Evaluating Different Counter Stains in Fluorescent Staining Technique for Detecting Acid Fast Bacilli: Best Amongst The Better

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Tuberculosis is a global emergency needing an early and accurate diagnosis for management and control. Smear microscopy is a rapid diagnostic method for detecting acid fast bacilli (AFB) and improvising this method would contribute in better detection rates. The present study compares the efficacy of various counter stains used in Auramine O staining method in examining sputum smears under fluorescence microscope. This was a prospective study conducted in the Department of Microbiology from January to March, 2017. 102 sputum specimens were collected during this period from patients clinically suspected with pulmonary tuberculosis. Five smears were prepared from each specimen, where each smear was stained with Auramine O staining technique using five different counter stains, 0.1% potassium permanganate, 0.1% methylene blue, 0.1% toluidine blue, 0.1% malachite green and undiluted blue ink. Among the 102 sputum specimens, 50(49.01%), 50(49.01%), 49(48.03%), 44(43.13%) and 48(47.05%) smears showed positive results using potassium permanganate, methylene blue, toluidine blue, malachite green and blue ink respectively. On comparing the degree of fluorescence exhibited by debris in these smears, using potassium permanganate, methylene blue, toluidine blue, malachite green and blue ink, fluorescence was observed in 22(21.6%), 11(10.8%), 46(45.1%), 0(0%) and 31(30.4%) smears respectively. When the counter stains were analyzed, there was a statistically significant difference for debris getting fluoresced (p<0.001). In conclusion, methylene blue as a counter stain has almost equivalent effect compared to routinely used potassium permanganate. It has shown to have the best sensitivity and specificity, reduced debris fluorescence and better contrast in appreciating AFB.

Keywords: Tuberculosis, Acid Fast Bacilli, Light-Emitting Diode,
Fluorescence Microscope, Counter stain.

Tuberculosis (TB) is one of the deadliest menace of the developing world[1]. In 1993, World Health Organization (WHO) declared Tuberculosis as a Global Emergency[2]. Accounting to the depredations caused by the disease, especially over the last several hundred years, it has earned the epithet, 'The captain of all these men of death'. As reported by WHO in the year 2016, 10.4 million people around the world were infected with TB and 1.7 million cases attributed to TB related deaths, 95% cases from low and middle income countries.

64% of new cases have been reported from high burden countries like India, Indonesia, China, Philippines, Pakisthan, Nigeria and South Africa, with India taking a lead in the number of cases[3].

Tuberculosis control aims at reducing the transmission rate thereby decreasing the number of cases, which eventually lessens morbidity and mortality[4]. Accurate and efficient diagnosis of infection plays a significant role in managing the disease burden. Despite the recent development of more sensitive technologies, most of the developing countries still continue to rely on sputum smear microscopy which is an uncomplicated, economical and accessible tool for

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pulmonary TB diagnosis and monitoring treatment in positive cases[5]. Recently, WHO has endorsed XpertMTB RIF assay as a frontline testing for TB replacing smear microscopy. However, this endorsement is less implemented in most of the resource limited countries owing to high cost involved. Hence, replacement of smear microscopy with any other testing method is not likely in the near future[6]. Detecting one to two AFB in a smear is suggestive of a positive result[7].

Two microscopic systems are used in demonstrating AFB in sputum smears to diagnose pulmonary tuberculosis, Bright field or Ordinary Microscopy and Fluorescence Microscopy[8]. Bright field microscopy uses carbol fuschin method which involves Ziehl-Neelsen technique and Fluorescence Microscopy comprises fluorochrome procedure using Auramine O or auramine-rhodamine stain. A standardized technique of fluorescence staining method was recommended by the International Union against Tuberculosis and Lung Disease (IUATLD) in 1978. Fluorescence staining basically utilizes the same approach as Ziehl-Neelsen's staining, but carbol fuschin is replaced by a fluorescence dye (Auramine O, auramine-rhodamine). Fluorescence microscopy has an added advantage of the stained slides getting examined at a lower magnification, thus allowing the examination of a larger area in a shorter duration. If time consumed in screening an area is 10 minutes under a light microscope, the same would be achieved in 2 minutes under a fluorescent microscope[8]. In 2011, WHO declared a new policy on Light -Emitting Diode (LED) based Fluorescence Microscopy (FM) for diagnosing TB where it recommended a phased approach to change from bright field microscopy to LED based FM across the microscopy network.

The present study was conducted with an objective of comparing the efficacy of various counter stains in Auramine O staining method enabling better detection of acid fast bacilli in sputum smears.

MATERIALS AND METHODS

This prospective study was conducted for a period of three months from January 2017 to March 2017 in the Department of Microbiology, Kasturba Medical College, Manipal, a tertiary care teaching hospital of coastal Karnataka. The study was reviewed and approved by the Institutional Ethics Committee, Kasturba Hospital, Manipal, Karnataka. The clinical samples included in the study were anonymized and the confidential handling of data by staff was followed.

Preparation of sputum smear

Sputum samples were collected from clinically suspected cases of pulmonary tuberculosis. Five smears were prepared from each specimen. The smears were prepared on grease free glass slides, air dried and heat fixed for the staining procedure.

Reagents used in Auramine O staining technique

The different reagents used in Auramine O staining technique were 0.3% Auramine O , 1% acid alcohol as decolorizer and various counter stains like 0.1% potassium permanganate, 0.1% methylene blue, 0.1% toluidine blue, 0.1% malachite green and undiluted blue ink[9].

Procedure of Auramine O staining technique

Sputum smears were stained as per Revised National Tuberculosis Control Programme (RNTCP) guidelines[9]. Slides were stained with freshly prepared 0.3% auramine-phenol for 10 minutes and were washed in running tap water. Later, the smears were decolorized with 1% acid alcohol for two minutes following which they were again washed under running tap water. Counterstaining was performed using five different stains (potassium permanganate, methylene blue, toluidine blue, malachite green and blue ink) for 45 seconds, each smear being stained by a different dye.

Observation of results

The stained smears were observed under Light-Emitting Diode Fluorescence Microscope (Zeiss Primo star): Transmitted light microscope of compact design, for the presence of AFB and grading of positive smears was done as per standard guidelines[9]. The efficacy of various counter stains in Auramine O staining technique was evaluated.

Confirmation of positive results using Ziehl-Neelsen's staining method

The positive result for the presence of AFB was confirmed using ZN technique which includes strong carbol fuschin as primary stain, 20% sulphuric acid as decolorizer and 0.1% methylene blue as counter stain[7].

RESULTS

Among the 102 sputum samples evaluated for the presence of AFB, positivity shown was almost similar using five different counter stains. Potassium permanganate and methylene blue showed maximum positivity (49.01%) while malachite green showed the least (43.13%) (Table 1). The sensitivity and specificity of potassium permanganate and methylene blue was 100% as there were no false positive or false negative results whereas toluidine blue, malachite green and blue ink showed few false negative results, malachite green with least sensitivity (88%) (Table 2). Quantification of positive smears with grading 3+, 2+, 1+ and scanty showed comparable results with potassium permanganate, methylene blue, toluidine blue and blue ink. However, using malachite green, the number of bacilli detected in smears were comparatively lesser showing decrease in the number of smears as they were graded from scanty to 3+ (Table 3).

Smears were also evaluated for the amount of fluorescence taken up by debris which is depicted in Table 4. Methylene blue counter stained smears showed lesser fluorescence of debris (10.8%) compared to potassium permanganate, toluidine blue and blue ink (21.6%, 45.1%, and 30.4%). Total absence of debris fluorescence in smears counter stained with malachite green was observed because of the dark background. Comparing the stains in terms of quenching debris fluorescence, Cochran's Q test indicated a statistically significant difference in the proportion of debris fluorescence (p<0.001). However, paired comparison between potassium permanganate and methylene blue showed no significant difference.

DISCUSSION

Tuberculosis is known to be one of the deadliest infectious disease claiming millions of lives. It is a major public health threat, and its control has become a challenge in developing countries, including India. Early and accurate diagnosis is the mainstay in curbing this disease and several techniques have been devised as diagnostic modalities. As this infection mainly targets resource poor settings, implementing a rapid, accurate and cost effective method becomes very important[10].

Microscopic examination of sputum samples remains significant for the rapid presumptive diagnosis of tuberculosis because of slow growth of *Mycobacterium tuberculosis* in culture. The early diagnosis of active tuberculosis still depends on the presence of AFB in stained sputum smears[11]. This study highlights on improvising the already established Auramine O staining method with minor modifications in counter staining and evaluating the efficacy of these counter stains. This would contribute in developing better fluorescent staining method in diagnosing AFB in sputum smears.

Standard fluorescence staining method using Auramine O stain was introduced in 1978. Currently, several laboratories with a privilege of fluorescent microscope have implemented this method for rapid screening of clinical specimens for the presence of mycobacteria. Fluorescence microscopy has been proved to be at least 10% more sensitive than traditional light microscopy by various studies conducted[12] and can be beneficial in paucibacillary specimens. Time required to read a fluorescent stained smear is significantly lesser than smears stained with conventional methods[8].

The first fluorescence staining technique performed by Hagemann in 1938 did not comprise a counterstain[13]. Without the counterstain, the background debris retained the primary fluorescence stain, making it difficult to differentiate the bacilli from the debris resulting in false positive results. Thus, incorporating counter stains in the staining technique is very essential. Routinely used counter stain presently is potassium permanganate ($KMnO_4$) which aids in bright fluorescence of AFB and is a good quencher of artefact fluorescence[14].

In the present study, we compared the efficacy of five different counter stains, all of them in a concentration of 0.1%, in Auramine O method using Light-Emitting Diode Fluorescence Microscopy. Positive cases detected was almost similar except malachite green which showed less number of positive smears. Statistically, there was no significant difference for the positive and negative results. Results obtained with each counter stain was compared with the routinely used potassium permanganate.

The specificity and sensitivity of $KMnO_4$ counter stained smears was 100% but the debris also getting fluoresced was a drawback. This

was unlike the study conducted in 2010 where they reported good quenching activity but the background was too dark to visualize AFB[14]. Toluidine blue had good contrasting power but the debris got fluoresced to the maximum making it difficult to differentiate between the bacilli and the debris. When malachite green was used for counter staining, the background appeared too dark to appreciate fluorescence of the bacilli, altering the results and the grades for positive smears making it an inefficient counter stain. Blue ink used, had a good contrasting power but the bacilli failed to take up good fluorescence. Methylene blue as counter stain showed 100% specificity and sensitivity with good contrasting power, the debris showing comparatively less fluorescence. All these features highlight the significance of using methylene blue as counter stain compared to routinely used KMnO$_4$.

Van Deun A et al (2010) in their study comparing the background staining property of blue ink, potassium permanganate and methylene blue have reported that methylene blue had the best sensitivity of 95.6% compared to the other two. This was concordant with the finding of our study where methylene blue showed sensitivity of 100%. Also, they have concluded that in their study methylene blue was better than potassium permanganate and blue ink as a counter stain, with added advantages of availability and cost[14]. In another study conducted in 1995, comparing acridine orange and Auramine O as primary stains, the importance of methylene blue component in the destaining reagent has been highlighted, which provided reduced background fluorescence and better contrast making AFB easily visible[15]. In our study methylene blue stain showed comparable results with potassium permanganate, toluidine blue and blue ink with regard to positivity and quantification of smears but showed better result in quenching debris fluorescence.

Table 1. Frequency of positive and negative results from sputum specimens

Counter stain	Positive results (%)	Negative results (%)
Potassium permanganate	50 (49.01)	52 (50.99)
Methylene blue	50 (49.01)	52 (50.99)
Toluidine blue	49 (48.03)	53 (51.97)
Malachite green	44 (43.13)	58 (56.87)
Blue ink	48 (47.05)	54 (52.95)

Table 2. Sensitivity and specificity of five different counter stains

Counter stain	True positive	False positive	True negative	False negative	Sensitivity (%)	Specificity (%)
Potassium permanganate	50	0	52	0	100	100
Methylene blue	50	0	52	0	100	100
Toluidine blue	49	0	52	1	98	100
Malachite green	44	0	52	6	88	100
Blue ink	48	0	52	2	96	100

Table 3. Comparison of counter stains in grading the positive smears

Grade	Potassium permanganate	Methylene blue	Toluidine blue	Malachite green	Blue ink
Scanty	14	15	15	17	14
1+	9	6	6	11	7
2+	10	12	12	15	11
3+	17	17	16	1	16
Negative	52	52	53	58	54

Table 4. Frequency of debris fluorescence using
different counter stains

Counter stain	Number of smears showing debris fluorescence (%)
Potassium permanganate	22 (21.6)
Methylene blue	11 (10.8)
Toluidine blue	46 (45.1)
Malachite green	0
Blue ink	31 (30.4)

CONCLUSION

Methylene blue stain with best sensitivity and specificity, improved background clearance and good contrast, enabling better detection of AFB can be a better substitute for potassium permanganate as counter stain considering the availability and cost involved.

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