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COMBINATION OF BLEACH AND FLOURESCENT MICROSCOPY: A MILESTONE IN THE DIAGNOSIS OF SMEAR NEGATIVE TUBERCULOSIS

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ABSTRACT

Background: The reliability of direct smear microscopy for diagnosis of tuberculosis has frequently been questioned due to low sensitivity. Treatment of sputum with sodium hypochlorite (NaOCI) has been used to increase sensitivity in many settings. However, no study has established the effect of NaOCI on fluorescent microscopy.
Objective: To establish whether NaOCI concentration method enhances positivity of fluorescent microscopy smear negative sputum for diagnosis of tuberculosis.
Design: A prospective study.
Setting: Mbagathi District Hospital and Centre for Respiratory Diseases Research, Kenya Medical Research Institute.
Results: Forty five (22%) specimens were culture positive. Fluorescent microscopy sensitivity was 28.9% and 22.2% after centrifugation and sedimentation with 3.5% NaOCI, respectively ($P > 0.05$). Sensitivity was 24.4% and 17.8% after centrifugation and sedimentation with 5% NaOCI, respectively ($P > 0.05$). Although there was no statistical significance difference between the two NaOCI concentration methods, 3.5% NaOCI with centrifugation indicated a higher yield.
Conclusion: Use of NaOCI significantly enhances positivity of smear negative sputum for diagnosis of tuberculosis when used with fluorescent microscopy. This approach could be recommended for screening all tuberculosis suspects especially in settings with potential smear negative tuberculosis.

INTRODUCTION

Fluorescent microscopy (FM) staining is on average 10% more sensitive than the Ziehl Neelsen (ZN) staining method (1-4) with similar high specificity as far as diagnosis of tuberculosis (TB) is concerned. However, FM sensitivity remains lower than that of culture since the latter is able to detect as few as 10 bacilli per milliliter of sputum (5) compared to at least 5000 bacilli per milliliter of sputum required for FM (6). A major disadvantage with culture is that it takes long before results are available.

The most commonly cited advantage of FM is the possibility to scan a sputum smear at 250x magnification compared to that of 1000x used in ZN microscopy, allowing a theoretical reduction of

examination time of the same area to one sixteenth as the surface increases by the square of the diameter (7). The examination time is reduced about 10-fold (8). Despite its high sensitivity compared to ZN, FM is mainly used as a research tool and in selected settings where more than 25 specimens are examined daily (9).

Since the advent of HIV, the annual incidence of TB has more than doubled in some African countries (10,11). Subsequently, HIV infection has been associated with sputum smear negative TB (11). Although there is limited data on the use of FM to diagnose TB in HIV co-infected patients the available information suggests that FM maybe a promising diagnostic tool in this population (3,4).

Detection of smear positive cases is the highest priority in a TB control programme, as these cases are infectious and contribute substantially to the transmission of disease (10).

Direct smear microscopy for AFB, specifically using ZN method, is still the cornerstone of TB diagnosis especially in resource-limited settings but has a low sensitivity. More sensitive techniques need to be developed and established if patients with active TB are to be accurately and promptly diagnosed. It has been documented that sensitivity of smear microscopy using ZN method can be significantly increased after treating sputum with NaOCI followed by either centrifugation or sedimentation (12-17). However, no study has addressed the effect of NaOCI on FM. This study was, therefore, designed to determine whether NaOCI enhances the sensitivity of FM, particularly in smear negative TB, whose rates are currently on the increase (18).

MATERIALS AND METHODS

Sputum specimens/study site: Of the ZN routine smear negative sputum specimens collected from patients suspected to have pulmonary tuberculosis (PTB) attending Mbagathi District Hospital (MDH) Nairobi; two hundred and four were confirmed to be FM smear negative. MDH is a public hospital that serves as a TB referral centre for Nairobi province. From each patient, sputum was aseptically pooled from the three specimens that are usually collected for routine microscopy to make a volume of at least 10ml per patient. Specimens that did not attain this volume were excluded.

Laboratory procedures: Specimens were securely transported to Kenya Medical Research Institute, Center for Respiratory Diseases Research (KEMRI-CRDR) TB laboratory which is about half an hour drive from MDH, making delivery of specimens easy and prompt.

All ZN smear negative specimens were homogenized with sterile glass beads using a vortex mixture. Direct smears were prepared in accordance with methods previously described (19) and stained for FM using a standard staining procedure previously described (20) to determine the status. Briefly, using a pipette, smears were made on pre-labeled slides, air dried, heat fixed using a slide drier for at least 2 hours to ensure they remained firmly fixed during staining. They were then loaded in bulks of 48 slides and stained with auramine 0 phenol using an automatic staining machine (Shandon-Elliot) (2,21). The staining reagents were replenished either on weekly basis or after staining 200 slides whichever was earlier. The staining jars were appropriately cleaned before replacement of reagents to avoid carryovers. Since auramine stain precipitates, the stain was filtered before use.

All FM confirmed smear negative specimens were then aliquoted into five equal portions. Each portion represented an individual specimen. One portion was treated with sodium hydroxide (NaOH) for culture on Lowenstein Jensen (LJ) media. Two portions were treated each with equal volumes of 3.5% NaOCI and the other two each treated with equal volumes of 5% NaOCI. The 3.5% NaOCI used in this study was obtained in this concentration commercially from local supermarkets. The product is usually produced for domestic purposes and also used in Kenyan health-care settings as a disinfectant. The 5% concentration was selected for comparison, since it has been used in a number of studies for the ZN method (12,13,16,17).

The 5% NaOCI concentration was then reconstituted from 12.5% commercially available bleach. The appropriate concentration of NaOCI was determined by checking the available chorine on weekly basis using United States Pharmacopoeia (USP) method of analysis of NaOCI (22).

All portions treated with NaOCI were left to stand for 30 minutes at room temperature in a safety cabinet for liquefaction to take place (17). Two portions, containing 3.5 and 5% NaOCI, respectively, were centrifuged at 3,000 Relative Centrifugal Force for

15 minutes and the other two portions sedimented at room temperature for 15 hours. The supernatant of each portion was carefully pippeted off; the deposit was mixed by vortex. Smears were made and stained as described above.

Smears for each NaOCI concentration and each method, were examined by four independent technicians and graded according to methods previously described (2,23). Sensitivity and specificity of NaOCI treated smears for the two concentration methods were determined using L-J conventional culture as a Gold standard. Culture was done according to methods previously described (2).

Quality control: Quality control for microscopy and culture was employed for KEMRI laboratory procedures and respective results were interpreted accordingly. Positive and negative control specimens and smears were used in every batch of culture and staining. Blinded reading of the slides was done by another group of four independent technicians. An experienced microscopist independent from the two groups, read an arbitrary 10% positives and 5% negatives selected randomly (17). In addition to this, the same microscopist re-read all the smears with discrepant results. All reagents and media were prepared in accordance with standard operating procedures (SOPs) used at KEMRI-CRDR, TB laboratory.

Statistical analysis: Data was double entered using Microsoft excel (Ms office 2000) and then was transferred to SPSS version 11.5 for Windows (SPSS Inc.) for analysis, McNemar's test was used to assess the level of significance in the comparison of the four tests. The discordant pairs were used in the computation of the chi-square test, with one degree of freedom. The sensitivity, specificity, positive and negative predictive values (PPV and NPV) were computed including their 95% confidence intervals (CI).

RESULTS

Of the 204 confirmed direct FM smear negative specimens, 45 (22%) were culture positive. Of these, 13 (28.8%) were both culture and smear positive after treatment with 3.5% NaOCI with centrifugation, 10 were both culture and smear positive after treatment with 3.5% NaOCI with sedimentation, 11 were both culture and smear positive after treatment with 5% NaOCI with centrifugation and eight were both culture and smear positive after treatment with 5% NaOCI with sedimentation (Table 1). Two specimens were culture negative but smear positive after treatment with 3.5% NaOCI with centrifugation. Concordance among the two groups

Table 1

Sensitivity, specificity, negative and positive predictive values, 95% confidence intervals of fluorescence microscopy using 3.5% and 5% sodium hypochlorite followed by either centrifugation or sedimentation

Method	%NaOCI concentration	Smear and culture positive specimens (n = 42)	% Sensitivity (95% CI)	% Specificity (95% CI)	% NPV (95% CI)	% PPV (95% CI)
Centrifugation	3.5	13	28.9 (16-44)	98.7 (95-99)	83.1 (77-88)	86.7 (59-98)
Sedimentation	3.5	10	22.2 (11-37)	100.0 (97-100)	82.0 (75-87)	100.0 (69-100)
Centrifugation	5.0	11	24.4 (12-39)	100.0 (97-100)	82.4 (76-87)	100.0 (71-100)
Sedimentation	5.0	8	17.8 (8-32)	100.0 (97-100)	81.1 (75-86)	100.0 (63-100)

PPV = Predictive value of positive smear, NPV = Predictive value of Negative smear,
CI = Confidence intervals

Table 2

Comparison of sensitivities for 3.5% and 5% NaOCI followed by either centrifugation or sedimentation

Concentration	Sensitivities	Z-value	P-value
3.5c v 5c	28.9 vs. 24.4	0.483	P>0.05
3.5c v 3.5s	28.9 vs. 22.2	0.693	P>0.05
5c v 5s	24.4 vs. 17.8	0.77	P>0.05
3.5s v 5s	22.2 vs. 17.8	0.523	P>0.05

3.5c = 3.5% NaOCI with centrifugation; 3.5s = 3.5% NaOCI with sedimentation; 5c = 5% with centrifugation; 5s = 5% NaOCI with sedimentation; Standardised Normal Deviate (n = 45)

of microscopists was 98%, 97%, 100% and 98% with four sets of slides, respectively. The smears with discrepant results were confirmed as true positives / negatives using culture as a gold standard.

Sensitivity varied between 17.8% and 28.9%. The sensitivity of 3.5% NaOCI with centrifugation (28.9%) was the highest compared to that of 3.5% NaOCI with sedimentation (22.2%), that of 5% NaOCI with centrifugation (24.4%) and that of 5% NaOCI with sedimentation (17.8%). However, the difference was not statistically significant ($P > 0.05$). Specificity varied from 98.7% to 100%. NPV varied between 81.1% and 83.1%. PPV varied from 86.7% to 100%. With exception of 3.5% NaOCI with centrifugation which had a lower level of specificity (98.7%) and PPV (86.7%), all the rest indicated similar specificity (100%) and PPV (100%) (Table 1). There were no significant statistical differences in sensitivities for both NaOCI concentrations followed by either sedimentation or centrifugation ($P > 0.05$) (Table 2).

DISCUSSION

This is the first time NaOCI concentration method is being used with FM. It is also the first time this method is used to determine whether NaOCI improves sensitivity of FM smear negative sputum specimens for diagnosis of PTB. Furthermore, it is the first study to compare different concentrations and different methods of NaOCI on direct smear microscopy.

According to WHO guidelines, a smear negative PTB case diagnostic criteria should include at least three sputum smear negative for AFB and radiographic abnormalities consistent with active PTB and no response to a course of broad-spectrum antibiotic; and a decision by a clinician to treat the patient with a full course of anti-TB drugs or positive culture but negative AFB examination (24). Although the focus of this study was based on confirmed direct FM smear negative sputum specimens, and not necessarily following the WHO criteria for defining smear negative PTB, 22% of the analysed specimens were culture positive. Accordingly, these cases would initially be regarded as smear negative PTB. It is important to note that in many settings not all smear negative suspects are screened according to WHO guidelines and in the absence of systematic approach, these cases would, therefore, pose a great threat to the community if left undetected. For this reason, use of NaOCI on all smear negative sputum specimens with FM would significantly enhance case detection in this category of patients.

Generally, there was a significant increase in sensitivity using both concentrations of NaOCI, with smears positivity ranging from 1+ (10-99 / 100 fields) to 3+ (>10 / field) (23). No smear had 1-9 / 100 fields. These findings are in contrast with observations made in a review article where the authors indicated that the end results of using bleach may be to turn smears classified as 'scanty' into positives (25). In this study, majority of smears were graded as 1+, despite the fact that sputum specimens used were already confirmed and declared smear negative before using the NaOCI concentration method. Since none of the previous studies that have used the bleach method has focused on only smear negative specimens, expressions made by Ramsay and associates may be justified (25). Results from this study also showed a high specificity of between

98.7% and 100% regardless of NaOCI concentration. These results are in agreement with observations in a recent systematic review by Steingart and colleagues where it was shown that the specificity of FM in detection of AFB is similar to that of ZN (4). Nevertheless, these findings are in contrast with earlier data by Kubica indicating lingering doubt of specificity when using FM (1).

The number of positive specimens following centrifugation was higher than that when sedimentation method was used regardless of NaOCI concentration used. This may partly be due to the fact that centrifugation yields a more solid sediment that is not easily disturbed during removal of supernatant as compared to the sediment formed when sedimentation method is used. This approach further facilitates appropriate smear preparation, staining and scanning making the process user friendly. 3.5% NaOCI treated specimens showed a higher number of positive specimens than those treated with 5% NaOCI after centrifugation. This may, partly, be due to some smears being washed off the slides during staining as a result of over digestion of sputum with a higher concentration of 5% NaOCI leading to non-adherence of smears on the slides. However, no attempt was made to confirm that this was the case in this study although only two specimens that were smear positive after treatment with 3.5% NaOCI followed by centrifugation were found to be culture negative. On one hand, since the smears had grades of between 1+ and 2+, they could have been true positives that were either not picked by culture or patients were already on treatment. On the other hand, it could be argued that the use of a staining machine would enhance the positivity due to cross contamination by carryovers. In practice this may not be possible for such high positivity being observed in only two slides. To ascertain this was not the case, at least four negative control slides were included in each staining rack holding 48 slides. None of these was positive throughout the entire study. This observation was also made in a previous study (2).

The apparent advantage of FM is compromised by the fact that skill is essential to distinguish between genuine AFB from fluorescent artifacts, the failure of which could lead to high false positivity rate (2). This was demonstrated in this study where concordance among the two sets of experienced independent microscopists ranged between 97%-100%. The difference occurred in smears that had grade of 1+. It is, therefore, advisable to subject all doubtful smears for counter checking by experienced technicians. In addition to this, it has previously been suggested that restaining FM stained slides with ZN method to confirm presence of AFB would be beneficial when using FM method. However, the same principle cannot be applicable to the ZN stained smears (2). Furthermore, this approach has been a subject of controversy, since FM is superior to ZN, thus, a possibility of false ZN negative. Accordingly, culture would be the most appropriate confirmatory approach in this scenario.

World Health Organization (WHO), Expert consultation on improving the diagnosis of TB through optimization of sputum smear microscopy has recommended that FM may be considered at all levels of the health system in high HIV prevalence countries seeking to improve the sensitivity of sputum smear microscopy and reduce workload (26). To date, only one study has reported that fluorochrome staining is more efficient in detecting cases associated with HIV seropositivity especially paucibacillary cases (27). Although this study did not directly address the impact of HIV infection on the results, the fact that 'smear negative' is mainly associated with TB/HIV co infection whose rates range between 50% and 60% in the study area (18), it can be assumed that this aspect was indirectly addressed. Only one study using NaOCI has specifically addressed this issue (16). Therefore, more work needs to be done to elucidate the use of NaOCI with FM in TB/HIV co-infection.

In conclusion, results from this study have shown for the first time that NaOCI significantly enhances positivity of FM smear negative sputum for diagnosis of TB. However 3.5% NaOCI with centrifugation indicated a higher yield. The technique can be used to process only initially direct FM smear negative specimens excluding smear positive specimens. Nevertheless, for practicability the method could be recommended for screening all TB suspects.

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