

## Comparison of Variants of Carbol Fuchsin & Phenol in Ziehl Neelsen Staining to Detect AFB

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### Abstract

**Introduction:** Sputum smear microscopy is the mainstay in the rapid diagnosis of pulmonary tuberculosis. Ziehl Neelsen staining is popularly used smear microscopy techniques.

**Method:** We evaluated the use of 0.1% Basic Fuchsin, 5% phenol containing primary staining reagent (Solution 'A') and 0.1% Basic Fuchsin, 7.5% phenol (Solution 'B') in the diagnosis of pulmonary tuberculosis. Results are compared with standard.

**Results:** Out of 196 sputum samples smear positivity were 9.7%, 7.6% and 9.2% respectively with standard method and Solution 'A' and Solution 'B'.

**Conclusion:** Even though with some reduction in expenditure, reagents concentrations should not be altered.

**Keywords:** ZN staining; Tuberculosis (TB); Carbol Fuchsin (CF); Basic Fuchsin (BF)

### Introduction

Tuberculosis (TB) is a world pandemic, a bacterial disease caused by *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*). Microbiological diagnosis is the main stay for the effective treatment of pulmonary TB (PTB) [1]. Because of rapidity and low cost, PTB is diagnosed by identifying Acid Fast Bacilli (AFB) in sputum smears by Ziehl Neelsen (ZN) staining. Revised National Tuberculosis Control Programme (RNTCP) guidelines [2] recommend use of 1% basic fuchsin (BF) in ZN staining. Guidelines of World Health Organization (WHO) [3] and International Union against Tuberculosis and Lung Disease (IUATLD) [4] recommend use of 0.3% Carbol fuchsin (CF) as primary staining reagent.

Selvakumar et al. [5] showed that ZN staining by using 0.1% BF detected only 75% of the smears found positive by ZN using 1% BF. Whereas in another study by Selvakumar et al. [6] showed that use of 0.3% of BF may result in 20% smear positive patients being missed. Later Pawan et al. [7] explained that the reduced smear positive results in Selvakumar et al. [6] studies is due to lowered concentration of phenol to ~ 1.7%. But in the standard ZN [8] staining the phenol concentration is 5% and CF 1%.

As per Pawan et al. [7] it is very clear that reducing the concentration of phenol automatically reduces the smear positivity. By keeping Pawan et al. [7] report in mind we have taken the present research project on ZN staining by changing the concentration of phenol to 7.5% and BF to 0.1%.

### Material and Methods

The present study was conducted in the department of Microbiology, GSL Medical College Rajahmundry over a period of 2 months i.e. 1<sup>st</sup> April to 31<sup>st</sup> May 2013. Twelve hundred bedded GSL Medical College teaching hospital is a referral center for TB treatment and a microscopy center for TB diagnosis under RNTCP.

The study was approved by the Institutional Ethics Committee. Informed written consent was taken from all the volunteers. Individuals

with >2 weeks of cough and aged 14 years and above were included in the study. After collection of clinical history, the participants were explained the importance of submission of sputum rather than saliva and the visual difference between sputum and saliva was shown practically. Subsequently collection of good quality sputum sample was demonstrated [9], by taking three deep breaths, followed by a deep cough. All the participants are requested to submit five ml of sputum sample by giving marked container. During the study period one hundred and ninety six sputum samples were processed.

First macroscopic findings of each specimen were recorded. New unscratched slides were labeled with study number and used for smear preparation. Three smears were prepared with each sample. Smears were stained by ZN staining as per the RNTCP [2], the procedure is given below. For the first slide 1% BF solution was used as primary staining reagent, solution A for the second slide and solution B was used for the third slide as primary staining reagent. All the stained slides were graded as per RNTCP guidelines [2] and the results of the first slide were reported.

Before microscopy, the study numbers were covered with wrap around stickers. With this the lab technicians (LTs) were blinded to smear results, bias was avoided [10]. Stickers were removed by another LT before entering the smear results. As a part of internal quality control, all the positive slides and randomly 30% of negative slides were screened by the Microbiologist.

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## Preparation of reagents

I. Standard CF solution (1%): BF 10 g was dissolved in 100 ml ethanol and 50 ml molten phenol in a flask maintained at 60°C in a water bath. The solution was made up to 1000 ml with distilled water.

II. Solution 'A' contains 0.1% of BF and 5% phenol. Stock solution of 1% BF was prepared by dissolving 1 g basic fuchsin in 100 ml ethanol. CF solution 0.1% was prepared by mixing 10 ml of 1% BF solution with 90 ml of 5% phenol solution.

III. Solution 'B' contains 0.1% BF and 7.5% phenol. Stock solution of 1% BF was prepared by dissolving 1 g basic fuchsin in 100 ml ethanol. CF solution 0.1% was prepared by mixing 10 ml of 1% basic fuchsin solution with 90 ml of 7.5% phenol solution.

IV. Sulphuric acid (25%): Concentrated sulphuric acid 250 ml was slowly added to 750 ml distilled water.

V. Methylene blue (0.1%): Methylene blue 1 g was dissolved in 1000 ml distilled water.

## ZN staining

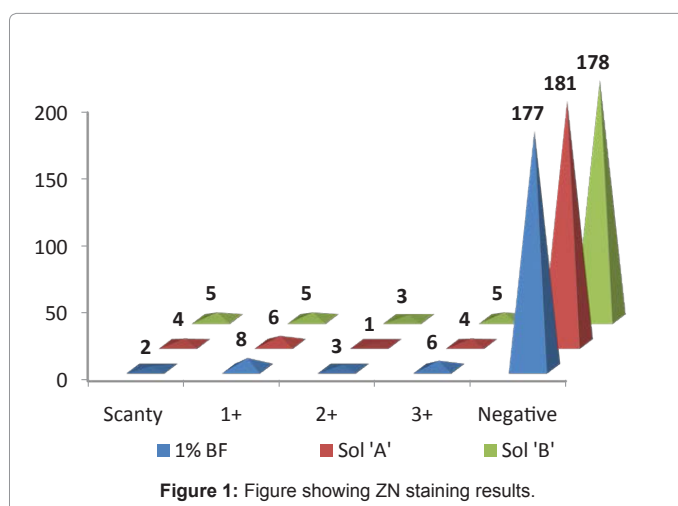
Smears, flooded with filtered CF and heated until it was steaming, were left for five minutes. After rinsing the slides with a gentle stream of water, 25% sulphuric acid was used to decolorize the smears for 2 to 3 minutes. The slides were rinsed as above and counterstained with 0.1% methylene blue for 30 seconds. The slides were washed, air dried before examination under a light binocular microscope.

## Grading of smears

The smears were graded using 100 x oil immersion objective as per RNTCP guidelines [2]. Scanty=1-9 AFB in 100 oil immersion fields; 1+=10-99 AFB in 100 fields; 2+=1 to 9 AFB per field in at least 50 fields; 3+=10 or more AFB per field in at least 20 fields; Neg=no AFB in 100 fields.

## Results

In the current study 196 sputum samples were tested by ZN staining to identify AFB. The smear positivity was 9.7% when stained with 1% CF (Figure 1). Whereas the smear positivity fall down to 7.6% (Figure 1) with solution 'A' and there was significant improvement of smear positivity (Figure 1), 9.2% when solution 'B' was used as primary staining reagent. All 177 smears which were negative with standard CF



were also negative with solution A & Solution B as primary staining reagents.

## Discussion

TB is a major public problem, majority of TB cases occur in low and middle income countries [11]. In high TB burden countries, infrastructure for the diagnosis is not adequate; ZN staining is the only diagnostic technique. In spite of new technologies such as TB culture, Line probe Assay and Gene X pert, WHO recommends use of Sputum Smear Microscopy (SSM) for the diagnosis and treatment monitoring. SSM is a cost effective tool, provides not only a preliminary confirmation of the disease, but also quantitative estimation of AFB.

As per WHO or RNTCP, PTB patient is defined as an individual with at least one sputum smear positive for AFB or culture positive for tubercle bacilli. In limited resources and high TB burden countries like India culture facility is not adequately available. Hence majority of TB cases are diagnosed based on SSM.

The quest for rapidity and efficacy has resulted in several modifications to simplify ZN staining. But none of these modified methods gained wide acceptance and are not used now. It was proved very clearly that reduction in phenol concentration significantly reduces the sputum smear positivity [6].

The chunk of the study is increasing the concentration of phenol and reduction in concentration of BF, which reduces the reagents expenditure (10% reduction in BF and no significant change in phenol).

In the current study also there is a significant fall in sputum smear positivity even with 50% increase in Phenol concentration and 0.1% BF. Hence the reagents concentration should not be reduced (BF<0.3%) to get ideal sputum smear results by ZN staining for the diagnosis of TB.

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