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Role of Bleach Concentration Method for Detection of Acid-fast Bacilli (AFB) in Sputum using Conventional Ziehl-Neelsen (ZN) Staining Technique

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Abstract

Sputum microscopy is the most important tool for the detection of *Mycobacterium tuberculosis* in peripheral laboratories. Sodium hypochlorite concentration technique prior to sputum microscopy may improve the detection of AFB bacilli over direct Ziehl Neelsen's staining method. A prospective study was conducted at Kasturba Medical College, Manipal. Consecutive sputum samples were collected for 3 months. ZN stained smears were made directly of fresh specimens and of specimens that were processed with 3.5% sodium hypochlorite (NaOCl), from each patient. Then, ZN stained smears were observed under light microscopy. A total of 239 samples were collected. The yield of positive smears with ZN staining after NaOCl centrifugation was higher than that with microscopy after direct ZN stain. The percentage increase yield of ZN staining after NaOCl centrifugation over direct ZN staining was 3.3% (8/239). Bleach centrifugation can help in increasing the yield of positivity in sputum smear.

Keywords: Ziehl-Neelsen Staining, Sputum, *Mycobacterium tuberculosis*, Sodium Hypochlorite

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INTRODUCTION

With millions of people being affected annually, tuberculosis (TB) is reckoned as a global health crisis for both developing and developed countries.[1] Tuberculosis is one of the oldest diseases known to mankind and a significant cause of mortality and morbidity around the world is caused by *Mycobacterium tuberculosis* complex.[2]

In developing countries such as India, sputum microscopy with Ziehl-Neelsen (ZN) staining is used to identify tuberculosis because it is simple, inexpensive and provides rapid results.[3,4]

Bleach (sodium hypochlorite, NaOCl) can be used as an ideal chemical processing agent in a resource-poor country. To enhance the yield of AFB for diagnosing pulmonary tuberculosis, digestion of sputum sample with bleach is suggested by some researchers.[5,6] This agent is commonly available, affordable, and its disinfectant properties could enhance infection control in laboratories lacking proper biosafety equipment.[7]

MATERIALS AND METHODS

This study was conducted from February 2019 to April 2019 in the Department of Microbiology, Kasturba Medical College, Manipal, Karnataka.

Patients presented with productive cough lasting more than 2 weeks duration were included in this study. The pre-analytical processing of sputum sample and sample anonymization had been done. Smear was made from each specimen for direct smear microscopy and was stained using the hot ZN method. The remainder of the specimen was transferred to a 15-mL disposable plastic conical tube with an equal volume of 3.5% commercial bleach (Emplura) added to the tube and was vortexed for 2-3 sec. The tube was then allowed to stand for 10-15 minutes at room temperature and then shaken again intermittently every 5 minutes. An equal quantity of distilled water was then added to the tube. The same was centrifuged at 3000 rpm for 15 minutes. After discarding the supernatant, the pellet was suspended in a few drops of the remaining fluid. By using a sterile glass pipette, 1-2 drops were transferred to a glass slide. Using the ZN stain for light microscopy, a smear was made and stained.

RESULTS

The study included a total of 239 consecutive sputum samples as per the inclusion criteria. Out of which, 18.8% (45/239) were mucopurulent, 38.5% (92/239) were purulent, 5.4% (13/239) were blood-tinged and 37.2%

Table 1. Increased positivity by ZN staining in different types of sputum sample after bleach centrifugation (n = 239)

Sputum type (n)	Direct ZN Staining	ZN Staining after Concentration	Increased percentage
Mucopurulent (45) (18.8%)	6 (13.3%)	7 (15.6%)	2.3%
Purulent (92) (38.5%)	7 (7.6%)	7 (7.6%)	0
Blood tinged (13) (5.4%)	1 (7.7%)	2 (15.4%)	7.7%
Mucoid (89) (37.2%)	6 (6.7%)	12 (13.5%)	6.8%
Total (239)	20 (8.4%)	28 (11.7%)	3.3%

Table 2. Comparison between ZN staining of mucoid samples with and without hypochlorite (n = 89)

	ZN staining without hypochlorite (Positive)	ZN staining without hypochlorite (Negative)	Total
ZN staining with hypochlorite (Positive)	6	6	12
ZN staining with hypochlorite (Negative)	0	77	77
Total	6	83	89

Results of Mc Nemar test: Chi-squared statistic = 4.167, P value = 0.0412

Table 3. Comparison between ZN staining of mucopurulent samples with and without hypochlorite (n = 45)

	ZN staining without hypochlorite (Positive)	ZN staining without hypochlorite (Negative)	Total
ZN staining with hypochlorite (Positive)	6	1	7
ZN staining with hypochlorite (Negative)	0	38	38
Total	6	39	45

Results of Mc Nemar test: Chi-squared statistic = 0.000, P value = 1.000

Table 4. Comparison between ZN staining of blood-tinged samples with and without hypochlorite (n = 13)

	ZN staining without hypochlorite (Positive)	ZN staining without hypochlorite (Negative)	Total
ZN staining with hypochlorite (Positive)	1	1	2
ZN staining with hypochlorite (Negative)	0	11	11
Total	1	12	13

Results of Mc Nemar test: Chi-squared statistic = 0.000, P value = 1.000

Table 5. ZN stain grading increase with use of hypochlorite decontamination (n=18)*

Sample type	No change in grading	Increase in grading	Remarks
Mucoid	3	3	Scanty to 3+: One 1+ to 2+: One 1+ to 3+: One
Mucopurulent	2	4	Scanty to 2+: One 1+ to 3+: One 2+ to 3+: Two
Blood tinged	0	1	1+ to 3+: One
Purulent	3	2	1+ to 2+: One 1+ to 3+: One

No. of samples in which grading increased = 10
No. of samples in which grading increased by at least two steps = 6
* Positive, 3+ samples (direct ZN staining) excluded from analysis since no increase in grading to be documented

(89/239) were mucoid (Table 1) 8.4% (20/239) were positive by direct ZN staining while 11.7% (28/239) were positive by ZN staining following NaOCl concentration. 3.3% more positivity was detected after NaOCl concentration. For mucoid samples, significantly higher numbers (p=0.0412) were detected after NaOCl concentration (Table 2). Increase in detection with NaOCl concentration was insignificant (p> 0.05) for mucopurulent and blood-tinged samples (Table 3, Table 4). For purulent samples there was no difference in detection rates between direct ZN staining and NaOCl concentration method. Though increased detection following concentration is not significant for all types of samples yet half or more of positive samples of each category have shown increase in grading (Table 5). Ten samples showed increased in grading following concentration, out of which 6 shows at least 2 steps increased in grading. The cost per test for bleach centrifugation was INR 1/ sample. Bleach centrifugation technique provided a clear background, facilitating better visualization.

DISCUSSION

In India, TB remains to be a major public health problem. It is one of the leading causes of morbidity and mortality worldwide.[2] In developing countries, sputum microscopy is the fastest, cheapest and the most widely used screening method for detection of TB in peripheral laboratories.[8] Application of 3.5% bleach can inactivate the bacteria without altering their structure so that even if the bacteria get killed, they can be stained and observed under microscope. This provides a greater safety for laboratory personnel.[3]

Bleach has an oxidative effect, which disrupts the enzymatic processes of Mycobacteria, hence act as an effective disinfectant.[9] It enhances biosafety by reducing aerosolization.[10] Additionally, bleach centrifugation is known to increase smear sensitivity due to multiple factors. After the treatment with bleach, there are attributable changes in the surface properties (like, charge and hydrophobicity) of the mycobacteria. The process of denaturation of sputum constituents also leads to flocculation with subsequent increase in the sedimentation rate of mycobacteria.[11]

The standard method for concentration is the NALC-NaOH (N-acetyl-L-cysteine-sodium hydroxide) method which is technically demanding for a remote laboratory, and costs about 1 USD (INR 80 approx) per clinical specimen.[12] NaOCl is however available at prices almost negligible (INR 1 / sample) as compared to NALC-NaOH method. At such cheap prices this easy concentration technique may be used for rapid and safe screening of tuberculosis in the remote areas. It does not require any in house reagent preparation and storage. But, this method is not suitable if culture is contemplated since hypochlorite kills acid fast bacilli.

In our study, no such hindrance to detection was noted. All positive cases by direct ZN staining were also detected by bleach centrifugation. Bleach centrifugation detected additional positives which were missed by direct ZN staining. Increase in positivity was found to be most for mucoid samples. For purulent, mucopurulent and blood-tinged samples, it did not make any significant difference. This finding is however in contrary to that of Bonnet M et al., who found that bleach centrifugation technique produced significant increase in positivity for purulent samples only among HIV patients.[13] In the study conducted by Angeby KA et al. NaOCl concentration method yielded significantly more number of positive cases when compared with direct smear microscopy but differences between mucoid, purulent and mucopurulent samples was not evaluated.[14] Bruchfeld J et al. reported similar results to Angeby KA et al. but on HIV patients.[15] Our disagreement with Bonnet M et al. could be influenced by the difference in patient profile (HIV vs non-specific) but such a finding requires further corroboration from other studies.[13]

As noted by Saxena S et al., we also found that bleach sedimentation technique provided a clearer background for better observation. This, along with concentration by centrifugation may have contributed to improved grading after bleach centrifugation technique.[16]

Bleach centrifugation technique increases acid fast bacilli detection in sputum samples but as per this study it does so significantly in mucoid samples only. Bleach sedimentation provides clearer background for visualization and improves smear grading. Being a much cheaper and easier alternative, it may be implemented for high volume AFB screening at remote areas, though its effectiveness beyond mucoid samples may be disputed.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

PP carried out the study, collected the data and framed the manuscript. SVP contributed in scientific planning and review of the manuscript. PB wrote the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, Kasturba Medical College and Kasturba Hospital, Manipal, Karnataka, India (IEC: 24/2019).

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