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**Comparison of Various Concentrations of Carbol Fuchsin Solutions in Ziehl-Neelsen Staining for Detection of Acid-Fast Bacilli in Sputum Samples**Iduh, M.U.<sup>1\*</sup>, Spencer, T.H.I.<sup>1</sup>, Mohammed, K.<sup>1</sup>, Garba, M.K.<sup>1</sup>, Nataala, S.U.<sup>1</sup>, Ashcroft, O.F.<sup>1</sup>, Abdulrashid, L.<sup>2</sup>Department of Medical Microbiology, School of Medical Laboratory Science/College of Health Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria <sup>1</sup>, Department of Medical Laboratory Science, Kebbi Medical Centre, Kalgo Kebbi State, Nigeria <sup>2</sup>.**Corresponding author: E-mail:** unataiduh@gmail.com./ +234-806-605-4527**Abstract**

This study was carried out to evaluate Ziehl-Neelsen staining using various concentrations of carbol fuchsin solution. Duplicate smears from 173 sputum samples were made and stained with 1%, 0.3% and 0.1% carbol fuchsin solution. The smear results were recorded semi quantitatively according to the WHO guidelines. A comparative analysis of the results obtained from each carbol fuchsin solution was carried out the McNemar test and p-value was determined. P-value of <0.05 was considered significant. Comparison of 1% and 0.3% carbol fuchsin solution shows close relationship with McNemar chi square of 3.0 and p-value of 0.39. The agreement from these results had a kappa value of 0.934. Twenty-five (25) of the 173 samples were positive and detected by 1% and 0.3% concentrations, 25(14%) and 25(14%) respectively. Comparison of 1% and 0.1% carbol fuchsin solution indicates a very poor relationship with a McNemar of 18 and a significant p-value of 0.012. Twenty-five (25) of the 173 samples were positive which was detected by 1% 25(14%) and 0.1% detected only 20(11%). The sensitivity of ZN using 0.3% and 1% basic fuchsin was comparable, while it was reduced using 0.1% compared to 1% basic fuchsin. Reducing the concentration of basic fuchsin below 0.3% in ZN staining was found to significantly reduce its sensitivity.

**Keywords:** Tuberculosis, Carbol fuchsin, Ziehl-Neelsen stain, Sputum, Patient. Ziehl-Neelsen stain, Sputum, Patient.

**Introduction**

Tuberculosis (TB) remains an important public health problem. The world health organization (WHO) estimates that 8 million new TB cases are reported annually and are the cause of death in 2-3 million patients. Each untreated pulmonary TB patient is responsible for the spread of the disease to 10-15 humans over a year. This makes TB one of the most important causes of the death from an infectious agent (WHO, 2007 and Corbett *et al.*, 2003).

The most effective means of protection is early diagnosis and treatment of the disease. Preliminary diagnosis is based on clinical findings, but definite diagnosis is by laboratory methods (Drobniewski *et al.*, 2003).

Pulmonary Tuberculosis is diagnosed by the detection of acid-fast bacilli (AFB) in sputum smears by the Ziehl-Neelsen (ZN) method, which is commonly used in many high TB burden countries due to its simplicity and low cost. This conventional method plays a key role in the diagnosis and monitoring of TB treatment. Its major disadvantage is its low sensitivity, ranging from 20% to 43% (Kurup and Chester, 2014; Daniel, 1989). Microscopy has many advantages when it comes to speed feasibility, and if its sensitivity could be improved, it has the potential to become an even more valuable tool for TB control programs around the world.

A lot of modifications have been tried to improve its sensitivity since Robert Koch first described it in 1882. The World Health Organization (WHO) and the International Union against Tuberculosis

and Lung Disease (IUATLD) guidelines recommended 0.3%. The reasons for reducing the concentration from 1% to 0.3% are not known. The need to document the adequacy of 0.3% over 1% basic fuchsin in ZN staining has been stressed (Angra *et al.*, 2003).

## Materials and Method

### Study area and Subject

This research work was carried out in School of Medical Laboratory Science, Usmanu Danfodiyo University Sokoto in collaboration with Sokoto State Specialist Hospital. The study sites and participating hospital have enabling environment to carry out this study.

Sokoto State is located extreme North West of Nigeria near the confluence of Sokoto River and the Rima River with an average annual temperature of 28.3°C (82.9°F), Sokoto is on the whole a very hot area. However, maximum day time temperatures are for most of the year generally under 40°C (104°F). The warmest months are February to April when daytime temperature can exceed 45°C (113.0°F). The rainy season is from June to October during which showers are daily occurrence. There are 2 major seasons, wet and dry which are distinct and characterised by high and low malarial transmission respectively. Report from the 2007 National Population Commission indicated that the state had a population of 3.6 million. The metropolis is estimated to have a population of 427,760 (NPC/FRN, 2007) made up of Hausa and Fulani majority and a minority of Zabarmawa and other non- indigenous settlers.

There are two major languages in this state which are Hausa and Fulfulde spoken among the Fulani. The main occupation of the people is grain production and animal husbandry. Majority of the indigenous people practice agriculture. Crops produced includes commercial crops like millet, sorghum, beans, rice and maize. Other occupations commonly practiced are dying, blacksmithing, weaving, carving, trading, and cobbling. Socio cultural characteristics is homogenous as majority of its indigenes and inhabitants are Muslims, therefore the doctrine of Islam provides the singular code of conduct and behavioral characteristics generally across the state.

### Eligibility criteria

All patients with suspicious pulmonary TB (chest radiography, positive tuberculin skin test, clinical presentation) or TB patients under anti-TB therapy are enrolled to participate in the study.

### Exclusion criteria

All patients without clinical presentation for TB are not eligible to take part in the study.

### Consent and Ethical approval

Verbal informed consent was sought from participants in this study. Ethical clearance was also obtained from ethical committee in Sokoto State Specialist Hospital.

### Sample size

A total of one hundred and seventy-three (173) sputum samples were collected from Specialist Hospital Sokoto and used in the study.

### Preparation of chemicals and reagents

Ethanol, phenol, sulphuric acid, methylene blue and basic fuchsin were used in the study. The WHO guidelines for the preparation of smears and reagents, and for the staining, examination and grading of direct smears were followed.

### Carbol-fuchsin

#### (i) Carbol-fuchsin solution (1%)

Basic fuchsin 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) Carbol-fuchsin solution (0.3%)

A stock solution of 3% basic fuchsin was prepared by dissolving 3 g basic fuchsin in 100ml ethanol. A stock solution of 5% phenol was then prepared by adding 25 ml molten phenol to 475 ml distilled water. Carbol fuchsin (0.3%) solution was prepared by adding 10 ml of 3% basic fuchsin solution to 90 ml of 5% phenol solution.

#### (iii) Carbol-fuchsin solution (0.1%)

A stock solution of 1% basic fuchsin was prepared by dissolving 1 g basic fuchsin in 100 ml ethanol. Carbol fuchsin solution (0.1%) was then prepared by mixing 10ml of 1% basic fuchsin solution with 90 ml of 5% phenol solution.

**Decolorizing solvent***Sulphuric acid (25%)*

Concentrated sulphuric acid 250 ml was slowly added to 750 ml distilled water.

**Counter stain***Methylene blue (0.1%)*

Methylene blue 1 g was dissolved in 1000 ml distilled water.

**Sample collection and processing**

A sputum sample was aseptically collected from the study participants into a cleaned dried plastic capped sample container and duplicate smears were made from each sample. One set was stained with 1% carbolfuchsin, the second set with 0.3% carbolfuchsin and third set with 0.1% carbolfuchsin.

**Ziehl-Neelsen staining method**

**Principle:** Mycobacteria, which do not stain well by Gram stain, are stained with carbol fuchsin combined with phenol. The phenol carbol fuchsin stain is heated to enable the dye to penetrate the waxy mycobacterial cell wall. The stain binds to the mycolic acid in the mycobacterial cell wall which resist decolorization with 25% sulphuric acid or 3% acid alcohol.

Ziehl- Neelsen staining method was used to stain all the smears. A smear was made on a clean grease free slide about 2-3cm in diameter. The prepared smears were allowed to air dried completely. The smear was heat fixed. Flooded with carbolfuchsin solution, underneath of the slide was heated gently until steam rises. Boiling of the stain was avoided, and stained for 5 minutes. The film was washed in a gentle and

indirect stream of tap water until no color appears in the effluent. The stained smear was decolorized with the 25% sulphuric acid until the film becomes yellowish and washed immediately with water. The smear was counter stained with methylene blue for 2 minutes and washed with tap water. It was then air dried. The stained smears were examined under oil immersion lens. About 100 fields was examined

**Result:**

Acid fast bacilli appeared red.

Other organisms and cellular materials appeared blue (Ochei and Kolhatkar, 2007).

**Statistical Analysis**

The results obtained were analyzed using SPSS version 20 and the statistical significance of observed differences in smear results between the different staining methods was determined using McNemar's test. A  $p$ -value  $< 0.05$  was considered significant. The level of agreement in smear results between the ZN methods was determined using Kappa statistics.

**Result**

Comparison of smear results obtained from ZN staining using 0.3% carbol fuchsin solution and 1% carbol fuchsin solution revealed close agreement (Kappa= 0.93): respectively 25 (14%) and 25 (14%) of the 173 samples were positive (Table 1).

The comparison of smear results obtained from ZN staining using 0.1% and 1% carbol fuchsin showed a significant difference ( $p= 0.012$ ): 20 (11%) and 25 (14%) respectively of the 173 smears were positive (Table 2).

**Table 1. Smear results obtained from ZN methods using 0.3% carbolfuchsin compared to 1% carbol fuchsin.**

	1% Carbol fuchsin						Total
	Scanty	1+	2+	3+	Any Positive	Any Negative	
<b>0.3% Carbol fuchsin Scanty</b>	8	1	0	0	9	0	9
<b>1+</b>	0	5	1	0	6	0	6
<b>2+</b>	0	0	5	1	6	0	6
<b>3+</b>	0	0	0	4	4	0	4
<b>Any Positive</b>	8	6	6	5	25	0	25
<b>Any Negative</b>	0	0	0	0	0	148	148
<b>Total</b>	8	6	6	5	25	148	173

McNemar=3.0, p- value=0.39

3+= > 10 AFB/oil immersion field in at least 20 fields; 2+= 1–9 AFB/oil immersion field in at least 50 fields; 1+= 10–99 AFB/100 oil immersion fields; Scanty =1–9 AFB/100 oil immersion fields. ZN = Ziehl-Neelsen; AFB = acid-fast bacilli.

**Table 2. Smear results obtained from ZN methods using 0.1% carbolfuchsin compared to 1% carbol fuchsin.**

	1% Carbol fuchsin						Total
	Scanty	1+	2+	3+	Any Positive	Any Negative	
<b>0.1% Carbol fuchsin scanty</b>	4	3	1	0	8	0	8
<b>1+</b>	0	2	5	3	10	0	10
<b>2+</b>	0	0	0	1	1	0	1
<b>3+</b>	0	0	0	1	1	0	1
<b>Any Positive</b>	4	5	6	5	20	0	20
<b>Any Negative</b>	4	1	0	0	5	148	153
<b>Total</b>	8	6	6	5	25	148	173

McNemar=18, p value=0.012

3+ => 10 AFB/oil immersion field in at least 20 fields; 2+ = 1–9 AFB/oil immersion field in at least 50 fields; 1+ = 10–99 AFB/100 oil immersion fields; Scanty = 1–9 AFB/100 oil immersion fields. ZN = Ziehl-Neelsen; AFB = acid-fast bacilli.

### Discussion

A total of 173 sputum samples were used in the research work, of which 25 (14.5%) samples were positive and detected by both 1% and 0.3% carbol fuchsin. This is lower compared to previous reports [Mani *et al.* (2017) (20.0%); Annam *et al.* (2009) (33.3%) and Gangane *et al.* (2008) (27.0%)]. However, it is higher than result obtained by Chandrasekhar and Prayaga (2012) (12.5%). Simplification of the ZN staining method in order to achieve rapidity and efficacy has resulted in several modifications (Selvakumar *et al.* 2002b). Nevertheless, it's only the cold staining method that has been widely accepted unlike the others (Balows *et al.*, 1991). In this research work, ZN staining using 0.3% basic fuchsin seems to be as sensitive and specific as that using 1% basic fuchsin. The cost of staining can be reduced and more so wastage of basic fuchsin avoided by the usage of this reagent. However, it was discovered that the sensitivity of ZN was affected by reducing the concentration of basic fuchsin below 0.3%.

In a study carried out by Selvakumar *et al.* (2002a) it was revealed that 21% of smears detected as positive by ZN using 1% basic fuchsin were missed when diluted carbol-fuchsin (0.3% basic fuchsin and 1.7% phenol) was used. This indicates that, lowering the concentration of phenol in carbol fuchsin solution will significantly affect the detection of smear-positive cases.

In this work, the mixing of basic fuchsin and phenol solutions was done before staining according to WHO guidelines (Kurup and Chester, 2014; Wayer, 1998). However, in the field, this will give some operational and technical problems as many microscopy centers lack measuring cylinders, and an ideal mixing of stock solutions cannot be guaranteed in these settings.

### Conclusion

The results obtained revealed that lowering the concentration of carbol fuchsin below 0.3% significantly reduces the sensitivity of ZN staining and consequently, more positive smear(s) may not be seen. The reagents concentration should not be reduced (basic fuchsin should not be less than 0.3%) to get ideal sputum smear results by ZN staining for the diagnosis of TB.

### Recommendations

1. Standard procedure(s) in the preparation of reagents and grading of smear result should be adhered to.
2. 0.3% carbol fuchsin should be used as it was found to provide same result as 1% carbol fuchsin, using this reagent can avoid wastage of basic fuchsin and reduce the cost of staining.
3. Carbol fuchsin solution less than 0.3% should not be used in ZN staining for AFB as positive smears may be missed.

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