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IMPROVED DIAGNOSIS OF ZIEHL-NEELSEN SMEAR NEGATIVE TUBERCULOSIS USING SODIUM HYPOCHLORITE SEDIMENTATION METHOD
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IMPROVED DIAGNOSIS OF ZIEHL-NEELSEN SMEAR NEGATIVE TUBERCULOSIS USING SODIUM HYPOCHLORITE SEDIMENTATION METHOD

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ABSTRACT

Background: **Bacteriological diagnosis of tuberculosis (TB) is largely dependent on Ziehl-Neelsen (ZN) microscopy. This method has a low sensitivity. Although concentration of sputum with sodium hypochlorite (NaOCl) followed by sedimentation increases the sensitivity of direct smear microscopy, no study has focused on the effect of NaOCl on smear negative sputum specimens.**
Objective: **To establish whether 3.5% NaOC1 sedimentation method specifically improves the diagnosis of Ziehl-Neelsen smear negative tuberculosis.**
Design: **A prospective study.**
Setting: **Mbagathi District Hospital and Center for Respiratory Diseases Research, Kenya Medical Research Institute.**
Subjects: **Two hundred and thirty confirmed direct ZN smear negative sputum specimens from new TB suspects were analysed.**
Results: **Seventy (30.4%) specimens were culture positive. Of these, 19 were ZN smear positive. The ZN sensitivity, specificity, positive and negative predictive values were 27.1%, 99%, 95% and 76%, respectively, after sedimentation with 3.5% NaOC1.**
Conclusion: **Overnight sedimentation using 3.5% NaOC1 significantly improves diagnosis of ZN smear negative TB. This technique has potential to improve diagnosis in TB diagnostic services especially in settings with high burden of dual TB/HIV infection.**

INTRODUCTION

Bacteriological diagnosis of tuberculosis (TB) is to a large extent dependant on direct smear microscopy of sputum after Ziehl-Neelsen (ZN) staining. This method is rapid, specific and reasonably easy to perform, but its sensitivity is low ranging from 8.8-46.6% in most African laboratories (1). Since it is likely to diagnose the most infectious patients, World Health Organization (WHO) recommends the method for screening patients with cough lasting for more than two weeks for TB disease (2). Smear microscopy has many advantages in terms of speed and feasibility and if sensitivity could be improved, it has the potential to become an even more valuable tool for TB diagnosis for National Tuberculosis Programmes (NTPs) worldwide especially in the resource limited settings.

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 Programme (NLTP) in the last ten years (3). This dramatic increase has been attributed largely to HIV infection and rising poverty levels. However, along this increase in TB cases, the increase in smear negative pulmonary

tuberculosis (PTB) has been disproportionately large (4). There is, therefore, an urgent need to establish more sensitive, safe and fairly rapid methodologies that could confirm diagnosis particularly of smear negative PTB patients.

In the last decade, many researchers have suggested that the performance of sputum smear microscopy can be significantly improved if sputum is liquefied using sodium hypochlorite (NaOC1) and then concentrated by either centrifugation or sedimentation prior to staining (5-10). However, the majority of these studies have focused on using NaOC1 with centrifugation. Furthermore, no study has addressed the effect of NaOC1 on specimens that are classified as smear negative TB. A recent report by WHO TB consultation in September 2005 (11), indicates that there is not enough evidence to support implementation of NaOC1 sedimentation methods until results from further studies, particularly those involving long sedimentation times have been carried out. Accordingly, this study was carried out to establish whether NaOC1 overnight sedimentation method specifically improves diagnosis of smear negative TB.

MATERIALS AND METHODS

Sputum specimens/site: Two hundred and thirty two direct ZN smear negative sputum specimens from new TB suspects attending Mbagathi District Hospital (MDH) in Nairobi province were collected. MDH is a public hospital, which serves as the TB referral centre in Nairobi province.

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

Sputum direct smears were prepared from all the specimens in accordance with methods previously described (12) and stained with ZN technique to confirm their status. Only smear negative sputum specimens were included in this study. The specimens were homogenised using a vortex mixture and aliquoted into two equal portions. One portion was processed for culture using standard methods previously described (13). The other portion was treated with an equal volume of 3.5% NaOC1, and left overnight at room temperature for 15 hours after which the supernatant was carefully pippeted off. Smears were made from the sediment, air-dried, heat fixed and stained with ZN technique. Smears were examined using a bright field microscope under oil immersion (X 1000), and reported according to standard methods previously described (13,14). The sensitivity and specificity of direct and NaOC1 sediment smear method were determined by using the conventional culture as the Gold standard (13,15).

Quality control: Known positive and negative sputum specimens were included in every batch of specimens processed. After initial examination of smears (direct and NaOC1 treated) were securely stored in slide boxes. An arbitrary 10% of the positive smears and 5% of the negative smears were selected at random (10) and re-examined by an independent microscopist from KEMRI-CRDR TB laboratory. The microscopist was blinded to the initial results. All reagents and media were prepared in accordance with standard operating procedures (SOPs) used at KEMRI-CRDR., TB laboratory. NaOC1 was reconstituted on a weekly basis.

Data analysis: The data were entered in Microsoft excel (Ms 2000). Analysis was done using SPSS version 11.5 for Windows (SPSS Inc.). McNemar's test was used to assess the level of significance for the two test procedures on the same sample. The test statistic for testing for the level of significance was the chi-square test. The test assesses for heterogeneity of the association between the two study methods. Other measures calculated were sensitivity, specificity, positive and negative predictive values (PPV and NPV). A test for normalisation of proportions was used in order to test for differences in sensitivity/specificity tests.

RESULTS

Of the 230 initially confirmed direct ZN smear negative sputum specimens, 70 (30.4%) were culture positive. This means that nearly one third of the sample screened using the direct ZN method were missed. However, using ZN sedimentation method, 20/230 (8.7%) specimens were smear positive. Of these, 19 were also culture positive with a sensitivity of 27.1%. One hundred and sixty specimens were culture negative.

Table 1

Sensitivity, specificity, negative and positive predictive values, and 95% confidence intervals of Ziehl-Neelsen staining method using 3.5% sodium hypochlorite followed by sedimentation

Method	%NaOC1 (concentration)	% sensitivity (95% C1)	% specificity (95% C1)	% PPV (95% C1)	% NPV (95% C1)
Sedimentation	3.5	27 (17-39)	99 (96-99)	95 (75-99)	76 (69-81)

PPV = Predictive value of positive smear, NPV = Predictive value of negative smear

The sedimentation method had a misclassification of 1/160 (0.63%) specimens. The smear positivity ranged from 1+ (19 specimens) to 3+ (1 specimen).

There was a significant increase in sensitivity using ZN sedimentation method ($Z = 4.7$, $p<0.001$), Pearson Chi-square $X^2 = 43.129$, $p = 0.0001$.

Statistical analysis using McNemars test showed that there was a significant difference between the culture and the smear method ($p < 0.00001$).

Table 1 summarises sensitivity, specificity, positive and negative predictive values, and 95% confidence intervals of ZN staining method after treatment with 3.5% NaOC1 followed by sedimentation.

DISCUSSION

This was the first study to use NaOC1 sedimentation method specifically on ZN negative smear specimens using long sedimentation period. In this study, 3.5% NaOC1 was the concentration of choice because it is the most common formulation of NaOC1 in the Kenyan market and was successfully used in a recent study (16).

Results showed that there was a significant increase in sensitivity using 3.5% NaOC1. Taking into consideration that the 230 specimens included in this study were already confirmed direct ZN smear negative, the increase of 8.7% smear positivity with a sensitivity of 27.1% using 3.5% NaOC1 with overnight sedimentation is a very encouraging finding. In addition to this, except for one specimen, all the other specimens that were smear positive after treatment with 3.5% NaOC1 were also culture positive further proves the reliability of this technique. Considering a number of factors that include duration, cost and expertise involved in the culture process, it can be concluded that use of NaOC1 in smear microscopy although not comparable to culture, could be supplementary in the diagnosis of TB. This, however, does not exclude the principle usefulness of culture as a Gold standard and for identification and drug susceptibility testing for the management of TB.

It is suggested that NaOC1 digests the sputum which when followed by concentration of bacilli by either centrifugation or sedimentation greatly increases the number of bacilli per microscopic field and hence the increase in sensitivity (6).

In an attempt to increase sensitivity of ZN smear microscopy, different concentrations of NaOC1 followed by either sedimentation or centrifugation have been used in previous studies (5,6,9,10). Nevertheless, none of these studies has either addressed the aspect of smear negative specimens or documented the use of 3.5% NaOC1 sedimentation method. For instance, in a study in New Delhi, 1% NaOC1 was used and the bacilli concentrated by floatation on a layer of xylene before staining (17). Five per cent NaOC1, has been used by several investigators (5,6,9,10) and their findings have indicated contradicting results. In South Africa where between 4% and 5% NaOC1 was used, findings indicated that there was no increase in sensitivity (18). Other studies using 5% NaOC1 showed sensitivity between 70% (6) and 100% (5). A study in Ethiopia using 5% NaOC1 showed an increase in sensitivity from 54.2% to 63.1% in HIV negative patients and 38.5% to 50% in HIV-positive patients (9). In these studies, specimens analysed were from all TB suspects whereas in our study the focus was on smear negative specimens. Furthermore, in those studies, bacilli were concentrated using centrifugation method only. Centrifugation has a serious limitation because it requires access to a centrifuge, which may not be available in many peripheral laboratories in most resource-limited countries.

NaOC1 overnight sedimentation method has several advantages. These include the ease of seeing the bacilli against a clear background under the

microscope since all cells and debris in sputum are digested by NaOC1 and cleared during staining process leaving only the AFB. There is a significant reduction in time for diagnosis of ZN smear negative TB, that usually takes a long process to ascertain (19). This method will lead to early diagnosis of smear negative TB for prompt treatment thereby lowering the rate of transmission within the community and eventually reducing mortality and morbidity due to TB. In addition to this, overnight sedimentation may necessitate reduced chances of laboratory acquired infections since the 3.5% NaOC1 has potential to immobilise most of the mycobacteria (20). Taking into consideration that between 1000 and 5000 bacilli per 1 ml of sputum is required for a ZN positive smear (14), findings from this study in which 19/20 initially smear negative but culture positive specimens had yielded 1+ smear positivity clearly indicates that NaOC1 sedimentation method has potential to improve TB diagnosis. This approach also confirms that smear positivity is not just a boost of specimens which are already scanty into positives (21), but indeed actual detection of AFBs not detected by direct ZN smear microscopy. Accordingly, the technique can be used to process only initially direct ZN smear negative specimens excluding smear positive specimens. Nevertheless, since this study has now proved that use of 3.5% NaOC1 sedimentation method increases the sensitivity of smear negative specimens, application of this technique for screening all TB suspects may be recommended in the diagnosis of TB.

CONCLUSIONS

A core element of any effective TB control programme is to diagnose cases with active disease promptly and initiate effective therapy, to reduce individual morbidity and mortality that can result from delays in diagnosis and also break the cycle of disease transmission. Using 3.5% NaOC1 with overnight sedimentation significantly increases the sensitivity of direct smear microscopy by ZN method. This approach has the potential to improve diagnosis in TB diagnostic services in resource limited countries and should be recommended in settings with high TB/HIV prevalence especially where ZN microscopy is a core diagnostic tool. However, further studies to determine the cost effectiveness of the technique are needed.

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