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Comparative Study of Different Staining Techniques - Ziehlneelsen Stain, Gabbet's Stain, Fluorochrome Stain for Detecting of Mycobacterium Tuberculosis in the Sputum.

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Abstract

Objectives*:*
comparison of results with bright-field and Fluorescence microscopy for detection of acid-fast bacilli (AFB) in sputum.

Methodology*:*
Three smears from 200 consecutive sputum specimens between March 2012 and august 2012 were prepared and stained by the Ziehl-Neelsen, Gabbet's and Fluorescence staining method.

Results*:*
The findings showed that the both Z-N, Gabbet's and Fluorescence staining preparations each showed positive for AFB in 37 (18.5%), 33 (16.5%), 47 (23.5%), respectively. The sensitivities for the Gabbet's and Fluorescence stain were 89.18% and 94.87% respectively. The positive agreement between Z-N, Gabbet's (94.28%) and Z-N and Fluorescence stain (97.69%) were good. The Fluorescence microscopy showed higher sensitivity than Z-N staining in detecting AFB in clinical specimens. Smears were read lower magnification than Z-N smear reading (20-40vs 100x), thus smears were read more quickly and efficiently. Fluorescence microscopy has taken less time than Z-N for smear examination.

Conclusion*:*
The results obtained with one technique are highly reproducible by the others. Two step Gabbet's cold staining method was less time consuming and easier to perform in the field. Fluorochrome microscopy appears to be more likely to detect in tuberculosis than bright-field microscopy, and it more than halves the required examination time.

Keywords*: acid-fast bacilli; auramine-0; fluorescence; microscopy; tuberculosis; Ziehl-Neelsen.*

INTRODUCTION

WHO in 1993 declared, Tuberculosis (TB) as a global emergency that has affecting mankind since early times, it is prevalent in India and one of the leading causes of death. Sputum examination is cornerstone to diagnosis of pulmonary tuberculosis. Sputum microscopy is helpful to assess the response to treatment and to establish cure or failure at the end of treatment .There are two microscopic systems used to demonstrate the acid-fast bacilli(AFB), in the sputum to diagnosis pulmonary tuberculosis :bright field or ordinary microscopy and fluorescence microscopy.

Ziehl-Neelsen (Z-N) Sputum smear microscopy is usually low cost procedure but poses problem in the field. The Z-N staining method is cumbersome because it requires the heat application during the carbol-fuchsin staining resulting necessity for a flame source (1) to eliminating heating step of this technique there have been develop a cold method for demonstration of AFB ,for example Kinyoun used higher concentration of basic fuchsin and phenol (2)While tax thiam Hok devised a method (3) by combining the staining technique of kinyon and Gabbet(4). In the Gabbet's staining method, methylene blue act as decolorizer and counter stain, it has been advocated as an alternative staining technique (1).

A Standardization technique Fluorescence staining method was recommended by the International Union Against Tuberculosis and Lung Disease (IUATLD) in 1978 (5). fluorescence staining, utilizes basically the same approach as Z-N staining, but carbo fuchsin is replaced by a fluorescent dye (auramine-O, rhodamine) The advantages of Fluorescence method is that slides can be examined at lower magnification thus allowing the examination of a much larger area per unit of time. In fluorescence microscopy, the same area that needs examination for 10 min with a light microscope can be examined in 2 min.

The present study compares Gabbet's staining and Fluorochrome methods against the Z-N method, in the field conditions.

MATERIALS AND METHODS:

Two hundered sputum samples were collected from suspected tuberculosis cases at Santhiram General Hospital Nandyal Andhrapradesh India .Three smears were prepared

from each sample of the 200 sputum samples, first one is using for Z-N staining second and third for Gabbet's and fluorescence staining respectively.

THE Z-N STAINING METHOD:

The staining solutions were prepared for Z-N method , 1% carbol-fuchsin was prepared with 1 gm of basic fuchsin dissolved in 50 ml molten phenol; 100 ml of ethanol (95%) was added to the fuchsin – phenol mixture. The solution was diluted with distilled water to make a volume of 1,000 ml then it was filtered. Decolorizing agent sulphuric acid (25%) was prepared with 250ml concentrated sulphiric acid which was slowly added to 750 ml distilled water. Methylene blue (0.1%) was prepared with 1 gram methylene blue dissolved in 100 ml distilled water. Procedure of Z-N staining as per RNTCP guidelines heat fixed smears flooded with 1% carbol-fuchsin and heat applied until steam rises but not boiling for 5 minutes. After cooling of slide the smear washed with tap water and decolorize step done by the 25% sulphuric acid for 4 minutes. the slides washed in tap water ,then counter stain with 0.1% methylene blue for 30 seconds, finally smear slides were washed, then air dried.

THE GABBET'S COLD STAINING METHOD:

The staining solutions were prepared for Gabbet's staining method the fuchsin phenol solution was prepared in the same way as the Z-N method. The Gabbets methylene blue was prepared with 1 gram methylene blue, 20 ml sulphuric acid, 30ml 95% ethanol, and 50 ml distilled water. Procedure of Gabbet's cold staining method was carried out as follows. The smears were air dried, but not heat fixed. Smears are flooded with basic fuchsin phenol solution and allowed to stand for 10 minutes without heat application. Next ,the smear washed with the tap water, then decolorized and counter stained with Gabbets methylene blue for 2 minutes finally, smear slides were washed and airdried.

FLUORESCENT AURAMINE-O STAINING METHOD:

The staining solutions were prepared for fluorescence staining method. The 3% stock solution of phenol: was prepared with 3 g of phenol crystals dissolving in 87 ml Distilled water Auramine phenol solution: was prepared with warm 100 ml stock of three percent phenol to 40^{o}c. To this add gradually 0.3 gm of Auramine with vigorous shaking for 10 minutes, and it was filtered and stored in a dark brown bottle. Acid alcohol: was prepared with 0.5 gm sodium chloride dissolve in 25 ml Distilled water ,add 0.5 ml concentrated hydrochloric acid , mix with 75ml absolute alcohol and stored in amber colored bottle .0.1% potassium permanganate: 1 gm of $Kmno_4$ is added to 100 ml of distilled Water. The fluorescence staining method was carried out as follows. Mucopurulent portion sputum was taken for smear, near to the flame by using a broom stick. On a clean or fresh glass slide , at room temperature smear was allowed for air dried, and heat fixed have done by passing the slide over flame 2-3 times for about 2-3 seconds. Flooded the slides with freshly filtered auramine – phenol kept for 20 minutes without heat application .next the smears were washed with tap water, next decolorized by covering completely with acid alcohol for 3 minutes. next washed with tap water then counter stain with 0.1% $KMno_4$ for 1minute and the slides gently rinsed with water and drain.

Three smears were prepared of each to the 200 sputum sample and stained by the Z-N Gabbets and fluorescent staining and randomly numbered. All Carbol- fuchsin Stained smears were observed under oil immersion by an experienced examiner. Z-N and Gabbet's stained slides were screened for a minimum of 5 min under compound microscope. The smears

Graded as per RNTCP guidelines like 3+ = more than 10 AFB/ oil immersion field; 2+ = 1-10 AFB per oil immersion field; 1+ = 10-99 AFB 100 oil immersion field; Scanty = 1-9 AFB per 100 oil immersion field; Negative = no AFB per 100 oil immersion field.

Auramine –o stained smears observed under LED fluorescent microscope in linear pattern approximately a minimum of 2 min for 100 fields, or three horizontal sweeps. The fluorescent stained smears were examined at much lower magnifications (typically 250x) than used for Z-N stained smears (1000x) each field examined under fluorescence microscopy therefore has a large area than that seen with bright field microscopy. Thus a report based on a fluorchrome stained smear examined at 250x may contain much larger number of bacilli than a similar report from the same specimen stained with carbolfuchsin and examined at 1000x .The smears graded According to WHO manual .3+ = more than 100 AFB field after examination of 20 fields ;2+ = 11-100 AFB per field after examination of 50 fields;1+ =1-10 AFB per field after examination of 100 fields; doubtful = 1-3 AFB per 100 fields; Negative = No AFB per 100 fields.

Table-1-Cross comparison of the Gabbet's cold and Fluorescent staining methods with the Z-N methods.

Grade	*Z-N method*					
	3+	**2+**	**1+**	**Scanty**	**negative**	**Total**
Gabbet's Method						
3+	6	-	-	-	-	6
2+	1	4	-	-	-	5
1+	1	2	7	-	-	10
Scanty	-	-	3	9	-	12
Negative	-	-	-	4	163	167
Total	8	6	10	13	163	200
F.S. Method						
3+	8	3	-	-	-	11
2+	-	3	4	-	-	7
1+	-	-	6	5	-	11
Scanty	-	-	-	6	12	18
Negative	-	-	-	2	151	153
Total	8	6	10	13	163	200

RESULTS

A comparison of the smear results obtained with Gabbet's and fluorescence staining Method against Z-N method is shown in Table 1, 200 sputum samples 37 (18.5%) were positive for AFB with the Z-N method, 33 (16.5%) were positive for AFB with the Gabbet's staining and 47 (23.5%) were positive for AFB with the fluorescent staining method. All specimens positive for AFB with the Gabbet's staining

procedures were positive by Z-N Method. Four of 37 samples positive by the Z-N method were negative by Gabbet's staining method. Ten samples positive by the Fluorescence staining method were negative by the Z-N method. All samples are read negative by the Z-N and Gabbet's staining method but positive by the fluorescence staining method were found to only have scanty AFB with the fluorescence staining method. Fourteen samples positive by the Fluorescence staining methods were read as negative by the Gabbet's method. None of the samples positive by the Gabbet's method were read as negative by the Fluorescence staining method.

The Gabbet's and Fluorescence staining methods are cross compared with the Z-N method in Table 2. The sensitivities of Gabbet's cold staining method and Fluorescence staining method were 89.18% and 94.87% respectively, and the specificities of the Gabbet's cold and Fluorescence staining method were 100% and 93.7%, respectively. The Positive agreement between Z-N and Gabbet's was (94.29%) and between the Z-N and fluorescence staining method was (97.69) indicating good agreement.

Table 2- Smear results by Z-N, Gabbet's and fluorescence staining methods

		Z-N Method	
		Positive	Negative
Gabbets Methods	Positive	33	-
	Negative	4	163
F.S Method	Positive	37	10
	Negative	2	151

DISCUSSION:

Through out the country in all Primary health centers sputum smear microscopic examination successfully implemented by the RNTCP. Due to the presence of unsaponable wax substances in the cell wall of the tubercle bacilli the Z-N method shows major difficulty in staining it requires heat application to the microscopic slide for the uniform penetration of dye in to the cell wall through its waxy barrier. However for this operation possess problem like fairly precise control of the temperature to the slide ,and regular supply of the alcohol or liquid propane gas (LPG) is require for the heating and fixing steps with the Z-N staining method. A desire to develop an alternate staining procedure has resulted in several modifications of the Z-N staining method (8,9,10,11,12,13,14,) to over come these drawbacks. Numerous attempts have been made to develop a cold staining procedure for acid fast bacteria (2,3,6) however Gabbet's cold staining method has been previously evaluated (1) and the Auramine o technique which agrees with the finding of other previous study (5) which concluded both Z-N and Fluorescence Staining can be used for the diagnosis of TB. Previous studies (6) the fluorescence staining is economical in terms of both time and expense. Thus it was recommended for laboratories handling large number of sputum specimens and that fluorescent microscopy is more reliable than Z-N method. The Gabbet's method describes corbol fuchsin longer exposure helps the uniform penetration of the dye through the cell wall the staining solution concentration is same as that used in the Z-N method and no extra cost is involved.

Fluorescence staining utilizes basically the same approach as Z-N staining but carbolfuchsin is replaced by fluorescent dye (Auramine-o), the advantage of Auramine –o techniques is that slides can be examined at a lower magnification and allows the examination of much larger area per unit of time. In fluorescence microscopy the same area that needs examination for 10 min with a light microscope can be examined in 2 minutes.

CONCLUSION

In the present study Gabbet's cold staining and fluorescence staining methods were compared against Z-N method, Gabbet's and fluorescence staining methods showing lower sensitivity, but 100% specificity with the Gabbet's and 93.7% with the fluorescence staining. The Z-N was superior to the Gabbets cold staining method in our study but there was a good agreement between them.

The tubercle bacilli morphology in the Gabbets staining method appear more delicate and closer to their morphology but are also fainter of than those seen with the Z-N stain, which may be reason for the false negative results compared by the Z-N method. In the conventional Ziehl-Neelsen method there is a better penetration of stain through the complex cell surface structure due to the heating therefore the organism appears brighter against back ground .Alcohol or LPG required for heating process In Z-N stain, which may be cumbersome or hazardous however two step process is easier to perform than a three step process which gives the Gabbet's cold staining method a selective advantage.

The Conclusion in the study there was a highly significant relation ship between the Z-N and Auramine o techniques in the detection of AFB than Gabbet's method. The Fluorescence staining method used here had a better sensitivity than Z-N and Gabbet's methods in the detection of AFB. In this study both Z-N and Auramine-o techniques can be used in the detection of AFB. The fluorescence microscopy is better than bright field microscopy and there was a good agreement between them but Fluorescence staining method is quite economical in terms of both time and expense. However fluorescence staining technique a method of choice in this study population whenever dealing with large samples and it is more reliable than Z-N.

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