

*Full Length Research Paper*

# **Cold acid fast staining method: Efficacy in diagnosis of *Mycobacterium tuberculosis***

**Anita Pandey\*, Molly Madan, Ashish K. Asthana, Ritu Kansal, Anupam Das**

Department of Microbiology, Subharti Medical College, Swami Vivekanand Subharti University, Meerut, Uttar Pradesh, India.

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Ziehl Neelson, a carbol fuchsin based stain, using the 3 component hot process is a standard staining technique for staining sputum smears for detection of *Mycobacterium tuberculosis*. We evaluated a 2 step cold staining method for detection of acid fast bacilli in sputum smears. 1836 sputum samples from patients of pulmonary tuberculosis were studied. Smears were prepared from each sample and slides were simultaneously stained by both methods and examined. The 2 step cold stain method was found to be equally sensitive as the ZN method detecting 20% of cases, when the primary stain was kept for a period of 20 min. The CS method also has the advantage of being simple, economical and less cumbersome and in the procedure of staining, heating, a much too precise step in ZN method for large scale application, is eliminated. The CS method may therefore prove as a good alternative for diagnosis of *M.tuberculosis*, particularly in smaller laboratories and peripheral centers where there is limitation of the funds and equipments.

**Key words:** Cold stain, acid fast bacilli, *Mycobacterium tuberculosis*.

## **INTRODUCTION**

Demonstration of acid fast bacilli in the sputum is the easiest, quickest and a reliable tool for the diagnosis of pulmonary tuberculosis (WHO, 1974). Ziehl Neelsen, a carbolfuchsin based stain (hot process) is a standard staining technique for staining of sputum smears for detection of *Mycobacterium tuberculosis*. The other method used for AFB staining using carbolfuchsin is the cold stain method (Madan et al., 1999). Modification of this traditional ZN stain Kinyoun stain (ZN - cold method) has been developed to cut short the processing time and increase the laboratory productivity. However, improvements and simplifications have been continuously sought, to further simplify and speed up the AFB smear staining process (Sonnerwirth, 1980). CS method using Gabbet's

methylene blue as decolouriser and counter-stain has been advocated as an alternative staining technique by various workers in the past (Gokhale et al., 1990; Madan et al., 1999).

In the present study, we decided to evaluate the 2 step cold AFB stain to assess its performance for sensitivity, simplicity and rapidity over the traditional ZN method, so that it may prove as a good alternative for diagnosis of *M. tuberculosis*.

## **MATERIALS AND METHODS**

A total of 1836 early morning sputum samples from suspected cases of pulmonary tuberculosis, received at Designated Microscopy Centre (DMC) of Subharti Medical College, Meerut, Uttar Pradesh, India were screened for AFB using the traditional ZN hot method and the 2 step CS method.

\*Corresponding author. E-mail: [sideyedoc@yahoo.com](mailto:sideyedoc@yahoo.com) or [ashuashuasthana@rediffmail.com](mailto:ashuashuasthana@rediffmail.com).

## **Preparation of the smears and staining procedure**

A set of 3 slides were prepared from the representative portions of each sputum sample. The slides were coded, air dried, heat fixed and stained. Concentration method was not used for making the smears.

### **Z-N method**

One slide was stained by the traditional ZN hot method (Mackie and McCartney, 1989). The sputum smears were flooded with filtered 1% carbol fuchsin and heated until steaming for 3 - 5 min. The slides were rinsed with water and 25% sulphuric acid was added to the slide to decolourise the smears for 2 - 4 min. The process of decolourising had to be repeated several times until the film is only very faintly pink (decolourisation generally requires contact with sulphuric acid for a total time of at least 10 min). The slides were rinsed and counterstained with 0.1% methylene blue for 30 s. They were then washed, air-dried and examined using an oil immersion objective (1000x).

### **C-S method**

The other 2 slides were stained by the 2 step cold stain method. The CS method was done by using 2 different time duration, that is, with duration of primary stain (carbofuchsin) kept for 10 min (CS 10 min) and primary stain kept for 20 min (CS 20 min) simultaneously using Gabbett's methylene blue modification (Madan et al., 1999).

### **Reagents for CS method**

1. Carbofuchsin 1% (Qualigens fine chemicals, India), which was same as for the standard ZN method.
2. Gabbett's methylene blue (Sonnerwirth, 1980) the constituents of which were methylene blue 25 gm, distilled water 1250 ml, absolute alcohol 750 ml, sulphuric acid 500 ml supplied by Qualigens fine chemicals, India.

The cold stain was performed as follows, the smears were flooded with cold carbofuchsin and allowed to stand at room temperature for 10 min (CS 10 min) and 20 min (CS 20 min) respectively. The smears of CS methods were washed with water after their respective time. After washing with water the slides were then decolorized and counterstained simultaneously by Gabbett's methylene

blue for 2 min. The slides were finally washed with tap water, air dried and examined under oil immersion objective, (1000x)

### **Examination of slides**

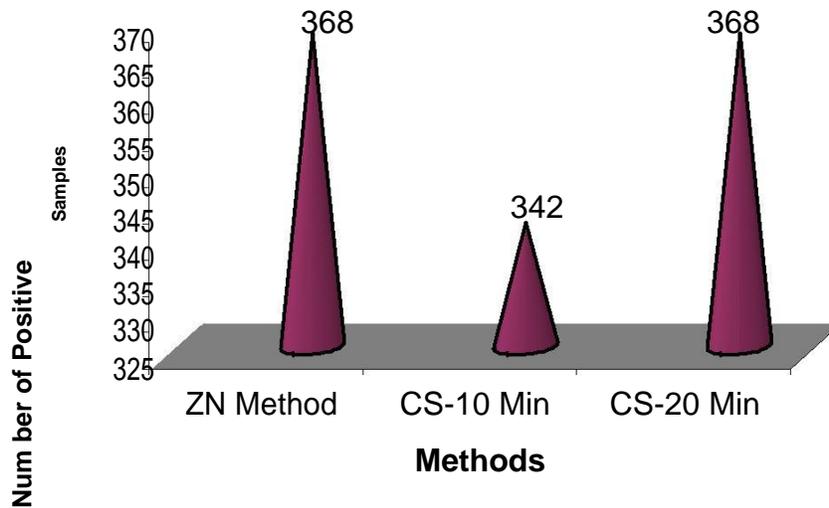
The coded slides were examined independently by 2 groups of experienced technicians. Both group of laboratory technician had no information on the staining method used for each slide and the result of the other microscopists, so as to reduce the bias. The results were submitted after the slides were reported as per the recommendation of the American Thoracic Society, 1981 (Tan, 1962). However to ensure correct grading, a senior technician crosschecked all the positives and 25% of the negative slides. The smears results were finally decoded and matched for comparison.

## **RESULTS**

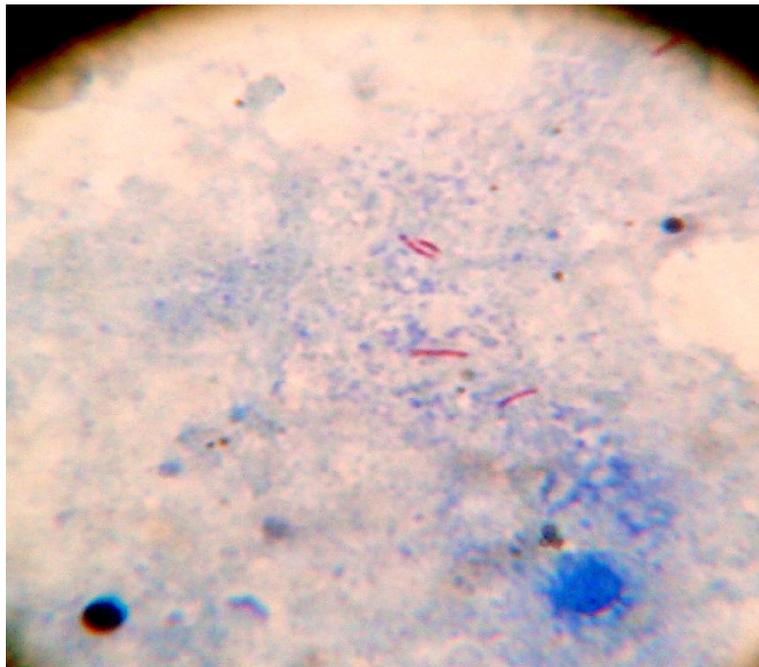
Out of 1836 samples examined, AFB was detected by traditional ZN method in 368 (20.0%). Interestingly, the CS method was equally sensitive in detecting all the 368 samples when the primary stain was kept for a period of 20 min (that is, CS-20). However, when the primary stain was kept for 10 min (that is, CS -10) as performed by Gokhale et al. (1990), we could detect only 342 (18.62%) samples positive (Figure 1). In each of the 26 samples which were missed by CS -10 min method, the AFB smear showed lower concentration of bacteria (that is, scoring 1+ and scanty bacilli).

## **DISCUSSION**

Implementation of the Tuberculosis Control Programme at the level of general health institutions requires sputum smear examination to be performed at large number of institutions throughout the country. A staining method for AFB, which can be practiced even at remote areas, is desirable for successful implementation of National Tuberculosis Control Programme, provided it is technically adequate and simple to perform as the ZN method. The 2 step cold acid fast stain, is an improved alternative method over the traditional ZN hot stain, due to its sensitivity, simplicity and rapidity. CS-20 min method was found to be equally sensitive in detecting AFB as the traditional ZN method. Our study too gave 100% agreement between the 2 techniques as regards positive results. Similar findings were observed by various



**Figure 1.** Smear positive results by Ziehl Neelson and cold staining methods.



**Figure 2.** Acid fast bacilli (delicate and slender) as seen by Cold stain method (CS-20 min)

other workers. (Selvakumar et al. 2002; Madan et al., 1999; Vasantha et al., 1986; Deshmukh et al., 1996). However, the CS-10 min method could not detect 26 positive samples with lower bacterial count in our study. Similarly Gokhle et al. (1990) also missed 18 of their

samples with lower count when the dye was kept for a period of 10 min. However, we could achieve equal sensitivity using CS method when the dye penetration was achieved by doubling the staining time from 10 to 20 min. Doubling the time has definitely increased the

sensitivity in our case which is an important observation. This standardization of time needs to be studied in larger number of cases and in many centers before being implemented.

The decolorizer used in the Gabbett's methylene blue includes both acid and alcohol, making it superior to the conventionally used only acid as decolorizer in the hot method (Sonnerwirth, 1980; Mackie and McCartney, 1989). This is useful as it eliminates the false positive smears due to the non pathogenic and contaminant mycobacteria which are only acid fast. These are occasionally recovered from urine samples and tap water but are of no clinical significance.

It was also observed that by CS method the AFB appeared to be more delicate and slender, which is closer to their natural morphology (Figure 2). It may be because avoidance of heat reduces the chances of alteration in the morphology in this method. Similar observation was seen by other workers too (Gokhale et al., 1990).

However, we found that the CS method (CS-20 min) has the advantages of being highly sensitive, economical and easy to learn and simple to perform as compared to the traditional ZN hot stain method and should be recommended for routine AFB sputum staining. Similar observations were noted by many other authors in the past (Tripathi et al., 2001; Deshmukh et al., 1996; Gokhale et al., 1990; Vasantha et al., 1986).

To conclude, the 2 step cold stain is an improvement over the traditional ZN hot stain. There are some organizational problems associated with performing Z-N staining as a routine procedure in peripheral microscopy centers:

- a) The carbol fuchsin stain has to be filtered everyday before use, which largely depends on the supply of clean glass/ plastic funnels and filter papers.
- b) Heating/flaming carbol fuchsin on slides requires spirit/ LPG gas connection: supply of a sufficient quantity of spirit poses technical and organizational problems and many centers are not provided with LPG connections (Sujatha et al., 1991).
- c) Decolourisation with 25% sulphuric acid is a critical step and the time of decolourisation should be sufficiently increased while staining thick smears.

The 2 step cold staining method used in this study does not include the above 3 steps and thus removes the above associated problems. The time taken for the staining is also considerably reduced. Due to so many advantages, the CS method can be:

- a) Routinely adopted for AFB putum staining especially

in settings with high workloads or can be used as an alternative staining method which can be practiced even at remote areas due to its sensitivity, simplicity and rapidity.

- b) This method could also be included in the curriculum, for the undergraduate medical students, who would find it an easier procedure to learn and perform.

- c) It can form part of the Tuberculosis Control Programme in developing countries for detection of those cases that are a source of infection, as it can be performed in remote areas and at periphery, where laboratory facilities are poor.

However, a large scale multi-centric studies in different conditions needs to be conducted to assess its efficacy in diagnosis of *M. tuberculosis*.

## Note

All authors contributed equally.

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