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Improved Ziehl-Neelsen Microscopy: Bleach Sputum Smear Negative Specimens after Centrifugation

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Abstract: Background: Direct ZN (Ziehl-Neelsen) sputum smear microscopy for diagnosis of TB (tuberculosis) has low sensitivity, especially in TB/HIV co-infected patients. Sputum concentration by bleach (NaOCl) with sedimentation has been used to increase the sensitivity of sputum smear microscopy in many settings but with varying results. Objective: To determine whether bleach plus centrifugation significantly improves the detection of AFB (acid-fast bacilli) in ZN smear-negative sputum specimens. Methods: Three hundred and seventy sputum specimens were collected from new TB suspects attending a Nairobi referral district hospital and processed for direct microscopy using ZN technique and culture on Lowenstein Jensen Media. All smear-negative specimens were treated with 3.5% bleach and left to stand for 30 min before centrifugation. The bleach treated smears were processed and examined using ZN technique. Results: Of the 370 specimens, 200 (54%) were positive culture. The number of sputum samples that were smear-positive by direct ZN was 138 (37.2%), with a sensitivity of 66%. After treatment of direct ZN smear-negative specimens with 3.5% bleach and centrifugation, the total number of AFB smear-positive samples increased to 171 with an increase in sensitivity of 66% to 81.1% (15.1%). Conclusion: In this study, bleach with centrifugation significantly increased the yield of sputum smear microscopy. Further evaluation of these techniques in routine programmes is required especially in settings where the burden of TB/HIV is high.

Key words: Diagnosis, smear-negative TB, centrifugation, Ziehl-Neelsen microscopy.

1. Introduction

TB (tuberculosis) is the leading cause of death among acquired immunodeficiency syndrome (HIV/AIDS) patients in sub-Saharan Africa. The WHO (World Health Organization) estimates that up to 80% of TB patients in the region are living with HIV [1]. In 2008, an estimated 9.4 million people developed TB; almost 2 million people died from TB, including 500,000 HIV co-infected individuals [2]. More than 90% of TB patients live in low and middle-income countries [3]. Despite this tremendous global burden, case detection rates continue to be low [4]. Rates of smear-negative PTB (pulmonary tuberculosis) and EPTB (extrapulmonary tuberculosis) have been raised in countries with human immunodeficiency virus (HIV) epidemics [5]. The mortality rate among HIV co-infected TB patients is higher than that of non co-infected TB patients, particularly among those with smear-negative PTB and EPTB [5]. In addition, delayed diagnosis may be an important cause of excess mortality in HIV co-infected individuals with smear-negative PTB and EPTB [5].

Because of limited diagnostic tools and the lack of laboratory capacity in many rural, resource-poor settings, the detection of smear-negative TB, EPTB and drug-resistant TB is difficult [6]. Direct smear microscopy using Ziehl Neelsen technique remains the mainstay for diagnosis of PTB especially in resource-limited settings. This technique has been in use for decades to detect smear-positive patients who

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are the most potent sources of transmission of *M. tuberculosis* in the community [7]. However, the effectiveness of direct smear microscopy is limited by its low sensitivity, particularly in areas with high rates of HIV co-infection. Rates of active TB detection by smear microscopy in people with HIV can be as low as 20% in adults, and as low as 5% in children [6]. Despite a substantial (though still not adequate) increase in TB diagnostics research, there are, as of yet, no new laboratory tests that are simple or sensitive enough to replace smear microscopy in the under-resourced peripheral laboratories in the developing world.

Due to the enormous global burden of TB and limitation of existing methods, there remains a tremendous need to develop TB diagnostic techniques that are rapid, accurate, safe, inexpensive and convenient. However, for now, in most resource-limited countries, microscopy will remain the primary means of microbiological diagnosis of TB for the foreseeable future and strategies that optimize microscopy methods urgently need to be explored [8]. In the last decade, studies have shown that liquefaction of sputum with bleach and concentration of bacilli through centrifugation increases the sensitivity of direct microscopy [9-11]. Using this bleach method, there is an average 13% increase in sensitivity of ZN smear microscopy [12]. It has been observed that the sensitivity of acid-fast smears is directly related to the relative centrifugal force achieved while concentrating the specimen [13]. However, no study has focused on the incremental yield of bleach with centrifugation using 3.5% concentration when used after direct smear microscopy. However, a study in Kenya [14] showed that there was increase in sensitivity of smear-negative specimens using fluorescent microscopy. Therefore, this study was carried out to establish whether use of 3.5% bleach with centrifugation will significantly increase the yield after direct microscopy is done using ZN technique.

2. Materials and Methods

2.1 Sputum Specimens/Site

Three hundred and seventy sputum specimens from new TB suspects attending a TB referral district hospital in Nairobi province were collected. These specimens were one specimen from each patient regardless of whether the specimen was spot or early morning.

2.2 Laboratory Procedures

Direct sputum smears were prepared from all the specimens in accordance with International Union against TB and Lung Disease [15] stained with ZN technique and examined for presence of AFB. Only direct smear-negative sputum specimens were treated with 3.5% bleach. Direct smear-negative specimens were homogenized using a vortex mixture and treated with an equal volume of 3.5% bleach, left for 30 min at room temperature for liquefaction to occur, centrifuged at 3,000 RCF for 15 min after which the supernatant was carefully removed by pipette. Smears were made from the sediment, stained with ZN technique, examined using a bright field microscope under oil immersion (× 1,000) and reported according to standard methods [16].

The sensitivity and specificity of direct and bleach sediment smear method were determined using conventional culture as the gold standard.

2.3 Quality Control

Known positive and negative sputum specimens were included in every batch of specimens processed. After initial examination, all smears (direct and bleach treated) were securely stored in slide boxes. An arbitrary 10% of the positive smears and 5% of the negative smears were selected at random and re-examined by an independent microscopist from KEMRI-CRDR TB laboratory blinded to the initial results. All reagents and media were prepared in accordance with SOPs (standard operating procedures)

used at KEMRI-CRDR TB laboratory. NaOCl was reconstituted on a weekly basis.

2.4 Data Analysis

The data were double entered and analysis done using SPSS version 11.5 for Windows (SPSS Inc.). The sensitivity, specificity, positive and negative predictive values of the sputum smear examinations were calculated by using the sputum culture results as the "gold standard".

3. Results

Of the 370 specimens, 200 (54%) were culture positive, 132 (35.9%) were both culture and smear-positive, and six were culture negative and smear-positive (Table 1). The total number of direct smear-positive specimens by direct ZN was 138 (37.2%) with a sensitivity of 66% among culture positive samples. The number of AFB positive specimens increased to 171 (46.2%) with a sensitivity of 80% after treatment of direct ZN smear-negative specimens with 3.5% bleach followed by centrifugation (Table 2).

Of the 232 smear-negative specimens treated with 3.5% bleach with centrifugation, 33 (14.3%) became ZN smear-positive with a sensitivity of 42% (Table 2). However, 5 (2.1%) of the bleach with centrifugation ZN smears were culture negative but smear-positive. There was 15.1% increase in sensitivity when 3.5% bleach was used with centrifugation on originally smear negative sputum specimens.

4. Discussion

This study was designed to assess whether there is increase in sensitivity of direct ZN smear microscopy if smear negative specimens are treated with 3.5% bleach followed by centrifugation after getting the results of direct smear microscopy. The choice of 3.5% bleach used in this study was because it is the most common formulation of bleach in the Kenyan market and had been successfully used in three recent studies [14, 17, 18].

Findings from this study showed that direct ZN smear microscopy has a sensitivity of 66% compared with TB cultures; this result is consistent with those from previous studies in Kenya where direct ZN smear microscopy ranged from 60% to 65% [19, 20]. Six specimens, when using direct smear microscopy, in the current study were culture negative but smear-positive, and graded between 1+ and 3+. In addition, four specimens were culture negative but smear positive after treating the ZN smear negative specimens with 3.5% bleach and three were graded 1+ and one 2+. These specimens were classified as false

Table 1 Number of positive and negative specimens identified by direct Ziehl Neelsen and culture.

		Culture		
		Positive	Negative	Totals
Direct ZN microscopy	Positive	132	6	138
	Negative	68	164	232
	Totals	200	170	370

Table 2 Overall sensitivity, specificity, positive and negative predictive values of 370 specimens using Ziehl-Neelsen after treatment of negative specimens with 3.5% bleach with centrifugation.

Method	Total No. of smear positive specimens	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Method*	138	66	97.1	96.4	78
Method**	33	42.9	98.1	90.9	79.7
Method***	171	80	94.12	95	81

The total samples are 370.

Method*: Direct ZN microscopy; Method**: 3.5 % bleach + centrifugation (n = 232); Method***: Direct ZN microscopy + 3.5% bleach treated specimens followed by centrifugation.

PPV: predictive value of positive smear; NPV: predictive value of negative smear.

positives. However, they could have been true positives that were either not picked by culture or from patients on treatment.

The increase in sensitivity of direct ZN smears from 66% to 80% in this study after treating the smear-negative specimens with 3.5% bleach by any standard was high taking into consideration that it is low in direct smear microscopy and ranges from 8.8% to 46.6% in most laboratories in Africa compared to mycobacterial culture [21, 22]. This increase sensitivity is very encouraging bearing in mind that it only takes one extra hour to process specimens using the bleach centrifugation method. Therefore, adding bleach centrifugation to routine direct microscopy should substantially change a patient's waiting time (for the results for 2 days) and travelling costs.

In addition, the increase of 15.1% smear positivity from initially direct smear-negative specimens treated with 3.5% bleach is a very encouraging finding. Furthermore, except for the four specimens graded as 1+ and 2+ after treatment with 3.5% bleach, all the other specimens that were smear-positive after treatment with 3.5% bleach were also culture positive further proving the reliability of this technique.

Though these "incremental improvements" do not address all of the limitations of direct smear microscopy for the diagnosis of TB, in high-burden countries even small improvements in the test's sensitivity and specificity could save thousands of lives. A TB working group has estimated that a rapid and accessible test for TB with sensitivity for smear-positive and negative cases greater than 85% and specificity of 97% could save approximately 400,000 lives a year [23]. Although the sensitivity of a combination of direct smear microscopy with bleach centrifugation of smear-negative specimens did not quite reach 85%, a sensitivity of 80% with a specificity of 95% suggests that this approach should improve the detection of TB over current methods while we await for a more sensitive and specific test. Results from the current study showed that 33 specimens found to be direct ZN smear-negative became smear-positive after treatment of the sputum specimen with 3.5% with centrifugation. This is a relatively high number of cases which were missed and are infective to the community. Therefore, it may be concluded that the increasing trend of smear-negative cases in Kenya may partly be as a result of missed cases by direct smear microscopy as shown in this study.

In a recent systematic review by Steingart and colleagues, results suggested that centrifugation with any of the several chemical methods including bleach combined with smear microscopy is more sensitive with a similar specificity compared with standard direct smear microscopy [12]. In their review it was shown that the average incremental yield of smear positives was 13% in six studies where bleach with centrifugation was used and 11% in a study involving HIV patients. The findings of an increase in sensitivity of 15.1% using bleach centrifugation for smear-negative specimens over standard smear microscopy are consistent with these results. However, it is important to note that the sensitivity of direct smear microscopy in our study was relatively high (66%) compared to previously reported studies [24, 25].

Although this study did not focus on HIV infection, it may be assumed that since smear-negative TB is mainly associated with HIV co-infection, this aspect was appropriately taken care of taking into consideration that this study was carried out in a high TB/HIV co-infected population with a range of between 50% and 60% [26].

In an attempt to increase sensitivity of ZN smear microscopy, different concentrations of bleach followed by either sedimentation or centrifugation have been used in previous studies [10-11, 27]. Nevertheless, none of these studies used 3.5% bleach with centrifugation after direct smear microscopy to improve on the sensitivity of ZN technique. Two Kenya studies used 3.5% bleach followed by

concentration of bacilli using overnight sedimentation before staining [17, 18] while another used 3.5% and 5% bleach centrifugation methods respectively with fluorescent microscopy rather than the ZN technique.

Bleach centrifugation method is advantageous because it is easier to see the AFB bacilli against a clear background under the microscope since all of the cells and debris in the sputum are digested and cleared during staining process. As with direct smear microscopy, the patient can get the results in the same day. This approach of combining smear microscopy with bleach centrifugation should improve the current diagnostic algorithms and shorten the time required to establish the diagnosis of PTB in some patients who would otherwise have been smear-negative.

The limitations of this study include lack of clinical data to ascertain clinical diagnosis of smear negative TB and lack of information on HIV status to establish a correlation between smear negativity and HIV infection. A requirement of access to a centrifuge, which may not be available in many peripheral laboratories in most resource-limited countries, is another limitation. However, studies have shown that the use of a table top centrifuge which may not necessarily be costly would produce similar results.

5. Conclusion

In this study bleach with centrifugation significantly increased the yield of sputum smear microscopy. Further evaluation of these techniques in routine programmes is required especially in situations where the burden of TB/HIV is high.

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