Comparison of ZN Stain (RNTCP) Versus Fluorescent Microscopy and Modification of Cold Stain to Detect Acid Fast Bacilli from Sputum Sample

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ABSTRACT

Introduction: Microbiological diagnosis is the crux for the effective treatment of pulmonary TB (PTB). The search for rapid and efficient method has resulted in several modification of ZN stain. With this background this study was planned to compare the results of Revised National Tuberculosis Control Programme (RNTCP) advocated method of ZN stain with modified cold method using 7.5%phenol, 0.3% CF as recommended by WHO and IUATLD and 20% sulfuric acid as decolorizer. Objective of the research was to compare the results of ZN stain (RNTCP) with the modified cold method, Compare the results of ZN stain (RNTCP) with Fluorescent microscopy.

Material and methods: A prospective interventional study was carried using 266 sputum samples received at DMC. All the 266 samples were subjected to three methods- ZN stain modified cold stain and FM. They were compared for sensitivity and specificity in terms of qualitative results and grades of smear as recommended by RNTCP.

Results: The smear positivity rate was 27.65%, 24.90% and 27.44% in Ziehl-Neelsen stain, modified cold stain and fluorescent microscopy respectively. Compared to ZN stain the proposed modified cold method has sensitivity of 90.41% and specificity of 100%. The concordance of qualitative result between ZN and modified cold method was 97.34%. Comparison of RNTCP grades of smear with ZN and modified cold stain has Kappa with Linear Weighting as 0.877 and Kappa with Quadratic Weighting as 0.877.

Conclusion: Modified cold method cannot replace the ZN stain. However as it is economical and safe, can be adopted for training medical and paramedical students

Keywords: Acid fast bacilli, Fluorescent microscopy, *Mycobacterium tuberculosis,* Sputum smear, Ziehl-Neelsen stain

INTRODUCTION

Tuberculosis is caused by *Mycobacterium tuberculosis* complex. It is the most common disease affecting the low socio-economic group in developing countries like India. World Health Organization (WHO) statistics as of 2011 estimates gives global incidence of 8.7 million cases, about 2.2 million from India. About 40% of Indian population is infected with tuberculosis.[1]

Tuberculosis commonly affects lungs but can also be extra-pulmonary. Hence microscopic examination of sputum for detection of Acid Fast Bacilli (AFB) is of utmost importance. Early detection can prevent further complication. As per WHO or Revised National Tuberculosis Control Programme (RNTCP), an individual with at least one sputum smear positive for AFB or culture positive for tubercle bacilli is labeled to be suffering from Pulmonary Tuberculosis (PTB). In developing countries like India with deficit of resources compared to high TB burden, culture facility is not adequately available. Hence most of the TB cases are diagnosed based on Sputum Smear Microscopy (SSM).

The search for rapid and efficient method has resulted in several modification of Ziehl-Neelsen stain (ZN). Microbiological diagnosis is crucial for the effective and prompt treatment of pulmonary TB (PTB).[2] Diagnosis of pulmonary tuberculosis by demonstration of Acid Fast Bacilli (AFB) in sputum smears by ZN staining is known to be economical and less time consuming. The decolorizing agent used is 25% H_2SO_4 in the ZN staining. But in modified cold stain we propose to use 20% H_2SO_4 and heating of slide is not done. The limitations for ZN stain use in remote area are i) Shortage of trained and experienced technician. ii) Availability of spirit for heating. iii) It may be dangerous to use 25% of acid and fire. Recently under RNTCP some of designated Microscopy Centre (DMC) use only Fluorescent microscopy for screening, cross checking only the positive slides by ZN stain.

Revised National Tuberculosis Control Programme (RNTCP) guidelines[3] recommend use of 1% basic fuchsin (BF) in ZN staining. Guidelines of World Health Organization (WHO)[4] and International Union against Tuberculosis and Lung Disease (IUATLD)[5] advocates use of 0.3% Carbol fuchsin (CF) as primary staining reagent.

Selvakumar et al. showed that use of 0.3% of BF may result in 20% smear positive patients being missed.[6] The reduced smear positive result was found to be due to lowered concentration of phenol to~1.7%.[7] But in the standard ZN staining the phenol concentration is 5% and CF 1%.[3]

Reducing the concentration of phenol automatically reduces the smear positivity.[7] In another study, there was a significant fall in sputum smear positivity when the concentration of phenol was 7.5% and BF0.1%.[8]

The standard text books of microbiology advocate the use of acid-alcohol mixture or use of 20% sulfuric acid.[9,10] In a study of the 171 smears that were positive in the ZN stain (RNTCP) only 2 were negative in the cold method using

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Gabbett's methylene blue containing 20% sulfuric acid and phenol and Carbol fuchsin same as in ZN stain, indicating almost 100% correlation between the two methods. There was no difference in the intensity of staining in both methods.[11] Now RNTCP has brought in Fluorescent microscopy for screening, cross checking only the positive slides by ZN stain, time may not be a constraint. But availability of spirit as fuel for hot method maybe a limiting factor in some centres and it may be dangerous to use acid (25%) and fire. With this back ground this study is planned to compare the results of RNTCP advocated method of ZN stain with modified cold method using7.5% phenol, 0.3% CF as recommended by WHO and IUATLD and 20% sulfuric acid as decolorizer.

MATERIAL AND METHODS

A prospective interventional study was undertaken with permission from state tuberculosis officer. Institutional ethical committee clearance obtained. We could collect 266 samples for our study. All the sputum samples were received at Designated Microscopy Centre [DMC] at Government tertiary care hospital during the study period.

Inclusion criteria: All adults of both the gender suspected to be a case of pulmonary tuberculosis as per RNTCP guidelines

Exclusion criteria: Samples other than sputum, Samples macroscopically resembling saliva
Sample size: 266 (The average number of sputum samples received at our DMC is 150 per month, since ICMR short term project study period is 2 months)

Sample collection method
All sputum samples received for fluorescent microscopy at the DMC were screened for presence of Acid Fast Bacilli (AFB) by ZN stain (RNTCP) and modified cold method as described below. All the smears were prepared by the same, trained RNTCP technician thereby ensuring quality of smear. All smears were examined microscopically by using an oil-immersion microscope. Routine Fluorescent microscopy results at Designated Microscopy Centre of our hospital as recorded by the trained RNTCP technician were recorded. All positive slides and 20% of the negative slides at random were reviewed by the microbiologist to eliminate any error in screening.
ZN staining: Heat-fixed smear on glass slides was flooded with filtered CF and heated until steaming, left for five minutes. After rinsing the slides with stream of water, 25% Sulphuric acid is used to decolorize the smears for 2 to 3 minutes. The slides rinsed as above and counter stained with 0.1% methylene blue for 30 seconds.The slides are washed, air dried before examination under a oil immersion of binocular microscope.[3]
Modified Cold method: Heat fixed smears on microscope slide flooded with solution B (contains 0.3% BF[6] and 7.5% phenol[8] and allowed to stand at room temperature for 10 minutes. The smears were then washed in running water, counterstained with 0.1% methylene blue for 2 minutes, and subsequently washed in tap water and air-dried.
Grading of smears: The smears were graded using 100x oil immersion objective as per RNTCP guidelines.[3] Scanty = 1-9 AFB in100 oil immersion fields; Grade 1+ = 10-99 AFB in 100 oil immersion fields; Grade 2+ = per oil immersion field an atleast 50 fields.
field in at least 50 fields; Grade 3+= 10 or more AFB per field in at least 20fields; Negative = no AFB in 100 fields.

STATISTICAL ANALYSIS

Data collected were analysed using SPSS software, kappa value and p value were calculated.

RESULTS

A total of 266 sputum smear samples received at the Designated Microscopy Centre were subjected to Ziehl-Neelsen stain, modified cold stain and fluorescent microscopy during the study period of two months. Two smears stained with ZN stain and one smear stained by modified cold method were of poor quality hence not included in the study. Comparison of the qualitative result of the above three microscopic methods is depicted in table1.The smear positivity rate is 27.65%, 24.90% and 27.44% in Ziehl-Neelsen stain, modified cold stain and fluorescent microscopy respectively. Compared to ZN stain the proposed modified cold method has sensitiv-

Staining method / **Smear microscopy result**	**Ziehl Neelsen stain N= 264**	**Modification of cold stain N= 265**	**Fluorescent Microscopy N= 266**
Positive	73	66	73
Negative	191	199	193

Table-1: Comparison of qualitative results of Ziehl- Neelsen method, Modified cold staining method and Fluorescent microscopy.

	ZN stain method							
Modified Cold Stain Method	**Grade of positive smears**	**3+**	**2+**	**1+**	**Scanty**	**Negative**	**Any positive**	**Total**
	3+	11	-	-	-		11	11
	2+	10	12	-	-		22	22
	1+	4	5	14	-		23	23
	Scanty	-	-	4	6		10	10
	Negative	-	-	2	5	191	7	198
	Any positive	25	17	18	6	-	66	66
	Total	25	17	20	11	191	73	264

Table-2: Cross tabulation of Positive smear grades by Ziehl-Neelsen stain and Modified cold stain method

Study	Slide +ve rate by ZN	Slide +ve rate by FM	Sample size
Prasanthi et al[16]	50%	69%	38
Ulukanligil et al[17]	9.89%	12.47%	465
Golia S et a1[1]	10.41%	16.56%	634
Suria et al[19]	12.4%	19.1%	225
Jayachandra et al[8]	9.7%	Not done	196
Our study	27.65%	27.44%	266

Table-3: Comparison of slide positivity rate between ZN and FM in various studies

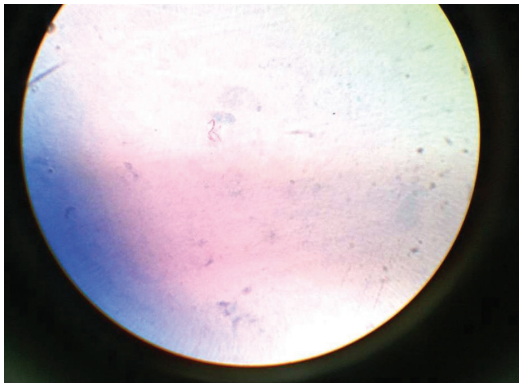

Figure-1: Photomicrograph of Modified cold stain demonstrating Acid Fast Bacilli (1000x) as pink colored, beaded or barred forms, while the tissues cells and other organism are stained blue

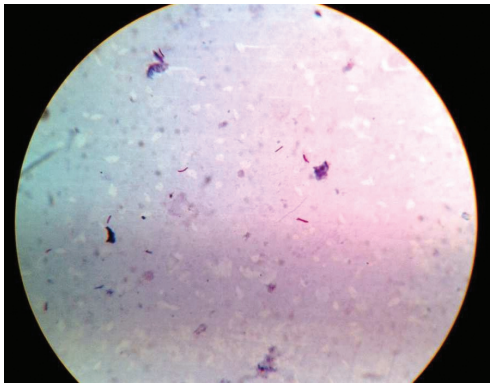

Figure-2: Photo micrograph Ziehl-Neelsen stain of sputum smear showing AFB (1000x) as bright pink colored, beaded or barred forms, while the tissues cells and other organism are stained blue

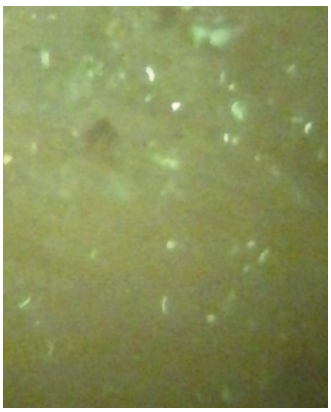

Figure-3: Photomicrograph of Auramie- O stain sputum smear by LED microscopy (400x). The bacilli are seen as yellow luminous organism in a dark field

Comparative statistics of Modified cold method against Ziehl-Neelsen method

Sensitivity=90.41% p value for 0.5 is 0.014019
Specificity=100% for 1.0 is 0.023342
Positive predictive value =100% kappa value smear positivity is 0.877
Negative predictive value = 96.46% kappa value for smear grading is 0.872
False negative = 9.5%
False positive = 0%
Smear positivity rate for ZN = 27.65% & modified cold method = 24.9%

ity of 90.41%, specificity and positive predictive value of 100%, Negative predicted value of 96.46%, p value at 95% confidence interval is 0.014019. Comparison of qualitative results (in terms of slide positive and negative for AFB) of modified cold stain method with ZN stain has kappa value of 0.9317 with 95% confidence limits (0.8818-0.9816, Standard error {SE} 0.0255).The p value is 0.014019.Comparison of RNTCP grades of smear with ZN and modified cold stain has Un weighted Kappa of 0.7417 with 95% confidence limits (0.6547-0.8287, SE0.0444), Kappa with Linear Weighting is 0.877 with 95% confidence limits (0.8373-0.9167, SE0.0202) and Kappa with Quadratic Weighting is 0.877 with 95% confidence limits (0.8373-0.9167). Out of 11 smears graded as scanty by ZN stain, 6 were graded scanty by modified cold method. All these 6smears had bacilli count between 5 to 9/100 oil immersion fields. The remaining 5 smears had bacilli count <5/oil immersion field by ZN stain

DISCUSSION

The slide positivity rate of ZN stained smears was minimally (0.21%) better than fluorescent microscopy (FM). The slide positivity rate of Modified cold method is slightly lower than ZN stain (2.75%) and FM (2.64%). This finding is similar to that seen in other study.[8] The color contrasts of the smears stained by both the methods were visually identical in agreement with other studies.[12]

The specificity of the qualitative result of modified cold method is cent percent against ZN stain. There were no false positives reported from modified cold method. It is well documented in other studies comparing ZN stain with variant cold methods; cold staining method is at least as specific as ZN although falls short of it in terms of sensitivity. The ZN Method is also known to give a few false positive results (Toman1979), perhaps due to the heating step involved in staining.[13] The sensitivity is of 90.41%. The p value obtained is statistically significant. The concordance of qualitative result between ZN stain and modified cold method is 97.34%. In other study employing 2.5% of basic fuchsin the Concordance between ZN and cold stain was 90% (kappa 0.7).[14] Comparison of qualitative results (in terms of slide positive and negative for AFB) of modified cold stain method with ZN stain has kappa value of 0.9317.

Comparison of RNTCP grades of smear with ZN and modified cold stain has Kappa with Linear Weightingas0.877and-KappawithQuadraticWeighting as0.877.According to Landis and Koch's kappa value between 0.81-1.0 is interpreted as perfect agreement.[15]

The smears stained by ZN method can detect bacilli when the concentration of bacilli is 10^5/mL of sputum, whereas a more sensitive staining technique like FM stain detects the bacilli when the bacillary load is 10^4/mL of sputum.[18]

The qualitative result of the proposed modified cold method of staining was found to be in perfect agreement with the ZN stain. But the performance of the proposed modified cold stain method was slightly inferior to the standard ZN stain method by (2.75%). So we propose that modified cold method cannot replace the ZN stain. However as it is economical and safe, can be adopted for training medical and paramedical students.

The qualitative result of FM was found to be slightly inferior to the ZN unlike in other studies. This could be due to the FM and ZN smear being screened by different persons. ZN smear and Modified Cold stain smear was preliminarily screened by the investigator. So there is a possibility of thorough screening. The FM was done by the RNTCP laboratory technician as part of his routine work. No incentive was provided to him. We understand that if sample load at a DMC is low then definitely the performance of ZN stain matches that of FM. Limitations: FM should have been done by the investigator itself but because of time constraints it could not be done.

CONCLUSION

The qualitative result of the proposed modified cold method of staining was found to be in perfect agreement with the ZN stain. But the performance of the proposed modified cold stain method was slightly inferior to the standard ZN stain method by (2.75%). So we propose that modified cold method cannot replace the ZN stain. However as it is economical and safe, can be adopted for training medical and paramedical students. We suggest the performance of modified cold method may be evaluated by extending the time of staining with carbolfuschin in order to not to miss the scanty number of AFB.

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