51(100%)	51(52.0%)	51(0.0%)	
Negative	13(27.65%)	47(47.95%)	34(72.34%)	0.0001
Total	**64(65.30%)**	**98(100%)**	**98(34.69%)**	

conventional Z-N method, 13 cases were found to be positive in modified Bleach method. There was no such case that Z-N method was found positive but the Bleach method was found negative (Table 3).

After the direct comparison, this study conducted grading between two methods. Table 4 shows the comparison of AFB grading by routine Z-N and modified Bleach method according to a sensitive scale of the American Thoracic Society (ATS). The no. of the cases that were positive in grading had shown upgraded result grade in the modified bleach method. Among the 47 negative cases of Z-N method, 13 (25.5%) cases were shown positive in bleach method (10 cases were "scanty" and 2 cases were "1+" in grading scale). Also 39 (39.8%) cases were graded as scanty in routine Z-N method, among them 25 (64.1%) cases were shown 1+ bleach method. And those cases who were in grade 1+ and 2+ in Z-N staining method (10 and 2 cases respectively) also found in next upgrade grade in modified bleach method. An interclass correlation test (ICC test) showed substantial agreement (colour coated on table 4) between the two methods (Table 4).

Discussion

This cross-sectional study was carried out first time in Bangladesh to compare between a newer 'modified bleach method' and traditional Z-N staining method for improved structural and morphological detection of Acid-Fast Bacilli (AFB) in tertiary as well as peripheral low-cost settings. In the study, it was observed that 52.06% were female and 46.94% were males and female to male ratio was 1: 0.88. As females in Asian countries are exposed to unhygienic and crowded environment and they do not get proper healthcare access, less treatment adherence, nutritional deprivation, immunological differences leading them to more vulnerable to tuberculosis[21-22].

In Bangladesh most affected patients of tubercular lymphadenitis were from the city where slums, unhygienic and crowded living are common[23,24]. This study also found that most patients were from Dhaka, Gazipur, Tangail, Narayanganj and other city corporation areas which are mostly densely populated cities in Bangladesh.

Not only treatment but also diagnosis of tuberculous lymphadenitis has lot of branches and diversities. Some of them are proved their accuracy and demand within underdeveloped countries and some are still in scepticism state in clinical practice. The usefulness of

Table 1: Cytomorphological pattern and AFB positivity on routine Z-N staining and Modified Bleach Method

Routine Z-N stain	Bleach method					
	Negative	Scanty	1+	2+	3+	Total
Negative	0	10/13 (76.9%)	3/13 (23%)	0	0	13/47 (27.6%)
scanty	0	11/39 (28.2%)	25/39 (64.1%)	3/39 (7.6%)	0	39/51 (76.4%)
1+	0	0	2/10 (20%)	7/10 (70%)	1/10 (10%)	10/51 (19.6%)
2+	0	0	0	0	2/2 (100%	2/51 (3.92%)
3+	0	0	0	0	0	0
Total		**21/63 (33.3%)**	**30/63 (47.6%)**	**10/63 (15.8%)**	**3/63 (4.7%)**	**63/98**

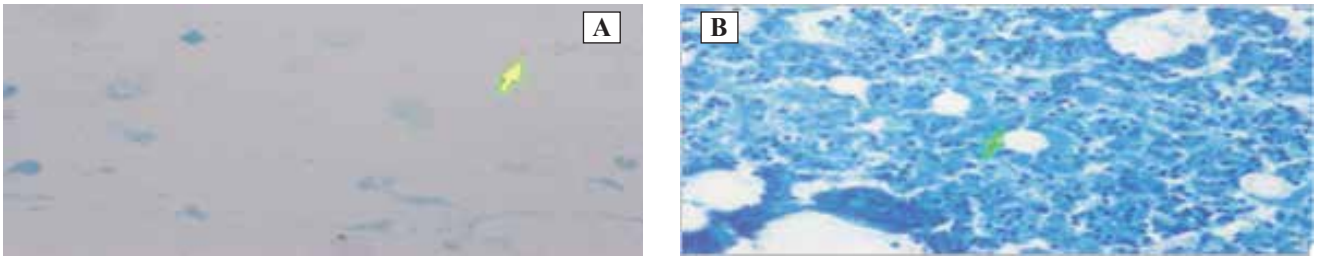

Figure I (case 18): Photomicrograph (a) showing bleached process smear containing multiple AFB against clearer background (Bleach processed Z-N stain, 400x); (b) showing AFB in dense necrotic polymorphous background (Z-N stain 400x)

clinical examination, ESR, and chest x-ray in the diagnosis of tuberculous cervical lymphadenopathy is limited. Although excision biopsy of lymph nodes was rarely required, FNAC seemed to be the investigation of choice[25,26].

The bleach method has the potential to enhance the number of positive suspicions for MTB. Some researchers have advised sodium hypochlorite (NaOCl) as a powerful disinfectant (bleach) to improve sensitivity and assumed that it was safe to be used in tuberculosis diagnosis. Bleach has been shown to improve smear microscopy sensitivity by digesting the mucus and debris in the exudate, resulting in a clean and efficient microscopy field. When compared to the results of direct microscopy, a comprehensive review found modest evidence supporting the efficacy of bleach processing followed by centrifugation the sensitivity was improved 13 percent with 95 percent confidence interval[27,28].

Most importantly, this study found that Bleach method has promising result in case of detection of AFB than Z-N staining method. The number of AFB-positive cases in Bleach method was 64(64.29%) and number of AFB-positive cases in Z-N staining method was 51(52%) out of total 98 cases which was statistically significant.

Household bleach, which is composed of 3.5 percent sodium hypochlorite, is commonly available, cheaper, and may be obtained straight from a local distributor. As a result, supply is rather consistent when compared to laboratory chemicals that are frequently purchased from far-flung or overseas providers. Alternatives to NaOCl have been suggested, including chitin and N-acetyl-l-cysteine. They are, however, not as readily available as home bleach[29].

In this study, AFB were seen in clearer, transparent and thinner background and the morphology of AFB were more distinct and well-preserved in Bleach method. Also, we found that due to the mechanism of digestion and flocculation of the substances present in the pus the background can be more visible. This newer modified Bleach method has the capacity to clear the background requiring lesser effort for visibility and exposing the AFB with strong morphology under the microscope[30,31,32,33]. AFB could be seen in clumps with thin background and AFB morphology could be preserved in sedimentation with bleach after centrifugation which could make the screening process easier, faster and less strenuous. Mycobacteria have a low specific gravity and may remain buoyant during centrifugation in bleach method[34].

The modified bleach method not only has improved detection rate of AFB but also has the meticulous identification capability of those AFB which were being missed by other traditional Z-N method. This meticulous study also found that Bleach method has the capability to identify those AFB which were not detected by conventional ZN method. The 47 cases of the study which were negative in ZN method, among them 13 cases (27.65%) were being positive in bleach method.

However, this bleach method to detect AFB was already studied in several countries in several times except Bangladesh. The countries who have successfully trialled this method are India, Nigeria, Zambia, South Africa, Congo, Laos, Kenya, Ethiopia, Singapore, Papua new Guiana, Cambodia, Australia, Jamaica, Myanmar and many other under developed countries like Bangladesh[35].

This method can save money and effort in national TB control programme as because we don't have enough high machinery facilities in our root level where we fail to diagnose approx. 27 TB patient per day[36].

American thoracic society (ATS) scaling and grading system are recognized worldwide for the categorization of AFB bacillary load by any method. And ATS scaling and grading were used in several studies to compare between ZN method and Bleach method[37,38]. All the scales measured by this study showed that bleach method have improved scoring by both grading and staging than Z-N method in each individual case.

In the study, out of 47 negative cases in Z-N method 13 cases (27.6%) were positive in bleach method, among these 13 cases there were 10 cases (76.9%) were fell into scanty group and 3 cases (23%) were fell into 1+ group and so on. Increased sedimentation rate of the AFB along with clearer background, there was higher density of the bacilli per microscopic field obtained by the bleach method and subsequent improvement in the ATS scaling and grading system.

The bleach microscopy approach is an excellent example of a low-cost technique for improving case detection in low economic countries that is unlikely to be promoted by commercial initiatives. The bleach-treated sediment can be used for smear preparation in rural laboratories that do not have access to electricity. Centrifugation can be done in laboratories with dependable electricity.

When dealing with M. tuberculosis-infected specimens, the danger of cross-infection is an important factor to consider. The direct contact of

laboratory employees with infectious pathogens, laboratory acquired infection is reasonably a matter of plausible fact. Because most of the laboratories of Bangladesh lack a biosafety cabinet, bleach processing of specimen is believed to be a safer alternative[39]. Because, this approach has the advantage of rendering the bacilli nonviable while keeping them acid fast. As a result, using NaOCl reduces the risk of spreading infection in laboratory[40].

Many academics believe that the bleach microscopy method has a lot of potential and it should be researched further, along with its potential drawbacks. They also agreed that, the international organizations like World Health Organization and the European Union should come forward to promote, evaluate and documentation of the studies upon bleach method to ensure that this method could be a promising technique in national program settings. Thus, the Bleach method will be identified and recommended as a technique that can truly improve and strengthen our laboratory services[41].

Conclusion

This study is not only an intricate work in diagnosis of AFB operated by a method that have been conducted for the first time in Bangladesh for extra-pulmonary sample but also it can be an important diagnostic aid requiring minimal methodical and structural set-up in a developing country like Bangladesh. The application of the bleach method clearly improves microscopic detection and can be a useful adjunct to routine cytology. This would be of an advantage to the patients in receiving early and effective treatment of tuberculous lymphadenitis.

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Conflict of Interest
The authors have no conflicts of interest to disclose.

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Authors' contributions
The authors confirm their contribution to the paper as follows: study conception and design: Md. Shajjad Hossain, Saequa Habib. Data collection: Md. Shajjad Hossain. Analysis and interpretation of results: Md. Shajjad Hossain, Mohosina Sultana, Saumitra Chakravarty. Draft manuscript preparation: Md. Shajjad Hossain, Md. Shahiror Nahid. All authors reviewed the results and approved the final version of the manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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ORCID
Md. Shajjad Hossain: https://orcid.org/0009-0003-0622-1728
Saequa Habib: https://orcid.org/0000-0001-8703-1333
Saumitra Chakravarty: https://orcid.org/0000-0002-4829-3397
Md. Shahrior Nahid: https://orcid.org/0009-0004-9858-9753
Mohosina Sultana: https://orcid.org/0000-0001-7594-2076

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