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RESEARCH ARTICLE

EVALUATION OF BLEACH AND PHENOL AMMONIUM SULPHATE AS SPUTUM PRETREATMENT METHODS FOR *MYCOBACTERIUM TUBERCULOSIS* DETECTION IN SUSPECTED PULMONARY TUBERCULOSIS PATIENTS

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ABSTRACT

Introduction: Significant mortality and morbidity is caused by Tuberculosis (TB) in developing countries like India. Direct ZiehlNeelsen (ZN) sputum smear microscopy for the detection of AFB remains the most important diagnostic test for TB. But the sensitivity of this method in diagnosing pulmonary TB is discouragingly low. One approach to the improvement of sputum smear microscopy is the application of chemical pretreatment to disrupt sputum structure, separate clumps of mycobacteria, and concentrate bacilli, thereby increasing the probability of their detection. **Aims and Objective:** To compare the efficacy of Sodium Hypochlorite and Phenol ammonium sulphate pretreatment methods with direct sputum smear microscopy to detect AFB from sputum samples of suspected pulmonary TB patients. **Methods:** A total of 300 samples from 300 suspected pulmonary TB patients were studied. The sputum samples were divided into 3 parts and used for direct ZN staining, pretreatment with 5% sodium hypochlorite by centrifugation and sedimentation and phenol ammonium sulphate. The smears were stained by ZN technique and seen under 100 X oil immersion lens. The results were compared with culture on LJ media, as gold standard. **Results:** The sensitivity of direct microscopy was seen to be the lowest of 37.50%, followed by PhAS 45.83%, Bleach by sedimentation 50% and highest by Bleach by centrifugation method 52.08%. **Conclusion:** Pretreatment by sodium hypochlorite centrifugation method is a better method than PhAS for improving sputum smear microscopy.

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INTRODUCTION

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. It is a major global health problem, responsible for ill health among millions of people each year. TB ranks as the second leading cause of death from an infectious disease worldwide, after Human Immunodeficiency Virus (HIV). Of the estimated 9 million people who developed TB in 2013, more than half (56%) were in the South-East Asia and Western Pacific Regions. A further one quarter was in the African Region, which also had the highest rates of cases and deaths relative to population. India and China alone accounted for 24% and 11% of total cases respectively. India is the highest TB burden country accounting for one fifth of the global incidence (WHO, Global tuberculosis report, 2014 and TB India, 2011).

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Most deaths due to TB are preventable if people can access health care for a diagnosis and the correct treatment is provided. In 2012, out of the estimated global annual incidence of 8.6 million TB cases, 2.3 million were estimated to have occurred in India (TB India, 2014). The Government of India adopted the DOTS (Directly Observed Treatment Short Course) under RNTCP which was launched in a phased manner in 1993 (Mukhopadhyay *et al.,* 2013). The vision of RNTCP for the national strategic plan (2012–2017) "reaching the unreached" is a 'TB free India, through achieving universal access by providing quality diagnosis and treatment for all TB patients in the community'. However, the primary obstacle to control the spread and incidence of the disease is poor case finding which still remains less than 70% in some South- East Asian countries such as India (61.3%). When more cases will be identified, more cases will be cured and less transmission will occur, which will reduce the incidence of TB (Srikanth *et al.,* 2009 and Dye *et al.,* 1998). Culture for AFB on Lowenstein-Jensen (LJ) medium is considered as the gold standard test for detection of PTB (sensitivity ranging from 70-80%), but is time consuming and takes about 6 to 8 weeks.

Also it requires 10 to 100 AFB bacilli per ml of sputum to be culture positive (Toman, 2004 and Colebunders *et al.*, 2000). The microbiological diagnosis of PTB by direct sputum smear microscopy plays a key role in routine diagnosis of TB and treatment follow up in TB Control Programs in India. For a smear to be positive, at least 5000-10,000 bacilli per ml of sputum must be present (Kashyap *et al.*, 2012). The simplicity, inexpensiveness and predictive power of Ziehl-Neelsen (ZN) sputum smear microscopy makes it the applicable laboratory diagnostic tool of choice for TB in low resource settings but, the sensitivity of this method is low (43-60%) when compared with that of the cultures (Tadesse *et al.,* 2014. Lawson *et al.*, 2006 and Makunde *et al., 2007*). There are several other methods that can be used to improve sensitivity of detection of *M.tuberculosis* such as culture (LJ, MGIT, other liquid media), but these methods are limited by a long processing time and high cost. Newer molecular technique like Polymerase Chain Reaction (PCR), though rapid, is too expensive to be widely applied in resource limited settings. An alternative to culture is optimization of the sputum smear process. It has been shown that sensitivity of smear microscopy can be improved if the sputum sample is liquefied with one or more chemical reagents and then concentrated by centrifugation or sedimentation before acid fast staining. Various sputum concentration methods have been tried to increase the yield of sputum smear microscopy; for example oxalic acid, sulphuric acid, sodium hydroxide, N-acetyl L-cysteine-NaOH (NALC- NaOH) methods and newer methods such as PhAS (PhAS) method, chitin sedimentation, bleach centrifugation and sedimentation methods (Best *et al.*, 1990). The current study evaluated the performance of three pretreatment procedures for sputum smear microscopy (bleach sedimentation & centrifugation and PhAS methods) and compared their effectiveness with Lowenstein-Jensen (LJ) culture.

MATERIALS AND METHODS

The present prospective observational study was conducted in the Department of Microbiology, PGIMS Rohtak in collaboration with Department of TB and Respiratory Medicine, PGIMS, Rohtak over a period of one year. The study included 300 patients with clinical suspicion of Pulmonary TB. The patients who attended the RNTCP lab for the first time with clinical suspicion of PTB with any age group and sex were randomly included in the study. The patients who were already on anti-tubercular treatment were excluded from the study. Two sputum samples were collected from each patient. The first sample was on the spot sample and the second was early morning sample. All the specimens were transported immediately to the microbiology laboratory and were processed for ZN staining and culture in class II Biological Safety Cabinet by using Personal Protective Equipments (PPE). The on the spot sputum sample was used for inoculating LJ media after homogenisation and processing, by modified Petroff's method using 4% NaOH. Cultures were examined first after 48 to 72 hours to detect gross contamination. Thereafter, cultures were examined weekly upto 8 weeks on a specified day of the week. Growth was seen usually at the end of 4 to 8 weeks. Cultures were reported negative if no growth was seen at the end of 8 weeks. Formation of discrete, raised, irregular, dry and wrinkled colonies which were creamy white initially and then became buff coloured were considered as growth of *M.tuberculosis.*

The early morning sputum sample was homogenised and then used to make smear on 2 fresh and unscratched slides for direct sputum smear microscopy without any optimisation and stained by ZN technique. The remaining early morning sputum sample was divided into 3 equal parts.

- The first part was used for bleach optimization by centrifugation method**:**
  An equal amount of freshly prepared household bleach (5% NaOCl) was added to the sputum sample in a test tube and shaken for 30 seconds. The tube was left on the table top for 15 minutes at room temperature and hand shaken regularly to ensure homogenization. Then, double the amount of distilled water was added and the sample was centrifuged at 3000 rpm for 20 minutes. The supernatant was poured off and the smears were prepared using the sediment (Srikanth *et al.,* 2009).
- The second part of sputum was used for bleach optimization by sedimentation method:
  An equal amount of bleach (5% NaOCl) was added to the sputum sample and left at room temperature overnight (12-14 hours). The supernatant was poured off and the smears were prepared using the sediment (Srikanth *et al.,* 2009).
- The third part of sputum was used for optimisation by Phenol Ammonium Sulphate (PhAS) sedimentation method:
- To the fifth part of sputum sample an equal volume of PhAS reagent was added.

The PhAS reagent was prepared by dissolving 50 grams of phenol crystals (TM Media) and 40 grams of ammonium sulfate (Rankem) in 950 ml of distilled water. The reagent was prepared and kept in the laboratory until use (3 to 5 days). The sample was mixed well with PhAS reagent and left to stand overnight at room temperature. Next morning, the supernatant was poured off and the smears were prepared using the sediment (Selvakumar *et al.,* 2002). All the slides were air dried, heat fixed, stained by ZN technique and examined under 100X oil immersion field. The smears were graded using RNTCP guidelines.

Statistical Analysis: The data was collected and analysed by using Chi-square test. A `p' value <0.05 was considered as statistically significant. Evaluation was done by calculating the sensitivity, specificity, positive predictive value and negative predictive value.

RESULTS

The present study included a total of 300 patients with the clinical suspicion of PTB. The study population included both male (194) and female (106) with mean age of 45.12±17.47 years. Out of the 300 specimens processed, a total of 48 isolates were obtained in the LJ culture technique Table 1 compares the results of direct sputum smear microscopy , bleach sedimented smear, bleach centrifuged smear and PhAS sedimented smear with culture (gold standard). Of the culture positive samples, 18(37.5%) were positive by direct microscopy, 24(50%) were positive by bleach sedimentation, 25(52.08%) were positive by bleach centrifugation and 22(45.83%) were positive by PhAS sedimentation methods. Interestingly, we have observed that all the direct AFB smear positive specimens showed growth on LJ media. The sensitivity, specificity, Positive Predictive Value and Negative Predictive Value of all the methods investigated in this study are summarized in Table 2.

Table 1.

METHOD	DIRECT		BLEACH S[a]		BLEACH C[b]		PhAS[c]	
CULTURE	POS[d]	NEG[e]	POS[d]	NEG[e]	POS[d]	NEG[e]	POS[d]	NEG[e]
POS[d]	18	30	24	24	25	23	22	26
NEG[e]	0	252	2	250	2	250	5	247

[[a]: Bleach by sedimentation, [b]: Bleach by centrifugation, [c]: Phenol ammonium sulphate, [d]: positive, [e]: Negative.]

Table 2. Comparison of sensitivity / specificity / NPV / PPV of direct AFB smear by ZN staining after various sputum pretreatment methods

Pretreatment method	Sensitivity (%)	Specificity (%)	Negative predictive value (%)	Positive predictive value (%)
Direct microscopy	37.50	100	89.32	100
Bleach sedimentation	50	99.20	91.24	92.30
Bleach centrifugation	52.08	99.20	91.57	92.59
PhAS	45.83	98.01	90.47	81.48

DISCUSSION

Despite renewed efforts to control the epidemic, tuberculosis remains a public health emergency predominantly affecting the poorest countries of the world. Though India is the second-most populous country in the world one fourth of the global incident TB cases occur in India annually. As per WHO Global TB Report, 2015, out of the estimated global annual incidence of 9.6 million TB cases, 2.2 million were estimated to have occurred in India (WHO Global tuberculosis report, 2016).

Proper identification of cases is the pillar of TB control programs. Serial sputum smear microscopy is the only available test to confirm TB that is suitable and affordable for implementation at lower levels of the health service. Unfortunately, microscopy is associated with low and variable sensitivity (20-60%) (Bonnet *et al.,* 2011). The gold standard for diagnosing PTB is culture of sputum on LJ medium. However, due to lack of access to culture facilities and the long turn-around times involved with sputum culture, most programmes use direct ZN microscopy for detection of AFB in sputum smears (Hepple *et al.*, 2010). The frequency of smear negative PTB is also increasing which may be due to the low sensitivity of the direct AFB smear or the overloaded nature of TB laboratories.

The above mentioned issues raise the need of optimisation and sputum pretreatment methods with readily available reagents for diagnosis of Pulmonary TB, particularly in high prevalence and resource limited settings like ours**.** Therefore, the present study was carried out to assess the performance of two short duration chemical pretreatment procedures (PhAS and NaOCl methods) to diagnose Pulmonary TB suspected cases. In the present study, a total of 48 isolates were obtained on LJ culture out of 300 specimens processed. All the direct AFB smear positive specimens showed growth on LJ media, while all bleach pretreated smear positive specimens showed growth on LJ media except 2 which might be due to paucibacillary nature of these samples. With PhAS maximum number of false positives were seen which might be due to low specificity of the method. The present study shows that direct microscopy is least sensitive but has highest specificity among all the methods. The data suggests that PhAS is more sensitive than direct microscopy but least specific of all the methods. Bleach centrifugation and sedimentation methods were found to equally specific while bleach centrifugation method is more sensitive than the sedimentation method. So, bleach centrifugation is a better sputum pretreatment method among all the methods done. Also this method reduces the turn around time as compared to the sedimentation method which delays the result by 24 hours. Phenol ammonium sulphate pretreatment by sedimentation also increases the reporting time by overnight. Ammonium-sulfate prevents the formation of hydrogen bonds of proteins with water and facilitates the interaction of proteins with each other to form aggregates. This causes the mucus and other proteins in the sputum to precipitate and later sediment by the ‘‘salting out’’ phenomenon. Bleach is widely available and inexpensive, and its disinfectant properties could improve infection control in laboratories lacking adequate biosafety facilities (Best *et al.*, 1990). Bleach has been reported to increase the sensitivity of smear microscopy primarily through digestion of the mucus and debris in sputum, resulting in a clearer microscopy field (Bonnet *et al.*, 2008). This chemical has a few limitations like instability of the bleach can be prevented by storing them in dark containers and cool areas. In summary, the sensitivity of PhAS and bleach centrifugation methods are noticeably higher than the direct AFB smear in the diagnosis of suspected cases of Pulmonary TB. Further, bleach centrifugation method was found to be efficient to diagnose suspected cases of pulmonary tuberculosis.

Conflict of interest: None declared

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