

# COMPARATIVE EVALUATION OF TWO COLD STAINING METHODS WITH THE ZIEHL-NEELSEN METHOD FOR THE DIAGNOSIS OF TUBERCULOSIS

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**Abstract.** In developing countries pulmonary tuberculosis is usually diagnosed by detecting acid-fast bacilli (AFB) in sputum using a Ziehl-Neelsen (Z-N) staining method. However, in the field the traditional method of staining is difficult to carry out. This study evaluates the efficiency of two cold staining methods, namely Gabbet's and a modified two reagent cold staining method compared with the Z-N taken as the gold standard. Triplicate smears were prepared from 267 sputum samples and stained by the Z-N, Gabbet's, and a modified cold staining method, the smears were positive for AFB in 21 (7.87%), 18 (6.74%) and 19 (7.12%), respectively. The sensitivities for the Gabbet's and modified cold stain were 85.7% and 90.5%, respectively. The positive agreement between the Z-N and Gabbet's (92.3%) and Z-N and modified cold stain (95%) were good. The modified cold staining method was less time consuming and easier to perform in the field.

## INTRODUCTION

Tuberculosis (TB) is a disease that has affected mankind since early times. It is prevalent in India and a leading cause of death. Demonstration of acid-fast bacilli (AFB) in the sputum is a method used to diagnose pulmonary tuberculosis throughout the world (Selvakumar *et al*, 2002).

Sputum smear microscopy is a low cost procedure suited to developing countries but poses problems in the field. The Ziehl-Neelsen (Z-N) method is used in the Directly Observed Treatment, Short-course (DOTS) as per Revised National Tuberculosis Control Program (RNTCP) guidelines initiated in 1993 (Rao, 2007). The Z-N staining method

is cumbersome because it requires the application of heat during carbol-fuchsin staining resulting in the necessity for a flame source (Gokhale *et al*, 1990). To achieve success in tuberculosis control in developing countries, modifications to eliminate technical and administrative problems in an affordable and practical way should be considered (Jagannath, 1990).

Cold staining using Gabbet's methylene blue as a decolorizer and counter stain has been advocated as an alternative staining technique (Gokhale *et al*, 1990). Even a novel two step cold staining method for detecting AFB in sputum smears has been developed (Reuben, 1989; Tripathi DG *et al*, 2001), eliminating heating and combining the decolorizing and counter staining stages. However, its use in peripheral laboratories has not been adequately studied.

The present study compares Gabbet's cold staining method and a modified two

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step cold staining method against the Z-N method in field conditions.

## MATERIALS AND METHODS

Two hundred sixty-seven sputum samples were collected from suspected tuberculosis cases at Kasturba Hospital, Manipal, India. Smears were prepared in triplicate of each of the 267 sputum samples, one using Z-N staining, a second using Gabbet's cold staining and a third using a modified two reagent cold staining method.

### The Z-N staining method

The stains for the Z-N method were prepared as follows. The 1% carbol fuchsin solution was prepared with 1 gram 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. The sulphuric acid (25%) was prepared with 250 ml concentrated sulphuric acid which was slowly added to 750 ml distilled water. The methylene blue (0.1%) was prepared with 1 gram methylene blue dissolved in 100 ml distilled water.

The Z-N stain was carried out following RNTCP guidelines. The smears were air dried and heat fixed by flaming. The slides were then flooded with filtered 1% carbol fuchsin and heated until steaming but not boiling for 5 minutes. Next, the smears were washed in tap water and decolorized with 25% sulphuric acid for 4 minutes. Finally, the slides were rinsed in a gentle stream of tap water, then counter stained with 0.1% methylene blue for 30 seconds, Washed, then air dried.

### The Gabbet's cold staining method

The stains for the Gabbet's cold staining method were prepared as follows. The fuchsin-phenol solution was prepared in the

same way as the Z-N method. The Gabbet's methylene blue was prepared with 1 gram methylene blue, 20 ml sulphuric acid, 30 ml 95% ethanol, and 50 ml distilled water. The Gabbet's cold staining method was carried out as follows. The smears were air dried, but no heat fixation was done. The slides were then flooded with basic fuchsin-phenol stain and allowed to stand at room temperature for 10 minutes. Next, the smears were washed with tap water, then decolorized and counter stained with Gabbet's methylene blue for 2 minutes. Finally, the slides were washed and air dried.

### Two reagent modified cold staining method

The stains for the two reagent modified cold staining method were prepared as follows. The fuchsin-phenol solution prepared in the same way as the Z-N and Gabbet's methods. The decolorizing counterstain solution was prepared with 0.25 gram methylene blue, 25 ml 95% ethanol, 10 ml glycerol, 0.01 gram KOH, 4.5 ml glacial acetic acid, 3 ml HCl, and distilled water to make a volume of 100 ml.

The two reagent modified cold staining method was carried out as follows. The smears were air dried but not heat fixed. The slides were then flooded with fuchsin-phenol stain for 5 minutes. Next, the smears were washed in a gentle stream of water and counter stained with a modified decolorizer counter stain for 3 minutes. Finally, the slides were washed and air dried.

Triplicate smears were made of each of the 267 sputum samples and stained by the Z-N, Gabbet's and modified two reagent cold staining methods and randomly numbered so the examiner was blinded to the staining method. All smears were read under oil immersion by an experienced examiner. Each slide was screened for a minimum of 5 minutes. After the smears were read the results were documented. The smears were

Table 1  
Cross comparison of the cold staining methods with the Z-N method.

Grade	Z-N method					Total
	3+	2+	1+	Scanty	Negative	
Gabbet's method						
3+	4	-	1	-	-	5
2+	2	7	1	-	-	10
1+	-	-	1	1	-	2
Scanty	-	-	-	1	-	1
Negative	-	-	-	3	246	249
Total	6	7	3	5	246	267
Modified cold stain						
3+	3	-	1	-	-	4
2+	2	6	1	-	-	9
1+	1	1	-	1	-	3
Scanty	-	-	1	2	-	3
Negative	-	-	-	2	246	248
Total	6	7	3	5	246	267

Table 2  
Smear results by Z-N method and cold staining methods.

		Z-N method	
		Positive	Negative
Gabbet's method	Positive	18	-
	Negative	3	246
Modified cold stain	Positive	19	-
	Negative	2	246

graded per RNTCP guidelines: 3+ = more than 10 AFB per oil immersion field; 2+ = 1-10 AFB per oil immersion field; 1+ = 10-99 AFB per 100 oil immersion field; scanty = 1-9 AFB per 100 oil immersion fields; negative = no AFB per 100 oil immersion fields.

## RESULTS

A comparison of the smear results obtained with the Gabbet's and a two step modified cold staining methods against the

Z-N method is shown in Table 1.

Of the 267 samples, 21 (7.87%) were positive for AFB with the Z-N method, 18 (6.74%) with the Gabbet's staining and 19 (7.12%) with the two step modified cold staining method. All specimens positive for AFB with the cold staining procedures were positive by Z-N method. Three of the 21 samples positive by the Z-N method were negative by Gabbet's cold staining method, and 2 positive by the Z-N method were negative by the modified cold staining method. All

samples read negative by the two step cold staining method but positive by the Z-N method were found to only have scanty AFB with the Z-N method. Two samples positive by the modified cold staining method were read as negative by the Gabbet's method and one sample positive by the Gabbet's method was negative by the modified cold staining method.

The two cold staining methods are cross compared with the Z-N method in Table 2. The sensitivities of the Gabbet's cold staining method and the two step modified cold staining method were 85.71% and 90.48%, respectively, and the specificities were 100% for each. The positive agreement between the Z-N and Gabbet's methods was 92.3% and between the Z-N and modified cold stain methods was 95%, indicating good agreement.

## DISCUSSION

For successful implementation of the RNTCP, sputum smear microscopy is performed in the primary health centers throughout the country. Even remote rural areas perform Z-N staining. However, the Z-N method poses problem like regular supply of the alcohol or liquid propane gas (LPG) used in 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 (Padmanabha *et al*, 1966; Noack, 1979; Mathew *et al*, 1994; Deshmukh *et al*, 1996; Pathan and Arain, 2002; Tansuphasiri and Kladphuang, 2002; Selvakumar *et al*, 2005) to overcome these drawbacks. However, none of these methods has gained wide acceptance except for the cold staining method (Selvakumar *et al*, 2005).

The cold staining techniques have omitted the decolorization step and heating of

carbol fuchsin. Gabbet's cold staining method, has been previously evaluated (Gokhale *et al*, 1990) and so is a modified two step cold staining method available commercially from Tulip diagnostics (Tripathi *et al*, 2001; Selvakumar *et al*, 2002). The previous evaluations have been promising.

In the present study, Gabbet's cold staining and a modified two step cold staining method were evaluated against the Z-N method, both showing lower sensitivity but 100% specificity. The Z-N method was superior to the cold staining methods in our study, but there was good agreement between them.

With the cold staining methods, the AFB appear more delicate and closer to their original morphology but are also fainter than those seen with the Z-N stain, which may be the reason for the false negative results compared with the Z-N method. In Z-N method there is a better penetration of stain through the complex cell surface structure due to heating, therefore, the organism appears brighter against background though there may be a slight change in morphology.

Performing the Z-N stain required alcohol or LPG for the heating process, which may be cumbersome or hazardous. Moreover the two step process is easier to perform than a three step process, which gives the cold staining method a selective advantage.

The modified cold staining method used here had a better sensitivity than the Gabbet's staining method and was less time consuming (<10 minutes) allowing more time for examination of the smears.

The ease of performance, cost effectiveness and time saved give the two step modified cold staining method an advantage. Large scale multicentric studies need to be performed for further evaluation of this method.

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