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Evaluation of Rapid AFB Cold (RAC) Staining Method for Sputum Smears Treated with Bleach Ammonium Sulphate

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In the present study, a total of 570 smears were prepared from 190 sputum samples treated with 5% Bleach ammonium sulphate(BAS), which were stained using Rapid – AFB Cold (RAC) and Hot Ziehl – Neelsen (HZN) staining methods. With Direct control (DC) used as gold standard, RAC smear microscopy demonstrated a sensitivity and specificity of 78% and 95% respectively, compared to 79% and 92% respectively for the HZN method. Comparison of smear results obtained from RAC and HZN methods were statistically highly significant (P < 0.0001). RAC staining method was found to be a suitable alternative to the HZN staining method in diagnosing Tuberculosis (TB) by smear microscopy in peripheral TB centers.

Key words: Cold stain, Ziehl- Neelsen stain, Bleach Ammonium Sulphate, Tuberculosis.

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is an infectious disease that has plagued humans, since the Neolithic times. Physicians in ancient Greece called this illness "phthisis" to reflect its wasting character. In more recent times, TB has been called "consumption". Koch in 1882 demonstrated the tubercle bacilli as a mold like pellicle in liquid cultures and used a dye that was capable of staining the mycolic cell wall of the tubercle bacilli. He employed three important factors in staining of tubercle bacilli namely the use of mordant, heating and decolorization. Gokhale *et al.,* (1990) and Chandrasekaran *et al.,* (1991) demonstrated that Ziehi-Neelsen (ZN) method is still the most extensively used procedure for AFB staining. However, in the developing countries, its applicability at peripheral centre's in outlying areas appears to pose operational problems due to short supply or non-availability of alcohol or spirit for heat fixing, heating and decolourisation steps. Hence, it has become necessary to look for procedures where the use of alcohol could be avoided completely.

Noack (1976) studied the cold staining of acid fast bacilli and found that it is qualitatively and quantitatively better than the conventional ZN method and was found to be suitable under field conditions. Kochhar (2002) suggested that the simplified concentration methods using cold stain technique are suitable alternative for demonstration of mycobacteria in busy clinical laboratories. Hence, the present study evaluates the alternate Rapid AFB cold (RAC) with conventional Hot Ziehl Neelsen (HZN) stain for staining smears prepared from sputum samples concentrated by 5% Bleach Ammonium Sulphate (BAS).

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MATERIAL AND METHODS

5% Bleach Ammonium Sulphate (BAS)

The BAS reagent was prepared by dissolving 5 grams of bleaching powder (Saceanu, *et al.,* 1993) and 4 grams of Ammonium sulphate (E.Merc, Mumbai, India) in 100 ml of distilled water (Selvakumar *et al.,* 2002; Vasanthakumari R, 1988). It was prepared fresh everytime (Chandrasekar *et al.,* 2008).

A total of 190 sputum samples were collected from the Institute of Thoracic Medicine and Tuberculosis Hospital, Chetpet, Chennai as per the guidelines (Sherafin Jancy Vincy *et al.,* 2007). A Direct control (DC) smear was taken from each collected sputum sample. All smears were prepared by spreading about 0.01 ml of sputum over a 2 cm^2 area on a clean glass slide using a 5 mm diameter wire loop (Hi media loop), the smear preparations was carried out in laminar air flow chamber. Then, the smears were air dried and heat fixed by passing the slides through the blue cone of the spirit lamp flame, stained by conventional HZN stain and stored in cardboard box. The remaining sputum sample was treated with 5% BAS.

Sputum Digestion

An equal amount of 5% BAS reagent was added to the remaining sputum sample in container and was kept overnight for sedimentation of the bacilli (Hakan Miorner, *et al*, 1996). Then the supernatant was discarded, sputum deposits were vortexed for 5 minutes and two smears were prepared. One smear was stained with HZN and the other with RAC stain.

Hot Ziehl – Neelsen (HZN) Stain

The HZN staining was performed by using staining kit from Hi Media Laboratories Pvt. Ltd. Mumbai. BAS deposit smears were flooded with filtered 1% carbol fuchsin, heated until steaming and left for 5 minutes. Then, the slides were gently rinsed with the tap water to remove the excess carbon fuchsin and tilted to drain off excess water. Decolorizer was added for 2-4 minutes, till the red color was almost completely disappeared from the smear. The slides were rinsed as above, making sure that the smear itself was not washed away and the slides were tilted to drain off the water. Methylene blue was added for 30 seconds, and gently rinsed as above. Then the slides were washed, air dried and examined (Smithwick, 1976; Selvakumar *et al.,* 2002).

Rapid-AFB Cold Staining

The Rapid AFB cold staining was performed by using staining kit from Bio Lab Diagnostics Co Pvt Ltd, Boisar. Reagent 1(Red Stain) was added to BAS deposit smears and allowed to stand for 4-5 minutes, then washed for a minute and was tilted to drain off the water. Reagent 2 (Green Stain) was added exactly for 1 minute, then the slides were washed, air dried and examined (Selvakumar *et al.,* 2002).

Examination of Smear

A total of 570 stained smears (190 DC +190 HZN+190 RAC) were coded according to International guidelines by senior technologist. Two trained technologists independently graded the coded slides without any bias. Quantitative screening of each slide was done by counting the number of bacilli for 100 fields and recorded in standardized forms containing 100 boxes. Screening was performed using oil immersion objective of binocular light microscope (Magnus MLX-BI, Olympus, India). Immersion oil from Merck Specialties Private Ltd., Mumbai was used. If no bacilli were observed in 100 fields, an additional 200 fields were examined before recording the result as negative for Acid Fast Bacilli (AFB). If AFB were detected, the slides were recorded as positive and the numbers of organisms present were classified using standard criteria for quantifying AFB (Nguyen, *et al.,* 1999).

Statistical Analysis

The DC results from the Institute of Thoracic Medicine and Tuberculosis Hospital, Chennai were considered gold standard. The diagnostic accuracy and reliability of the tests compared to the gold standard were assessed by their sensitivities, specificities and 95% confidence intervals. Mc Nemar's test was performed to determine the statistical significance of the difference observed between the smear results.

RESULTS AND DISCUSSION

The smear results for 570 smears (190 DC +190 HZN+190 RAC) were presented in two-way table (Table 1 and 2). With DC used as gold

standard, RAC smear microscopy demonstrated a sensitivity and specificity of 78% and 95% respectively, compared to 79% and 92% respectively for the HZN method. Comparison of smear results obtained from RAC and HZN methods were statistically highly significant ($P < 0.0001$).

Out of 190 slides, 3 were found to be 3+, 1 was 2+, 14 were 1+, 1 was scanty and 136 were negative for both DC & HZN methods (Table 1). Out of 190 slides, 3 were found to be 3+, 1 was 2+, 16 were 1+, 3 were scanty and 137 were negative for both DC and RAC methods (Table 1). Out of 190 slides, 4 were found to be 3+, 2 were 2+, 18 were 1+, 1 was scanty and 138 were negative for both HZN and RAC methods (Table 2).

Liquefaction and concentration of sputum by bleach before ZN staining improves yield and also makes examination of smears rapid and convenient. It is inexpensive, easily available in hospitals; additional advantage is that as a

Table 1. Comparison of Rapid- AFB cold (RAC) stained smear and Hot Ziehl –Neelsen (HZN) stained smear results with Direct control (DC) smear results

Smear Methods		Direct Control						
**HZN Stain	*Smear Results	3+	2+	1+	Scanty	Any Positive	Negative	Total
	3+	3	-	-	-	3	-	3
	2+	-	1	1	1	3	-	3
	1+	1	1	14	3	19	5	24
	Scanty	-	-	7	1	8	7	15
	Any Positive	4	2	22	5	33	12	45
	Negative	-	-	5	4	9	136	145
	Total	4	2	27	9	42	148	190
***RAC Stain	3+	3	-	1	-	4	-	4
	2+	-	1	1	1	3	-	3
	1+	-	1	16	4	21	1	22
	Scanty	-	-	5	3	8	6	14
	Any Positive	3	2	23	8	36	7	43
	Negative	-	-	3	7	10	137	147
	Total	3	2	26	15	46	144	190

Table 2. Comparison of Rapid- AFB cold (RAC) stained smear with Hot Ziehl -Neelsen stained smear results

Smear Methods		****RAC Stain						
	*Smear Results	3+	2+	1+	Scanty	Any Positive	Negative	Total
	3+	4	-	-	-	4	-	4
	2+	-	2	-	-	2	-	2
	1+	-	1	18	5	24	3	27
****HZN Stain	Scanty	-	-	2	1	3	6	9
	Any Positive	4	3	20	6	33	9	42
	Negative	-	-	1	9	10	138	148
	Total	4	3	21	15	43	147	190

*Smear results: 3+, more than 10 AFB per oil immersion field in at least 20 fields; 2+, 2 to 10 AFB per oil immersion field in at least 50 fields; 1+, 1 to 99 AFB in 100 oil immersion fields; scanty, 1 to 9 AFB in 100 oil immersion fields.
**For the HZN stained smear reading versus Direct control (DC) smear reading, sensitivity was 79%, and specificity was 92%.
*** For the RAC stained smear reading versus Direct control (DC) smear reading, sensitivity was 78%, and specificity was 95%.
****For the RAC stained smear reading versus HZN smear reading, sensitivity was 77%, and specificity was 94% ($P < 0.0001$).

disinfectant it reduces the risk of laboratory acquired infections and its application has been proved in the diagnosis of pulmonary TB (Saxena *et al.,* 2001; Khubani and Munjal 2005; Aung *et al.,* 2001).

The implementation of bleach method can be a useful contribution in the National TB control program (Mutha *et al.,* 2005). It is recommended for implementation to enable rapid and sensitive laboratory diagnosis of pulmonary TB, especially in resource – poor settings, where culture is not possible (Angeby *et al.,* 2000) and for use in routine laboratory in developing countries (Gebre, 2003).

The alternate RAC method is qualitatively and quantitatively better than the conventional HZN method. In a similar study done by Selvakumar *et al.,* 2002 showed that the two – reagent cold staining method is sensitive and specific as the ZN method. Deep to pale red colored bacilli with dark bluish green background and bold pink bacilli with blue cells background were observed when stained with alternative RAC and conventional HZN methods respectively. New red bacilli with dark bluish green background gives a relaxation to technicians eyes from the usual pink bacilli with blue background in busy laboratories. The RAC staining method includes only two steps, thus removes all associated problems such as, usage of sprit, cotton, etc. The time taken for staining is considerably reduced, but the overall time for complete procedure is increased due to sputum treatment with 5% BAS which requires overnight sedimentation.

This study proves that RAC method is as efficient as HZN method, when the sputum samples were treated with 5% BAS for the diagnosis of pulmonary TB. Reasons stated for preferring the RAC method by the two technologists included longer shelf life, ready made reagents and ease of processing. It can be supplied to peripheral laboratories in programme conditions.

Hence, RAC staining method was found to be a suitable alternative to the conventional HZN staining method. It can be recommended for the preparation of sputum slides for the diagnosis of pulmonary TB in peripheral laboratories where there is poor laboratory settings. However, evaluation of RAC staining method for other concentration techniques such as PhAS, NALC and NaOH need to be conducted to assess its efficacy in the diagnosis of pulmonary TB.

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