

## Comparative evaluation of direct Ziehl-Neelsen (ZN) smear and modified ZN against fluorescent technique in the detection of acid-alcohol fast bacilli in lymph node aspirates

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### ABSTRACT

**Background:** Tuberculosis is a major public health problem in Kenya as well as other developing countries. Diagnosis of extra-pulmonary tuberculosis is a common challenge especially where direct Ziehl Neelsen (ZN) technique is employed.

**Objective:** This study was aimed to establish whether modification of ZN by use of 3.5% NaOCl-Xylene Floatation would improve the detection of acid-alcohol fast bacilli (AAFBs) from lymph node aspirates.

**Design:** This was a descriptive cross-sectional study.

**Setting:** Kenyatta National Hospital, Kenya.

**Subjects:** One hundred cases suspected of clinically having TB with lymphadenopathy referred for fine needle aspiration cytology (FNAC) in FNA Clinic at Kenyatta National Hospital.

**Methodology:** The study was approved by Kenyatta National Hospital/ University of Nairobi/ Ethics and Research Committee (KNH/UON/ERC) and informed consent sought from patients. Analysis was done using SPSS version 12.0 for Windows (SPSS Inc.). McNemar's test was used to assess the level of significance for the two test procedures. Chi-square was used to assess for the heterogeneity of the association between the two study methods.

**Results:** Thirty (30%) of the specimens were positive with fluorescent microscopy, 17% were modified ZN positive while 6% were direct ZN positive. The sensitivity, specificity, positive and negative predictive values for the modified technique (3.5% NaOCl-Xylene Floatation) were 53.3%, 98.6%, 94.1%, and 83.1% respectively.

**Conclusions:** Liquefaction of the aspirated specimens with NaOCl followed by Xylene floatation significantly increased the yield of AAFBs. This finding is of great interest in developing countries where smear-negative for acid-alcohol fast bacilli (AAFBs) has become common.

**Recommendation:** Local settings should consider adopting use of modified ZN technique in order to increase the sensitivity and detection rate of AAFBs from lymph node aspirates.

### INTRODUCTION

Tuberculosis (TB) is on the rise globally. Nearly one-third of the world's population is infected with *Mycobacterium tuberculosis*. Three to four million new cases are estimated to occur yearly<sup>1</sup>. Developing countries are experiencing an increase in the burden of TB, with a major public health problem compounded by the emergence of multi-drug-resistant tuberculosis (MDR-TB)<sup>2</sup>. Kenya is one of the twenty two high TB burden countries. The trend is still rising with an average annual increase of 16% cases (all forms) notified to National Leprosy and Tuberculosis Program (NLTP) in the last ten years<sup>3</sup>.

Extra-pulmonary tuberculosis (EPTB) continues to be a major health problem

in developing countries<sup>4</sup>. Tuberculous lymphadenitis (TB-L) is the most common form of extra-pulmonary TB<sup>5,6</sup>. It occurs with an increased frequency in patients with human immunodeficiency virus (HIV)<sup>7</sup>. Traditionally, the diagnosis of TB-L is established by histopathology and smear microscopy or by culture. Over the past decade, fine-needle aspiration (FNA) cytology has assumed an important role in the evaluation of peripheral lymphadenopathy as a possible non-invasive alternative to surgical excision biopsy<sup>7-9</sup>.

Diagnosis of tuberculous lymphadenitis (TB-L) is however, still a challenge in sub-Saharan Africa, where there is a high rate of human immunodeficiency Virus (HIV) infection<sup>10</sup>. Ziehl-Neelsen method

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is recommended by World Health Organization (WHO) for screening patients. Though conventional Ziehl-Neelsen (ZN) staining method plays a key role in the diagnosis and monitoring of treatment in TB, its major disadvantage is low sensitivity, ranging from 20% to 43%<sup>11</sup>. Mycobacterial culture is the reference method for isolation of *tubercle bacilli*, but it is time-consuming and requires specialized safety procedures in laboratories. Serological techniques have the disadvantage of low sensitivity and specificity<sup>11</sup>. While fluorescent stain (FS) has been proven to be superior to the Ziehl-Neelsen's (ZN) stain, especially in paucibacillary cases, its use is still limited in developing countries. In recent times, PCR has been found to be the most sensitive technique for rapid diagnosis of *M. tuberculosis*<sup>12</sup>. However, it is not applicable in routine use because of cost implications.

Due to the above mentioned limitations, various attempts have been made to improve the sensitivity of ZN microscopy. Modifications of ZN using sodium hypochlorite (NaOCl, bleach), or sodium hydroxide to liquefy the specimen and then concentration by either centrifugation or sedimentation prior to staining have been shown to increase the sensitivity in most studies<sup>3,13</sup>. However, majority of these studies have focused on using NaOCl with centrifugation. Only a few studies have shown that liquefaction of sputum by sodium hypochlorite (NaOCl) and concentration of bacilli through layering on Xylene will significantly increase the sensitivity of direct microscopy. Furthermore no study has addressed the effect of use of NaOCl and concentration by xylene floatation in lymph node aspirates. Hence, this study of using the bleach NaOCl-Xylene Floatation method in fine needle aspiration cytology (FNAC) of lymph nodes was performed to demonstrate advantages in combining two superior investigative methods not previously used.

## MATERIALS AND METHODS

**Samples:** A total of 100 Fine needle aspirates were obtained from patients who presented with signs and symptoms of Tuberculosis after consent was given.

**Sample processing:**

(i) FNA material from lymph nodes were applied to prior labeled slides directly and direct smears prepared for ZN staining.

(ii) Leftover material in the hub of the needle was rinsed in one milliliter (ml) normal saline and transferred into Bijou bottles. To this, one ml of 3.5% NaOCl was added and the mixture incubated at room temperature for 15 minutes while shaking at regular intervals. One half of a ml of xylene was added and the mixture let to stand for 15 minutes undisturbed to float the bacilli. The creamy layer was carefully scooped using a wire loop and smears prepared on prior labeled, albumin coated microscope slides. The slides were air-dried, heat fixed and stained by the ZN method and Auramine respectively.

**Direct ZN:** FNA material obtained were smeared on slides and directly stained with strong Carbol fuchsin and steamed for five minutes. The slides were then washed with tap water and decolorized with 1% acid alcohol until clear. Subsequent rinsing in tap water followed. Counter staining was done in Methylene blue for three minutes and finally rinsed in tap water and left to air dry. Examination was done using high power (X100) and reported using WHO format.

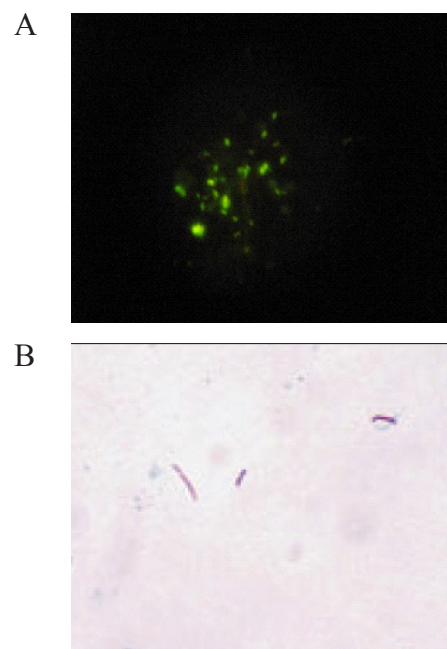
**Modified ZN (NAOCL-Xylene Floatation method):** Heat fixed smears were stained with strong Carbol fuchsin and steamed for five minutes. The slides were then washed with tap water and decolorized with 1% acid alcohol until clear. Subsequent rinsing in tap water followed. Counter staining was done in Methylene blue for three minutes and finally rinsed in tap water and left to air dry. Examination was done using a light microscope at high power (X100) and reported using WHO format.

**Fluorescent method:** Heat fixed smears were stained with Auramine stain for fifteen minutes. The smears were rinsed in distilled water followed by decolourisation in 0.5% acid- alcohol for two minutes. The smears were again rinsed in distilled water followed by counter staining in potassium permanganate for two minutes. The slides were rinsed in distilled water and allowed to air dry. Examination was done using a Fluorescent microscope at high power (X40) and reported using WHO format.

## RESULTS

### Microscopy results

*Photomicrographs of results obtained from the patient specimens.*



(a) Golden yellow bacilli in a dark background by fluorescent microscopy (x40); (b) Few AAFBs seen under high power magnification (x100) by modified ZN- light microscopy.

*Performance characteristics of direct and modified ZN (3.5% NaOCL-xylene floatation):*

Based on fluorescence microscopy as the reference method, the sensitivity of direct ZN and modified ZN was 20% and 53.3% respectively. The specificity was 100% for direct ZN and 98.6% for modified ZN microscopy. The positive predictive value (PPV) of the two tests was 94.1% by modified ZN microscopy and 100% by direct smear microscopy. The negative predictive value (NPV) was 74.5% by direct ZN microscopy and 83.1% by modified ZN microscopy (Table 1).

**Table 1:** Performance characteristics of direct ZN and the modified ZN

Tests	Sample size	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
Direct smear	100	20	100	74.5	100
Modified ZN	100	53.3	98.6	83.1	94.1

Concordant results with those of fluorescence microscopy were obtained in (76%) by direct smear microscopy and (85%) by modified ZN microscopy. The Kappa Statistics was 25.9% (P = 0.0001) by direct smear microscopy and 59.2% by modified ZN microscopy (P = 0.001) (Table 2).

**Table 2:** The concordance and Kappa results for the test methods

Test	Sample size	Concordant Results	Kappa %	Kappa P value
Direct smear	100	76	25.9	0.0001
Modified ZN	100	85	59.2	0.001

Overall, modified ZN performed better than direct ZN since it demonstrates a fair agreement of 59.2% with the reference method compared to that of 25.9% by direct ZN. It also demonstrated 85% concordance with the reference method.

## DISCUSSION

This study was aimed at establishing the performance of 3.5% NaOCL-Xylene floatation in the detection of AAFBs in lymph node aspirates in suspected tuberculous lymphadenitis. In this study the proportion of AAFB detected varied depending on the test method employed which ranged from 6% by direct ZN method, 17% by modified ZN method and 30% by fluorescent microscopy method. This finding is similar to other studies showing superiority of fluorescent microscopy over light microscopy of ZN stained smears (50% by ZN versus 69% by FM) for the detection of acid-fast bacilli<sup>14</sup>. The results from this study correlates with the results of Githui *et al*<sup>13</sup> (65% by ZN, 80% by FM), and Ulukanligil *et al*<sup>15</sup> (67.6% by ZN, 85.2% by FM)<sup>15</sup> although they used sputum

samples and centrifugation rather than floatation to concentrate the bacilli.

From this study, the smear positivity increased from 6% (6/100) conventional ZN method to 30% (30/100) by the modified method results that were comparable to results obtained by Annam *et al*<sup>17</sup> [33.33% (31/93) by conventional ZN method to 63.44% (59/93) by the bleach method]<sup>16</sup>. Khubnani and Munjal<sup>17</sup>, also observed an increased positivity from 21.8% cases by conventional ZN staining to 70.90% cases positive for AAFB by the bleach method. The improved recovery of AAFBs after treatment with NaOCl might be due to changes in the surface properties of the AAFBs (i.e., charge and hydrophobicity). Also, the increased smear positivity by the bleach method is attributable to the higher density of bacilli per microscopic field obtained by this method and reduction of debris, leaving a clean field for microscopy<sup>18</sup>.

According to Frimpong *et al*<sup>19</sup>, a study done on sputum samples generated the sensitivity rates of 71.8% by (1% NaOCL-xylene floatation) and 66.3% by direct smear methods. This improvement in sensitivity is thus correlates with our findings despite the fact that our study was on lymph node aspirates. The specificity rate was 95.9 % for both methods based on culture as the gold standard<sup>19</sup>. From this study, the specificity for Modified ZN was slightly lower (98.6%) than that of conventional ZN (100%) probably due to the different reference techniques and type of samples used. In yet another study by Habeenzu *et al*<sup>20</sup>, the use of NaOCl was found to increase the smear sensitivity from 43.4% to 76.3% with the specificity of 100%. These findings also correlate well with findings from this study. However, direct comparison is a challenge owing to the fact that different techniques are used as gold standard techniques and that the specimen types used in these studies are different. Githui *et al*<sup>13</sup> also demonstrated an increase in sensitivity using 3.5% bleach on sputum samples. The sensitivity, specificity, positive predictive values and negative predictive values were: 27.1%, 99%, 99% and 76% respectively after sedimentation; culture used as the gold standard. In comparison to our study, the sensitivity of modified ZN improved from 20% by direct ZN to 53.3% without much loss of specificity 100% by direct ZN to 98.6% by the modified ZN using Fluorescence microscopy as the reference technique. However, it is noted that the sensitivity obtained by Githui *et al*<sup>13</sup> is lower probably due to the fact that they were evaluating reported smear negative sputa using culture as the reference technique. All the same, the negative and positive predictive values were comparable.

Results concordant or test accuracy improved from 76% by direct smear microscopy to 85% by modified ZN microscopy. The Kappa Statistics also improved from 25.9% by direct smear microscopy to 59.2% by modified ZN microscopy. These results indicate that the modified ZN microscopy has superior accuracy and agrees better with the fluorescence



microscopy compared to the routine direct smear method. The McNemar P value was 0.0001 for direct ZN and P value of 0.001 for modified ZN microscopy, further strengthening our conclusion that modified ZN had better performance compared to direct smear microscopy. This better performance by modified ZN calculated using both the concordance/ accuracy and by McNemar has been reported in various studies. From the results obtained by this study, it is evident that modified ZN increases the sensitivity and detection rate of Acid-alcohol fast bacilli in lymph node aspirates. Local settings should therefore consider adopting use of this method to improve the diagnostic accuracy of TB lymphadenitis.

## ACKNOWLEDGEMENTS

Sincere acknowledgement to the CEO Kenyatta National Hospital, Head TB National reference laboratory, all the staff of both UoN/KNH cytology and TB reference laboratories for their great support and Musa Ong'ayo for data analysis.

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