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HYDROCHLORIC ACID VS SULPHURIC ACID – AN ECONOMICAL DESTAINING REAGENT FOR ZIEHL NEELSEN STAINING TO DETECT ACID FAST BACILLI IN SPUTUM SMEARS

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Abstract: Ziehl Neelsen (ZN) staining is one of the simplest, rapid diagnostic tests to detect Acid fast bacilli in sputum smears. To compare 25% Sulphuric acid (H_2SO_4) and 6% Hydrochloric acid (HCl) in alcohol to differentiates acid fast bacilli in sputum smears. Department of Microbiology, GSL Medical College, Rajahmundry. A total of 187 sputum samples were stained by ZN acid and ZN acid alcohol method. Smear positivity was 31.6% for both the methods. HCl 6% in alcohol is recommended over 25% H_2SO_4 due to less cost, easy to dilution.

Key Words: Ziehl Neelsen (ZN) staining, Acid fast bacilli (AFB), Sulphuric acid (H_2SO_4), Hydrochloric acid (HCl).

INTRODUCTION

Mycobacterium tuberculosis (MTB) complex, an Acid Fast Bacilli (AFB) is the causative agent of tuberculosis (TB). TB is diagnosed by identifying AFB in sputum smears (SS) by Ziehl Neelsen (ZN) staining [1]. ZN staining is one of the simple, economical, rapid diagnostic techniques. Due to rapidity and ease of the technique, majority of National TB Control Programmes (NTPs) recommend sputum smear microscopy (SSM) for the diagnosis of lung TB. The only diagnostic technique for TB, suitable to peripheral levels of health services, is serial SSM with ZN staining[1]. In spite of its few disadvantages such as inability to identify drug resistance, limited utility in the diagnosis of TB in human immunodeficiency virus (HIV) patients, World Health Organization (WHO) and Revised National Tuberculosis Control Programme (RNTCP) insist SSM [2].

ZN staining contain three steps: primary staining decolorization and counter staining with basic fuchsin, dilute acid and methylene blue respectively. RNTCP recommend 25% Sulphuric acid (H_2SO_4) as destaining agent. Whereas WHO recommend 3% hydrocholoric acid (HCl) in alcohol as decolorizer in ZN staining. The apprehension with acid decolorizer is lowering the smear positivity for AFB due to unclean smears[3]. However destaining with H_2SO_4 makes to identify AFB more easily even form thick smears due to removal of some of the back ground material. Both the decolorizers i.e. H_2SO_4 and HCl in alcohol have merits and demerits. Sulphuric acid is costly, bulk quantities dilution is difficult due to heaviness and viscosity, in addition to the considerable heat which is generated during dilution. But acid alcohol is economical, easy to handle, easy to transport except the difficulty in procurement of large quantities.

Researchers worked on different decolorizing agents in the field conditions to detect AFB in ZN staining[4]. By keeping the above studies in mind in the current study we want to identify the economical destaining reagent. This study may be useful for the teaching institutions like Medical Colleges who spent significant amount of money on ZN staining decolorizing reagent especially for undergraduate practical.

MATERIAL AND METHODS

The study was conducted in the department of Microbiology, GSL Medical College, Rajahmundry from 1 April 2014 to 30 June 2014. Study was approved by the Institutional Ethics Committee. An informed written consent in the presence of witness was taken from all the volunteers who participated in the study. Both the genders and aged 18 yrs / above with suspected lung TB were included in the study.

All the individuals were explained about the importance of submission of the sputum sample and how to submit the good quality of sputum sample in their local language as patient education is imperative for obtaining correct sputum sample[15]. The visual difference between sputum and saliva was demonstrated. Finally they were explained to provide 5 ml of sputum sample.

Immediately after collection, two smears were prepared with each sample on new glass slides and heat fixed. One slide was stained with standard ZN technique as per RNTCP guidelines [6] and the second smear was also stained by ZN method but 6% acid alcohol was used as decolorizer. After ZN staining

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slides were observed under oil immersion objective of binocular microscope. All the positive slides and 25% of the negative slides were screened by the senior author.

Sputum sample collection

Initially patient should inhale deeply 2 to 3 times with mouth open, cough out deeply from chest, open the sterile, new, leak proof sample container, spit out the sputum in to it and close the container tightly.

Smear preparation

A new unscratched slide was selected for smear preparation. Smear was prepared with sterile loop. A good smear is spread evenly, over a size of 2 X 3 cm and is neither too thick nor too thin. This was allowed to air dry for 15-30 min and fixed by passing it over a blue flame 3-4 times[6].

Acid alcohol (6%)

To 940 ml of alcohol in a jar, 60 ml of concentrated HCl was added.

ZN staining[6]

Smears, flooded with filtered 1% carbol fuchsin (CF) were heated until it was steaming and left to steam for 5 min. After rinsing the slides with a gentle stream of water, 25% H_2SO_4 was used to decolorize the smears for 2-4 min and if necessary decolorization step may be repeated for another 1-3 min. The slides were rinsed as above and counterstained with 0.1% methylene blue for 30 seconds. The slides were then washed, air dried and examined under oil immersion.

Grading of smear

The smears were graded using 100 × oil immersion objective as per the RNTCP technical manual. The smears will be graded using 100 x oil immersion objective as per the RNTCP technical manual[6]: 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

During the study period 187 sputum samples were stained by ZN acid and ZN acid alcohol methods. The sputum smear positivity was 59 (31.6%) for both the methods except some difference in the smear grading (Table). This difference in smear grading does not have any role in treatment.

DISCUSSION

TB is a major health problem in low and middle income countries[7]. In high TB burden countries, the infrastructure for the diagnosis is not adequate; ZN staining is the only diagnostic technique[8]. After primary staining step, strong acids are used for destaining so that AFB differentiated from other acid fast microbes. But, AFB resists to decolorize with acids. This is the principle of ZN staining.

During the study period one hundred and eighty seven (n= 187) sputum samples were processed. The smear positivity was 31.6% (59) respectively with 25% H_2SO_4 and 6% HCl in alcohol (Table). M. Gomathi Shekar *et al.*, reported that sensitivity and specificity were 79% and 89% respectively for ZN acid (25% H_2SO_4) or ZN acid (3% HCl) alcohol methods [3]. Because of less false positivity, KJM Aung *et al.*, concluded that 6 – 10% acid in alcohol is being recommended for ZN destaining above H_2SO_4 [4].

Mokhtari *et al.*, did not observe any difference between 20% H_2SO_4 in water or alcohol solution [9]. A 10% proportional increased sensitivity compared to ZN was contradicted by Devulder *et al.*, for the cold primary staining, Kinyoun staining/Tam Thiam Hok modification [10, 11, 12, 13].

In the original ZN staining technique, H_2SO_4 in water was preferred over nitric acid by Neelsen[14]. Due to the advantages like easy availability, procurement, storage etc. most of the NTPs use 25% H_2SO_4 as destaining reagent in ZN staining. Whereas procurement of alcohol require a huge official procedures and it has difficult storage due to threat of theft.

In academic institutions / teaching hospitals the administration set up is somewhat different. Especially in private institutions always there is some monitoring. Hence the most important threat, theft may not possible. In teaching institutions there is significant wastage of reagents due to training of the students. This wastage is minimum in NTPs due to involvement of trained staff.

Due to less cost and with other added advantages like safety, easy dilution 6% HCl in alcohol is recommended rather than 25% H_2SO_4 as destaining reagent in ZN staining especially in teaching hospitals.

Table: Positive smear results from ZN acid (25% H_2So_4) and ZN acid alcohol (6% HCl in alcohol) techniques.

		ZN acid				
		Scanty	**1+**	**2+**	**3+**	**Positive**
ZN alcohol	Scanty	4	7	0	0	11
	1+	6	9	7	2	24
	2+	0	6	9	2	17
	3+	0	1	2	4	7
	Positive	10	23	18	8	59

REFERENCES

1. T Jaya Chandra. Same day sputum smear microscopy approach for the diagnosis of pulmonary tuberculosis in a microscopy center at Rajahmundry. Ind. J. Of. Tuber.2012; 59: 141 – 144.

2. Chandra TJ, Raj RS, Sharma YV. Same day sputum smear microscopy approach with modified ZN staining for the diagnosis of pulmonary tuberculosis in a microscopy centre at Rajahmundry. Indian J Med Microbiol 2014; 32: 153-6.

3. M Gomathi Shekar, Fatima Rehman, Vanaja Kumar, N Selvakumar. Equialence of acid alone or acid alcohol as decolourizing agent in Ziehl Neelsen method. Indian J Tuber 2012; 59: 219 - 223.

4. KJM Aung, P Nandi, A Hamid Salim, A Hossain, A Van Deun. Hydrochloric vs. sulphuric acid in water for Ziehl-Neelsen staining of acid-fast bacilli. Int J Tuberc Lung Dis 2011; 15(7):955 - 958.

5. T Jaya Chandra. Same day sputum smear microscopy approach for the diagnosis of pulmonary tuberculosis in a microscopy center at Rajahmundry. Indian Journal of Tuberculosis 2012; 59: 141 - 144.

6. RNTCP Central TB Division. Manual for Laboratory Technicians. New Delhi, India: Directorate General of Health Services, Ministry of Health and Family Welfare, 1998. Available from: http://www.tbcindia.org/LABMANUAL. pdf (Last accessed date on 2014 Jan 22).

7. World Health Organization, Global tuberculosis control; epidemiology, strategy, financing. Geneva, World Health Organization. WHO/HTM/ 2009; 411.

8. Jaya Chandra T, Selvaraj R, Sharma YV. Comparison of variants of Carbol Fuchsin & Phenol in Ziehl Neelsen staining to detect AFB" J Mycobac Dis 3: 131. doi:10.4172/2161-1068.1000131

9. Mokhtari Z, Larbaoui D. Comparison of two methods of decolouration of Mycobacterium tuberculosis. Additional results. Bull Int Union Tuberc 1973; 48: 42–44. [French].

10. Kinyoun JJ. A note on Uhlenhuths method for sputum examination, for tubercle bacilli. Am J Public Health 1915; 5: 867–870.

11. Tan Thiam Hok. A simple and rapid cold-staining method for acid-fast bacilli. Correspondence. Am Rev Respir Dis 1962; 85: 753–754.

12. Allen JL. A modified Ziehl-Neelsen stain for mycobacteria. Med Lab Sciences 1992; 49: 99–102.

13. Devulder B. Coloration des bacilles acido-alcoolo-résistants par la technique de Tan Thiam Hok modifi ée. Ann Inst Pasteur Lille 1963; 14: 229–231. [French].

14. Bishop PJ, Neumann G. The history of the Ziehl-Neelsen stain. Tubercle 1970; 51: 196–206.

15. Chandra TJ, Dash S, Srinivas G, Prabhakara Rao PV. A study on rapid confirmation of pulmonary tuberculosis in smear negative for AFB cases by using Fiberoptic Bronchoscopy, done through trans oro pharyngeal spacer. J. Fam. Community Med 2012; 19 (1): 43 – 46.

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