Original Article

EQUIVALENCE OF ACID ALONE OR ACID-ALCOHOL AS DECOLOURIZING AGENT IN ZIEHL - NEELSEN METHOD

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Summary

***Background*:** Microscopists opine that acid-alcohol decolourized slides may enhance acid-fast bacilli (AFB) smear positivity, and published documents on equivalence of acid and acid-alcohol in ZN staining method are not easily accessible.

***Setting*:** National Institute for Research in Tuberculosis, Chennai, India.

***Objective*:** To document the equivalence of 25% sulphuric acid (ZN-acid method) and 3% hydrochloric acid-alcohol (ZN-alcohol method) as decolourizing agents in ZN method for detection of acid-fast bacilli.

***Methods*:** Two smears from each of 253 sputum samples from pulmonary tuberculosis patients, prepared and allocated, one to ZN-acid method and another to ZN-alcohol method were read blind. All the specimens were cultured for *Mycobacterium tuberculosis* by modified Petroff's method. Culture of *M. tuberculosis* was gold standard.

***Results*:** The concordance between the methods was 85% (kappa 0.68), and the sensitivity (79%) and specificity (89%) were same for both the methods.

***Conclusion*:** In conclusion, the common belief that acid-alcohol decolourized slides give enhanced smear positivity stands void. **[*Indian J Tuberc 2012; 59:* 219-223]**

***Key words*:** AFB, Decolourizing agent, *Mycobacterium tuberculosis,* Ziehl-Neelsen

INTRODUCTION

Detection of acid fast bacilli (AFB) in sputum or any biological sample is diagnostic of tuberculosis[1]. It is achieved generally by staining the smears by hot Ziehl-Neelsen (ZN) method all over the world. ZN method generally involves staining, decolourizing and counter-staining the smears, respectively, by basic fuchsin solution, dilute acids or acid-alcohol solution and methylene blue solution to achieve best results[2]. However, apprehension persists in the field on the recommended use of 25% sulphuric acid over 3% acid-alcohol as decolourising agent in ZN method (non-documented feedback from the field). The apprehension is that decolourization with acid presumably gives unclean smears for examination under the oil immersion microscopes and reduces AFB smear positivity rate. It is also opined that acid-alcohol decolourized slide gives clean smears and enhances the smear positivity. In addition, lack of accessible documented evidences on the equivalence of acid and acid-alcohol in the ZN method[3], especially in this era of information technology, and recent scientific interest in the investigation of efficiency of dilute hydrochloric acid as a decolourizing agent in the ZN method[4], prompted us to document our observations on the equivalence of acid and acid-alcohol as decolourizing agents in ZN method.

MATERIAL AND METHODS

Consecutive two hundred and fifty three sputum samples from pulmonary tuberculosis suspects, assessed for admission into the controlled clinical trials at National Institute for Research in Tuberculosis (NIRT), were selected. Of these, 33 were diagnostic samples collected before initiating treatment, 94 were from patients during treatment or follow up period and the remaining 126 were from patients who were assessed for admission into clinical trials but not included in clinical trails for different reasons including the previous history of treatment. Patient's consent was obtained before admitting them in controlled clinical trials and the Institutional ethics committee's clearance was obtained. From each of the samples, two direct smears were made and heat fixed on hot plate. One

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smear was allotted to hot ZN method using 3% hydrochloric acid-alcohol (ZN-acid alcohol method) as a decolourizing agent and other to ZN method using 25% sulphuric acid (ZN-acid method). All the smears were coded and read blind. For the ZN-acid method, the preparation of direct smears and reagents, and staining, examination and grading protocols were followed as per RNTCP laboratory manual.[5] All positives and 20% of negative slides were checked by a senior technician. Referee reading resolved any discrepancy in smear results. The results of the referee reading were taken as final. The smear results were decoded and used for analysis. All the sputum samples were processed by modified Petroff's method and cultured on solid Lowenstein Jensen (LJ) medium. The *M. tuberculosis* isolated were identified by phenotypic methods followed in NIRT.[6] The sensitivity and specificity were calculated against culture of *M. tuberculosis* as the gold standard. The agreement between ZN-acid and ZN-acid alcohol methods was studied using kappa statistics.

PREPARATION OF THE REAGENT

Acid alcohol (3%): To 485 ml of alcohol (Tamil Nadu State Government supply) in a flask, 15 ml of concentrated hydrochloric acid (Qualigens, India) was added.[1]

Sulphuric acid (25%): To 375 ml of distilled water, 125 ml of concentrated sulphuric acid (Qualigens, India) was added keeping the flask in cold water. Two batches of reagents were prepared and used. The other reagents, 1% carbol fuchsin and 0.1% methylene blue were prepared as per RNTCP guidelines.[4]

RESULTS

For all the samples studied, agreement of smear results between ZN-acid and ZN-acid alcohol method was 85% (*k value* = 0.68) (Table 1). Out of 253 sputum samples, 11 were eliminated; three as

Table 1: Smear results obtained from ZN method using 25% sulphuric acid (ZN-acid) and 3% acid alcohol (ZN-alcohol)

		ZN -acid						
		Scanty*	**1+**	**2+**	**3+**	**All positives**	**Negative**	**Total**
ZN - alcohol	**Scanty**	2	3	0	0	5	9	14
	1+	4	19	8	5	36	7	43
	2+	0	4	3	4	11	1	12
	3+	0	1	6	16	23	1	24
	All positives	6	27	17	25	**75**	**18**	93
	Negative	11	5	0	0	**16**	**144**	160
	Total	17	32	17	25	91	162	253

* 3 +: More than 10 AFB per oil immersion field in at least 20 fields; 2 +: 1–9 AFB per oil immersion field in at least 50 fields; 1+: 10–99 AFB in 100 oil immersion fields; Scanty: 1–9 AFB in 100 oil immersion fields.

contaminants and eight as non-tuberculous mycobacteria (NTM). Out of three contaminated samples, one each was positive in ZN-acid and ZN-acid alcohol method. Among the eight samples that yielded NTM, two were smear positive in ZN-acid alcohol method only. Of the remaining 242 samples which were analysed, 91 were culture positives. The sensitivity and specificity were 79% and 89% respectively either for ZN-acid or for ZN-acid alcohol method (Table 2). In this study, 17 culture negative samples (15 from follow-up /

Table 2: Comparison of smear results obtained from ZN using 25% sulphuric acid (ZN-acid) and ZN using 3% acid alcohol (ZN-alcohol) with culture results

	Smear results**	**Culture results***						
		Cols	**1+**	**2+**	**3+**	**All positive**	**Negative**	**Total**
ZN - acid	**Scanty**	0	3	6	1	10	6	16
	1+	2	9	4	8	23	9	32
	2+	1	1	7	7	16	1	17
	3+	0	1	2	21	24	1	25
	All positives	3	14	19	37	**73**	**17**	90
	Negative	7	7	4	1	**19**	**133**	152
	Total	10	21	23	38	92	150	242
ZN-alcohol	**Scanty**	2	1	2	0	5	8	13
	1+	3	8	10	12	33	8	41
	2+	0	2	3	7	12	0	12
	3+	0	0	5	18	23	1	24
	All positives	5	11	20	37	**73**	**17**	90
	Negative	5	10	3	1	**19**	**133**	152
	Total	10	21	23	38	92	150	242

*3+: confluent growth; 2+: innumerable number of colonies; 1+: >20 to 100 colonies; Cols: 1–19 colonies; all positive: total positives; Negative: no growth of *M. tuberculosis*;

**** As shown in the previous table**

assessment patients and two from new cases) were smear positive in either of the methods.

DISCUSSION

The higher smear positivity rate (79.3%; 73/92) observed in this study could be due to the selective referral of pulmonary tuberculosis suspects from the peripheral clinics. The reduced specificity (89%; 133/150) could be attributed to the inclusion of samples collected during treatment and follow up period. It is known that 'smear positive and culture negative' samples could range from 20-25% among samples collected and studied during treatment [7] and in this study 15 of 17 'smear positive and culture negative' samples were from patients on treatment/follow up.

National Tuberculosis Programmes (NTP) recommend the use of 25% sulphuric acid as decolourizing agent in hot ZN method although WHO and UNION (International Union Against Tuberculosis and Lung Disease) recommend either 25% sulphuric acid or 3% acid-alcohol [1, 2]. NTPs prefer the use of acid, as procurement and preparation of 25% sulphuric acid is less cumbersome for the district TB programme officials compared to the lengthy regulated administrative procedures needed to procure alcohol, and avoid stock outs in their stores. In addition, the stringent storage conditions and safety precautions to prevent theft in the health facility are added risks to the use of alcohol in peripheral health settings.[8]

The anticipated advantage of using acid-alcohol in ZN method could be that *M. smegmatis* can be decolourised with alcohol in biological samples, especially in urine samples.[9] Therefore, the diagnosis of renal tuberculosis in urine samples should be unequivocally confirmed with ZN-acid alcohol method as *M. smegmatis,* excreted in urine as result of transient colonization in urinary tract, will not be stained and mis-diagnosed for tuberculosis. In India, where the causative organism of pulmonary and extra-pulmonary tuberculosis is predominantly *M. tuberculosis*, the use of 25% sulphuric acid in hot ZN method will meet all the requirements and the use of acid-alcohol in the ZN method is not warranted for sputum samples in the diagnosis of pulmonary tuberculosis. The present study also, though small in its magnitude, reveals that acid-alcohol didn't show any added advantages over sulphuric acid in the detection of AFB in sputum samples. Nevertheless, the quality of smears, especially the staining characteristics in both the methods, which was not documented in the study, might have given some inputs over cleanliness of smears.

CONCLUSION

In conclusion, the findings reveal that 25% sulphuric acid is as good as 3% hydrochloric acid-alcohol as a decolouriser in ZN method for detection of AFB in sputum samples, and the common belief in the field that the acid-alcohol could yield enhanced smear positivity stands void.

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REFERENCES

1. World Health Organization. Laboratory services in tuberculosis control. Part II: microscopy. WHO/TB/98.258. Geneva, Switzerland: WHO, 1998.
2. International Union against Tuberculosis and Lung Disease. Technical guide. Sputum examination for tuberculosis by direct microscopy in low income countries. Paris, France: IUATLD, 2000.
3. Bogdănescu V, Iacob L, Martinas O. Comparative study of the use of alcohol-hydrochloric acid and of sulfuric acid in Ziehl-Neelsen staining. *(Romanian)* 1981; **30**: 157-64.
4. Aung KJM, Nandi P, Hamid Salim A, Hossain A, Van Deun A. Hydrochloric *vs.* sulphuric acid in water for Ziehl-Neelsen staining of acid-fast bacilli. *Int J Tuberc Lung Dis* 2011; **15**: 955-8.
5. Central TB Division. Manual for Laboratory Technicians. Directorate General of Health Services, Ministry of Health and Family Welfare. RNTCP Training Module for Laboratory Technician. New Delhi, India: 2005. pp.19. (http://tbcindia.nic.in/pdfs/Module%20for%20Laboratory%20Technician.pdf) Accessed on 22/02/2012.

6. Standard operating procedure for mycobacteriology. Department of Bacteriology.Version1.1, November 2010. http://www.trc-chennai.org/pdf/sop.pdf. Accessed on 05/03/2012.
7. Kim TC, Balckman RS, Heatwole KM, Kim T, Rochester DF. Acid fast bacilli in sputum smear of patients with pulmonary tuberculosis. Prevalence and significance of negative smears pretreatment and positive smears post treatment. *The American Review of Respiratory Disease* 1984; **129**: 264-8.
8. Sujatha Chandrasekaran, Sreenivas TR, Chaudhuri K. Modified sputum microscopy techniques sparing the use of alcohol for field application. *Indian J Tuberc* 1991; **38**: 87-90.
9. Davies EM.and Lewis DA. *In: Medical Bacteriology practical approach*, 2nd edn, Vol 8, ed. Hawkey, P. and D. Lewis: Oxford University, Press. 2004. pp. 8.