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Application of Bleach Method to Improve Smear Microscopy for the Diagnosis of Pulmonary and Extrapulmonary Tuberculosis.

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ABSTRACT

Background: The microbiological diagnosis of pulmonary TB by direct sputum smear microscopy plays a key role in routine diagnosis of TB and treatment follow up in Tuberculosis Control Programs in India. Direct smear preparation of clinical samples is hazardous for technicians working in centers without a bio-safety hood. Studies have 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. The present study aimed to quantify the gain in positivity of microscopic detection and to test its implementation in a peripheral laboratory participating. **Methods:** This is a prospective observational study were conducted in the Department of Microbiology, Kamineni Institute of Medical Sciences, Narketpalli, Nalgonda, Telangana, India. A total of 100 patients, of all age groups and sex, presenting to the OPD for the first time, with clinical suspicion of pulmonary or extrapulmonary TB was included in the study. All the demographic details of the patients were noted and consent was taken from patients. Salivary samples were immediately removed and a new sample was requested. The time between sputum collection and analysis was recorded. **Results:** In present study the 100 patients enrolled, the male/female ratio was 1.32, the mean age was 57 years (range 10–85), and 92% of patients were sampled for TB case detection. Only one patient had known HIV infection. Overall, AFB were detected on 44 smears prepared by the direct method (12.3%) and 55 smears prepared by the bleach method (16.0%), a statistically significant difference (P = 0.0006), giving an increase in positivity. The semi-quantitative results highlighted a significant gain in positivity (P < 0.001) with the bleach method. **Conclusion:** Our study, conducted in a population with a low prevalence of HIV/AIDS, confirms the benefits of the bleach method for the microscopic case detection of pulmonary TB. This rustic, simple and inexpensive method could easily be integrated into the routine of a peripheral laboratory after a short training period and strengthening of quality assurance.

Keywords: Bleech Methods, Pulmonary Tuberculosis, Direct Method, HIV.

INTRODUCTION

Tuberculosis (TB) is one of the biggest public health challenges confronting the world today despite the fact that its causative organism Mycobacterium tuberculosis was discovered more than a century ago.[1] Out of 8.8 million TB cases that occurred globally in 2010, 59% occurred in Asia, 26% in the African Region, 7% in the Eastern Mediterranean Region, 5% in the European Region, and 3% in the American Region.[2] India is one of the 22 high-burden countries. It bears the share of 26% of global cases with TB incidence of 2.5 million as notified cases in 2011. Though, about 80% of TB patients suffer from pulmonary tuberculosis, the incidence of extra-pulmonary manifestations is also high (1 in 5 patients).[3] India ranks 2nd in the world and accounts for about 10% of the global burden of HIV associated TB.[4]

The microbiological diagnosis of pulmonary TB by direct sputum smear microscopy plays a key role in routine diagnosis of TB and treatment follow up in Tuberculosis Control Programs in India. For a smear to be positive, at least 5000-10,000 bacilli per ml of sputum must be present.[5] The simplicity, inexpensiveness and predictive power of Ziehl – Neelsen (ZN) sputum smear microscopy makes it the applicable laboratory diagnostic tool of choice for tuberculosis in low resource settings but, the sensitivity of this method is low (43-60 %) when compared with that of the cultures.[6-8] The sensitivity of this technique is further reduced in paediatric and HIV (20-35%) patients because HIV mediated immunosuppression leads to impaired granuloma formation, resulting in both ineffective containment of M. tuberculosis bacilli and diminished formation of pulmonary cavities and lower concentrations of bacteria in sputum. Frequent

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smear negative cases exacerbate the difficulty of detecting HIV associated TB resulting in the death. The sensitivity of direct smear microscopy is low in children because their sputa harbour lower number of acid fast bacilli.[9-14]

Direct smear preparation of clinical samples is hazardous for technicians working in centers without a bio-safety hood.[15] The technicians sometimes may not prepare sputum smears properly owing to fear of the possibility of getting the infection. Thus, it is desirable to introduce improvisations in the direct microscopy methods for effective TB diagnosis in TB control programs.

Studies have 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.[16] 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 (Phenol ammonium sulphate) method, Chitin sedimentation, Bleach centrifugation and sedimentation methods.[5]

The present study aimed to quantify the gain in positivity of microscopic detection and to test its implementation in a peripheral laboratory participating.

MATERIALS AND METHODS

This is a prospective observational study were conducted in the Department of Microbiology, Kamineni Institute of Medical Sciences, Narketpalli, Nalgonda, Telangana, India.

Inclusion criteria

A total of 100 patients, of all age groups and sex, presenting to the OPD for the first time, with clinical suspicion of pulmonary or extrapulmonary TB was included in the study. All the demographic details of the patients were noted and consent was taken from patients.

Exclusion criteria

- Patients already taking antitubercular drugs and/or quinolone.

Sample Processing

Sputum samples were first classified according to their macroscopic aspect in the laboratory. Salivary samples were immediately removed and a new sample was requested. The time between sputum collection and analysis was recorded. A 20–30 mm smear was made on a new slide with a wooden applicator. After heat fixation, hot Ziehl-Neelsen (ZN) staining was performed: carbol fuchsin 0.3%, slow heating until steaming, rinsing after 5 min, destaining with 25% sulfuric acid, rinsing, counterstaining with methylene blue 0.3% for 1 min, rinsing and drying. The remainder of the specimen was concentrated following the bleach method, as described by Gebre et al.17 A solution of 5% NaOCl was prepared weekly by dilution in distilled water of household bleach manufactured in Thailand (6% NaOCl) and added to an equal volume in the sputum container. The mixture was homogenised by shaking and then incubated for 15 min at room temperature. A volume of 2–15 ml was transferred to a disposable plastic conical tube with an equal volume of distilled water. After centrifugation at 2000 rpm for 15 min, a drop of the pellet was transferred onto a slide, dried, heat-fixed and stained as the first smear.

Microscopic examination was performed by two experienced technicians for 20 min per smear for 200 high-power microscopic fields (HPF). The results were expressed as per the quantitative scale of The Union.18

Quality control

The anonymous slides were coded with numbers carried in a notebook kept by the investigator. All the smears, regardless of their method of preparation, were read separately by the two microscopists. After unblinding and comparison, results with agreement were validated. Discordant results were read a third time by the two technicians to reach a consensus. The concentration of the NaOCl solution was checked to 4.75% by the iodometric method.

RESULTS

In present study the 100 patients enrolled, the male/female ratio was 1.32, the mean age was 57 years (range 10–85), and 92% of patients were sampled for TB case detection. Only one patient had known HIV infection [Table 1].

Table 1: Demographic profile of patients

Demographic profile	Number	Percentage
Sex		
Male	57	57%
Female	43	43%
Age Groups		
10-20 yrs	7	7%
21-40 yrs	28	28%
41-60 yrs	30	30%
>60 yrs	35	35%
Case findings (TB)	92	92%
HIV positive case	1	1%

Overall, AFB were detected on 44 smears prepared by the direct method (12.3%) and 55 smears prepared by the bleach method (16.0%), a statistically significant difference ($P = 0.0006$), giving an increase in positivity. The semi-quantitative results highlighted a significant gain in positivity ($P < 0.001$) with the bleach method.

Positivity rates were higher using the bleach method, regardless of study site, the aspect of the sputum and the delay between sampling and analysis.

All TB patients detected by the direct method were also detected by the bleach method. Regarding the only HIV-positive patient enrolled in this study, nine serial sputum samples were tested over an 8-day period. Only one was AFB-positive by the direct method vs. six by the bleach method, providing a positive result 1 week earlier.

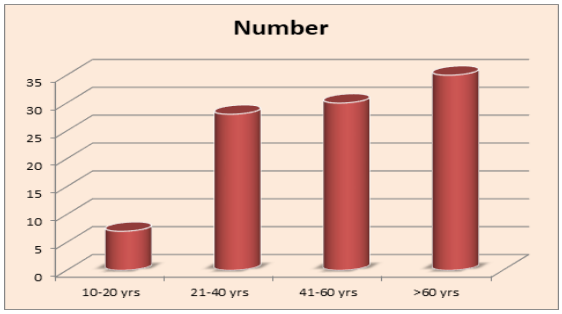

Figure 1: Age wise distribution of patients.

Table 2: Distribution of results obtained on each sample by the direct method and the bleach method according to the semi-quantitative scale of The Union.

	Direct methods						P-value
	Negative	±	+	++	+++	Total	
Bleach method negative *	300	0	0	0	0	300	<0.0001
±	9	2	0	0	0	11	
+	5	5	0	0	0	10	
++	0	0	3	0	2	5	
+++	0	1	7	7	17	32	
Total	314	8	10	7	19	358	

DISCUSSION

The bleach method was compared to the direct method on a large number of sputum samples from two different sites, one central and one peripheral. It can be introduced into routine laboratories after a brief training period for technicians responsible for AFB sputum smear microscopy.

The bleach method increases the effectiveness of TB case finding, detecting 11 patients who were not detected by the direct method. It may also reduce delays in diagnosis and treatment; for two patients with serial sputum samples, including one with HIV co-infection, the bleach method provided positive results respectively 5 and 7 days before the direct method.

In the absence of a gold standard, i.e., mycobacterial culture, it was not possible to compare the sensitivity and the specificity of the two methods. Furthermore, the prevalence of HIV co-infection among the patients enrolled was too small to assess the benefits of the method in people living with HIV/AIDS.

Several methodological parameters may explain such a wide range: the target population; the numbers of patients enrolled and samples collected; whether the comparison was performed on patients or on individual samples; the exclusion of salivary samples; the source, preparation and conservation of the NaOCl solution; the incubation time; the power and duration of centrifugation (or duration of sedimentation); blinded reading; the minimum duration of smear reading; and the positivity criteria.[19]

Although the often-mentioned lack of standardization and quality assurance are unacceptable defects in biological analysis, it is difficult to demand the same level of performance in a well-equipped central laboratory and a poorly equipped peripheral laboratory. Bleach digestion followed by centrifugation is a rustic method. If specific technical adjustments have been made in some centres, they are not likely to affect the results if the key parameters are met: 2–5% NaOCl concentration of the bleach solution, incubation at room temperature for 15 min and 15–30 min centrifugation. As the latter does not require high speed, it can be replaced by sedimentation for 12–18 h.[20]

Centrifugation is often seen as a major obstacle because of the cost of the initial investment and consumables, the fact that power cuts are frequent and the risk of reusing disposable tubes,[21] which is why some centres prefer sedimentation.[9] Quality assurance of sputum microscopy is a mandatory component of any NTP.[18] Implementing the bleach method requires training in situ, which provides an excellent opportunity to strengthen the procedures in all laboratories participating in the NTP.[22,23]

CONCLUSION

Our study, conducted in a population with a low prevalence of HIV/AIDS, confirms the benefits of the bleach method for the microscopic case detection of pulmonary TB. This rustic, simple and inexpensive method could easily be integrated into the routine of a peripheral laboratory after a short training period and strengthening of quality assurance.

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