

Special Feature: Short communication**Same day sputum smear microscopy with an economical destaining step for the diagnosis of pulmonary tuberculosis****T. Jaya Chandra,¹ R. Selvaraj,² Y. V. Sharma³***Departments of ¹Microbiology, ³Pathology, GSL Medical College, Rajahmundry and**²Centre for Laboratory Animal Technology and Research (CLATR), Sathyabama University, Chennai***ABSTRACT**

Back ground: Acid fast bacilli (AFB) positivity using modified Ziehl Neelsen (MZN) staining and jar method was studied in sputum samples obtained by spot morning (SM) and same day (SS2) approaches.

Methods: Three specimens (spot, second spot and morning) were collected in 252 patients, duplicate smears were prepared and stained by MZN staining and jar method.

Results: Smear positivity was 34 (13.5%) and 33 (13%) for MZN staining and jar method respectively ($p>0.99$) for the SM approach; and 34 (13.5%) and 32 (12.7%) for MZN staining and jar method ($p>0.99$) respectively for SS₂ approach.

Conclusion: Jar method in view of lesser cost and similar diagnostic yield can be useful for training purposes.

Key words: Spot morning approach, Same day approach, modified Ziehl Neelsen staining

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INTRODUCTION

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB). Several diagnostic techniques are available for the diagnosis of TB. Due to rapidity and low cost, most of the National Tuberculosis Control Programmes follow sputum microscopy for the diagnosis of TB. Hence microbiological diagnosis is the main stay for effective treatment of pulmonary TB.¹⁻³

As per the Revised National Tuberculosis Control Programme (RNTCP) guidelines, two sputum samples obtained as spot and overnight sample collected in the morning (SM) approach is the sample collection method for the diagnosis of pulmonary TB. The same day (SS2) approach where two sputum samples are collected with a gap of one hour has been observed to have similar sensitivity to that of

the SM approach in the diagnosis of pulmonary TB.⁴⁻⁶

Several modifications of ZN staining have been reported in the literature; some researchers increased the duration of primary staining step,⁵ others have changed the concentration of the ingredients used in the preparation of primary staining solution, 1% Carbol fuchsin⁷⁻⁹ and some others performed primary staining step in sputum containers.¹⁰ With these modifications two aspects of ZN staining technique were only satisfied: improved sensitivity and minimizing the risk of occupational hazard to laboratory technicians (LTs) while performing smear microscopy. However, studies on cost effective aspect on ZN staining are limited. On cost-effectiveness aspect of ZN technique, researchers either reduced the concentration of ingredients or

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changed the destaining solution. It was reported that the concentration of the reagents should not be changed for reduction of expenditure.¹¹ In the available literature also we did not find studies on economical decolourization step by using sulphuric acid (H_2SO_4), which is a costly chemical used in ZN staining technique.

Therefore, this study was taken with the following objectives: (i) sputum smears staining by ZN method with prolonged primary staining step [modified ZN (MZN) staining] (ii) decolourization of sputum smears by dipping in coplin jar containing 25% H_2SO_4 ; primary staining as well as counterstaining steps were similar to that of MZN staining (jar method); and (iii) comparison of the proportion of acid-fast bacilli (AFB) positivity in MZN staining and jar methods for the SM and the SS2 approaches.

MATERIAL AND METHODS

The study was conducted in the Department of Microbiology, GSL Medical College, Rajahmundry, from October 2014 to February 2015. The study protocol was approved by Institutional Research and Ethics Committees. An informed written consent was obtained from all the study participants. Individuals aged 18 years or above were studied. All the individuals were explained in local language about the importance of submission of a proper sputum sample. Visual difference between sputum and saliva and how to produce good quality sputum sample were demonstrated practically.

All the individuals were informed to provide three sputum samples, spot (S) sample at the time of first visit to the hospital, second (S2) spot was collected 1 hour after the S sample; early morning (M) sample was collected after getting up from bed early in the morning. After collecting two spot samples (S1 and S2), patients were provided with prelabelled sample containers to collect morning sample at home.

Duplicate smears were prepared with each sputum sample. One smear was stained by MZN⁵ staining technique and second was stained by jar method. After staining, the data on slides was covered with a wrap, so that the microscopist would not be aware of the staining technique, thus avoiding misinterpretation of smear reading and bias. All the stained slides were observed and graded as per RNTCP guidelines.¹² As a part of internal quality control, all the positive slides and randomly 25% of negative slides were read by the senior author. In case of any discrepancy in smear reading, senior author's decision was considered final.

MZN staining

Smears were flooded with filtered 1% carbol fuchsin and heated until they were steamed and left to steam for 5 minutes.⁵ After rinsing the slides with a gentle stream of water, 25% H_2SO_4 was used to decolourize the smears for 2-4 minutes, and if necessary, the decolorization step was repeated for another 1-3 minutes. The slides were rinsed as described earlier and counterstained with 0.1% methylene blue for 30 second. The slides were then washed, air dried and examined under oil immersion.

Jar method

Smears were flooded with filtered 1% carbol fuchsin and heated until they were steamed and left to steam for 15 minutes. After rinsing the slides with a gentle stream of water, slides were dipped in coplin jar containing 25% H_2SO_4 for 2-4 minutes and if necessary decolorization step was repeated for another 1-3 minutes. The slides were rinsed as above and counter stained with 0.1% methylene blue for 30 second. The slides were then washed, air-dried and examined under oil immersion.

Grading of sputums smear

The sputums smear was graded as¹² scanty = 1-9 AFB in 100 oil immersion fields; 1+ = 10-

99 AFB in 100 oil immersion fields; 2+ = 1-9 AFB per field in at least 50 oil immersion fields; 3+ = 10 or more AFB per field in at least 20 oil immersion fields; and negative = no AFB seen in 100 fields.

The expenditure incurred for carrying out the procedures was also calculated.

Statistical Analysis

Statistical analysis was carried out using Microsoft Excel. Fisher's Exact test was used to study the proportion smear of positive cases between the approaches and staining techniques. A p-value less than 0.05 was considered to be statistically significant.

RESULTS

During the study period, sputum samples from 252 patients were processed (Table 1). For SM approach, smear positivity was 34 (13.5%) and 33 (13%) for MZN staining and jar methods, ($p>0.99$) respectively. For SS2 approach, smear positivity was 34 (13.5%) and 32 (12.7%) for MZN staining and jar methods respectively, ($p>0.99$).

In this study, the recurring expenditure on decolourizer was 38% less for jar method

(₹ 4563/- versus ₹ 2838/- respectively for MZN staining and jar methods).

DISCUSSION

According to the WHO expert group, biological safety cabinets are not mandatory to perform direct smear microscopy. It was found by the expert group that with good microbiological technique, direct sputum microscopy carries low risk in generating infectious aerosols, and these procedures can be performed on an open bench.¹³ Hence, with good microbiological practice, modifications were not felt required in ZN technique for the safety of LTs.

In this study, smear positivity was similar for MZN staining and jar methods in both approaches (13.5% and 12.7% respectively for SM and SS2 approach), except some differences in smear grading (Table 1). However, differences in smear grading do not influence initiation of anti-TB treatment, because as per the RNTCP guidelines, smear positivity is the only criteria to start anti-TB treatment. Statistically there may be insignificant difference between MZN staining and jar methods ($p>0.99$), but, ideally it may be better not to miss even a single case of smear-

Table 1: Sputum smear-positivity

Grading		MZN staining													
		SM approach								SS ₂ approach					
		Scanty	1+	2+	3+	Any positive	Neg	Total	Neg	Any positive	3+	2+	1+	Scanty	
Jar method	Scanty	2	2	0	0	4	0	4	12	0	12	2	0	3	7
	1+	1	5	0	1	7	0	7	10	0	10	0	4	3	3
	2+	0	1	10	3	14	0	14	5	0	5	0	5	0	0
	3+	0	0	3	4	7	0	7	6	0	6	6	0	0	0
	Any positive	3	8	13	8	32		32	33	0	33	8	9	6	10
	Neg	2	0	0	0	2	0	2	1	1	0	0	0	0	1
	Total	5	8	13	8	34	0	34		1	33	8	9	6	11

MZN=modified Ziehl-Neelsen staining; SM approach=spot-morning approach, SS2 approach=same day approach; Neg=negative

positive TB because one individual with active TB can spread disease to ten almost individuals per year.¹⁴ But this technique has the potential to be implemented in medical college teaching hospitals during undergraduate training as cost on the reagents can be minimized significantly.

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