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Evaluation of modified bleach technique for the detection of acid fast bacilli in lymph node aspirate at the University of Gondar Comprehensive Specialized Hospital, Northwest Ethiopia

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ABSTRACT

Background: Tuberculous Lymphadenitis (TBLN) is the commonest form of extrapulmonary tuberculosis. Definitive diagnosis of TBLN is difficult due to the low efficacy of the routine diagnostic techniques as compared to culture.
Objective: To determine whether prior bleach concentration can improve the detection of acid-fast bacilli when compared with conventional Ziehl-Neelsen (ZN) staining by using culture as the gold standard.
Methods: Cross-sectional study was conducted from March 01, 2015 to May 30, 2015. The study subjects were clinically suspected TBLN patients. Fine needle aspiration (FNA) was performed. Two different smears were prepared directly from the first needle pass for cytology and ZN staining. The remaining aspirate was used for the modified bleach concentration. The second needle pass aspirate was transferred into a sterile container containing sterile normal saline. The sediment was inoculated on the Lowenstein Jensen (LJ) medium. Measurement parameters for reliability and validity were used to analyze the results.
Result: A total of 93 participants were included in the study. Fifty-six out of the 93 (60.2 %) cases were positive for mycobacterium tuberculosis complex on LJ culture. The detection rates of direct ZN staining and modified bleach method were 20.4 % and 44.1 %, respectively. 73.1 % of the cases showed cytomorphological features consistent with TBLN by cytologic examination. The sensitivities of direct ZN staining and modified bleach method and cytomorphology were 32.0 %, 67.8 %, and 92.8 %, respectively.
Conclusion: Implementation of bleach concentration increases the detection rate of AFB over the direct ZN method. The bleach method can also be easily performed and provide a safe working environment by reducing infections.

1. Introduction

Tuberculosis (TB) is the leading cause of mortality and morbidity among infectious diseases around the globe. According to the 2021 global TB report of the World Health Organization (WHO), an estimated 10 million people fall ill with TB, and 1.5 million die from TB every year [1]. The burden of TB is higher in resource-limited countries due to factors like poverty, undernutrition, and even HIV, which propagate its spread and complicates its control [2]. Ethiopia ranks 7th among the 22 high TB burden countries and third in Africa [3].

Extrapulmonary tuberculous (EPTB) affects different organs of the body whereas tuberculous lymphadenitis (TBLN) is the most common manifestation of all EPTB. The most commonly involved lymph nodes were cervical, axillary, inguinal, abdominal, and supraclavicular sites. Cervical lymph nodes are the most commonly affected group of nodes [4]. The prevalence of TBLN in northern Ethiopia was reported to be 65.7 % [5].

Different methods are in use for the diagnosis of EPTB such as smear

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microscopy, culture, histopathology, and tuberculin skin test. However, a definitive diagnosis of TBLN is often difficult as most of the available techniques are low either in their sensitivity or specificity. Even though clinical features are indicative of EPTB, they are not adequate for making a definitive diagnosis [6–8]. Microscopy has been a diagnostic tool for TB for over a century and is still currently the most rapid diagnostic method. Standard light microscopy (LM) and fluorescent microscopy (FM) are the common methods. The WHO has recommended light-emitting diodes fluorescent microscopy (LED FM) as an alternative to LM in resource-limited settings [9,10]. Although it is expensive nucleic acid amplification tests offer an alternative robust approach for detecting tuberculosis from EPTB specimens with good diagnostic accuracy [11]. Mycobacterium tuberculosis secretory proteins have gained more attention as TB biomarkers, for the early diagnosis and treatment of TB [12]. Another sensitive method for the detection of *M. tuberculosis* is culture. Culture can detect as few as 10 to 100 viable bacteria /ml but it takes weeks to perform and requires a biosafety level-III facility [13]. Fine needle aspiration cytology (FNAC) being a simple outpatient diagnostic procedure is well accepted by patients and rapid diagnostic technique but is characterized by low specificity [14,15].

Conventional ZN stain is in most circumstances the only available confirmatory diagnostic method in tuberculous lymphadenitis. It is rapid, reasonably priced, specific, and capable of identifying the most infectious cases of TB, but its sensitivity in EPTB cases is limited to 20–43 % [16]. Moreover, the detection rate of acid fast bacilli (AFB) depends on the cytomorphology of inflammation [17,18]. Studies have shown that the sensitivity of ZN can be improved if the aspirate is liquefied with one or more chemical reagents and then concentrated by centrifugation before ZN staining. The most widely studied procedure is the liquefaction of sputum with NaOCl, usually known as household bleach [19–21]. Previous studies done in Ethiopia showed the efficacy of the bleach method in enhancing the case detection rate of pulmonary TB [22–24]. Despite the potential benefits, concentration techniques have not been made into routine practice in resource-poor settings where culture and other effective and sensitive diagnostic methods like PCR are not routine practice [20]. The concentration of lymph node aspirate improves the detection rates of AFB by increasing the sensitivity to 60–70 % [21,26,27].

Despite the tremendous TB burden, case detection rates continue to become low. This demands the development of diagnostic tests that provide a rapid, sensitive, specific, and cost-effective approach for effective management of cases, especially in resource-limited settings. Therefore, this study was designed to evaluate the performance of the bleach concentration method for the detection of acid-fast bacilli in lymph node aspirates at the University of Gondar Comprehensive Specialized Hospital, Northwest Ethiopia.

2. Methods and materials

2.1. Study area, design, and period

Laboratory-based comparative cross-sectional study was conducted at the University of Gondar Comprehensive Specialized Hospital from March 01, 2015, to May 30, 2015. The Hospital is one of the biggest tertiary level referral and teaching hospitals in Amhara National regional state located 739 km from Addis Ababa. The hospital provides treatment and diagnostic services at the directly observed treatment short-course (DOTS) clinic and Tuberculosis diagnostic center, respectively. The hospital also provides FNAC examinations for over 500 suspected TBLN patients in the pathology department per year.

2.2. Sample size and sampling technique

A total of ninety-eight clinically suspected TBLN patients who visited the Pathology laboratory of the University of Gondar Comprehensive Specialized Hospital during the study period were included in the study.

Consecutive sampling techniques were employed to recruit the study participants. Patients on anti-TB treatment at the time of the lymph node aspiration were excluded from the study.

2.3. Specimen collection and laboratory processing

Varying sites of Lymphadenopathy were aspirated using a 22 gauge needle attached to 10 ml disposable syringe under strict aseptic precaution by an experienced Pathologist. Before lymph node aspiration, the overlying area was cleaned with 70 % alcohol. Two-needle passes were used while FNA is performed. The affected lymph node was punctured and the FNA was performed with several passes. The first needle pass was used to prepare smears for FNAC and conventional ZN staining. The remaining aspirates from the first needle pass were transferred into 5 ml sterile tubes containing 1 ml of normal saline for the modified bleach concentration. The aspirate from the second needle pass was poured into 5 ml sterile tubes containing 1 ml of normal saline for culture. The samples were kept at 4 °C until transported to Bahir Dar Regional Laboratory for culture (28).

2.3.1. Cytology examination (FNAC)

The gross appearance of the lymph node aspirate was noted by a pathologist during specimen collection. After characterizing the aspirates as caseous for cheese-like or yellow-white aspirates and purulent/non-caseous for greenish-yellow or yellow aspirates, smears were prepared on the slide. The slide was air-dried. The dried smear was kept on a staining rake, covered with freshly filtered Wright's stain, and buffered with distilled water. After 6 min, the slides were washed with tap water and air-dried. Finally, the slides were examined by a pathologist to evaluate whether the cytomorphology was consistent with TBLN or not [28,29].

2.3.2. Ziehl-Neelsen staining

Direct smear microscopy for the detection of AFB was done immediately after the FNA specimen is collected. A drop of the aspirate was placed on a clean labeled slide to make a direct smear. The standard ZN staining procedure was applied after allowing the smear to air dry. After the addition of carbolfuchsin, the heat was applied to penetrate the waxy mycobacterial cell wall. Following decolonization with acid alcohol, the smear was counterstained with methylene blue providing a contrast color against red AFB. Stained smears were examined by an experienced laboratory technologist for the presence of AFB under oil immersion (100x) using a light microscope [30].

2.3.3. Modified bleach concentration technique

The lymph node aspirate sample in a 5 ml sterile test tube containing 1 ml of normal saline was mixed with 2 ml of 5 % sodium hypochlorite (NaOCl). Incubation of the mixture was made at room temperature for 15 min by shaking at regular intervals for bleach digestion and then centrifuged in the test tube containing the mixture at 3000 rpm for 15 min after the addition of 2 ml distilled water. The supernatant was discarded and 1 ml of sediment was transferred with a sterile pipette on to sterile slide for smear preparation. After allowing to air dry, the slide will undergo the conventional ZN staining, and an examination was undertaken by a laboratory technologist for the presence of AFB under oil immersion (100x) using a light microscope [24,25].

2.3.4. LJ culture

Upon arrival at the Bahir Dar Regional laboratory, the tube containing 2–3 ml of aspirate was centrifuged and the sediment was inoculated onto Lowenstein-Jensen (LJ) slants. In brief, the sediment was distributed using plastic Pasteur pipettes and about 0.2–0.4 ml of it was inoculated over the entire surface of the slant. Inoculated slant was incubated at 37 °C. A culture was considered negative when no colony was seen after 8 weeks of incubation [13]. Differentiation of MTB complex from non-tuberculous mycobacteria (NTM) was done by

Capilia TB test which detects the MPT64 antigen-specific to M. tuberculosis complex strains.

2.4. Data analysis and interpretation

The data were entered into epidemiological information statistical software (*Epi* Info) and transferred to statistical package for social sciences (SPSS) statistical software version 16 for analysis. Descriptive statistics were employed. Sensitivity, specificity, and positive and negative predictive values of the modified bleach concentration method were calculated as compared to LJ culture, ZN stationing, and cytological examination.

2.5. Ethical considerations

The Ethical review committee of the school of Biomedical and Laboratory Science, College of Medicine and Health Sciences, University of Gondar approved the study. Letters of permission were obtained from the University of Gondar Comprehensive Specialized Hospital. All patients or guardians were requested for written consent before enrolment in the study and they were informed that all the information and results of their sample were kept confidential.

3. Results

3.1. Characteristics of the study participants

At first, a total of 98 patients who had presumptive TBLN were included in this study. Out of the 98 patients, the culture result of five of them was found contaminated. Thus, only 93 of the patients were included in this study. The majority, 53.7 % (50/93) were females. The ages of the participants range from 3 to 80 years, with a mean age of 30.7 years. Nearly half, 51.6 % (48/93) of the study participants were within the age range of 15 to 30 years. The proportion of suspected TBLN patients who were presented with anterior cervical, supraclavicular, and inguinal lymphadenopathy were 20 %, 19.3 %, and 17.2 %, respectively. The presence of lymph node scar was observed in 16 % (15/93) of the cases. The gross appearance of the lymph node aspirate was hemorrhagic in 45.2 % (42/ 93) of the cases.

3.2. The detection rate of M. Tuberculosis by the different methods

The lymph node aspirates were inoculated on LJ media and the mycobacterium tuberculosis complex was isolated in 60.2 % (56/93) of the specimens. The cytological examination of the aspirates revealed that 73.1 % (68/93) of the cases were diagnosed as TBLN. The microscopic examination of the specimens using the ZN staining technique showed that 20.4 % (19/93) of the suspected cases were positive for acid-fast bacilli. The detection rate of acid-fast bacilli by using the modified bleach concentration technique was 44.1 % (41/93). The modified bleach concentration method detected 22 extra patients with an incremental yield of 23.7 % (22/93), which was statistically significant (P = 0.0001). The sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy of the modified bleach method were 100 %, 70.3 %, 46.3 %, 100 %, and 76.3 %, respectively, as compared to the ZN staining technique (Table 1).

Out of the 56 culture-positive lymph node aspirates, 38 (67.8 %) were positive for acid-fast bacilli with the modified bleach concentration method, whereas out of the 37 culture-negative samples, 3 (8.1 %) were positive for AFB. As compared with the culture method, the modified bleach concentration method showed 67.8 % sensitivity, 91.9 % specificity, 92.7 % positive predictive value, and 65.4 % negative predictive value. The overall accuracy of the modified bleach technique was 77.4 % (95 % CI: 67.5 to 85.5). Nearly-one-third, 32.1 % (18/56) of the culture-positive lymph node aspirates were positive for acid-fast bacilli by using the conventional ZN staining. The sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy of the conventional ZN staining were 32.0 %, 97.3 %, 94.7 %, 48.7 %, and 58.1 %, respectively, as compared to the culture technique (Table 2).

Out of the 93 lymph node aspirate, 68 (73.1 %) of them were classified as TBLN on cytomorphological examination. Of these, AFB was detected in 54.4 % (37/68). The sensitivity, specificity, positive predictive value negative predictive value, and overall accuracy of the modified bleach method were 54.4 %, 80.0 %, 88.1 %, 39.2 %, and 61.3 %, respectively, as compared to the cytomorphological examination (Table 3).

From the culture positives, 92.8 % (52/56) showed cytomorphological features consistent with TBLN. Similarly, from the culture negatives, 43.2 % (16/37) were classified as TBLN by cytomorphological examination. On the other hand, 76.4 % (52/68) of the lymph node aspirates which were positive by culture were diagnosed as TBLN on cytological examination. As compared to the culture technique, the cytological examination showed a sensitivity of 92.8 %, specificity of 56.8 %, and positive predictive value of 76.5 %, a negative predictive value of 84 %, and an accuracy of 78.5 % (Table 4).

3.3. Cytomorphologic characteristics and modified beach technique

Cytomorphological features of tuberculous lymphadenitis were clearly observed in 68 participants. Out of these, 54.40 % (37/68) were positive for AFB by the modified bleach method. More than half, 55.9 % (38/68) of the lymph node aspirates with cytomorphological features consistent with TBLN had only caseous necrosis. Of these, 68.4 % (26/38) were positive for acid-fast bacilli by the modified bleach technique. Out of the 25, lymph node aspirates with cytomorphologic features non-consistent with TBLN, 16 % (4/25) were positive for acid-fast bacilli. Among different factors assessed for association with smear positivity on concentration method, lymph node scar was statistically associated with the presence of a scar [p-value = 0.0001, OR = 3.05, 95 % CI = 1.5–5.9] (Table 5).

4. Discussion

In resource-poor countries, like Ethiopia, the conventional diagnostic tool for TBLN mainly relies on FNA cytology and direct smear microscopy as a confirmatory test. FNAC has limited specificity because of the presence of cytologic features such as epithelioid cell aggregates,

Table 1
Comparison of the modified bleach method with the conventional Ziehl–Neelsen method for detection of acid-fast bacilli in lymph node aspirate.

Method	Conventional ZN technique		Total
	Positive	Negative	
Modified bleach technique			
Positive	19	22	41
Negative	0	52	52
Total	19	74	93

Table 2
Comparison of the modified bleach technique and ZN staining with the mycobacterium culture for detection of acid-fast bacilli in lymph node aspirate.

Method	Mycobacterium culture		Total
	Positive	Negative	
Modified bleach technique			
Positive	38	3	41
Negative	18	34	52
Total	56	37	93
Conventional ZN method			
Positive	18	1	19
Negative	38	36	74
Total	46	37	93

Table 3
Comparison of the modified bleach technique with the cytomorphologic examination for detection of acid-fast bacilli in lymph node aspirate.

Method	Cytomorphologic features		Total
	consistent	not consistant	
Modified bleach technique			
Positive	37	5	42
Negative	31	20	51
Total	68	25	93

Table 4
Comparison of the cytomorphologic examination with the mycobacterium culture for the diagnosis of TBLN in lymph node aspirate.

Method	Mycobacterium culture		Total
	Positive	Negative	
Cytomorphologic features			
Consistent with TBLN	52	16	68
Not consistent with TBLN	4	21	25
Total	56	37	93

Table 5
Cytomorphologic characteristics of lymph node aspirates and detection rate of AFB using modified bleach technique.

Cytomorphologic features	Total	Acid fast bacilli	
		Positive	Negative
Cytopathology features consistent with TBLN	**68**	**37**	**31**
Epithelioid granuloma with necrosis	3	1	2
Epithelioid granuloma without necrosis	9	3	6
Mainly necrosis with degenerated epithelioid histiocyts	38	26	12
Necrosis with degenerated epithelioid histiocytes and scattered neutrophils against fluidly proteinaceous background.	7	4	3
Suppurative inflammation with granuloma	11	3	8
Cytopathology features non-consistent with TBLN	**25**	**4**	**21**
Suppurative inflammation without granuloma	15	4	11
Reactive lymphoid hyperplasia	10	0	10
Total	93	41	52

necrotic lesions, and other diseases associated with TB histological features such as leprosy, sarcoidosis, and other inflammatory conditions [31]. Moreover, FNAC requires highly trained pathologists which is not feasible to practice at district health facilities.

Culture is the gold standard for the diagnosis of TBLN. However, the availability and affordability of this method in resource-limited settings like Ethiopia require other techniques with added value over direct ZN microscopy. In such settings concentrating on the FNA sample can improve the diagnosis of TBLN. The nature of the specimen; the inadequate lymph node aspirates and inappropriate aspiration could be the main factor for the decreased sensitivity of direct smear. Many reports have shown that liquefaction of clinical samples followed by centrifugation significantly increases the smear sensitivity up to 72 % [21,27,31], our findings verified comparable results to this observation.

As compared with the culture method the sensitivity of the conventional ZN staining was 32.0 % and it increased to 67.8 % on the modified bleach concentration method. Three of the positive cases on the modified bleach method were culture negative. This may be because the AFB positivity rate on the modified bleach concentration method was highest when the cytomorphological feature was caseous necrosis. Whereas caseous lesion is the death of the majority of the tubercle bacilli, therefore, these non-viable bacilli are unable to grow on culture. Cytomorphological features should be used in conjunction with the modified bleach concentration method as the best alternative for direct ZN microscopy to manage such cases in clinical practice. AFB positivity rate by direct and concentration method was highest when the cytomorphological features of FNAC were necrosis without epithelioid cell aggregates. By the concentration method, there is an increase in AFB smear positivity from 33 % in those with epithelioid granuloma without necrosis to 68.4 % when only necrosis was seen in cytology.

Similarly, a previous study [21] observed the highest rate of AFB positivity in smears showing necrosis alone and decreased smear positivity rates with the appearance of epithelioid granuloma. This is probably because the central necrotic portion of the tubercle contains more bacilli. The sensitivity of the modified bleach method in the present study was slightly lower (67.8 %) compared to that reported in a previous study (72 %) [27], but relatively closer to another study (66 %) [21]. The improved detection rate of AFB by using the modified bleach concentration method might be due to changes in the lipid coat surface properties of the bacilli [31]. Because there is a common belief that due to their lipid coat, mycobacteria remain floating during centrifugation. On the contrary, the bleach technique has been able to allow the deposition of bacilli at the bottom of the test tube after centrifugation which can increase the bacterial load and can have a useful contribution to routine cytology [31].

Out of 15 cases classified as suppurative inflammation on cytology, TBLN was diagnosed in 4 cases by the modified bleach method and in 2 cases by direct ZN microscopy. The possible explanation for the misdiagnosis of specimens as a suppurative abscess on cytology may be the absence of characteristic features within abundant mixed inflammatory superinfections by other bacteria. In the current study TBLN suspects with lymph node scars were 3.5 times more likely to yield a positive result on the concentration method as compared to those patients without a scar. This could be due to an infection of the lymph node, the caseous material penetrates the deep fascia and escapes into the superficial fascia resulting in collar stud abscess formation, the abscess may present with persistent discharging sinus and finally develop in the scar.

In conclusion, the detection rate and sensitivity of the modified bleach concentration method are much higher in comparison with conventional ZN microscopy. The highest AFB positivity rate was observed in cytomorphological features consistent with caseous necrosis. The modified bleach concentration method is a simple technique that requires no expertise; affordable and safe because NaOCl kills the mycobacterium, due to this it cannot be used on samples intended for culture. The modified bleach concentration method increases the positivity rate by making AFB easily visible and detectable in a thin background, making the screening process easier, faster, and less exhausting. The modified bleach concentration method can be implemented in routine laboratory practice together with conventional cytology to provide an efficient TBLN diagnosis.

5. Ethical considerations

The Ethical review committee of the school of Biomedical and Laboratory Science, College of Medicine and Health Sciences, University of Gondar approved the study. Letters of permission were obtained from the University Of Gondar Comprehensive Specialized Hospital. All patients or guardians were requested for written consent before enrolment to the study and they were informed that all the information and results of their sample were kept confidential.

CRediT authorship contribution statement

Firehiwot Mulugeta: Conceptualization, Methodology, Writing – original draft. **Moges Tiruneh:** Supervision. **Bewketu Abebe:** Visualization, Investigation. **Gashaw Yitayew:** Visualization, Investigation. **Zimam Ayehubizu:** Visualization, Investigation. **Muluwork Getahun:** Writing – review & editing. **Aschalew Gelaw:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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